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Affinity Chromatography: A Valuable Strategy to Isolate Substrates of Methionine Sulfoxide Reductases?

Lionel Tarrago,[1–4,*] Sylvie Kieffer-Jaquinod,[5] Tiphaine Lamant,[4] Marlène Marcellin,[5] Jérôme Garin,[5] Nicolas Rouhier,[4] and Pascal Rey[1–3]

Abstract

Reactive oxygen species fulfill key roles in development and signaling, but lead at high concentration to damage in macromolecules. In proteins, methionine (Met) is particularly prone to oxidative modification and can be oxidized into Met sulfoxide (MetO). MetO reduction is catalyzed by specialized enzymes, termed methionine sulfoxide reductases (MSRs), involved in senescence and protection against diseases and environmental constraints. The precise physiological functions of MSRs remain often elusive because of very poor knowledge of their substrates. In this study, affinity chromatography was used to isolate partners of *Arabidopsis thaliana* plastidial methionine sulfoxide reductase B1 (MSRB1). Twenty-four proteins involved in photosynthesis, translation, and protection against oxidative stress, as well as in metabolism of sugars and amino acids, were identified. Statistical analysis shows that the abundance of MSRB1 partners in chromatography affinity samples is proportional to Met content. All proteins, for which structural modeling was feasible, display surface-exposed Met and are thus potentially susceptible to oxidation. Biochemical analyses demonstrated that H_2O_2 treatment actually converts several MSRB1-interacting proteins into MSRB substrates. In consequence, we propose that affinity chromatography constitutes an efficient tool to isolate physiological targets of MSRs. *Antioxid. Redox Signal.* 16, 79–84.

Introduction

Proteins undergo oxidative modifications, particularly in the methionine (Met) residue. Reactive oxygen species oxidize Met into two diastereoisomers of methionine sulfoxide (MetO). This modification is reversible by methionine sulfoxide reductases (MSRs) A and B, specific for *S*- and *R*-diastereoisomers, respectively, and present in almost all living organisms (8). Several lines of evidence argue for MSR involvement in aging and protection against oxidative stress (4,5). However, the biochemical mechanisms underlying the genetic evidence of MSR critical functions are still elusive because of poor knowledge of their substrates, that is, proteins displaying MetO reduced by MSRs. Met oxidation, which occurs in many proteins, can lead to damage and could also participate in peroxide scavenging or act as a regulatory switch. Very few proteins have been validated as MSR targets *in vivo*, except the Ffh component of *Escherichia coli* signal recognition particle (2). Only one report describes the direct trapping in cell extracts of five proteins interacting with MSRs using co-immunoprecipitation (Co-IP) in *Helicobacter pylori* (1).

Regarding higher plants, which possess numerous MSRs (8), only three potential substrates are known from *in vitro* studies: one plastidial heat shock protein and two proteins involved in targeting photosynthetic components to thylakoids (5). Consistently, an *Arabidopsis* mutant deficient for both plastidial methionine sulfoxide reductase B (MSRB) isoforms, MSRB1 and MSRB2, displays reduced growth and impaired photosynthesis under environmental constraints (5). To isolate partners of *Arabidopsis thaliana* plastidial MSRB1 in plant extracts, we developed a strategy based on affinity chromatography and mass spectrometry.

Isolation of AtMSRB1 partners

Leaf soluble proteins were applied to a column where recombinant MSRB1 was immobilized. After stringent washes, elution was performed using 500 m*M* NaCl to unbind

[1]Lab Ecophysiol Molecul Plantes, CEA, DSV, IBEB, Saint-Paul-lez-Durance, France.
[2]CNRS, UMR Biol Veget and Microbiol Environ, Saint-Paul-lez-Durance, France.
[3]Aix-Marseille Université, Saint-Paul-lez-Durance, France.
[4]Unité Mixte de Recherches 1136 INRA Nancy University, Interactions Arbre-Microorganismes, IFR 110 EFABA, Vandoeuvre Cedex, France.
[5]Laboratoire d'Etude de la dynamique des Protéomes Grenoble, CEA, DSV, iRTSV, Grenoble, France.
*Current affiliation: Division of Genetics, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts.

