The *Rhodobacter sphaeroides* methionine sulfoxide reductase MsrP can reduce *R*- and *S*-diastereomers of methionine sulfoxide from a broad-spectrum of protein substrates.

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Running title: *Broad spectrum substrates for the non-stereospecific RsMsrP*

Abstract

Methionine (Met) is prone to oxidation and can be converted to Met sulfoxide (MetO), which exists as *R*- and *S*-diastereomers. MetO can be reduced back to Met by the ubiquitous methionine sulfoxide reductase (Msr) enzymes. Canonical MsrA and MsrB were shown as absolutely stereospecific for the reduction of *S*- and *R*-diastereomer, respectively. Recently, the molybdenum-containing protein MsrP from *Escherichia coli* was shown to be able to reduce MetO of periplasmic proteins without apparent stereospecificity. Here, we describe the substrate specificity of the *Rhodobacter sphaeroides* MsrP. Proteomics analysis coupled to enzymology approaches indicate that it reduces a broad spectrum of periplasmic oxidized proteins. Moreover, using model proteins, we demonstrated RsMsrP preferentially reduces unfolded oxidized proteins and we confirmed that this enzyme, like its *E. coli* homolog, can reduce both *R*- and *S*-diastereomers of MetO with similar efficiency.

Introduction

Aerobic life exposes organisms to reactive oxygen species (ROS) derived from molecular oxygen, such as hydrogen peroxide (H_2O_2) or singlet oxygen ($^1O_2$). Bioenergetics chains are important sources of intracellular ROS, with H_2O_2 principally produced during respiration (Messner and Imlay, 1999) and $^1O_2$ arising from photosynthesis (Glaeser et al., 2011). These oxidative molecules act as signaling messengers playing major roles in numerous physiological and pathological states in most organisms and their production and elimination are tightly regulated (Ezraty et al., 2017; Schieber and Chandel, 2014). However, numerous stresses can affect ROS homeostasis and increase their intracellular concentration up to excessive values leading to uncontrolled reaction with sensitive macromolecules (Imlay, 2013). For instance, photosynthetic organisms, such as plants or the purple bacteria *Rhodobacter sphaeroides* can experience photo-oxidative stress in which unbalance between incident photons and electron transfer in photosynthesis generates detrimental accumulation of $^1O_2$ due to energy transfer from excited pigments, such as bacteriochlorophyll, to O_2 (Ziegelhoffer and Donohue, 2009). Moreover, production of ROS could be used advantageously by several organisms in a defensive strategy against potential pathogenic invaders. For instance, mammalian phagocytic cells, such as macrophages and neutrophils, produce the strong oxidant hypochlorite (ClO^-) from hydrogen peroxide and chlorine ions to eliminate bacteria and fungi (Ezraty et al., 2017; Winterbourn and Kettle, 2013). Because of their abundance in cells, proteins are the main targets of oxidation (Davies, 2005). Methionine (Met), one of the two sulfur containing amino acid with cysteine, is particularly prone to oxidation and the reaction of Met with oxidant leads to the formation of Met sulfoxide (MetO), which exists as two diastereomers *R* (Met-*R*-O) and *S* (Met-*S*-O) (Sharov et al., 1999). Further oxidation can form Met sulfone ($MetO_2$) *in vitro*, but evidences of its existence *in vivo* are very scarce (Vogt, 1995). Contrary to most oxidative modifications on

amino acids, the formation of MetO is reversible, and oxidized proteins can be repaired thanks to methionine sulfoxide reductases (MSR) enzymes, which exist principally in two types, MsrA and MsrB. These enzymes, present in almost all organisms, did not evolve from a common ancestor gene and possess an absolute stereospecificity toward their substrates. Indeed, MsrA can reduce only Met-*S*-O (Ejiri et al., 1979; Lowther et al., 2002; Moskovitz et al., 2002; Sharov et al., 1999; Vieira Dos Santos et al., 2005) whereas MsrB acts only on Met-*R*-O (Grimaud et al., 2001; Kumar et al., 2002; Lowther et al., 2002; Moskovitz et al., 2002; Vieira Dos Santos et al., 2005). This strict stereospecificity of both enzymes was enzymatically demonstrated using Met-*R*-O and Met-*S*-O chemically prepared from racemic mixtures of free MetO or using HPLC methods allowing discrimination of both diastereomers, and was structurally explained by deciphering the mirrored pictures of their active site, in which only one MetO diastereomer can be accommodated (Lowther et al., 2002).

