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Import of cytochrome *c* into mitochondria

Cytochrome *c* heme lyase

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The import of cytochrome *c* into mitochondria can be resolved into a number of discrete steps. Here we report on the covalent attachment of heme to apocytochrome *c* by the enzyme cytochrome *c* heme lyase in mitochondria from *Neurospora crassa*.

A new method was developed to measure directly the linkage of heme to apocytochrome *c*. This method is independent of conformational changes in the protein accompanying heme attachment. Tryptic peptides of [^{35}S]cysteine-labelled apocytochrome *c*, and of enzymatically formed holocytochrome *c*, were resolved by reverse-phase HPLC. The cysteine-containing peptide to which heme was attached eluted later than the corresponding peptide from apocytochrome *c* and could be quantified by counting ^{35}S radioactivity as a measure of holocytochrome *c* formation. Using this procedure, the covalent attachment of heme to apocytochrome *c*, which is dependent on the enzyme cytochrome *c* heme lyase, could be measured. Activity required heme (as hemin) and could be reversibly inhibited by the analogue deuterohemin. Holocytochrome *c* formation was stimulated 5–10-fold by NADH > NADPH > glutathione and was independent of a potential across the inner mitochondrial membrane. NADH was not required for the binding of apocytochrome *c* to mitochondria and was not involved in the reduction of the cysteine thiols prior to heme attachment. Holocytochrome *c* formation was also dependent on a cytosolic factor that was necessary for the heme attaching step of cytochrome *c* import. The factor was a heat-stable, protease-insensitive, low-molecular-mass component of unknown function.

Cytochrome *c* heme lyase appeared to be a soluble protein located in the mitochondrial intermembrane space and was distinct from the previously identified apocytochrome *c* binding protein having a similar location. A model is presented in which the covalent attachment of heme by cytochrome *c* heme lyase also plays an essential role in the import pathway of cytochrome *c*.

Cytochrome *c* is a component of the respiratory chain in the mitochondrial intermembrane space where it is reversibly associated with cytochrome *c* reductase and cytochrome *c* oxidase at the outer face of the inner membrane [1–4]. It is imported into mitochondria by a pathway which is distinct from that of other nuclear-coded mitochondrial proteins (for review see [5–10]). Apocytochrome *c*, the precursor of holocytochrome *c*, differs from its mature counterpart in that it does not contain covalently bound heme and has a different conformational arrangement. Apocytochrome *c* is a nuclear gene product which is translated on free cytoplasmic ribosomes (for review see [11]) and is released, without a pre-piece [12–17], as a soluble protein into a cytoplasmic pool [18]. It is then post-translationally transferred into mitochondria [12, 16, 18, 19] via specific receptors [20–22] and converted to holocytochrome *c* [12, 16, 18, 20] by covalent attachment of heme by the enzyme cytochrome *c* heme lyase [20, 23–26]. Unlike most other imported mitochondrial proteins, but similar to those which must pass only the outer membrane [27–29], cytochrome *c* import and maturation is independent of a membrane potential [21].

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Abbreviations. Buffer A: 250 mM sucrose, 10 mM Mops/KOH pH 7.2, 2 mM EDTA, 1 mM PMSF; hemin, Fe protoporphyrin IX Cl; PMSF, phenylmethylsulfonyl fluoride; NEM, *N*-ethylmaleimide.

The covalently attached heme of cytochrome *c*, which plays a crucial role in its function as an electron carrier, also appears to be important for events in the import pathway of cytochrome *c*. When heme attachment is inhibited by the analogue deuterohemin, the import of apocytochrome *c* is blocked at the stage of high-affinity receptor binding [20]. Apocytochrome *c* is tightly associated with mitochondria at this point but is in equilibrium with the soluble form since it can be displaced from its binding sites by incubation of the mitochondria with excess apocytochrome *c*. Upon reversal of deuterohemin inhibition with protohemin, the bound apocytochrome *c* is converted to holocytochrome *c* and the protein is translocated completely across the outer mitochondrial membrane. This translocation appears to be intrinsically coupled to the covalent attachment of heme. A likely mechanism is that formation of the thioether linkages between the heme vinyl groups and the thiols of cysteines 18 and 21 of apocytochrome *c* (from *Neurospora crassa*, cysteines 14 and 17 by universal numbering) initiates conformational changes along the polypeptide chain which result in driving the translocation of the protein across the outer membrane. This model is particularly attractive since there is no known energy requirement, such as a membrane potential or ATP, for the import of cytochrome *c*. Because of the obvious importance of the covalent attachment of the heme group to apocytochrome *c* in the import pathway itself, the enzyme which catalyses this event, namely cytochrome *c* heme lyase (following recommendations of the IUPAC/IUB Commission

on Enzyme Nomenclature), plays a central role in the assembly of cytochrome *c* into mitochondria.

We report here on the activity of cytochrome *c* heme lyase from the mitochondria of *N. crassa*. The similarities and differences of the enzyme compared to that reported in yeast are discussed. A model is presented which pools our knowledge to date on the import and maturation of cytochrome *c* in mitochondria.

MATERIALS AND METHODS

Cell growth

Neurospora crassa, wild-type 74A (Fungal Genetic Stock Center no. 262), was grown for 14 h at 25°C with vigorous aeration in Vogel's minimal medium supplemented with 2% (w/v) sucrose [30]. The cultures were inoculated with 10^9 conidia/l and grown under bright illumination. Where indicated, cells were radioactively labelled by growth under the same conditions except that sulfate was reduced to 0.08 mM and 3 mCi $^{35}SO_4$ (800 – 1300 Ci/mmol, Amersham, Buckinghamshire, England) were added per liter of culture.

Isolation of mitochondria

Unless otherwise indicated, all procedures were performed on ice or at 4°C. Hyphae were harvested by filtration and immediately homogenized by grinding with quartz sand in Percoll medium [30% (v/v) Percoll (Pharmacia, Uppsala, Sweden), 180 mM KCl, 3% (w/v) bovine serum albumin, 1 mM EDTA, 1 mM PMSF, 10 mM Mops/KOH, pH 7.2] at a ratio of 3 ml Percoll medium and 1.5 g quartz sand per g (wet weight) of hyphae. Sand and cellular debris were removed by centrifugation for 5 min at $1900 \times g$ (Beckman JA-20 rotor), then again for 5 min at $3000 \times g$. A gradient of Percoll was established in the resulting supernatant by centrifugation at $165000 \times g$ (Beckman Ti70 rotor) for 15 min. The mitochondrial band was removed with a pasteur pipette and diluted with 20 vol. buffer A (250 mM sucrose, 10 mM Mops/KOH, pH 7.2, 2 mM EDTA, 1 mM PMSF added immediately before use from a 200 mM stock in ethanol). Mitochondria were sedimented by centrifugation for 12 min at $17400 \times g$ (Beckman JA-20 rotor) and washed by resuspending them in the same volume of fresh buffer A. Mitochondria were again sedimented at $17400 \times g$ for 12 min and resuspended at a protein concentration of 5 mg/ml in buffer A or at 8 mg/ml for digitonin fractionation.

Subfractionation of mitochondria with digitonin

Digitonin, recrystallized from ethanol, was prepared as a 1% (w/v) stock solution in buffer A and was used within 15 min. Dilutions were prepared with buffer A such that the concentrations were twofold the required final concentrations. Equal volumes (0.2 ml each) of the digitonin solution and mitochondrial suspension (at 8 mg protein/ml) were combined for 60 s at 0°C, then diluted with 0.5 ml buffer A. The mixture was layered on a 0.3-ml cushion of 0.7 M sucrose, 30 mM Tris/HCl, pH 7.5, and spun for 10 min at $48400 \times g$ (Beckman JA-20 rotor with plastic adapters for 1.5-ml Eppendorf tubes). Both layers of the supernatant were carefully removed and the pellet was resuspended in 0.9 ml buffer A plus 0.3 ml of 0.7 M sucrose, 30 mM Tris/HCl pH 7.5 to mimic buffer conditions of the supernatants. The enzymatic activity of adenylate kinase [31] and fumarase [32] were determined as previously described. The fractionation of porin, cytochrome *c*, 20-kDa protein, ADP/ATP carrier and isocitrate dehydrogenase (α and β subunits) were determined by resolving the samples on SDS/polyacrylamide gels [33] and Western blotting to nitrocellulose membranes [34, 35]. The membranes were incubated with a mixture of specific antibodies (against the above-mentioned proteins) and decorated with ^{14}C-labelled protein A (prepared by reductive methylation in the presence of [^{14}C]formaldehyde as described previously for apocytochrome *c* [22]). The proteins were visualized by autoradiography and were quantified by cutting out the bands and determining radioactivity.

