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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 transported across the outer membrane to reach its functional location in the intermembrane space where it serves as an electron carrier between cytochrome *c* reductase (complex III) and oxidase (complex IV).

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)[1] (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 a 2% (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 *Bal*31 (Biolabs), ligation into the *Sma*I 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-[^{35}S]cysteine (1100–1400 Ci/mmol, Amersham Corp.) as previously described (26). Aliquots of the

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

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 hemin, and 25 μl of [^{35}S] cysteine-labeled reticulocyte lysate. The reaction was started by adding freshly prepared sodium dithionite to a final concentration of 1 mg/ml (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 $K_3(Fe(CN)_6)$ in experiments where it was important to stop the reaction abruptly.

Measurement of Holocytochrome c Formation—The amount of holocytochrome *c* synthesized was determined essentially as described before (26). Samples were immunoprecipitated using an antiserum directed against holocytochrome *c*. The immunocomplexes were dissociated in 8 M urea and digested with trypsin (type XIII from bovine pancreas, Sigma) for 60 min at 37 °C. The resulting tryptic peptide mixtures were then resolved by reverse-phase HPLC. The cysteine-containing peptide from holocytochrome *c* was collected and its radioactivity determined.

In experiments where the amount of apocytochrome *c* was to be determined as well, minor modifications were made. The samples were immunoprecipitated with a mixture of antisera directed against both apo- and holocytochrome *c*. At the end of the tryptic digestion, further trypsin activity was inhibited by adding 75 μg of soybean trypsin inhibitor (we have observed that continued tryptic digestion does not affect the holocytochrome *c* cysteine-containing peptide but eventually degrades the equivalent apocytochrome *c* peptide). Both peptides were collected from the HPLC runs and their radioactivity was determined.

Analysis of Binding of Apocytochrome c to Mitochondria—Purified apocytochrome *c* was radiolabeled by reductive methylation in the presence of [^{14}C]formaldehyde (Du Pont-New England Nuclear), as previously described (27), to yield ^{14}C-apocytochrome *c* having a specific activity of 1000 cpm/pmol. Binding to mitochondria was determined as before (27) with minor changes. Conversion to holocytochrome *c* during the binding assay was prevented by omitting reducing agents instead of preincubation of mitochondria with deuterohemin. Mitochondria (60 μg of protein) were incubated for 30 min at 0 °C in a mixture (final volume 215 μl) containing 250 mM sucrose, 2 mM EDTA, 10 mM Mops/KOH, pH 7.2, 1% (w/v) bovine serum albumin (fatty acid free from type V, Sigma), 0.02% (w/v) holocytochrome *c*, and varying amounts (0.25–80 pmol) of ^{14}C-apocytochrome *c* in 1.5-ml centrifuge tubes which had been precoated by incubation in a 3% (w/v) solution of bovine serum albumin for 30 min at 0 °C. Following the binding incubation, the mitochondria were reisolated by centrifugation for 15 min at 27,200 × *g* (Beckman JA-20 rotor). An aliquot of the resulting supernatant was removed to determine unbound (free) ^{14}C-apocytochrome *c* radioactivity. The mitochondrial pellet was resuspended in SEM buffer, transferred to new tubes, and sedimented by centrifugation for 20 min at 48,400 × *g*. The supernatant was discarded and the pellet containing bound ^{14}C-apocytochrome *c* was lysed in 1% (w/v) SDS, 0.1 M Tris/HCl, pH 8.0 and its radioactivity determined. The resulting binding data are presented as Scatchard plots.

Pretreatment of Mitochondria with Proteases—Freshly isolated mitochondria were suspended in 1.5 ml of SEM buffer at a protein concentration of 1 mg/ml and cooled to 0 °C. Either trypsin (type XIII from bovine pancreas, Sigma), chymotrypsin A4 (from bovine pancreas, Boehringer Mannheim), or proteinase K (from *Tritrachium album*, Boehringer Mannheim) were added at the indicated concentrations and incubated at 0 °C for 30 min. Digestion was halted by adding 0.75 ml of SEM buffer containing 1 mM PMSF and 1 mg/ml of soybean trypsin inhibitor (type I-S, Sigma) to all samples. After a further 5 min of incubation at 0 °C, the mitochondria were reisolated by centrifugation at 17,400 × *g* for 12 min. The mitochondrial pellet was then suspended in fresh SEM buffer. Controls were treated in an identical manner except that no protease was present. By this procedure, no residual proteolytic activity was detectable in the resulting mitochondrial preparations (not shown).

