The disulfide relay system of mitochondria is connected to the respiratory chain

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All proteins of the intermembrane space of mitochondria are encoded by nuclear genes and synthesized in the cytosol. Many of these proteins lack presequences but are imported into mitochondria in an oxidation-driven process that relies on the activity of Mia40 and Erv1. Both factors form a disulfide relay system in which Mia40 functions as a receptor that transiently interacts with incoming polypeptides via disulfide bonds. Erv1 is a sulfhydryl oxidase that oxidizes and activates Mia40, but it has remained unclear how Erv1 itself is oxidized.

Introduction

The biogenesis of proteins requires the folding of newly synthesized polypeptide chains. In vivo, this process is assisted by molecular chaperones that control the folding of their client proteins in an energy-dependent reaction (for reviews see Hartl and Hayer-Hartl, 2002; Bukau et al., 2006). In the cytosol of bacteria and eukaryotes most of these chaperones rely on the hydrolysis of ATP as an energy source. In the periplasmic space of bacteria and the ER of eukaryotes, chaperone systems exist that stabilize the three-dimensional structure of their client proteins by oxidation, i.e., by the controlled formation of disulfide bridges between cysteine residues of the proteins (Collet and Bardwell, 2002; Kadokura et al., 2003; Ellgaard, 2004; Gross et al., 2004; Ellgaard and Ruddock, 2005). In the bacterial periplasm, two components are critical for protein oxidation, DsbA and DsbB. DsbA is a soluble protein that directly interacts with the client proteins and transfers the electrons to the membrane protein DsbB. DsbB transfers the electrons further via the ubiquinone pool and the electron transport chain of the inner membrane to molecular oxygen from which water is then finally produced (Kobayashi et al., 1997; Bader et al., 1999).

Recently, the intermembrane space (IMS) of mitochondria was found to harbor a large number of proteins containing disulfide bonds (for reviews see Herrmann and Hell, 2005; Koehler et al., 2006; Herrmann and Koll, 2007). Like in the periplasm of bacteria, proteins in the IMS of mitochondria are efficiently oxidized by a specific redox mechanism that is structurally not related to the bacterial system. The IMS contains two components, Mia40 and Erv1, that are essential for protein oxidation and the viability of eukaryotes (Lisowsky, 1994; Lange et al., 2001; Chacinska et al., 2004; Naoe et al., 2004; Hofmann et al., 2005; Terziyska et al., 2005). Mia40 functions as an import receptor in the IMS. It transiently interacts with newly imported polypeptides, thereby converting them from a reduced and import-competent state to an oxidized, stably folded one (Allen et al., 2005; Mesecke et al., 2005; Rissler et al., 2005; Tokatlidis, 2005). During this interaction, Mia40 is reduced but subsequently reoxidized by the sulfhydryl oxidase Erv1 (also named augmenter of liver regeneration [ALR] in humans). Erv1 is a flavin adenine dinucleotide–binding protein that in vitro can directly pass its electrons on to molecular oxygen, giving rise to the production of hydrogen peroxide (Lee et al., 2000). Interestingly, Farrell and Thorpe (2005) showed that, at least in vitro, the human homologue of Erv1, ALR, is able to reduce oxidized cytochrome c. This observation led to the interesting hypothesis that the mitochondrial disulfide relay system might, like that of bacteria, interact with the electron transport chain of the inner membrane (Allen et al., 2005).

Here, we show that Erv1 passes its electrons on to molecular oxygen via interaction with cytochrome c and cytochrome c oxidase. This connection to the respiratory chain increases the efficient oxidation of the relay system in mitochondria and prevents the formation of toxic hydrogen peroxide. Thus, analogous to the system in the bacterial periplasm, the disulfide relay in the intermembrane space is connected to the electron transport chain of the inner membrane.

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Abbreviations used in this paper: ALR, augmenter of liver regeneration; IMS, intermembrane space.

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cytochrome c. As a consequence, the import of proteins into the IMS via the disulfide relay system depends on the energetic state of the respiratory chain. We show that this connection to cytochrome c not only facilitates efficient reoxidation of Mia40 but also prevents the formation of potentially harmful hydrogen peroxide in the IMS of mitochondria.

