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Structural insights of proteins in sub-cellular compartments: In-mitochondria NMR

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ABSTRACT

Many eukaryotic proteins exert their physiological function in specific cellular compartments. Proteins of the inter-membrane space (IMS) of mitochondria, for example, are synthesized in the cytoplasm and translocate to the IMS, where they are further processed to their mature form. In-cell Nuclear Magnetic Resonance (NMR) has proven to be an ideal approach to investigate eukaryotic proteins at the atomic level, inside the cytoplasm. Here we show that proteins inside intact mitochondria isolated from human cells can be structurally characterized by NMR (in-mitochondria NMR). By this approach, we characterized the folding and maturation state of two human proteins in the IMS, SOD1 and Mia40. Both observed proteins were in the folded state. Mia40 was in the oxidized, functional state, while SOD1 disulfide bond formation was promoted by increasing the level of the SOD1 chaperone, CCS, in the IMS.

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1. Introduction

Eukaryotic proteins which exert their function in organelles are synthesized in the cytosol, and subsequently targeted to their physiological destination by the cellular translocation machinery. Most of these proteins rely on specific targeting sequences. Secreted proteins, for example, have a N-terminal targeting sequence for the endoplasmic reticulum (ER) which acts in a co-translational manner, causing the ribosome to migrate and continue protein synthesis on the ER translocation channels [1]. Mitochondria, which provide the main energy supply in eukaryotic cells through the oxidative phosphorylation pathway, are an unusual case. They are surrounded by two membranes, likely a legacy of their ancient origin from endosymbiontic single-cell organisms, and possess their own DNA [1,2]. The vast majority of mitochondrial proteins is however encoded by nuclear DNA, and has to be targeted towards its final mitochondrial compartment [3–5]. Soluble mitochondrial proteins can be localized either in the mitochondrial matrix or in the inter-membrane space (IMS). Matrix proteins are usually targeted by a N-terminal targeting sequence [6,7], whereas multiple pathways exist for IMS proteins [8–10]. Moreover, some cytosolic proteins were also reported to partially localize in the IMS, like superoxide dismutase 1 (SOD1), its copper chaperone (CCS), and glutaredoxin 1 [11–13].

It has been previously shown that nuclear magnetic resonance (NMR) applied to living cells (in-cell NMR) can provide atomic-level data in living eukaryotic cells, thus allowing the characterization of eukaryotic proteins in their physiological environment [14–21]. For this goal, our approach relies on transiently overexpressing and selectively labelling a protein of interest in cultured human cells, and proved to be especially suitable for characterizing proteins localized in the cytosol and the processes in which they are involved. By this approach we characterized intracellular processes such as chaperone-mediated protein maturation involving multiple metal binding events, and redox-dependent protein folding [20,21]. Here we show that structural information of a protein inside mitochondria can be obtained by solution NMR directly on intact mitochondria isolated from human cells (in-mitochondria NMR).

In order to increase the mitochondrial protein levels and overcome the intrinsic sensitivity limit of NMR, protein overexpression has to be induced by transiently transfecting cells with the corresponding cDNA. A direct consequence of high level protein expression in a relatively short time is that the newly synthesized proteins often accumulate in the cytosol even if naturally targeted to mitochondria, likely due to the cellular machinery which regulates mitochondrial protein import being overloaded. Therefore, while high cytosolic levels are easily reached, proteins may not be correctly localized. It was recently shown that the mitochondrial oxidative import pathway occurs with

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slower kinetics compared to the pathway relying on N-terminal mitochondrial targeting sequences (MTS) [22]. To overcome this limitation, we employed a minimal MTS of 53 amino acids from the N-terminal region of the human Smac/DIABLO protein, which was reported to efficiently deliver fused exogenous proteins to the IMS of mitochondria [23].

By inducing overexpression of both native proteins and MTS-fused constructs, we investigated the structural properties of the human proteins copper–zinc superoxide dismutase 1 (Cu,Zn-SOD1) and Mitochondrial intermembrane space Import and Assembly protein 40 (Mia40) in the inter-membrane space (IMS) of intact mitochondria isolated from human cells.