Import of F_1-ATPase Subunit β and Cytochrome c_1—The precursors of F_1-ATPase subunit β ($F_1\beta$) and cytochrome *c* were synthesized in rabbit reticulocyte lysate as above except that L-[^{35}S]methionine (Amersham Corp.) was used. Synthesis was directed by transcripts from plasmids prepared as described (cytochrome c_1, 31; $F_1\beta$[2]). The [^{35}S]methionine-labeled reticulocyte lysates were combined and used together for import studies.

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 $MgCl_2$, 16 mM potassium ascorbate, pH 7, 0.4 mM *N,N,N′,N′*-tetramethylphenylenediamine, 5 mM NADH, 10 mM Mops/KOH, pH 7.2, and 25 μl of the pooled [^{35}S]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 $F_1\beta$ and of intermediate-size plus mature-size cytochrome c_1 are expressed in arbitrary units.

Miscellaneous Methods—Treatment of membranes with aqueous perturbants (100 mM Na_2CO_3, pH 11.5, or 4 M urea) was performed as previously described (32) at a protein concentration of 0.25 mg/ml. Published methods were used for the preparation of holocytochrome *c* and apocytochrome *c* and production of specific antibodies in rabbits (27), immunoprecipitation and preparation of samples for SDS-polyacrylamide gel electrophoresis (26), SDS-polyacrylamide gel electrophoresis (33), protein determination (34), radioactivity determination, fluorography, treatment of mitochondria with digitonin (26), measurement of adenylate kinase activity (35), and measurement of fumarase activity (36).

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 Na_2CO_3, 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* that 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 Na_2CO_3, pH 11.5, or with 4 M urea. Under conditions where holocytochrome *c* formation was

[2] F. Rassow and W. Neupert, manuscript in preparation.

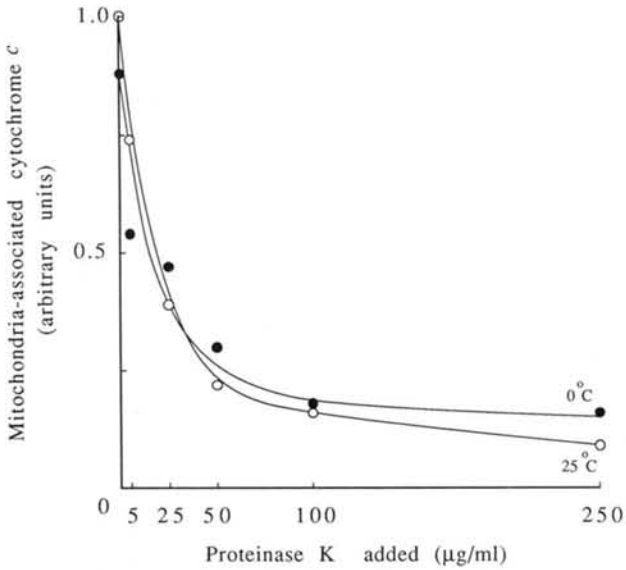

FIG. 1. **Apocytochrome *c* bound to mitochondria is accessible to low concentrations of externally added proteinase K.** Apocytochrome *c* from [^{35}S]cysteine-labeled reticulocyte lysate (25 μl) was bound to mitochondria for 10 min at either 0 °C (●) or 25 °C (○) in SEM buffer (200 μl final volume) 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.

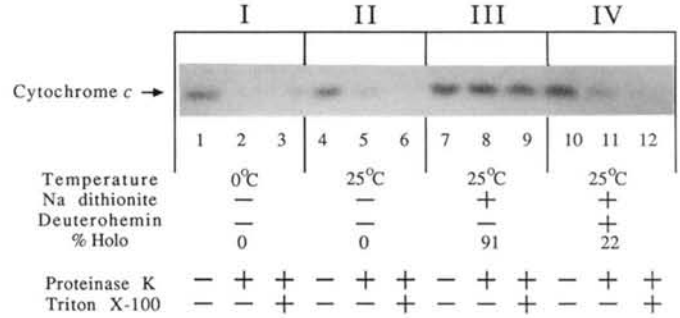

FIG. 2. **Transport of cytochrome *c* to a protease-resistant location is coupled to covalent heme attachment.** Four pools were prepared in multiples equivalent to 75 μg mitochondrial protein in SEM buffer (200 μl final volume) and containing apocytochrome *c* from 25 μl of [^{35}S]cysteine-labeled 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 deuterohemin. The pools were incubated for 10 min at either 0 °C (*I*) 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).

