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Brief Communication

Visualization of Redox-Controlled Protein Fold in Living Cells

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SUMMARY

Most mitochondrial proteins are encoded by nuclear DNA, synthesized in the cytoplasm, and imported into mitochondria. Several proteins of the intermembrane space (IMS) are imported and localized through an oxidative process, being folded through the formation of structural disulfide bonds catalyzed by Mia40, and trapped in the IMS. To be imported, these proteins need to be reduced and unfolded; however, no structural information in situ exists on these proteins in the cytoplasm. In humans, Mia40 undergoes the same mechanism, although its folding state in the cytoplasm is unknown. We provide atomic-level details on the Mia40 folding state in the human cell cytoplasm through in-cell nuclear magnetic resonance. Overexpressed cytoplasmic Mia40 is folded, and its folding state depends on the glutaredoxin 1 (Grx1) and thioredoxin 1 (Trx1) systems. Specifically, increased Grx1 levels keep most Mia40 unfolded, while Trx1 is less effective.

INTRODUCTION

The majority of the human proteins are produced by nuclear DNA and released in the cytoplasm and/or in the endoplasmic reticulum (ER). These proteins then need to complete their folding and maturation process, which could involve several steps, from cofactor binding to cysteine oxidation to other posttranslational modifications. Furthermore, if the protein destiny is a cellular compartment other than the cytoplasm, some or all maturation and folding steps may take place in the final cellular localization. This is particularly true for a large share of proteins present in the intermembrane space (IMS) of mitochondria, but which do not feature any target sequence for this organelle (Banci et al., 2009b; Longen et al., 2009; Neupert and Herrmann, 2007). They were proposed to be in an unfolded state in the cytoplasm and thus can enter mitochondria, thanks to their conformational flexibility that allows them to go through the translocase of the outer membrane (TOM) channel (Neupert and Herrmann, 2007). Once they have entered the IMS, they fold to their native form, thus being blocked in a defined, more rigid conformation, which prevents them from crossing back to the outer membrane (Chacinska et al., 2004; Lu et al., 2004; Mesecke et al., 2005). It is therefore evident that the folding state of a protein depends on the cellular compartment where the protein is located and on its properties. It has been shown that the import of some of these IMS proteins is in fact modulated by cytosolic thiol-disulfide regulation systems (Durigon et al., 2012). However no detailed, atomic resolution study has confirmed such findings.

In this work we show, by exploiting in-cell nuclear magnetic resonance (in-cell NMR; Banci et al., 2013; Inomata et al., 2009; Ogino et al., 2009; Reckel et al., 2007; Selenko et al., 2008), that indeed the properties of the cell compartment in terms of redox-regulating components influence the folding state of one of such proteins, whose oxidation state is compartment-dependent. We have specifically characterized Mia40, a hub protein for the mitochondrial protein import process. Mia40 is an oxidoreductase that catalyzes in the IMS the formation of internal disulfide bonds on its protein substrates through the intermediate formation of a mixed disulfide bond between the substrate and its catalytic CPC motif (Banci et al., 2009a; Chacinska et al., 2004; Grumbt et al., 2007; Naoé et al., 2004). Together, these proteins constitute the disulfide relay system of the IMS (Mesecke et al., 2005; Tokatlidis, 2005). Upon interaction with Mia40 and subsequent formation of their disulfide bonds, the substrates of Mia40 become folded and are trapped in the IMS (Banci et al., 2010; Gabriel et al., 2007). Interestingly, Mia40 itself obtains its final structure in the IMS upon the formation of two internal disulfide bonds, likely by acting as a substrate of itself (Chacinska et al., 2008; Grumbt et al., 2007). Like the other substrates of the disulfide relay system, Mia40 has to cross the outer mitochondrial membrane in an unfolded, reduced state. Glutaredoxin 1 (Grx1) and thioredoxin 1 (Trx1) are cytoplasmic oxidoreductases involved in the regulation of protein thiol groups and in the cellular defense against oxidative stress (Holmgren, 1989; Meyer et al., 2009). Trx1 was recently shown to be responsible for facilitating the mitochondrial import of the small Tim proteins (Durigon et al., 2012). It can be hypothesized that other small proteins of the IMS sharing the same import mechanism, including Mia40, are regulated by such thiol-regulating proteins.