SOD1 exerts a major role in the cellular defence against oxidative stress. Endogenously expressed SOD1 is mainly localized in the cytosol, but a small fraction is localized in the IMS of mitochondria, which is critical to prevent mitochondrial damage caused by reactive oxygen species [24,25]. The homodimeric protein needs to bind a Cu(I) and a Zn(II) ion per subunit and to form an intramolecular disulfide bond to reach its mature, functional state. Mia40 is the hub protein involved in the oxidative folding pathway of a large set of proteins of the IMS of mitochondria [9,26–28]. It catalyzes the formation of an intramolecular disulfide bond on its protein substrates, inducing their folding and their trapping in the IMS [28,29]. Like its own substrates, Mia40 crosses the outer mitochondrial membrane in an unfolded, reduced state, and obtains its final structure in the IMS with the formation of two structural disulfide bonds [21,30].

2. Materials and methods

2.1. Constructs

To obtain the MTS-Mia40 construct, the cDNA of human Mia40 (amino acids 1–142, Uniprot Q8N4Q1) was extended at the N-terminal end with a series of overlapping primers by sequential PCR with the N-terminal sequence of human Smac/DIABLO (amino acids 10–59, Uniprot Q9NR28). Between the MTS and Mia40 sequence, the restriction site BamHI was inserted (encoding two additional amino acids G S). The MTS-Mia40 DNA was amplified by PCR and subcloned in pHLsec vector [31] between the restriction sites EcoRI and XhoI. The clone was confirmed by DNA sequencing. To obtain MTS-CCS, the cDNA encoding human CCS was cloned between the restriction sites BamHI and XhoI of the pHLsec_MTS-Mia40 vector previously digested to remove the Mia40 cDNA. The clones were verified by DNA sequencing. The pHLsec vectors containing human SOD1 and human Mia40 cDNAs had been obtained previously [20].

2.2. Cell culture and transfection

HEK293T cells were cultured and transfected as previously described [20]. For coexpression of SOD1 and MTS-CCS, cells were transfected with plasmids containing the constructs in a 1:1 ratio. Commercial DMEM medium was used for unlabeled samples; BioExpress6000 medium (CIL) was used for [U–^{15}N] labelling in-mitochondria samples; for selective [^{15}N]cysteine labelling, a reconstituted medium was prepared following the DMEM (Sigma)-reported composition, in which [^{15}N]cysteine was added together with all the other unlabeled components. For mitochondrial samples containing SOD1, Zn(II) was supplemented as $ZnSO_4$ to a final concentration of 10 μM immediately after transfection, and Cu(II) as $CuCl_2$ to a final concentration of 50 μM after 48 h of protein expression.

2.3. Extraction of mitochondria and NMR samples preparation

For each in-mitochondria NMR sample, mitochondria were extracted from 4 T75 flasks of transfected HEK293T cells. Mitochondrial fractions were isolated from cell extracts using a mitochondria isolation

