Role of Cytochrome c Heme Lyase in the Import of Cytochrome c into Mitochondria*

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The import of cytochrome c into Neurospora crassa mitochondria was examined at distinct stages in vitro. The precursor protein, apocytochrome c, binds to mitochondria with high affinity and specificity but is not transported completely across the outer membrane in the absence of conversion to holocytochrome c. The bound apocytochrome c is accessible to externally added proteases but at the same time penetrates far enough through the outer membrane to interact with cytochrome c heme lyase. Formation of a complex in which apocytochrome c and cytochrome c heme lyase participate represents the rate-limiting step of cytochrome c import. Conversion from the bound state to holocytochrome c, on the other hand, occurs 10-30-fold faster. Association of apocytochrome c with cytochrome c heme lyase also takes place after solubilizing mitochondria with detergent. We conclude that the bound apocytochrome c, spanning the outer membrane, forms a complex with cytochrome c heme lyase from which it can react further to be converted to holocytochrome c and be translocated completely into the intermembrane space.

Most mitochondrial proteins are synthesized as precursors containing amino-terminal targeting sequences which specifically direct them to mitochondria (for review see Refs. 1 and 2). Import is dependent on both a membrane potential and on ATP and occurs at sites of close contact between inner and outer membranes (3-8). During or following import, the targeting sequence is removed by a specific peptidase located in the mitochondrial matrix (9, 10). Cytochrome c, on the other hand, differs markedly from these proteins in its import pathway: it is not synthesized with a removable amino-terminal prepiece (11-16), it does not require a membrane potential to drive import (17-19), and it must only be translocated completely into the intermembrane space.

Apocytochrome c, the precursor of cytochrome c, has relatively little detectable secondary structure and lacks covalently attached heme compared to its mature counterpart holocytochrome c (20, 21). It is a nuclear gene product which is synthesized on free cytoplasmic ribosomes and then released into a cytoplasmic pool (22). Its import into mitochondria is mediated by specific binding sites (17, 23, 24). During import, heme is covalently attached via thioether linkages to cysteine residues, near the amino terminus of the apocytochrome c precursor, in a reaction which is catalyzed by the enzyme cytochrome c heme lyase (CCHL) (23, 25, 26). The process requires heme in the reduced state (19) and is coupled to the transport of cytochrome c across the outer mitochondrial membrane (see below).

Here we have examined separately several distinct stages of cytochrome c import into mitochondria, namely, specific recognition and high affinity binding of the apocytochrome c precursor, covalent attachment of the heme moiety, and translocation of the protein across the outer membrane. In contrast to other imported mitochondrial proteins which, because of complex import pathways, appear to involve a relatively large number of mitochondrial constituents to facilitate import (i.e., surface receptors, contact site elements, processing peptidases etc.), we demonstrate that cytochrome c follows a relatively simple import pathway. An explanation for this exceptional situation is discussed on an evolutionary basis.

EXPERIMENTAL PROCEDURES

Cell Growth and Subcellular Fractionation—Neurospora crassa (wild type 74A) was grown for 14–16 h at 25 °C with bright illumination and vigorous aeration as previously described (27). The cyt-2-1 mutant was grown under identical conditions except that cultures were incubated for 36–40 h.

Mitochondria were isolated by differential centrifugation essentially as described before (3) in a buffer containing 250 mM sucrose, 2 mM EDTA, 10 mM Mops/KOH, pH 7.2 (SEM buffer) plus 1 mM PMSF added from a freshly prepared stock solution in ethanol. PMSF was omitted from the SEM buffer in experiments where protease treatment was involved.

Solubilization of Mitochondrial Proteins—Cytochrome c heme lyase was solubilized from mitochondria at 0 °C by combining equal volumes of a mitochondrial suspension (at 5 mg protein/ml) with 5 mM (w/v) solution of n-octyl glucoside (Boehringer Mannheim) in SEM buffer. The mixture was shaken briefly at 1-min intervals for 5 min. The preparation was then spun for 15 min at 226,000 × g (Beckman Ti-50 rotor) and the upper two-thirds of the resulting supernatant was retained.

