Characterization and Solution Structure of Mouse Myristoylated Methionine Sulfoxide Reductase A*

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**Background:** Mammalian methionine sulfoxide is myristoylated, but the function of myristoylation is unknown.

**Results:** Although most characteristics of methionine sulfoxide reductase are unchanged by myristoylation, protein-protein interaction is enhanced.

**Conclusion:** The myristoyl group is not buried but is docked on the protein surface in a “myristoyl nest.”

**Significance:** The myristoyl nest of methionine sulfoxide A enhances its ability to participate in protein-protein interactions.

Methionine sulfoxide reductase A is an essential enzyme in the antioxidant system which scavenges reactive oxygen species through cyclic oxidation and reduction of methionine and methionine sulfoxide. The cytosolic form of the enzyme is myristoylated, but it is not known to translocate to membranes, and the function of myristoylation is not established. We compared the biochemical and biophysical properties of myristoylated and nonmyristoylated mouse methionine sulfoxide reductase A. These were almost identical for both forms of the enzyme, except that the myristoylated form reduced methionine sulfoxide in protein much faster than the nonmyristoylated form. We determined the solution structure of the myristoylated protein and found that the myristoyl group lies in a relatively surface exposed “myristoyl nest.” We propose that this structure functions to enhance protein-protein interaction.

All organisms living in an aerobic atmosphere are subjected to oxidative stress from reactive oxygen and nitrogen species. Proteins are a notable target of these species, and mechanisms have evolved to intercept them and deal with proteins that do undergo oxidative modification (1). If the covalent modification is not reversible then the protein is targeted for selective degradation. Repair systems have evolved for the reversible modifications, including oxidation and reduction of the sulfur-containing amino acids cysteine and methionine. The latter is relatively readily oxidized to methionine sulfoxide. Virtually all organisms from bacteria to mammals have several methionine sulfoxide reductases (msr) which catalyze the reduction of methionine sulfoxide to methionine (2). Reduction of methionine residues in proteins allows them to react again with reactive species, creating a system with catalytic efficiency in scavenging potentially damaging species. Thioredoxin and thioredoxin reductase act sequentially to regenerate active msr, with the net result being the catalytic scavenging of reactive oxygen species at the expense of NADPH. A scheme of the system is shown in Ref. 3.

Oxidation of methionine to methionine sulfoxide creates a chiral center, and the S- and R-epimers are substrates for specific msr (2, 4). In most organisms the S-epimer is reduced by msrA and the R-epimer by msrB, both using thioredoxin as the source of reducing equivalents. Recently, msrA was shown to also catalyze the stereospecific oxidation of free methionine and methionine residues in proteins to S-methionine sulfoxide, suggesting that it may participate in redox signaling via oxidation and reduction of methionine residues (5). In mammals one gene with two initiation sites encodes two forms of msrA, one targeted to the cytosol and the other to mitochondria (6). The cytosolic form is myristoylated on its amino-terminal residue, Gly-22 (3). Myristoylation is required for membrane binding of certain proteins, but it is not sufficient to direct them to the membrane (7). A second structural element is required, and if that element can be modulated then membrane binding of the protein becomes reversible. None of the second elements known to support membrane binding has been found in msrA, and various stimuli have failed to promote translocation of msrA from the cytosol to the plasma membrane or endoplasmic reticulum (6). With the goal of elucidating the role of myristoyla-

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2 The abbreviations used are: msr, methionine sulfoxide reductase; HSQC, heteronuclear single-quantum correlation; msrA, methionine sulfoxide reductase A; r.m.s.d., root mean square deviation.

3 We follow the usual practice in the field and number residues beginning with Met-1. Studies in this paper were performed with the cytosolic form whose amino-terminal residue is Gly-22.
Myristoylated Methionine Sulfoxide Reductase A

EXPERIMENTAL PROCEDURES

Recombinant myristoylated and nonmyristoylated msrA was produced, analyzed, and assayed as described (6). For NMR analyses, 13C- and 15N-labeled msrA was produced in cells grown in minimal medium with [15N]ammonium chloride and [13C]glucose as the sole nitrogen and carbon sources, supplemented with 0.25 mM sodium pyruvate, for myristoylated msrA.

Size exclusion chromatography was performed on a Tosoh G3000SWXL column (Tosoh Bioscience, King of Prussia, PA). MsrA, 10 μg, was injected onto the column run at 0.5 ml/min with 50 mM sodium phosphate buffer, pH 7.2, and 100 mM NaCl. Fluorescence intensity spectra were recorded at 20 °C using a QM-6SE spectrophotometer (PTI, London, ON). Measurements were performed with 280-nm excitation and Glan polarizers at magic angle condition. Thermal denaturation was performed in a Jasco J-715 spectropolarimeter (Jasco, Easton, MD), monitoring the ellipticity at 222 nm.