We show here that Mia40, even in the reducing environment of the cytoplasm, is largely in the oxidized, folded state when it is overexpressed, thus indicating that the high cytoplasmic level of reduced glutathione is not sufficient alone to maintain the reduced state of Mia40. Co-expression of Grx1 (Meyer et al., 2009; Sagemark et al., 2007) keeps Mia40 mostly in the unfolded, reduced state, while co-expression of Trx1, which has a similar role in keeping protein thiols reduced in the

cytoplasm (Holmgren, 1979; Meyer et al., 2009), affects the oxidation state of Mia40 to a lesser extent. Additionally, Grx1 does not catalyze the reduction of Mia40 in the presence of reducing agents in vitro, implying some effect of the cytoplasmic environment that is not reproduced in vitro. Overall, these results indicate that Mia40, and likely other mitochondrial proteins that share the same structural motif and import pathway, tend to reach the oxidized, folded conformation in the absence of specific proteins, even in the reducing environment of the cytoplasm, and therefore require intervention of the cytoplasmic thiol-disulfide regulation mechanisms, especially those of Grx1, to reach the outer mitochondrial membrane in the reduced, import-competent state.

RESULTS

Transient expression of human Mia40, either alone or together with Grx1 or Trx1, was induced in human embryonic kidney (HEK293T) cells, and the relative expression levels were modulated by transfecting different amounts of DNA for each gene. The expression levels of each protein were determined with western blot analysis of the cell extracts (Figure 1). The concentration of overexpressed Mia40 remained constant in most of the samples, around 60 ± 10 μM. Grx1 was co-expressed in various amounts, ranging from 20 ± 10 μM to 100 ± 30 μM. Trx1 was co-expressed in two amounts: 35 ± 4 μM and 70 ± 15 μM. We assessed the cellular distribution of the overexpressed proteins in isolated cytosolic and mitochondrial fractions. Irrespective of the total protein abundance, >96% of Mia40 and >99% of either Grx1 or Trx1 were present in the cytosolic fraction (Figure S1A available online). Endogenous Mia40 remained undetected (<20 nM, data not shown), while endogenous levels of Grx1 were 60 ± 20 nM, ~10% of which was found in mitochondria, and 5 ± 2 μM of Trx1 was localized in the cytoplasm only, as measured on extracts of untransfected cells (Figure S1B).

Mia40, overexpressed alone in the cytoplasm of human cells, took a folded conformation, as monitored through ^{1}H-^{15}N and ^{1}H NMR spectra, which corresponds to the functional state of Mia40 that is normally found in the IMS (Figure 2A), and is the same conformation as that of oxidized Mia40 in vitro ($Mia40^{2S\text{-}S}$; Figure S2A). The central region of the protein is stably folded in two α helices while the N- and C-terminal regions are intrinsically unstructured. The cross-peaks of the folded part were well dispersed in the spectrum, while the peaks of the unstructured regions appeared as intense overlapped signals, which fell in the central part of the spectrum. Analysis of the methyl region of the ^{1}H NMR spectrum, which provides more sensitivity to assess the relative amount of folded Mia40 (Figure 2B), indicated that the folded protein was ~85% of the total protein amount (Figure 4). When it was co-expressed with various amounts of Grx1, a large fraction of Mia40 was present in the reduced, unfolded state. This effect was confirmed by a decrease of the methyl signal and also reflected in the ^{1}H-^{15}N NMR spectra, in which the cross-peaks of the folded part of Mia40 were barely detected as expected, while the cross-peaks of the unfolded parts were still visible, both in intact cells (Figures 2C and 2D) and in cell extracts (Figures 3C and 3D). In the presence of increasing amounts of Grx1, oxidized Mia40 ($Mia40^{2S\text{-}S}$) in the cytoplasm decreased from ~85% to ~25% of the total

