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Import of cytochrome *c* into mitochondria: Reduction of heme, mediated by NADH and flavin nucleotides, is obligatory for its covalent linkage to apocytochrome *c*

(cytochrome *c* heme lyase/mitochondrial biogenesis)

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ABSTRACT **The covalent attachment of heme to apocytochrome *c*, and therefore the import of cytochrome *c* into mitochondria, is dependent on both NADH plus a cytosolic cofactor that has been identified to be FMN or FAD. NADH in concert with flavin nucleotides mediates the reduction of heme. Heme in the reduced state is a prerequisite for its covalent attachment to apocytochrome *c* by the enzyme cytochrome *c* heme lyase and thus for subsequent translocation of cytochrome *c* across the outer mitochondrial membrane during import.**

The import of cytochrome *c* into mitochondria follows a pathway that differs in many regards from that of the majority of other imported mitochondrial proteins (for review, see refs. 1 and 2). Cytochrome *c* is a nuclear gene product that is synthesized in the cytosol (3) as apocytochrome *c*, which differs from its mature counterpart (holocytochrome *c*) in conformation and by the absence of covalently bound heme. It does not contain an N-terminal prepiece (4–6) and does not require a membrane potential for import (7, 8).

The covalent attachment of heme to apocytochrome *c* requires NAD(P)H and a cofactor present in a cell cytosol fraction (8–10). In this report, we have examined the NADH-dependent stage of cytochrome *c* import into *Neurospora crassa* mitochondria. NADH was found not to be necessary for specific binding of apocytochrome *c* to mitochondria, but it was required for holocytochrome *c* formation and therefore for import. NADH-dependent import also required the presence of a cytosolic cofactor. Our data suggest this cofactor to be a flavin nucleotide that mediates the reduction of heme by NADH. Furthermore, we demonstrate that the reduction of heme is an obligatory step which must precede the covalent attachment of heme to apocytochrome *c* by the enzyme cytochrome *c* heme lyase.

MATERIALS AND METHODS

Cell Growth and Subcellular Fractionation. Growth of *N. crassa* (wild-type 74A) and isolation of mitochondria by Percoll gradient centrifugation and of a cytosol fraction were carried out as described (10, 11). *Saccharomyces cerevisiae* wild-type D273-10B was grown in 2% lactate and mitochondria were isolated as described (12). Cytochrome *c* heme lyase was solubilized from mitochondria by combining mitochondria (at 5 mg/ml) with an equivalent volume of 2% (wt/vol) *n*-octyl glucoside in buffer A [250 mM sucrose, 10 mM Mops/KOH (pH 7.2), 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride]. After shaking for 10 min at 4°C, the preparation was spun at 48,400 × *g* for 20 min and the upper two-thirds of the resulting supernatant was retained.

Measurement of Cytochrome *c* Heme Lyase Activity. Apocytochrome *c* was synthesized in rabbit reticulocyte lysates (13) in the presence of L-[^{35}S]cysteine (specific activity, 1100–1400 Ci/mmol; 1 Ci = 37 GBq; Amersham) as described (10). Synthesis was directed by *N. crassa* poly(A)-containing RNA or apocytochrome *c*-specific transcripts derived from a cDNA clone (14).

Import from [^{35}S]cysteine-labeled reticulocyte lysate. Mitochondria (75 μg), or an octyl glucoside extract from the equivalent amount of mitochondrial protein, was incubated for 10 min at 25°C in the presence of 50 μl of [^{35}S]cysteine-labeled reticulocyte lysate plus other additions, as indicated, in a total volume of 200 μl of buffer A. When mitochondria were used, they were reisolated by centrifugation at 17,400 × *g* for 12 min after incubation. Holocytochrome *c* formation was determined by immunoprecipitating total cytochrome *c* (holo- plus apo-), dissociation of the immunocomplexes with urea, digestion with trypsin, and analysis of the resulting peptides by reverse-phase HPLC (10).

Chase of prebound apocytochrome c *to holocytochrome* c. Cytochrome *c* was bound to mitochondria in the absence of conversion to holocytochrome *c* (by withholding NADH) in mixtures containing 75 μg of mitochondrial protein and 50 μl of [^{35}S]cysteine-labeled reticulocyte lysate that were incubated for 10 min at 25°C in 200 μl of buffer A (final volume). The mitochondria were reisolated by centrifugation for 12 min at 17,400 × *g* and resuspended in fresh buffer A. The bound apocytochrome *c* was chased to holocytochrome *c* for 10 min at 25°C in the presence of 3 μM hemin and additions (i.e., 5 mM NADH) in a total volume of 200 μl of buffer A.

