Subcellular localization of methionine sulfoxide reductase A (MsrA): evidence for mitochondrial and cytosolic isoforms in rat liver cells

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Proteins are sensitive to reactive oxygen species, and the accumulation of oxidized proteins has been implicated in the aging process and in other age-related pathologies. In proteins, methionine residues are especially sensitive to oxidation, leading to *S*- and *R*-methionine sulfoxide diastereoisomers, the reversion of which is achieved by the peptide methionine sulfoxide reductases MsrA and MsrB respectively. The MsrA enzyme, in addition to its role in repair, forms part of the reactive oxygen species scavenging systems that are important in cellular antioxidant defence. MsrA is present in most living organisms, and the mammalian enzyme has been detected in all tissues investigated. In the present study, we investigated the subcellular distribution of MsrA in rat liver cells. Since it seemed likely that MsrA may be localized in areas where reactive oxygen species are produced, rat liver mitochondrial matrix and cytosolic extracts were prepared. The presence of MsrA was assayed in these

subcellular compartments by monitoring peptide methionine sulfoxide reductase enzymic activity, by Western blotting and by *in situ* immunolocalization by electron microscopy using a specific antibody. Moreover, MsrA was identified by MS in a partially purified cytosolic fraction and in a mitochondrial matrix crude extract. Rat MsrA isoforms are encoded by a single gene, and it is suggested that the precursor of the mitochondrial form contains an N-terminal cleavable signal sequence that localizes the MsrA to this organelle. Finally, two-dimensional gel electrophoresis followed by Western-blot analysis of partially purified MsrA from the cytosol and mitochondria, and comparison with the two-dimensional patterns of oxidized recombinant MsrA, revealed oxidative modifications of cysteine residues.

Key words: isoform, methionine sulfoxide reductase, post-translational modification, protein oxidation.

INTRODUCTION

The mitochondria have been described as the major source of reactive oxygen species (ROS) in eukaryotic cells. Indeed, a small percentage of oxygen reacting in the respiratory chain is incompletely reduced, and forms ROS that are released either into the cytosol or into the mitochondria [1,2]. ROS attack leads to the oxidative modification of biomolecules such as lipids, DNA and proteins. Within proteins, the sulphur-containing residues (methionine and cysteine) are the most sensitive to oxidation by ROS. The mild oxidation of cysteine and methionine residues results in the formation of intra- and inter-molecular disulphide bridges and methionine sulfoxide respectively. Oxidation of other amino acids leads to the formation of either hydroxy or carbonyl derivatives. Methionine oxidation can regulate protein function, as observed with the α_1 -antitrypsin inhibitor and calmodulin [2,3]. For instance, activation of the plasma membrane Ca-ATPase channel by calmodulin is regulated by the oxidation of two specific methionines [3]. However, oxidation of methionine residues within proteins has generally been associated with a loss of biological activity, as reported for HIV-2 protease [4]. To prevent their accumulation in the cell, oxidized proteins can be either repaired by specific enzymic repair systems or degraded through the proteasome pathway [5–7]. When the balance between the formation of oxidized proteins and their removal is disrupted, oxidized proteins accumulate, as observed in normal aging and in age-related pathologies, such as pulmonary emphysema, cataract formation and Alzheimer's disease [8–10].

In this context, in *Escherichia coli* as well as in mammalian cells, it should be noted that only disulphide bridges and methionine sulfoxide resulting from the oxidation of sulphur-containing amino acids can be changed back to their reduced forms by enzymic systems which thereby protect the cell against oxidative damage. Specially, methionine oxidation leads to the formation of *S*- and *R*- free or peptide-bound methionine sulfoxide species, which are reduced by the methionine sulfoxide reductase system. In *Escherichia coli*, methionine sulfoxide reductase A (MsrA) and methionine sulfoxide reductase B (MsrB) can reduce specifically *S*- and *R*- free and peptide-bound methionine sulfoxide [Met-*S*-(O) and Met-*R*-(O)] respectively [11,12]. In addition, free Met-*S*-(O) and Met-*R*-(O) are reduced by free SMsr and free RMsR respectively [13,14]. In humans, MsrA activity has been attributed to a single enzyme, while MsrB activities are carried by two distinct proteins, SelX and Cbs-1 [15–18]. SelR, a SelX homologue, has also been characterized in the mouse and in *Drosophila melanogaster* [17,19]. In addition to its repair role, MsrA participates in ROS elimination by reducing surface-exposed methionine residues of proteins that act as ROS scavengers, as described for glutamine synthetase [1,2].