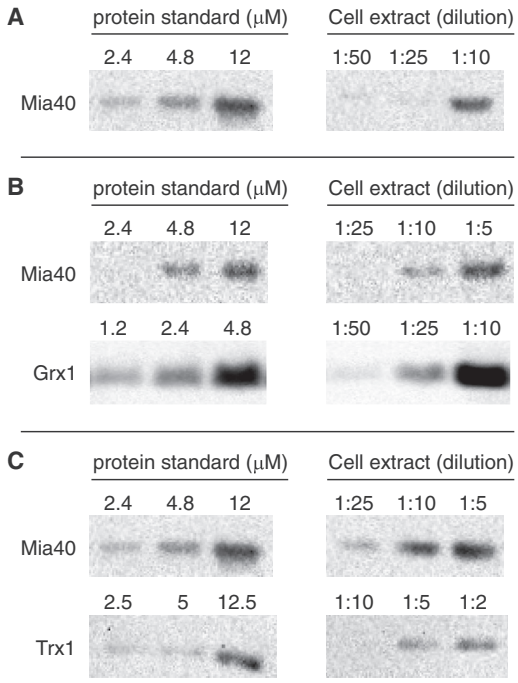

Figure 1. The Total Amount of Mia40, Grx1, and Trx1 in the Cell Extracts Is Measured with Western Blot Analysis

Samples of cell extracts were blotted at increasing dilutions together with samples of pure Mia40, Grx1 and Trx1 at known concentrations.

(A) Extract from cells expressing Mia40 alone.

(B) Extract from cells co-expressing Mia40 and Grx1 (1:0.25:2 *hMia40:hGrx1*:PEI ratio).

(C) Extract from cells co-expressing Mia40 and Trx1 (1:1:2 *hMia40:hTrx1*:PEI ratio) in selenium-supplemented medium. A calibration curve was obtained for each blot from the intensities of the standard Mia40 samples.

The concentration of Mia40 in the cell extracts was calculated for each dilution from the calibration curve and averaged.

See also Figure S1.

Mia40 (Figure 4). Co-expressed Grx1 was in the fully reduced and active state (Figure S1C), consistent with its reported redox potential (E_0 = −230 mV, compared to −290 mV for the GSH/GSSG couple in the cytoplasm; Sagemark et al., 2007). Grx1 is invisible in the in-cell NMR spectra because its signals are broadened beyond detection as a consequence of its slow tumbling rate, which is likely due to interactions with the cellular environment (a similar effect has been reported for several proteins overexpressed in *E. coli* cytoplasm, including wild-type ubiquitin [Sakai et al., 2006], cytochrome c [Crowley et al., 2011], *Escherichia coli* thioredoxin, and FKBP [Reckel et al., 2012]). This behavior was confirmed by the empty ^{1}H-^{15}N in-cell NMR spectra when Grx1 alone was expressed (Figure S3A), whereas Grx1 signals became visible upon cell lysis (Figure S3B). The NMR properties of cytoplasmic Grx1 therefore allowed us to obtain ^{1}H-^{15}N NMR spectra of Mia40 free of interference from Grx1 signals.