Import of Cytochrome c into Mitochondria—A full-length cDNA coding for apocytochrome c was isolated from a N. crassa library (28).

Cloning, shortening of the 5′ end of the cDNA with Bal31 (Biolabs), ligation into the Smal site of pGEM3 (Promega), and transformation into Escherichia coli strain DH1 were performed essentially as described (29). Clones oriented in the SP6 direction were selected. Isolated plasmids were transcribed with SP6 RNA polymerase, and the capped transcripts were used to direct protein synthesis. Apocytochrome c was then synthesized in nuclease-treated rabbit reticulocyte lysates (30) in the presence of L-[35S]cysteine (1100–1400 Ci mmol, Amersham Corp.) as previously described (36). Aliquots of the

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1The abbreviations used are: CCHL, cytochrome c heme lyase; PMSF, phenylmethylsulfonyl fluoride; Mops, 3-(N-morpholino)propanesulfonic acid; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate.

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post-ribosomal supernatant were stored at −80 °C under nitrogen gas.

Unless otherwise indicated, binding and import were performed in reaction mixtures in SEM buffer (200 μl of final volume) containing 75 μg of mitochondrial protein (or an octyl glucoside extract from an equivalent amount of mitochondria), 3 μM henin, and 25 μl of [35S]cysteine-labeled reticulocyte lysate. The reaction was started by adding freshly prepared sodium dithionite to a final concentration of 1 mM (19) and incubating for 10 min at 25 °C. The reaction was stopped by cooling the samples to 0 °C and lysing them with 1 ml of buffer containing 1% (w/v) Triton X-100, 300 mM NaCl, and 10 mM Mops/KOH, pH 7.2. The buffer also contained 10 mM K3Fe(CN)6 in experiments where it was important to stop the reaction abruptly.

Mitochondria with Proteases—Freshly isolated mitochondria were incubated for 30 min at 25 °C in a mixture (total volume 100 μl) containing 3% (w/v) bovine serum albumin, 70 mM KCl, 220 mM sucrose, 2.5 mM potassium ascorbate, pH 7, 0.4 mM N,N,N′,N′-tetramethylphenyl-

Mitochondria (50 μg of protein) were incubated for 30 min at 25 °C in a mixture (total volume 100 μl) containing 3% (w/v) bovine serum albumin, 70 mM KCl, 220 mM sucrose, 2.5 mM potassium ascorbate, pH 7, 0.4 mM N,N,N′,N′-tetramethylphenyl-

denediamine, 5 mM Na2HCO3, 10 mM Mops/KOH, pH 7.2, and 25 μl of the pooled [35S]methionine-labeled reticulocyte lysates. Following incubation, the mixtures were cooled to 0 °C and treated with 20 μg of proteinase K/ml for 30 min at 0 °C. The samples were diluted with SEM buffer containing 1 mM PMSF; mitochondria were reisolated by centrifugation at 17,400 × g for 12 min (Beckman JA-20 rotor), lysed with SDS-containing sample buffer, and resolved on SDS-polyacrylamide gels. Bands in the dried gel were visualized by fluorography and quantified by laser densitometry of the resulting film. The amount of protease-resistant mature size F0 and of intermediate-size plus mature-size cytochrome c, were in arbitrary units.

RESULTS

Apocytochrome c Bound to Mitochondria Is Accessible to Externally Added Proteases—In the absence of reductants (i.e. NADH or sodium dithionite), formation of holocytochrome c from apocytochrome c added to isolated mitochondria does not occur (26). As a consequence, the binding of apocytochrome c to mitochondria could be examined independently of subsequent import steps. The positioning of apocytochrome c bound to the mitochondrial surface was examined by its accessibility to proteases, and by its extractability with aqueous perturbants such as Na2CO3, pH 11.5, or urea.