Reduction of Methionine Sulfoxide in Peptides—Peptides were kindly donated by Kris Gevaert (Ghent University, Ghent, Belgium). To convert the methionine peptides into their corresponding sulfoxides, we incubated 200 nmol of each peptide with 0.5% (v/v) H2O2 (163 mM) in 1.5 ml of 0.5% (v/v) trifluoroacetic acid (TFA) for 30 min at 30 °C. Buffer and excess reagents were removed using Sep-Pak Classic C18 Cartridges (Waters, Milford, MA). Cartridges were stripped with 10 ml of 50% (v/v) CH3CN, 0.1% (v/v) TFA and then washed with 10 ml of 0.1% (v/v) TFA. The sample was loaded onto the column followed by a washing step with 20 ml of 0.1% (v/v) TFA. Elution of the peptides was carried out with 4 ml of 80% (v/v) CH3CN, 0.1% (v/v) TFA. Eluates were dried in a vacuum centrifuge and then redissolved in 200 μl of 50 mM Tris-HCl, pH 7.6. For reduction of methionine sulfoxide by msrA, 20 nmol of each methionine sulfoxide peptide was dissolved in 200 μl of 50 mM Tris-HCl, pH 7.6, containing 25 mM DTT. The mixture was incubated at 37 °C for 5 min followed by the addition of 20 pmol of msrA (enzyme:substrate ratio of 1:1000). After 2.0 min, the reaction was stopped by adding 20 μl of 10% (v/v) TFA. 20 μl of the acidified mixture was loaded onto a Zorbax 300 SB-C18 reverse phase column, 2.1 × 50 mm, 3.5 μm (Agilent) connected to an Agilent 1200 Series HPLC system. A linear gradient was applied, starting at 0 min with 100% solvent A (0.1% (v/v) TFA in water), 0% solvent B (0.1% (v/v) TFA in CH3CN), and going to 60% (v/v) solvent B at 45 min. The flow rate was 80 μl/min. The fractional reduction of methionine S-sulfoxide was calculated from the areas of the reduced and the oxidized peaks, integrated using Chemstation (Agilent). All experiments were run in triplicate.

Preparation of Apolipoprotein A-I-containing Methionine S-Sulfoxide—Apolipoprotein A-I was kindly provided by Alan Remaley (NHLBI, National Institutes of Health, Bethesda, MD). Two mg of apoA-I was dissolved in 0.1% (v/v) TFA and treated with 0.5% (v/v) H2O2 (163 mM) for 30 min at 30 °C. Removal of hydrogen peroxide was accomplished with a Sep-Pak Classic C18 cartridge as described above. The dried apoA-I was then dissolved in 500 μl of 50 mM Tris-HCl, pH 7.6, with 25 mM DTT. The protein concentration was determined using the Bradford assay (8). Eight μg of recombinant His-tagged Escherichia coli msrB was added for 4 h at 37 °C to convert the oxidized apoA-I into its S-sulfoxide form. The msrB was removed by a Ni2+ chelate column, and the solution of apoA-I was stored at −20 °C until used. 200 μg of S-sulfoxide apolipoprotein was used for each incubation with msrA. The volume was adjusted to 500 μl with 50 mM Tris-HCl, pH 7.6, with 25 mM DTT. The sample was incubated for 5 min at 37 °C followed by the addition of 0.4 μg of msrA (enzyme:substrate ratio of 1:1000). Aliquots with 20 μg of apoA-I were withdrawn at 0, 2, 5, 10, 15, 25, 30, 45, 60, and 90 min and mixed with 50 μl of 1% (v/v) TFA to stop the reduction. The acidified samples were separated on the same Zorbax column noted above. After isocratic flow with 20% (v/v) solvent B (0.1% (v/v) TFA in CH3CN) for 2 min, a gradient of 2% (v/v) solvent B/min was developed. The areas of the reduced and oxidized peaks were again obtained with the Chemstation integrator as for the peptides. All experiments were run in triplicate.