These canonical MsrA and MsrB are thiol oxidoreductases using the reducing power coming from NADPH, generally through thioredoxin or glutaredoxin systems, to reduce their substrates (Tarrago et al., 2009; Tarrago and Gladyshev, 2012). While MsrA can reduce Met-*S*-O, whether as a free amino acid or included in proteins, MsrB is specialized in the reduction of protein-bound Met-*R*-O, and both are more efficient on unfolded oxidized proteins (Tarrago et al., 2012; Tarrago and Gladyshev, 2012).

Eukaryotic cells address MSRs in each subcellular compartment (using several genes or alternative splicing) to protect proteins form oxidation (Kim, 2013). In bacteria, MsrA and MsrB are generally located in the cytoplasm (Ezraty et al., 2017), except for *Neisseria* or *Streptococcus* species, for which MsrA and MsrB enzymes can be addressed to the envelope (Saleh et al., 2013; Skaar et al., 2002). Because of their catalytic activity, they are important actors in oxidative stress protection, aging and neurodegenerative diseases in animals (Kim,

2013), and during environmental stresses (Laugier et al., 2010) or seed longevity in plants (Châtelain et al., 2013). They play a role in the protection against oxidative stress and as virulence factors in bacteria (Ezraty et al., 2017).

Beside these stereotypical MSRs found in all kind of organisms, several other enzymes can catalyze MetO reduction, principally in bacteria. For instance, numerous bacteria as well as unicellular eukaryotes, such as *Saccharomyces cerevisiae*, possess another type of MSR, called free-*R*-MSR (fRMSR) or MsrC, specialized in the reduction of the free form of Met-*R*-O, which is also absolutely stereospecific (Le et al., 2009; Lin et al., 2007). The lack of such enzyme renders mammals unable to reduce free Met-*R*-O (Lee et al., 2008). As MsrA and MsrB, fRMSR uses thiol-based chemistry and the reducing power coming from the NADPH to reduce its substrate (Le et al., 2009; Lin et al., 2007).

In bacteria, several molybdenum cofactor containing enzymes were also shown to be able to reduce oxidized Met. Particularly, the biotin sulfoxide reductase BisC, or its homolog TorZ/BisZ, were shown to specifically reduce the free form of Met-*S*-O, in *Escherichia coli* cytoplasm and *Haemophilus influenza* periplasm, respectively (Dhouib et al., 2016; Ezraty et al., 2005). Moreover, the DMSO reductase of *E. coli* can reduce a broad spectrum of substrates, among which MetO (Weiner et al., 1988), and the *R. sphaeroides* homolog was shown as absolutely stereospecific towards *S*-enantiomer of several alkyl aryl sulfoxides (Abo et al., 1995). Finally, another molybdoenzyme, MsrP (formerly known as YedY) was recently identified as a key player of MetO reduction in the bacterial periplasm (Gennaris et al., 2015; Melnyk et al., 2015). MsrP was shown to be induced in gram negative bacteria by exposure to the strong oxidant hypochlorite (ClO^-). The corresponding cistron, *msrP*, belongs to an operon together with the cistron encoding the transmembrane protein MsrQ, which is responsible for electron transfer from the respiratory chain to the molybdoreductase (Gennaris et al., 2015). Alternatively, the cytosolic flavin reductase Fre was proposed as potential carrier of electrons

