Mechanism of Iron Transport to the Site of Heme Synthesis inside Yeast Mitochondria*

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The import of metals, iron in particular, into mitochondria is poorly understood. Iron in mitochondria is required for the biosynthesis of heme and various iron-sulfur proteins. We have developed an in vitro assay to follow the uptake of iron into isolated yeast mitochondria. By measuring the incorporation of iron into porphyrin by ferrochelatase in the matrix, we were able to define the mechanism of iron import. Iron uptake is driven energetically by a membrane potential across the inner membrane but does not require ATP. Only reduced iron is functional in generating heme. Iron cannot be preloaded in the mitochondrial matrix but rather has to be transported across the inner membrane simultaneously with the synthesis of heme, suggesting that ferrochelatase receives iron directly from the inner membrane. Transport of iron is inhibited by manganese but not by zinc, nickel, and copper ions, explaining why in vivo these ions are not incorporated into porphyrin. The inner membrane proteins Mmt1p and Mmt2p proposed to be involved in mitochondrial iron movement are not required for the supply of ferrochelatase with iron. Iron transport can be reconstituted efficiently in a membrane potential-dependent fashion in proteoliposomes that were formed from a detergent extract of mitochondria. Our biochemical analysis of iron import into yeast mitochondria provides the basis for the identification of components involved in transport.

The acquisition of iron by eukaryotic cells involves various transport systems that ensure the regulated uptake of this essential metal (for review, see Refs. 1–6). Most of the cellular iron is utilized within mitochondria, where it is required for the generation of heme by ferrochelatase in the matrix (7) and for several iron-sulfur cluster-containing proteins (Fe/S proteins)1 of both the matrix (e.g. aconitase and homoaconitate hydratase) and the inner membrane (e.g. Rieske Fe/S protein; see Refs. 8 and 9). The import of iron into mitochondria must be tightly regulated, as hardly any accumulation of iron is observed in mutants defective in heme biosynthesis (10). Limiting the iron content would impair the metabolic and respiratory activity of the organelle, whereas excess iron may exert toxic effects by generation of an oxidative stress through radical formation (11). Free iron ions might be particularly harmful in mitochondria, where free reactive oxygen species are generated as a side reaction of the electron transport (see, e.g. Ref. 12). Three proteins have recently been described as being important for iron homeostasis within mitochondria: the yeast ABC transporter Atm1p of the inner membrane (13, 14), and the matrix proteins frataxin (yeast Yfh1p; 15, 16) and Saq1p (17). Defects in any of these proteins cause large increases in the mitochondrial iron content. Mutations in the human homolog of Atm1p, hABC7, may cause a form of sideroblastic anemia (14, 18). Frataxin is mutated in patients with Friedreich's ataxia (19). Saq1p, a member of the 70-kDa heat shock protein family, was proposed to be required for correct processing of frataxin (17) even though recent findings indicate that this relation may be more complex (20). In any case, the distinct function of these proteins in iron homeostasis within mitochondria remains to be determined.

Despite the central importance of iron for mitochondrial biogenesis, the molecular basis of its import into the organelles is not understood. Earlier studies have attempted to investigate iron uptake into mammalian mitochondria by assessing the iron associated with the organelles (summarized in Refs. 11 and 21). Contradicting results have been achieved concerning the energy dependence of the import reaction. Although some reports found no obvious requirement for a potential across the mitochondrial inner membrane (22, 23), evidence has been presented for iron uptake occurring in both a membrane potential-dependent and an independent fashion (24). No conclusive evidence is available in what form (free, chelated, reduced, or oxidized) iron must be presented to mitochondria to be competent for import (for review, see Ref. 21). A serious shortcoming of previous reports has been the failure to localize the iron associated with mitochondria to one of the organelar compartments; i.e. it was not possible to differentiate truly imported iron from iron unspecifically associated with mitochondrial membranes. Using isolated yeast mitochondria we also failed to distinguish between imported and unspecifically bound iron. The reason for these obstacles is the avid association of iron with biological membranes.