The number of high-affinity binding sites for apocytochrome *c* was determined by Scatchard analysis essentially as previously described [22]. Mitochondria, or pellets from digitonin fractions, were suspended at a protein concentration of 1 mg/ml (starting material) in buffer A containing 50 µM deuterohemin, and were incubated for 5 min at 25°C. The samples were spun at $48400 \times g$ for 10 min (Beckman JA-20 rotor) and the resulting pellets were resuspended at 0.3 mg protein/ml in 0.25 M sucrose, 50 mM NaCl, 2 mM EDTA, 14 mM 2-mercaptoethanol, 1% (w/v) bovine serum albumin, 0.02% (w/v) holocytochrome *c*, 10 mM Mops/KOH, pH 7.2, in silicone-coated glass minivials. The samples were incubated with increasing amounts of ^{14}C-labelled apocytochrome *c*, prepared by reductive methylation [22], for 20 min at 25°C. Pellets were re-isolated by centrifugation as above, and the unbound apocytochrome *c* was measured in the supernatant by radioactivity determination. The pellets were lysed in 0.5 ml of 1% (w/v) SDS, 100 mM Tris/HCl pH 8.0 and the bound apocytochrome *c* was also quantified by radioactivity determination.

Cytosol fraction

A post-ribosomal supernatant was prepared in the following manner. Hyphae were harvested by filtration and homogenized by grinding in quartz sand and buffer A at a ratio of 1 ml buffer A and 1.5 g quartz sand per g hyphae (wet weight). In preparations that were tested for protease sensitivity, PMSF was not included in buffer A but was added after protease treatment. A post-mitochondrial supernatant was prepared to remove sand, cellular debris and most cellular organelles, by centrifugation at $27200 \times g$ for 15 min (Beckman JA-20 rotor). The resulting supernatant was then spun for 60 min at $226000 \times g$ (Beckman Ti 50 rotor). The upper two-thirds of the supernatant of this preparation was carefully removed and taken as the cytosol fraction.

Measurement of cytochrome c *heme lyase activity*

Principle of the method. Apocytochrome *c* from *N. crassa* contains two cysteines at positions 18 and 21 (positions 14 and 17 according to standardized nomenclature) which form thioether bridges with the vinyl groups at positions 3 and 8 (IUPAC numbering) of protoheme, respectively, to produce holocytochrome *c*. Although it is difficult to resolve apo- and holocytochrome *c* by conventional techniques, their respective tryptic peptide patterns can be clearly distinguished by reverse-phase HPLC. The only peptide which is affected is the cysteine-containing peptide which has a far greater retention time when heme is covalently attached to it. By incorporating [^{35}S]cysteine into apocytochrome *c*, the rate of formation of a radiolabelled peptide with a longer retention time (owing to the heme group) can be used as a measure of cytochrome *c*

heme lyase activity. To do this, samples were incubated with the post-ribosomal supernatant of reticulocyte lysates which had been programmed by *N. crassa* poly(A)-containing RNA and were translated in the presence of [^{35}S]cysteine. Cytochrome *c* was purified from these incubations by immunoprecipitation and digested with trypsin. The resulting peptide mixture was resolved by reverse-phase HPLC and the heme/cysteine-containing peptide was collected and its radioactivity measured.

Incubation mixtures. Samples, containing up to 100 μg protein, were pre-incubated for 5 min at 25 °C in the presence of 6.7 mM NADH plus any additions, in a total volume of 150 μl buffer A. The reaction was started by adding 50 μl of post-ribosomal supernatant of reticulocyte lysate that had been translated in the presence of [^{35}S]cysteine (therefore 200 μl final incubation volume, 5 mM NADH). Following a further incubation for 10 min at 25 °C, the reaction was halted by cooling the samples to 0 °C. In experiments using whole mitochondria, the samples were diluted with 1 ml buffer A and mitochondria were re-isolated by centrifugation for 12 min at 17400 × *g*. The pellets were prepared for immunoprecipitation by resuspending them in 1 ml of 1% (w/v) Triton X-100, 0.3 M NaCl, 10 mM Tris/HCl pH 7.2. Other samples were directly dissociated in 1 ml of this buffer.

Immunoprecipitation of cytochrome c. Samples were shaken for 15 min at 4 °C and were then clarified by centrifugation for 15 min at 27200 × *g* (Beckman JA-20 rotor). During this time, immunoglobulins from antisera raised against *Neurospora* holocytochrome *c* were pre-bound to protein-A–Sepharose by mixing for 15 min at 4 °C. Depending on the titre, usually 50 μl serum was mixed with 7.5 mg protein-A–Sepharose (Bioprocessing, London, England). The supernatants from the clarified samples were mixed with the antibody/protein-A–Sepharose complexes and shaken for 60 min at 4 °C. Sepharose beads were collected by centrifugation at 8900 × *g* for 1 min in an Eppendorf centrifuge. The pellets were washed (by shaking for 1 min followed by centrifugation at 8900 × *g*) twice with 1 ml of 1% (w/v) Triton X-100, 0.3 M NaCl, 10 mM Tris/HCl pH 7.2, once with 1 ml of 1 mM $K_3[Fe(CN)_6]$ in 10 mM Tris/HCl pH 7.0 (to oxidize cytochrome *c* for uniform tryptic digestion [36]), and twice more with 1 ml 10 mM Tris/HCl pH 7.0. The cytochrome *c* was eluted from the immunocomplexes by shaking the beads for 15 min in 60 μl of 8 M urea at room temperature followed by 30 min at 56 °C and additional shaking for 15 min at room temperature. The protein-A–Sepharose was sedimented by centrifugation at 8900 × *g* for 3 min and the supernatant was retained. The efficiency of elution by this method was 60–70%.

Digestion with trypsin. The eluted immunoprecipitates (55 μl) were incubated in a total volume of 300 μl containing 60 mM NH_4HCO_3, 25 μg of purified *Neurospora* holocytochrome *c* (for a marker) and 50 μg trypsin (type XIII from bovine pancreas, treated with tosylphenylalanylchloromethane, 10000 U/mg, Sigma, St. Louis MO) for 90 min at 37 °C. Under these conditions, the diluted urea (approximately 1.5 M) did not affect trypsin activity. Following incubation, the samples were cooled to room temperature and made 0.1% (v/v) in trifluoroacetic acid (Sigma) by adding 6 μl of a 5% (v/v) stock.

HPLC analysis. The tryptic peptide mixtures were resolved by reverse-phase HPLC on a Waters Associates (Milford, MA) μBondapak phenyl column (3.9 mm × 30 cm, 10 μm particle size, see [37]). For analytical runs, the column was equilibrated at a flow rate of 0.8 ml/min in solvent A (0.1%, v/v, CF_3CO_2H). Following sample injection, the peptides were eluted with a linear gradient of 0–50% solvent B (0.1% CF_3CO_2H, 9.9% H_2O, 90% CH_3CN; v/v/v) as described in Fig. 1.

Using this protocol, the cysteine-containing tryptic peptide from apocytochrome *c* eluted at 37.5 min following injection while the cysteine-containing peptide from holocytochrome *c* eluted at 58.2 min. The total run time, including column re-equilibration, was 90 min. During routine analysis, an abbreviated program was used in which the column was equilibrated in 80% solvent A/20% solvent B and eluted by a linear gradient of 20–50% solvent B. Following this protocol, the apo-cysteine peptide eluted shortly following the column void volume whereas the holo-cysteine peptide was retained for 29.0 min. Total run time, including re-equilibration, was 45 min. All HPLC runs were performed at a constant flow rate of 0.8 ml/min at room temperature. For determination of cytochrome *c* heme lyase activity, the holocytochrome *c* cysteine-containing tryptic peptide was collected (by monitoring the marker holocytochrome *c* peptides at 415 nm) and its radioactivity measured.

