Cofactor-dependent maturation of mammalian sulfite oxidase links two mitochondrial import pathways

Julian M. Klein and Guenter Schwarz*

Institut für Biochemie, Department Chemie und Zentrum für Molekulare Medizin Köln, Universität zu Köln, Zülpicher Str. 47, 50674 Köln, Germany

*Author for correspondence (gschwarz@uni-koeln.de)

Accepted 25 June 2012
Journal of Cell Science 125, 4876–4885
© 2012. Published by The Company of Biologists Ltd
doi: 10.1242/jcs.110114

Summary

Sulfite oxidase (SO) catalyses the metabolic detoxification of sulfite to sulfate within the intermembrane space of mitochondria. The enzyme follows a complex maturation pathway, including mitochondrial transport and processing, integration of two prosthetic groups, molybdenum cofactor (Moco) and heme, as well as homodimerisation. We have identified the sequential and cofactor-dependent maturation steps of SO. The N-terminal bipartite targeting signal of SO was required but not sufficient for mitochondrial localization. In the absence of Moco, most of the SO, although processed by the inner membrane peptidase of mitochondria, was found in the cytosol. Moco binding was required to induce mitochondrial trapping and retention, thus ensuring unidirectional translocation of SO. In the absence of Moco, the import of SO requires the chemical precursor, starts out in mitochondria (Teschner et al., 2010) with three subsequent cytosolic steps (Schwarz and Mendel, 2006). Given the labile chemical nature of Moco, it is assumed to become immediately associated to apo-enzymes following the completion of its biogenesis in the cytosol (Schwarz, 2005). Electrons derived from sulfite oxidation are transferred via the SO heme cofactor to the final electron acceptor cytochrome c. In order to become active, SO requires homodimerization mediated by the C-terminal dimerization domain (Kisker et al., 1997). The mitochondrial localization of SO is driven by an N-terminal targeting signal, which directs the protein in an ATP-dependent manner via the translocase of the outer membrane (TOM) complex to the intermembrane space (Ono and Ito, 1984). The first 80 residues of chicken SO are absent in the mature mitochondrial protein and are believed to be processed by mitochondrial peptidases (Kisker et al., 1997; Ono and Ito, 1984). The exact import mechanism and the involved peptidases were however not known so far. Given the different maturation steps of SO, mitochondrial translocation, Moco and heme cofactor insertion and dimerization require a spatially and temporally precisely organized process.

In this study, we have identified Moco as a central component of the SO maturation process, which is first a prerequisite for heme integration and dimerization, and second essentially required for mitochondrial localization of SO. We have characterized the mitochondrial import of SO as being dependent on a bipartite mitochondrial targeting signal and processed by the inner membrane peptidase (IMP) complex. However, the N-terminal targeting peptide of SO is not sufficient for mitochondrial localization, because Moco is additionally required for mitochondrial retention of SO. In absence of Moco, the import of

Introduction

Mitochondria are multifunctional organelles that are crucial for a multitude of essential cellular processes including oxidative phosphorylation, fatty acid β-oxidation, urea cycle, iron-sulfur cluster synthesis and various detoxification and catabolic reactions. One of these processes is catalysed by the molybdenum- and heme-containing sulfite oxidase (SO) converting toxic sulfite to nonhazardous sulfate in the intermembrane space of mitochondria (Cohen et al., 1972). Sulfite, mainly produced as a final product of the hepatic cysteine catabolism, is a strong nucleophile and hence able to severely damage a multitude of cellular components including membrane proteins and sulfolipids (Schwarz et al., 2009). In absence of functional SO enzymes, sulfite accumulates in the liver, the primary organ of cysteine catabolism (Stipanuk et al., 2006), and spreads out the body including the brain due to its ability to cross the blood–brain barrier. The corresponding disease, human SO deficiency, is characterized by rapidly progressing neurodegeneration and death in early childhood (Tan et al., 2005). Neurons appear to be particularly susceptible to the severe effects of sulfite accumulation as illustrated by the rapid degeneration of mainly the cortex within the first months of life and therefore constitute the main phenotypic site of the disease.

For the oxidation of sulfite, SO requires two prosthetic groups. The catalytic site is formed by the pterin-based molybdenum cofactor (Moco), which sequentially transfers two electrons derived from sulfite oxidation to the N-terminal cytochrome-β5-type heme cofactor. Both cofactors are synthesized in multi-step reaction cascades. Synthesis of heme is initiated and completed in mitochondria, with four intermediate steps occurring in the cytosol (Ajioka et al., 2006). The synthesis of Moco, derived from GTP as

Key words: Folding trap, Intermembrane space, Molybdenum cofactor, Sulfite oxidase, Heme

4876 Research Article
SO becomes arrested within the TOM complex with the C-terminus of SO being exposed to the cytosol. In aggregate, our data support a model in which Moco incorporation constitutes the driving force for a vectorial translocation of SO into the intermembrane space. Therefore, SO maturation provides a novel link between the stop-transfer sorting pathway and folding trap mechanisms of mitochondrial intermembrane space protein import.

Results

Sulfite oxidase mislocalizes to the cytosol in absence of Moco

As free Moco is unstable in a protein-free environment (Schwarz and Mendel, 2006), we asked the question of how Moco and SO, both being synthesized in the cytosol, become imported into and associated with each other within mitochondria. Following the completion of the Moco biosynthesis by gephyrin (Smolinsky et al., 2008), the cofactor was expected to require an immediate association with the respective apo-protein (Schwarz et al., 2009), which would suggest a rapid interaction between Moco and SO in the cytosol. However, cytosolic folding of SO would interfere with the mitochondrial import machinery. To assess a possible mutual dependence between the mitochondrial localization of SO and Moco, we expressed full-length SO carrying the proposed mitochondrial targeting sequence in wild-type (WT) and Moco-deficient human cells and determined the subcellular localization by immunocytochemistry and western blot analysis using SO or myc-tag-specific antibodies, respectively (Fig. 1; supplementary material Fig. S1).