We have developed an in vitro assay for the import of iron into the matrix of yeast mitochondria to study the biochemical mechanism of iron transport. The assay circumvents previous technical difficulties by following the uptake of iron through the generation of radioactively labeled heme from [55Fe]iron and a porphyrin substrate. This reaction is catalyzed by ferrochelatase, a protein of the mitochondrial matrix peripherally associated with the inner membrane (7, 25). Because this procedure detects only biochemically competent iron that has crossed the inner membrane, well known problems with pre-
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or with mitochondria isolated from a yeast strain (Δhem15) in which the gene of ferrochelatase has been deleted (29). This suggests that the incorporation of iron into DP did not occur spontaneously under our experimental conditions but rather was catalyzed by ferrochelatase. In keeping with this, heme formation was inhibited strongly by the addition of zinc ions, known to serve as a competitive inhibitor of the ferrochelatase enzyme activity (38, 39; see below). The integrity of the isolated mitochondria was estimated by both the activity of citrate synthase (40) and the protease accessibility of inner membrane and matrix proteins (37) and was higher than 98% (not shown). This indicates that the generation of heme in intact isolated mitochondria can be taken as a measure of iron import into mitochondria. The assay system was used to analyze the biochemical mechanism of this process.

Iron Import into Mitochondria Requires a Membrane Potential—First, we tested whether the uptake into mitochondria of the substrates of ferrochelatase requires the presence of a membrane potential. When isolated mitochondria were energized by the addition of NADH or ethanol, efficient synthesis of 55Fe-radiolabeled heme was observed (Fig. 2A). Comparatively slow synthesis of heme occurred in the absence of these compounds, which feed electrons into the respiratory chain. Hardly any heme was formed upon depleting the membrane potential by the addition of the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP; Fig. 2A). Similar results were obtained by inhibiting the formation of the membrane potential by the addition of antimycin A (inhibitor of complex III), the ionophore valinomycin, and oligomycin (inhibitor of the F1,F0-ATPase) to the reaction (not shown; see below). Thus, the transport of iron and/or DP to matrix-localized ferrochelatase requires a membrane potential in isolated mitochondria.

To define which of the substrates of ferrochelatase requires a membrane potential for import, we investigated the transport of the hydrophobic DP. To this end, we incubated mitochondria with or without DP in the presence or absence of antimycin A, valinomycin, and oligomycin. Under all conditions, DP avidly associated with mitochondria, even at 0 °C (not shown). After removal of free DP by reisolation of the mitochondria, the soluble matrix fraction was isolated and used as the source of DP in a heme synthesis assay employing purified ferrochelatase. The matrix fraction obtained from mitochondria that were incubated with DP supported 5-fold higher heme synthesis than the matrix fraction derived from mock-treated mitochondria (Fig. 2B). The stimulation of heme formation by the matrix extract was independent of the presence of a membrane potential during the import of DP into mitochondria. It appears from these data that DP can be imported into the mitochondrial matrix independently of a membrane potential. Together with data presented below, we conclude that DP can permeate the inner membrane in the absence of a potential under our experimental conditions. This suggests that the requirement of a membrane potential for in organello heme synthesis is related to the uptake of iron rather than porphyrin.

To analyze whether iron import into mitochondria requires a membrane potential in vivo, we added radiolabeled 55Fe-iron to wild-type yeast cells in the presence or absence of the uncoupler CCCP (41). After labeling for 1 h, cells were washed with citrate and EDTA to remove iron that had not been incorporated by the cells. A cell lysate was prepared by breaking yeast cells with glass beads, and a postmitochondrial supernatant and a crude mitochondrial fraction were obtained. The 55Fe-heme generated during the labeling period was extracted by n-butyl acetate and quantitated. In the presence of CCCP, only 25% of heme was formed compared with untreated cells (Fig. 3A). This strong impairment of cellular heme synthesis was not the result of defective uptake of iron into CCCP-treated cells because compared with wild-type cells even slightly higher amounts of total 55Fe-iron were observed in the cell lysate and the postmitochondrial supernatant (Fig. 3B). We also noticed in this in vivo experiment that similar amounts of radiolabeled iron associated with mitochondria independently of the existence of a membrane potential. This strengthens our view that iron association is not a reliable measure for mitochondrial import even under in vivo conditions (see above). Together, these data demonstrate a membrane potential dependence of heme synthesis in vivo, suggesting that the import of iron into mitochondria requires energized organelles.