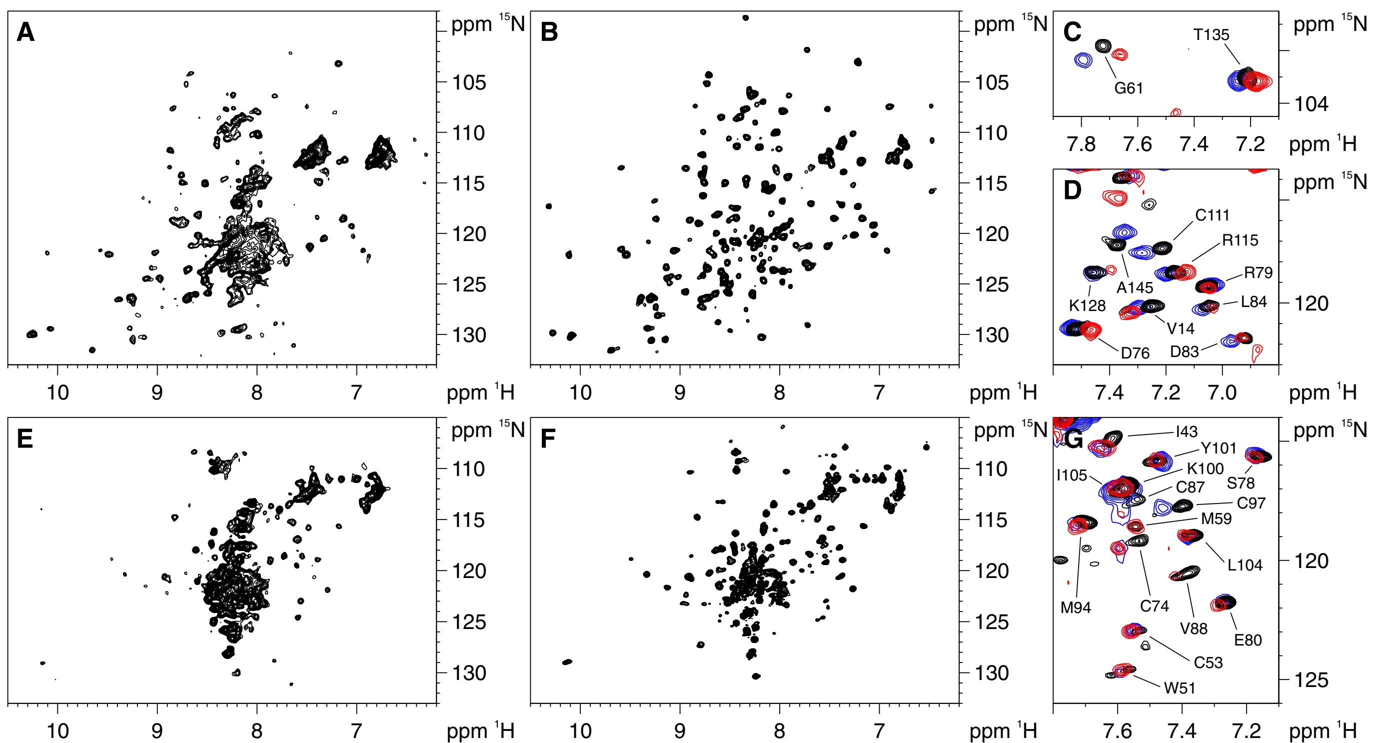

Fig. 1. In-mitochondria NMR. ^{1}H–^{15}N SOFAST-HMQC spectra were recorded from samples of intact mitochondria isolated from cells expressing [U–^{15}N] labelled WT SOD1 (A), [U–^{15}N] labelled MTS-Mia40 (E). Both spectra were processed by subtracting a spectrum of mitochondria isolated from untransfected cells acquired within the same experimental conditions. ^{1}H–^{15}N SOFAST-HMQC spectra of *in vitro* E,Zn-SOD1^{SH} (B) and Mia40^{2S-S} (F) are shown for reference (adapted from [36] and [21], respectively). Detailed comparison of in-mitochondria (red), cytosolic (blue) and *in vitro* (black) NMR spectra of SOD1 (C, D) and Mia40 (G) shows that most crosspeaks can be identified, with minor perturbations between the samples (spectra of cytosolic SOD1 and Mia40 are adapted from [20] and [21], respectively).