As expected, SO exclusively localized to mitochondria in HEK-293 cells (Fig. 1A). In contrast, when expressed in Moco-deficient fibroblasts (supplementary material Fig. S2) derived from a patient with a mutation in the MOCS1 gene, SO was found to be diffusely distributed throughout the entire cell body with no clear mitochondrial localization (Fig. 1B). No nuclear exclusion of the SO staining was observed, which would point to an apparent size of less than 60 kDa as seen for other proteins that are able to passively enter the nucleus (Nigg, 1997).

Considering the presence of the mitochondrial targeting signal in SO, which was sufficient to drive GFP to mitochondria independently of Moco (supplementary material Fig. S3), the observed mislocalization of SO in Moco-deficient fibroblasts appeared to be unexpected. To probe if Moco binding to SO is required for mitochondrial targeting of the enzyme, we designed a mutant variant of SO (SO-R367H-K380R), which should not be able to bind Moco as concluded from the crystal structure of chicken SO (Kisker et al., 1997). We recombinantly expressed and purified SO-R367H-K380R from *E. coli* and determined the Moco content by high-pressure liquid chromatography (HPLC) FormA analysis to be less than 1%, while WT-SO was saturated with at least 40% Moco (Fig. 1C). Using size exclusion chromatography, we observed that Moco-free SO-R367H-K380R was unable to dimerize efficiently, as observed by the large monomeric peak in comparison to the corresponding dimer peak of WT-SO (Fig. 1D,E).

Following the expression of SO-R367H-K380R in HEK-293 cells, which are capable of Moco synthesis, a diffuse cellular distribution was found for this variant (Fig. 1F), as seen with WT-SO in Moco-deficient cells (Fig. 1B). To quantitatively assess the degree of colocalization of mitochondrial and SO variants, the Pearson correlation coefficients (PCC) were determined, which generate values from +1 (complete colocalization) to −1 (separate localization), with 0 representing a random and uncorrelated distribution (Manders et al., 1992). Under WT conditions, SO and mitochondria showed an expected average PCC of +0.97 (Fig. 1G). For the Moco-deficient variant, an average PCC value of +0.59 was determined (Fig. 1G). As a negative control, a cytosolic variant of SO lacking residues 1–80 (supplementary material Fig. S4) was used and revealed an average PCC value of +0.52 (Fig. 1G), indicating that the theoretically expected reading...
of ‘0’ is not reached. Therefore, the determined PCC values for Moco-deficient SO are in well agreement with the negative control lacking the mitochondrial targeting sequence.

To provide additional biochemical evidence for the observed mislocalization of SO in absence of Moco, WT-SO as well as the Moco-deficient SO variant R367H-K380R, each containing a C-terminal myc tag, were expressed in HEK-293 cells. Mitochondrial and cytosolic fractions were separated by discontinuous density gradient centrifugation and SO variants were detected in both fractions by anti-myc western blotting (Fig. 1H). Consistent with our microscopic observations, WT-SO was exclusively detected in the mitochondrial fraction, while the Moco-deficient SO-R367H-K380R variant was mainly present in the cytosolic pool. Band intensities were quantified and revealed nearly complete localization of the Moco-containing SO with mitochondria, while only 30% of the Moco-deficient variant was found in the mitochondrial fraction and approximately 70% remained in the cytosol. Appropriate separation of mitochondrial and cytosolic components was confirmed using voltage-dependent anion channel (VDAC) and gephyrin as respective markers (Fig. 1H). Consistent with these distributions, a major part of SO-R367H-K380R was sensitive to external proteinase K (PK) treatment, while WT-SO was protected from digestion in respect to its mitochondrial localization (supplementary material Fig. S5).

**SO is imported into mitochondria independent of Moco and is processed by the inner membrane peptidase complex**

To unravel the underlying mechanisms determining the Moco-dependent localization of SO, we first asked for the impact of Moco on the mitochondrial import process of SO, as the diffuse distribution of SO observed in absence of Moco may result from an impaired import into mitochondria. Mammalian SO contains a predicted N-terminal mitochondrial targeting sequence, which is absent in the mature protein and, as other known N-terminal mitochondrial targeting peptides, cleaved after mitochondrial import (Ono and Ito, 1984). Usually, this cleavage results in a shift of the electrophoretic mobility, which serves as a marker for successful import of the protein into mitochondria. WT-SO as well as the Moco-deficient SO-R367H-K380R variant were expressed in HEK-293 cells, while the unprocessed full-length variant was heterologously expressed in *E. coli*. A western blot against the C-terminal myc tag of SO revealed a similar processing of both, the WT and the Moco-deficient SO-R367H-K380R variant, detected at the same apparent size of ~53 kDa, which was clearly below the size of the full-length, non-processed SO (Fig. 2A). Consequently, mitochondrial import of SO is not dependent on the presence of Moco and occurs efficiently in a Moco-deficient background.