The effect of Trx1 on the Mia40 state was also investigated by inducing co-expression of Trx1 with Mia40. Similar to Grx1, Trx1 in the human cell cytoplasm is not detectable with NMR, consistent with what was reported in *E. coli* (Reckel et al., 2012; Figure S3C). The intracellular activity of Trx1 depends on the

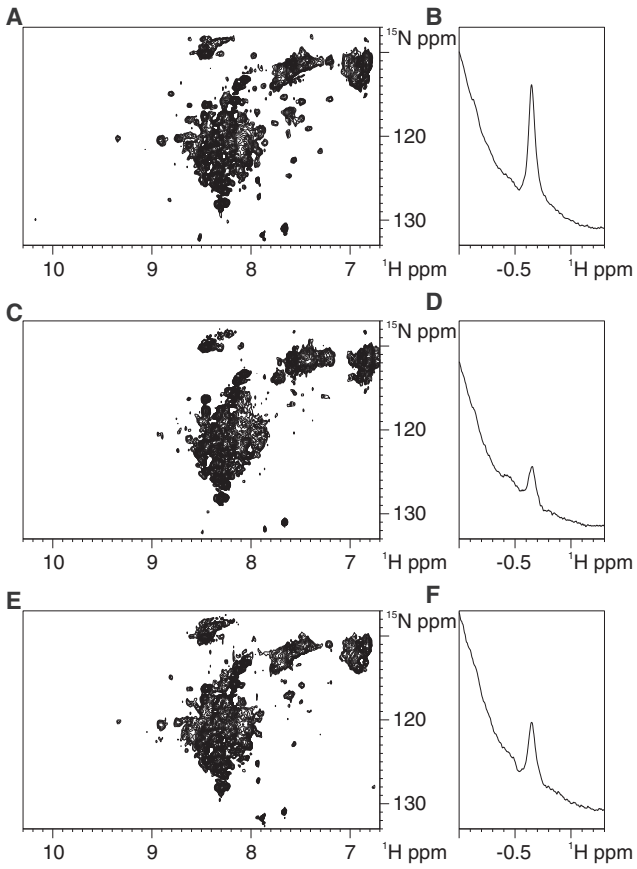

Figure 2. The Folding State of Mia40 in the Cytoplasm Depends on the Presence of Different Redox-Regulating Proteins

NMR spectra were acquired on human cells expressing uniformly ^{15}N-labeled Mia40 in the cytoplasm.

(A and B) Spectra of cells expressing Mia40.

(C and D) Spectra of cells co-expressing Mia40 and Grx1.

(E and F) Spectra of cells co-expressing Mia40 and Trx1 in selenium-supplemented medium.

(A, C, and E) ^{1}H-^{15}N SOFAST HMQC spectra. The strong, overlapped cross-peaks between 8.0 and 8.5 ppm (^{1}H) correspond to the unfolded regions, while the weaker, dispersed cross-peaks belong to the residues of the folded region of Mia40 (see also Figure S2).

(B, D, and F) Aliphatic region of ^{1}H spectra showing the ^{1}Hγ peak of Ile 53, which is a marker of the folded conformation of Mia40 and falls in a cellular background-free region.