**Results and discussion**

**The redox state of Mia40 is influenced by the oxygen concentration**

Reduced and oxidized forms of Mia40 can be easily separated on nonreducing SDS gels (Mesecke et al., 2005). This can be used to monitor directly the functionality of the disulfide relay system in isolated yeast mitochondria. In wild-type mitochondria ~80% of the endogenous Mia40 is present in the oxidized, active form (Fig. 1 A). When the levels of Erv1 in the mitochondria are down-regulated, Mia40 is shifted to its reduced form, which shows a lower mobility on SDS-PAGE. Conversely, when the protein levels of Erv1 are up-regulated, basically all Mia40 is oxidized. Thus, the activity of Erv1 influences the redox state of Mia40.

Molecular oxygen was shown to serve as the final electron acceptor of Ero1 (Tu and Weissman, 2002). To test whether the redox relay in the mitochondrial IMS is influenced by oxygen, we assessed the redox state of Mia40 under oxygen-depleted and -saturated conditions at different glutathione concentrations. The precise glutathione concentration in the IMS is not known but the large diffusion limit of the porin channels in the outer membrane (~5,000 D) should lead to similar levels of glutathione as in the cytosol, which in yeast is ~13 mM (Ostergaard et al., 2004). In the experiment shown in Fig. 1 (B and C), we assessed the redox state of Mia40 at glutathione concentrations from 0 to 40 mM. Under oxygen-depleted conditions, Mia40 is significantly more susceptible to reduction by glutathione than under oxygen-saturated conditions. It should be mentioned that the oxygen-depleted conditions used were ~5–10% of fully saturated oxygen levels (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200707123/DC1). This is equivalent to physiological oxygen concentrations in animal mitochondria and still allows respiration (Fig. S2). In summary, the oxygen concentration in mitochondria has a direct influence on the redox state of Mia40, suggesting that in the mitochondrial disulfide relay system molecular oxygen serves as the final electron acceptor.

**The redox state of Mia40 depends on the activity of respiratory chain complexes**

To address the dependency of the disulfide relay system on enzymes of the respiratory chain, Mia40 redox states were examined in mitochondria of different yeast mutants. The respiratory chain of yeast mitochondria (Fig. 2 A) contains two proton-pumping enzymes, cytochrome c reductase (complex III) and cytochrome c oxidase (complex IV). Mitochondria were isolated from yeast strains lacking activity of either cytochrome c reductase (Fig. 2 A, Δcyt1 and Δcor1) or oxidase (Δcox19 and Δcox23). In addition, mitochondria were prepared from a strain in which both mitochondrial cytochrome c isoforms (Fig. 2 A, Δcyt1/Δcyt7) were simultaneously deleted as well as from mutants lacking Atp1 or Atp10, respectively, which are subunits of the F,F1 ATPase. We observed that the different mutants affected the redox state of Mia40 in different directions; in mitochondria devoid of cytochrome c or cytochrome c oxidase activity, Mia40 was considerably less oxidized than in wild-type mitochondria (Fig. 2 A). This suggests that oxidized cytochrome c stimulates oxidation of Mia40. In contrast, the loss of cytochrome c reductase activity shifted Mia40 to its oxidized state. The respiratory activity per se was not critical as the ATPase mutants did not influence Mia40. This is consistent with a specific function of oxidized cytochrome c for oxidation of Mia40.
The Mia40 dependence on respiratory chain complexes was further studied in detail. In mitochondria of two exemplary yeast strains lacking activity of cytochrome c reductase (Δcyt1) or oxidase (Δcox19) the influence of externally added glutathione on the redox state of Mia40 was tested (Fig. 2, B and C). In wild-type mitochondria Mia40 remained largely oxidized even in the presence of up to 30 mM of reduced glutathione. The loss of cytochrome c reductase activity even slightly increased the levels of oxidized Mia40. Conversely, in Δcox19 mitochondria, Mia40 was rapidly reduced when glutathione was added, and at glutathione concentrations >15 mM virtually no oxidized Mia40 protein was found. This again points to a direct influence of the respiratory chain on the redox state of Mia40.

To exclude side effects in the various mutants, we tested the effect of inhibitors of the respiratory chain on the redox state of Mia40. To this end, wild-type mitochondria were incubated in the presence of 25 mM glutathione and subjected to antimycin A or potassium cyanide, which completely inhibit cytochrome c reductase and oxidase activity, respectively. The addition of antimycin A increased the fraction of oxidized Mia40, whereas inhibition of cytochrome c oxidase by cyanide led to a decrease of the oxidized form (Fig. 2 D). These shifts were not found in mutants lacking cytochrome c (Fig. 2 E). This again indicates that cytochrome c oxidase activity influences the state of Mia40.