Ono and Ito reported an ATP-dependent import of SO into mitochondria through the TOM and translocase of the inner membrane (TIM) machineries, while the involved peptidases and thus the import mechanism remained uncharacterized (Ono and Ito, 1984). Mature mouse SO lacks the first 80 residues and contains a predicted (MITOPROT II) matrix processing peptidase (MPP) cleavage site at position 19. The remaining part of the leader sequence contains a hydrophobic, predicted transmembrane stretch at position 56–72 (Kyte and Doolittle, 1982). This signal sequence structurally resembles the mitochondrial targeting motifs of *Saccharomyces cerevisiae* cytochrome *b* and the mammalian diablo protein (Fig. 2B). Both proteins have been shown to be imported based on bipartite mitochondrial targeting signals and processed by the inner membrane peptidase (IMP) complex (Burri et al., 2005; Glick et al., 1993), a hetero-oligomer composed of the two catalytic subunits IMP1 and IMP2 and a third non-catalytic subunit. Deletion of any of the two catalytic subunits destabilizes and catalytically silences the complex (Gakh et al., 2002; Nunnari et al., 1993). In respect to the topological similarities of the N-terminal presequences of SO compared to cytochrome *b* and diablo, we asked if the IMP complex may also process SO. Thus, we knocked down the human IMMP1 protein by shRNA in HEK-293 cells and examined the processing of co-transfected, myc-tagged SO (Fig. 2C). Significant knockdown of IMMP1 was confirmed by western blotting, revealing only 20% residual expression after shRNA treatment. The decreased IMP levels resulted in the appearance of an additional band for endogenous diablo, representing the unprocessed precursor form. SO of untreated cells appeared to be completely processed, while the shRNA-mediated IMMP1 knockdown resulted in an accumulation of unprocessed SO (Fig. 2C). We thus conclude that the IMP complex processes SO in the mitochondrial intermembrane space.

**SO undergoes a retrograde translocation to the cytosol in absence of Moco**

Successful cleavage of the mitochondrial leader sequence demonstrated an efficient mitochondrial import of SO in absence of Moco. However, ~70% of the protein accumulated outside of mitochondria. Therefore, we asked if a retrograde translocation to the cytosol after mitochondrial import may explain the observed mislocalization of apo-SO.
The TOM complex is known to allow bidirectional translocation of unfolded, small proteins (<20 kDa) (Chacinska et al., 2004; Lutz et al., 2003). The import of those proteins is converted into a vectorial process by the integration of metal cofactors or the formation of internal disulfide bonds, thus being trapped by folding within the intermembrane space. We asked if SO may also be subject to a passive and retrograde translocation to the cytosol in absence of Moco. Therefore, we designed a mutant variant, which cannot be proteolytically processed by the IMP complex and should remain anchored at the inner mitochondrial membrane. We exchanged a short peptide sequence (residues 66–86) of SO, which we expected to contain the cleavage site for the IMP complex, for the topologically similar part of the TIM50 protein (Fig. 3A). While TIM50 is not processed by the IMP complex, it follows an import pathway (Mokranjac et al., 2003) similar to that proposed for SO. This chimeric fusion prevented IMP-mediated processing of SO and its full release into the intermembrane space, as demonstrated by the accumulation of unprocessed protein in crude cell extracts (Fig. 3B).

After ensuring that the chimeric SO–TIM50 variant remains catalytically active (supplementary material Fig. S6), we expressed the latter in Moco-deficient human fibroblasts. Notably, anchoring of the protein in the inner mitochondrial membrane resulted in a complete mitochondrial localization even in absence of Moco (Fig. 3C). Therefore, we propose that SO undergoes a reverse translocation from the intermembrane space to the cytosol in absence of Moco. Consequently, we conclude that Moco integration into SO initiates folding and thereby trapping of SO within mitochondria.

A reverse translocation from mitochondria to the cytosol has also been described for fumarase (Knox et al., 1998; Sass et al., 2001). In contrast to small intermembrane space proteins, which undergo reverse translocations via the TOM complex in the event that native folding is prohibited, fumarase has been shown not to completely cross the TOM complex before reverse translocation occurs (Knox et al., 1998). We asked if SO completes its translocation across the outer mitochondrial membrane in absence of Moco, or if Moco integration may be required as an additional driving force to pull the protein entirely across the outer mitochondrial membrane. Therefore, we expressed SO–TIM50 as well as the Moco-deficient variant SO–TIM50–R367H–K380R, each labelled with a C-terminal myc tag, in HEK-293 cells. Mitochondria were enriched by differential centrifugation and PK treatment, resulting in the hydrolysis of peptide sequences (including the myc tag) being outside of mitochondria. While diablo (intermembrane space control) levels remained unaffected following PK application, the cytosolic protein gephyrin became entirely digested (Fig. 3D). The SO–TIM50 chimera, capable of integrating Moco, was neither affected by PK treatment (Fig. 3D), whereas 30–40% of the C-termini of the Moco-deficient variant SO–TIM50–R367H–K380R were accessible to PK digestion (Fig. 3D,E). Since the latter variant was indeed localized to mitochondria (supplementary material Fig. S7), we conclude that the import of SO is not efficiently completed in absence of Moco but arrested in the TOM complex. Therefore, at least part of the C-terminal myc tag remained exposed to the cytosol and sensitive to PK treatment. However, anchoring of the SO–TIM50–R367H–K380R chimera in the inner mitochondrial membrane constituted an artificial driving force towards the intermembrane space, which may explain why only 30% of the chimeras were digested by PK, while 70% of SO–R367H–K380R were detected outside mitochondria (Fig. 1H).

Heme is integrated after Moco and dispensable for mitochondrial trapping of SO

Mammalian SO, apart from Moco, requires cytochrome b₅ type heme as a second cofactor (Kisker et al., 1997). Because heme cofactors have been shown to contribute to trapping of proteins in
the intermembrane space of mitochondria (Dumont et al., 1988) and heme binds at the N-terminal end of SO, we asked for the function of heme in the mitochondrial maturation process of SO.