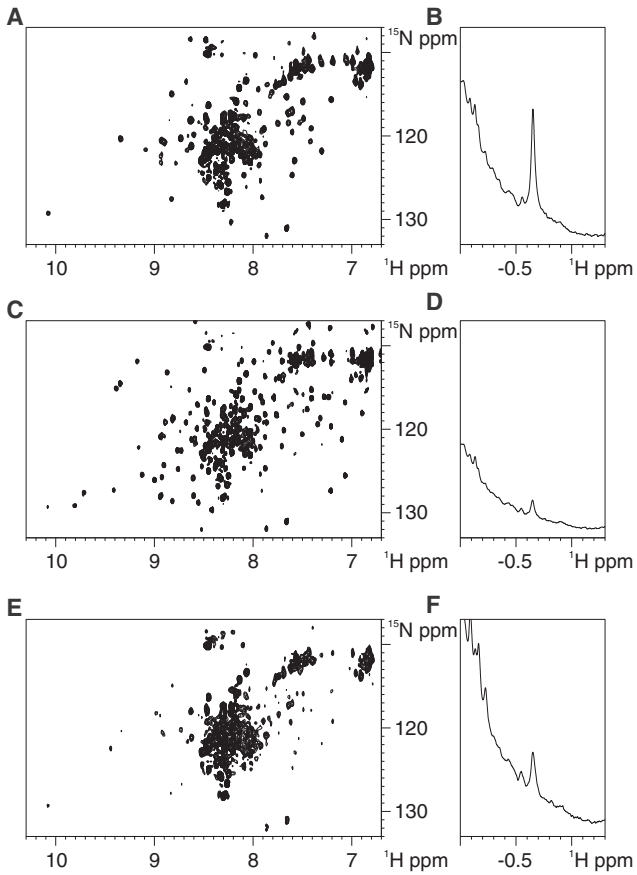

Figure 3. NMR Spectra Acquired on the Cell Extracts Corresponding to the Samples in Figure 2

NMR spectra were acquired on the cell extract of samples expressing uniformly ^{15}N-labeled Mia40. The relative amounts of the two Mia40 folding states remained unchanged upon cell lysis.

(A and B) Spectra of the cell extract containing Mia40.

(C and D) Spectra of the cell extract containing Mia40 and Grx1.

(E and F) Spectra of the cell extract containing Mia40 and Trx1 in selenium-supplemented medium.

Upon cell lysis, both Grx1 and Trx1 became visible in the ^{1}H-^{15}N spectra (C and E; see also Figure S3). Glutathionylation of unfolded Mia40 was excluded with mass spectrometry (see also Figure S4).

availability of selenium, which is required for the activation of thioredoxin reductase (TrxR; Ueno et al., 2007). Indeed, Trx1 co-expression in cells grown with basal amounts of selenium did not affect the folding state of Mia40 (data not shown). To ensure the complete activation of TrxR, and consequently active Trx1, sodium selenite was supplemented to cells co-expressing Trx1 and Mia40. In these conditions, co-expressed Trx1 was fully reduced (Figure S1C) and active, consistent with the reported Trx1 redox state at endogenous levels (Watson et al., 2003). Co-expression of active Trx1 affected the folding state of Mia40 to a lesser extent than Grx1 when expressed at similar levels because ~50% of cytoplasmic Mia40 was still in the folded, oxidized state (Figures 2E, 2F, 3E, and 3F). Therefore, the two thiol-regulating proteins have different efficacy in keeping Mia40 reduced, despite being reported to have overall similar functions in the cytoplasm (Figure 4).

In contrast to what was observed in the cells, fully reduced Grx1 had no effect on the Mia40 redox state in vitro. U-^{15}N Mia40$^{2S\text{-}S}$ was incubated in reduction conditions, either in the presence of 20 mM dithiothreitol (DTT) or 20 mM reduced glutathione (GSH), with increasing concentrations of unlabeled, fully reduced Grx1, and each step was monitored with NMR. No change in the ^{1}H-^{15}N NMR spectrum occurred upon addition of up to 2 eq of Grx1 to Mia40$^{2S\text{-}S}$ and 48 hr incubation, thus excluding a direct mechanism of reduction of Mia40 by Grx1. In a control experiment, U-^{15}N Mia40$^{2S\text{-}S}$ (Figure S2A) was completely reduced by heat denaturation at 95°C in buffer containing either 20 mM DTT or 20 mM GSH. No protein degradation occurred. The protein remained reduced in both reductants when cooled down at 25°C (Figure S2C). In these conditions,