However, it should be noted that even in the absence of cytochrome c or cytochrome c oxidase activity, Mia40 is not completely reduced, and thus cytochrome c and cytochrome c oxidase are not essential for the oxidation of Mia40 under the conditions tested.

Yeast Erv1 interacts with cytochrome c
It has been shown that in vitro cytochrome c can function as an electron acceptor for ALR (Farrell and Thorpe, 2005). In general cytochrome c accepts electrons in vitro from a variety of sulfhydryl oxidases, including those that are not present in mitochondria. However, Erv1 and cytochrome c are both located in the mitochondrial IMS, making a direct interaction both feasible and physiologically reasonable. Because the direct transfer of electrons by sulfhydryl oxidases to molecular oxygen yields hydrogen peroxide, a physiological interaction with cytochrome c might protect the cell against oxidative damage. First, we tested whether yeast Erv1 is able to interact with cytochrome c in vitro like its human homologue. To this end, we incubated Erv1 and oxidized cytochrome c with DTT, which serves as a substrate for Erv1 (Lee et al., 2000; Levitan et al., 2004), and measured the reduction of cytochrome c in a spectrophotometer at 550 nm. In the presence of Erv1, cytochrome c was efficiently reduced (Fig. 3 A, squares). This was not caused by a direct
explicitly, we assessed the redox states of Mia40 in wild-type or more a certain degree. To test the relevance of cytochrome c oxidized in vivo even in the absence of cytochrome c, fractions of Mia40 were separated by nonreducing SDS-PAGE and analyzed. All samples were treated with iodoacetamide. Oxidized and reduced fractions of Mia40 were separated by nonreducing SDS-PAGE and analyzed.

In the wild type, the addition of 7.5 mM glutathione affected the long as the oxygen concentration is high. Mia40 also remains largely oxidized even in cytochrome c-deficient mitochondria (Fig. 3 B, left). The addition of low amounts of glutathione (7.5 mM) did not considerably influence the state of Mia40, indicating a stable state of the redox relay system as calculated and plotted against time (squares). For comparison, control samples lacking Erv1 (circles) or both Erv1 and DTT (triangles) are shown. (B) Wild-type and Δcyc1/Δcyc7 mitochondria were incubated in the presence of 7.5 mM glutathione under oxygen-saturated or -depleted conditions. All samples were treated with iodoacetamide. Oxidized and reduced fractions of Mia40 were separated by nonreducing SDS-PAGE and analyzed by Western blotting.

interaction of DTT with cytochrome c, as cytochrome c remained almost entirely oxidized when Erv1 was omitted (Fig. 3 A, circles). Thus, Erv1 can efficiently shuttle electrons from DTT to cytochrome c.

In contrast to Erv1 and Mia40, cytochrome c is not essential for the viability of yeast cells, indicating that Erv1 can be oxidized in vivo even in the absence of cytochrome c, at least to a certain degree. To test the relevance of cytochrome c more explicitly, we assessed the redox states of Mia40 in wild-type or Δcyc1/Δcyc7 double deletion mutants under oxygen-saturated and -depleted conditions. Upon saturation with atmospheric oxygen, Mia40 was largely oxidized even in cytochrome c-deficient mitochondria (Fig. 3 B, left). The addition of low amounts of glutathione (7.5 mM) did not considerably influence the state of Mia40, indicating a stable state of the redox relay system as long as the oxygen concentration is high. Mia40 also remains largely oxidized in both strains under low oxygen conditions. In the wild type, the addition of 7.5 mM glutathione affected the redox state of Mia40 only slightly (Fig. 3 B, right). In contrast, in the Δcyc1/Δcyc7 mitochondria, Mia40 was almost completely shifted to its reduced form. This suggests that cytochrome c is especially important under oxygen-limiting conditions.