The essential role of MsrA in the maintenance of the cellular reduction/oxidation balance is supported by experiments in which null mutants of the *msrA* gene in *Escherichia coli*, *Saccharomyces cerevisiae* or mice have been shown to exhibit an increased sensitivity to oxidative stress [20–22]. Moreover, during aging and in Alzheimer's disease, two situations whereby

Abbreviations used: dabsylMet(O), 4-dimethylaminoazobenzene-4'-sulphonylmethionine sulfoxide; 1D, one-dimensional; 2D, two-dimensional; DTT, dithiothreitol; EGFP, enhanced green fluorescent protein; MsrA, methionine sulfoxide reductase A; MsrB, methionine sulfoxide reductase B; MTS, matrix targeting signal; LC-MS/MS, liquid chromatography–tandem MS; ROS, reactive oxygen species.

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oxidatively modified proteins tend to accumulate, i.e. a decrease in peptide methionine sulfoxide reductase activity and decreased MsrA protein and mRNA levels, have been reported [10,23]. In addition, it has been shown recently that overexpression of MsrA in *Saccharomyces cerevisiae* and *Drosophila melanogaster* contributes to a higher resistance against oxidative stress and increases the lifespan of those organisms [22,24].

The importance of these repair enzymes in cellular defence against oxidative stress has been demonstrated, but their antioxidant action has yet to be localized at the subcellular level. Combined with the thioredoxin system, the MsrA enzyme is thought to be present in all subcellular areas where ROS are produced. Indeed, in *Arabidopsis thaliana*, five *msrA*-like genes encode MsrA proteins that are located in the cytosol and plastids [25], and a recent report showed that human MsrA fused to enhanced green fluorescent protein (EGFP) and transfected into several human cell lines was located only in the mitochondria [26]. In the present study, we addressed the precise location of endogenous MsrA in rat liver cells. After subcellular fractionation of cytosol and mitochondria, we investigated peptide methionine sulfoxide reductase activity and tested for the presence of MsrA in each cellular compartment using Western blotting, *in situ* immunolocalization by electron microscopy analysis and MS. Our results showed that MsrA is present both in the cytosol and in the mitochondrial matrix of rat liver cells. Both enzymes exhibited acidic isoforms, probably corresponding to the presence of oxidized cysteine residues within the protein.

EXPERIMENTAL

Isolation of rat liver mitochondria

All operations were performed at 4 °C. A total of 25 rat livers were cut into pieces, rinsed with 2 mM Hepes buffer, pH 7.4, containing 220 mM mannitol, 0.1 mM EDTA, 70 mM sucrose and 0.5 % (w/v) BSA (buffer A) and homogenized using the Ultra-Turrax system. The homogenate was centrifuged at 600 g for 10 min. To remove cell debris, the supernatant was filtered and centrifuged twice at 8000 g for 10 min. The supernatant, corresponding to the cytosolic fraction, was kept at -70 °C (2 g of total protein). The pellet was washed twice, first with buffer A and then with buffer A without BSA. The pellet resuspended in buffer A without BSA constituted the mitochondrial fraction (750 mg of total protein). Polarographic measurements were performed to assess the respiratory capacity of the mitochondria. The rate of oxygen consumption was assayed in the presence of 50 mM succinate by an oxypolarographic method using a Clark-type electrode, as described by Aprille and Asimakis [27]. State 4 respiration was measured first in the absence of ADP, and state 3 respiration was measured in the presence of ADP to determine the maximal rate of coupled ATP synthesis. The respiratory control ratio was calculated using the ratio between state 3 and state 4 respiration. The integrity of the isolated mitochondria was checked by electron microscopy. The mitochondrial fraction was sonicated and centrifuged at 100 000 g for 45 min. The supernatant contained the matrix proteins (540 mg of total protein), and the pellet contained the membranes.