Using this procedure, 25–50% of the available apocytochrome *c* could be taken up by mitochondria. Over 90% of apocytochrome *c* that had been prebound to mitochondria could be chased to holocytochrome *c* and translocated to a protease-resistant location, where it was determined to be soluble in the intermembrane space (data not shown). Because of the close coupling of heme addition with membrane translocation, import in intact mitochondria was monitored by holocytochrome *c* formation (8, 14).

Measurement of Heme Reduction. The concentration of reduced heme was determined by difference spectroscopy. A mixture (4 ml in buffer A) containing either 0.375 mg of mitochondrial protein per ml (or the equivalent amount of octyl glucoside extract), 3 μM hemin, and (where indicated) 2 μM FMN was divided into two aliquots. To start the reaction, NADH or sodium dithionite was added to one sample (reduced) and buffer A was added to the other (oxidized). After 10 min at 25°C, the difference spectrum was recorded. The difference in absorbance between the maximum at 570 nm and the minimum at 635 nm ($\Delta A_{570-635}$) was proportional to the concentration of reduced heme. An excess of sodium dithionite was added to the reduced half of the sample and the spectrum was rerecorded to obtain the $\Delta A_{570-635}$ of the fully reduced sample from which the con-

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centration of heme that was reduced during the incubation could be calculated. The difference extinction coefficient ($\Delta\varepsilon_{570-635}$) of the sodium dithionite-reduced minus oxidized samples was 2.35 $mM^{-1} \cdot cm^{-1}$.

Electrochemical Reduction of Heme. Hemin [ferroprotoporphyrin (protoheme IX) Cl; Porphyrin Products, Logan, UT] was dissolved in 1 M KOH at a concentration of 123 mM and was then neutralized by dilution to a final concentration of 1 mM in 100 mM Tris·HCl (pH 7.5). Immediately before use, the hemin solution was further diluted to 6 μM in 0.1 M KCl, 10 mM Mops/KOH (pH 7.2). The electrochemical properties of the hemin solution (2 ml) were first examined by cyclic voltammetry using a mercury pool cathode, a platinum anode, and a saturated calomel electrode (SCE) as a reference (15). When the potential was swept between −200 mV and −600 mV (vs. SCE), a single cathodic peak was detected at approximately −425 mV (data not shown). (The buffer alone did not contribute to the wave pattern in the potential range used for these experiments.) A potential of −505 mV (vs. SCE) was selected for subsequent electrochemical reduction of the hemin.

To prepare electrochemically reduced heme, 2 ml of 6 μM hemin (in the KCl/Mops buffer described above) was stirred in a standard electrochemical cell having a mercury pool electrode and with catholyte and anolyte separated by a frit (Vycor tip) under anaerobic conditions. A potential of −505 mV (vs. SCE) was then applied for 5 min. The initial current was ≈100 μA and decreased to zero with a half-time of 36 sec. The total charge transported was estimated to be 24.9 μA·min, corresponding to 15.6 nmol of single-electron transfer steps. This was in good agreement with the 12.0 nmol of hemin present in the solution. Over 95% of the heme was reduced by this procedure, as measured by difference spectroscopy.

Quantitation of FMN and FAD. Flavin nucleotides were extracted from samples with ice-cold $HClO_4$ and half of each extract was incubated at 100°C for 30 min to convert FAD to FMN as described (16, 17). FMN was quantified by the bacterial luciferase reaction (16) in a 1-ml mixture containing 100 mM KP_i (pH 6.8), 70 mM 2-mercaptoethanol, 1.4 mM NADH, 0.16 mM *n*-decyl aldehyde, and 40 μg of luciferase (type V from *Vibrio fischeri*; Sigma). The reaction was started by adding the FMN-containing extract to the mixture and measuring light emission in a chemiluminescence detector. Total phosphoflavin (FAD plus FMN) was determined by the light emission of the samples treated at 100°C. FMN was determined in the unheated samples and the FAD content was calculated by subtracting the FMN content from the total phosphoflavin content.