Miscellaneous methods

Apo- and holocytochrome *c* were purified from *N. crassa* and specific antibodies were raised in rabbits as previously described [30]. Preparation of rabbit reticulocyte lysates and cell-free protein synthesis directed by *N. crassa* poly(A)-containing RNA [38] was performed essentially as described before [39] except: (a) L-[^{35}S]cysteine (specific activity 1100–1400 Ci/mmol, Amersham) was used in an amino acid mixture which lacked unlabelled cysteine; (b) following translation, a post-ribosomal supernatant was prepared by centrifugation for 60 min at 226000 × *g* (Beckman Ti 50 rotor) which was then made iso-osmotic (for mitochondria) by adding sucrose to a final concentration of 0.25 M; (c) during translation, the mixture contained either 13 μM hemin (10 μM in the sucrose-diluted post-ribosomal preparation) or 5 mM cAMP [40].

Radioactivity determinations were performed by scintillation counting in either 1 ml of 1% (w/v) SDS, 100 mM Tris/HCl, pH 8.0 plus 10 ml ACS II (Amersham) for samples from the HPLC, or in 0.5 ml of 1% (w/v) SDS, 100 mM Tris/HCl pH 8, plus 12 ml of a cocktail containing 60% (v/v) toluene, 40% (v/v) methylglycol, 6 g/l Permablend III (Packard, Zürich, Switzerland) for all other samples. Protein was determined by the method of Bradford [41]. SDS/polyacrylamide gel electrophoresis was performed using standard techniques [33]. Immunoprecipitates were dissociated by incubation at 95 °C for 5 min in SDS-sample buffer [2% (w/v) SDS, 0.35 M 2-mercaptoethanol, 50 mM Tris/HCl pH 6.8, 10% (v/v) glycerol, 0.005% (w/v) bromophenol blue]. Gels were prepared for fluorography by soaking them for 30 min in Amplify (Amersham) prior to drying. Bands in the original gel were quantified by scanning for radioactivity on a thin-layer chromatograph linear analyzer (Berthold, Wildbad, FRG).

RESULTS

Measurement of cytochrome c *heme lyase activity*

A method for directly measuring the covalent attachment of heme to apocytochrome *c* was developed which was independent of related changes in the protein molecule (i.e. conformational). Apo- and holocytochrome *c* could be separated by reverse-phase HPLC regardless of conformation

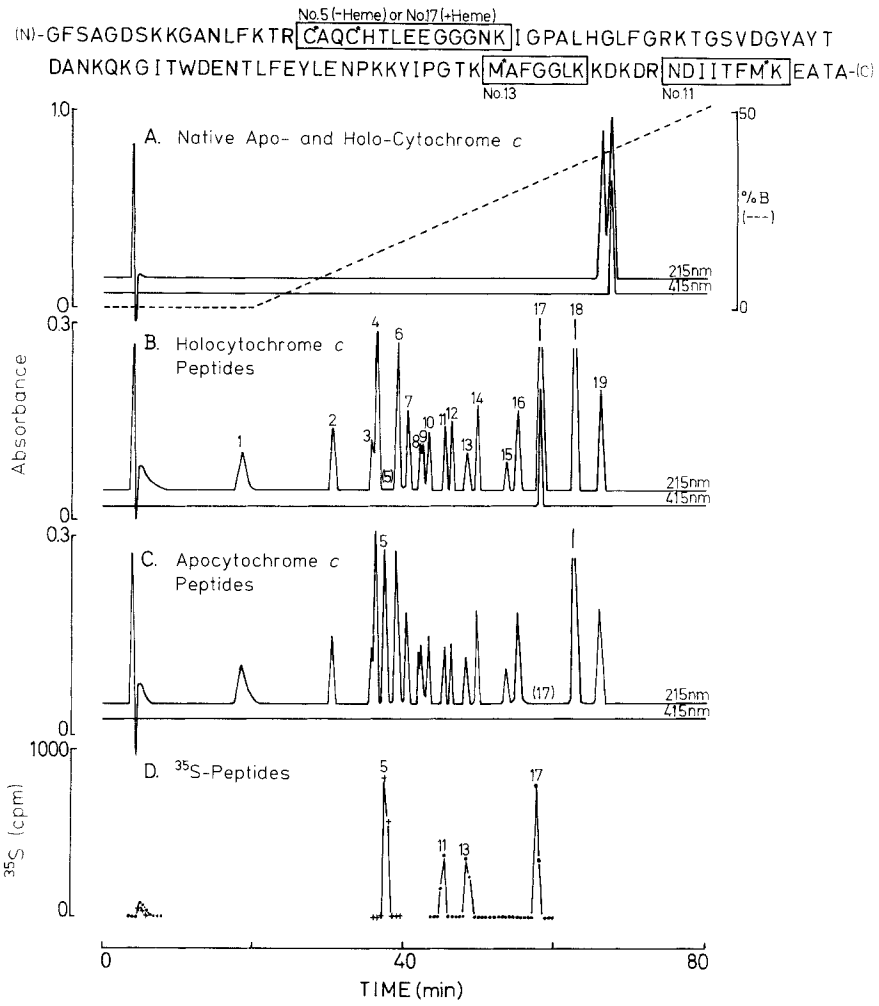

Fig. 1. *Resolution of tryptic peptides of cytochrome* c *by reverse-phase HPLC.* Samples were applied to a Waters μBondapak phenyl column equilibrated in 0.1% (v/v) CF_3CO_2H and eluted with a linear gradient of solvent B (0.1% CF_3CO_2H, 9.9% H_2O, 90% CH_3CN, v/v/v) as indicated in A. Chromatographs were monitored at 215 nm and 415 nm (A–C) or fractions were collected at 0.5-min intervals (D) and counted for radioactivity. (A) Mixture containing 100 μg each of apocytochrome *c* and holocytochrome *c*. (B) Holocytochrome *c* tryptic peptides: 200 μg holocytochrome *c* were digested in a total volume of 50 μl 60 mM NH_4HCO_3 with 10 μg trypsin for 15 h at 37 °C. (C) Apocytochrome *c* tryptic peptides: as B except with 200 μg apocytochrome *c*. (D) Identification of cysteine-containing peptides (fractions not presented contained no detectable radioactivity): (+) apocytochrome *c* was immunoprecipitated from 25 μl of [^{35}S]cysteine-labelled reticulocyte lysate, immunoprecipitates were eluted with urea and digested with trypsin; (●) holocytochrome *c* was immunoprecipitated from 100 μg mitochondrial protein from cells that were grown in the presence of $^{35}SO_4$, immunoprecipitates were eluted then digested with trypsin. The amino acid sequence of *N. crassa* cytochrome *c* is presented above A (see [42, 43])

(i.e. under native or denaturing conditions) but not with adequate resolution for use in routine analysis (Fig. 1A). Although the effect of the heme group on the retention time of the whole cytochrome *c* molecule was only slight, the effect on a smaller peptide fragment was expected to be considerably greater. The peptides generated by the digestion of cytochrome *c* with trypsin results in a mixture containing one peptide (amino acids 18–31) in which the heme attachment sites (cysteines 18 and 21) are located. This fragment has the advantageous feature of containing the only two cysteines in the *Neurospora crassa* cytochrome *c* molecule. Using [^{35}S]cysteine-labelled cytochrome *c*, the migration of the cysteine-containing peptide could be specifically determined as a means for measuring holocytochrome *c* formation.