Iron Import into Mitochondria Occurs Independently of ATP—The requirement for nucleotides, in particular for ATP, for iron import was tested with isolated mitochondria in the presence of ascorbate. No stimulatory effect on the generation of heme was observed by inclusion of 100 μM adenosine nucleotides in the assay (Fig. 4A). Higher amounts inhibited the
of all fractions were used to determine the amount of 55Fe-radiolabeled heme by extraction with 20 mM Hepes-KOH, pH 7.4. They were incubated with or without 20 μM CCCP (dissolved in dimethyl sulfoxide) for 15 min at 30 °C. Inclusion of CCCP specifically inhibited protein import into mitochondria (41). Radiolabeled 55FeCl3 (2 × 107 cpm) was added in the presence of 4 mM ascorbate, and incubation was continued for 1 h at 30 °C. Cells were washed twice with 20 mM Hepes-KOH, pH 7.4, 50 mM citrate, and 1 mM EDTA. They were resuspended in 2 ml of SoH buffer. A cell lysate was prepared by breaking yeast cells with glass beads and removing the cell debris. Mitochondrial (Mitos) and postmitochondrial supernatant (PMS) fractions were obtained by centrifugation (10 min at 15,000 × g). Aliquots of all fractions were used to determine the amount of [55Fe]iron by liquid scintillation counting (panel A) and the total content of [55Fe]iron by liquid scintillation counting (panel B). The content of radiolabeled heme or iron is given per g of wet cells.

reaction (Fig. 4B). Presumably, this inhibition is caused by chelation of iron by nucleotides because similar effects were seen upon the addition of citrate, an efficient chelating agent, or of branched chain α-keto acids, which serve as siderophores in certain bacteria (Fig. 4A and Ref. 42). To test the requirement for intramitochondrial ATP, mitochondria were treated with apyrase before assaying for the synthesis of radiolabeled heme (43). Depletion of ATP by apyrase had no effect on heme synthesis, also when new synthesis of ATP by the F,Fo-ATP synthase was precluded by adding the inhibitor oligomycin (Fig. 4B). Thus, neither extramitochondrial nor internal ATP is required for the uptake of iron into mitochondria. Likewise, the addition of ADP and AMP does not appear to stimulate the transport of iron to ferrochelatase, as this has been reported earlier for the association of iron with mammalian mitochondria (22, 44).

Iron Must Be Reduced before Transport to Ferrochelatase—In our standard assay using isolated mitochondria energized with NADH, iron is maintained in a reduced state by the addition of ascorbate. When this reducing agent was omitted, hardly any radiolabeled heme was generated by isolated mitochondria (Fig. 5A). Efficient synthesis of heme could be restored when iron was reduced by inclusion of dithiothreitol or reduced glutathione in the assay. Both reagents were capable of reducing iron in vitro (not shown). The requirement for reduction of iron could not be replaced by the inclusion of various chelating compounds such as adenine nucleotides, α-keto acids, or citrate, which prevent the precipitation of ferric iron out of solution (Fig. 4A, Ascorbate).

In the absence of NADH as a substrate of the electron transport chain, isolated mitochondria synthesized about 5-fold less heme compared with the presence of NADH, presumably because of a residual membrane potential (Fig. 5B; see also Fig. 2A). Similar to the reaction in fully energized mitochondria, synthesis of heme required the reduction of iron by the addition of ascorbate, dithiothreitol, or reduced glutathione (Fig. 5B). The reduction of iron could not be performed by isolated mitochondria themselves even when they were energized with the reducing compound NADH. A comparable requirement for reduction of iron by ascorbate, dithiothreitol, reduced glutathione, or sodium dithionite was observed under anaerobic conditions using mitochondria that were energized by the addition of 0.1 mM ATP (not shown). In support of these findings, we found relatively weak inhibition of heme formation by adding the chelator desferrioxamine, which binds ferric iron with an affinity constant of 1020 (Fig. 5C). This indicates that it is not ferric iron that is transported across the mitochondrial inner membrane. We conclude from these observations that iron has to be in a reduced state before its uptake into mitochondria to be
competent in the ferrochelatase reaction.