Immunogold electron microscopy

The anti-MsrA [23] labelling was checked using purified mitochondria according to the method described by Verbeke et al. [28]. After incubation with the anti-MsrA antibody, mitochondria were incubated in the presence of Protein A-gold complex

(10 nm; Biocell). The electron microscopy views were realized to a final magnification of $\times 10\,000$ or $\times 16\,000$.

Purification of MsrA from cytosolic and mitochondrial matrix fractions

Both cytosolic and mitochondrial fractions were precipitated with ammonium sulphate cut at 30 % and 70 % saturation. Proteins were then dialysed against PBS and loaded on an anti-MsrA affinity chromatography column equilibrated with the same buffer. The affinity column consisted of CNBr-activated Sepharose 4B (Amersham Biosciences) coupled to anti-MsrA antibody. The MsrA protein was eluted with 0.1 M glycine, pH 2.8, the collected fractions were immediately neutralized with 1.5 M Tris/HCl, pH 8.8, and 20 mM dithiothreitol (DTT) was added.

One-dimensional (1D) and two-dimensional (2D) gel electrophoresis and Western-blot detection of MsrA

Analysis of MsrA by 1D or 2D gel electrophoresis was performed in duplicate, with one gel for Coomassie Blue G-250 or silver staining [29] and the other for Western-blot analysis. For 1D gel electrophoresis, proteins were separated on SDS/PAGE [12 % (w/v) gel; 20 cm $\times$ 20 cm] and then electrotransferred on to a nitrocellulose membrane (HybondTM ECLTM; Amersham Biosciences). The membrane was incubated with anti-MsrA polyclonal antibodies [23] at a dilution of 1 : 1000. Blots were developed using SuperSignal[®] West Pico Chemica-luminescent Substrate (Pierce). Films were scanned and the amount of MsrA was quantified by densitometric analysis using Image Master 1D quantification software (Amersham Biosciences).

For 2D gel electrophoresis, the first dimension was performed using ReadyStrips IPG strips (pH 5–8; length 17 cm; Bio-Rad) and the Multiphor II isoelectric focusing system (Amersham Biosciences). An aliquot of purified cytosolic MsrA was diluted in sample buffer [9 M urea, 2 % (w/v) CHAPS, 2 % (v/v) Biolytes, 20 mM DTT and Bromophenol Blue]. Two strips were rehydrated in this solution overnight at room temperature and focused over 23 h for a total of 50 000 V-h. The strips were then equilibrated with gentle shaking for 10 min with 50 mM Tris/HCl, pH 6.8, 6 M urea, 30 % (v/v) glycerol, 1 % (w/v) SDS and 2 % (w/v) DTT, followed by a 10-min equilibration with the same buffer containing 2.5 % (w/v) iodoacetamide. Strips were then placed on top of SDS/PAGE [12 % (w/v) gel; 20 cm $\times$ 20 cm]. Western-blot analyses of proteins separated by 2D gel electrophoresis were carried out as for those separated on the 1D gel.