In a first approach, apocytochrome c was bound to mitochondria and the mitochondria were then treated with varying concentrations of proteinase K. Apocytochrome c was bound to mitochondria was digested with low concentrations of proteinase K (Fig. 1). Since these concentrations of proteinase K were not sufficient to penetrate the outer membrane (see below), at least a part of the bound apocytochrome c molecule was exposed to the outer surface of the outer membrane. While total apocytochrome c binding was slightly reduced at lower temperature (the amount bound at 0 °C was 88% of that bound at 25 °C), its sensitivity to protease treatment was the same. Approximately 10−15% of mitochondria-associated cytochrome c could not be digested with even the highest concentration of proteinase K tested. This may in part be due to low levels of holocytochrome c formation and the resulting transport across the outer membrane. The protease-resistant population of cytochrome c could not, however, represent the only import-competent precursor species since 90% of the bound apocytochrome c could be chased and converted to holocytochrome c (see below).

The mitochondria-bound apocytochrome c could also be released from the membrane fraction by aqueous perturbation (not shown). When apocytochrome c was bound to mitochondria at either 0 or 25 °C, all of it could be subsequently extracted with 100 mM Na2CO3, pH 11.5, or with 4 M urea. Under conditions where holocytochrome c formation was...
stimulated by the presence of sodium dithionite, similar extraction was observed.

We conclude that apocytochrome c which is bound to mitochondria remains exposed to the outer surface of the outer mitochondrial membrane prior to subsequent import steps. In addition, it appears to be situated in a hydrophilic membrane environment.

Translocation of Cytochrome c across the Outer Mitochondrial Membrane Is Dependent on Covalent Attachment of Heme—Translocation of cytochrome c across the outer membrane was assessed by protection from digestion by externally added protease K. Whereas apocytochrome c bound to mitochondria was accessible to externally added proteases (Fig. 2, lanes 1–6; also see Fig. 1), transport to a protease-resistant location occurred only under conditions where holocytochrome c was formed (Fig. 2). In the presence of sodium dithionite, 91% of the mitochondria-associated cytochrome c was in the holo-form and an equivalent proportion was resistant to digestion by externally added protease K (lanes 7–9). When the formation of holocytochrome c was inhibited by the analogue deuterohemin, the translocation of cytochrome c to a protease-resistant location was inhibited in parallel (lanes 10–12).

Thus, the attachment of heme appears to be a prerequisite for complete translocation of cytochrome c across the outer membrane to the intermembrane space, where it is not accessible to externally added protease K (i.e. the steps of holoformation and translocation could not be separated experimentally). Therefore, translocation appears to be tightly coupled to or dependent on the covalent attachment of the heme group.

**Apocytochrome c Which Is Bound to Mitochondria Forms a**

**Salt-resistant Complex with Cytochrome c Home Lyase—**To examine the positioning of bound apocytochrome c with respect to the CCHL enzyme, apocytochrome c was bound to mitochondria in the presence or absence of varying concentrations of KCl and then chased to holocytochrome c (by adding sodium dithionite), also in the presence or absence of varying concentrations of KCl (Fig. 3). When the binding of apocytochrome c to mitochondria was performed in the presence of KCl, substantially reduced import during the chase (as measured by holocytochrome c formation) was observed. This was due to inhibition of apocytochrome c association with mitochondria (Fig. 3, inset). If, on the other hand, binding of apocytochrome c to mitochondria was allowed to occur in the absence of KCl and then KCl was added following the binding incubation, the subsequent formation of holocytochrome c during the chase was unaffected. Identical results were obtained with the detergent-solubilized CCHL enzyme (Fig. 3).

In summary, these findings indicate that the bound apocytochrome c forms a complex with CCHL which is sufficient for covalent heme addition provided that heme is available in the reduced form. Formation of the complex can be impeded by salt, but it cannot be dissociated with salt once it is formed.