to MsrQ (Juillan-Binard et al., 2016). MsrP was shown to reduce MetO on several abundant periplasmic proteins in *E. coli* (Gennaris et al., 2015) or on a Met-rich protein, apparently dedicated to scavenge oxidant in the periplasm of *Azospira suillum* (Melnyk et al., 2015). A most striking feature of the *E. coli* MsrP (EcMsrP) is that, contrary to all known methionine sulfoxide reductases, it seems capable of reducing both Met-*R*-O and Met-*S*-O (Gennaris et al., 2015). The *msrPQ* operon is conserved in the genome of most gram-negative bacteria, suggesting that the MsrP/Q system is very likely a key player for the general protection of the bacterial envelop against deleterious protein oxidation (Gennaris et al., 2015; Melnyk et al., 2015). *R. sphaeroides* MsrP (RsMsrP) shares 50% identical amino acids residues with EcMsrP and transcriptomic analyses evidenced that *RsmsrP* is strongly induced under high-light conditions, suggesting a putative role in protecting the periplasm against singlet oxygen generated by photo-oxidative stresses in photosynthetic bacteria (Glaeser et al., 2007).

In this paper, we describe the biochemical characterization of MsrP from the photosynthetic bacterium *R. sphaeroides* regarding its specificity of substrate. Using kinetics activity experiments and mass spectrometry analysis, we show that RsMsrP is a very efficient protein-bound MetO reductase. Proteomics analysis indicate that it can reduce a broad spectrum of proteins in *R. sphaeroides* periplasm, and that Met sensitive to oxidation and efficiently reduced by RsMsrP are found in clusters. Moreover, using model proteins, we demonstrate that RsMsrP can reduce both Met-*R*-O and Met-*S*-O and preferentially reduces unfolded oxidized proteins.

Results

The R. sphaeroides *MsrP is an efficient protein-MetO reductase.*

Previous characterization of the RsMsrP (formerly known as YedY) demonstrated its abitity to reduce dimethylsulfoxide (DMSO), trimethylamine oxide (TMAO) and the free form of MetO (Loschi et al., 2004; Sabaty et al., 2013). However, the low apparent affinity (high K_M values) determined for these molecules and the homology with the EcMsrP prompted us to test its ability to reduce MetO-containing proteins and peptides, as potential physiological substrates. RsMsrP was first expressed and purified as described in (Sabaty et al., 2013) and the DMSO reductase activity was characterized in buffers of different pH. This experiment indicated that RsMsrP efficiency was optimal at pH 5.0 (Figure S1). Subsequent activity assays were performed at pH 6.0 to avoid denaturation of the oxidized proteins used as substrates. We next chose the free amino acid MetO, a synthetic tripeptide Ser-MetO-Ser and the bovine β-casein as model substrates. The β-casein contains six Met, it is intrinsically disordered, and was shown as efficient substrate for the yeast MsrA and MsrB, after oxidation (Tarrago et al., 2012, see also figure S5). The β-casein was oxidized with H_2O_2 and mass spectrometry analysis confirmed an increase in mass of 96 Da for each peak, very likely corresponding to the addition of 6 oxygen atoms on the Met residues (Figure 1). The numerous peaks observed, even on the untreated β-casein, are due to the fact that 13 genetic variants exist and that the commercial β-casein is purified from milk which contains a mixture of these variants.