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 proteinase 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 proteinase 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 proteinase 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 Heme 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

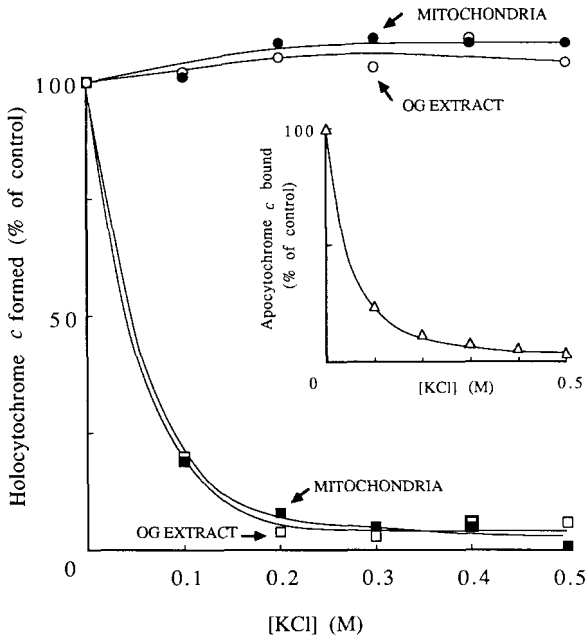

FIG. 3. **Apocytochrome *c* forms a salt-resistant complex with cytochrome *c* heme lyase from which it can be chased to holocytochrome *c*.** Two approaches were taken to examine the salt-dependence of binding of apocytochrome *c* to CCHL in whole mitochondria (●, ■) and in octyl glucoside solubilized extracts (○, □) 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 (■) or an octyl glucoside (*OG*) extract from the equivalent amount of starting material (□) 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 [^{35}S]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 [^{35}S]cysteine-labeled reticulocyte lysate (25 μl), was preincubated with either 75 μg of mitochondrial protein (●) or an octyl glucoside (*OG*) extract (○) 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 (△, *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 saturation within 2.5 min (Fig. 5*A*). At 0 °C, formation of the complex was only 20–30% less efficient as

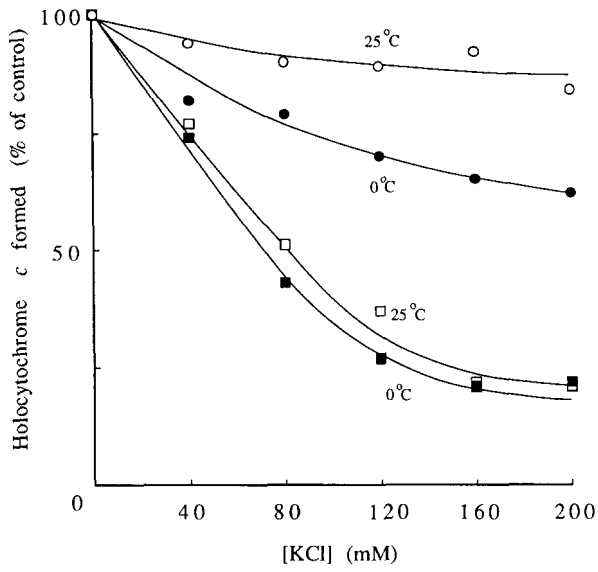

FIG. 4. **Formation of the salt-resistant complex between apocytochrome *c* and cytochrome *c* heme lyase is only partly impeded at low temperature.** Binding of apocytochrome *c* to mitochondria in the absence of KCl, then chasing to holocytochrome *c* in the presence of KCl (○, ●) was compared to the alternate approach where both binding and import were performed in the presence of KCl (□, ■) as described in Fig. 3, except that the binding incubation was at either 25 °C (○, □) or 0 °C (●, ■). In addition, incubation at the given temperature was extended for 5 min in the presence of all additions except sodium dithionite. After adding sodium dithionite (1 mg/ml final concentration), all samples were chased for 10 min at 25 °C and then the holocytochrome *c* formed was determined.