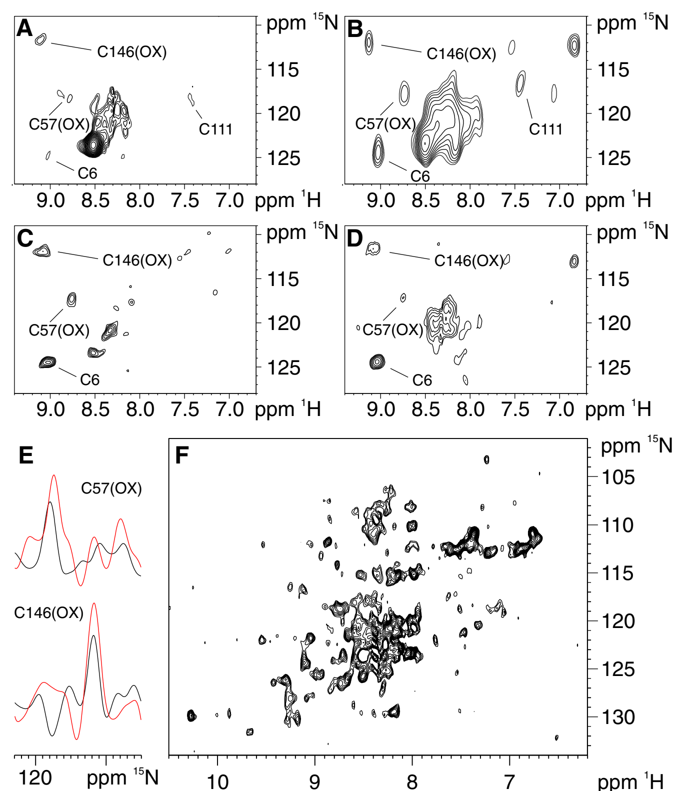

Fig. 2. In the presence of CCS, SOD1 in the IMS is fully in the oxidized state. $^{1}H–^{15}N$ SOFAST-HMQC spectra were recorded from samples of intact mitochondria and IMS extracts from cells expressing [^{15}N]cysteine labelled SOD1 either alone (A, B) or together with MTS-CCS (C, D). Panels (A, C) show spectra of intact mitochondria; panels (B, D) show spectra of IMS extracts. SOD1 cysteine crosspeaks are labelled, and the oxidation state of Cys57 and Cys146 is indicated. 1D projections along the ^{1}H dimension of Cys57 and Cys146 crosspeaks are shown in (E). The black curves correspond to the spectrum in (A), the red curves to the spectrum in (C). A $^{1}H–^{15}N$ SOFAST-HMQC spectrum was recorded from a sample of intact mitochondria coexpressing [U–^{15}N] labelled SOD1 together with MTS-CCS (F). The spectrum was processed by subtracting a spectrum of mitochondria isolated from untransfected cells acquired within the same experimental conditions. Several crosspeaks from SOD1 can still be detected, although at lower S/N ratio than when only SOD1 is overexpressed.

kit for cultured cells (Thermo Scientific). The final pellet containing the mitochondria was washed once with mitochondrial buffer (250 mM sucrose; 10 mM Tris–HCl; 0.15 mM $MgCl_2$; 1 mM EDTA, pH 6.7) and resuspended in mitochondrial buffer + 10% D_2O to a final volume of 120 μL. The suspension was then placed in a 3 mm Shigemi tube for NMR analysis.

For the IMS separation, mitochondria isolated from 4 T75 flasks were carefully resuspended in 2 mL of hypotonic buffer (1 mM KPi; 0,5 mM EDTA, pH 7) and kept in ice for 40 min, followed by centrifugation at 10.000 *g* for 10 min. The supernatant containing the IMS extract was collected and concentrated for NMR analysis. To extract the mitochondrial matrix, the pellet from the osmotic shock containing the mitoplasts was resuspended in 2 mL of hypotonic buffer and sonicated. After centrifugation at 10.000 *g* for 20 min, the supernatant containing the mitochondrial matrix extract was collected and concentrated for NMR analysis.

2.4. NMR experiments

NMR spectra were acquired at a 950-MHz Bruker Avance III spectrometer equipped with a CP TCI CryoProbe. 1D ^{1}H and 2D $^{1}H–^{15}N$ SOFAST HMQC spectra [32] were acquired at 305 K. The total time to acquire 1D and 2D spectra on each sample was around 2 h. To remove the background signals, the $^{1}H–^{15}N$ SOFAST HMQC spectra of [U–^{15}N] labelled intact mitochondria and IMS extracts were processed by subtracting spectra of mitochondria and the corresponding IMS extract from untransfected cells, obtained in the same experimental conditions.