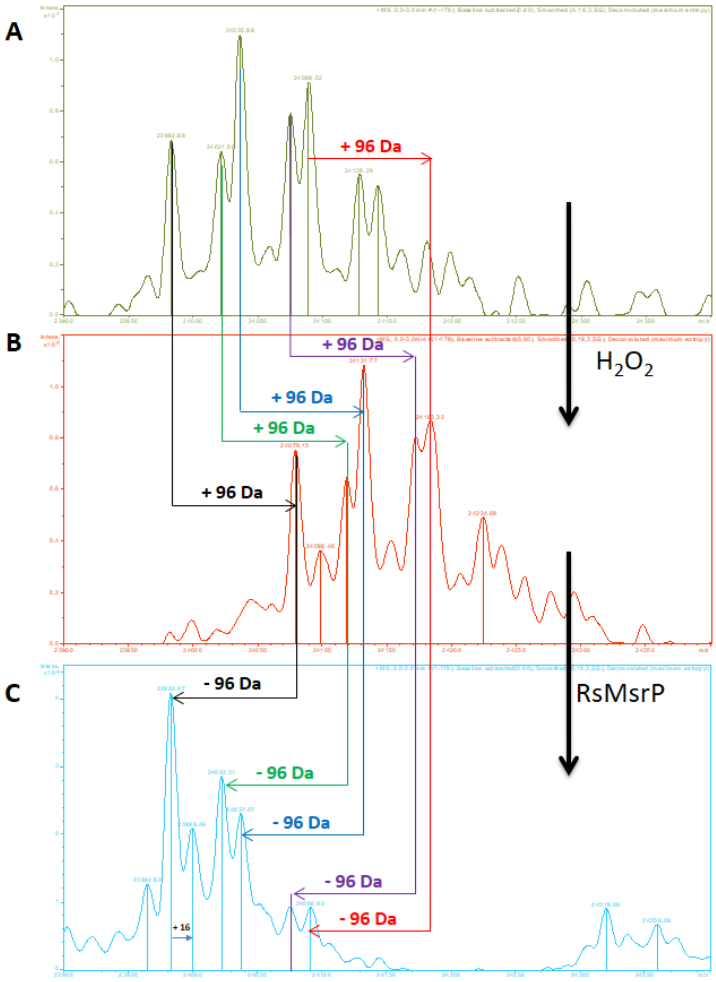

Figure 1. Mass spectrometry spectrum of β-casein non-oxidized (A), oxidized with H_2O_2 (B) and repaired by RsMsrP (C). A) Commercial β-casein exists as mixture of genetic variants (7 in our batch). β-casein was analyzed by ESI-MS. B) β-casein was oxidized with 50 mM H_2O_2 before MS analysis. All major peaks undergone an increase of 96 Da compared to the non-oxidized sample. C) Oxidized β-casein was incubated with RsMsrP (440 nM) in presence of benzyl viologen (0.8 mM) and sodium dithionite (2 mM) as electron donors. All major peaks had masses corresponding of the non-oxidized β-casein, showing the ability to reduce all MetO in this protein. Note the presence of a peak with an increase of 16 Da compared to the main reduced peak, indicating an incomplete reduction of the total protein pool.

Using benzyl viologen as electron donor and following its oxidation spectrophotometrically in activity assays, we determined the catalytic parameters of the RsMsrP with these substrates (Table 1). Using the free oxidized amino acid, we measured a k_{cat} value of ~ 122 s^{-1} and a K_M of ~ 115,000 µM, yielding a catalytic efficiency (k_{cat}/K_M) of ~ 1,000 $M^{-1}.s^{-1}$. With the Ser-MetO-Ser tripeptide, the k_{cat} and the K_M values were ~ 108 s^{-1} and ~ 13,000 µM, and thus the catalytic efficiency (k_{cat}/K_M) was ~ 8,300 $M^{-1}.s^{-1}$. Compared to the free amino acid, the ~ 8-fold increase in catalytic efficiency is explained by the lower value of K_M, and thus this indicates that the involvement of the MetO in peptide bonds increases its ability to be reduced by the RsMsrP. With the oxidized β-casein, the k_{cat} and the K_M were ~ 100 s^{-1} and ~ 90 µM, respectively. The catalytic efficiency was thus ~ 1,000,000 $M^{-1}.s^{-1}$. This value was 4 orders of magnitude higher than the one determined with the free amino acid, indicating that the oxidized protein was a far better substrate for the RsMsrP as evidenced by the striking difference in K_M values. Moreover, even assuming that all MetO in the oxidized β-casein were equal substrates for the RsMsrP and thus multiplying the K_M value by 6, the catalytic efficiency obtained (~ 175,000 $M^{-1}.s^{-1}$) remained ~ 175-fold higher for the oxidized protein than for the free amino acid. These results indicated that the RsMsrP acts effectively as a protein-MetO reductase, as demonstrated for its homologs in *E. coli* (Gennaris et al., 2015) and *A. suillum* (Melnyk et al., 2015).