Innovation

Methionine sulfoxide reductases (MSR), enzymes reducing the oxidized form of methionine, methionine sulfoxide (MetO), are present in almost all organisms and have important functions in the protection against oxidative stress conditions, in aging, as well as in neurodegenerative diseases. However, the biochemical mechanisms underlying the genetic evidence of MSR critical functions are still elusive because of poor knowledge about their substrates, that is, proteins displaying MetO reduced by MSRs. Up to now, very few substrates of MSRs have been identified, almost all being discovered using targeted approaches. The authors developed a nontargeted approach based on affinity chromatography to isolate potential substrates of a plant plastidial MSR. Twenty-four proteins mainly involved in photosynthesis, translation, and protection against oxidative stress have been identified, 13 being likely relevant substrates because of their localization. Statistical, structural modeling, and biochemical analyses, coupled to literature survey, showed that many MSRB-interacting partners likely constitute physiological substrates. The authors' strategy based on affinity chromatography constitutes an innovative, easy-to-develop, valuable approach, which can be applied to all organisms, to trap substrates of MSRs and thus decipher the roles of these enzymes.

proteins tightly associated with MSRB1. A second elution using dithiothreitol (DTT) was performed to unbind proteins possibly interacting through disulfide bonds, such as MSR reductants. This approach associated with mass spectrometry allowed identifying 24 proteins (Table 1, Supplementary Table S1, and Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline.com/ars). Except raffinose synthase (RS6) and catalase 3 (CAT3), all proteins were eluted without DTT, indicating that the interaction with MSRB1 does not involve a disulfide bond.

Many of the 13 plastidial MSRB1-interacting proteins directly participate in photosynthetic processes, such as ATP synthase α- and β-subunits, large RuBisCO subunit, RuBisCO activase (RCA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and phosphoribulokinase (PRK). The other identified proteins are involved in amino acid metabolism (ferredoxin-dependent glutamate synthase and aspartate kinase/homoserine dehydrogenase), sugar metabolism (RS6 and ADP-glucose pyrophosphorylase), translation (elongation factor tu [EFtu]), and protein folding (chaperonin 60β [CPN60β]).

Among the 11 nonplastidial proteins, several share functions similar to those of plastidial partners, such as vacuolar ATP synthase , glyceraldehyde-3-phosphate dehydrogenase GAPC, elongation factor EF2, and one heat shock cognate of 70 kDa. The other MSRB1-interacting proteins are two catalases (CAT2 and CAT3), one actin (ACT2), two clathrin heavy chains, an *S*-adenosyl-L-homocysteine hydrolase 1, and a putative 26S proteasome regulatory subunit.

Very interestingly, some homologs of MSRB1 partners, such as catalase and CPN60β, have been reported as MSRB partners in *H. pylori* using a Co-IP strategy (1). Another one, *Synechocystis* GADPH, interacts with MSRA in yeast two-hybrid assays (7). The fact that related proteins are found to bind MSRs using distinct approaches in three organisms gives high credence to the validity of all these results. In other respect, among MSRB1 partners, 17 possess human homologs and 12 display conserved Met, which are sensitive to oxidation in their related proteins (3) (Supplementary Fig. S1).

We also performed Co-IP using immobilized antibodies against MSRB1 incubated with leaf extracts of wild-type or MSRB1 overexpressing plants, in parallel of two negative controls using *msrB1*$^{-}$/*msrB2*$^{-}$ double knockout plants or gel without antibodies. Only one protein, missing in negative controls, was identified by mass spectrometry. Very interestingly, this plastidial* protein, RS6, was also isolated by affinity chromatography (Table 1), strongly suggesting that this plastidial protein constitutes a physiological partner of MSRB1.