We designed a mutant variant of SO, in which the two heme-coordinating histidine residues His119 and His144 were replaced by alanine. First, SO-H119A-H144A was expressed in E. coli, purified and analysed for heme binding and oligomerization using size exclusion chromatography. While dimerization was not affected, the very low heme-specific absorption at 413 nm demonstrated that heme cofactor integration was severely reduced (Fig. 4A). HPLC FormA analysis revealed a Moco saturation of around 50% and thus similar to WT-SO, indicating that heme depletion does not interfere with Moco incorporation (Fig. 4B). Expression of SO-H119A-H144A in HEK-293 cells resulted in a complete mitochondrial localization, demonstrating that the heme cofactor is dispensable for mitochondrial trapping and localization of SO (Fig. 4C).

The integration of heme represents a folding event, which was shown to be involved in the transfer of cytochrome b$_2$ across the outer mitochondrial membrane and its retention in the mitochondrial intermembrane space (Esaki et al., 1999). Heterologous expression of the Moco-deficient SO-R367H-K380R in E. coli revealed the presence of heme (Fig. 1E), while no retention of this variant was observed in mitochondria (Fig. 1F). Hence, we asked if the SO heme cofactor is principally capable for trapping the heme domain within the intermembrane space. To uncouple Moco insertion from heme integration, we expressed the isolated heme domains of WT as well as H119A-H144A SO variants in HEK293 cells. While the WT heme domain localized to mitochondria (Fig. 4D), the heme-deficient H119A-H144A variant showed a diffuse distribution (Fig. 4E) similar to full-length SO in absence of Moco (Fig. 1F). We conclude that the SO heme cofactor is competent for trapping the heme domain in the intermembrane space and following heme incorporation, reverse translocation from mitochondria is prohibited. Because SO is not efficiently trapped in mitochondria in absence of Moco, we therefore conclude that the heme cofactor is not inserted efficiently in vivo in absence of Moco, pointing to a hierarchy of cofactor insertion starting with Moco followed by heme integration.

**SO dimerization is not involved in retaining the protein in the intermembrane space**

Dimerization comprises a third event in the SO maturation process, which could also contribute to trapping of SO in the intermembrane space. To integrate SO dimerization into the hierarchy of processes leading to SO maturation, we created a monomeric SO variant. In a SO-deficient patient, a substitution of glycine to aspartate at position 473 has been found and it was assumed to form a monomeric and hence not functional SO variant (Kisker et al., 1997). Therefore, we replaced the corresponding Gly531 residue of mouse SO to aspartate. Consistent with the authors proposal (Kisker et al., 1997), size exclusion chromatography of SO-G531D expressed and purified from E. coli demonstrated a monomeric appearance of this variant. In addition, the heme content was similar to WT-SO (Fig. 5A) and HPLC FormA analysis demonstrated that Moco incorporation was neither affected in SO-G531D (Fig. 5B). Finally, expression of SO-G531D in HEK-293 cells resulted in

---

**Fig. 4.** The heme cofactor is not involved in trapping and is integrated after Moco in vivo. (A) Size exclusion chromatography of purified SO-H119A-H144A variant monitored by absorption at 280 nm (blue trace) and 413 nm (red trace), demonstrating a very low saturation of heme for the SO-H119A-H144A variant. The displayed profiles are representative of three independent experiments. (B) Quantification of Moco saturation in 100 pmol of purified WT-SO and SO-H119A-H144A variant using HPLC FormA analysis. Error bars represent standard deviations of three independent experiments. (C–E) Expression of the heme-deficient SO-H119A-H144A variant (C), of the isolated heme domain (SO-heme) with the leader sequence (D) and the heme-deficient SO-heme-H119A-H144A variant (E) in HEK-293 cells as described in Fig. 1. Scale bars: 10 μm. Cartoons illustrate the status of cofactor saturation and oligomerization: L, leader sequence; H, heme; Mo, Moco; DD, dimerization domain.

**Fig. 5.** Dimerization is not involved in mitochondrial SO trapping. (A) Size exclusion chromatography of purified SO-G531D variant monitored by absorption at 280 nm (blue trace) and 413 nm (red trace), demonstrating that SO-G531D is predominately monomeric. The displayed spectra are representative of four independent experiments. (B) Quantification of Moco saturation in 100 pmol of purified WT-SO and SO-G531D variant using HPLC FormA analysis. Error bars represent standard deviations of four independent experiments. (C) Expression of monomeric SO-G531D variant in HEK-293 cells as described in Fig. 1. Scale bar: 10 μm.
mitochondrial localization (Fig. 5C), demonstrating that SO dimerization is dispensable for mitochondrial trapping.

As shown in Fig. 1D, no proper dimerization does occur in absence of Moco, which appears to be a prerequisite for dimerization. Hierarchically, dimerization therefore is supposed to occur after Moco integration. Heme integration and dimerization seem to proceed independent of each other in no defined hierarchic order. While the heme-deficient variant SO-H119A-H144A allows dimerization, the monomeric SO-G531D variant efficiently integrates the heme cofactor.

The mitochondrial import of Moco and its incorporation into SO is highly organized

We have shown that the association of Moco with apo-SO is required to shift the equilibrium of SO maturation to the intermembrane space. Given the known instability of Moco in a protein-free environment (Deistung and Bray, 1989), the process of Moco translocation to mitochondria and insertion into apo-SO must be synchronized. For cytosolic Moco enzymes (xanthine and aldehyde oxidase), a co-translational maturation is proposed (Schwarz et al., 2009), while in autotrophic organisms Moco storage proteins have been identified, which permit cofactor insertion into the respective apo-enzymes (Fischer et al., 2006; Kruse et al., 2010).