RESULTS

Import of Cytochrome *c* into Mitochondria Is Dependent on NADH. When apocytochrome *c* was imported directly into mitochondria from reticulocyte lysate, the formation of holocytochrome *c* was dependent on the presence of NADH (Fig. 1). The binding of apocytochrome *c* to mitochondria did not require NADH and, in fact, was inhibited at NADH concentrations >5 mM.

When apocytochrome *c* was prebound to mitochondria in the absence of NADH and the mitochondria were reisolated (conditions under which only negligible holocytochrome *c* formation occurred), the formation of holocytochrome *c* from the bound apocytochrome *c* was also dependent on NADH (Fig. 1). No inhibition was observed at higher (e.g., >5 mM) NADH concentrations. NADH is therefore required for steps in the cytochrome *c* import pathway that occur after the initial binding event. In addition, by manipulating the NADH concentration it was possible to separate the binding of

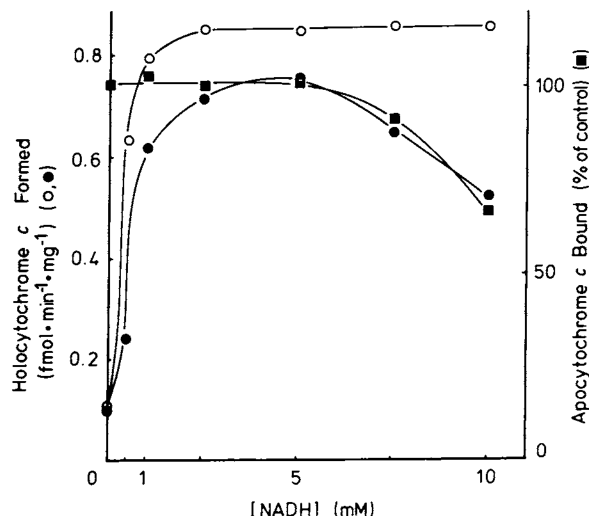

FIG. 1. Holocytochrome *c* formation depends on NADH, but the binding of apocytochrome *c* to mitochondria does not. Apocytochrome *c* was imported directly into mitochondria in the presence of various concentrations of NADH and then the holocytochrome *c* formed was determined (●). In parallel samples, apocytochrome *c* binding to mitochondria was determined in the presence of 25 μM deuterohemin (to block holocytochrome *c* formation) (■). The amount of apocytochrome *c* bound in the absence of NADH is set at 100%. The effect of NADH on holocytochrome *c* formation from prebound apocytochrome *c* was determined by first binding apocytochrome *c* to mitochondria in the absence of NADH and then chasing to holocytochrome *c* for 10 min at 25°C in the presence of 3 μM hemin, 25% (vol/vol) unlabeled reticulocyte lysate, and various concentrations of NADH. The holocytochrome *c* formed was then determined (○).

apocytochrome *c* to mitochondria from its conversion to holocytochrome *c* and subsequent import into mitochondria.

NADH-Dependent Holocytochrome *c* Formation Requires a Cytosolic Cofactor That Can Be Substituted for by FAD or FMN. Apocytochrome *c*, which was prebound to mitochondria, was inefficiently chased to holocytochrome *c* in the presence of NADH unless the incubation mixtures were supplemented with either unlabeled reticulocyte lysate (as in Fig. 1) or a cell cytosol fraction (Fig. 2*A*, open circles). A cofactor present in these preparations is apparently necessary for the NADH-dependent step in holocytochrome *c* formation. In an earlier study (10), this cofactor was found to be heat stable, nonproteinaceous, and readily dialyzable. When FAD or FMN was used instead of the cytosol fraction, equivalent stimulation of holocytochrome *c* formation occurred at concentrations between 0.1 and 10 μM flavin nucleotide (Fig. 2*B*). The activity in the presence of FAD or FMN was also dependent on NADH.

The cytosolic cofactor, like FMN, was sensitive to light (Fig. 2*A*). When preparations of either FMN or cytosol were illuminated, their ability to stimulate NADH-dependent holocytochrome *c* formation from prebound apocytochrome *c* was abolished or reduced. In the presence of 10 μM FMN, neither the light-treated nor control cytosol fractions stimulated activity further.

This suggests that the cytosolic cofactor necessary for cytochrome *c* import is a flavin nucleotide. In support of this, we determined the FAD and FMN concentrations in the cytosol fraction. The total phosphoflavin content (FAD plus FMN) in the cytosol fraction was 2.38 μM; the FMN concentration was 1.63 μM; FAD (by difference) was 0.75 μM. These values are in close agreement with the concentrations of flavin nucleotides that support holocytochrome *c* formation and confirm the identification of the cofactor.