Met content in identified proteins

The Met percentage in all MSRB1 partners varies from 1.5% to 5.1% (Table 1). We calculated the Pearson correlation coefficient between Met percentage and protein abundance in elution fractions. We first normalized the number of peptides for each protein as a function of abundance in leaf extracts and of propensity to tryptic digestion. To this purpose, we used the normalized abundance factor (NAF) experimentally determined (9). This factor represents the abundance of each protein in leaves, taking into consideration the number of theoretical and relevant tryptic peptides. We estimated the relative abundance of proteins in affinity chromatography samples as the absolute value of the product of the protein NAF by the number of peptides identified by mass spectrometry (Supplementary Table S2). A positive correlation between this estimation of protein abundance in eluted fractions and the Met content was observed when calculating the Pearson correlation coefficient value (0.621; $p < 0.05$). Most interestingly, no significant correlation was observed for any of the 19 other amino acids (Supplementary Table S2). These data clearly indicate that proteins exhibiting a high Met content have been preferentially isolated and argue for a specific interaction between MSRB1 and partners through oxidized Met during the affinity chromatography procedure.

We then determined whether Met residues are surface-exposed and accessible for oxidation in MSRB1 partners by analyzing their modeled tridimensional structures. Reliable models were obtained for 16 proteins (Supplementary Table S3). All display exposed Met (Table 1 and Supplementary Fig. S1), indicating that they could indeed constitute substrates for MSRB1.

Activity of plastidial MSRBs on oxidized partners

Two plastidial proteins were selected for further characterization: RS6, found in both affinity chromatography and Co-IP, and EFtu, proposed to be an MSR substrate in *E. coli* (6). Oxidized recombinant proteins were assayed as MSR substrates. The activity was measured as the initial rate of nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidation in the presence of plastidial MSRBs, MSRB1 or MSRB2, and their specific reducing systems. Almost no activity was detected when using nonoxidized RS6 or EFtu

*(http://ppdb.tc.cornell.edu/gene.aspx?id=22888).

Table 1. Partners of Methionine Sulfoxide Reductase B1 Identified by Affinity Chromatography

Protein name	TAIR acc.	Number of peptides[a]		Number and % of Met in mature protein	
		E1 (−DTT)	E2 (+DTT)	Total (%)	Exposed (%)[b]
Plastidial					
RuBisCO activase (RCA)[c]	At2g39730	7	2	19–4.6/5.1	
Elongation factor tu (EFtu)	At4g20360	5	1	13–3.2	5–38.5
ATP synthase subunit β (ATPB)	AtCg00480	10		15–2.8	8–61.5
Ferredoxin-dependent glutamate synthase (FdGOGAT)	At5g04140	5	3	36–2.3	
Raffinose synthase 6 (RS6)[d]	At5g20250		1	17–2.2	
ATP synthase subunit α (ATPA)	AtCg00120	3	2	10–2.0	4–40.0
Phosphoribulokinase 2 (PRK2)	At1g32060	2	2	7–2.0	
Glyceraldehyde-3-phosphate dehydrogenase B (GAPB)	At1g42970	4	3	8–2.0	4–57.1
Aspartate kinase/homoserine dehydrogenase (AK-HSDH II)	At4g19710	2		16–1.8	3–18.8
ADP-glucose pyrophosphorylase (ADG2)	At5g19220	2		8–1.7	4–57.1
Glyceraldehyde-3-phosphate dehydrogenase A (GAPA)	At3g26650	5	2	6–1.7	4–80.0
RuBisCO large chain (RBCL)	AtCg00490	10	5	7–1.5	2–28.6
Chaperonin 60 β (CPN60B)	At1g55490	2		8–1.5	6–85.7
Other subcellular localization					
Elongation factor 2 (EF2)	At1g56070	8	5	38–4.5	21–55.3
Actin 2 (ACT2)	At3g18780	4	1	16–4.3	10–62.5
S-Adenosyl-L-homocysteine hydrolase 1 (SAHH1)	At4g13940	5		16–3.3	9–56.3
Vacuolar-type H^+-ATPase subunit A (VHA-A)	At1g78900	2		17–2.7	
Clathrin heavy chain putative 1 (CHC1)	At3g08530	22	2	47–2.8	
Clathrin heavy chain putative 2 (CHC2)	At3g11130	22		47–2.8	
26S proteasome regulatory subunit, putative (RPN2)	At2g32730	3		22–2.2	
Heat shock cognate 70-kDa protein 3 (HSC70-3)	At3g09440	3		14–2.2	9–81.8
Glyceraldehyde-3-phosphate dehydrogenase C (GAPC)	At3g04120	3		7–2.1	
Catalase 2 (CAT2)	At4g35090	3		10–2.0	4–40
Catalase 3 (CAT3)	At1g20620		4	8–1.6	4–57.1