Mia40-mediated protein import depends on the activity of respiratory chain complexes

Next we examined whether the variation of the redox states of Mia40 observed in respiration-deficient mitochondria affects protein import into the IMS. It has been shown that the depletion of Erv1 renders protein import highly sensitive to DTT (Mesecke et al., 2005). Import experiments were performed under oxygen-depleted conditions with mitochondria isolated from wild-type cells and respiratory chain mutants. Low amounts of DTT (2 mM) reduced protein import of Cox19 into isolated wild-type mitochondria to ~50% and almost completely blocked import into mitochondria lacking cytochrome c oxidase (Fig. 4 A, Δcox18). In contrast, import into mitochondria of a cytochrome c reductase-deficient strain (Δrip1) was less affected than in wild-type mitochondria (Fig. 4 A). Similar results were obtained with other IMS proteins such as Tim10 (unpublished data).

Import into Δcyc1/Δcyc7 mitochondria was hypersensitive to DTT similarly to import into mitochondria lacking cytochrome c oxidase activity (Fig. 4 B). Moreover, the import of Tim10 was tested in the presence of antimycin A or potassium cyanide. Blockage of cytochrome c oxidase by potassium cyanide rendered the import of Tim10 more sensitive to the addition of DTT, whereas the inhibition of cytochrome c reductase with antimycin A had the opposite effect (Fig. 4 C). In addition, the DTT sensitivity of the import correlated with the DTT sensitivity of the binding of Mia40 to the newly imported Tim10 (Fig. 4 C, Mia40 ● Tim10). This suggests that the activity of the respiratory chain complexes influences the activity of the Mia40 receptor and, as a consequence, the efficiency of protein import. In summary, the observed effects on the protein import into the IMS correlate with the redox levels of Mia40 found in the mutants: increased oxidation of cytochrome c renders protein import more resistant toward DTT, whereas lower levels of oxidized cytochrome c impair the import process.

The presence of cytochrome c prevents the production of hydrogen peroxide by Erv1

Does the interaction of Erv1 with cytochrome c really prevent the formation of hydrogen peroxide? To address this question, we developed an assay to monitor the Erv1-dependent production of hydrogen peroxide. To this end, we incubated purified recombinant Erv1 with its substrate DTT in the presence of Amplex red. This compound reacts in a 1:1 ratio with hydrogen peroxide, thereby forming resorufin, which can be easily detected by fluorescence. As shown in Fig. 5 A, mixing DTT and Erv1 led to rapid generation of hydrogen peroxide (top). Interestingly, the addition of oxidized cytochrome c considerably delayed the production of hydrogen peroxide in a dose-dependent manner (Fig. 5 A, middle and bottom). This was not caused by
a potential quenching effect of cytochrome c because the fluorescence generated by hydrogen peroxide in the presence or absence of cytochrome c was identical (Fig. 5 B). We thus conclude that electron transfer from Erv1 to cytochrome c and cytochrome c oxidase leads to the generation of water instead of harmful hydrogen peroxide.

Conclusions
In this paper, we describe a pivotal role of the respiratory chain for the activity of the disulfide relay system in the IMS of mitochondria. Based on our observations we propose a physical interaction of Erv1 with cytochrome c (Fig. 5 C). This interaction directly connects the redox relay system to the respiratory chain that prevents the generation of hydrogen peroxide. The following observations support this model: (a) Erv1 efficiently reduces cytochrome c in vitro; (b) in the absence of oxidized cytochrome c, Mia40 is shifted to its reduced state; (c) the increase of oxidized cytochrome c increases the oxidized form of Mia40; (d) cytochrome c prevents the Erv1-dependent formation of hydrogen peroxide; and (e) protein import in the absence of cytochrome c or cytochrome c oxidase is hypersensitive to DTT.

Although oxidized cytochrome c clearly facilitated oxidation of Mia40, it was found to be nonessential for this process at least under the conditions tested. However, under oxygen-limiting conditions, cytochrome c efficiently prevented the reduction of Mia40 by glutathione. This suggests that the interaction of the disulfide relay to the respiratory chain might be particularly important under low-oxygen conditions. Allen et al. (2005) reported that the single deletion of the cytochrome c isoform Cyc1 impairs growth under oxygen-depleted conditions. Low oxygen concentrations are common in many tissues of animals and humans (for reviews see Nathan and Singer, 1999; Erecinska and Silver, 2001). It is very conceivable that the interaction of the disulfide relay system with the respiratory chain might be particularly important for multicellular organisms. The prevention of hydrogen peroxide production is presumably more critical for higher eukaryotes than for yeast cells.
Therefore, it will be interesting to address the physiological relevance of the Erv1–cytochrome c interaction in mammalian tissues in the future.