Two approaches were used to identify the cysteine-containing tryptic peptide from apo- and holocytochrome *c*. First, the purified proteins were digested with trypsin and the resulting peptides were resolved by reverse-phase HPLC. In the case for both apo- and holocytochrome *c*, 18 fragments could be distinguished (theoretically, there are 17 tryptic cleavages sites in *Neurospora* cytochrome *c*). Holocytochrome *c* (Fig. 1B) contained a single peptide absorbing at 415 nm (owing to the heme group) which had a retention time of 58.2 min (peptide 17). This peptide was not present in the tryptic digests of apocytochrome *c*. Likewise, apocytochrome *c* (Fig. 1C) contained a unique peptide (no. 5) which had a retention time of 37.5 min and was not present in tryptic digests of holocytochrome *c*. To confirm that peptide 5 was the cysteine-containing peptide of apocytochrome *c* and that peptide 17 was the corresponding peptide from holocytochrome *c* but with covalently attached heme, a second approach was used (Fig. 1D). Apocytochrome *c* was immunoprecipitated from the post-ribosomal supernatant of reticulocyte lysates [programmed by *N. crassa* poly(A)-containing RNA] which had been translated in the presence of [^{35}S]cysteine. The immunoprecipitates were eluted and digested with trypsin, then the resulting peptides were resolved by reverse-phase HPLC. A single radioactive peak cor-

responding to the position of peptide 5 was observed. Holocytochrome *c* was immunoprecipitated from isolated mitochondria prepared from *Neurospora* cultures that were metabolically labelled by growth in the presence of $^{35}SO_4$, and processed in the same way. In this case, a major radioactive peak corresponding to peptide 17 was observed. In addition, two smaller peaks containing [^{35}S]methionine were also resolved (peptide 11 containing methionine 102, and peptide 13 containing methionine 84). This confirmed that the cysteine-containing tryptic peptide of cytochrome *c* corresponded to peptide 5 without covalently attached heme (i.e. from apocytochrome *c*) and to peptide 17 with covalently attached heme (i.e. from holocytochrome *c*) and formed the basis of an HPLC-based assay system for the measurement of holocytochrome *c* formation by the activity of cytochrome *c* heme lyase.

Following the incubation of [^{35}S]cysteine-containing reticulocyte lysates with *Neurospora* mitochondria, appearance of radioactivity co-migrating with peptide 17 occurred with a concomitant decrease in the radioactivity in peak 5. Under optimal conditions, up to 30% of the cysteine radioactivity in peak 5 (from apocytochrome *c*) could be converted to peak 17 (holocytochrome *c*). The assay was linear for 12 min at 25 °C (the optimal temperature) and up to 100 μg mitochondrial protein. Activity was optimal at pH 7.0 and exhibited a broad pH range with 50% activity remaining at pH 5.5 or pH 8.5 (data not presented). The error between replicate samples was less that 5%. Using this system, the activity of cytochrome *c* heme lyase could be measured directly and did not rely on secondary changes such as those required for distinguishing between apo- and holocytochrome *c* with specific antibodies or by differential protease sensitivity.

Cytochrome c *heme lyase is dependent on heme and can be reversibly inhibited by the analogue deuterohemin*

The preparation of reticulocyte lysates normally used in the measurement of cytochrome *c* heme lyase activity contains hemin, which is added to inhibit the phosphorylation of initiation factor 2 during translation. To examine the requirement for heme (as hemin) by cytochrome *c* heme lyase, cAMP was substituted to perform this function [40]. Cytochrome *c* heme lyase activity could then be measured using hemin-free reticulocyte lysates. Holocytochrome *c* formation was dependent on exogeneously added hemin (Fig. 2A) and was optimal at 2 μM hemin (equivalent to approximately 5 nmol hemin/mg mitochondrial protein). Activity was progressively inhibited at higher concentrations, explaining the observation that hemin added to incubation mixtures using normal hemin-containing reticulocyte lysates inhibits activity. When the heme analogue deuterohemin (which does not contain the vinyl groups at positions 3 and 8 of the porphyrin ring) was added to incubation mixtures using hemin-containing reticulocyte lysates (2.5 μM final concentration of hemin contributed by the reticulocyte lysate), cytochrome *c* heme lyase activity was inhibited (Fig. 2B). Deuterohemin only marginally affected the binding of apocytochrome *c* to mitochondria (i.e. 13.4% reduced at 50 μM deuterohemin, data not presented). Activity was inhibited by 50% at approximately 2.5 μM deuterohemin (the same concentration as hemin from the reticulocyte lysate) suggesting that the enzyme has equivalent affinity for deuterohemin and for hemin. Activity was reduced by 93% at 25 μM deuterohemin and nearly completely abolished at 50 μM. This inhibition by deuterohemin could be reversed with hemin, but only under

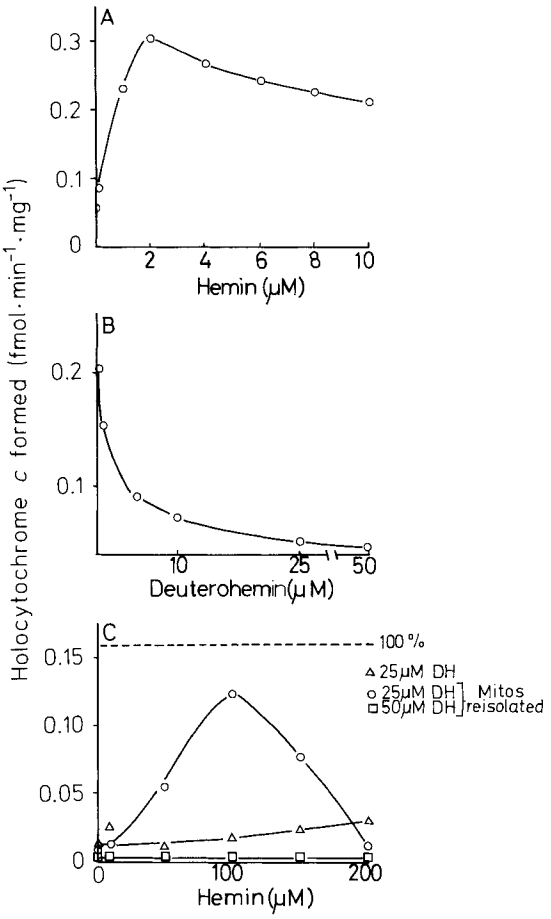

Fig. 2. *Dependence of cytochrome* c *heme lyase on hemin, and reversible inhibition of activity by deuterohemin.* (A) Activity was determined (as described in Methods) in samples containing 75 μg mitochondrial protein using [^{35}S]cysteine-labelled reticulocyte lysates that had been translated in the presence of cAMP instead of hemin. Hemin was added as indicated. (B) Activity was measured in mitochondria (75 μg protein) using normal reticulocyte lysates (containing hemin) with increasing concentrations of deuterohemin. The final concentration of hemin in the assay mixture (contributed by the reticulocyte lysate) was 2.5 μM. (C) Mitochondria (75 μg) were pre-incubated with either 25 μM (○) or 50 μM (□) deuterohemin (DH) for 5 min at 25 °C in a mixture containing 6.7 mM NADH in a total volume of 150 μl buffer A. After adding 50 μl [^{35}S]cysteine-labelled reticulocyte lysate, the samples were incubated for 10 min more at 25 °C, then mitochondria were re-isolated by diluting the samples in 1 ml buffer A and sedimenting them for 12 min at 17400 × *g*. The mitochondria were suspended in 200 μl buffer A containing 5 mM NADH, 50 μl unlabelled reticulocyte lysate and varying concentrations of hemin. Following further incubation for 10 min at 25 °C, holocytochrome *c* formation was determined. In a parallel experiment, samples were incubated with 25 μM deuterohemin as above but mitochondria were not re-isolated before hemin addition (△). Hemin was added at the concentrations indicated and the samples were incubated 10 min more at 25 °C. Holocytochrome *c* formation was then determined. Control mitochondria were not treated with deuterohemin or hemin but otherwise were processed the same (– – – –)

limited conditions (Fig. 2C). In samples that were inhibited with 25 μM deuterohemin, 78% of the activity was recovered with 100 μM hemin; however, inhibition could not be reversed when activity was inhibited with 50 μM deuterohemin. Furthermore, mitochondria had to be re-isolated following incubation with deuterohemin otherwise only a small proportion of the original activity could be regained. In summary,

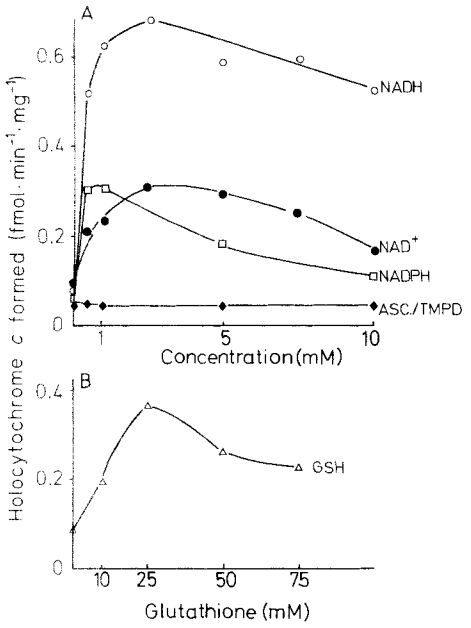

Fig. 3. Effect of reducing agents on cytochrome c *heme lyase activity.* Mitochondria (75 μg protein) were incubated in a standard mixture containing various concentrations of: (○) NADH; (●) NAD^+; (□) NADPH; (◆) ascorbate plus 0.25 mM tetramethylphenylenediamine (Asc./TMPD); (△) glutathione (GSH, reduced form)

under defined conditions the enzymatic activity of cytochrome *c* heme lyase, which was dependent on hemin, could be competitively and reversibly inhibited by the analogue deuterohemin.