[a]Mass spectrometry parameters of identified proteins are presented in Supplementary Table S1.
[b]Met was defined as exposed when sulfur atom is on the predicted molecular surface. The percentage of exposed Met was calculated in function of the number of Met present in the model. Note that Met present in N-terminal and C-terminal parts are generally not included in models (Supplementary Table S3) and thus not counted in the number of exposed Met.
[c]Two distinct forms of RCA exist in *Arabidopsis thaliana* and the identified peptides do not allow distinguishing them.
[d]RS6 was also identified by Co-IP.
TAIR acc., accession numbers from The Arabidopsis Information Resource (www.arabidopsis.org); E1, first elution without DTT; E2, second elution with 2 m*M* DTT; Met, methionine; Co-IP, co-immunoprecipitation; DTT, dithiothreitol.

(Fig. 1). In contrast, using oxidized RS6, specific activities of 1148 and 2614 nmol oxidized NADPH min^{-1} mg $MSRB^{-1}$ were measured with MSRB1 and MSRB2, respectively. Similarly, incubation of oxidized EFtu resulted in specific activities of 1882 and 5838 nmol oxidized NADPH min^{-1} mg $MSRB^{-1}$ for MSRB1 and MSRB2, respectively. These data indicate that oxidized RS6 and EFtu are substrates for MSRBs. Besides, similar assays revealed that two other partners (RCA and CPN60β) are also efficiently reduced by MSRB2 after oxidation (Supplementary Fig. S2).

Reduction of oxidized EFtu by MSRBs

After H_2O_2 treatment, EFtu migration during sodium dodecyl sulfate–polyacrylamide gel electrophoresis was shifted compared with that of the reduced form (Fig. 2). Subsequent incubation with MSRB2 partially restored the migration profile. From mass spectrometry analysis, nonoxidized EFtu exhibits a measured mass (49,097 Da) very close to the theoretical mass (49,096 Da) (Table 2). After 10 m*M* and 50 m*M* H_2O_2 treatments, increases of 140 and 207 Da, which could correspond to the addition of ~9 and ~13 oxygen atoms, were observed. When subsequently incubating the oxidized protein with MSRBs, both mass increments were reduced by ca. 50%, showing that MSRB treatment leads to the reduction of the half of MetO residues (Table 2). The remaining oxygen atoms are very likely incorporated in MetO *S*-diastereoisomers, which are reduced only by MSRA isoforms. Altogether, these data demonstrate that EFtu is efficiently reduced by plastidial MSRBs *in vitro*.

Concluding Remarks and Future Directions

Affinity chromatography allowed isolating 13 plastidial proteins constituting potential physiological substrates of *Arabidopsis* MSRB1 and 11 other proteins, which could interact with nonplastidial MSRB isoforms. Several homologs of plant MSRB1 partners are reported to interact with bacterial MSRs (1,6,7) and/or to possess Met sensible to oxidation in human cells (3), arguing for the relevance of the strategy we developed. Moreover, *in vitro* assays show that four MSRB1-interacting proteins are converted into MSRB substrates when oxidized (Fig. 1, Supplementary Fig. S2). The *A. thaliana* double mutant deficient for both MSRB1 and MSRB2 shows a delay in growth