Iron Is Supplied to Ferrochelatase from the Inner Membrane—We tested whether iron can be preloaded into isolated mitochondria before starting the ferrochelatase reaction by the addition of DP. We took advantage of the fact that the import of iron can be inhibited efficiently by the addition of EDTA, a chelator that is not capable of entering mitochondria (Fig. 6A, left). [55Fe]Iron was incubated with energized mitochondria for 4 min, before further import of iron was inhibited by chelating with EDTA those iron ions that were still outside of the organelles. When the reaction was supplemented with DP, and samples were incubated further, no significant heme formation was observed compared with the simultaneous addition of iron and DP (Fig. 6A, right). Thus, iron cannot be imported into mitochondria before the addition of DP. We conclude from these data that iron present in the mitochondrial matrix does not form a pool that can be used by ferrochelatase. Rather, iron is supplied to ferrochelatase directly from the inner membrane without transient passage across the matrix.

When DP was preincubated with mitochondria in the absence of iron, and excess DP was removed by reisolation of the organelles before the addition of [55Fe]iron, the amounts of radiolabeled heme generated were similar to those in the one-step incubation (Fig. 6B). The initial association of DP with mitochondria did not require the presence of a membrane potential across the inner membrane (Fig. 6B; see above). This supports our view that DP can associate with mitochondria in a manner that is competent for the generation of [55Fe]heme upon the subsequent addition of radiolabeled iron.

The Putative Mitochondrial Metal Transporters Mmt1p and Mmt2p Are Not Required for Delivery of Iron to Ferrochelatase—Transport of iron into yeast mitochondria has been suggested to involve two highly homologous proteins of the inner membrane, Mmt1p and Mmt2p (45). To analyze whether these putative metal transporters mediate the delivery of iron to ferrochelatase, we constructed a yeast mutant in which both the MMT1 and MMT2 genes were deleted. These mutant cells (strain Δmmt1Δmmt2) displayed hardly any growth defect on standard media (45; not shown). Mitochondria isolated from the mutant cells contained similar amounts of cytochromes and harbored the same activity of the mitochondrial Fe/S protein aconitase as did wild-type cells (not shown). Mutant mitochondria showed wild-type activity in the generation of [55Fe]-radiolabeled heme (Fig. 7). We conclude that Mmt1p and Mmt2p are not required for the transport of iron to ferrochelatase.

Transport of Iron to Ferrochelatase Can Be Reconstituted in Proteoliposomes—We attempted to dissolve the mitochondrial membranes in detergent and reconstitute proteoliposomes that faithfully harbor the activity to generate heme. To this end, mitochondria were lysed in dodecyl-maltoside, and aggregated material was removed by ultracentrifugation. The clarified extract was supplemented with a phospholipid mixture, diluted below the critical micellar concentration, and the proteoliposomes formed were reisolated. They were capable of synthesizing ATP upon the generation of a membrane potential by NADH (not shown), i.e. the components of oxidative phosphorylation were at least partially reintegrated into the lipid bilayer in a functional fashion. When the proteoliposomes were tested for their ability to generate heme from added [55Fe]iron and DP, they synthesized heme at almost the same efficiency as intact mitochondria (Fig. 8). The generation of radiolabeled heme was dependent upon the addition of NADH (not shown) and was inhibited by antimycin A or CCCP (Fig. 8), i.e. iron import required a membrane potential in a manner similar to that observed with intact mitochondria. In contrast, by using the supernatant of the reconstitution procedure (Fig. 8, middle panel) or purified ferrochelatase (not shown), no effects of antimycin A or CCCP were detectable for the synthesis of heme.