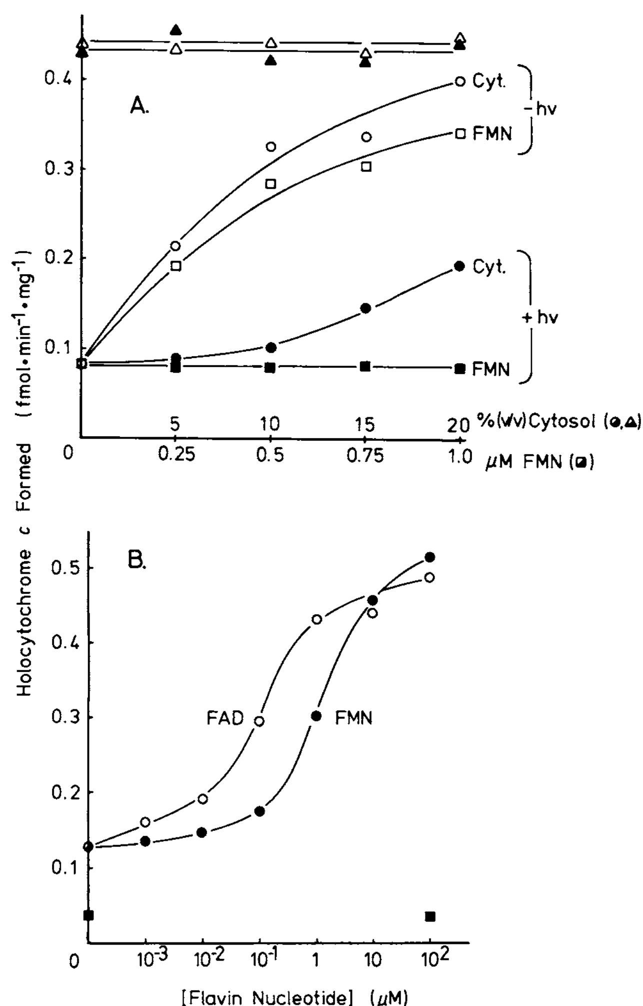

FIG. 2. Dependence of NADH-mediated holocytochrome c formation on a factor from cytosol or on flavin nucleotides. (A) A 0.3-ml aliquot of either cytosol fraction (●) or 100 μM FMN in buffer A (■) was adjusted to pH 8.0 with KOH and exposed to bright fluorescent illumination overnight at 4°C with constant stirring. Any loss of volume owing to evaporation was compensated for by adding water and the samples were then adjusted to pH 7.2 with HCl. Nonilluminated controls (○, cytosol fraction; □, FMN) were treated identically except that they were kept in the dark. Apocytochrome c was prebound to mitochondria in the absence of NADH and then chased to holocytochrome c in the presence of 3 μM hemin, 5 mM NADH, and various amounts of the cytosol fraction or FMN preparations for 10 min at 25°C. The holocytochrome c formed was then determined. Formation of holocytochrome c was also determined in the presence of 3 μM hemin, 5 mM NADH, plus various amounts of either the control cytosol (△) or light-treated cytosol (▲) (i.e., the same as ○ and ●, respectively) except that 10 μM FMN was also present. ±hv, With or without illumination; Cyt, cytosol fraction. (B) Apocytochrome c was prebound to mitochondria and chased to holocytochrome c in the presence of NADH as described for A except that untreated FAD (○) or FMN (●) was used instead of the cytosol fraction. Controls without NADH (■) were incubated in the presence or absence of FMN.

It has been previously reported that the import of cytochrome c into yeast mitochondria also depends on a cytosolic cofactor and that it can be substituted for by an NADPH-regenerating system (18). In our hands, the chase of prebound apocytochrome c to holocytochrome c in the presence of NADPH was only marginally stimulated by a regenerating system (1.4-fold in *N. crassa*, 1.7-fold in yeast) (Table 1), although these levels are comparable to those described previously in yeast (2.2-fold; see ref. 19). The import of *N. crassa* apocytochrome c into yeast mitochondria, however, showed nearly identical characteristics compared to the requirements in *N. crassa* mitochondria, including marked stimulation by either cytosol fraction or FMN. We suggest that the cofactor required by yeast mitochondria is also FMN or FAD (or both).