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. 5*B*). 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. 5*A*, *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

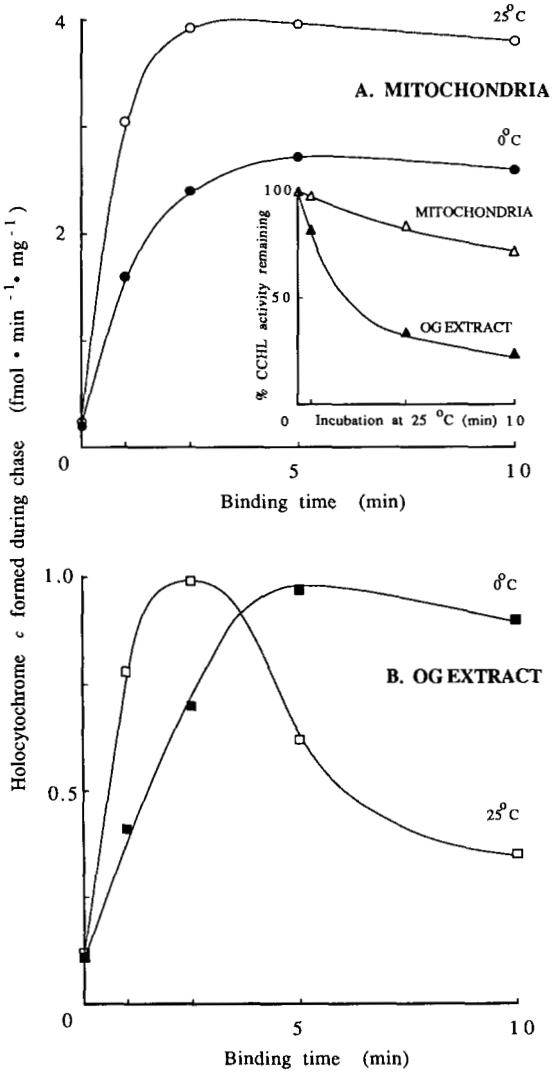

FIG. 5. **Kinetics of formation of a salt-resistant complex between apocytochrome *c* and cytochrome *c* heme lyase.** Apocytochrome *c*, from [^{35}S]cysteine-labeled reticulocyte lysate (25 μl), was mixed with either mitochondria (75 μg protein) (*panel A*) or an ocytl glucoside (*OG*) extract (*panel B*), from an equivalent amount of starting material, in a final volume of 100 μl of SEM buffer. Following incubation at either 25 °C (○, □) or 0 °C (●, ■) for the indicated time intervals, further association of apocytochrome *c* with CCHL was blocked by adding 20 μl of 3 M KCl (in SEM buffer). The salt-resistant CCHL-associated apocytochrome *c* was then chased to holocytochrome *c* by adding hemin (3 μM), sodium dithionite (1 mg/ml), and SEM buffer to a final volume of 200 μl and incubating for a further 10 min at 25 °C. The final KCl concentration after these additions (0.3 M) was sufficient to block any further interaction between apocytochrome *c* and CCHL (see Fig. 3). Holocytochrome *c* formed was then determined as described under "Experimental Procedures." The temperature sensitivity of the CCHL enzyme (*panel A, inset*) was tested by preincubating either mitochondria (△) or the octyl glucoside extract (▲) for the indicated periods of time at 25 °C. The remaining activity was then determined by measuring holocytochrome *c* formation during a second incubation for 10 min at 25 °C in the presence of [^{35}S]cysteine-labeled reticulocyte lysate, hemin, and sodium dithionite as described under "Experimental Procedures."

with CCHL. It also suggests the possibility that CCHL itself may be involved in the specific binding of apocytochrome *c* to mitochondria. It cannot be excluded from these experiments, however, whether a distinct binding protein might exist which could bind apocytochrome *c* in a salt-sensitive manner but then be able to transfer the precursor to CCHL (for holocytochrome *c* formation) independently of the salt concentration.

Apocytochrome c Bound to Mitochondria Is Efficiently Converted to Holocytochrome c—Apocytochrome *c* which was bound to mitochondria was very rapidly converted to holocytochrome *c* upon addition of reducing agents (Fig. 6). At 25 °C, over 85% of the bound apocytochrome *c* was converted to holocytochrome *c* within 10 s. This occurred at a somewhat slower rate at lower temperatures but was nevertheless much faster than the preceding binding step. At all temperatures, approximately 90% of the mitochondria-bound apocytochrome *c* could be converted to holocytochrome *c* (at 0 °C this required 120 s to reach 87.5%; not shown).

We conclude that the majority of apocytochrome *c* is bound to mitochondria in a highly specific import-competent manner. Furthermore, although apocytochrome *c* binding to mitochondria occurs rapidly (compared to that of other imported mitochondrial precursor proteins (37, 38)), the conversion of apocytochrome *c* in the bound state to holocytochrome *c* occurs approximately 10–30-fold faster. Import-competent binding to mitochondria therefore probably represents the rate-limiting step in cytochrome *c* import.