Materials and methods

Yeast strains
Saccharomyces cerevisiae yeast strains Δrip1, Δcox18, Δatp10, and Δcyc1/Δcyc7 were isogenic to the wild-type strain W303 (Mat α, ade2-1, his3-11,15, leu2-3,112, trp1-1, and ura3-1; Sherman et al., 1986). The cytochrome c double deletion strain was provided by A. Barrientos (University of Miami, Miami, FL). Other mutants used in this study (Δcor1, Δcry1, Δcox19, and Δcox23) and the corresponding wild-type BY4742 (Mat α, his3Δ1, leu2Δ10, lys2Δ0, and ura3Δ0) were obtained from the yeast deletion collection (Winzeler et al., 1999). All strains were grown in YP medium consisting of 10 g/liter of yeast extract and 20 g/liter of peptone adjusted to pH 5.5, to which 2% galactose was added. The GAL-ERV1 mutant (Mesecke et al., 2005) was grown in liquid lactate medium (Herrmann et al., 1994) in the presence of 0.1% glucose or galactose to repress or induce the GAL10 promoter, respectively. Mitochondria were isolated as described previously (Herrmann et al., 1994).

Analysis of the redox state of Mia40 and protein import
Mitochondria were incubated for 30 min at 25°C in SH buffer (0.6 M sorbitol and 20 mM Hepes/KOH, pH 7.2). Depending on the experiments, glutathione, 10 μM KCN, or 100 μg/ml antimycin A were added as indicated. Samples were diluted 15-fold into SH buffer containing an excess of 100 mM iodoacetamide to trap free thiol groups. After incubation for 30 min, mitochondria were reisolated and lysed in a nonreducing sample buffer. For oxygen-depleted conditions, experiments were performed in a nitrogen-flushed glove bag (Sekuroka; Carl Roth). All buffers used were degassed for 15 min using a water-jet vacuum pump (Carl Roth) and subsequently flushed with nitrogen. An oxygen electrode (Oxygraph; Hansatech Instruments) was
present in the glove bag to control oxygen levels that were \(\sim 5\%\) to full saturation. Western blotting signals of Mia40 were quantified using image analyzer software (AIDA; Raytest). Import of radiolabeled proteins into mitochondria was performed as described previously (Mesecke et al., 2005).

**Cytochrome c reduction by Erv1**

Recombinant Erv1 [expression plasmid provided by T. Lisowsky, University of Düsseldorf, Düsseldorf, Germany] was purified as described previously (Lee et al., 2000). Reduction of horse heart cytochrome c was measured at 550 nm in a UV/visible light spectrophotometer (Ultrospec 2100 pro; GE Healthcare). The reaction was started by addition of 2 mM DTT (GERBU Biochemicals) to 8 mM of purified Erv1 in 0.5 mM EDTA (Merck) and 50 mM potassium phosphate buffer, pH 7.4. Software (Swift II; GE Healthcare) was used for data collection and quantification.

**Reactive oxygen species measurements**

The production of hydrogen peroxide by Erv1 was measured using the fluorescence dye Amplex red (10-acetyl-3,7-dihydroxyphenoxazine) according to the manufacturer's instructions (Invitrogen). 2 mM of purified Erv1 was incubated in 100 mM potassium phosphate buffer, pH 7.4, with 50 \(\mu\)M Amplex red and 1 U/ml horseradish peroxidase (Sigma-Aldrich). Erv1 was activated with the artificial substrate DTT (Levitan et al., 2004). Cytochrome c from horse heart (Sigma-Aldrich) was added in the concentrations described. Fluorescence was recorded in a spectrofluorometer (FluoroMax-2; HORIBA Jobin Yvon) with excitation at 550 nm and emission at 610 nm using a 1-nm slit. The integration time was 600 ms and the data was collected every 600 ms.

**Online supplemental material**

Fig. S1 shows that the oxygen concentration in the oxygen-depleted conditions used in this study is equivalent to \(\sim 5\%\) to full saturation. Fig. S2 shows that these oxygen concentrations still allow respiration and growth of cells on nonfermentable carbon sources. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200707123/DC1.

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