Mitochondrial Ccs1 contains a structural disulfide bond crucial for the import of this unconventional substrate by the disulfide relay system

Dominik P. Groß[a], Caroline A. Burgard[a], Silvia Reddehase[a], Jeffry M. Leitch[b], Valeria C. Culotta[b], and Kai Hell[a]

[a]Adolf-Butenandt-Institut, Lehrstuhl für Physiologische Chemie, Ludwig-Maximilians-Universität München, 81377 München, Germany; [b]Department of Environmental Health Sciences, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD 21205

ABSTRACT **The copper chaperone for superoxide dismutase 1 (Ccs1) provides an important cellular function against oxidative stress. Ccs1 is present in the cytosol and in the intermembrane space (IMS) of mitochondria. Its import into the IMS depends on the Mia40/Erv1 disulfide relay system, although Ccs1 is, in contrast to typical substrates, a multidomain protein and lacks twin Cx_nC motifs. We report on the molecular mechanism of the mitochondrial import of *Saccharomyces cerevisiae* Ccs1 as the first member of a novel class of unconventional substrates of the disulfide relay system. We show that the mitochondrial form of Ccs1 contains a stable disulfide bond between cysteine residues C27 and C64. In the absence of these cysteines, the levels of Ccs1 and Sod1 in mitochondria are strongly reduced. Furthermore, C64 of Ccs1 is required for formation of a Ccs1 disulfide intermediate with Mia40. We conclude that the Mia40/Erv1 disulfide relay system introduces a structural disulfide bond in Ccs1 between the cysteine residues C27 and C64, thereby promoting mitochondrial import of this unconventional substrate. Thus the disulfide relay system is able to form, in addition to double disulfide bonds in twin Cx_nC motifs, single structural disulfide bonds in complex protein domains.**

Monitoring Editor
Thomas D. Fox
Cornell University

Received: Apr 4, 2011
Revised: Aug 11, 2011
Accepted: Aug 12, 2011

INTRODUCTION

Mitochondria are the major source of reactive oxygen species (ROS) within the cell. Because ROS are deleterious for cells, mechanisms of protection have evolved, such as ROS-converting enzymes. A class of these enzymes is made up of the superoxide dismutases (Sods), which catalyze the disproportionation of superoxide anions to hydrogen peroxide and oxygen (Fridovich, 1975). There are two superoxide dismutases in mitochondria, the Cu, Zn–dependent superoxide dismutase 1, which is present in the intermembrane space and in the cytosol, and the Mn-dependent superoxide dismutase 2 in the mitochondrial matrix (Weisiger and Fridovich, 1973; Sturtz *et al.*, 2001). Besides harboring a zinc and a copper ion, each monomer of the dimeric Sod1 forms one intramolecular disulfide bond (Fridovich, 1975). For its maturation in yeast, Sod1 requires the copper chaperone for Sod1 (Ccs1), which promotes the formation of the disulfide bond and the incorporation of the copper ion (Culotta *et al.*, 1997). Ccs1 is a multidomain protein of three domains containing conserved cysteine residues (Lamb *et al.*, 1999; Schmidt *et al.*, 1999; Rae *et al.*, 2001). The amino-terminal domain I of 74 residues of Ccs1 in *Saccharomyces cerevisiae* harbors a CxxC motif and has structural homology to the copper chaperone Atx1 that has the ability to bind copper ions (Pufahl *et al.*, 1997). In addition, the amino-terminal domain contains two cysteine residues at positions 27 and 64. Domain II, ranging from residue 79 to 223, has homology to Sod1 and mediates docking between Ccs1 and Sod1 through heterodimerization (Lamb *et al.*, 1999, 2000). Domain III comprises the C-terminal 26 residues harboring a CxC motif that is essential for the activation of Sod1 (Schmidt *et al.*, 1999; Lamb *et al.*, 2001; Rae *et al.*, 2001). It was suggested that the cysteine residues of this motif are required for the transfer of the copper ion and for the formation of the intramolecular disulfide bond in Sod1. Consistent with its function, Ccs1 is localized in the same subcellular compartments as

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E11-04-0296) on August 24, 2011.

Address correspondence to: Kai Hell (hell@med.uni-muenchen.de).

Abbreviations used: AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; Ccs1, copper chaperone for Sod1; DTT, dithiothreitol; GST, glutathione *S*-transferase; HA, hemagglutinin; IAA, iodoacetamide; IMS, intermembrane space; PMSF, phenylmethylsulfonylfluoride; Sod, superoxide dismutase; Tim, translocase of the inner membrane; TOM, translocase of the outer membrane.

Sod1, both in the cytosol and in the intermembrane space (IMS) of mitochondria (Sturtz *et al.*, 2001). Of interest, Ccs1 promotes the mitochondrial localization of Sod1 (Field *et al.*, 2003). Following transport of Sod1 in its reduced and unfolded form across the translocase of the outer membrane, Sod1 has been suggested to fold in the IMS by Ccs1-mediated formation of the disulfide bond and incorporation of the copper ion (Field *et al.*, 2003). Because folded Sod1 cannot pass the translocase of the outer membrane (TOM) complex, it is trapped in the IMS.

The import of Ccs1 into the mitochondrial IMS depends on the Mia40/Erv1 disulfide relay system that drives import of proteins by formation of disulfide bonds in substrate proteins (Kawamata and Manfredi, 2008; Khalimonchuk *et al.*, 2008; Reddehase *et al.*, 2009). The disulfide relay system consists of the oxidoreductase Mia40 (Tim40) and the FAD-dependent thiol oxidase Erv1 (Chacinska *et al.*, 2004; Naoe *et al.*, 2004; Terziyska *et al.*, 2005; Stojanovski *et al.*, 2008; Deponte and Hell, 2009; Koehler and Tienson, 2009; Endo *et al.*, 2010; Sideris and Tokatlidis, 2010; Riemer *et al.*, 2011). First, Mia40 uses its catalytically active intramolecular disulfide bond to form a mixed disulfide intermediate with the substrate protein and to subsequently transfer the disulfide bond to the substrate (Chacinska *et al.*, 2004; Terziyska *et al.*, 2005, 2009; Grumbt *et al.*, 2007; Banci *et al.*, 2009; Bien *et al.*, 2010). Then, reduced Mia40 is reoxidized by Erv1, which shuttles electrons via cytochrome *c* to cytochrome oxidase and molecular oxygen or to cytochrome *c* peroxidase (Allen *et al.*, 2005; Farrell and Thorpe, 2005; Mesecke *et al.*, 2005; Rissler *et al.*, 2005; Bihlmaier *et al.*, 2007; Dabir *et al.*, 2007).

The small Tim proteins required for protein sorting and cytochrome oxidase assembly factors, such as the copper chaperone Cox17, are examples of typical substrates of the Mia40/Erv1 system. These substrates are characterized by highly conserved cysteine residues that are arranged in either a twin Cx_3C motif or in a twin Cx_9C motif (Stojanovski *et al.*, 2008; Deponte and Hell, 2009; Koehler and Tienson, 2009; Sideris and Tokatlidis, 2010; Riemer *et al.*, 2011). The twin Cx_3C and Cx_9C segments are connected by two disulfide bonds between the inner and the outer pair of the cysteine residues (Curran *et al.*, 2002; Lu *et al.*, 2004; Arnesano *et al.*, 2005; Webb *et al.*, 2006). The disulfide bonds link two antiparallel helices and thereby stabilize the proteins.

Although Ccs1 is a multidomain protein and does not contain a twin Cx_nC motif, it is a substrate of the disulfide relay system (Kawamata and Manfredi, 2008; Reddehase *et al.*, 2009). However, it is not known how Ccs1 and probably other, so-far-unknown substrates without the twin Cx_nC motif are imported by the Mia40/Erv1 disulfide relay system. How do they interact with Mia40? Are disulfide bonds formed in these substrates?

Here we report on the mitochondrial import mechanism of the copper chaperone Ccs1. Although Ccs1 does not contain cysteine residues arranged in twin Cx_nC motifs, cysteines in Ccs1 are, nonetheless, important for its Mia40/Erv1–mediated import into the intermembrane space of mitochondria. In absence of the cysteine residues 27 and 64 the amount of Ccs1 was strongly decreased in mitochondria. We demonstrate that these residues form a stable structural disulfide bond in mitochondria. Mia40 interacts with cysteine residue 64 of Ccs1, generating a disulfide intermediate. Thereby, the disulfide relay system appears to allow efficient import of Ccs1 into mitochondria and to control the distribution of Ccs1 between the IMS of mitochondria and the cytosol. Enhanced Ccs1 levels then lead to an increase in the levels of active Sod1. In conclusion, the disulfide relay system not only forms double disulfide bonds in substrates harboring twin Cx_nC motifs, but it also has the ability to introduce single disulfide bonds into complex protein domains. Thus our results define a novel class of substrates of the disulfide relay system.