The Specificity of Iron Transport Precludes Access of Other Metals to Ferrochelatase—We investigated the metal specificity of the iron transport reaction by comparing the metal ion
inhibition of heme synthesis by intact mitochondria and by free ferrochelatase. Submicromolar concentrations of zinc ions strongly inhibited heme synthesis by free or purified ferrochelatase (see Ref. 38) but hardly affected the reaction occurring in intact mitochondria (Fig. 9A). Only at higher concentrations of zinc was the reaction in intact mitochondria inhibited (see Fig. 1B). Control samples contained 1 mM EDTA from the beginning of the reaction. Heme formed in the one-step reaction in the absence of EDTA was set as 100%. Panel B, heme formation was performed as in panel A (One Step). For the two-step reaction, 100 µg of mitochondria was first incubated with 1 µM DP in 500 µl of buffer A for 5 min at 25 °C. Mitochondria were reisolated by centrifugation (10 min, 15,000 × g, 2 °C), washed once with buffer A, and resuspended in 500 µl of buffer A containing 2 mM NADH. After 2 min, the iron import was started by the addition of 1 mM ascorbate and 0.2 µM 55FeCl3. Samples were incubated for 5 min at 25 °C. Further treatment was as in panel A. Heme formed in the one-step reaction was set as 100%.

FIG. 6. Iron reaches ferrochelatase without transient passage across the matrix. Panel A, the synthesis of 55Fe-radiolabeled heme was performed for 5 min at 25 °C as described in Fig. 1B (One Step). In other samples (Two Steps) mitochondria were energized with 2 mM NADH in 500 µl of buffer A for 2 min at 25 °C. 1 mM ascorbate (Asc) and 0.2 µM 55FeCl3 were added, and samples were incubated for 4 min at 25 °C. Further import of iron was prevented by chelation with 1 mM EDTA followed by incubation for 1 min. Synthesis of heme was started by the addition of 1 µM DP. Samples were incubated for 5 min at 25 °C; the reaction was stopped, and heme was quantitated as described in Fig. 1B. Control samples contained 1 mM EDTA from the beginning of the reaction. Heme formed in the one-step reaction in the absence of EDTA was set as 100%. Panel B, heme formation was performed as in panel A (One Step). For the two-step reaction, 100 µg of mitochondria was first incubated with 1 µM DP in 500 µl of buffer A for 5 min at 25 °C. Mitochondria were reisolated by centrifugation (10 min, 15,000 × g, 2 °C), washed once with buffer A, and resuspended in 500 µl of buffer A containing 2 mM NADH. After 2 min, the iron import was started by the addition of 1 mM ascorbate and 0.2 µM 55FeCl3. Samples were incubated for 5 min at 25 °C. Further treatment was as in panel A. Heme formed in the one-step reaction was set as 100%.

FIG. 7. The putative mitochondrial metal transporters Mmt1p and Mmt2p are not required for the transport of iron to ferrochelatase. Heme synthesis was measured and quantitated as described in Fig. 1B using mitochondria isolated from wild-type and Δmmt1Δmmt2 yeast cells. Data are given as the amount of [55Fe]heme formed/mg of mitochondrial protein/min.

FIG. 8. Import of iron can be reconstituted in proteoliposomes in a membrane potential-dependent fashion. Mitochondria (10 mg/ml) were solubilized in buffer B (40 mM Hepes-KOH, pH 7.4, 100 mM KCl) containing 1% (w/v) dodecyl-maltoside for 20 min at 0 °C. The solution was centrifuged for 1 h at 100,000 × g to remove aggregated material. Before a 33-fold dilution with buffer B, a phospholipid mixture from sheep brain (1 mg/ml final concentration; Sigma) was added to the clarified supernatant. The formed proteoliposomes were collected by centrifugation (100,000 × g, 30 min). Aliquots of the pellet and the supernatant (equivalents of 100 µg of mitochondrial protein) or 100 µg of intact mitochondria (in buffer A) were used to measure the synthesis of [55Fe]heme as described in Fig. 1B. Where indicated, the membrane potential was depleted by addition of 8 µM antimycin A or 40 µM CCCP. The concentration of NADH was 0.2 mM for heme synthesis in proteoliposomes. The data were normalized to heme synthesis observed with intact mitochondria in the absence of inhibitors.
obtained using purified ferrochelatase (not shown). The reactions were formation by intact mitochondria and free ferrochelatase. 

supernatant of the reconstitution procedure described in Fig. 8 ( synthesis was measured as in Fig. 1). Results similar to those with free ferrochelatase were obtained using purified ferrochelatase (not shown). The reactions were performed in the presence of the indicated amounts of ZnCl₂ (panel A) or MnCl₂ (panel B). 