Analytical Ultracentrifugation—Experiments were conducted at 20 °C using a Beckman Optima XL-1 analytical ultracentrifuge (Beckman, Palo Alto, CA) equipped with a four-hole An Ti-60 rotor and cells with 12-mm double-sector Epon centerpieces and sapphire windows. The sample volume was 0.4 ml. MsrA was dialyzed overnight into 50 mM sodium phosphate, pH 7.5, with 150 mM NaCl. After thermal equilibrium was reached at rest, the rotor was accelerated to 60,000 rpm. Interference and 280-nm absorbance scans were collected continuously until no further sedimentation boundary movement was observed. The partial specific volume of msrA and buffer density and viscosity were calculated using the SEDNTERP program (9). Data analysis was conducted using the c(s) method in the SEDFIT program (10). Menisci positions and frictional ratios were optimized during the fitting procedure, and distributions were regularized using a maximum entropy method and a 0.68 confidence interval. All final fits had a S.D. < 0.01 unit.

NMR Spectroscopy—Myristoylated and nonmyristoylated 13C/15N msrA were studied in a Bruker 800 MHz spectrometer equipped with a cryoprobe. Backbone resonance assignment was done by standard techniques using CBCA(CO)NH and HNCACB experiments at 300 K. 15N-edited NOESY spectra (tmix = 100 ms) were measured for structure determination whereas 15N heteronuclear single-quartum correlation (HSQC) spectra were measured for chemical shift perturbation analysis. These were performed with 13C-labeled samples.

Structure Determination—Backbone dihedral phi and psi restraints were obtained with TALOS (11) from the observed Ca, Cβ, Hα, and N frequencies. We used only dihedrals predicted with high confidence, with ranges equal to twice the TALOS uncertainty. NOE-derived distance restraints were obtained for assigned NOE cross-peaks using peak intensities (heights) normalized by the corresponding diagonal peak.
intensities. Normalized intensities were converted to distances by calibrating with a subset of nonoverlapped cross-peaks arising from close intra, sequential, and medium range HN-HN and Ha-HN distances in regions of well defined secondary structure. These yielded \( d(\AA) = 2.02^* f^{-0.226} \), where \( d \) is distance and \( f \) is the normalized cross-peak intensity. The observed variation in cross-peak volumes corresponding to particular distances was a factor of 10, so that

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\begin{align*}
\frac{u(\AA)}{H_11005} & = 2.02 \left( \frac{I}{3.162} \right)^{0.226}, \\
\frac{l(\AA)}{H_11005} & = 2.02 \left( I \times 3.162 \right)^{-0.226}
\end{align*}
\]

where \( u \) and \( l \) are the upper and lower distance bounds in angstroms. Two hundred structures were generated using the backbone angle and distance restraints with a standard simulated annealing protocol in the XPLOR-NIH program (12). Floating chirality was employed for diastereotopic methylene and methyl groups (13). The myristoyl group definition in the XPLOR topology file was generated using analogous XPLOR peptide, methylene, and methyl parameters. The 20 structures with lowest experimental restraint violations were superimposed, and the cartesian average structure of the ensemble was minimized using MacroModel (Schrödinger, New York) with the OPLS 2005 force field in implicit solvent.

RESULTS

Biophysical Characterization—Size exclusion chromatography showed that myristoylated msrA eluted distinctly later than nonmyristoylated msrA, consistent with a smaller Stokes radius or nonideal interaction of the hydrophobic myristoyl group with the gel filtration medium (Fig. 1A). Analytical ultracentrifugation revealed identical distributions for both forms with an average sedimentation coefficient \( S_{20,w} \) of 2.59 ± 0.05 S and an estimated molecular mass of 23.8 kDa (Fig. 1B). Thus, both the myristoylated and nonmyristoylated msrA are monomeric in solution.

To probe for effects of myristoylation on secondary structure or conformational dynamics, we examined the tryptophan fluorescence and thermal stability of the two forms of msrA. The tryptophan fluorescence emission spectra were identical, indicating that myristoylation has no effect on the local environments of the three tryptophans in msrA (Fig. 1C). In contrast to the stabilizing effect of myristoylation on protein kinase A (14), lipidation decreased the thermal stability of msrA (Fig. 1D), suggesting that the myristoyl group in msrA is not bound in a deep hydrophobic pocket as it is in protein kinase A.

Effect of Myristoylation on the Rate of Reduction of Peptides—We previously established that the kinetic constants for reduction of free methionine sulfoxide were not altered by myristoylation (6). It was also demonstrated that the rate of reduction of peptides by nonmyristoylated msrA varied with the sequence near the methionine sulfoxide residue (15). We therefore determined the rates of reduction of peptides by myristoylated and nonmyristoylated msrA. One set of peptides had the identical sequence except for the residue preceding the methionine sulfoxide. Another set of peptides had sequences predicted to enhance reduction (15). Although we observed considerable differences in rate among the peptides, myristoylation did not affect the rates (Fig. 2).