Apocytochrome c Binding Is Not Mediated by a Protease-sensitive Structure (Receptor) on the Surface of Mitochondria But Does Require a Protein Which Is Accessible to Membrane-active Proteases—It has been well established for a number of precursor proteins that treatment of the outer mitochon-

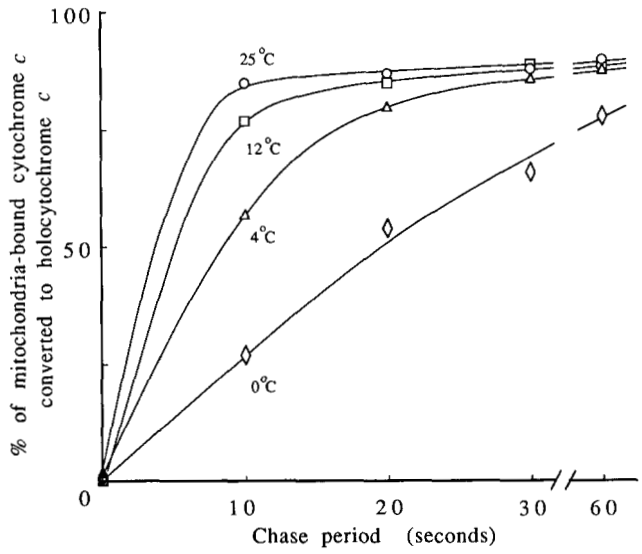

FIG. 6. **Kinetics of conversion of mitochondria-bound apocytochrome *c* to holocytochrome *c*.** Apocytochrome *c* was bound to mitochondria for 10 min at 0 °C in a pool having the equivalent of 75 μg of mitochondrial protein and 25 μl of [^{35}S]cysteine-labeled reticulocyte lysate in a final volume of 200 μl in SEM buffer. The mitochondria were reisolated by centrifugation for 12 min at 17,400 × *g* and were resuspended in fresh SEM buffer. The bound apocytochrome *c* was then chased to holocytochrome *c* at either 0 °C (◇), 4 °C (△), 12 °C (□), or 25 °C (○) in mixtures equivalent to 75 μg of mitochondrial protein (containing bound apocytochrome *c*), 3 μM hemin, and 1 mg sodium dithionite/ml in a final volume of 200 μl in SEM buffer. The chase was started by the addition of the sodium dithionite to the pool at the indicated temperature. The chase was halted by withdrawing a 200-μl aliquot and immediately dispersing it in 1 ml of buffer (at 0 °C) containing 1% (w/v) Triton X-100, 300 mM NaCl, 10 mM Tris/HCl, pH 7.2, and 10 mM $K_3(Fe(CN)_6)$. Total cytochrome *c* was immunoprecipitated from the samples, and the amount of apo- and holocytochrome *c* was determined as described under "Experimental Procedures." Results are given as the percentage of the total mitochondria-associated cytochrome *c* which had been converted to holocytochrome *c* during the chase. In this experiment, 100% represents 0.932 fmol of [^{35}S]cysteine-cytochrome *c*.