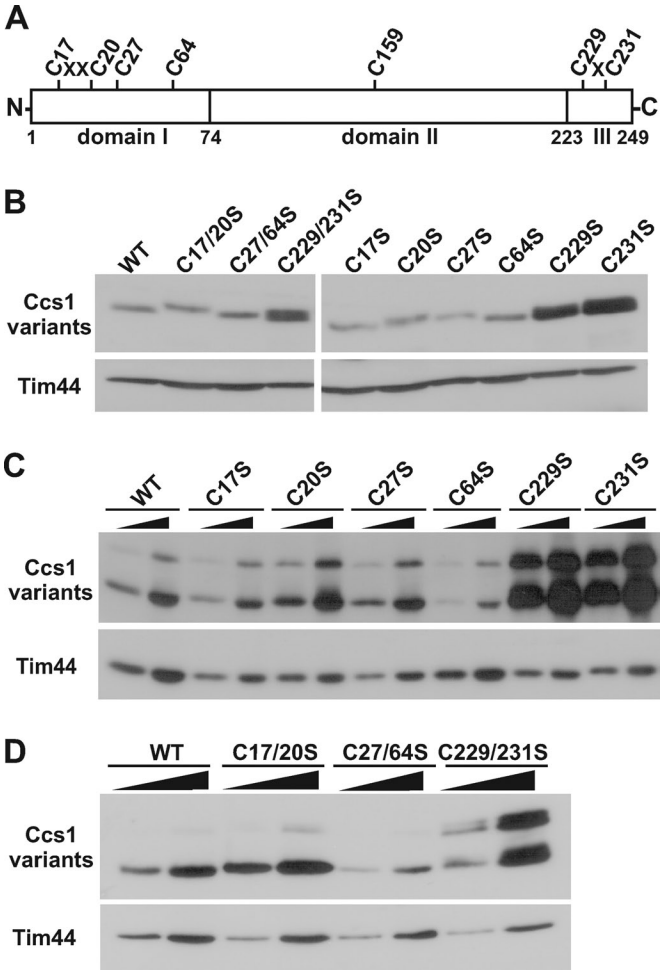

FIGURE 1: Distinct cysteine residues in Ccs1 determine the cellular localization of Ccs1. (A) Schematic overview of the domains of *S. cerevisiae* Ccs1 and of the position of its cysteine residues. III, domain III. (B) Total cell extracts were prepared from cells expressing the indicated cysteine-to-serine exchange variants and wild-type (WT) Ccs1. Cellular proteins were analyzed by SDS–PAGE and immunoblotting with antibodies against Ccs1 and Tim44. Tim44 was used as a control for equal amounts of proteins loaded. (C, D) Mitochondria (12.5, 25 µg) were isolated from cells expressing the indicated single (C) and double (D) cysteine variants of Ccs1 and WT Ccs1. Mitochondrial proteins were analyzed as described earlier. The Ccs1 proteins were expressed with two HA tags. The faster-migrating form of Ccs1 in C and D was not detectable with antibodies against the HA tag (unpublished data), suggesting that these tags are prone to proteolytic removal.

RESULTS

The mitochondrial localization of Ccs1 depends on specific cysteine residues

In *S. cerevisiae*, Ccs1 contains seven cysteine residues. As depicted in Figure 1A, four of them reside in a CxxC motif or a CxC motif, whereas the other cysteine residues are not part of a cysteine motif. We analyzed various cysteine-to-serine exchange variants for their possible roles in mitochondrial localization. The variants were expressed as fusion proteins harboring a hemagglutinin (HA) epitope tag under control of the endogenous *CCS1* promoter in cells lacking

a chromosomal copy of the *CCS1* gene. The HA epitope tag did not compromise the function of the Ccs1 proteins (unpublished data; Culotta *et al.*, 1997, Schmidt *et al.*, 1999). Their expression levels were tested in total cell extracts with antibodies against Ccs1 (Figure 1B). All variants had similar expression levels in the cell, with the exception of Ccs1-C229S, Ccs1-C231S, and Ccs1-C229/231S, which were present in increased amounts compared with wild-type Ccs1, consistent with previous results (Schmidt *et al.*, 1999). This higher expression level may reflect the role of these cysteines in copper-dependent turnover of the protein (Caruano-Yzermans *et al.*, 2006). The nonconserved C159S domain II variant behaved like the Ccs1 wild-type protein in all aspects tested and is therefore not included in the figures (unpublished data). Next the Ccs1 levels were analyzed in isolated mitochondria. Compared to wild-type Ccs1, the amounts of the C17S, C20S, and C27S variants were not significantly altered in mitochondria (Figure 1C). In contrast, the amount of the C64S variant was strongly reduced. This was consistent with results obtained analyzing the double mutants (Figure 1D). The amount of the C27/64S variant in mitochondria was reduced compared with those of wild-type Ccs1 and the C17/20S variant. As observed in total cell extracts, the single and the double variants of the cysteine residues C229 and C231 were present in higher amounts, suggesting an unaltered ratio between the cytosolic and the mitochondrial fractions compared with wild-type Ccs1. Whereas the C17/20S variant also showed an unaltered distribution between these fractions, the mitochondrial fractions of the C27/64S and the C64S variants were strongly decreased. In summary, the exchange of the cysteine residue 64 strongly affects the amount of Ccs1 in mitochondria, pointing to a crucial role of this residue in the import of mitochondrial Ccs1.

Distinct cysteine residues are required for the Mia40-dependent import of Ccs1

Next we asked whether certain cysteine residues of Ccs1 are crucial for the Mia40-dependent import. To analyze this, Ccs1 double mutants were expressed in cells harboring *MIA40* under a regulatable promoter and in corresponding wild-type cells. As observed for the wild-type Ccs1 protein, the protein levels of the C17/20S and the C229/231S variants were increased upon overexpression of Mia40 (Figure 2A). In contrast, no increase was detected for the C27/64S variant. Thus Mia40 appears not to be a limiting factor for the residual mitochondrial import of this Ccs1 variant. Next we depleted Mia40 from these cells and analyzed the effects on the mitochondrial protein levels of the Ccs1 variants. Like wild-type Ccs1, the variants C17/20S and C229/231S were present in reduced amounts in mitochondria depleted of Mia40 (Figure 2B). The amounts of the variant C27/64S were also reduced in Mia40-depleted mitochondria, albeit to a smaller extent. In summary, cysteine residues 27 and 64 of Ccs1 mediate the Mia40-dependent mitochondrial import of Ccs1.

Mia40 interacts with the cysteine residue C64 of Ccs1

Because Mia40 interacts via disulfide bonds with Ccs1, we asked which cysteine residues of Ccs1 are important for the formation of such disulfide intermediates. To this end, radiolabeled Ccs1 wild-type and variant proteins were incubated with isolated mitochondria harboring histidine (His)-tagged Mia40. Mia40 was isolated with nickel–nitrilotriacetic acid (Ni-NTA) agarose beads after lysis of the mitochondria. An intermediate of imported Ccs1 and Mia40 was isolated, as described previously (Figure 3A; Reddehase *et al.*, 2009). Whereas the intermediate was also detected upon import of the C17/20S and the C229/231S variants, it was lacking for the

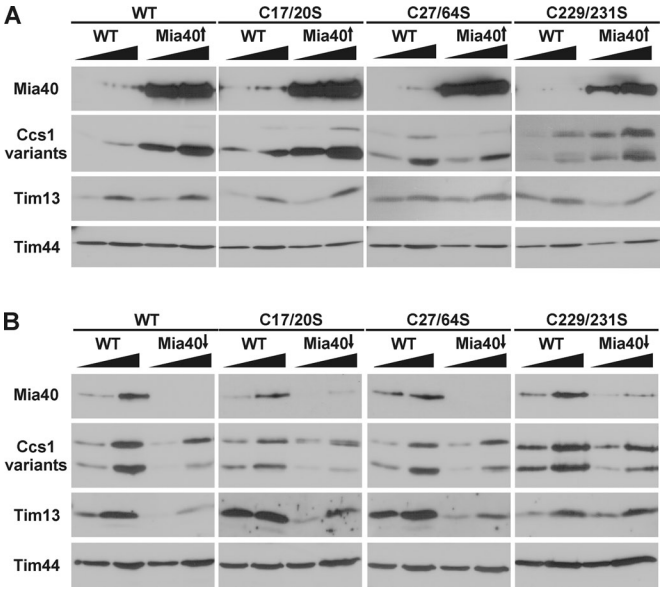

FIGURE 2: The Mia40-dependent import of Ccs1 depends on distinct cysteine residues. Mitochondria were isolated from cells (A) overexpressing Mia40 (Mia40↑) or (B) depleted of Mia40 (Mia40↓) and from the corresponding wild-type cells (WT). Isolated mitochondria, 12.5 and 25 µg, were analyzed by SDS–PAGE and immunodecoration with antibodies against the indicated proteins. Different times of exposure were chosen for the Ccs1 variants to allow best comparison of the protein levels in WT and Mia40↑, as well as in WT and Mia40↓ mitochondria. Mia40 was more than eightfold overexpressed in Mia40↑ mitochondria. On down-regulation, Mia40 was depleted to at least 10% of the amount present in wild type. The depletion was less prominent in the strain harboring the Ccs1 C229/231S variant. This might explain why the levels of the known Mia40 substrate Tim13, used as a control, were not yet decreased in this mutant, in contrast to the levels in Mia40↓ mitochondria harboring the other Ccs1 variants. However, the levels of Ccs1-C229/231S variant were already reduced. As previously reported, Tim13, present solely in mitochondria, was not affected by the increased Mia40 levels (Reddehase *et al.*, 2009). Tim44, a control for mitochondrial proteins, was present in similar amounts in all mitochondria.