Protein digestion by trypsin and endoproteinase AspN, and MS analysis

After 1D (or 2D) gel electrophoresis, the MsrA-containing bands (or spots) identified by Western blotting were excised. Proteins were reduced with 10 mM DTT and alkylated with 55 mM iodoacetamide in 0.1 M ammonium carbonate, and then subjected to in-gel digestion with trypsin or endoproteinase AspN (sequencing grade; Roche) according to Shevchenko et al. [29]. After extraction, peptides were desalted using ZipTip C18 (Millipore). Peptides were dried and resuspended in 5 μ l of 1 % (v/v) formic acid. Peptides were then analysed by MS. Digestion of mitochondrial matrix proteins (100 μ g) with 1 : 20 (w/w) trypsin was performed for 18 h at 37 °C in 0.1 M Tris/HCl, pH 8.5. Liquid chromatography–tandem MS (LC-MS/MS) analyses were performed with an electrospray ion-trap mass spectrometer (LCQ Advantage; ThermoFinnigan)

coupled on-line with a Surveyor HPLC system (ThermoFinnigan). A 100 mm $\times$ 0.18 mm ThermoHypersil Betabasic-18 column (5 μ m particle diameter; 150 Å pore size) with a mobile phase of solvent A [0.1 % (v/v) formic acid in water] and solvent B [0.1 % (v/v) formic acid in acetonitrile] was used with a gradient of 5 to 50 % of mobile phase B over 20 min at a flow rate of 300 μ l/min. The flow was split with a precolumn, and 3 μ l/min was directed to the column. The electrospray needle was operated with a voltage of 3.5 kV, and the heated desolvation capillary was held at 220 °C. Nitrogen was used as the sheath gas. All scans were acquired in positive ion mode. The mass spectrometer operated in a data-dependent MS/MS mode, i.e. a full-scan mass spectrum (m/z range 400–1500) is followed by a tandem mass spectrum. The isolation width of the parent ions was set to 3 m/z units with 35 % normalized collision energy. An m/z ratio for an ion that had been selected for fragmentation was placed in a list and dynamically excluded from further fragmentation for 1 min. Proteins were identified automatically by the computer program TurboSEQUEST (ThermoFinnigan).

Derivatization of cysteine residues of MsrA with iodoacetic acid

The recombinant, cytosolic and mitochondrial proteins were denatured and reduced for 30 min at 37 °C in 10 mM Tris/HCl, pH 8, supplemented with 1 mM EDTA, 5 mM TCEP [Tris-(2-carboxyethyl)phosphine] and 8 M urea. An alkylation reaction was performed with 50 mM iodoacetic acid to derivatize the cysteine residues in 250 mM Tris/HCl, pH 8, and 250 mM KOH for 15 min at room temperature. The reaction products were analysed by 2D gel electrophoresis followed by a Western blot using the anti-MsrA antibody.

Oxidation of recombinant MsrA

A 50 μ g aliquot of recombinant MsrA in PBS, pH 7.4, was incubated for 30 min at room temperature in the presence of 0.1 mM H₂O₂. The hydrogen peroxide was eliminated by dialysing MsrA against water. The oxidized MsrA was analysed by 2D gel electrophoresis as described above.

Enzymic assay

Peptide methionine sulfoxide reductase activity was determined by monitoring the reduction of the synthetic substrate 4-dimethylaminoazobenzene-4'-sulphonylmethionine sulfoxide [dabsylMet(O)], as described previously by Petropoulos et al. [23].

RESULTS

Evidence for peptide methionine sulfoxide reductase activity in cytosol and mitochondrial matrix

Cytosolic and mitochondrial matrix extracts were prepared from rat liver after homogenization and subcellular fractionation, as described in the Experimental section. Western-blot analysis with monoclonal antibodies directed against cytosolic (proteasome α -subunit) and mitochondrial (citrate synthase) proteins revealed that there was no cross-contamination between the cytosolic and mitochondrial fractions (results not shown). Moreover, electron microscopy views showed that mitochondria resulting from this subcellular fractionation were intact (Figure 1A), and polarographic measurements showed normal respiratory function of the mitochondria (respiratory control ratio = 4).