2.5. Western blot analysis

Mia40 was stained with a rabbit polyclonal anti-Mia40 antibody (Abcam: ab87033, diluted to 0.5 mg/mL); SOD1 with a rabbit polyclonal to superoxide dismutase 1 antibody (Abcam: ab16831, diluted to 0.5 mg/mL); and CCS with a mouse monoclonal [(3A1)] to superoxide dismutase 4 (Abcam: ab16964, diluted to 0.5 mg/mL). Goat anti-rabbit IgG (whole molecule)-peroxidase secondary antibody (Sigma:A0545) diluted at 1:80,000 (for Mia40 and SOD1) and goat anti-mouse IgG (Fc Specific)-peroxidase secondary antibody (Sigma:A0168), diluted at 1:80,000 (for CCS) were used for detection. To check the purity of the cytoplasmic and mitochondrial fractions, antibodies against a cytoplasmic marker (rabbit polyclonalanti-GAPDH antibody, Abcam: ab9485, diluted at 1:2000) and a mitochondrial marker (rabbit polyclonal anti-COX IV, Abcam: ab16056, diluted at 1:5000) were used. For the mithocondrial matrix, antibody against Pyruvate Dehydrogenase E1-alpha subunit (rabbit monoclonal [EPR11098] to Pyruvate Dehydrogenase E1-alpha subunit, Abcam: ab168379, diluted at 1:2000) was used. For detection, LiteAblot EXTEND chemiluminescent substrate (EuroClone) was used.

3. Results and discussion

3.1. Native SOD1 and Mia40 accumulated in the cytosol

Native wild-type SOD1 and Mia40 were overexpressed by transient transfection, and their subcellular distribution was determined by subcellular fractionation followed by Western blot analysis. In accordance to what previously shown [20,21], both proteins were mostly localized in the cytosolic fraction, and only a relatively small amount (1–3% of total protein) was localized in the mitochondrial fraction (Supplementary Figure S1). In the case of SOD1 this distribution is consistent with the reported localization of endogenous SOD1 [11]. Conversely, the localization of overexpressed Mia40 was far from the native distribution, which is reported to be exclusively mitochondrial (and specifically localized in the IMS). Despite this, the level of mitochondrial Mia40 upon overexpression was increased with respect to the endogenous by at least 50 fold (Supplementary Figure S1).

3.2. MTS-Mia40 mostly localized in the mitochondrial fraction

In order to restore the correct localization of Mia40, we created the MTS-Mia40 construct by fusing the 50 amino acid MTS derived from the Smac/DIABLO protein [23] to the N-terminus of the human Mia40 gene. Although the MTS-Mia40 construct was expressed at much lower levels in transiently transfected HEK293T cells compared to native Mia40, the former was mostly localized in the mitochondrial fraction (Supplementary Figure S1). Moreover, it was correctly processed by the mitochondrial processing peptidase, as it ran at the same height as native Mia40 on SDS-PAGE (Supplementary Figure S2), indicating that the mitochondrial import pathway for the MTS-fused construct is fully functional, and was not overloaded by transient overexpression.

In the case of SOD1, while most of the protein remained in the cytoplasm, the levels of mitochondrial protein were sufficient for in-mitochondria NMR experiments; therefore the native protein, without targeting sequence, was expressed.