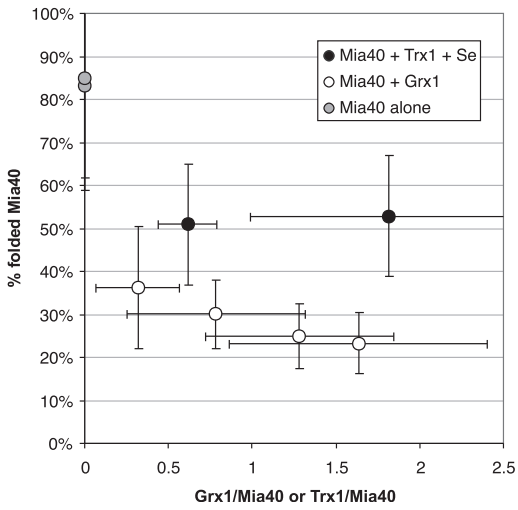

Figure 4. Effect of Grx1 and Trx1 on the Mia40 Folding State in the Cytoplasm

The ratio of folded Mia40 over total Mia40 was determined in cell samples with varying relative amounts of co-expressed Grx1 or Trx1. The amount of each protein was measured on cell extracts with western blot analysis. The amount of folded Mia40 was measured on the cell extracts by NMR. When only Mia40 was expressed, it was largely found in the folded state in the cytoplasm (gray circles). Co-expression of Grx1 caused a remarkable decrease of folded Mia40, which also occurred at substoichiometric amounts relative to Mia40 (white circles). Co-expression of Trx1 in the presence of selenium affected the folding state of Mia40 to a lesser extent (black circles).
Error bars represent SDs; n = 3.

the ^{1}H-^{15}N cross-peaks of the α-helical region disappeared, analogous to what was observed in the cytoplasm in the presence of Grx1. The alkylation reaction with 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) depicted with SDS-PAGE confirmed the complete reduction of all cysteines (Figures S2B and S2D). Upon removal of the reducing agent and exposure to air, Mia40 rapidly reverted back to the folded, oxidized conformation. To test whether Grx1 could bind fully reduced Mia40 and prevent its oxidation, 2 eq of Grx1 was added to a sample of U-^{15}N labeled, unfolded Mia40 in presence of 20 mM GSH. Upon exposure to air, Mia40 rapidly folded, thus indicating that Grx1 did not protect reduced Mia40 from oxidation. Grx1-catalyzed glutathionylation of reduced Mia40, either in vitro in presence of GSH or in the cytoplasm, was excluded by mass spectrometry analysis of the in vitro Mia40 samples and of Mia40 isolated from cell extracts (Figure S4).

DISCUSSION

The current model of Mia40 maturation pathway requires that the protein, which is natively expressed in the cytoplasm from nuclear mRNA, crosses the outer mitochondrial membrane through the TOM channel in a reduced and unfolded conformation. No Mia40 has been reported to reside in the cytoplasm, except for the time required to translocate to the mitochondria (Hofmann et al., 2005). However, when Mia40 was overexpressed, it did not translocate quantitatively to the mitochondria, and close to all of the Mia40 remained in the cytoplasm. This effect could be explained with the limited availability of the import machinery on the outer mitochondrial membrane (i.e., the TOM channel), which determines an upper limit to the import level of Mia40. Consequently, unimported Mia40 accumulates in the cytoplasm. Our results show that in these conditions, cytoplasmic Mia40 reached the folded state, which is thus a thermodynamically favored conformation in the cytoplasm. Therefore, in physiologic conditions there is the need for a mechanism to keep Mia40 reduced until it reaches the outer mitochondrial membrane because the amount and ratio of cytoplasmic GSH are apparently not sufficient for that purpose.

Overexpression of Grx1 allowed most Mia40 to remain in the reduced, unfolded state. This effect also occurred with substoichiometric amounts of Grx1, consistent with a catalytic role of Grx1. Conversely, when oxidized Mia40 was incubated in vitro with Grx1 in the presence of GSH, it did not change its redox state and the reduction of the structural disulfide bonds was only possible upon heat denaturation in reducing conditions. Moreover, Grx1 did not prevent oxidation of heat-denatured Mia40 when added in the presence of GSH and molecular oxygen, and neither partially oxidized Mia40 nor glutathionylated Mia40 were formed. The lack of a direct interaction of Grx1 with Mia40 suggests that the effect of Grx1 on the oxidation state of intracellular Mia40 is mediated by some other component of the cytoplasm.