RsMsrP can reduce a broad spectrum of periplasmic proteins.

To identify potential periplasmic substrates of RsMsrP and gain insight into its substrate specificity, we applied a high-throughput shotgun proteomic strategy taking advantage of the latest generation of tandem mass spectrometer incorporating an ultra-high-field Orbitrap analyzer. Periplasmic proteins from *msrP*$^-$ *R. sphaeroides* mutant were

188 extracted, oxidized with NaOCl, then reduced *in vitro* with the recombinant RsMsrP in the
189 presence of reduced benzyl viologen as electron donor. Untreated periplasmic proteins,
190 oxidized periplasmic proteins and RsMsrP-treated oxidized periplasmic proteins were
191 analyzed by semi-quantitative nanoLC–MS/MS. All experiments were done systematically
192 for 3 biological replicates and resulted in the identification of a total of 362,700 peptide-to-
193 spectrum matches. From all the 11,320 individual peptide sequences, we identified a total of
194 2,553 unique Met belonging to 720 proteins. The overall percentage of Met oxidation were ~
195 35%, ~ 71% and ~ 40% for the periplasm extract, the periplasm oxidized with NaOCl and the
196 RsMSRP-repaired periplasmic proteins, respectively (Table S1). This first result indicates that
197 the RsMsrP is very likely able to reduce the methionine sulfoxides of numerous proteins and
198 to restore an oxidation rate similar to that of the periplasmic extract that has not undergone
199 any oxidation.

200 The identification of preferential RsMsrP substrates requires the precise comparison of
201 the oxidation state of Met residues from periplasmic proteins before and after the action of the
202 enzyme. After tryptic digestion, since most of the Met/MetO-containing peptides were found
203 in low abundance (*i.e.* with very low spectral counts), we focused on the proteins robustly
204 detected in all samples. We selected the Met-containing peptides for which at least 10 spectral
205 counts were detected in two replicates for each condition (*i.e.* untreated periplasm, oxidized
206 periplasm and repaired oxidized periplasm) and at least 7 spectral counts were found in the
207 third replicate. This restricted the dataset to 202 unique Met belonging to 70 proteins (Table
208 S2). Overall percentage of Met oxidation (calculated as the number of spectral counts for a
209 MetO-containing peptide vs. the total number of spectral count for this peptide) varied from
210 2% to 87%, from 9% to 100% and from 4% to 91% in periplasm, oxidized periplasm and
211 repaired oxidized periplasm, respectively. Comparison of Met-O containing peptides between
212 oxidized and RsMsrP treated samples indicates that the percentage of reduction varied from

100 % to no reduction at all. Eleven MetO were not reduced and 22 were reduced at more than 75 % (only 2 at 100 %). The percentage of reduction for the remaining majority of MetO was almost distributed uniformly between inefficient (less than 25 %) to efficient (75% or more) reduction (Figure 2A).

Among these 70 identified proteins, thirty-four are annotated as related to transport of amino acids/peptides, (oligo)nucleotides or other solutes, such as the α-keto acids transporter TakP. This high proportion is likely related to the abundance of these proteins in the periplasm. Five are potentially implicated in electron transport chain, among which the DMSO reductase and both cytochromes c2 and c'. No clear evidence of sequence or structure characteristic arose from this list of proteins, neither in term of size or in Met content (Table S2). The periplasmic chaperone SurA, the peptidyl-prolyl cis-trans isomerase PpiA, the thiol-disulfide interchange protein DsbA, the spermidine/putrescine-binding periplasmic protein PotD and the ProX protein were previously proposed as potential substrates of the EcMsrP (Gennaris et al., 2015). All these proteins contain at least one MetO among the most efficiently reduced by the RsMsrP (Table S2), indicating that they are potential conserved substrates of MsrP enzymes in *E. coli* and *R. sphaeroides*, and very likely in numerous gram-negative bacteria.