C27/64S variant. In addition, we analyzed the interaction of Ccs1 variants with Mia40 in vivo. To do so, we performed a Ni-NTA pull-down experiment from mitochondria harboring a His-tagged form of Mia40, as well as the various Ccs1 variants, under nonreducing conditions. As previously reported, a covalently linked adduct of wild-type Ccs1 and Mia40 was detected in the eluate fraction (Figure 3B; Reddehase *et al.*, 2009). The same was observed for the Ccs1 variants C229/231S and C17/20S, whereas virtually no adduct with Mia40 was detected with the C27/64S variant. To determine the contribution of each of the cysteine residues at positions 27 and 64, single-cysteine variants were analyzed likewise. The formation of the disulfide adduct was extremely increased for the C27S and reduced for the C64S variant. Thus Cys-64 appears to mediate the covalent interaction with Mia40, whereas Cys-27 is dispensable for the covalent intermediate. Moreover, the disulfide intermediate was trapped in the C27S variant, obviously because cysteine residue C27 was not available to attack the intermolecular disulfide bond of Mia40 with C64, thereby converting the intermolecular disulfide bond into an intramolecular disulfide bond in Ccs1. Because a small amount of Mia40-Ccs1 intermediate was trapped in the C64S variant despite low mitochondrial levels of Ccs1 in this variant, the cysteine residue at position 27 was most likely able to mediate the

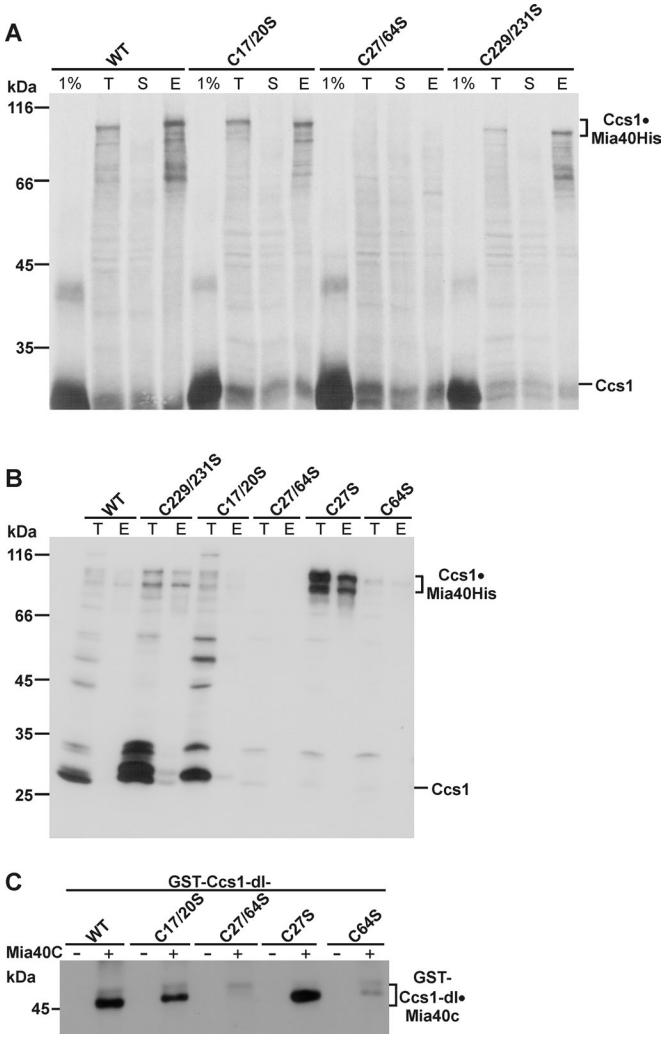

FIGURE 3: Ccs1 forms a disulfide intermediate via its cysteine residue C64 with Mia40. (A) Radiolabeled precursors of wild-type (WT) Ccs1 and Ccs1 variants were incubated with mitochondria overexpressing Mia40 with an octahistidinyl tag and subsequently treated with IAA. Mitochondria were lysed in Triton X-100–containing buffer and the supernatants were incubated with Ni-NTA agarose beads. Subsequently, beads were washed and bound proteins were eluted. Samples were analyzed by nonreducing SDS–PAGE and autoradiography. It should be noted that the import efficiency was low (<1%). The signal of imported monomeric Ccs1, therefore, could not be distinguished from the unspecific background signal obtained in the import assays. One percent of the amount of radiolabeled precursors used was loaded as input control. E, bound material (100%); S, unbound material (10%); T, total material after lysis (10%). (B) Mitochondria expressing Mia40 with an octahistidinyl tag and the indicated Ccs1 variants were treated with IAA. Mitochondria were lysed, and the extracts were incubated with Ni-NTA agarose beads. Total and bound material was analyzed by SDS–PAGE and immunodecoration with antibodies against Ccs1. (C) The GST-tagged recombinant domains I (Ccs1-dI) of the indicated Ccs1 variants were incubated together with the C-terminal fragment of Mia40 (Mia40C, amino acid residues 284–403) and treated with IAA. Samples were analyzed by nonreducing SDS–PAGE and immunodecoration with antibodies against Mia40.

interaction with Mia40 in absence of the cysteine residue 64, although obviously less efficiently. Next we confirmed the crucial role of the C64 cysteine residue for the interaction with Mia40 in vitro. To this end, we purified the first domain of Ccs1 (Ccs1-dI) and cysteine-to-serine exchange variants of domain I as glutathione *S*-transferase (GST) fusion proteins. The fusion proteins were bound to GST beads and incubated with the C-terminal fragment of Mia40. On reisolation of the beads, Mia40 was coisolated with Ccs1-dI-WT and Ccs1-dI-C17/20S, but not with Ccs1-dI-C27/64S. Mia40 was also coisolated with the Ccs1-dI-C27S variant but hardly with the Ccs1-dI-C64S variant, supporting the results obtained in vivo and in organello (Figure 3C). In summary, the cysteine residue C64 of Ccs1 is the residue that forms a disulfide intermediate with Mia40.

Ccs1 is present in an oxidized state in mitochondria

Next we asked whether mitochondrial Ccs1 contains disulfide bonds. Therefore, we determined its oxidation state using the thiol-alkylating reagent 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS). AMS binds to free thiols of cysteine residues and thereby increases the molecular mass of the protein. This results in a decrease of the migration velocity in SDS gel electrophoresis. When mitochondria were treated with AMS, slower-migrating forms of Ccs1 were observed, indicating the presence of free thiols that were modified by AMS (Figure 4A, top left, lane 2). However, not all thiols were accessible under these conditions. The detection of several modified forms of Ccs1 indicates that cysteine residues were at least partially oxidized. Moreover, some cysteine residues were present in an oxidized state, since an even slower-migrating form of Ccs1 (Ccs1 part.red.) was generated upon treatment with dithiothreitol (DTT) at room temperature (Figure 4A, top left, lane 3). However, not every disulfide bond in wild-type Ccs1 was reduced at room temperature, since another shift in migration was observed upon treatment with DTT at 95°C (Figure 4A). This slower-migrating form (Ccs1 red.) probably represents Ccs1 protein with all seven cysteine residues modified. Obviously, heat denaturation was required to open a disulfide bond, leading to the modification of two additional cysteine residues. The partially reduced Ccs1 obtained upon DTT treatment without heat denaturation appeared to be modified by five molecules of AMS because fully reduced double variants that contain only five cysteine residues showed the same migration behavior (Figure 4A). In conclusion, two cysteine residues form a disulfide bond that is very stable against reducing agents. The pattern of AMS modification of the double-mutant variants of Ccs1 provides evidence for the nature of the stable disulfide bond. The C17/20S and the C229/231S variants were still generating an additional shift upon incubation with DTT at 95°C, indicating the presence of the stable disulfide bond (Figure 4A, bottom, lanes 3 and 4). The C27/64S variant did not show an additional shift; all five cysteine residues appeared to be reduced upon DTT treatment at room temperature (Figure 4A). In conclusion, cysteine residues 27 and 64 form a stable disulfide bond. In most of the C17/20S variant proteins and, less prominently, in the C229/231S variant proteins an additional disulfide bond was observed. These bonds were redox sensitive at room temperature, as indicated by the mobility shift of these variants upon DTT treatment at 25°C. Thus, besides C27 and C64, other cysteine residues appear to be at least partially present in an oxidized form in isolated mitochondria.