Differential effects of zinc and manganese ions on heme formation by intact mitochondria and free ferrochelatase. Heme synthesis was measured as in Fig. 1B using intact mitochondria or the supernatant of the reconstitution procedure described in Fig. 8 (Free ferrochelatase). Results similar to those with free ferrochelatase were obtained using purified ferrochelatase (not shown). The reactions were performed in the presence of the indicated amounts of ZnCl₂ (panel A) or MnCl₂ (panel B).

FIG. 9. Differential effects of zinc and manganese ions on heme formation by intact mitochondria and free ferrochelatase. Heme synthesis was measured as in Fig. 1B using intact mitochondria or the supernatant of the reconstitution procedure described in Fig. 8 (Free ferrochelatase). Results similar to those with free ferrochelatase were obtained using purified ferrochelatase (not shown). The reactions were performed in the presence of the indicated amounts of ZnCl₂ (panel A) or MnCl₂ (panel B).

Iron import into isolated mitochondria was dependent on a membrane potential as an energy source. A similar requirement for energized mitochondria was observed in vitro. This appears to be the only driving force, because iron import occurred independently of adenine nucleotides both inside and outside of mitochondria. It is therefore unlikely that the transport of iron is mediated by a transport ATPase. Further studies are needed to define whether the electrochemical or the pH gradient at the inner membrane drives import of iron. 

Only the reduced (ferrous) form of iron was found to be competent for transport to ferrochelatase. Iron could not be reduced by isolated mitochondria even when the organelles were supplied with NADH and thus exhibited a high reducing capacity. Transport of reduced iron to ferrochelatase across the inner membrane might provide a mechanistic advantage because ferrochelatase is known to utilize specifically ferrous iron for incorporation into the porphyrin ring (38). Thus, ferrochelatase appears to be supplied with the proper iron substrate. 

Iron preloaded into mitochondria does not serve as a pool for ferrochelatase activity. Rather, iron had to be delivered to ferrochelatase directly from the inner membrane to be competent in heme synthesis. It seems unlikely that iron, upon entry into the matrix, becomes rapidly incompetent, e.g., by oxidation, because the mitochondrial matrix represents a reducing environment. We therefore propose that ferrous iron reaches the active site of ferrochelatase directly after its passage across the inner membrane without transient movement through the matrix. This raises the interesting question of whether the transport of this metal across the inner membrane provides a clear explanation for this problem. In contrast to what is found for these metals, manganese ions efficiently inhibit heme synthesis in organello at concentrations that do not impair soluble ferrochelatase. Thus, manganese ions interfere directly with iron transport. However, inhibition occurs only at rather high concentrations that may not elicit any significant inhibition of iron transport to ferrochelatase in vivo.

Heme synthesis by intact mitochondria and by ferrochelatase in solution displays differential sensitivities to various metal ions. Zinc, copper, and nickel do not appreciably inhibit ferrochelatase function in intact organelles at concentrations that block free ferrochelatase almost completely. Apparently, the iron transporter of the mitochondrial inner membrane is highly selective and efficiently excludes zinc, copper, and nickel ions from being transferred to ferrochelatase. Zinc has long been known to represent a strong competitive inhibitor of free ferrochelatase because it becomes incorporated into the porphyrin ring instead of iron (38, 39). An unsolved question therefore was how the formation of zinc protoporphyrin IX is precluded in vivo. The observed selectivity of iron transport across the mitochondrial inner membrane provides a clear explanation for this problem. In contrast to what is found for these metals, manganese ions efficiently inhibit heme synthesis in organello at concentrations that do not impair soluble ferrochelatase. Thus, manganese ions interfere directly with iron transport. However, inhibition occurs only at rather high concentrations that may not elicit any significant inhibition of iron transport to ferrochelatase in vivo.