Holocytochrome c *formation is dependent on NADH*

Mitochondria incubated with reticulocyte lysates in the absence of reducing agents converted very little apocytochrome *c* to holocytochrome *c*. In the presence of NADH, however, holocytochrome *c* formation was stimulated up to 10-fold (Fig. 3A). The optimal concentration varied between mitochondrial preparations but ranged between 1 mM and 10 mM NADH. Stimulation of activity was also observed with NADPH or NAD^+, up to 45% of the maximum activity observed with NADH. When NAD^+ was added to incubation mixtures in the presence of an NADH-regenerating system (either lactate dehydrogenase plus L-lactate, or glucose-6-phosphate dehydrogenase plus D-glucose 6-phosphate), the stimulation of holocytochrome *c* formation by NAD^+ was nearly identical to the stimulation by NADH alone. No increase in activity was observed when a membrane potential was generated by *N,N,N',N'*-tetramethyl-*p*-phenylenediamine plus ascorbate (Asc./TMPD). Similarly, the stimulation of activity by NADH was unchanged when the membrane potential was either dissipated by valinomycin (0.5 μM) plus K^+, or when its generation was blocked by the combination of antimycin A plus oligomycin (8 μM and 20 μM, respectively). Reduced glutathione also enhanced holocytochrome *c* formation but was required at significantly higher concentrations than observed for NADH (Fig. 3B). Preliminary experiments indicate that glutathione stimulates activity by reversing the reaction catalyzed by glutathione reductase to form NADH. NADH was not required for the binding of apocytochrome *c* to mitochondria and, in fact, it progressively reduced binding at concentrations greater than 5 mM (i.e. 17.5% reduction of apocytochrome *c* binding at 25 mM NADH, data not presented).

Covalent attachment of heme to apocytochrome c *is blocked by* N*-ethylmaleimide*

When mitochondria were pre-treated with varying concentrations of *N*-ethylmaleimide (NEM) then reisolated (so NEM would not react with apocytochrome *c* when added), holocytochrome *c* formation was unaffected (Fig. 4B). This indicated that none of the import machinery for cytochrome *c* has essential free sulphydryl groups. NEM pretreatment of apocytochrome *c* in reticulocyte lysates, however, resulted in nearly complete loss of conversion to holocytochrome *c* by mitochondria. This inhibitory affect was not due to reduction of the ability of NEM-treated apocytochrome *c* to bind to mitochondria (Fig. 4A) since NEM did not significantly affect binding (except at 20 mM where a 25% reduction in mitochondria-associated apocytochrome *c* was observed). When apocytochrome *c* was pre-bound to mitochondria and then treated with NEM, conversion to holocytochrome was inhibited. The concentrations of NEM required for inhibition were similar to those required for inhibition of holocytochrome *c* formation from NEM-treated apocytochrome *c* in the reticulocyte lysate using untreated mitochondria, except for a slight latency which may reflect a reduced accessibility of NEM to the pre-bound apocytochrome *c*. Since the pre-bound apocytochrome *c* was able to react with NEM (which blocks only free SH groups) in the absence of NADH, then the apocytochrome *c* cysteines are reduced throughout the import pathway and this suggests that NADH is not involved in their reduction.

A factor found in reticulocyte lysate or cell cytosol is necessary for holocytochrome c *formation*

When mitochondria were incubated with reticulocyte lysate in varying total volumes, the conversion of apo- to holocytochrome *c* was considerably reduced in dilute samples while the binding of apocytochrome *c* to mitochondria was only marginally affected (Fig. 5A). This effect was observed regardless of whether the absolute concentration of hemin was kept constant (Fig. 5A) or the ratio of hemin to mitochondrial protein was held constant (not shown). This implied that a factor, other than hemin, which was present in the reticulocyte lysate was necessary for holocytochrome *c* formation. To examine whether a similar factor was present in *Neurospora* cytosol, apocytochrome *c* was pre-bound to mitochondria in the absence of NADH, the mitochondria were re-isolated and washed, and the dependence of holocytochrome *c* formation on a cytosolic fraction was determined. In the presence of all other known requirements for cytochrome *c* heme lyase activity (apocytochrome *c* pre-bound to mitochondria, NADH and hemin), conversion of apocytochrome *c* to holocytochrome *c* was low in the absence of cytosol but was stimulated five-fold when the cytosolic preparation was included (Fig. 5B). When holocytochrome *c* formation was assayed this way in the presence of non-radioactive reticulocyte lysate instead of cytosol, a similar effect was also observed. The cytosolic fraction alone, in the absence of mitochondria, did not catalyse holocytochrome *c* formation. Stimulation by cytosol was also dependent on NADH (i.e. no increase in activity was observed in the absence of NADH) and the effect of the cytosolic fraction on activity could not be substituted for by an NADH-regenerating system. When