In case of mammalian SO, a co-translational association with Moco needs to be prevented to ensure efficient mitochondrial targeting of the unfolded precursor. To probe the ability of SO to bind Moco in the cytosol, we expressed full-length SO as well as a cytosolic variant lacking the N-terminal targeting signal (supplementary material Fig. S4), each containing a C-terminal His tag, in HEK-293 cells. Following affinity purification, SO activities of both variants were determined (Fig. 6A). Both, the mitochondrial as well as the cytosolic variants showed equal activities, indicating that Moco and heme were incorporated into SO in the cytosol in absence of the mitochondrial targeting signal (Fig. 6A).

Since the association of full-length SO and Moco is prevented in the cytosol, we next asked for the presence of a Moco-stabilizing and/or -storing component in mitochondria, which would bind and transfer Moco to apo-SO. For this purpose, we purified heterologously expressed mouse SO from E. coli (Fig. 6B) and mitochondria from murine liver by discontinuous density gradient centrifugation. Purity of the separated fractions was ensured by anti-VDAC and anti-gephyrin western blotting as markers for mitochondrial and cytosolic fractions, respectively (Fig. 6C). SO activities of mitochondria and purified SO were determined and the fraction of transferable Moco was measured by the nit-1 assay, which is based on the reconstitution of apoprotein reductase derived from a Moco-deficient Neurospora crassa cell extract (Nason et al., 1971) (Fig. 6D,E). For both, SO and mitochondria, a dose-dependent enzyme activity as well as cofactor activity was found, respectively. The fraction of transferable Moco per unit SO activity was determined and we found an ~50% higher content of transferable Moco in mitochondria than with purified SO. This SO independent pool of free Moco might be a fraction, which is ‘in transit’ to apo-SO. However, the mitochondrial amidoxime-reducing component mARC (Havemeyer et al., 2006) also requires Moco and consequently contributes to the fraction of SO independent cofactor.

Discussion

The formation of functional SO implies a complex, multi-step maturation procedure, which is believed to be highly coordinated. The transport of SO and Moco to mitochondria, the mitochondrial processing of SO, the integration of Moco and heme as well as the homodimerization need to occur in a defined order to ensure efficient SO maturation.

We identified Moco as the key component of SO assembly, which does not only ensure mitochondrial localization but also heme integration and dimerization. In absence of Moco, about 70% of SO was not present in mitochondria but instead mislocalized to the cytosol, although processing of the N-terminal targeting peptide revealed that the protein was first imported into mitochondria. Since anchoring of SO in the inner mitochondrial membrane resulted in mitochondrial localization in absence of Moco, we propose that unfolded, Moco free SO undergoes a passive backshift to the cytosol (Fig. 7).

Cofactor-dependent mitochondrial localization has been described earlier for cytochrome c, which requires the heme
mitochondrial import of SO fundamentally differs from all other intermembrane proteins, that use the presequence pathway (Neupert and Herrmann, 2007), in which the presence of the N-terminal targeting motif is entirely sufficient for mitochondrial localization.

A subpopulation of fumarase, a mitochondrial matrix protein becoming imported based on a conservative N-terminal targeting motif, was shown to move in a retrograde fashion to the cytosol after processing, thus resulting in a dual localization of the protein (Knox et al., 1998). This particular subpopulation, destined as the cytosolic pool, therefore does not completely cross the TOM complex, while a C-terminal folding event outside of mitochondria constitutes a reverse driving force towards the cytosol (Sass et al., 2001).

According to the SO size of 50 kDa, a passive and bidirectional diffusion across the TOM complex, as described for small and unfolded proteins of the intermembrane space, seemed to be unlikely. In analogy to the maturation of fumarase, we rather asked if SO may not completely cross the TOM complex in absence of Moco, but instead remains arrested in the latter before undergoing a reverse translocation in absence of Moco as the folding trap. This hypothesis was supported by the expression of the Moco-deficient chimera SO–TIM50-R367H-K380R, a fraction of which was C-terminally accessible to protease treatment. We assume that Moco incorporation is required to initiate SO folding and to permit an additional and essential driving force to pull SO across the TOM complex. Therefore, the mitochondrial import of SO provides a novel and unique connection between the folding-trap mechanism and the presequence pathway, in which elements of two different and so far not connected pathways are essentially required to ensure an efficient mitochondrial localization. SO combines the N-terminal bipartite targeting peptide and the ATP-dependent translocation across the inner mitochondrial membrane of the members of the presequence pathway with a cofactor-dependent trapping mechanism known for members of the folding-trap/ Mia40 import pathway.

Interestingly, the heme cofactor did not contribute to mitochondrial trapping of SO, while it is absolutely essential for the mitochondrial localization of cytochrome b$_2$ (Esaki et al., 1999) and cytochrome c (Dumont et al., 1988). In respect to the observations of Esaki and co-workers (Esaki et al., 1999), we expected the non-covalently bound heme cofactor of SO to be suitable for mitochondrial trapping, which was indeed the case for the isolated SO heme domain. We conclude that the cytochrome b$_3$ type heme of SO is competent for mitochondrial trapping, but in vivo it is not incorporated efficiently in absence of Moco and therefore does not rescue the mitochondrial localization of SO in absence of Moco.