The rate of heme synthesis in our in vitro experiments is about 1.5 nmol of heme/mg of mitochondrial protein/h. Yeast cells contain about 100 nmol of total heme/g of dry weight (7, 46). Assuming that 30% of dry weight corresponds to protein and 1/10 of that represents mitochondrial protein, a total of 3.3 nmol of heme/mg of mitochondrial protein has to be synthesized per generation time. With an average doubling time of 2 h, the estimated in vivo heme requirement of a growing yeast cell (about 1.7 nmol of heme/mg of mitochondrial protein/h) nicely matches the amounts of heme produced in our in vitro experiments. Clearly, our experimental system using isolated mitochondria faithfully and efficiently reproduces the in vivo reaction. Previous studies have suggested that ferrochelatase is present in yeast cells in amounts exceeding the needs of a living cell (7, 46). Our studies on detergent solubilization and reconstitution of heme synthesis confirm these observations and provide an explanation for the previous measurements. A 5-fold higher enzyme activity was observed using solubilized (free) ferrochelatase compared with the reaction in intact organelles. Apparently, direct access of iron to ferrochelatase results in significantly higher activity in heme synthesis.

DISCUSSION

The goal of the present communication is a mechanistic characterization of iron import into yeast mitochondria. We have used the in organello synthesis of heme to monitor the transport of this metal across the inner membrane thereby avoiding well known problems resulting from the unspecific and membrane potential-independent association of iron with mitochondria. Despite the increased complexity of the assay involving the transport of both iron and porphyrin, it was possible to define the requirements for iron transport. This was due to the observation that porphyrin is transported across the mitochondrial membranes in a spontaneous fashion. Porphyrin accumulated within mitochondria at 0 °C in the absence of a membrane potential and could be provided for heme synthesis before iron was added. Apparently, under our in vitro conditions this compound is able to permeate the membranes by virtue of its hydrophobicity.

Iron import into isolated mitochondria was dependent on a membrane potential as an energy source. A similar requirement for energized mitochondria was observed in vitro. This appears to be the only driving force, because iron import occurred independently of adenine nucleotides both inside and outside of mitochondria. It is therefore unlikely that the transport of iron is mediated by a transport ATPase. Further studies are needed to define whether the electrochemical or the pH gradient at the inner membrane drives import of iron.

Only the reduced (ferrous) form of iron was found to be competent for transport to ferrochelatase. Iron could not be reduced by isolated mitochondria even when the organelles were supplied with NADH and thus exhibited a high reducing capacity. Transport of reduced iron to ferrochelatase across the inner membrane might provide a mechanistic advantage because ferrochelatase is known to utilize specifically ferrous iron for incorporation into the porphyrin ring (38). Thus, ferrochelatase appears to be supplied with the proper iron substrate.

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results suggest that iron import is rate-limiting for heme formation in intact mitochondria.

Two proteins of the inner membrane, Mmt1p and Mmt2p, have been claimed to function as mitochondrial iron transporters (45). However, transport has not been measured directly. Our quantitative analysis of heme synthesis in mitochondria isolated from Δmmt1Δmmt2 cells shows that these proteins are not required for the transport of iron to ferrochelatase. These data fit well the observation of unchanged amounts of cytochromes in these cells relative to wild-type cells. Our study does not experimentally address the question of whether these two proteins might be involved in the transport of iron into the matrix to provide iron for the biogenesis of Fe/S proteins such as aconitase. This seems unlikely though because the activity of mitochondrial aconitase was unchanged upon simultaneous deletion of the MMT1 and MMT2 genes (not shown). Together, these data demonstrate that Mmt1p and Mmt2p are not essential for iron import into mitochondria.

Iron transport into mitochondria can be reconstituted efficiently in proteoliposomes. As found with intact mitochondria, the reaction is dependent on a membrane potential and requires reduced iron. The possibility of dissolving the mitochondrial membranes in detergent and forming proteoliposomes that retain the activity of transporting iron across the lipid bilayer breaks the grounds for isolation and characterization of the components involved in this process. Our biochemical characterization of the mechanism of iron transport into mitochondria represents a crucial step toward better understanding of mitochondrial iron homeostasis. Our study will also facilitate the elucidation of the function of the ABC transporter Atm1p and of frataxin in maintaining proper mitochondrial iron levels.

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