To determine whether apocytochrome c was able to gain access to and interact with CCHL under conditions where the fluidity of the membrane lipids was reduced by low temperature (i.e. 0 °C), the formation of the salt-resistant complex at 25 and at 0 °C was compared (Fig. 4). When apocytochrome c was bound to mitochondria at 25 °C in the absence of salt (as described for Fig. 3 above), it was efficiently converted to

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**Fig. 1.** Apocytochrome c bound to mitochondria is accessible to low concentrations of externally added protease K. Apocytochrome c from [35S]cysteine-labeled reticulocyte lysate (25 μl) was bound to mitochondria for 10 min at either 0°C (C) or 25°C (O) in SEM buffer (200 μl final volume) containing 75 μg of mitochondrial protein. After cooling all samples to 0°C, proteinase K was added at the indicated concentrations and incubation was continued for 30 min at 0°C. The samples were then diluted with 1 ml of SEM buffer containing 1 mM PMSF, and mitochondria were reisolated by centrifugation for 12 min at 17,400 × g. The mitochondrial pellets were lysed, and apocytochrome c was immunoprecipitated as described under "Experimental Procedures." The immunoprecipitates were dissociated in SDS-containing sample buffer and resolved on SDS-polyacrylamide gels. The cytochrome c bands from a fluorogram of the resulting gel were quantified by laser densitometry.

**Fig. 2.** Transport of cytochrome c to a protease-resistant location is coupled to covalent heme attachment. Four pools were prepared in multiples equivalent to 75 μg mitochondrial protein in SEM buffer (200 μl final volume) and containing apocytochrome c from 25 μg of [35S]cysteine-labeled reticulocyte lysate plus 3 μM hemin. In addition, pools III and IV contained sodium dithionite (1 mg/ml concentration), and pool IV also contained 50 μM deuterohem. The pools were incubated for 10 min at either 0°C (1) or 25°C (II–IV) and then cooled to 0°C. The samples were diluted with 1 ml SEM buffer, and the mitochondria were reisolated by centrifugation for 12 min at 17,400 × g. The resulting pellets were resuspended in SEM buffer at 1 mg of mitochondrial protein/ml. Each pool was divided into four aliquots (75 μg of protein each). Triton X-100 was added to one aliquot (0.3% (w/v) final concentration) and then proteinase K (200 μg/ml) as indicated. The samples were incubated for 30 min at 0°C and then lysed with 1% Triton X-100, 300 mM NaCl, 10 mM Tris/HCl, pH 7.2, 1 mM PMSF. Total cytochrome c was immunoprecipitated, dissociated in SDS-containing sample buffer, and resolved on SDS-polyacrylamide gels. The cytochrome c bands from the resulting fluorogram are shown. From the fourth aliquot, mitochondria-associated apo- and holocytochrome c were determined as described under "Experimental Procedures." Note: even in the presence of detergent, not all cytochrome c was digested by proteinase K (lanes 9 and 12) owing to the resistance of holocytochrome c to proteolytic degradation in the presence of reducing agents. This protease-resistant species, however, cofractionated with soluble intermembrane space markers following digitonin treatment (not shown).
Two approaches were taken to examine the salt-dependence of binding of apocytochrome c to CCHL in whole mitochondria (1, 2) and in octyl glucoside solubilized extracts (3, 4) by varying the sequence of additions. In the first approach (binding and import in the presence of varying concentrations of KCl), the following procedure was used. Either 75 μg of mitochondrial protein (3) or an octyl glucoside (OG) extract from the equivalent amount of starting material (4) were incubated for 5 min at 25 °C in a total volume of 100 μl of SEM buffer (to mimic the preincubation of the second approach). The mixtures were then supplemented with varying concentrations of KCl (added first) at the indicated concentrations, 3 μM hemin, 25 μl of [35S]cysteine-labeled reticulocyte lysate (added last), and SEM buffer so that the total volume was 200 μl. Holocytochrome c formation was then initiated by adding sodium dithionite (1 mg/ml final concentration), and the mixtures were incubated for 10 min at 25 °C. In the second approach (binding apocytochrome c to CCHL before KCl addition) the following procedure was used. Apocytochrome c, synthesized in [35S]cysteine-labeled reticulocyte lysate (25 μl), was preincubated with either 75 μg of mitochondrial protein (3) or an octyl glucoside (OG) extract (4) from the equivalent amount of starting material for 5 min at 25 °C in a total volume of 100 μl in SEM buffer. The mixtures were then supplemented with KCl (at the indicated concentrations), hemin (3 μM), and SEM buffer to that the total volume was 200 μl. The apocytochrome c which had interacted with CCHL was then chased to holocytochrome c by adding sodium dithionite (1 mg/ml final concentration) and incubating the mixture for a further 10 min at 25 °C. The samples from both approaches were lysed, immunoprecipitated, and holocytochrome c formed was determined as described under "Experimental Procedures." Results are expressed as a percentage of the controls which did not receive any KCl. The total association of cytochrome c with mitochondria in the presence of KCl (3, inset) was determined as described for the first approach except that the mitochondria were diluted with SEM buffer (1 ml) following the import incubation, mitochondria were reisolated, and then immunoprecipitated with antisera directed against both apo- and holocytochrome c. The immunocomplexes were dissociated with SDS-containing sample buffer and resolved on SDS-polyacrylamide gels. The bands corresponding to cytochrome c in the resulting fluorogram were quantified by laser densitometry.