In contrast to the in vivo situation in HEK cells, overexpression of Moco-deficient SO in E. coli revealed the incorporation of heme, demonstrating that heme integration is not strictly dependent on prior Moco incorporation. However, recombinantly expressed SO is accumulating in E. coli and therefore constantly exposed to heme integration. In contrast, when expressed in mammalian cells, the reverse translocation from mitochondria in absence of Moco kinetically competes with heme integration. Assuming that heme integration is a kinetically less favoured event in absence of Moco, SO would integrate heme in E. coli due to high expression and accumulation, but would not incorporate heme in vivo where the reverse translocation occurs presumably faster than heme integration. Given the fact that 30% of the Moco-deficient SO did not become imported into mitochondria based on its N-terminal bipartite targeting signal (black and blue line). The N-terminus (black line) is directed to the matrix in an ATP-dependent manner until a hydrophobic anchor (blue line) in the second part of the targeting signal results in a stop of the transfer. The IMP complex of the inner mitochondrial membrane cleaves SO to release the soluble protein into the intermembrane space. In the absence of Moco, the import is not completed and the C-terminal part (green line) of SO remains exposed to the cytosol and heme is not able to bind efficiently. A major population of soluble SO lacking the N-terminal targeting sequence relocates to the cytosol and is not able to re-enter mitochondria. In the presence of Moco, which enters mitochondria independently of SO by a yet unknown mechanism (?), SO folding is initiated and thereby a retrograde translocation of SO to the cytosol is prevented, resulting in a vectorial translocation to the intermembrane space. Following Moco integration, the heme-binding site becomes available allowing efficient heme incorporation. Finally, SO dimerizes following Moco insertion. See text for details. IM, inner membrane; IMP, inner membrane peptidase; IMS, intermembrane space; TOM, translocase of the outer membrane.

Fig. 7. Assembly and maturation of mammalian SO. Cytosolic SO is targeted to the TOM complex in the outer mitochondrial membrane (OM) based on its N-terminal bipartite targeting signal (black and blue line). The N-terminus (black line) is directed to the matrix in an ATP-dependent manner until a hydrophobic anchor (blue line) in the second part of the targeting signal results in a stop of the transfer. The IMP complex of the inner mitochondrial membrane cleaves SO to release the soluble protein into the intermembrane space. In the absence of Moco, the import is not completed and the C-terminal part (green line) of SO remains exposed to the cytosol and heme is not able to bind efficiently. A major population of soluble SO lacking the N-terminal targeting sequence relocates to the cytosol and is not able to re-enter mitochondria. In the presence of Moco, which enters mitochondria independently of SO by a yet unknown mechanism (?), SO folding is initiated and thereby a retrograde translocation of SO to the cytosol is prevented, resulting in a vectorial translocation to the intermembrane space. Following Moco integration, the heme-binding site becomes available allowing efficient heme incorporation. Finally, SO dimerizes following Moco insertion. See text for details. IM, inner membrane; IMP, inner membrane peptidase; IMS, intermembrane space; TOM, translocase of the outer membrane.
was located to mitochondria, heme integration might have occurred in those cases preventing the retrograde backshift of a fraction of SO in absence of Moco. Therefore, we propose that the presence of the apo-SO Moco-binding domain slows down heme integration significantly and ultimately Moco insertion becomes the rate limiting step in the overall SO maturation process.

Apart from prosthetic groups, oligomerization events are also conceivable to contribute to trapping mechanisms of proteins in the intermembrane space. Given the fact that a monomeric variant of SO localized to the mitochondria, we conclude that oligomerization is not required for mitochondrial retention of SO. Since Moco-deficient SO mainly forms monomers, we hypothesize that Moco integration presents a prerequisite for dimerization, thus inducing structural rearrangements of the dimmerization domain allowing oligomerization. In contrast to heme integration, which can occur upon overexpression in E. coli in absence of Moco, efficient dimerization appears to be strictly dependent on Moco integration.

As Moco is required for trapping and mitochondrial retention of SO, a separate transport of the cofactor to the intermembrane space is required. The functional trapping of SO demonstrates that Moco associated with SO does not cross the TOM channel and remains inside mitochondria. In addition, the folded monomeric Moco-containing domain of SO comprises a diameter of ~4 nm (Kisker et al., 1997), while the diameter of the open pore of the TOM complex accounts for a maximum of only 2 nm (Ahling et al., 1999). A combined import of any Moco–SO complex across the TOM complex therefore appears to be very unlikely. However, in respect to the instability of Moco, fast association of Moco with its apo-proteins or stabilizing Moco-binding proteins has been proposed and could occur co-translationally in the cytosol (Schwarz et al., 2009). In case of SO, our data suggest that apo-SO does not associate with cytosolic Moco, thus allowing two conclusions: first, binding of Moco to apo-SO must be prevented in the cytosol and second, Moco needs to be transported separately from apo-SO to mitochondria.

In absence of the N-terminal leader sequence, SO accumulates in the cytosol being fully active. Therefore, the N-terminus seems to block spontaneous Moco insertion, probably by inducing the binding of chaperones that keep SO in a TOM-transport-competent state. Alternatively, SO may be imported to mitochondria in a co-translational manner to prevent premature cofactor insertion into SO. Accumulation of ribosomes on the mitochondrial surface and evidence for partial co-translational protein import into mitochondria were shown formerly (Kellems et al., 1975; Ades and Butow, 1980). Moco might be transported to mitochondria by a hitherto unknown carrier protein. In the green alga Chlamydomonas reinhardti, such a Moco carrier protein was identified (Fischer et al., 2006) and a novel class of Moco-binding proteins was recently reported for Arabidopsis thaliana (Kruse et al., 2010). Similar Moco-binding chaperons, which stabilize Moco and promote its insertion into apo-proteins, were not found in animals so far. However, our identification of a SO independent pool of Moco in mitochondria may either point to a Moco-stabilizing component or to an unexpected high stability of free Moco within the cell.