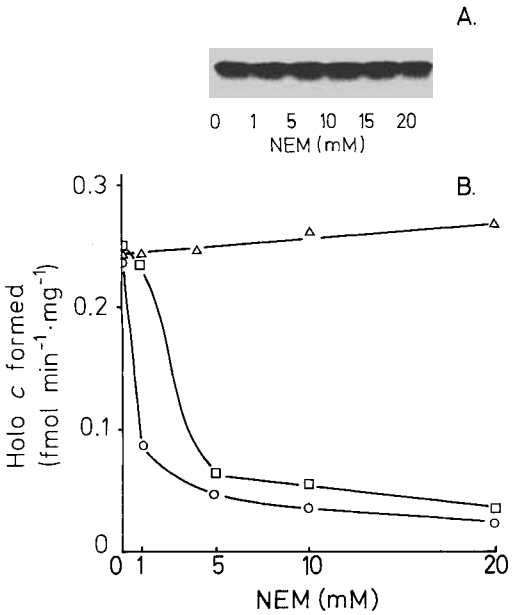

Fig. 4. *Effect of* N-*ethylmaleimide on the conversion of apocytochrome* c *to holocytochrome* c. (A) Effect of *N*-ethylmaleimide (NEM) on binding of apocytochrome *c* to mitochondria. [^{35}S]Cysteine-labelled reticulocyte lysates (50 µl) were treated with varying concentrations of NEM for 60 min at 0 °C in a total volume of 100 µl buffer A. Mitochondria (75 µg protein) were incubated with 50 µM deuterohemin for 5 min at 25 °C in a total volume of 100 µl, then combined with 100 µl of the reticulocyte lysate/NEM mixture and incubated for an additional 10 min at 25 °C. The samples were diluted in 1 ml buffer A and mitochondria were re-isolated by centrifugation for 12 min at 17400 × *g*. Cytochrome *c* was immunoprecipitated with a mixture of apo- and holocytochrome *c* antisera. The precipitates were dissociated in SDS-sample buffer and resolved on SDS/polyacrylamide gels. The gels were dried and visualized by fluorography. (B) Effect of NEM on holocytochrome *c* formation. Either 75 µg of mitochondrial protein (△) or 50 µl [^{35}S]cysteine-labelled reticulocyte lysate (○) were treated with NEM for 60 min at 0 °C in a total volume of 100 µl buffer A at the indicated concentrations. In the case of treatment of mitochondria with NEM, they were then re-isolated as above. Heme attachment was measured by adding 50 µl of untreated [^{35}S]cysteine-labelled reticulocyte lysate. In the case of treatment of the reticulocyte lysate with NEM, 75 µg mitochondrial protein was added. The total volumes in both cases were 200 µl in buffer A containing 5 mM NADH. The mixtures were incubated for 10 min at 25 °C, mitochondria were re-isolated and holocytochrome *c* formation was determined. In a third experiment (□), apocytochrome *c* from [^{35}S]cysteine-labelled reticulocyte lysate was pre-bound to mitochondria in the absence of NADH by incubating 75 µg mitochondrial protein with 50 µl of the lysate in a total volume of 200 µl buffer A for 10 min at 25 °C. Mitochondria were re-isolated as above and suspended in 100 µl buffer A containing varying concentrations of NEM. The samples were incubated for 60 min at 0 °C, then mitochondria were re-isolated. The mitochondria were incubated in a total volume of 200 µl buffer A containing 35 µl cytosol fraction, 5 mM NADH and 2 µM hemin for 10 min at 25 °C. Holocytochrome *c* formation was then determined. (△) Treatment of mitochondria with NEM; (○) treatment of reticulocyte lysate with NEM; (□) treatment of mitochondria containing bound apocytochrome *c* with NEM

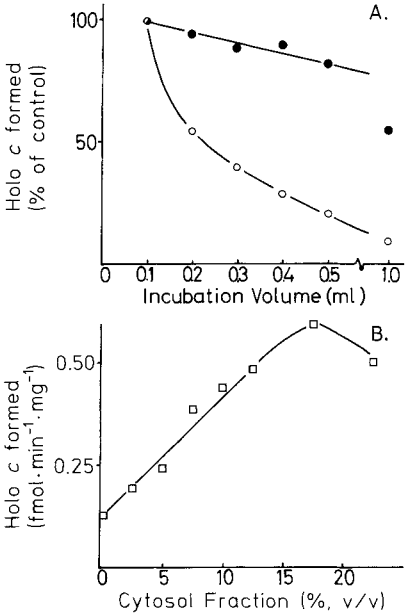

Fig. 5. *Dependence of cytochrome* c *heme lyase activity on a factor from reticulocyte lysate and cytosol.* (A) Dilution of reticulocyte lysate. In parallel samples, 75 µg mitochondrial protein was pre-incubated in varying volumes of buffer A (as indicated, minus 50 µl), 5 mM NADH and 5 µM hemin (final concentration after addition of reticulocyte lysate) for 5 min at 25 °C, then for an additional 10 min at 25 °C after adding 50 µl [^{35}S]cysteine-labelled reticulocyte lysate. The samples were cooled to 0 °C and diluted to a total volume of 1 ml with buffer A. Mitochondria were re-isolated by sedimenting them for 12 min at 17400 × *g*. Total cytochrome *c* associated with mitochondria (●) was measured by immunoprecipitating samples with a combination of antisera directed against apo- and holocytochrome *c*. Bands were quantified by scanning gels for radioactivity. Holocytochrome *c* formation was measured in the parallel samples (○). Values are expressed as percentage of the undiluted control (0.1 ml). (B) Titration of stimulation by cytosol. Apocytochrome *c* from [^{35}S]cysteine-labelled reticulocyte lysate was pre-bound to mitochondria in the absence of NADH (therefore no conversion to holocytochrome *c*) by incubating a mixture containing 750 µg mitochondrial protein and 500 µl [^{35}S]cysteine-labelled reticulocyte lysate in a total volume of 2 ml buffer A for 10 min at 25 °C. Mitochondria were re-isolated by centrifugation for 12 min at 17400 × *g* and resuspended in buffer A. Cytosolic stimulation of activity was determined by incubating a mixture containing 75 µg mitochondrial protein containing pre-bound apocytochrome *c*, 5 mM NADH, 5 µM hemin and varying concentrations of cytosol fraction in a total volume of 200 µl buffer A for 10 min at 25 °C. Holocytochrome *c* formation was then determined (□)

cytosol was added to standard incubation mixtures containing mitochondria, [^{35}S]cysteine-labelled reticulocyte lysate and NADH, activity was stimulated two-fold, suggesting that the stimulation of activity by the factor from reticulocyte lysate and cytosol were additive or that the content of the factor in the reticulocyte lysate was below the concentration necessary for optimal activity.

To examine the nature and possible role of this factor in holocytochrome *c* formation, cytosolic preparations were pre-treated under various conditions and then their ability to stimulate holocytochrome *c* formation from apocytochrome *c*, pre-bound to mitochondria, was determined (Table 1). The stimulating factor was a heat-stable, low-molecular-mass cytosolic component that was not sensitive to trypsin. When it was dialyzed out of cytosolic preparations, the resulting dialyzate could be concentrated and the stimulating activity recovered. To substantiate further that the factor was not hemin, dialyzed cytosol (which did not significantly affect activity) was supplemented with hemin and used to stimulate holocytochrome *c* formation. The dialyzed cytosol treated this way did not affect activity any more than the dialyzed cytosol alone. Equivalent volumes of unlabelled reticulocyte lysate (35 µl per 200 µl total volume) stimulated activity to 63% of that observed with untreated cytosol. Using 50 µl of

Table 1. *Stimulation of cytochrome* c *heme lyase activity by cytosolic fractions*

Apocytochrome *c* from [^{35}S]cysteine-labelled reticulocyte lysate was pre-bound to mitochondria in the absence of NADH as described in Fig. 5B. The mitochondria were reisolated and suspended in a mixture containing 75 μg mitochondrial protein, 5 mM NADH, 2 μM hemin and 35 μl of a cytosolic fraction or reticulocyte lysate in a total volume of 200 μl buffer A. The samples were incubated for 10 min at 25 °C and holocytochrome *c* formation was determined. In experiment I, cytosol fractions were pretreated in the following manner: (2) no treatment; (3) heating for 5 min at 95 °C; (4) five cycles of slow freezing to −20 °C, then thawing to 25 °C; (5) extensive dialyzing against buffer A overnight at 4 °C in Visking 8/32 tubing (Union Carbide, Chicago IL); (6) 40 μg trypsin was preincubated with 800 μg soybean trypsin inhibitor for 5 min at 25 °C; the mixture was then added to 1 ml cytosol and further incubated for 20 min at 25 °C; (7) incubation for 20 min at 25 °C with 4 μg trypsin/ml cytosol then for 5 min at 25 °C with 80 μg soybean trypsin inhibitor/ml; (8) as in (7) except with 40 μg trypsin/ml cytosol and 800 μg soybean trypsin inhibitor/ml; (9 and 10) no cytosol, but with 35 μl and 50 μl (respectively) of the post-ribosomal supernatant of reticulocyte lysate containing unlabelled cysteine instead of [^{35}S]cysteine. In experiment II, fractions (12) and (13) were treated as (2) and (5), respectively; (14) the dialyzate from (13) was saved, lyophilized, and suspended in H_2O in the same volume as the original sample; (15) the incubation mixtures containing the dialyzed cytosol (13) were supplemented with 2 μM hemin. The activity of the sample with untreated cytosol (2) and (11) were set at 100%

Expt	Condition	Activity
		fmol × min^{-1} × mg^{-1} (%)
I.	(1) no cytosol	0.0266 (10.8)
	(2) control	0.246 (**100**)
	(3) 5 min 95 °C	0.268 (109)
	(4) freeze/thaw	0.249 (101)
	(5) dialysis	0.0413 (16.8)
	(6) mock trypsin	0.208 (84.6)
	(7) 4 μg trypsin/ml	0.216 (87.8)
	(8) 40 μg trypsin/ml	0.201 (81.7)
	(9) 35 μl reticulocyte lysate	0.155 (63.0)
	(10) 50 μl reticulocyte lysate	0.209 (85.0)
II.	(11) no cytosol	0.0313 (16.6)
	(12) control	0.188 (**100**)
	(13) dialyzed cytosol	0.0402 (21.4)
	(14) dialyzate	0.159 (84.6)
	(15) dialyzed cytosol + hemin	0.0353 (18.8)

unlabelled reticulocyte lysate (equal to the amount of lysate normally used per incubation mixture) holocytochrome *c* formation occurred at 85% of the optimal rate observed with cytosol. The exact identity and role of this factor is unclear from these experiments, except that it is not required for the binding of apocytochrome *c* to mitochondria. It is therefore most likely required for the activity of cytochrome *c* heme lyase.