Table 1. Reducing conditions required for holocytochrome c formation in yeast and *N. crassa* mitochondria

Condition	Holocytochrome c formed, $fmol \cdot min^{-1} \cdot mg^{-1}$ (%)	
	N. crassa	*S. cerevisiae*
No addition	0.044 (2.1)	0.053 (4.9)
NADH	0.467 (22.0)	0.105 (9.7)
NADH + FMN	2.12 (100; set)	1.09 (100; set)
NADH + cytosol	1.90 (89.5)	0.975 (89.6)
NADPH	0.214 (10.1)	0.056 (5.2)
NADPH + FMN	1.62 (76.5)	0.639 (58.7)
NADPH + cytosol	1.31 (61.6)	0.487 (44.8)
NADPH + G6P/G6PDH	0.289 (13.6)	0.098 (9.0)
Sodium dithionite	2.05 (96.5)	1.25 (115)

Apocytochrome c from [^{35}S]cysteine-labeled reticulocyte lysate, programmed by *N. crassa* poly(A)-containing RNA, was prebound to either *N. crassa* or *S. cerevisiae* mitochondria in the absence of NADH. The mitochondria were reisolated and the apocytochrome c was chased to holocytochrome c in the presence of 3 μM hemin and other additions as indicated. The concentrations of additions were as follows: NADH, 5 mM; NADPH, 5 mM; FMN, 10 μM; cytosol fraction, 20% (vol/vol); D-glucose 6-phosphate (G6P), 20 mM; glucose-6-phosphate dehydrogenase (G6PDH), 1.2 units/ml; sodium dithionite, 1 mg/ml.

NADH Plus Flavin Nucleotides Are Required for Heme Reduction, and Reduced Heme Is Sufficient for the Cytochrome *c* Heme Lyase Reaction. Since NADH is not required for any import stages preceding covalent heme attachment, one possibility is that it is needed to reduce heme. In the presence of intact mitochondria, the rate of NADH-dependent heme reduction was markedly higher in samples containing FMN (0.182 $nmol \cdot min^{-1} \cdot mg^{-1}$) compared to samples not containing FMN (0.015 $nmol \cdot min^{-1} \cdot mg^{-1}$) (data not shown).

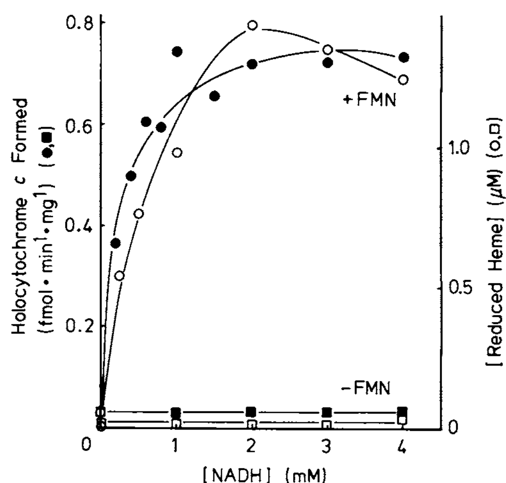

FIG. 3. NADH-dependent holocytochrome c formation corresponds to the levels of reduced heme. Apocytochrome c was prebound to mitochondria in the absence of NADH and then chased to holocytochrome c in a mixture containing 3 μM hemin and various concentrations of NADH in either the absence (■) or presence (●) of 2 μM FMN. The holocytochrome c formed was then determined after incubation for 10 min at 25°C. In parallel samples, heme reduction was measured in mixtures containing 0.375 mg of mitochondrial protein per ml, 10 μM hemin, and various concentrations of NADH in either the absence (□) or presence (○) of 2 μM FMN. The amount of reduced heme was determined after incubating for 10 min at 25°C.

To test whether NADH-dependent holocytochrome *c* formation in mitochondria corresponded to the concentration of reduced heme, holocytochrome *c* formation and heme reduction were measured in parallel incubation mixtures with various concentrations of NADH in the presence or absence of FMN (Fig. 3). Both the chase of prebound apocytochrome *c* to holocytochrome *c* and reduction of heme were dependent on about the same concentrations of NADH and occurred only in the presence of FMN.

Instead of NADH plus FMN, the reduction step necessary for holocytochrome *c* formation could also be driven by sodium dithionite (Fig. 4). In intact mitochondria there was a close correlation between the concentration of sodium dithionite required for heme reduction and that required for holocytochrome *c* formation (Fig. 4*A*).