To exclude redox reactions during isolation of mitochondria, cells harboring exclusively a mitochondrial form of Ccs1 were lysed under anaerobic conditions, and thiol modification experiments were performed with cell extracts. In these cells, Ccs1 and the Ccs1-C27/64S were expressed as fusion proteins with the cytochrome b_2 targeting signal (Cytb2-Ccs1), which directed the fusion proteins selectively to the mitochondrial IMS and was then proteolytically removed. In absence of reducing agent, wild-type Ccs1 was largely

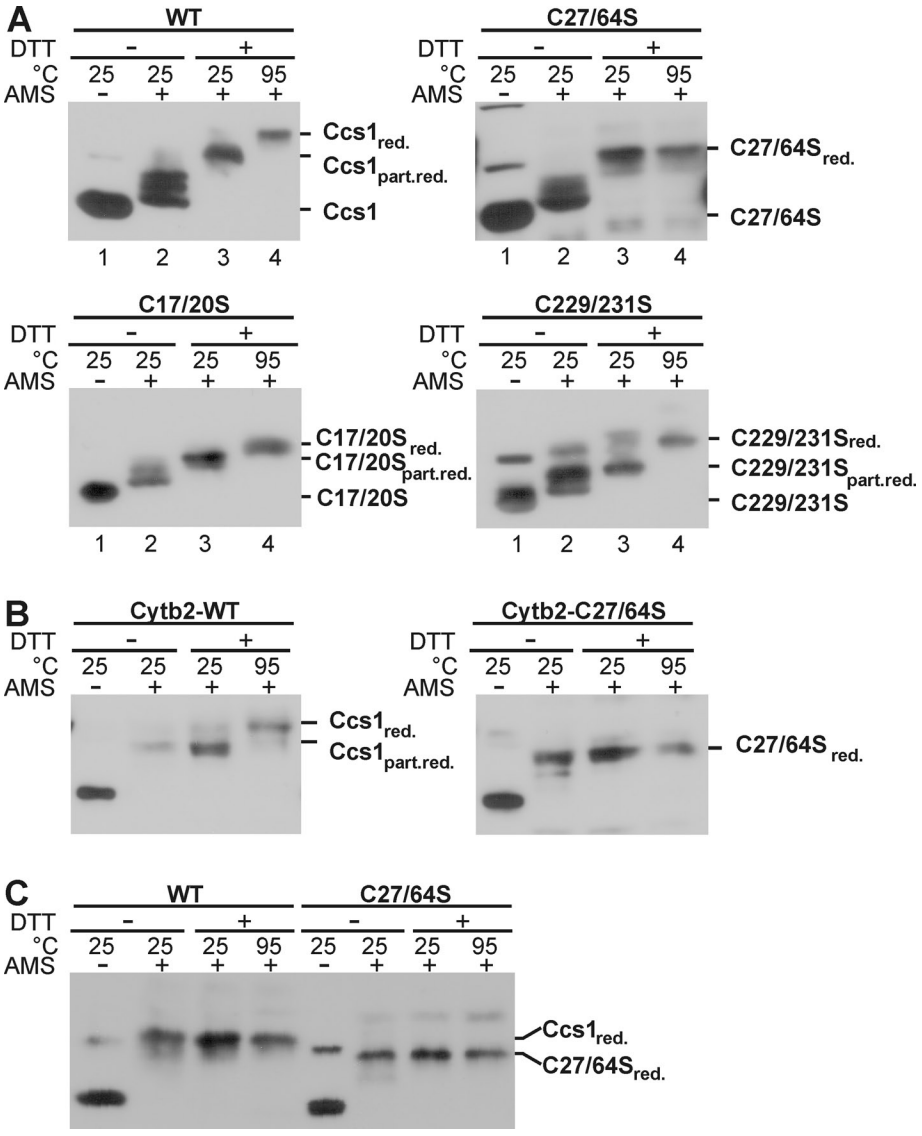

FIGURE 4: The cysteine residues C27 and C64 form a disulfide bond. (A) Mitochondria were isolated from cells expressing Ccs1 wild-type (WT) and variants of Ccs1. Mitochondria were incubated for 10 min in absence or presence of 15 mM DTT at 25 or 95°C. Proteins were precipitated with trichloroacetic acid, resuspended in buffer containing 2% SDS, and treated with 10 mM AMS or, when indicated (–AMS), left untreated. Samples were analyzed by SDS–PAGE and immunodecoration with antibodies against Ccs1. Different times of exposure were taken showing the distinct Ccs1 variants. part. red., partially reduced; red., reduced. (B, C) Cells harboring the indicated Ccs1 variants as fusion proteins with the cytochrome b_2 targeting signal (B) or the Ccs1 variants (C) were used. Cellular (B) and cytosolic (C) extracts were prepared and samples were treated and analyzed as in A.

modified with five molecules of AMS, indicating one disulfide bond (Figure 4B, left). The same was observed for cells harboring the Cytb2-Ccs1-C27/64S, indicating that these two cysteine residues were oxidized in the wild-type protein. When cell extract was treated with DTT at 95°C, a shift in the mobility of the protein was observed for the wild type but not for the C27/64S variant, as expected in presence of a stable disulfide bond between C27 and C64 (Figure 4B). Thus the stable disulfide bond was definitely formed in cells and not upon isolation of mitochondria.

Next we tested the cytosolic redox state of Ccs1 and the Ccs1 variant in cells. In the presence and absence of DTT most of the wild-type Ccs1 was fully reduced, indicated by the shift in migration reflecting modification of Ccs1 with seven molecules of AMS (Figure 4C). Only a minor fraction might be present in a redox-stable, partially reduced form at room temperature. The C27/64S variant showed a faster mobility, reflecting modification with five molecules of AMS (Figure 4C). This suggests that the disulfide bond between cysteine residues 27 and 64 was not efficiently formed in the cytosol. In conclusion, the cysteine residues that are crucial for mitochondrial import of Ccs1 form a disulfide bond in the mitochondrial IMS.

The C27/64S variant is more sensitive to proteolytic digestion

We demonstrated that formation of the disulfide bond between C27 and C64 in mitochondria drives the import of Ccs1 into mitochondria. Are there additional functions of this disulfide bond? To address this question, we tested the sensitivity of the Ccs1 and the C27/64S variant against protease in mitochondrial extracts. On treatment with trypsin, wild-type Ccs1 was degraded to a slightly smaller fragment, probably corresponding to domains I and II (Figure 5A; Schmidt *et al.*, 1999). In contrast, the C27/64S variant was more sensitive toward protease and largely degraded. A fragment of ~16 kDa was generated, most likely corresponding to domain II, based on work with recombinant proteins (Schmidt *et al.*, 1999). This suggests a stabilizing function of the disulfide bond connecting cysteine residues C27 and C64 in mitochondria.

Cysteine residues C27 and C64 of Ccs1 are important for localization of Sod1 to mitochondria

The mitochondrial fraction of Ccs1 was reported to be crucial for mitochondrial localization of Sod1 (Field *et al.*, 2003). Do cysteine residues C27 and C64 of Ccs1 have a role in targeting Sod1 to the mitochondrial IMS? To determine the amount of Sod1 in mitochondria of various Ccs1 double mutants, mitochondrial extracts were separated by SDS–PAGE and analyzed by immunodecoration with antibodies against Sod1. Compared to wild type, mitochondrial levels of Sod1 were reduced in the case of the C27/64S and C229/231S mutants (Figure 5B). Because Ccs1 is needed for the import of Sod1 into mitochondria, we conclude that reduced Sod1 levels in mitochondria of the Ccs1 C27/64S–expressing cells are caused by lower amounts of the C27/64S Ccs1 variant in these mitochondria.

In yeast, Ccs1 is essential for the enzymatic activity of Sod1. Applying an in-gel activity assay, we tested the activity of Sod1 in mitochondria isolated from the various double-mutant cells. Sod1 activity was not detected in mitochondria of Δccs1 cells (Figure 5C). The same was true for mitochondria harboring the C229/231S variant, confirming the requirement of the CxC motif for activation of Sod1. The C17/20S mutant displayed mitochondrial Sod1 activity similar

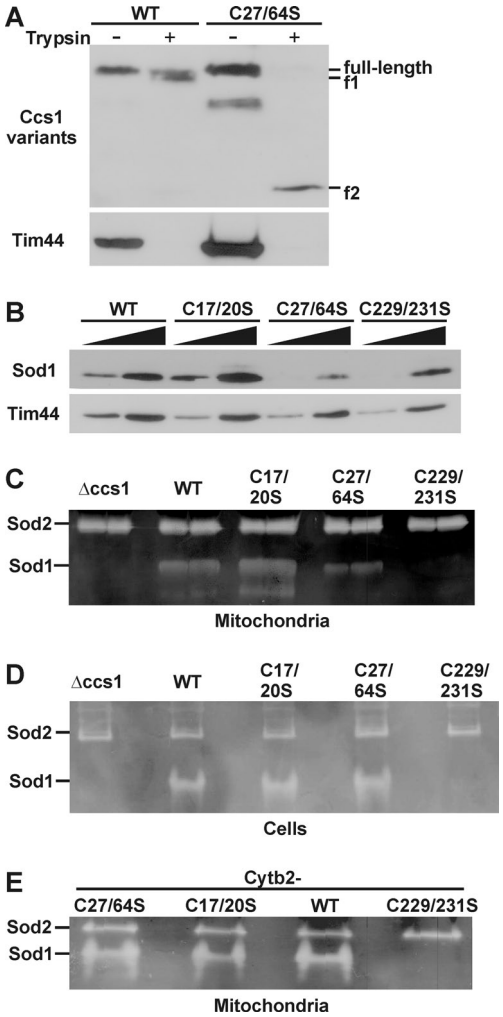

FIGURE 5: The Ccs1 cysteine residues C27 and C64 are crucial for mitochondrial localization of Sod1. (A) Mitochondria isolated from the indicated strains were lysed with Triton X-100 and then incubated on ice in the presence or absence of 200 μg/ml trypsin. After blocking of trypsin activity, samples were analyzed by SDS–PAGE and immunodecoration with antibodies against Ccs1. f1, f2 trypsin-resistant fragment of Ccs1. Three times more protein of the C27/64S mitochondria was loaded. (B, C) Mitochondria were isolated from cells expressing wild-type and the double-cysteine variants of Ccs1. Then they were analyzed by SDS–PAGE and immunoblotting with antibodies against Sod1 and Tim44 (B) and tested for Sod activity in a gel activity assay with nitro blue tetrazolium (C). Immunodecoration with antibodies against Tim44, a mitochondrial protein, indicated the presence of equal amounts of mitochondrial proteins loaded. (D) Activity of Sod1 was analyzed as earlier in extracts from cells expressing the indicated Ccs1 variants. (E) Mitochondria isolated from cells expressing fusion proteins of the cytochrome b_2–targeting signal and Ccs1 variants were tested for Sod1 activity as described in C.