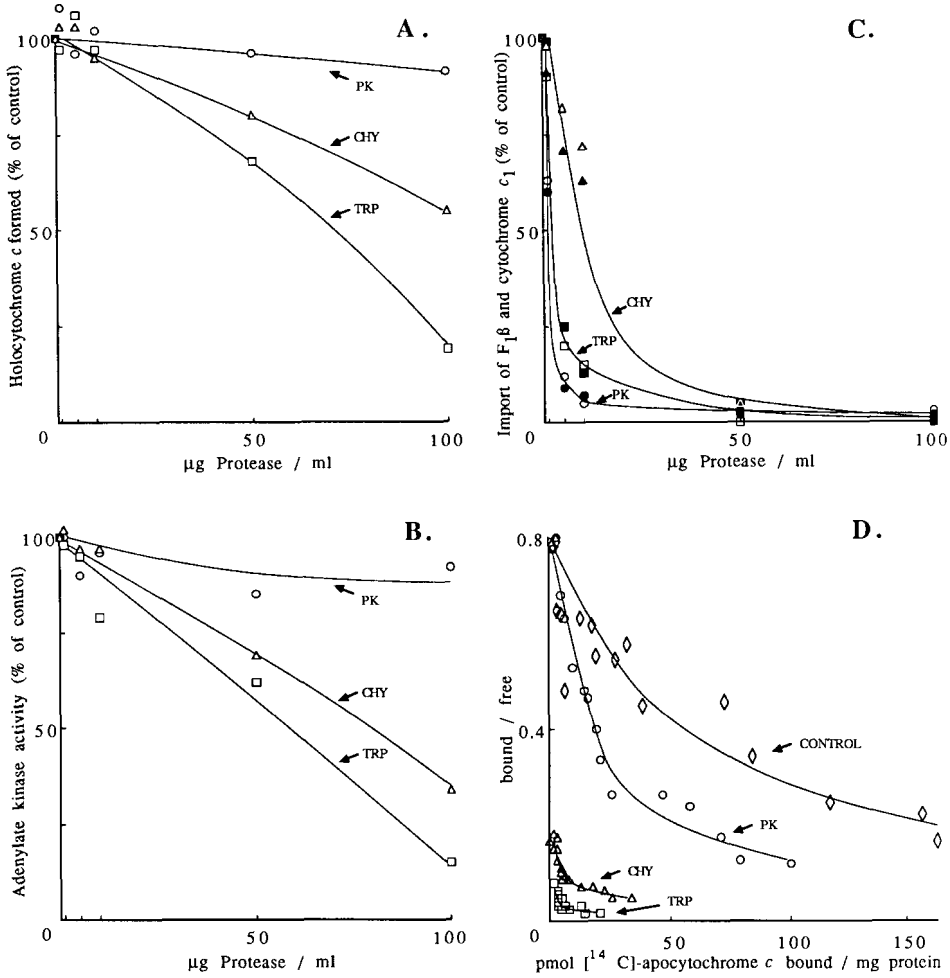

FIG. 7. **Inhibition of holocytochrome *c* formation and of apocytochrome *c* binding by protease treatment of mitochondria occurs only after perturbation of the outer membrane barrier.** Mitochondria were treated with either proteinase K (○), chymotrypsin (△), or trypsin (□) at the indicated concentrations 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 F_1-ATPase subunit β (*panel C, closed symbols*) and cytochrome c_1 (*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 ^{14}C-apocytochrome *c* (*panel D*) as described under "Experimental Procedures." Control mitochondria (◇) 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

Mitochondria were treated with either proteinase K or trypsin at a protease concentration of 100 µg/ml or were untreated (control) but otherwise processed the same, as described under "Experimental Procedures." The reisolated mitochondria were then tested for their ability to import cytochrome *c* (A), F_1-ATPase subunit β (D), and cytochrome c_1 (E), and were assayed for adenylate kinase activity (C). Portions of the treated mitochondrial preparations (at 5 mg protein/ml) were mixed with an equal volume of 2% (w/v) octyl glucoside in SEM buffer and the lysate was centrifuged for 60 min at 226,000 × *g*. The solubilized cytochrome *c* heme lyase activity in the resulting supernatants was then measured (B). Import and enzymatic activity are expressed as a percentage of the untreated control mitochondria which was set at 100%. Values in brackets for the soluble CCHL activity represent the percentage of activity remaining compared to the activity in whole mitochondria that had not been treated with protease nor lysed with octyl glucoside.

	Import or enzymatic activity after treatment with	
	Proteinase K	Trypsin
	% of control	
A. Holo *c* formed in mitochondria	93.8	2.8
B. Soluble CCHL activity	95.6 (74.0)	1.2 (0.9)
C. Adenylate kinase activity	94.3	3.4
D. ATPase $F_1\beta$ import	0	0
E. Cytochrome c_1 import	0	0

drial membrane with low concentrations of proteases abolishes their binding and import (38, 39). This demonstrates the presence of specific protease-sensitive "receptors" on the outer surface of mitochondria involved in recognizing precursor proteins and initiating import. Since mitochondria-bound apocytochrome *c* appears to interact with the CCHL enzyme, which is topographically exposed to the intermembrane space (see below), we have examined whether additional protease-sensitive components may exist on the surface of mitochondria for cytochrome *c* import.

Mitochondria that were pretreated with up to 100 µg of proteinase K/ml exhibited nearly normal levels of cytochrome *c* import (Fig. 7*A*). Treatment of mitochondria with either chymotrypsin or trypsin reduced holocytochrome *c* formation, although only at relatively high concentrations. In all cases, however, the concentrations of protease required to affect cytochrome *c* import were drastically higher than those required to abolish $F_1\beta$ or cytochrome c_1 import (*cf.* Fig. 7, *A* and *C*).