Overexpression of Trx1 resulted in a weaker effect because a sizable fraction of Mia40 reached the folded state. As with Grx1, the effect occurred also with a substoichiometric amount of Trx1, therefore suggesting that the effect of Grx1, and to a lesser extent that of Trx1, is not the consequence of a generic increase in reducing power in the cytoplasm, which would be concentration-dependent.

Overall, the data show that cytoplasmic redox-regulation systems, Grx1 in particular, have a specific catalytic—although likely indirect—role in keeping a sizable fraction of Mia40 unfolded in the cytoplasm, implying a link between these systems and the Mia40 maturation pathway.

SIGNIFICANCE

We characterized the folding state of Mia40 in the cytoplasm, obtaining atomic-level information in living human cells with NMR. We found that the folded form of Mia40, which cannot be imported into mitochondria, is thermodynamically favored in the cytoplasm. We also showed that the folding of Mia40 is controlled by the cytoplasmic Grx1 and Trx1 redox-regulation systems, Grx1 having a stronger effect. These results show the general relevance of atomic resolution studies performed in living cells, which provide a way to describe cellular physiologic processes such as the redox-controlled protein folding, and to understand how other involved pathways can affect and regulate such processes.

EXPERIMENTAL PROCEDURES

Overexpression of Mia40, Grx1, and Trx1 in human cells was performed by following a previously established protocol (Aricescu et al., 2006; Banci et al., 2013). Briefly, the cDNA sequences encoding Mia40 (amino acids 1–142, GenBank accession number: NP_001091972.1), Grx1 (amino acids

1–106, GenBank accession number: NP_001112362.1), and Trx1 (amino acids 1–105, GenBank accession number: NP_003320.2) were amplified with PCR and subcloned into the pHLsec vector (Aricescu et al., 2006; Banci et al., 2013) between EcoRI and XhoI restriction enzyme sites. The clones were verified by gene sequencing. Transient transfection was obtained by treating the cells with a DNA:polyethylenimine mixture. Cells were collected after 48 hr for in-cell NMR and western blot analysis. Intracellular distribution was assessed by separating the cytoplasmic and the mitochondrial fractions from cell extracts using a mitochondria isolation kit for cultured cells (Thermo Scientific).

Grx1 was co-expressed with Mia40, either unlabeled or with uniform ^{15}N labeling. Different ratios of DNA were chosen to obtain different protein ratios: 1:0.75:2; 1:0.5:2; 1:0.25:2; and 1:0.125:2 *hMia40:hGrx1*:PEI. Trx1 was co-expressed with Mia40 with the following ratios of DNA: 1:1:2 and 0.5:1:2 *hMia40:hTrx1*:PEI. To ensure complete activation of Trx1, sodium selenite (kindly provided by Prof. A. Arcangeli lab, University of Florence) was supplemented to the cell culture media to a final concentration of 100 nM, starting 24 hr before transfection.

The amount of folded Mia40 in the cell extracts was measured with NMR through the standard addition of pure, folded Mia40 at known concentration. The ^1H resonance at −0.7 ppm of Ile 53 $H\gamma$ was used as a marker of the folded conformation of Mia40. Total Mia40 was determined on the same cell extracts by western blot analysis, by using the same pure Mia40 sample at increasing dilutions as a reference; Grx1 and Trx1 concentrations in the cell extracts were determined as above by using samples of purified Grx1 and Trx1 as references. Mia40 was stained with a rabbit polyclonal anti-Mia40 antibody (Abcam: ab87033, diluted to 0.5 μg/ml); Grx1 with a rabbit polyclonal anti-glutaredoxin 1 antibody (Abcam: ab45953, 1.0 μg/ml); Trx1 with a rabbit polyclonal anti-thioredoxin/TRX antibody (Abcam: ab26320, 0.4 μg/ml). Goat anti-rabbit IgG (whole molecule)-peroxidase secondary antibody (Sigma: A0545) was used for detection, diluted at 1:160,000 (for Mia40) or 1:80,000 (for Grx1 and Trx1). For checking the purity of the cytoplasmic and mitochondrial fractions, antibodies against a cytoplasmic marker (rabbit polyclonal anti-GAPDH antibody, Abcam: ab9485) and a mitochondrial marker (rabbit polyclonal anti-COX IV, Abcam: ab16056) were used.