to wild type. In contrast, the activity of Sod1 was reduced in mitochondria harboring the C27/64S variant (Figure 5C). The assay also detected in parallel the activity of mitochondrial matrix Sod2. A similar activity of Sod2 was observed in all mitochondria, indicating that the Sod1 activity was specifically affected in the Ccs1 mutants. The reduction of the Sod1 activity in the C27/64S mutant mitochondria corresponds to the reduction in the level of Sod1 protein in mitochondria. To confirm that activation of Sod1 was not affected in the C27/64S mutant but instead that reduced amounts of Sod1 were responsible for reduction in Sod1 activity in mitochondria, the activity of Sod1 was measured in total cell extracts, in which comparable protein amounts of Sod1 were present. Indeed, similar Sod1 activities were determined in cell extracts of the C27/64S and the C17/20S mutants and in wild type (Figure 5D). Activity was not observed in the extracts of the C229/231S mutant and in the absence of Ccs1. Moreover, the mitochondrial targeted cytochrome b_2 fusion proteins of the Ccs1 variants were analyzed. The amounts of Ccs1 and Sod1 in mitochondria isolated from the C27/64S variant were similar to the amounts in wild type, and this was also true for the Sod1 activities (Figure 5E). Thus we conclude that mutations of the Ccs1 cysteine residues C27 and C64 do not appear to affect the activity of native Sod1 but rather the amount of Ccs1 in mitochondria. The reduced level of Ccs1-C27/64S in mitochondria then results in a decreased Sod1 activity due to the reduced protein level of Sod1. Thus the cysteine residues C27 and C64 of Ccs1 play an important role in the biogenesis of mitochondrial Sod1.

DISCUSSION

We elucidated the mechanism of import of an unconventional substrate of the disulfide relay system, Ccs1, into the IMS of mitochondria. The import of Ccs1 into yeast mitochondria strongly depends on the cysteine residues 27 and 64, which form a very stable disulfide bond in mitochondria. Our results suggest that Mia40 introduces this disulfide bond, thereby promoting efficient mitochondrial import of Ccs1 and controlling its distribution between mitochondria and cytosol. The results presented here shed light on the mechanistic diversity of the disulfide relay system. Whereas the disulfide relay system forms two disulfide bonds in typical substrates with the typical twin Cx_nC motif, it apparently introduces one disulfide bond into Ccs,1 indicating the ability of the system to form one or two disulfide bonds in natural substrates. Mia40 is also able to form single disulfide bonds in cysteine mutants of classic substrates (Banci *et al.*, 2009). In classic substrates with twin Cx_nC motifs, the two disulfide bonds covalently link two α-helices that are separated by a short loop. In Ccs1, the disulfide bond–forming cysteine residues are also present in α-helices (Lamb *et al.*, 1999). However, the helices are separated by more secondary structure elements—two β-sheets and three loops (Figure 6A). Ccs1 is a multidomain protein, and its ATX-like domain harboring the cysteine residues C27 and C64 is also more complex than are the small typical substrates (Lamb *et al.*, 1999). Thus the disulfide relay system has the ability to support the import of complex proteins and to form disulfide bonds in this class of proteins. The disulfide bonds provide a stabilizing element for Ccs1, as well as for the substrates with twin Cx_nC motifs, and are highly resistant against reducing conditions (Arnesano *et al.*, 2005; Webb *et al.*, 2006; Grumbt *et al.*, 2007).

How is Ccs1 recognized by Mia40? On one hand, Ccs1 clearly differs from other known substrates of Mia40; on the other hand, they have features in common. In both cases, one specific cysteine residue appears to be crucial for recognition. Our results demonstrate that the cysteine residue C64 of Ccs1 is important for the Mia40-dependent import into mitochondria and for the interaction with Mia40. It was postulated that typical substrates of the disulfide relay system are targeted to the IMS by specific import signal sequences that form an amphipathic helix with crucial hydrophobic residues (Milenkovic *et al.*, 2009; Sideris *et al.*, 2009). The mitochondrial intermembrane space sorting signal (MISS) obtained by analysis of small Tim proteins consists of nine amino acid residues with a most important hydrophobic leucine residue at position −4 relative to the cysteine residue (Milenkovic *et al.*, 2009). In another study, a signal sequence was defined as X[Ar]XX[Hy][Hy]XXC and termed

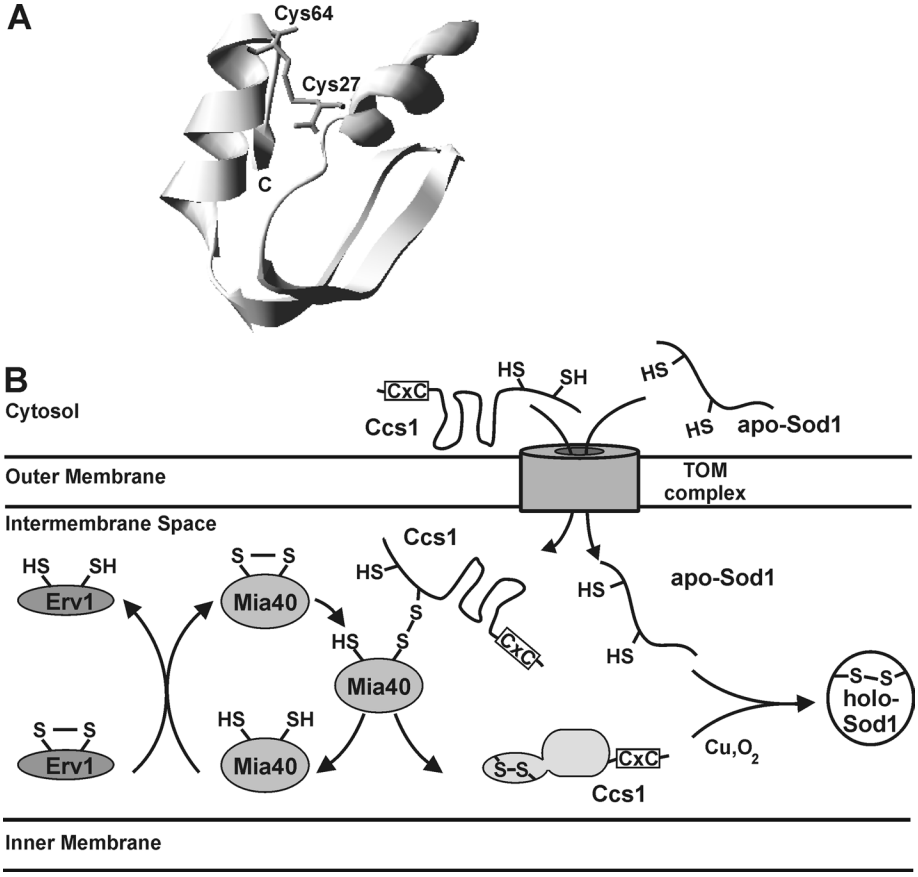

FIGURE 6: Model of the Mia40/Erv1 disulfide relay system–dependent import of Ccs1 and Sod1 into the IMS of mitochondria. (A) Structural representation in ribbon form of the secondary structure of amino acid residues 14–69 of Ccs1. The picture was generated using Swiss-PDB Viewer and PDB file 1qup (Lamb *et al.*, 1999). (B) Ccs1 crosses the TOM complex in the outer membrane of mitochondria in an unfolded state. Following translocation, Ccs1 interacts with oxidized Mia40, forming a disulfide intermediate employing the cysteine residue 64 of its amino-terminal domain. The intermolecular disulfide bond between Mia40 and Ccs1 is then transferred to Ccs1, thereby forming a stable intramolecular disulfide bond between cysteines C27 and C64 that probably promotes folding of Ccs1. On formation of oxidized Ccs1, Mia40 is released in its reduced form and is subsequently reoxidized by Erv1, regenerating oxidized Mia40. Folded Ccs1 mediates mitochondrial import of Sod1, which passes the TOM complex in its reduced apo form. Ccs1 activates apo-Sod1 to holo-Sod1, thereby trapping Sod1 in the IMS. The C-terminal CxC motif of Ccs1, but not the disulfide bond between C27 and C64, is needed for this activation. Copper and oxygen appear to be required as well. The thiol groups of C27 and C64 of Ccs1 are shown in the model.

the intermembrane space targeting signal (ITS; Sideris *et al.*, 2009). Of interest, the position of this latter sequence relative to the interacting cysteine residue, N- or C-terminal, and the orientation of the α-helix, N- to C-terminal or C- to N-terminal, appears to be flexible (Sideris *et al.*, 2009). Analysis of the flanking amino acid residues of the Ccs1 cysteine residues indicates that the sequence around C64 does not exactly match but has similarities to the consensus sequence of the MISS/ITS signal. A hydrophobic residue is present at positions −3 and +3: L61 and A68. Additional hydrophobic amino acid residues are present and, particularly in the case of the amino acid residues amino-terminal to C64, are located on one side of an amphipathic helix. We suggest that a cysteine-containing helix with one hydrophobic side is the general recognition and import signal of all substrates of the disulfide relay system. Because such a requirement is not very sequence specific, there might be many more potential substrates of the disulfide relay system to be discovered. The sequence around C27 might be a second, less efficient targeting signal within Ccs1. An isoleucine and a valine residue are located at positions −3 and +4, respectively, and the sequence N-terminal to C27 forms an amphipathic α-helix. Consistent with our import and interaction experiments, the flanking sequences of the other cysteine residues in Ccs1 do not resemble the IMS sorting signals.