Holocytochrome c during the chase period even in the presence of KCl. If the binding incubation was done at 0 °C, the amount of apocytochrome c that formed a salt-resistant interaction with CCHL was only slightly reduced (Fig. 4). The rate of formation of the salt-resistant complex at 25 °C was rapid and reached satiuation within 2.5 min (Fig. 5A). At 0 °C, formation of the complex was only 20–30% less efficient as compared to 25 °C and was marginally slower. This relatively high efficiency at low temperature would support the above mentioned suggestion that penetration of apocytochrome c through the outer membrane does not occur through a rigid lipid bilayer.

Rapid interaction between apocytochrome c and CCHL was also observed with detergent-solubilized preparations of the enzyme (Fig. 5B). Apparent saturation was reached after comparable binding periods as observed with intact mitochondria. If the binding time at 25 °C (prior to KCl addition and chase to holocytochrome c) exceeded 2.5 min, however, the amount of chaseable salt-resistant complex was reduced. This can be accounted for by the higher sensitivity of the detergent solubilized CCHL to thermal inactivation at 25 °C compared with the enzyme in whole mitochondria (Fig. 5A, inset). In all likelihood, the salt-resistant complex is formed in the early stages of the binding incubation but comes apart or is inactivated after prolonged incubation periods.

We conclude that apocytochrome c which is bound to mitochondria in an import-competent fashion forms a stable complex with the CCHL enzyme. Once formed, this complex is not dissociated by even high concentrations of KCl. The apocytochrome c bound in this manner can be chased to holocytochrome c by the addition of reducing agents. Formation of the complex in whole mitochondria is restricted at low temperatures (i.e. 0 °C) to about 70% of that formed at 25 °C, although this does not appear to be the case with the detergent-solubilized enzyme. Immobilization of mitochondrial membrane lipids and proteinaceous structures embedded in them may account for this observation.