Figure 1 Immunolocalization of MsrA enzyme in mitochondria

(A) Electron microscopy views of intact isolated mitochondria after subcellular fractionation of rat liver cells as described in the Experimental section. (B) The isolated mitochondria were incubated in the presence of anti-MsrA antibody, and were visualized by electron microscopy. MsrA (arrowheads) was detected in the matrix, but not in the membrane. Bars = 0.5 μ m.

Figure 2 Subcellular localization of MsrA in rat liver

Cytosolic and mitochondrial proteins were prepared by subcellular fractionation as described in the Experimental section. The mitochondrial matrix and membrane were obtained by sonication followed by centrifugation at 100 000 g for 45 min at 4 °C. Rat recombinant MsrA (0.1 μ g; lane 1) and protein extracts from the cytosol (50 μ g; lane 2), mitochondrial matrix (50 μ g; lane 3) and mitochondrial membrane (50 μ g; lane 4) were subjected to SDS/PAGE [12 % (w/v) gel], electrotransferred on to a nitrocellulose membrane, and immunoblotted with anti-MsrA antibody. The cytosolic and mitochondrial MsrA appeared as proteins with a slightly lower molecular mass than the recombinant MsrA. In lane 1 (rat recombinant MsrA), the upper band resulted from an incomplete cleavage of the His tag (1.9 kDa) by thrombin.

Peptide methionine sulfoxide reductase activity was monitored in both fractions using dabsylMet(O) as substrate. The specific activities measured in the cytosol and in the mitochondrial matrix were respectively 200.6 ± 12 and 210.6 ± 10 pmol of dabsylMet(O)/min per mg of protein. The chromophore substrate was a mixture of dabsylMet-S-(O) and dabsylMet-R-(O), which were reduced by either MsrA or MsrB. Therefore the presence of MsrA was assessed in both fractions by Western blotting with an anti-rat MsrA antibody. As shown in Figure 2, MsrA was present in the cytosol and in the mitochondrial matrix, but not in the membrane. In addition, it should be noted that the MsrA detected in the cytosol and mitochondria was slightly smaller than rat recombinant MsrA, as previously described by Petropoulos et al. [23]. The amounts of MsrA in the cytosol and the mitochondrial matrix were quantified by densitometric analysis. MsrA was estimated to be 3-fold more abundant in the cytosol than in the mitochondrial matrix, and represented 0.1 % and 0.03 % respectively of total protein. Localization of MsrA in the mitochondrial matrix was further confirmed by electron microscopic analysis of isolated mitochondria (Figure 1B).

Identification of cytosolic and mitochondrial MsrA isoforms by MS analysis

Purification of cytosolic MsrA was achieved in two steps. As described in the Experimental section, ammonium sulphate

Figure 3 Partial purification of cytosolic MsrA

A 300 mg aliquot of cytosolic extract was applied to affinity chromatography after ammonium sulphate precipitation. The MsrA protein was eluted with 0.1 M glycine, pH 2.8. Cytosolic extract (CE) and eluted fraction (EF) were analysed by SDS/PAGE (12% gel) and either stained with Coomassie Blue (lanes 1 and 2) or Western blotted with anti-MsrA antibody (lanes 3 and 4).

MTSASRRITLQ LLSSSIPVRM MGDSSSKVIS AEEALPGRTE SIPVAAKHHV
SGNRTVEFPF EGTQMAVEFGM GCFWGAERKF WLLKGVYSTQ VGFAGGYTRN
PTYKEVCSEK TGHAEVVRVV YRPEHVSFEE LLKVFENHND PTQGMRRQND
CGTQYRSADV PTSAVQMEAA LKSKEEYQKV LSKHGFGPIT TDIREGQVFF
YAEYHQQYL SKNPDGYCGL GGTGVSCPTA IKK

Figure 4 Identification of MsrA peptides by LC-MS/MS

MsrA peptides from mitochondrial matrix and cytosolic fractions, obtained by in-solution tryptic digestion, are underlined and emboldened respectively. Peptides from 2D gel electrophoresis separation of partially purified MsrA from cytosol and obtained by in-gel digestion with endoproteinase AspN are in italic.

precipitation followed by affinity chromatography enabled us to partially purify the MsrA protein. The eluted fraction was analysed by Western blot after SDS/PAGE with an anti-MsrA antibody (Figure 3). The recognized protein was in-gel digested with trypsin, and peptides were analysed by LC-MS/MS. Using this approach, 44% of the theoretical sequence was covered (Figure 4), confirming the presence of MsrA in the cytosol.