Ccs1 plays an important role in the biogenesis of mitochondrial Sod1. As previously reported and confirmed in our study, the cysteine residues 229 and 231 in the C-terminal domain of Ccs1 are essential for the activation of Sod1 and its import into mitochondria, in contrast to the residues C17 and C20 of the CxxC motif in the amino-terminal domain (Schmidt *et al.*, 1999; Lamb *et al.*, 2001; Rae *et al.*, 2001; Kirby *et al.*, 2008). We show that the cysteine residues C27 and C64 do not mediate the activation and the mitochondrial import of Sod1. Because Sod1 requires its intramolecular disulfide bond for activity (Furukawa *et al.*, 2004), the formation of this disulfide bond cannot depend on the disulfide bond between the cysteines C27 and C64 in Ccs1. Our present and previous data can be combined in a hypothetical model of the import of Ccs1 and Sod1 into mitochondria (Figure 6B). Reduced Ccs1 passes the TOM complex in its unfolded state. Then it interacts with Mia40 in the IMS, forming a disulfide intermediate that involves cysteine residue 64. The disulfide intermediate is prerequisite to form the stable disulfide bond between cysteine residues C27 and C64 in Ccs1. The Ccs1 folds and is thereby trapped in the IMS. Functional Ccs1 mediates the import of Sod1, whose apo form passes the TOM complex in a reduced, unfolded state. Activation by Ccs1, in particular insertion of the disulfide bond into Sod1, retains Sod1 in the IMS. Ccs1 uses the cysteine residues of the CxC motif, but not the disulfide residues C27 and C64, to generate the disulfide bond in Sod1 and to introduce the copper ion into Sod1. How the disulfide bond in Sod1 is formed with the help of the CxC motif of domain III has to be further elucidated. Copper and oxygen are required for the activation of Sod1 (Furukawa *et al.*, 2004). A disulfide intermediate found in the structure of the Ccs1–Sod1 docked complex between Ccs1 cysteine residue C229 of domain III and the Sod1 residue C57 might be the intermediate in formation of the intramolecular disulfide bond in Sod1 (Lamb *et al.*, 2001). There is no evidence so far that Mia40 plays a role in this process, although the possibility is not completely excluded. The effect of Mia40 on the import of Sod1 into mitochondria instead appears to be due to its effect on the import of Ccs1 and therefore on the amount of Ccs1 in mitochondria.

The formation of the disulfide bond within Ccs1 mediates the import of Ccs1 into mitochondria, making the import dependent on the Mia40/Erv1 disulfide relay system. Regulation of the activity of this system might affect the distribution of Ccs1 between the

cytosolic and the mitochondrial fractions and, thereby, also the distribution of Sod1. An increase of active Sod1 in mitochondria would probably improve protection against oxidative stress in mitochondria, as reported upon targeting of Ccs1 to the mitochondrial IMS, and thereby increasing mitochondrial levels of Sod1 (Sturtz *et al.*, 2001). Subsequently it was shown that Sod1 targeted to the IMS improves viability under conditions of mitochondrial oxidative stress in yeast and prevents biochemical and morphological defects in *Sod1* knockout mouse (Klöppel *et al.*, 2010; Fischer *et al.*, 2011). It can be speculated that regulation of the import of Ccs1 under certain physiological conditions may adapt the activity of Sod1 to the antioxidizing needs within the mitochondrial IMS. Such adaptation might be achieved by regulation of the activity of the disulfide relay system, which itself is linked to the activity of the respiratory chain (Allen *et al.*, 2005; Farrell and Thorpe, 2005; Bihlmaier *et al.*, 2007; Dabir *et al.*, 2007).

A fraction of mammalian Ccs1, CCS, and SOD1 is also present in the mitochondrial IMS in mammalian cells (Okado-Matsumoto and Fridovich, 2001; Kawamata and Manfredi, 2008). Of interest, the cysteine residues C27 and C64 of Ccs1 are not conserved in higher eukaryotes. Nonetheless, Mia40 has been shown to promote the import of CCS into mammalian mitochondria and to interact in an immunoprecipitation with CCS (Kawamata and Manfredi, 2008). Less efficient import into mitochondria, as also observed for the C27/64S variant in yeast, might occur in absence of these cysteine residues in mammalian CCS, and/or a different molecular mechanism of the mitochondrial import of CCS might function in mammalian cells. A noncovalent hydrophobic interaction might allow interaction of the unfolded CCS with Mia40, inducing folding of CCS on a Mia40 platform. Mia40, indeed, has been shown to function as molecular chaperone assisting in the folding of the typical twin Cx_nC substrates (Banci *et al.*, 2010). On the other hand, it is also possible that other cysteine residues, for example, additional cysteine residues in domain II of mammalian CCS, take over the function of yeast C27 and C64. The stabilizing effect of C27 and C64 on yeast domain I might be provided in human cells by hydrogen bonds formed by hydroxyl groups of polar amino acid residues, which replace these cysteine residues.

In conclusion, the Mia40/Erv1 disulfide relay system mediates the import of Ccs1 and, indirectly, of Sod1 into mitochondria by introducing a disulfide bond between the cysteine residues C27 and C64 of Ccs1. Thus the disulfide relay system has the ability to transfer a single disulfide bond to unconventional multidomain substrates, such as Ccs1, and, most likely, promotes mitochondrial import of additional intermembrane space proteins by this mechanism.

MATERIALS AND METHODS

Plasmids, yeast strains, and cell growth

The plasmid pHAL7-413 expressing Ccs1 fused to two copies of HA epitope tag (Culotta *et al.*, 1997) was used as a template to generate the Ccs1 cysteine-to-serine mutants by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA).

The regulatory sequences and the nucleotide sequences encoding the Ccs1 variants were inserted into the *TRP1*-containing vector pRS314 using the restriction sites *Apa*I and *Not*I. The plasmids pLS117 and pLS009 (Sturtz *et al.*, 2001) encoding the fusion protein of the first 88 amino acid residues of the cytochrome b_2 protein and wild-type Ccs1 or the Ccs1-C229/231S variant, respectively, were used to transfer the regulatory and the coding sequences to the *TRP1*-containing vector pRS314 using the restriction sites *Apa*I and *Not*I, generating pRS314-Cytb2-Ccs1 and pRS314-Cytb2-Ccs1-C229/231S. The plasmids pRS314-Cytb2-Ccs1-C17/20S and pRS314-Cytb2-Ccs1-C27/64S were generated by amplifying nucleotides 1–227 of the open reading frame of pRS314-Ccs1-C17/20S and pRS314-Ccs1-C27/64S by PCR and inserting the fragments into the plasmid pRS314-Cytb2-Ccs1 using the restriction sites *Nde*I and *Eco*RI.

The plasmids used to synthesize radiolabeled Ccs1 precursor proteins were constructed by amplifying the open reading frame of the Ccs1 variants by PCR. The fragments were then inserted into the pGEM4 vector using *Bam*HI and *Hin*dIII as restriction sites.

The plasmids expressing domain I of Ccs1 fused to GST were constructed by amplifying nucleotides 1–222 of the open reading frame of pRS314-Ccs1, pRS314-Ccs1-C17/20S, pRS314-Ccs1-C27/64S, pRS314-Ccs1-C27S, and pRS314-Ccs1-C64S by PCR and inserting the fragments into the plasmid pGEX6P1 using the restriction sites *Bam*HI and *Sal*I.

The deletion strain of *CCS1* (Δccs1) was generated by replacing the *CCS1* gene with the *kanMX* marker in the yeast strain YPH499 by homologous recombination (Wach *et al.*, 1994). The same procedure was used to delete *CCS1* in the GAL-MIA40 strain (Terziyska *et al.*, 2005), generating GAL-MIA40 Δccs1. The deletion strains were then transformed with the yeast expression plasmids encoding the Ccs1 variants.

Yeast strains were grown in minimal synthetic medium containing 2% lactate (SL) and 0.5% galactose or in minimal synthetic medium containing either 2% glucose (SD) or 2% galactose. To overexpress Mia40, cells of the GAL-MIA40 Δccs1 strain were grown in SL medium containing 0.5% galactose at 30°C. To deplete Mia40, cells were first grown in SL medium containing 0.5% galactose at 30°C, then shifted to SL medium containing 0.5% glucose and cultured for 30 h at 30°C.