 Taken together, these data indicate that while part of the bound apocytochrome c molecule is exposed to the outer surface of the outer membrane, at least part of it also penetrates through the outer membrane so that it can interact
Cytochrome c Biogenesis

A. MITOCHONDRIA

\[ \text{Heme lyase} \]

\[ \text{CCCHL activity remaining} \]

\[ \text{CCCHL activity} \]

\[ \text{Binding time (min)} \]

\[ \text{Holocytochrome c formed during chase} \]

\[ \text{Binding time (min)} \]

\[ \text{Mitochondria} \]

\[ \text{OG extract} \]

\[ \text{OG extract} \]

\[ \text{OG extract} \]

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B. OG EXTRACT

\[ \text{Holocytochrome c} \]

\[ \text{Holocytochrome c} \]

\[ \text{Holocytochrome c} \]

\[ \text{Holocytochrome c} \]

\[ \text{Holocytochrome c} \]

\[ \text{Mitochondria} \]

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Mitochondria were treated with either proteinase K (O), chymotrypsin (A), or trypsin (E) at the indicated concentrations as described under “Experimental Procedures.” Following inhibition of proteolytic activity with a combination of PMSF and soybean trypsin inhibitor, the mitochondria were reisolated by centrifugation and suspended in fresh SEM buffer. No residual digestive activity was detected following this treatment (not shown). The resulting mitochondrial preparations were then tested for their ability to catalyze holocytochrome c formation (panel A) and to import F1-ATPase subunit β (panel C, closed symbols) and cytochrome c1 (panel C, open symbols). As an indicator for possible damage to the outer mitochondrial membrane and release of or access to intermembrane space constituents, adenylate kinase activity (a marker for the intermembrane space) was measured (panel B). Apocytochrome c binding to mitochondria which had been treated with the highest concentration of proteases (100 μg/ml) was titrated using “C-apocytochrome c (panel D) as described under “Experimental Procedures.” Control mitochondria (O) were processed in an identical manner except that no protease was present. PK, proteinase K; CHY, chymotrypsin; TRP, trypsin.

**Table I**

*Holocytochrome c formation, inhibited in trypsin-treated mitochondria, cannot be restored by solubilization of cytochrome c heme lyase with detergent*

<table>
<thead>
<tr>
<th>Proteinase K after treatment with</th>
<th>Import or enzymatic activity</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>after treatment with</td>
<td></td>
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<tr>
<td></td>
<td>Proteinase K</td>
<td>Trypsin</td>
</tr>
<tr>
<td>A. Holo c formed in mitochondria</td>
<td>95.8</td>
<td>2.8</td>
</tr>
<tr>
<td>B. Soluble CCHL activity</td>
<td>95.6 (74.0)</td>
<td>1.2 (0.9)</td>
</tr>
<tr>
<td>C. Adenylate kinase activity</td>
<td>94.3</td>
<td>3.4</td>
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<tr>
<td>D. ATPase F1β import</td>
<td>0</td>
<td>0</td>
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<tr>
<td>E. Cytochrome c1 import</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

**Note:** The capacity of treated mitochondria to bind apocytochrome c decreased in the identical order (Fig. 7D).
Reduction of cytochrome c import following pretreatment of mitochondria with chymotrypsin or trypsin corresponded almost identically with perturbation of the outer membrane barrier, as measured by the loss of adenylate kinase activity (Fig. 7B). Therefore, high concentrations of proteases (especially membrane active proteases such as chymotrypsin and trypsin) apparently compromise the integrity of the outer membrane and thereby gain access to components, such as CCHL, which are necessary for holocytochrome c formation. Since apocytochrome c binding was also reduced, this suggests that either CCHL itself plays a critical role in the binding process or that another component which precedes CCHL in the import pathway, but which is also not exposed to the outer surface of the outer membrane, was degraded. As a control for whether the membrane-active behavior of chymotrypsin and trypsin per se accounted for reduced apocytochrome c binding to treated mitochondria, or whether their proteolytic activities was responsible, mitochondria were treated in an identical manner except that the proteases were first inactivated with PMSF and soybean trypsin inhibitor. Neither the inactivated proteases nor preincubated proteases (which were subsequently inactivated) affected either the binding of apocytochrome c to treated mitochondria or the formation of holocytochrome c (not shown).