Reduction of holocytochrome *c* formation in mitochondria which had been treated with high concentrations of proteases could be accounted for by the diminished ability of these mitochondria to bind apocytochrome *c* (Fig. 7*D*). Following treatment of mitochondria with various proteases (at 100 µg/ml, the highest concentration used), holocytochrome *c* formation decreased in the order: control/proteinase K/chymotrypsin/trypsin (Fig. 7*A*). The capacity of treated mitochondria to bind apocytochrome *c* decreased in the identical order (Fig. 7*D*).

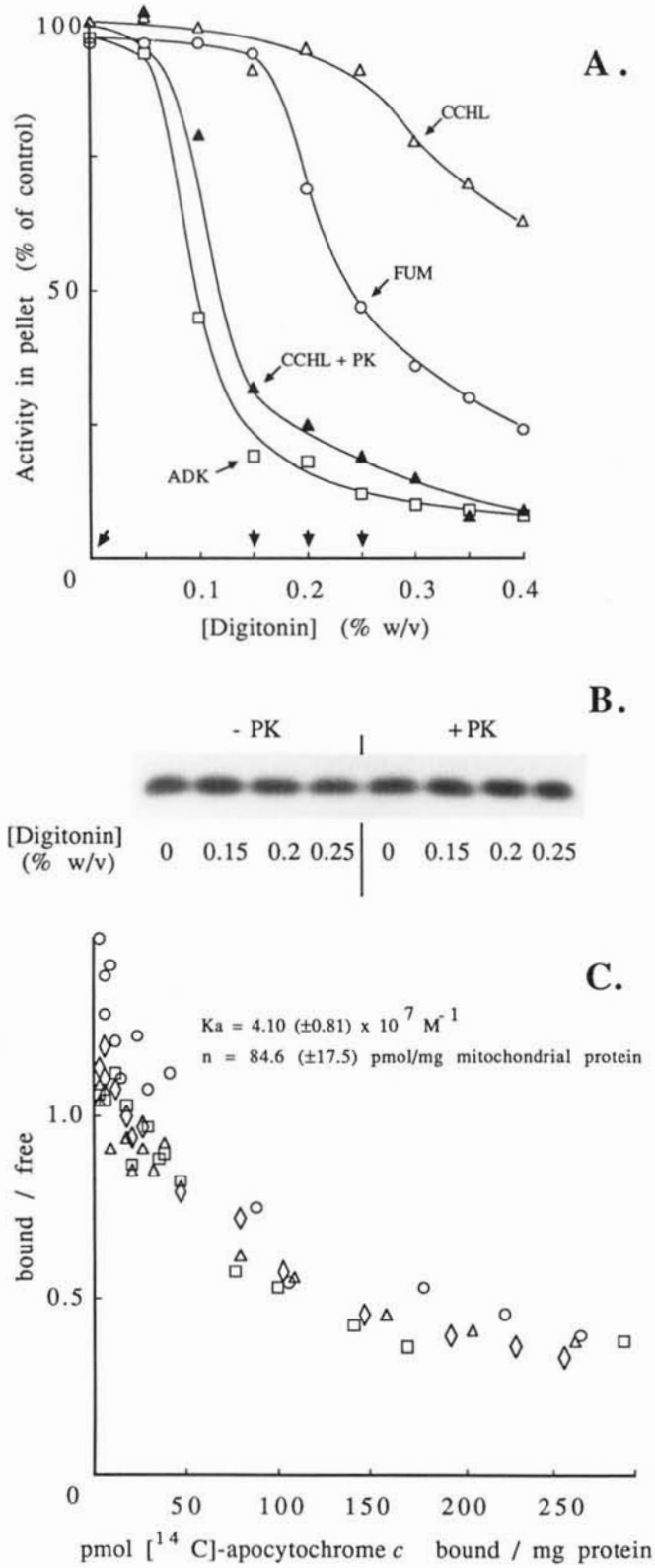

FIG. 8. **Cytochrome *c* heme lyase is a membrane-bound protein facing the intermembrane space and can be inactivated by proteinase K treatment without affecting apocytochrome *c* binding to mitochondria.** Freshly isolated mitochondria were suspended at a protein concentration of 8 mg/ml in SEM buffer and treated with varying concentrations of digitonin to successively open the intermembrane space and matrix as described under "Experimental Procedures." The samples were then diluted to a protein concentration of 2 mg/ml with SEM buffer and divided. One aliquot received proteinase K (*+PK*) at a final concentration of 50 μg/ml, while the controls (*−PK*) received SEM buffer. The concentration of proteinase K used was chosen since it did not perturb the outer membrane (see Fig. 7*B*) 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 enzyme 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 [^{35}S]cysteine-labeled reticulocyte lysate. Pellet fractions (from 75 μg of protein of treated mitochondria) were incubated with 25 μl of [^{35}S]cysteine-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 reisolated by centrifugation for 15 min at 48,400 × *g*. The pellets were lysed 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 ^{14}C-apocytochrome *c*. Following both digitonin and proteinase K treatment, the binding of ^{14}C-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 (K_a) 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.

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. 7*B*). 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 ocytyl 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 cytochrome *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 inactivated mitochondria.

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 ^{14}C-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 group to the cytochrome *c* precursor, it is remarkable that it comes into play at such an early stage of the import pathway. On the other hand, since linkage of the heme group is a prerequisite for translocation of cytochrome *c* across the outer mitochondrial membranes, the participation of CCHL during import processes (in addition to its catalytic role) seems reasonable.

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 mitochondria (43). The data we present here, however, would favor the location of CCHL to be in the vicinity of the outer membrane where it could participate in apocytochrome *c* binding without prior translocation of the entire precursor across the outer membrane. If the CCHL enzyme were in fact anchored to the inner membrane, it could only form a complex with apocytochrome *c* without complete translocation if the enzyme were situated in or near regions of close contact between the two membranes. Preliminary results indicate that this may indeed be the case.[3]