The sensibility to oxidation of the Met belonging to these 70 proteins, and their efficiency of reduction by the RsMsrP show a wide range of variation, from Met highly sensitive to oxidation and efficiently reduced to Met barely sensitive to NaOCl treatment and not reduced by RsMsrP (Table S2). Moreover, this diversity could be visible within a single protein, in which all Met may not be oxidized and reduced uniformly. For instance, the ABC transporter DdpA (YP_353887.1), along with another putative ABC transporter (YP_354805.1) (Figure 2B and C), contained one of the two only MetO found as fully reduced in the dataset (Met 230 and Met353, respectively), although DdpA also contained the

Met 243 that was neither efficiently oxidized or reduced. This is also illustrated by the case of the peptidyl-prolyl cis-trans isomerase (YP_352551.1), which possessed the Met which was found to have the higher decrease in oxidation in all the dataset (Met 172) but also a Met which was almost not reduced by the RsMsrP (Met 190) (Figure 2D). The Met 539 of the PQQ dehydrogenase XoxF (YP_352636.1) illustrates the case in which a Met was highly sensitive to NaOCl-oxidation and very efficiently reduced (Figure 2E). Twenty-one Met were oxidized at 50 % or more and reduced by 50 % or more by RsMsrP (Table S2). Altogether, these results show that RsMsrP can reduce a broad spectrum of apparently unrelated proteins (only 11 Met among 202 were not reduced). However, since all MetO were not reduced with similar efficiency, some structural or sequence determinants could drive the ability of MetO to be reduced by the RsMsrP, similarly to demonstrated for canonical MsrA and MsrB (Ghesquière et al., 2011; Tarrago et al., 2012).