While Moco may enter mitochondria by passive diffusion, the successful import of SO into mitochondria is dependent on an N-terminal targeting signal, a membrane potential and ATP (Ono and Ito, 1984). In the current study, we demonstrate that the incorporation of Moco is in addition required as a complementary driving force across the outer mitochondrial membrane resulting in a folding trap in the intermembrane space. In summary, we conclude that the N-terminus of SO is transported to the mitochondrial matrix until the hydrophobic anchor at position 56–72 stops further translocation at the inner mitochondrial membrane. Following the cleavage by the IMP complex, mature and soluble SO starting at residue 80 (Kisker et al., 1997) is released into the intermembrane space. After translocation, Moco is integrated, which subsequently increases the incorporation rate of the heme cofactor and finally dimerization completes the maturation process of SO.

Materials and Methods

Determination of SO content by the nit-1 assay

Isolated mitochondria and harvested human fibroblasts were homogenized by sonication on ice for 30 s and subsequently centrifuged at 21,000 g for 15 min. Purified SO was applied without any further treatment. Between 1 and 20 µl of mitochondrial or fibroblast extract or purified SO were incubated anaerobically with nit-1 extract for 12 h. Nitrate reductase activity was subsequently determined as described (Reiss et al., 2001).

Isolation of mitochondria

Crude mitochondria were obtained from murine liver or HEK-293 cells as previously described (Mattiuzzi et al., 2002). To obtain highly pure mitochondria, the crude fraction was loaded on a discontinuous density gradient of 80, 52 and 26% Percoll diluted in isolation buffer (220 mM mannitol, 70 mM sucrose, 2 mM EGTA, 0.1% BSA, 20 mM HEPES-KOH, pH 7.4). Samples were centrifuged at 23,000 rpm for 45 min in a Beckman MLS-50 rotor and the mitochondrial layer was aspirated from the 26–52% interface. The pure mitochondria were diluted to a total volume of 1.5 ml with isolation buffer in a 1.5 ml Eppendorf tube and centrifuged for 10 min at 16,000 g to remove residual Percoll. This washing step was repeated once and mitochondria were finally resuspended in isolation buffer.

Purification of mouse SO

Mouse SO was expressed in E. coli strain TP1000 (Palmer et al., 1996) for 48 h at 25°C. SO expression from pQE80 was induced after 2 hours incubation at 25°C with 100 µM IPTG. 150 µM sodium molybdate was added to the culture to achieve sufficient levels of Moco production. His-tagged SO was affinity purified by Ni-NTA according to the instructions of the manufacturer (Machery-Nagel). For further purification, Ni-NTA eluate was diluted 1:5 in buffer A (50 mM Tris pH 8.0) and separated on a Dr Maish SourceQ 15 anion exchange column to purity. The run was conducted applying a gradient of 0% buffer B (50 mM Tris pH 8.0, 1 M NaCl)/100% buffer A to 100% buffer B/0% buffer A.

SO activity assay

For SO activity determination, 53 ml of solution A (8 µl 1 M Tris/acetate pH 8.0, 10 µl 5 mM deoxycholic acid, 35 µl 0.5 mM KCl) and 120 µl of solution B [108 µl SO buffer (0.1 M Tris/acetate pH 8.0, 0.1 mM EDTA), 12 µl cytochrome c (10 mg/ml)] were mixed. 0–15 µl protein were added and the reaction was started by adding 12 µl 5 mM sodium sulfate. Reactions were conducted in 96-well plates and the A550 was recorded 30 times for 5 s each with an EL808 plate reader (BioTEK).

Expression constructs

Mouse SO was amplified from a mouse liver cDNA by PCR and cloned into pcDNA3.1 Myc/HisA (Invitrogen) using HindIII and EcoRI restriction sites and into pQE80 (Qiagen) using Sall and SrfI restriction sites, respectively. Mutant variants H119A-H144A, R367H-K380R and G531D were introduced by means of the QuikChange Site Directed Mutagenesis Kit (Stratagene) according to the instructions of the manufacturer. In the chimeric MSO–TIM50 variant, residues Arg64–Arg84 were replaced by residues Ile66–Phe86 of the murine Tim50 instructions of the manufacturer. In the chimeric MSO–TIM50 variant, residues Arg64–Arg84 were replaced by residues Ile66–Phe86 of the murine Tim50

Cell culture and transfection

HEK-293 and human fibroblasts were cultured in 10-cm dishes at 37°C and 5% CO2 in DMEM (PAA Laboratories). For confocal laser scanning microscopy, 1×106 HEK-293 cells and 3×106 human fibroblasts were seeded on collagenized coverslips in 12-well plates. After 24 h, transfection of HEK-293 cells and human fibroblasts was conducted with polyethylimine (PEI, 1 mg/ml, diluted in H2O, pH 7.0). For each well, 3.4 µl PEI were added to 66 µl DMEM in the absence of...
foetal calf serum. Following 5 min of incubation, 0.85 µg of DNA was added, followed by another 20 min of incubation. This mixture was added to each well and cells were grown for another 48 h. For biochemical studies using western blotting, 1.45×10^6 HEK-293 cells were seeded on 10-cm plates and transfected with PEI as described above but scaled linearly to the increased cell numbers. Cells were harvested 48 h after transfection.

**Antibody staining of cell culture preparations and confocal microscopy**

For mitochondrial staining, cells were incubated with MitoTracker Red CMXRs (Invitrogen) according to the instructions of the manufacturer. Cells were fixed with 4% paraformaldehyde for 20 min and subsequently permeabilized with 0.2% Triton X-100 (diluted in PBS) for 20 min. After incubation with 1% BSA for 2 min, the primary anti-SO antibody (Abcam) was applied for 1 hour at 37°C. Afterwards, cells were incubated with 5 µg/ml secondary antibody (Alexa Fluor 488 goat anti-mouse, Invitrogen) for 1 hour. Cells were finally washed twice with PBS and mounted on a microscope slide. Images of cells were taken with a Nikon Eclipse Ti confocal laser scanning microscope and processed with the EZ-C1 software (Nikon). Threshold Pearson coefficients were determined with the VOLocity software (PerkinElmer).