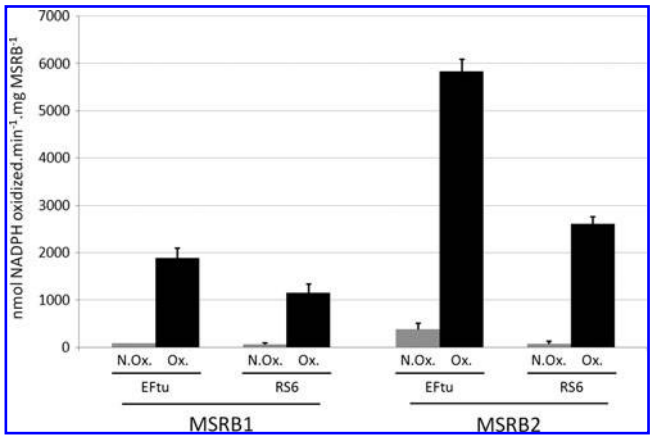

FIG. 1. Methionine sulfoxide reductase B (MSRB) activity using RS6 and EFtu as substrates. MSR activity was determined following NADPH oxidation using Grx and Trx systems as electron providers for MSRB1 and MSRB2, respectively. MSRB activity was tested using 20 μM RS6 and 20 μM EFtu, oxidized or not, as substrates. MSR was added after stabilization of NADPH oxidation rate due to the presence of the regeneration system, and basal rates were subtracted for calculation. N.Ox., nonoxidized; Ox., oxidized; NADPH, nicotinamide adenine dinucleotide phosphate reduced; RS6, raffinose synthase; EFtu, elongation factor tu.

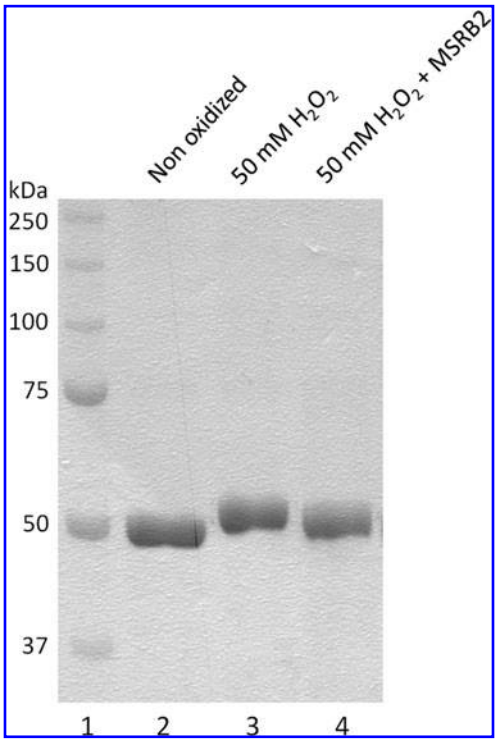

FIG. 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis mobility of oxidized and reduced EFtu. About 5 μg purified EFtu (lane 2), EFtu incubated with 50 mM H_2O_2 (lane 3), and EFtu incubated with 50 mM H_2O_2 and then with MSRB2 (lane 4) were loaded on 15% bisacrylamide (w/v) reducing gels. All samples were incubated with 5 mM dithiothreitol for 30 min to prevent cysteine oxidation.

TABLE 2. MOLECULAR MASSES (DA) OF ELONGATION FACTOR TU AFTER TREATMENTS WITH H_2O_2 AND METHIONINE SULFOXIDE REDUCTASE B

Untreated		*10 mM H_2O_2*		*10 mM H_2O_2+MSRB1 and MSRB2*		*50 mM H_2O_2*		*50 mM H_2O_2+MSRB1 and MSRB2*	
Measured 49097	Measured 49237	Increase (Nb. O atom) +140 (8.8)	Measured 49166	Increase (Nb. O atom) +69 (4.3)	Measured 49304	Increase (Nb. O atom) +207 (12.9)	Measured 49210	Increase (Nb. O atom) +113 (7.1)	

Purified recombinant EFtu was oxidized as described in the Supplementary Data. After removing excess H_2O_2, incubation with MSRB1 and MSRB2 was carried out in the presence of DTT, and as a control, DTT was added in untreated and oxidized EFtu samples. The recombinant EFtu protein possesses 14 Met.
MSRB, methionine sulfoxide reductase B.

during environmental constraints and a decreased photosynthetic capacity (5). The identity of the potential MSRB1 partners identified here is in full agreement with this phenotype, as many of them (ATPase subunits, RuBisCO, RCA, PRK, and GAPDH) participate in photosynthetic processes. We thus propose that the main function of plant plastidial MSRBs is related to the protection of the photosynthetic apparatus from oxidative damage. Further investigation is needed to get a complete overview of the nature and function of MSR partners in other kingdoms and also to compare whether MSRA and MSRB enzymes share similar substrates. The strategy described here will constitute a valuable tool to this aim, because it allows the direct trapping of MSR partners in cell extracts.