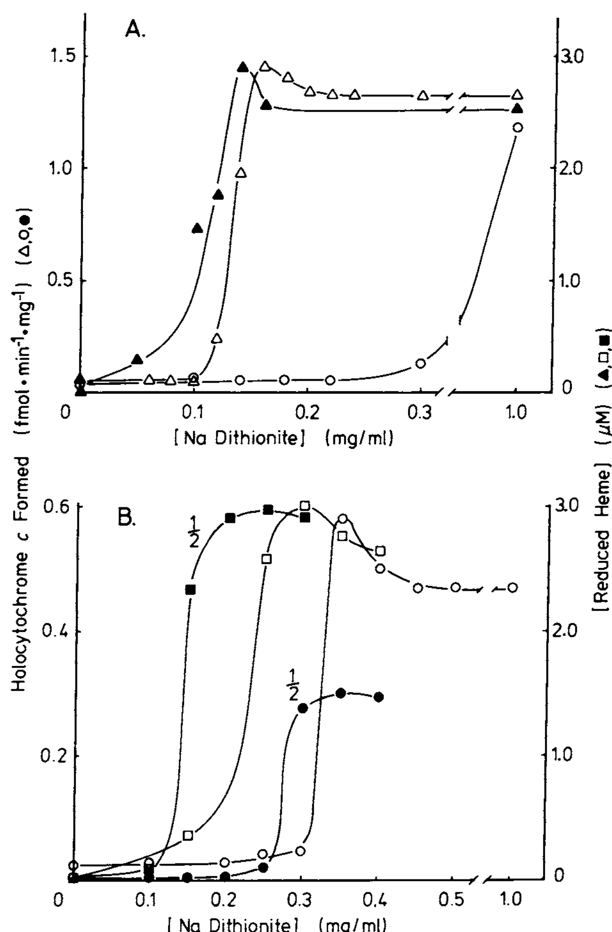

FIG. 4. Holocytochrome *c* formation corresponds to the levels of sodium dithionite reduced heme. (*A*) The formation of holocytochrome *c* was determined by chasing prebound apocytochrome *c* in the presence of 3 μM hemin plus various amounts of sodium dithionite for 10 min at 25°C (△). In parallel samples, heme reduction was determined in mixtures containing 0.375 mg of mitochondrial protein per ml, 3 μM hemin, plus various amounts of sodium dithionite, after incubation for 10 min at 25°C (▲). For comparison, holocytochrome *c* formation using an octyl glucoside extract instead of mitochondria was determined (○). (*B*) Holocytochrome *c* formation was determined in mixtures containing octyl glucoside-solubilized mitochondria (equivalent to 75 μg of original mitochondrial protein) (○) or one-half the amount (●) in the presence of 3 μM hemin, 50 μl of [^{35}S]cysteine-labeled reticulocyte lysate, and various amounts of sodium dithionite in a total volume of 200 μl. Heme reduction was measured after incubating for 10 min at 25°C in scaled-up but otherwise identical mixtures except that no reticulocyte lysate was present [equivalent to 0.375 mg of original mitochondrial protein per ml (□) or one-half the amount (■)].

When mitochondria were solubilized with octyl glucoside, cytochrome *c* heme lyase activity was completely recovered provided that dithionite was present in the assay. A comparable correlation between holocytochrome *c* formation and heme reduction was also observed (Fig. 4*B*). The concentration of dithionite at which heme reduction occurred was lower than that required for holocytochrome *c* formation. This is probably because reticulocyte lysate was present during the cytochrome *c* heme lyase assay but not during heme-reduction measurement. When half the amount of octyl glucoside extract was used, a shift toward lower sodium dithionite requirements was observed for both holocytochrome *c* formation and heme reduction.

In octyl glucoside extracts, holocytochrome *c* formation was not supported by NADH plus FMN (data not shown). In addition, FMN did not stimulate reduction of heme in the presence of NADH. Octyl glucoside apparently interferes with or disrupts the NADH/flavin nucleotide-dependent reduction of heme that occurs with intact mitochondria.

To directly determine whether the redox state of the heme alone accounts for the requirement for reducing agents during cytochrome *c* import, it was necessary to test for holocytochrome *c* formation in the presence of reduced heme but in the absence of other reducing reagents (i.e., NADH or sodium dithionite). To do this, heme was first reduced by electrochemical means and then added to a mixture containing octyl glucoside-solubilized mitochondria and apocytochrome *c* (Table 2). Under these conditions, electrochemically reduced heme was sufficient for holocytochrome *c* formation, which occurred at 90% of the rate observed in the control containing sodium dithionite.