U-^{15}N-labeled Mia40 for in vitro experiments and unlabeled Mia40 for NMR quantifications were produced as previously described (Banci et al., 2009b). Glutaredoxin 1 for in vitro interaction with Mia40 was produced as follows: a pTH34 vector containing the human Grx1 gene (N-term fused with His-tag and TEV recognition site) was transformed in *E. coli* BL21(DE3) gold competent cells. Cells were grown at 37°C in minimal medium to an optical density 0.6 and then induced with 0.5 mM IPTG for 16 hr at 25°C. Glutaredoxin 1 was purified by affinity chromatography using a nickel-chelating HisTrap (GE Healthcare) column. After digestion with AcTEV protease (Invitrogen) O/N at 25°C, the protein was separated from the affinity tag in a HisTrap column. The sample buffer was then exchanged with 50 mM potassium phosphate, 0.5 mM EDTA, pH = 7. Pure thioredoxin 1 for western blot analysis was prepared from HEK293T cells overexpressing Trx1. Cells were lysed in 20 mM potassium phosphate buffer (pH 7) and the cleared extract was incubated at 70°C for 4 min. After centrifugation (30 min at 16,000 × *g*), Trx1 was purified by anionic exchange chromatography using a HiTrap DEAE FF (Amersham Biosciences) column applying a linear gradient of potassium phosphate buffer (10–100 mM, pH 7).

Mia40 for mass spectrometry analysis was isolated from cell extracts by ionic exchange chromatography using a HiTrap DEAE FF (Amersham Biosciences) column applying a linear gradient of NaCl (0–500 mM NaCl in 20 mM TRIS buffer, pH 8). For both in vitro and cell extracts samples, the buffer was exchanged with 0.1% trifluoroacetic acid/ 50% acetonitrile by Zip Tip_{C18} tips (MILLIPORE). Mass spectrometry analysis was performed at a Bruker Ultraflex III MALDI TOF/TOF instrument, using an α-cyano-4-hydroxycinnamic acid matrix.

For thiol alkylation reaction with 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS), either cell or protein samples were precipitated with 10% trichloroacetic acid, washed with acetone, and resuspended in 100 mM Tris pH 7 + 2% SDS. The mixtures were then incubated 1 hr at 37°C with 20 mM AMS, and run on a nonreducing SDS-PAGE.

NMR experiments were acquired with a 950 MHz Bruker Avance III spectrometer equipped with a CP TCI CryoProbe. 1D ^1H and 2D $^1H,^{15}N$-SOFAST-HMQC (Schanda and Brutscher, 2005) spectra were acquired at 305K. The total acquisition time for each cell sample ranged from 1 to 2 hr. The supernatant of each cell sample was checked for protein leakage in the same experimental conditions. The same NMR spectra were also acquired on the cell extracts. Cell viability before and after NMR experiments was assessed with trypan blue staining (Freshney, 1987). Cell viability remained above 90% because damaged cells ranged from 3% before the experiments to 8% after the experiments. 2D $^1H,^{15}N$-SOFAST-HMQC NMR spectra of cell samples and extracts were processed with Bruker Topspin 3.1 software by subtracting a spectrum of untransfected cells/cell extract acquired within the same experimental conditions, thereby eliminating the interference of signals arising from unspecific ^{15}N labeling.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2013.05.007.

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