3.3. In-mitochondria NMR showed folded SOD1 and Mia40 in the IMS

Mitochondria were isolated from cultures of cells transfected with either WT SOD1 or MTS-Mia40, and were analyzed by NMR. $^{1}H–^{15}N$ heteronuclear experiments with sufficient S/N ratio could be acquired in less than 2 h. The sample preparation was optimized to increase the

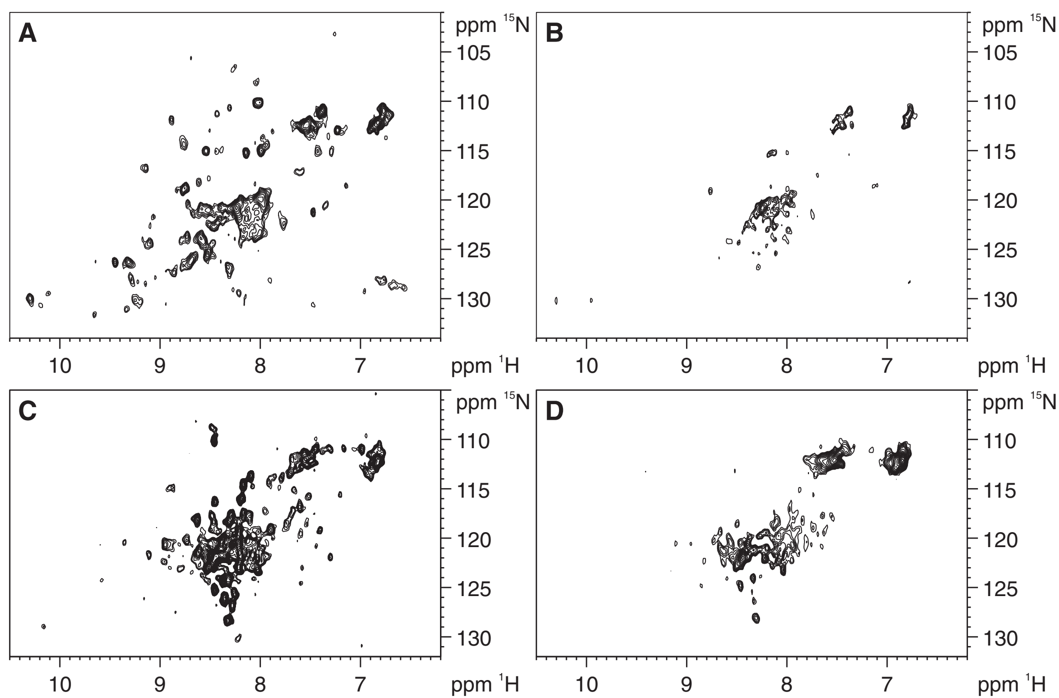

Fig. 3. SOD1 and Mia40 are correctly localized in the IMS. $^{1}H–^{15}N$ SOFAST-HMQC spectra were recorded from IMS and mitochondrial matrix extracts of mitochondria containing overexpressed [U–^{15}N] labelled SOD1 (A, B) and Mia40 (C, D). (A, C) IMS extracts; (B, D) mitochondrial matrix extracts. The spectra were processed by subtracting spectra of IMS and matrix extracts from untransfected cells acquired within the same experimental conditions. Signals originating from both Mia40 and SOD1 are visible in the IMS extracts, indicating that both proteins were correctly localized in the IMS, whereas the matrix extracts contain mostly residual signals from the background labelling.

stability of the mitochondrial NMR samples. In the best conditions the mitochondrial rupture was lower than 10%, after 2.5 h incubation at room temperature (Supplementary Figure S3). Crosspeaks arising from either SOD1 or Mia40 backbone amides could be clearly identified (Fig. 1), together with broad background signals centred around 8.3 ppm (^{1}H) arising from non-selective labelling of mitochondrial proteins. The background signals could be removed by subtracting the spectrum of mitochondria obtained from untransfected cells, and acquired in the same experimental conditions. Comparison with the NMR spectra of both *in vitro* and cytosolic SOD1 in various maturation states revealed that mitochondrial SOD1 was prevalently in the E,Zn-SOD1 form, i.e. a folded homodimer with one zinc ion bound to each monomer (Fig. 1 A–D). Analysis of the in-mitochondria NMR spectra of MTS-targeted mitochondrial Mia40 revealed that, after being correctly targeted to the IMS and cut by the mitochondrial processing peptidase, Mia40 was fully folded and oxidized (Mia40^{2S-S} i.e. with the two structural disulfide bonds correctly formed) and therefore in the final, functional state (Fig. 1 E–G). Most amide crosspeaks of both proteins could be identified, and minor chemical shift perturbations were observed between *in vitro*, cytosolic and mitochondrial proteins, which likely reflect the different composition of the cellular compartments (Fig. 1 C,D,G).