Apocytochrome *c* that is specifically bound to mitochondria exists in a membrane-spanning configuration. It is positioned in the outer membrane in such a way that part of it remains exposed to the outer surface, since it can be digested with externally added proteases, while at least some part of the apocytochrome *c* molecule penetrates through the outer membrane such that it can interact with the CCHL enzyme, which is topologically exposed to the intermembrane space. This transmembrane arrangement of the bound apocytochrome *c* may not involve the hydrophobic phase of the membrane lipids since apocytochrome *c* can be readily extracted with aqueous perturbants. Binding of apocytochrome *c* to mitochondria in such an import-competent configuration appears to be the rate-limiting step of cytochrome *c* import, although it occurs much faster than binding events for other imported mitochondrial proteins (37, 38).

How does mitochondria-bound apocytochrome *c* interact with CCHL? Can cytochrome *c* heme lyase alone account for both major stages of cytochrome *c* import (namely, specific binding of apocytochrome *c* and conversion to holocytochrome *c*), or are there two distinct components involved? Our experiments suggest the following possibilities. The binding of apocytochrome *c* is mediated by a protein moiety which is embedded in the mitochondrial membrane system and is not accessible to proteases from either side of the outer membrane. Apocytochrome *c* binds to this component in such a way that part of the apocytochrome *c* molecule remains exposed to the outer surface of the outer membrane. At the same time, some part of it forms a stable complex with CCHL that cannot be dissociated with even high concentrations of salt. Since CCHL is membrane bound, it is possible that a membrane-embedded domain of CCHL is responsible for the binding of apocytochrome *c* while an intermembrane-space exposed domain catalyzes holocytochrome *c* formation. This possibility is consistent with our recent observation that mitochondria of a mutant of *N. crassa* (*cyt-2-1*), which are deficient in CCHL activity, cannot bind apocytochrome *c* (40). On the other hand, this does not exclude the possibility that another protein is involved in the binding process which must precede the heme lyase step even in detergent-solubilized samples. The binding of apocytochrome *c* to this component may be sensitive to salt while the subsequent transfer of apocytochrome *c* from the binding component to CCHL may be resistant to salt. Studies of yeast mutants suggest that in addition to CCHL at least one more component, the *CYC2* gene product, may be involved in cytochrome *c* import (41). The mutant has not yet been characterized, but it does not encode either of the structural genes for apocytochrome *c* (*CYC1* and *CYC7* for iso-1 and iso-2, respectively) or the heme lyase activity (*CYC3*) (42). On the other hand, it is not known whether the *CYC2* gene product is even a mitochondrial constituent or whether it serves some other role in cytochrome *c* biogenesis.

In contrast to other imported mitochondrial proteins, the pathway for cytochrome *c* import is comparatively simple and

[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_2, 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 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 sequestered 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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