Since mitochondrial MsrA is less abundant than the cytosolic form, an alternative approach was developed to identify MsrA in the mitochondrial matrix. The extract of mitochondrial matrix proteins was subjected to digestion by trypsin and the resulting peptides were analysed by LC-MS/MS. Among the identified peptides, a 20-residue peptide of MsrA corresponding to residues 28–47 of the theoretical MsrA sequence was characterized (Figure 4). This peptide was identified previously by MS analysis of cytosolic MsrA-derived peptides. These results demonstrated that MsrA was localized both in the cytosol and in the mitochondrial matrix of rat liver cells.

Biochemical characterization of cytosolic and mitochondrial MsrA isoforms by 2D gel electrophoresis

To identify putative post-translational modifications, partially purified cytosolic and mitochondrial MsrA isoforms were analysed by 2D gel electrophoresis and Western blot. Two isoforms with different pI values were detected for both the recombinant and mitochondrial proteins (Figures 5A and 5C), while four cytosolic isoforms were visualized (Figure 5B). As observed on the 2D gels (Figures 5A–5C), these variations in pI were not associated with a detectable variation in molecular mass. LC-MS/MS analysis after in-gel digestion

Figure 5 2D gel patterns of recombinant, cytosolic and mitochondrial MsrA

Aliquots of 50 µg of recombinant MsrA (A), 200 µg of partially purified cytosolic MsrA (B), 150 µg of mitochondrial MsrA (C) and 200 µg of alkylated mitochondrial MsrA (D) were analysed by 2D gel electrophoresis as described in the Experimental section. Recombinant MsrA (A) was stained with Coomassie Blue G-250, and the MsrA-containing spots from the cytosolic (B) and mitochondrial (C and D) fractions were detected by Western-blot analysis with a polyclonal anti-MsrA antibody. IEF, isoelectric focusing.

with endoproteinase AspN confirmed the presence of MsrA in the four cytosolic spots (Figure 5B). Moreover, this analysis revealed peptides corresponding to the final 40 residues of MsrA, therefore identifying the C-terminal sequence of the protein (Figure 4).

Figure 2 shows that the mitochondrial MsrA was smaller than the recombinant MsrA. The recombinant MsrA contains five cysteine residues (Cys-72, Cys-107, Cys-151, Cys-218 and Cys-227; 233 total residues), with two residues localized near the C-terminal extremity. To determine whether the mitochondrial MsrA sequence lacked residues in the C- or N-terminal extremities, the content of cysteine residues was investigated using iodoacetic acid as a specific reagent of thiols. The reaction was carried out with partially purified mitochondrial MsrA. Products were separated by 2D gel electrophoresis, and six spots were revealed after Western-blot analysis using the anti-MsrA polyclonal antibody (Figure 5D). The more acidic spots appeared as a doublet, probably resulting from partial proteolytic degradation. Incomplete alkylation of the five cysteines was expected to lead to the six spots observed. Incomplete alkylation of only three cysteines would have generated a maximum of four spots. Therefore the mitochondrial MsrA most probably contains five cysteine residues, as in the recombinant MsrA, which strongly suggests that recombinant and mitochondrial MsrA differ at their N-terminal extremity.