NMR Spectroscopy—Assignments of resonances were made for 98% of the backbone of both the nonmyristoylated and myristoylated \(^{13}\text{C}/^{15}\text{N}\)-labeled msrA. NMR resonances for the amino-terminal amine are typically not observed for most proteins due to very fast solvent exchange. However, myristoylation converts the amine to an amide, which has slower solvent exchange. Thus, signals from the first residue, Gly-22, were observed from myristoylated msrA (Fig. 3A), as also reported for myristoylated ARF1 (16). In the CBCACONH spectrum, weak signals for the myristoyl C2 (38.0 ppm) and C3 (28.0 ppm) carbon resonances were detected via the Gly-22 amide. A weak peak corresponding to the degenerate resonances of the myristoyl C4–C11 methylene groups was observed in the \(^{13}\text{C}\) HSQC
with a $^1$H frequency 1.15 ppm and $^{13}$C frequency 31.9 ppm (supplemental Fig. S1) (17). Peaks for C12–C14 were too weak to be detected.

The backbone amide frequencies of the two forms of msrA were compared (Fig. 3B), revealing large chemical shift perturbations for residues 24–28. Thus, myristoylation causes a major structural change at the amino terminus. Smaller perturbations (<0.25 ppm) were seen for Gly-96, Gln-143, Ala-158, and Asp-192, suggesting that they lie near the myristoyl group or that their environments are indirectly altered by myristoylation (Fig. 3B). A comparison of $^{13}$C $\alpha$ and C$\beta$ frequencies also showed significant differences for residues 24–28. Those frequencies indicate a helical structure for the myristoylated form. No significant changes in C$\alpha$ and C$\beta$ frequencies were observed beyond the seventh residue from the amino terminus (Val-28).

The 15N NOESY spectra of myristoylated and nonmyristoylated msrA were compared, examining the through-space, proton NOE signals to backbone, and side chain 15N-attached protons. This comparison confirmed that there were no structural changes due to myristoylation. It also revealed 11 NOE signals for the myristoylated form not present in the spectrum of the nonmyristoylated form. Their frequencies were consistent with myristoyl resonances. Four of these signals were to the backbone amide of Gly-22 and are consistent with the reported C2 and C3 methylene group proton frequencies (17). NOE signals with the same proton frequency as the presumed C4–C11 methylene peak in the $^{13}$C HSQC spectrum were seen to the backbone amides of Ile-29, Gly-112, and Gly-144 and to the side chain He protons of Arg-156 and Arg-194. Two of the additional NOE signals had a proton frequency of 0.81 ppm, corresponding to the reported myristoyl C14 methyl 1H resonance (17) (supplemental Fig. S2). Unlike the $^{13}$C HSQC, the cross-peaks in the NOESY spectrum depend only on proton abundance, so that the C14 methyl proton resonance is observable.

The NOE spectra of both forms were also compared with a table of close (<5 Å) proton-proton distances generated from a homology model using the bovine nonmyristoylated msrA (18). No differences with the x-ray structure were seen for either form of mouse msrA, except for a minor difference in the turn region near Thr-162 which could be due to homology model generation.

**Structure Determination**—The three-dimensional structure of myristoylated msrA was determined using 2121 distance restraints from the 15N NOESY experiment and 349 backbone dihedral angles predicted using TALOS (11). The 20 lowest penalty structures had a backbone r.m.s.d. from average of 1.3 Å for residues 22–233 (Table 1). The ensemble and the minimized average structure are shown in Fig. 4, A and B. The central $\beta$-sheet (strands 1–3 and 6–8) was most precisely determined, with an average backbone r.m.s.d. from an average of 0.5 Å. Residues following Val-225 lack long range NOE signals and become progressively more disordered toward the carboxyl terminus. A solution structure determination of the nonmyristoylated form was not performed because of the near complete agreement between observed NOE signals.
and those predicted from the x-ray structure of nonmyristoylated msrA.

The myristoyl group lies extended in a shallow pocket at the msrA surface, adjacent to the residues with the largest chemical shift perturbations (Fig. 3B). The backbone for amino-terminal residues 24–27 adopts a helical structure on average. In contrast to that observed in protein kinase A (14), the myristoyl pocket is not particularly hydrophobic. Residues contacted by the myristoyl group include Lys-27, Val-28, Ile-29, Gly-96, His-97, Gly-112, Ala-114, Gly-144, Met-145, Thr-153, Arg-156, Thr-190, Asp-192, and Arg-194 (Fig. 4C). The myristoyl group is >14 Å away from the active site Cys-72 (Fig. 4B). As a consequence, the active site backbone resonances were only slightly perturbed by myristoylation (<0.05 ppm).