To confirm that protease treatment which resulted in the loss of adenylate kinase activity had also destroyed CCHL activity, mitochondria that had been exposed to 100 μg protease/ml were solubilized with octyl glucoside to dissociate CCHL from the membrane environment and measure activity without possibly deficient preceding import steps (Table I). Activity of the solubilized CCHL, however, corresponded identically with the ability of protease-treated whole mitochondria to import apocytochrome c. Therefore, reduction in holocytochrome c formation correlated well with the loss of CCHL activity and could not be restored by liberating the CCHL enzyme from treated mitochondria. K used was chosen since it did not perturb the outer membrane (see Fig. 7B) but degraded over 90% of detergent-solubilized CCHL (not shown). After 30 min at 0 °C, PMSF (2 mM final concentration) was added to all samples (to inhibit the proteinase K digestion), and they were separated into soluble and pelletable fractions by centrifugation (see under "Experimental Procedures"). A, topology of CCHL. The enzymatic activities of adenylate kinase (ADK), an intermembrane space marker, □, and fumarase (FUM, a matrix marker), ○, were determined in the samples not treated with proteinase K. (Marker enzymes activities in the pelletable fractions of proteinase K treated samples were identical; not shown.) Cytochrome c heme lyase (CCHL) activity was determined in both untreated (△) and proteinase K treated (▲) pellet fractions. B, binding of in vitro synthesized apocytochrome c. Pellet fractions of digitonin-treated mitochondria (at the digitonin concentrations indicated by the arrowheads in panel A) which had been treated, before centrifugation, with proteinase K (right half) or not (left half) were tested for their ability to bind apocytochrome c from [14C]labeled reticulocyte lysate. Pellet fractions (from 75 μg of protein of treated mitochondria) were incubated with 25 μl of [14C]labeled reticulocyte lysate in a final volume of 200 μl in SEM buffer for 5 min at 25 °C. The samples were diluted with 1 ml of SEM buffer, and the pellets were resolubilized for centrifugation at 15 min at 48,400 × g. The pellets were assayed in SDS-containing sample buffer and resolved by SDS-gel electrophoresis. The cytochrome c bands from the resulting fluorogram are shown. C, Scatchard analysis of the binding of [14C]apocytochrome c. Following both digitonin and proteinase K treatment, the binding of [14C]apocytochrome c to mitochondrial pellet fractions was titrated as described under "Experimental Procedures." The digitonin concentrations used to open the intermembrane space are indicated by the arrowheads in panel A and were control (○), 0.015% (△), 0.2% (□), and 0.25% ( □ ). The association constant (Kd) and number (n) of the high affinity binding sites was calculated for each of the four curves by computer-assisted graphic parameter fitting and is expressed as the average of the four determinations ± the standard deviation.
The Catalytic Activity of Cytochrome c Heme Lyase Can Be Proteolytically Degraded without Affecting the Binding of Apocytochrome c to Mitochondria—We have previously demonstrated that the CCHL enzyme is topologically exposed to the intermembrane space (26). Therefore, in order to examine the possible role of components facing the intermembrane space in the binding of apocytochrome c, mitochondria were treated with varying concentrations of digitonin to progressively open the intermembrane space, and then both apocytochrome c binding and CCHL activity were examined (Fig. 8). The activity of CCHL was not released but remained membrane-associated (A). If the samples were treated with proteinase K (following digitonin treatment) at concentrations which did not compromise the outer membrane, the protease action destroyed CCHL activity only when the intermembrane space was opened. This confirms that CCHL is membrane-associated and faces the intermembrane space. On the other hand, neither the binding of the in vitro synthesized apocytochrome c to mitochondria (B) nor the binding of 14C-apocytochrome c (C) was affected by this treatment.

We conclude that a protease-sensitive component is not present on the surface of mitochondria to recognize and bind apocytochrome c. Similarly, proteinaceous components facing the intermembrane space, including a moiety of CCHL responsible for or contributing to the catalytic activity, can also be removed without affecting apocytochrome c binding. Only membrane active proteases, such as chymotrypsin or trypsin, are able to gain access to and degrade the component(s) responsible for apocytochrome c binding to mitochondria. Therefore, the binding component of the cytochrome c import system appears to be associated with or embedded in the mitochondrial membranes.