Notes

Complementary information is proposed in Supplementary Data.

Plant material

A. thaliana (Columbia-0) plants were grown as previously described (5). Leaves from 5-week old plants were crushed in liquid nitrogen and the powder was suspended in 25 m*M* Tris(hydroxymethyl)aminomethane (Tris), pH 7.5, 100 m*M* NaCl or 50 m*M* Tris-HCl, pH 8.0, or 1 m*M* phenylmethylsulfonylfluoride for affinity chromatography or Co-IP experiments, respectively. After agitation (15 min) and centrifugation (14,000 *g*; 15 min; 4°C), the supernatant containing soluble proteins was collected. Protein concentration was determined using the BC Assay Reagent (Interchim).

Affinity chromatography

Ten milligrams of recombinant MSRB1 was coupled to the Hi-Trap NHS-activated HP 1-ml affinity column (GE Healthcare). Leaf proteins (175 mg at 2.75 mg.ml^{-1}) were applied by injection at a flow rate of 0.5 ml min^{-1}. The column was successively washed with 25 m*M* Tris (pH 7.5) buffers containing 100 and 350 m*M* NaCl (10 ml) at 0.5 ml min^{-1}. Elution was performed in 500 m*M* NaCl (10 ml) and then with the same buffer supplemented with 2 m*M* DTT. Washing and elution fractions were 10-fold concentrated using trichloroacetic acid precipitation.

Eluted proteins were identified by mass spectrometry (see Supplementary Data for details).

Co-immunoprecipitation

Co-IP was carried out using the Profound™ Co-Immunoprecipitation Kit (Thermo Fischer Scientific). About 0.4 mg purified antibodies was immobilized on AminoLink® Plus Coupling Gel (0.1 ml gel slurry). Leaf soluble proteins (2.5 mg in 300 μl) were incubated with immobilized antibodies for 2 h at room temperature to create antibody:MSRB1:partner complexes. Five washing and five elution steps were performed in modified phosphate-buffered saline and an acidic buffer, respectively. Eluted proteins were identified by mass spectrometry (see Supplementary Data text for details).

Protein oxidation and MSR activity assay

RS6 and EFtu (5–10 mg) were oxidized using H_2O_2 (10–50 m*M*) for 5 h at 25°C in 30 m*M* Tris-HCl (pH 8.0). Excess H_2O_2 was removed by desalting using an NAP-5 Sephadex G-25 column (GE Healthcare). MSR activity was measured using 20 μM protein and 5 μM MSR, following NADPH oxidation at 340 nm in the presence of a Grx reducing system for MSRB1 or of a Trx reducing system for MSRB2 as previously described (5). The reactions were carried out at 25°C in 250 μl of 30 m*M* Tris-HCl (pH 8.0) and the kinetics were recorded using a Cary 50 spectrophotometer (Varian).

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Address correspondence to:
Dr. Pascal Rey
Lab Ecophysiol Molecul Plantes
CEA, DSV, IBEB
Saint-Paul-lez-Durance 13108
France

E-mail: pascal.rey@cea.fr

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Abbreviations Used

ADG2 = ADP-glucose pyrophosphorylase
AK-HSDH II = aspartate kinase/homoserine dehydrogenase
CAT3 = catalase 3
CHC = clathrin heavy chains
Co-IP = Co-immunoprecipitation
CPN60β = chaperonin 60β
DTT = dithiothreitol
EFtu = elongation factor tu
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
HSC70-3 = heat shock cognate 70-kDa protein 3
Met = methionine
MetO = methionine sulfoxide
MSR = methionine sulfoxide reductase
MSRB = methionine sulfoxide reductase B
NADPH = nicotinamide adenine dinucleotide phosphate reduced
NAF = normalized abundance factor
PRK = phosphoribulokinase
RBCL = RuBisCO large chain
RCA = RuBisCO activase
RPN2 = putative 26S proteasome regulatory subunit
RS6 = raffinose synthase
Tris = Tris(hydroxymethyl)aminomethane
SAHH1 = *S*-adenosyl-L-homocysteine hydrolase 1

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