The minimized average structure from the NMR ensemble aligns with the x-ray structure of bovine msrA with a backbone pairwise r.m.s.d. of 1.3 Å for residues 29–228. Residues in the amino-terminal region before Val-29 and in the carboxyl-terminal region past Pro-228 were not observed in the x-ray structure. The regions where the average NMR structure differs most from the x-ray structure are not near the myristoyl group, centering instead at Arg-38 and Cys-217. These regions had fewer distance restraints than average, so it is unclear whether these regions truly differ from the x-ray structure. For residues 29–228, the pairwise r.m.s.d. values of structures within the NMR ensemble range from 1.4 Å to 1.8 Å, while compared with the x-ray structure they range from 1.4 Å to 1.9 Å, indicating that the x-ray structure lies within the precision of the NMR structure ensemble.

**Rate of Reduction of S-Methionine Sulfoxide in Apolipoprotein A-I**—As developed under "Discussion," the structure of the myristate binding site suggests that it may enhance protein-protein interaction and speed the reduction of methionine sulfoxide in the interacting protein. We tested this hypothesis with apolipoprotein A-I, the major protein in high density lipoprotein (HDL). Its methionine residues are sensitive to oxidation, the oxidized form is known to be present in vivo (19), and methionine oxidation induces conformational changes in the protein (20). Because msrA specifically reduces only S-methionine sulfoxide, we prepared apolipoprotein A-I with only that epimer as described under “Experimental Procedures.” Fig. 5 shows that myristoylated msrA reduced the methionine sulfoxides in apolipoprotein A-I four times faster than nonmyristoylated msrA.

**DISCUSSION**

Myristoylation is required for membrane binding of certain proteins, but it is not sufficient to direct them to the membrane (7, 21). A second structural element is required, and if that element can be modulated then membrane binding of the protein becomes reversible: the "myristoyl switch." Two second elements that can join with myristoylation to direct membrane binding are palmitoylation and a cluster of basic residues, but msrA has neither element. Myristoylated msrA has only been found in the cytosol of the tissues and cells examined to date, and various treatments failed to promote translocation to a membrane (6, 22–24).

The NMR spectra established that the overall structure of mouse myristoylated msrA is nearly the same as the nonmyristoylated form. The NMR solution structure of myristoylated msrA and the previously solved x-ray structure of bovine nonmyristoylated msrA are also quite similar (backbone r.m.s.d. of 1.3 Å). The only significant difference is in the amino-terminal residues, which are in a flexible random coil in the nonmyristoylated form and adopt a more helical conformation in the myristoylated form. The solution structure shows the myristoyl group interacting with a shallow pocket at the protein surface which is near the amino terminus but well removed from the active site. We refer to this site as the "myristoyl nest."

Comparison of the structure of myristoylated msrA with that of other myristoylated proteins revealed no similar binding sites. MsrA lacks a deep hydrophobic cavity into which the myristoyl group can be inserted reversibly, the myristoyl switch (7). These deep hydrophobic cavities are found in the HIV matrix protein which has an entropic switch (25), EF hand motif proteins which have a calcium switch (17), the Src kinase c-Abl which has a phosphotyrosine switch, and protein kinase A (14), which may have a phosphoserine switch (26). The HIV Nef protein is found both bound to membranes and in the cytosol, and its amino-terminal region is unstructured whether myristoylated or not (27).

MsrA shares some similarities with myristoylated GDP-bound ARF1. Its myristoyl group is in a shallow pocket at the protein surface, and its amino-terminal residues also have a helical propensity (16, 28, 29). However, the pocket in ARF1 is significantly more hydrophobic than the myristoyl nest in msrA. The less hydrophobic, shallow nest provides significant surface exposure of the myristoyl group in msrA. This characteristic, the lack of any known myristoyl switch, and the cytosolic location of myristoylated msrA lead us to suggest that the myristoyl nest serves as a docking site for protein-protein interaction.

Methionine sulfoxide is distinctly more hydrophilic than is methionine. Yet oxidation of a methionine residue within a protein generally renders that protein more hydrophobic (30), presumably because the sulfoxide perturbs folding and exposes hydrophobic patches that are normally buried. Those patches may dock at the myristoyl nest on msrA, after which the methionine sulfoxide can be reduced back to methionine by msrA.
Whereas the rate of reduction of methionine sulfoxide in unstructured peptides was unaffected by myristoylation, the rate of reduction in apolipoprotein A-I was faster with myristoylated msrA, providing experimental support for the proposal that the myristoyl nest structure enhances protein-protein interaction.

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