3.4. Coexpression of the copper chaperone for SOD1 promoted SOD1 oxidation in the IMS

The redox state of IMS-localized SOD1 was further investigated by analyzing mitochondria containing [^{15}N]cysteine labelled SOD1. Weak cysteine crosspeaks were detected, corresponding to the oxidized form of SOD1 (Fig. 2 A), suggesting that a fraction of the protein was oxidized in these conditions, at variance with the cytosolic E,Zn-SOD1 which is mostly reduced [20]. This behaviour would be consistent with the thiol-disulfide redox potential of the IMS being more oxidizing compared to that of the cytosol. The copper chaperone for SOD1 (CCS) [33] has an essential role in catalyzing the formation of SOD1 intramolecular disulfide bond [34,35], both in the cytosol and in the IMS [11]. A construct of mitochondrially targeted CCS (MTS-CCS) was coexpressed with SOD1, and mostly localized in the mitochondrial fraction, like MTS-Mia40 (Supplementary Figure S1). With increased levels of mitochondrial CCS, the crosspeaks of the oxidized species of [^{15}N]cysteine labelled SOD1 were stronger, indicating that indeed CCS promoted SOD1 disulfide formation in the IMS (Fig. 2 C,E). [U–^{15}N] labelled SOD1 was also detected in mitochondria when coexpressed with CCS, although with a lower S/N ratio than without coexpression of MTS-CCS (Fig. 2 F).

3.5. Mitochondrial fractionation confirmed the IMS localization of SOD1 and Mia40

After the NMR experiments, the mitochondria were collected and the content of the IMS was released by osmotic swelling and analyzed by NMR. Both SOD1 and Mia40 were found in the IMS fractions (Figs. 2 B,D, and 3 A,C), together with some background signals arising from other proteins of the IMS. In the IMS extract of the [^{15}N]cysteine labelled sample, fully oxidized SOD1 was clearly detected even with endogenous levels of CCS (Fig. 2 B), as the exposure to air likely caused the complete oxidation of reduced SOD1. The other mitochondrial fraction contained the mitoplasts, i.e. the mitochondria lacking the outer membrane and the IMS. The content of the mitoplasts was extracted by sonication and subsequently analyzed by NMR; only background signals were observed (Supplementary Figure S4 A,B, and Fig. 3 B,D). Signals of CCS were not detected in any of the extracts, likely due to lower concentration with respect to SOD1. The correct separation of the IMS content from that of the mitochondrial matrix was checked by Western blot analysis of the mitochondrial fractions (Supplementary Figure S5). Taken together, these results confirmed that the crosspeaks in the mitochondrial NMR samples containing either SOD1 or Mia40 originated only from proteins localized in the IMS.

4. Conclusions

The structural and functional characterization of proteins inside their physiological cellular compartments is an important step towards a true cellular structural biology approach. Here we report that solution NMR can be applied to investigate the structural properties of proteins in intact organelles, provided that sufficient protein levels and efficient isotopic labelling are achieved. These results show that isolation of mitochondria and in-mitochondria NMR is an effective and powerful approach for characterizing mitochondrial proteins in their physiological environment. Moreover, we show that the localization of the overexpressed proteins can be efficiently controlled by using a mitochondrial targeting sequence. The same strategy will allow, in principle, to obtain sufficient protein levels for NMR in other cellular compartments, such as the mitochondrial matrix and the endoplasmic reticulum. Therefore, we are confident that the in-mitochondria NMR technique described here will be the first of a series of *in organello* NMR approaches.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2014.06.009.

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