Cytochrome c *heme lyase is located in the mitochondrial intermembrane space and is distinct from the apocytochrome* c *binding protein*

Mitochondria were subfractionated by digitonin treatment in order to answer two questions, namely: (a) where is cytochrome *c* heme lyase localized and (b) is cytochrome *c* heme lyase distinct from the previously identified apocytochrome *c* binding protein (Köhler, Stuart and Neupert, unpublished results)? To address the second question, advantage was taken of the particular fractionation behaviour. The apocytochrome *c* binding protein cofractionates with intermembrane space markers in swelling and sonication experiments but exhibits some latency in release in the presence of increasing concentrations of digitonin. If cytochrome *c* heme lyase and the apocytochrome *c* binding protein were the same protein, then this delay in the release of cytochrome *c* heme lyase activity would be expected as well.

Mitochondria were carefully isolated by Percoll-gradient centrifugation and fractionated by digitonin (Fig. 6). High-affinity binding sites for apocytochrome *c* correlated with the amount of binding protein and fractionated as a hybrid between the outer membrane (porin) and intermembrane space (presumably associated with the inner face of the outer membrane). Cytochrome *c* heme lyase activity could not be detected in any of the supernatant fractions, but loss of activity from the pellets correlated with the loss of intermembrane space markers. The possibility that the loss of activity was due to inhibition of activity by digitonin was excluded by other experiments, such as fractionation by sonication and fractionation by swelling in hypotonic buffers. In all cases, loss of cytochrome *c* heme lyase activity correlated with the release of intermembrane space markers (unpublished experiments). The ability of the digitonin-treated mitochondria to bind apocytochrome *c* was not affected at low digitonin concentrations where holocytochrome *c* formation was reduced. Cytochrome *c* heme lyase, or some other component necessary for the covalent attachment of heme to apocytochrome *c*, appears to be localized in the mitochondrial intermembrane space and fractionates separately from the apocytochrome binding activity.

DISCUSSION

Previous methods for determining the covalent attachment of heme to apocytochrome *c* by the activity of cytochrome *c* heme lyase have been based on secondary changes in the protein molecule as a consequence of heme linkage. In our laboratory, we have made use of differential antibodies raised against apo- and holocytochrome *c* which do not cross-react under defined conditions [12, 18–22, 30]. In yeast, cytochrome *c* heme lyase activity has been examined by making use of the differential sensitivity of apo- and holocytochrome *c* to proteolytic digestion by trypsin under reducing conditions [23–26]. In the current study we have established a method for directly measuring the covalent linkage of heme to apocytochrome *c* which is totally independent of subsequent conformational changes in protein structure which may not occur under certain circumstances.

Formation of holocytochrome *c* was dependent on exogenously added hemin and, as observed previously [20], could be inhibited by the analogue deuterohemin. Deuterohemin apparently has similar affinity for cytochrome *c* heme lyase as the competent substrate hemin since 50% inhibition of activity occurred when the concentrations of both were equal. Blockage of holocytochrome *c* formation by deuterohemin could be reversed by protohemin. Very high concentrations of deuterohemin, however, abolished holocytochrome *c* formation irreversibly. It is possible that accumulation of high concentrations of heme compounds has perturbating effects on mitochondrial membranes and upsets other essential parts of the import pathway, such as receptor binding or presentation of the apocytochrome *c* cysteines to cytochrome *c* heme lyase. Indeed, reduction in the ability of

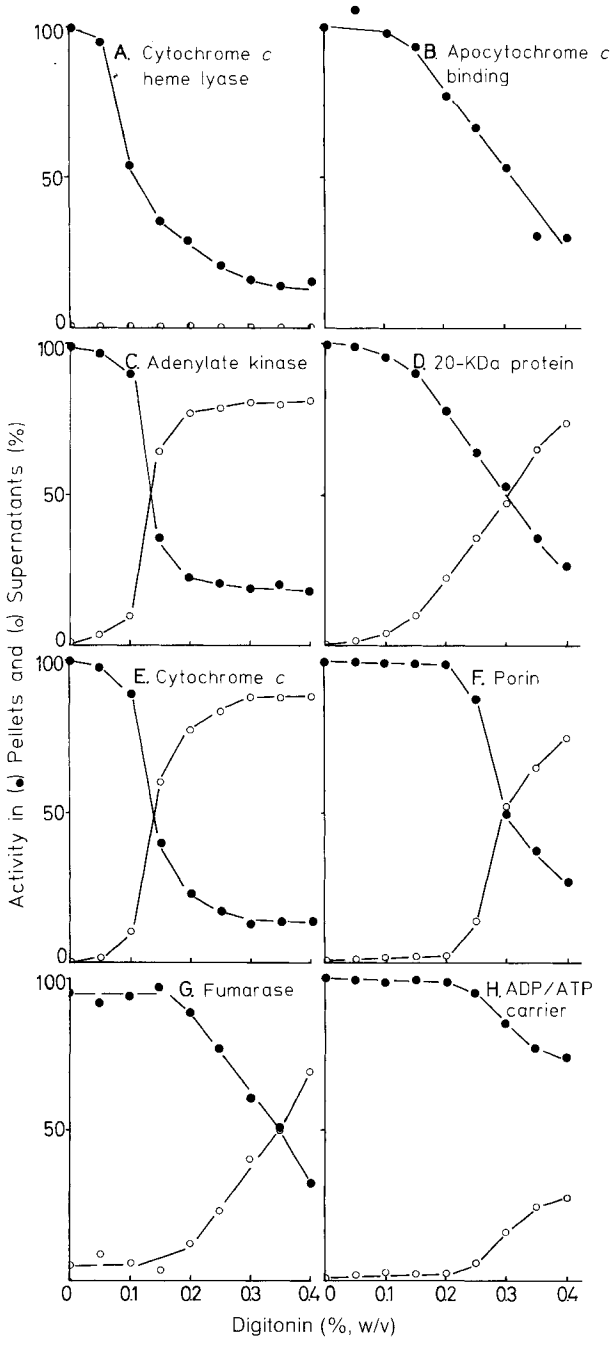

Fig. 6. *Submitochondrial localization of cytochrome* c *heme lyase by digitonin fractionation.* Mitochondria were fractionated with increasing concentrations of digitonin as described in Materials and Methods. Proteins were quantified by enzymatic or immunological methods: (●) Activity or content in pellets; (○) activity or content in supernatants. (A) Cytochrome *c* heme lyase activity; (B) number of high-affinity binding sites for apocytochrome *c*; (C) adenylate kinase activity (intermembrane space marker); (D) 20-kDa protein content; (E) holocytochrome *c* content (intermembrane space marker); (F) porin content (outer membrane marker); (G) fumarase activity (matrix marker); (H) ADP/ATP carrier content (inner membrane marker)

mitochondria to bind apocytochrome *c* was observed in the presence of high concentrations of deuterohemin, although displacement of pre-bound apocytochrome *c* did not occur (unpublished experiments).

Holocytochrome *c* formation could also be inhibited with *N*-ethylmaleimide which specifically and irreversibly reacts with free sulphydryl groups [44]. The data presented here indicate that the cysteine sulphydryl groups of the apocytochrome *c* in the reticulocyte lysate were reduced and therefore able to react with *N*-ethylmaleimide (NEM). This reaction appears to be the cause for NEM inhibtion of holocytochrome *c* formation, and not an inactivation of a component of the import machinery. Furthermore, since the conversion of apocytochrome *c* (which had been pre-bound to mitochondria before NEM treatment) to holocytochrome *c* was blocked by NEM, then the cysteines of bound apocytochrome *c* were also in the reduced state. Altogether, these data indicate that in the system used here the suphydryl groups of the cysteines of apocytochrome *c* remain reduced up to the stage where they are presented to cytochrome *c* heme lyase for covalent attachment of heme.