As shown in Figures 5(B) and 6(A), the four pI isoforms observed for the cytosolic MsrA may originate from a post-translational process resulting in acidic isoforms, without modification of the apparent molecular mass. Interestingly, the formation of a more acidic peroxiredoxin isoform has been attributed recently to oxidative modification of a cysteine residue of peroxiredoxin 2 and 3 in Jurkat cells [30,31]. Therefore the more acidic isoforms of cytosolic MsrA may also result from oxidation of cysteine residues. To further characterize the modification of MsrA, recombinant MsrA was oxidized with

Figure 6 Oxidation and cysteine alkylation of recombinant and cytosolic MsrA

Aliquots of 200 μ g of partially purified cytosolic MsrA before (A) and after (B) treatment with 50 mM iodoacetic acid were separated by 2D gel electrophoresis and detected by Western-blot analysis with a polyclonal anti-MsrA antibody, as described in the Experimental section. Aliquots of 50 μ g of recombinant MsrA treated with 0.1 mM H_2O_2 (C) and 100 μ g of recombinant MsrA treated with 50 mM iodoacetic acid (D) were analysed by 2D gel electrophoresis and stained with Coomassie Blue G-250 and silver nitrate respectively. IEF, isoelectric focusing.

0.1 mM hydrogen peroxide at pH 7.4, resulting in the formation of four spots detected by 2D gel electrophoresis (Figure 6C), reminiscent of the 2D gel pattern obtained with cytosolic MsrA. In addition, an alkylation reaction with iodoacetic acid was performed with both recombinant and cytosolic MsrA, and similar 2D patterns were obtained for the derivatized proteins (Figures 6B and 6D). A more complex pattern would have been generated if the pre-existing modifications of the cytosolic MsrA affected amino acids other than cysteine residues. Taken together, these data indicate that the more acidic spots observed for the cytosolic MsrA resulted from an additional negative charge on up to three cysteine residues, most probably due to oxidation of cysteine to sulphinic or sulphonic acid.

DISCUSSION

In the present study, we have demonstrated the dual localization of MsrA in the cytosol and mitochondria of rat liver cells. First, reductase activity against both *R*- and *S*-methionine sulfoxide diastereoisomers was observed in the cytosol and the mitochondrial matrix. The presence of MsrA was then demonstrated by Western-blot analysis, and the enzyme was identified by MS analysis, in both subcellular compartments. MsrA protein was estimated to represent 0.1 % of total cytosolic protein, and was 3-fold less abundant in the mitochondria. Moreover, as shown by *in situ* immunolocalization, the mitochondrial MsrA was localized only in the matrix. Finally, both cytosolic and mitochondrial enzymes were smaller than recombinant MsrA (233 residues), and were present as several isoforms with different pI values.

In a recent report, pioneering studies by Hansel et al. [26], using human MsrA fused to EGFP and transfected human neuroblastoma cells, provided evidence that MsrA is localized in

the mitochondria. To explain the observation that the localization of MsrA-EGFP was restricted to the mitochondria, Hansel et al. [26] proposed that MsrA enzyme, after being translocated to the mitochondria, might be exported back to the cytosol. The fusion of EGFP to MsrA would prevent any such movement to the cytosol. This hypothesis was supported by a single example, namely that of yeast fumarase [32]. In that case, after processing in the mitochondria, cytosolic and mitochondrial fumarase isoforms have the same molecular mass, with identical N-termini. However, in our present study, the cytosolic and mitochondrial MsrA forms did not have the same apparent molecular mass. This observation does not support a retroactive movement of MsrA from the mitochondria to the cytosol.