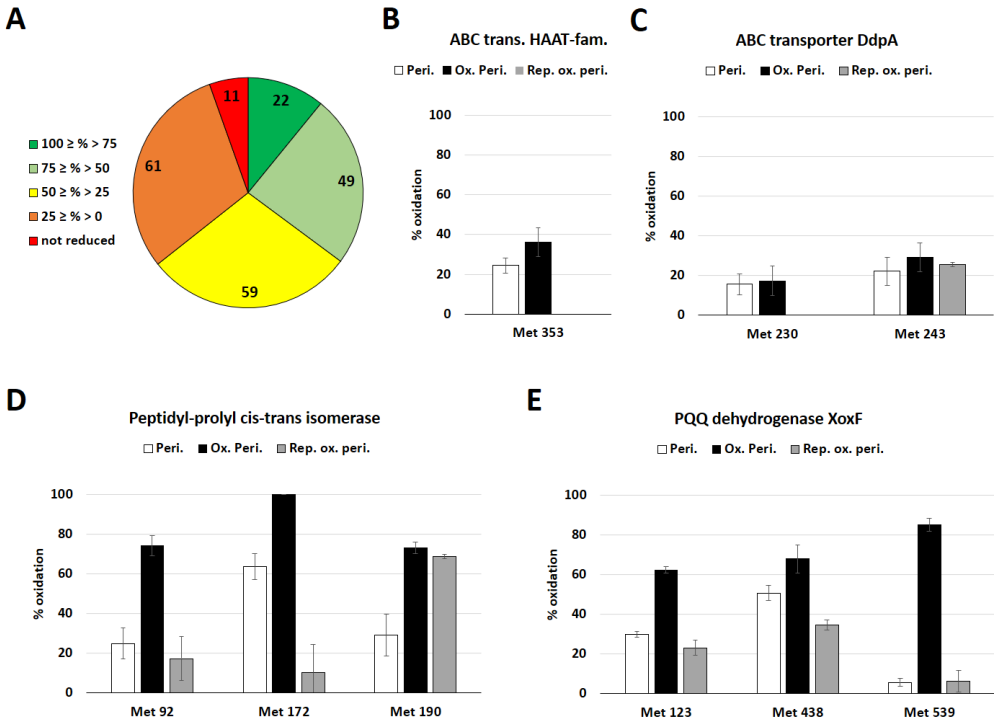

Figure 2. Characteristics of MetO reduction sites and oxidation state of Met in representative proteins. A) Repartition of the number of MetO per percentage of reduction. B) Percentage of oxidation of Met 353 of putative ABC transporter from HAAT family (Accession: YP_354805.1) in the 3 analyzed samples. C) Percentage of oxidation of Met 230 and 243 of ABC transporter DdpA (Accession: YP_353887.1). D) Percentage of oxidation of Met 92, 172 and 190 in the peptidyl-prolyl cis-trans isomerase (Accession: YP_352551.1). E) Percentage of oxidation of Met 123, 438 and 539 of the pyrroloquinoline quinone (PQQ) dehydrogenase XoxF (Accession: YP_352636.1).

The nature of the amino acids surrounding a MetO influences the efficiency of its reduction by RsMsrP.

Having in hands a relatively large dataset of oxidized and reduced Met prompted us to search for consensus sequences that could favor or impair either the oxidation of a Met or the reduction of a MetO by the RsMsrP. We extracted for all identified Met, the surrounding 5 amino acids on the N- and C-terminal sides to obtain an 11-amino acid sequence with the considered Met centered at the sixth position. We then performed a IceLogo (Colaert et al.,

2009) analysis aiming to identify whether some amino acid residues were enriched or depleted around the target Met. The principle is to compare a 'positive' dataset of peptides, to a 'negative' one. To find potential consensus sequence of oxidation, we first compared all unique MetO-containing peptides from both the untreated and the NaOCl-oxidized periplasmic extracts, our positive dataset, to the theoretical *R. sphaeroides* proteome used as negative dataset. The IceLogo presented in Figure 3A shows that MetO-containing sequences were mainly depleted of His and aromatic or hydrophobic residues (Trp, Phe, Tyr, Leu, Ile) and were mainly enriched of polar or charged amino acids (Asn, Gln, Asp, Glu and Lys). This suggests that Met in a polar environment, as commonly found at the surface of proteins, are very likely more susceptible to oxidation than those located in hydrophobic environments as in the core of a protein. This corroborated the results found in H_2O_2-oxidized human Jurkat cells (Ghesquière et al., 2011), or with model proteins (Tarrago et al., 2012). We then compared all these unique MetO-containing peptides to all the Met-containing peptides from the same samples (Figure 3B), and we observed that principally Trp, along with His, Tyr and Cys, were depleted around the potentially oxidized Met. Strikingly, the only amino acid significantly more abundant around an oxidized Met was another Met in position -2 and +2. These results indicate that oxidation sensitive Met might be found as clusters, and echo the results from a very recent analysis in epidermoid carcinoma A431 cells showing that Met sensitive to oxidation were surrounded by several other Met residues (Hsieh et al., 2017).