**DISCUSSION**

Apocytochrome c can be bound to mitochondria without further import by either inhibiting the heme-adding reaction with the analogue deuterohemin (23, 24) or by omitting reducing agents (this study). An intermediate along the cytochrome c import pathway can thereby be arrested at the stage of high affinity binding to the previously identified (24) saturable binding sites for apocytochrome c. When apocytochrome c is bound to these specific sites it exists within a salt-resistant complex that includes the CCHL enzyme. Since CCHL is a modifying enzyme that covalently attaches the heme, it is clear that part of it remains exposed to the outer surface of the outer membrane. At the same time, some part of it forms a stable complex with CCHL. Therefore, the binding component of the cytochrome c import system appears to be associated with or embedded in the mitochondrial membranes.

Whether the interaction between apocytochrome c and CCHL is bridged by a distinct binding protein within the salt-resistant complex, or whether a single protein is responsible for both apocytochrome c binding and heme addition remains to be ascertained.

It is clear that the precise localization and orientation of the CCHL molecule in mitochondria is of considerable importance in understanding how the mitochondria-bound apocytochrome c is positioned in the outer membrane and how it interacts to form a complex with CCHL. We demonstrate that the CCHL enzyme itself is topologically exposed to the intermembrane space but that it is membrane bound. Whether it is associated with the outer membrane or inner membrane is not yet clear. It has been reported that the enzyme is attached to the inner membrane in yeast mitochon-

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3 D. W. Nicholson and W. Neupert, unpublished observations.
does not involve the complex pathways required to import some other proteins. A good example of this difference is made by comparing the route taken by cytochrome c with that of prepiece-containing intermembrane space proteins (i.e. cytochrome c$_1$ and the Rieske Fe/S protein of bc$_1$ complex, and cytochrome b$_6$, 31, 44). All of these proteins function in the intermembrane space, the Fe/S protein and cytochrome c$_1$ being anchored to the inner membrane. During import, however, the prepiece-containing precursors are completely transferred into the matrix, where they are partially processed, and are then redirected back through to the intermembrane space side of the inner membrane. This seemingly complex pathway may be explained on evolutionarily grounds in which the prepiece-containing precursors are returned to remnants of their “ancestral” assembly pathways. These proteins may have retained this elaborate import pathway to take advantage of pre-existing mechanisms or because of stringent assembly requirements in which insertion through or into the inner membrane from the matrix side is necessary. On the other hand, cytochrome c is a small soluble protein of the mitochondrial intermembrane space, and it is less likely that the route it takes to get there is as important. This is reflected in the way in which its import pathway has evolved. Rather than making use of pre-existing mechanisms (in the bacterium Rhodopseudomonas capsulata, for instance, cytochrome c$_2$ is translocated across the bacterial plasma membrane with the help of a cleavable amino-terminal signal sequence (45)), mitochondrial cytochrome c is imported by a simpler mechanism where it is translocated only across the outer membrane. The conformational change accompanying the covalent attachment of heme has apparently been adapted during evolution to play a central role in import, and this may account for the importance of CCHL in several stages of cytochrome c biogenesis.

We suggest that the following sequence of events occur during cytochrome c import. Apocytochrome c binds with low affinity to anionic phospholipid head groups and spontaneously penetrates part way through the outer membrane (21, 46–50). The partially inserted precursor is then recognized and sequenced into a complex which includes cytochrome c heme lyase. In the presence of reduced heme (19), the bound precursor is rapidly converted to holocytochrome c. The conformational change, resulting from the covalent attachment of heme and the folding of the polypeptide around the heme moiety, pulls the protein completely across the outer membrane to its functional location in the intermembrane space. Purification of the CCHL enzyme and its reconstitution into liposomes will therefore be an important next step to ascertain which steps CCHL can account for in the absence of other mitochondrial constituents.

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