Thus MsrA protein is encoded by a single gene [17], but has more than one subcellular localization. Compared with the situation in *Saccharomyces cerevisiae*, where this process has been studied extensively [33], there are few instances of dual targeting from a single gene in higher eukaryotes; examples include rat fumarase [34] and goose liver malonyl-CoA decarboxylase [35]. The mechanism leading to the production of two MsrA proteins from a single gene has yet to be determined. However, it has been suggested by Petropoulos et al. [23] that the cDNA sequence of rat MsrA contains two initiation sites of translation, corresponding to Met-1 and Met-21 in the full-length recombinant protein sequence (residues 1–233). Transcription/translation *in vitro* from *msrA* cDNA in reticulocyte lysate leads to two proteins, probably resulting from translation from the two initiation sites (results not shown). Therefore the production of two proteins from a single gene may result from one or two mRNAs corresponding to an alternative translation initiation site, as reported for rat liver fumarase, or an alternative transcription initiation site, as reported for goose liver malonyl-CoA decarboxylase. As shown previously, both mitochondrial and cytosolic isoforms are shorter than recombinant MsrA. Therefore the cytosolic isoform is suggested to result from translation from Met-21, whereas the mitochondrial MsrA may be synthesized as a precursor protein starting at the Met-1 initiation site, and is then processed during import into the mitochondria.

Indeed, in order to be imported into the mitochondria, proteins should contain a signal peptide [36,37]. After translocation into the matrix, a mitochondrial processing peptidase removes this signal peptide [38,39]. According to the literature, the matrix targeting signal (MTS) can be localized in either the N-terminal or the C-terminal extremity [33]. For mitochondrial MsrA, localization of the signal peptide in the C-terminal extremity can be excluded, due to the observation that mitochondrial MsrA contains five cysteine residues, as in the recombinant protein. Cleavage of a C-terminal signal peptide would have resulted in the loss of two cysteine residues, Cys-218 and Cys-227, localized near the C-terminal extremity. Moreover, an MTS is predicted in the N-terminal extremity of rat recombinant MsrA, when its sequence is submitted to the Mitoprot software (<http://www.mips.biochem.mpg.de/cgi-bin/proj/medgen/mitofilter>). This peptide of 20 residues, containing basic and apolar residues and a high amphipathic potential, presents the features of an MTS [33]. After translocation of full-length MsrA into the mitochondria, the predicted signal peptide would be cleaved by a mitochondrial processing peptidase to leave mature mitochondrial MsrA. In conclusion, a putative signal peptide localized in the N-terminal extremity is postulated to target MsrA to the mitochondria.

The proteins in the cell are the targets of post-translational modifications that are implicated in the regulation of their function and/or in signal transduction. In addition, proteins can also be modified upon stress, such as oxidative stress. We have shown that

MsrA proteins in the cytosol and the mitochondria are modified. These modifications change the net charge of the protein, resulting in more acidic isoforms, but not the apparent molecular mass. The cytosolic MsrA was more sensitive to these modifications, since four cytosolic isoforms with different pI values were detected, compared with only two in the mitochondria. Recent reports showed that cysteine residues of peroxiredoxin 2 and 3 of Jurkat cells could be oxidized, resulting in a more acidic peroxiredoxin [30,31]. Cytosolic MsrA and recombinant MsrA treated with H₂O₂ showed the same pattern in 2D gel electrophoresis, suggesting that the acidic isoforms of cytosolic MsrA are due to oxidative modifications. Moreover, the 2D gel patterns of recombinant and cytosolic MsrA after derivatization by iodoacetic acid were also similar, indicating that the more acidic isoforms were derived from additional negative charges on cysteine residues. Thus the MsrA protein is most likely to be modified by oxidation of up to three of its five cysteine residues to sulphinic or sulphonic acid.

The oxidation of methionine residues within proteins leads to *R*- and *S*-methionine sulfoxide diastereoisomers in all subcellular locations. We showed that methionine sulfoxide can be reduced back to methionine in the cytosol and mitochondria. Along with the enzymic systems that repair oxidized cysteine residues, MsrA is implicated in the maintenance of the native protein pool in the cytosol and mitochondria. Moreover, by reducing exposed methionine residues acting as ROS scavengers, MsrA assists in detoxification in both subcellular compartments. MsrB enzymes that reduce the *R*-methionine sulfoxide diastereoisomer have recently been described [12,14,17–19]. Although their precise subcellular localization has not yet been investigated, their presence in both the cytosol and the mitochondria is highly probable.

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