**Western blotting and quantification**

Western blots were analysed with the Chemiluminescence DeVision HQQ camera system and the GEL-PRO ANALYZER software (Decon Science Tec). Band intensities were quantified with the ImageJ software (Decon Science Tec). Primary antibodies used were, anti-gephyrin (Synaptic Systems), anti-smap (Abgen), anti-gephyrin (Roche), anti-smac/diablo (Abcam), anti-SO (Abcam), anti-vdac (Abcam). Secondary antibodies (Santa Cruz) coupled to horseradish peroxidase were visualized by Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific) according to the instructions of the manufacturer.

**Detection of the Moco derivative FormA by high pressure liquid chromatography**

Purified SO (100 pmol) was diluted to a total volume of 140 µl with 100 mM Tris-HCl pH 7.2 and oxidized with 17.5 µM HClO4. After 30 min in the dark, the samples were diluted 1:10 in HPLC running buffer and equilibrated with HPLC running buffer. FormA fluorescence was recorded with an HPLC column (Dr Maisch GmbH) (10 mM KH2PO4 pH 3.0), 90 µM ascorbic acid was added. Finally, 70 µM of freshly prepared 1% ascorbic acid was added. Finally, 70 µM of freshly prepared 1% ascorbic acid was added. L-1.5/2.0% I2/KI solution + 1 µl 37% HCl at room temperature in the dark for 12 h. Samples were afterwards centrifuged at 21,000 g and to the supernatant 20 µl of freshly prepared 1% ascorbic acid was added. Finally, 70 µl 1 M Tris (unbuffered), 5 µl 1 M MgCl2 and 0.5 µl 1.5/2.0% I2/KI (Roche) were added and incubated for 30 min in the dark. After diluting the samples 1:10 in HPLC running buffer (10 mM KH2PO4 pH 3.0), 90 µl of the processed sample was injected onto a Dr Maisch 250×4.6 mm Reprosil 100 C 4, 5 µm HPLC column (Dr Maisch GmbH) equilibrated with HPLC running buffer. FormA fluorescence was recorded with an excitation at 370 nm and emission at 450 nm. Moco saturation of the protein was determined according to the FormA peak area and comparison to a FormA standard with known concentration.

**Acknowledgements**

We thank Thomas Langer and Takashi Tatsuta for helpful discussions. We are grateful to Joana Fischer for excellent technical assistance.

**Funding**

The work was supported by the Studienstiftung des Deutschen Volkes [to J.M.K.]; and Fonds der Chemischen Industrie [grant number 166652 to G.S.].

Supplementary material available online at http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.110114/-/DC1

**References**


Burri, L., Strahn, Y., Hawkins, C. J., Gentle, I. E., Purry, M. A., Verhagen, A., Callus, B., Vaux, D. and Lithgow, T. (2005). Mature DIABLO/Smac equilibrated with HPLC running buffer. FormA fluorescence was recorded with an excitation at 370 nm and emission at 450 nm. Moco saturation of the protein was determined according to the FormA peak area and comparison to a FormA standard with known concentration.


Fig. S1: Specificity of anti-myc and anti-SO primary antibodies

Myc-tagged SO was expressed in HEK-293 cells and total protein extract was loaded in each lane of the 12% SDS gel. After Western blotting, SO was detected either by anti-SO or anti-myc antibodies to ensure antibody specificity.
**Fig. S2: Moco content of MOCS1-/- Moco-deficient human fibroblasts**

Moco content of WT and MOCS1-/- Moco-deficient fibroblasts was determined using the *nit-1* assay. Different amounts of cell extract were each applied during the assay. The depicted value represents an average of the *nit-1* activities expressed as units per mg total protein.
Fig. S3: The SO leader sequence fused to GFP localizes to mitochondria independently of Moco

The SO leader sequence (residues 1-80, L) was fused to GFP and expressed in WT and MOCSI−/− Moco-deficient fibroblasts. Mitochondria were stained with MitoTracker Red as described in Figure 1. Bar, 10 µm.
Fig. S4: SO lacking the N-terminal targeting signal does not localize to mitochondria.

SO (Δ1-80) lacking the first 80 residues was expressed in HEK-293 cells. SO and mitochondria were stained as described in Figure 1. Bar, 10 µm.
Fig. S5: Moco-deficient SO is proteinase K sensitive.

WT SO and SO-R367H-K380R were expressed in HEK-293 cells for 48 h. Whole cell extracts were treated with or without proteinase K (PK) and loaded on a 12% SDS gel. Mitfusion 2 (MFN2 – marker for outer mitochondrial membrane) and diablo (marker for intermembrane space proteins) were detected as control proteins, SO variants were detected via their C-terminal myc-tags.
Fig. S6: The SO–TIM50 chimera is kinetically active.

WT SO and SO-TIM50 chimera were expressed in HEK-293 cells and SO activities in crude proteins extracts were determined using the cytochrome c-dependent SO assay. The depicted value corresponds to the total activity of SO in the cuvette (10 µg protein extract). As a control, untransfected HEK-293 cell extracts were used representing the intrinsic activity of HEK293 cell SO.
Fig. S7: Moco-deficient SO–TIM50 chimera localizes to mitochondria

SO-TIM50-R367H-K380R was expressed in HEK-293 cells. SO and mitochondria were stained as described in Figure 1. Bar, 10 µm.