Besides heme and apocytochrome *c*, holocytochrome *c* formation by mitochondria requires NADH and an unknown factor that is present in cytosolic fractions and reticulocyte lysate. The exact role of these cofactors is unclear. In the first case, it has been well established that import of cytochrome *c* does not require a potential across the inner mitochondrial membrane [21]. This was further substantiated here by experiments in which the membrane potential was dissipated (valinomycin/K^+) or its generation was blocked (antimycin A/oligomycin) and NADH-dependent holocytochrome *c* formation was unaffected. Similarly, when a membrane potential was generated in the absence of NADH (tetramethylphenylenediamine/ascorbate), only negligible holocytochrome *c* formation occurred. NADH was not required for the binding of apocytochrome *c* to mitochondria and the effects of *N*-ethylmaleimide suggest that the cysteine residues involved in heme attachment exist in their reduced state throughout import so that NADH is not required for their reduction prior to holocytochrome *c* formation. There is the possibility that NADH is required for reduction of the heme irons from Fe^{3+} to Fe^{2+} before enzymatic linkage of heme to apocytochrome *c* can occur. The exact role of NADH in the import of cytochrome *c*, however, remains unclear.

A cofactor that is present in both reticulocyte lysate and in *Neurospora* cytosolic preparations was also required for holocytochrome *c* formation (Fig. 5). A similar stimulatory effect by cytosolic factors on holocytochrome *c* formation has been described in yeast. With yeast this factor could be substituted for by an NADPH-regenerating system [45]. In our experiments, a regenerating system for either NADPH (isocitrate plus isocitrate dehydrogenase) or for NADH (lactate plus lactate dehydrogenase, or glucose 6-phosphate plus glucose-6-phosphate dehydrogenase) did not substitute for the requirement of the cytosolic cofactor. The factor appears to be a temperature-stable, non-protein component of *Neurospora* cytosol. It was readily dialyzable and may therefore be able to pass through the outer mitochondrial membrane to the intermembrane space via the channels formed by porin. The role of this cofactor in cytochrome *c* import is unclear. It is not involved in the binding of apocytochrome *c* to mitochondria and is presumably required for either the activity of cytochrome *c* heme lyase or for some events within the outer membrane which are required for presentation of apocytochrome *c* to the heme attaching enzyme.

The apparent location of cytochrome *c* heme lyase in the mitochondrial intermembrane space, as determined here, has been incorporated into our previous hypothesis on the

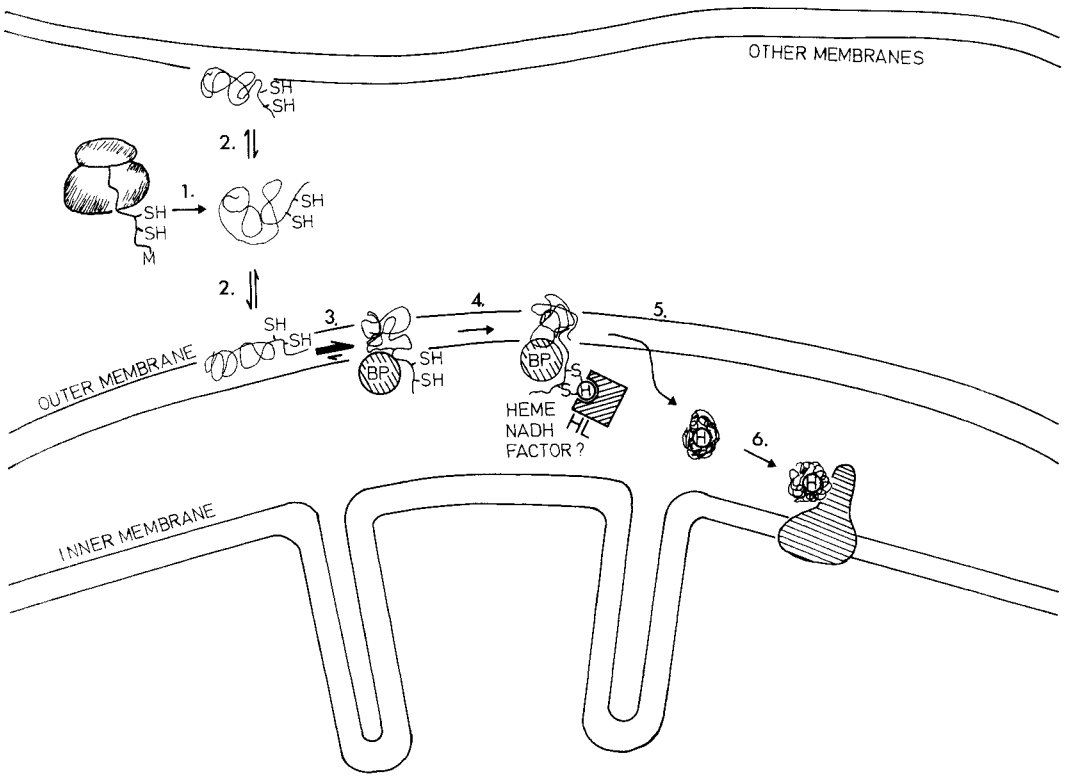

Fig. 7. *Pathway of cytochrome* c *import into mitochondria.* (1) Nuclear-coded apocytochrome *c* is synthesized on cytoplasmic ribosomes and released into a cytosolic pool. (2) The apocytochrome *c* protein interacts with cellular membranes, including the mitochondrial outer membrane, with low affinity and in a reversible manner, and spontaneously inserts into the lipid bilayer. (3) Apocytochrome *c* is bound with high affinity to a protein located at the inner face of the outer mitochondrial membrane. (4) Heme is covalently attached to cysteines 18 and 21 by the enzyme cytochrome *c* heme lyase with (5) concomitant translocation across the outer membrane by the resulting conformational change. (6) Holocytochrome *c* migrates to its functional location in association with cytochrome *c* reductase and cytochrome *c* oxidase at the outer face of the inner membrane. Abbreviations: BP, apocytochrome *c* binding protein; H, heme; HL, cytochrome *c* heme lyase

assembly of cytochrome *c* (Fig. 7). Nuclear-coded apocytochrome *c* is synthesized on free ribosomes in the cytosol and is released into a cytosolic pool. Other than the N-terminal methionine which is removed as a post-translational modification, the apoprotein does not contain a cleavable N-terminal targeting prepiece. The positively charged apocytochrome *c* is able to interact with anionic phospholipid head groups and can spontaneously insert, with low affinity, part way through the lipid bilayer of cell membranes [46–50]. In mitochondria, apocytochrome *c* is bound with high affinity by a binding protein from the intermembrane space. This complex is arranged in the outer membrane to facilitate the next step of transfer. At this stage, the bound apocytochrome *c* can still be displaced from mitochondria with an excess of externally added apocytochrome *c* [22]. Heme is then covalently attached to cysteines 18 and 21 of apocytochrome *c*, which are presumably exposed to the intermembrane space, by the enzyme cytochrome *c* heme lyase. The resulting conformational change pulls the protein completely through the lipid bilayer of the outer membrane and it is released into the intermembrane space as holocytochrome *c* where it subsequently migrates to its functional location in association with cytochrome *c* reductase and cytochrome *c* oxidase at the outer face of the inner membrane. It should be mentioned that, in yeast, cytochrome *c* heme lyase was reported to be membrane-bound [23], although the exact topological location was not determined.

Import into and maturation of cytochrome *c* in mitochondria appears to require at least two protein components, namely the apocytochrome *c* binding protein and cytochrome *c* heme lyase. In yeast, two similar functions have been identified by genetic analysis [51]. Mutations at two loci, unlinked to cytochrome *c* structural genes, appear to code for a heme attaching activity (CYC 3) and a protein which may facilitate apocytochrome *c* transport across the outer membrane (CYC 2).

In summary, cytochrome *c* heme lyase plays a central role in the transfer of apocytochrome *c* into mitochondria. It appears that the functional assembly of cytochrome *c*, namely covalent attachment of heme, is conveniently linked to the translocation of the protein across the outer mitochondrial membrane during import.

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