Analysis of the disulfide intermediate of Ccs1 and Mia40

For analysis of the intermediate between imported Ccs1 and Mia40, radiolabeled precursor proteins of Ccs1 and Ccs1 variants were synthesized in the presence of [^{35}S]methionine in an in vitro transcription/translation–coupled reticulocyte lysate system. The precursor proteins were first treated with 15 mM DTT for 5 min at 4°C, then precipitated in 2.7 M $(NH_4)_2SO_4$ solution and resuspended in 6 M urea, 5 mM DTT, and 20 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.2. After a clarifying spin (125,000 × *g*, 20 min, 4°C), supernatants were diluted in import buffer (Mesecke *et al.*, 2005) and incubated for 15 min at 25°C with mitochondria overexpressing octahistidinyl-tagged Mia40. Following addition of 80 mM iodoacetamide (IAA), samples were incubated for additional 15 min at 25°C. Nonimported precursor proteins were digested with trypsin (50 µg/ml). Mitochondria were isolated and lysed in buffer (20 mM Tris(hydroxymethyl)aminomethane [Tris], pH 7.4, 80 mM KCl, 20 mM imidazole, pH 8, 0.5% Triton X-100, 1 mM phenylmethylsulfonylfluoride [PMSF]) for 20 min at 4°C. After a clarifying spin (125,000 × *g*, 20 min, 4°C) supernatants were incubated with Ni-NTA agarose beads. Aliquots of the loaded and the nonbound material were taken. Subsequently, beads were washed with 0.05% Triton X-100–containing buffer, and bound proteins were eluted with Laemmli buffer containing 300 mM imidazole. Aliquots of the loaded and the nonbound material and the bound material were analyzed by nonreducing SDS–PAGE and autoradiography.

For analysis of the disulfide intermediate between endogenous Ccs1 and Mia40, mitochondria overexpressing an octahistidinyl-tagged Mia40 together with Ccs1 variants were incubated for 10 min at 25°C in the presence of 70 mM IAA. Mitochondria were lysed in a buffer (20 mM Tris, pH 7.4, 80 mM KCl, 20 mM imidazole, pH 8,

0.5% Triton X-100, 1 mM PMSF) containing EDTA-free protease inhibitor cocktail (Roche, Indianapolis, IN). The extracts were centrifuged (125,000 × *g*, 20 min, 4°C), and the supernatants were incubated with Ni-NTA agarose beads. Bound proteins were eluted with Laemmli buffer containing 300 mM imidazole. Total mitochondrial and bound proteins were analyzed by nonreducing SDS–PAGE and immunodecoration with antibodies against Ccs1.

For the in vitro interaction studies the GST-tagged fusion proteins were expressed in *Escherichia coli* XL1blue according to the protocol previously described (Grumbt *et al.*, 2007). The cells were harvested and lysed as described (Grumbt *et al.*, 2007). Fractions of the cell lysates containing the GST-tagged domain I of Ccs1 were incubated together with 30 µl of glutathione–Sepharose 4B beads (GE Healthcare, Piscataway, NJ) at 4°C for 1 h in buffer (20 mM Tris, pH 7.0, 200 mM NaCl) containing 5 mM DTT. The beads were washed three times with buffer without DTT and divided into two halves. The samples were further incubated for 10 min at 25°C, either in the presence or in the absence of 1 µg of purified C-terminal fragment of oxidized Mia40 (Grumbt *et al.*, 2007). Following addition of 50 mM IAA the beads were washed three times with buffer. The proteins were eluted with Laemmli buffer, and the eluates were analyzed by nonreducing SDS–PAGE and immunodecoration with antibodies against Mia40.

Protease digestion

A 60-µg amount of mitochondria was lysed for 20 min at 4°C in 40 µl of buffer (0.6 M sorbitol, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 0.5% Triton X-100) and incubated with or without 200 µg/ml trypsin for 15 min at 4°C. To stop trypsin, soybean trypsin inhibitor (3.3 mg/ml) was added, and the sample was diluted with Laemmli buffer. The proteins were analyzed by SDS–PAGE and immunodecoration with antibodies against Ccs1 and Tim44.

Preparation of protein extracts

To obtain cell extracts containing cytosolic and mitochondrial proteins, cells were grown in SD medium to an OD of 1. Ten OD units of cells were harvested and resuspended in a buffer containing 0.6 M sorbitol, 20 mM HEPES, 10 mM EDTA, and 1 mM PMSF. The following procedures were performed under anaerobic conditions in a nitrogen-filled glove box. A total of 200 µl of glass beads (0.75–1 mm in diameter) was added to the cells. To open the cells, samples were vortexed six times for 30 s, with a break of 30 s on ice between each step. Following centrifugation (1000 × *g*, 3 min, 4°C) the supernatant containing the cellular proteins was collected.

To prepare cytosolic extracts, cells were grown in SD medium to an OD of ~1. Cells from 50 ml of culture were harvested, washed, and incubated for 30 min at 30°C in buffer (1.2 M sorbitol, 20 mM KH_2PO_4 pH 7.4) with 0.35 mg/ml zymolyase to obtain spheroplasts. The following steps were performed in a nitrogen-filled glove box under anaerobic conditions. Spheroplasts were isolated by centrifugation (3220 × *g*, 5 min, 25°C) and resuspended in a buffer containing 0.6 M sorbitol, 10 mM Tris, pH 7.4, 1 mM EDTA, and 1 mM PMSF. They were opened with 30 strokes in a glass douncer. To remove cell debris and organelles, including mitochondria, samples were subjected to two centrifugation steps at 4°C—first for 5 min at 1700 × *g* and then for 10 min at 15,300 × *g*. The supernatant obtained was used in the thiol modification experiments and the Sod1 activity assay.

Thiol modification with AMS

Mitochondria (40 µg) or extracts prepared from 1 or 2.5 OD of cells were incubated at 25°C or 95°C for 10 min in buffer (0.6 M sorbitol, 20 mM HEPES) with or without 15 mM DTT. Following precipitation of the proteins with trichloroacetic acid, the pellet was washed with acetone and resolved in a buffer containing 2% SDS, 100 mM Tris, pH 8, 100 mM NaCl, and 10 mM EDTA. AMS, 10 mM (Invitrogen), or distilled water was added, and the samples were incubated for 60 min at 37°C in the dark. After addition of 50 mM IAA, Laemmli buffer was added, and the proteins were analyzed by SDS–PAGE and immunodecoration with antibodies against Ccs1.

Other procedures

Previously described procedures were used for preparation of total cell extracts for SDS–PAGE (Kushnirov, 2000) and for isolation of mitochondria (Daum *et al.*, 1982). The Sod1 activity assay was essentially performed by nondenaturing gel electrophoresis and staining with nitro blue tetrazolium as previously described (Flohe and Otting, 1984). Mitochondrial extracts used in the assay were prepared by lysis of mitochondria in a buffer containing 5% digitonin.

ACKNOWLEDGMENTS

We are grateful to Ulrike Wirth, Heiko Germeroth, and Marica Malesic for excellent technical assistance. We thank Walter Neupert for continuous support and comments on the manuscript and Andreas Ladurner for his support. This work was supported by grant SFB 594 (B13) from the Deutsche Forschungsgemeinschaft, by the University of Munich Förderprogramm für Forschung und Lehre, and by National Institutes of Health Grant GM 50016.

REFERENCES

Allen S, Balabanidou V, Sideris DP, Lisowsky T, Tokatlidis K (2005). Erv1 mediates the Mia40-dependent protein import pathway and provides a functional link to the respiratory chain by shuttling electrons to cytochrome *c*. J Mol Biol 353, 937–944.

Arnesano F, Balatri E, Banci L, Bertini I, Winge DR (2005). Folding studies of Cox17 reveal an important interplay of cysteine oxidation and copper binding. Structure 13, 713–722.

Banci L, Bertini I, Cefaro C, Ciofi-Baffoni S, Gallo A, Martinelli M, Sideris DP, Katrakili N, Tokatlidis K (2009). MIA40 is an oxidoreductase that catalyzes oxidative protein folding in mitochondria. Nat Struct Mol Biol 16, 198–206.

Banci L *et al.* (2010). Molecular chaperone function of Mia40 triggers consecutive induced folding steps of the substrate in mitochondrial protein import. Proc Natl Acad Sci USA 107, 20190–20195.

Bien M, Longen S, Wagener N, Chwalla I, Herrmann JM, Riemer J (2010). Mitochondrial disulfide bond formation is driven by intersubunit electron transfer in Erv1 and proofread by glutathione. Mol Cell 37, 516–528.

Bihlmaier K, Mesecke N, Terzyska N, Bien M, Hell K, Herrmann JM (2007). The mitochondrial disulfide relay system is connected to the electron transport chain. J Cell Biol 179, 389–395.

Caruano-Yzermans AL, Bartnikas TB, Gitlin JD (2006). Mechanisms of the copper-dependent turnover of the copper chaperone for superoxide dismutase. J Biol Chem 281, 13581–13587.

Chacinska A, Pfannschmidt S, Wiedemann N, Kozjak V, Sanjuan Szklarz LK, Schulze-Specking A, Truscott KN, Guiard B, Meisinger C, Pfanner N (2004). Essential role of Mia40 in import and assembly of mitochondrial intermembrane space proteins. EMBO J 23, 3735–3746.