We conclude that reduction of heme is required before it can be covalently attached to apocytochrome *c* by the enzyme cytochrome *c* heme lyase. Furthermore, when reduction is provided for by NADH, cytosolic flavin nucleotides are required to mediate the heme reduction step.

Table 2. Electrochemically reduced heme is sufficient for holocytochrome *c* formation

Condition	Holocytochrome *c* formed, fmol·min^{-1}·mg^{-1} (%)
Aerobic	
No sodium dithionite	0.255 (17.8)
Plus sodium dithionite	1.43 (100; set)
Anaerobic	
No electrochemical reduction of heme	0.360 (25.2)
Plus electrochemically reduced heme	1.30 (90.1)

Unless otherwise indicated, all buffers were degassed and purged with nitrogen prior to use, and all operations (including mitochondrial isolation) were performed anaerobically under a nitrogen atmosphere. Apocytochrome *c* was bound to mitochondria. The mitochondria were reisolated, resuspended, and then lysed with 1% (wt/vol) octyl glucoside in buffer A at a mitochondrial protein concentration of 0.75 mg/ml. Hemin was freshly prepared as a 6 μM solution in 0.1 M KCl, 10 mM Mops/KOH (pH 7.2). Each reaction was then performed in the following manner: An aliquot (200 μl) of the lysed mitochondrial suspension, containing prebound apocytochrome *c*, was diluted with 1.8 ml of buffer A. The suspension (2 ml) was then added to 2 ml of the heme solution and incubated for 5 min at 25°C. An aliquot was withdrawn and the holocytochrome *c* that was formed was determined. For the aerobic samples, the chase to holocytochrome *c* was performed without anaerobic precautions. In addition, one sample contained sodium dithionite (final concentration, 1 mg/ml) during the incubation. For the anaerobic samples, the reaction was performed directly in the electrochemical cell under anaerobic conditions. Prior to adding the lysed mitochondrial preparation to one sample, the heme was electrochemically reduced.

DISCUSSION

The covalent attachment of heme to apocytochrome *c* appears to occur at the inner face of the outer mitochondrial membrane. Before heme is attached, however, apocytochrome *c* is only partially inserted through the outer membrane since bound apocytochrome *c* can be displaced with an excess of added apocytochrome *c* (20) and since it is situated in a location where it is susceptible to exogenous proteases (8, 14). The translocation of cytochrome *c* across the outer membrane to a protease-resistant location is intrinsically coupled to covalent attachment of heme since it requires NADH and can be inhibited by the heme analogue deuterohemin (8, 14). In this way, maturation events that are required for the acquisition of functional components for cytochrome *c* (heme) are of central importance to the import mechanism as well.

We have shown here that cytochrome *c* heme lyase, which catalyzes the covalent linkage of heme to apocytochrome *c*, requires heme in the reduced state. *In vitro*, heme reduction can occur in the presence of NADH plus a flavin nucleotide. How and where does this reduction step occur *in vivo*? One possibility is that a specific reductase exists in mitochondria that reduces heme immediately prior to the cytochrome *c* heme lyase reaction. Another possibility is that cytochrome *c* heme lyase utilizes newly synthesized heme, which would probably be transiently in the reduced state since the final reaction in heme synthesis—namely, the insertion of iron into the porphyrin ring by the enzyme ferrochelatase—requires ferrous ions (21). The latter alternative does not appear to be the case for the *in vitro* NADH/flavin nucleotide requirement since holocytochrome *c* formation was also dependent on and supported by the addition of hemin to the import mixtures (10). It remains to be determined why reduced heme is specifically required for holocytochrome *c* formation. The electronic configuration of reduced heme may facilitate the nucleophilic attack of the cysteine thiols on the vinyl side chains. Or, binding of the heme to a specific site on the heme lyase enzyme may only be possible in the reduced state.

Most interestingly, NADH is also required for the import and maturation of cytochrome c_1 (8, 22, 23). A reduction step comparable to that required for cytochrome *c* import is also necessary prior to the covalent attachment of heme to the cytochrome c_1 apoprotein (24). Reduction of heme may therefore be a central requirement for the biogenesis of several mitochondrial cytochromes.

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