Culotta VC, Klomp LW, Strain J, Casareno RL, Krems B, Gitlin JD (1997). The copper chaperone for superoxide dismutase. J Biol Chem 272, 23469–23472.

Curran SP, Leuenberger D, Oppliger W, Koehler CM (2002). The Tim9p-Tim10p complex binds to the transmembrane domains of the ADP/ATP carrier. EMBO J 21, 942–953.

Dabir DV, Leverich EP, Kim SK, Tsai FD, Hirasawa M, Knaff DB, Koehler CM (2007). A role for cytochrome *c* and cytochrome *c* peroxidase in electron shuttling from Erv1. EMBO J 26, 4801–4811.

Daum G, Bohni PC, Schatz G (1982). Import of proteins into mitochondria. Cytochrome b_2 and cytochrome *c* peroxidase are located in

the intermembrane space of yeast mitochondria. J Biol Chem 257, 13028–13033.
Deponte M, Hell K (2009). Disulphide bond formation in the intermembrane space of mitochondria. J Biochem 146, 599–608.
Endo T, Yamano K, Kawano S (2010). Structural basis for the disulfide relay system in the mitochondrial intermembrane space. Antioxid Redox Signal 13, 1359–1373.
Farrell SR, Thorpe C (2005). Augmenter of liver regeneration: a flavin-dependent sulfhydryl oxidase with cytochrome *c* reductase activity. Biochemistry 44, 1532–1541.
Field LS, Furukawa Y, O'Halloran TV, Culotta VC (2003). Factors controlling the uptake of yeast copper/zinc superoxide dismutase into mitochondria. J Biol Chem 278, 28052–28059.
Fischer LR, Igoudjil A, Magrane J, Li Y, Hansen JM, Manfredi G, Glass JD, (2011). SOD1 targeted to the mitochondrial intermembrane space prevents motor neuropathy in the *Sod1* knockout mouse. Brain 134, 196–209.
Flohe L, Otting F (1984). Superoxide dismutase assays. Methods Enzymol 105, 93–104.
Fridovich I (1975). Superoxide dismutases. Annu Rev Biochem 44, 147–159.
Furukawa Y, Torres AS, O'Halloran TV (2004). Oxygen-induced maturation of SOD1: a key role for disulfide formation by the copper chaperone CCS. EMBO J 23, 2872–2881.
Grumbt B, Stroobant V, Terziyska N, Israel L, Hell K (2007). Functional characterization of Mia40p, the central component of the disulfide relay system of the mitochondrial intermembrane space. J Biol Chem 282, 37461–37470.
Kawamata H, Manfredi G (2008). Different regulation of wild-type and mutant Cu, Zn superoxide dismutase localization in mammalian mitochondria. Hum Mol Genet 17, 3303–3317.
Khalimonchuk O, Rigby K, Bestwick M, Pierrel F, Cobine PA, Winge DR (2008). Pet191 is a cytochrome *c* oxidase assembly factor in *Saccharomyces cerevisiae*. Eukaryot Cell 7, 1427–1431.
Kirby K, Jensen LT, Binnington J, Hilliker AJ, Ulloa J, Culotta VC, Phillips JP (2008). Instability of superoxide dismutase 1 of *Drosophila* in mutants deficient for its cognate copper chaperone. J Biol Chem 283, 35393–35401.
Klöppel C, Michels C, Zimmer J, Herrmann JM, Riemer J (2010). In yeast redistribution of Sod1 to the mitochondrial intermembrane space provides protection against respiration derived oxidative stress. Biochem Biophys Res Commun 403, 114–119.
Koehler CM, Tienson HL (2009). Redox regulation of protein folding in the mitochondrial intermembrane space. Biochim Biophys Acta 1793, 139–145.
Kushnirov VV (2000). Rapid and reliable protein extraction from yeast. Yeast 16, 857–860.
Lamb AL, Torres AS, O'Halloran TV, Rosenzweig AC (2001). Heterodimeric structure of superoxide dismutase in complex with its metallochaperone. Nat Struct Biol 8, 751–755.
Lamb AL, Wernimont AK, Pufahl RA, Culotta VC, O'Halloran TV, Rosenzweig AC (1999). Crystal structure of the copper chaperone for superoxide dismutase. Nat Struct Biol 6, 724–729.
Lamb AL, Wernimont AK, Pufahl RA, O'Halloran TV, Rosenzweig AC (2000). Crystal structure of the second domain of the human copper chaperone for superoxide dismutase. Biochemistry 39, 1589–1595.
Lu H, Allen S, Wardleworth L, Savory P, Tokatlidis K (2004). Functional TIM10 chaperone assembly is redox-regulated in vivo. J Biol Chem 279, 18952–18958.
Mesecke N, Terziyska N, Kozany C, Baumann F, Neupert W, Hell K, Herrmann JM (2005). A disulfide relay system in the intermembrane space of mitochondria that mediates protein import. Cell 121, 1059–1069.
Milenkovic D, Ramming T, Muller JM, Wenz LS, Gebert N, Schulze-Specking A, Stojanovski D, Rospert S, Chacinska A (2009). Identification of the signal directing tim9 and tim10 into the intermembrane space of mitochondria. Mol Biol Cell 20, 2530–2539.
Naoe M, Ohwa Y, Ishikawa D, Ohshima C, Nishikawa S, Yamamoto H, Endo T (2004). Identification of Tim40 that mediates protein sorting to the mitochondrial intermembrane space. J Biol Chem 279, 47815–47821.
Okado-Matsumoto A, Fridovich I (2001). Subcellular distribution of superoxide dismutases (SOD) in rat liver: Cu,Zn-SOD in mitochondria. J Biol Chem 276, 38388–38393.
Pufahl RA, Singer CP, Peariso KL, Lin SJ, Schmidt PJ, Fahrni CJ, Culotta VC, Penner-Hahn JE, O'Halloran TV (1997). Metal ion chaperone function of the soluble Cu(I) receptor Atx1. Science 278, 853–856.
Rae TD, Torres AS, Pufahl RA, O'Halloran TV (2001). Mechanism of Cu,Zn-superoxide dismutase activation by the human metallochaperone hCCS. J Biol Chem 276, 5166–5176.
Reddehase S, Grumbt B, Neupert W, Hell K (2009). The disulfide relay system of mitochondria is required for the biogenesis of mitochondrial Ccs1 and Sod1. J Mol Biol 385, 331–338.
Riemer J, Fischer M, Herrmann JM (2011). Oxidation-driven protein import into mitochondria: Insights and blind spots. Biochim Biophys Acta 1808, 981–989.
Rissler M, Wiedemann N, Pfannschmidt S, Gabriel K, Guiard B, Pfanner N, Chacinska A (2005). The essential mitochondrial protein Erv1 cooperates with Mia40 in biogenesis of intermembrane space proteins. J Mol Biol 353, 485–492.
Schmidt PJ, Rae TD, Pufahl RA, Hamma T, Strain J, O'Halloran TV, Culotta VC (1999). Multiple protein domains contribute to the action of the copper chaperone for superoxide dismutase. J Biol Chem 274, 23719–23725.
Sideris DP, Petrakis N, Katrakili N, Mikropoulou D, Gallo A, Ciofi-Baffoni S, Banci L, Bertini I, Tokatlidis K (2009). A novel intermembrane space-targeting signal docks cysteines onto Mia40 during mitochondrial oxidative folding. J Cell Biol 187, 1007–1022.
Sideris DP, Tokatlidis K (2010). Oxidative protein folding in the mitochondrial intermembrane space. Antioxid Redox Signal 13, 1189–1204.
Stojanovski D, Muller JM, Milenkovic D, Guiard B, Pfanner N, Chacinska A (2008). The MIA system for protein import into the mitochondrial intermembrane space. Biochim Biophys Acta 1783, 610–617.
Sturtz LA, Diekert K, Jensen LT, Lill R, Culotta VC (2001). A fraction of yeast Cu,Zn-superoxide dismutase and its metallochaperone, CCS, localize to the intermembrane space of mitochondria. A physiological role for Sod1 in guarding against mitochondrial oxidative damage. J Biol Chem 276, 38084–38089.
Terziyska N, Grumbt B, Kozany C, Hell K (2009). Structural and functional roles of the conserved cysteine residues of the redox-regulated import receptor Mia40 in the intermembrane space of mitochondria. J Biol Chem 284, 1353–1363.
Terziyska N, Lutz T, Kozany C, Mokranjac D, Mesecke N, Neupert W, Herrmann JM, Hell K (2005). Mia40, a novel factor for protein import into the intermembrane space of mitochondria is able to bind metal ions. FEBS Lett 579, 179–184.
Wach A, Brachat A, Pohlmann R, Philippsen P (1994). New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. Yeast 10, 1793–1808.
Webb CT, Gorman MA, Lazarou M, Ryan MT, Gulbis JM (2006). Crystal structure of the mitochondrial chaperone TIM9.10 reveals a six-bladed alpha-propeller. Mol Cell 21, 123–133.
Weisiger RA, Fridovich I (1973). Mitochondrial superoxide dismutaseSite of synthesis and intramitochondrial localization. J Biol Chem 248, 4793–4796.