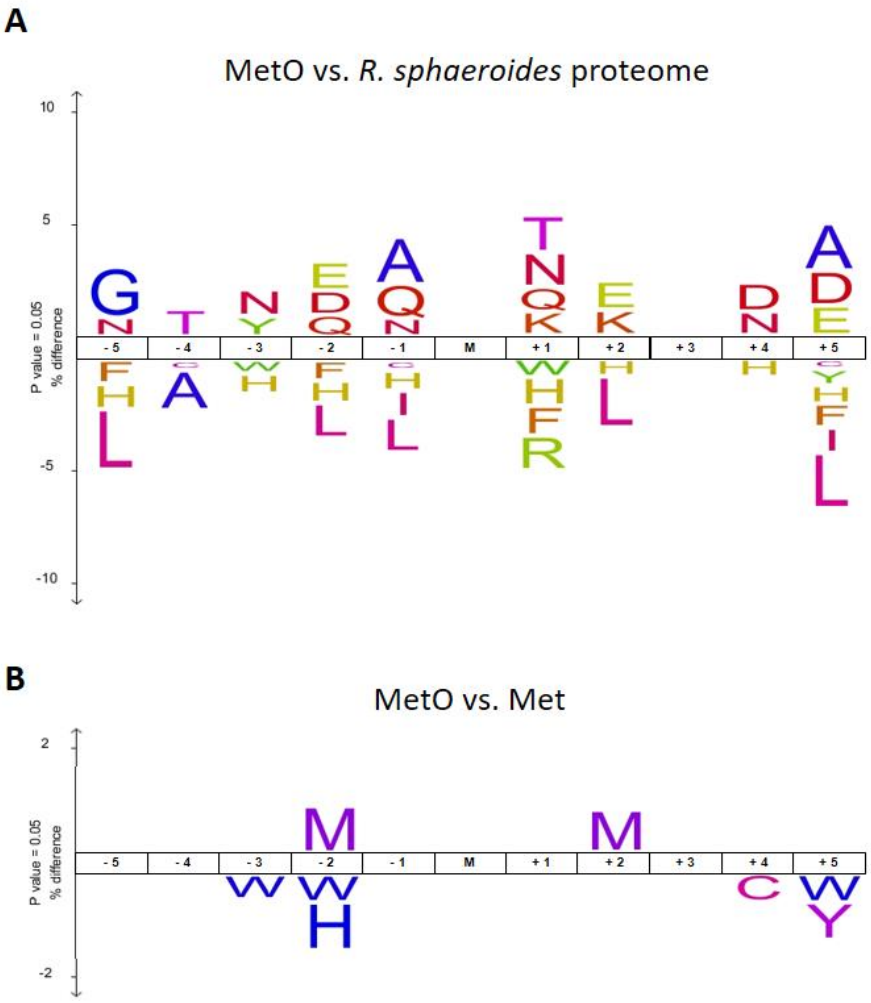

Figure 3. IceLogo representation of enriched and depleted amino acids around site of Met oxidation. A) Enrichment and depletion of amino acids around the oxidized Met (M) found in periplasmic extracts and oxidized periplasmic extracts by comparison with the theoretical proteome of *R. sphaeroides*. B) The same oxidized peptides were analyzed using the peptides containing a non-oxidized Met from the same samples (periplasm and oxidized periplasm extracts). Analysis were performed using the IceLogo server (http://iomics.ugent.be/icelogoserver/index.html) (Colaert et al., 2009).

In order to identify potential consensus sequence favorable to the reduction of a MetO by the RsMsrP, we performed a precise comparison of the percentage of oxidation before and after the action of the enzyme. We thus defined 2 criteria to characterize the reduction state of each Met: i) the percentage of reduction calculated using the formula described in Table S2 and based on the comparison of the percentages of oxidation in oxidized and reduced oxidized periplasm. For instance, a Met found oxidized at 25 % in the oxidized periplasm and at 5 % in the repaired oxidized periplasm was considered enzymatically reduced at 80 %. ii) the decrease in percentage of oxidation by comparison of the 2 samples. For instance, the same Met found oxidized at 25 % in the oxidized periplasm and 5 % in the repaired extract had a decrease in the percentage of oxidation of 20 %. This second criterion was used to avoid bias in which very little oxidized Met were considered as efficient substrate (*i.e.* a Met oxidized at 5 % in the oxidized periplasm extract and at 1 % in the repaired oxidized periplasm was reduced at 80 %, similarly to one passing from 100 % to 20 %, which intuitively appeared as a best substrate than the previous one). We selected as efficiently and inefficiently reduced MetO those for which both criterions were higher than 50 % and lower than 10 %, respectively. The comparison of the surrounding sequences of the efficiently reduced MetO to the theoretical proteome of *R. sphaeroides* showed no depletion of amino acid, but mainly enrichment of polar amino acids (Gln, Lys, and Glu) around the oxidized Met (Figure 4A). Similar analysis with the inefficiently reduced MetO indicated the enrichment of Thr and Ser in the far N-terminal positions (-5 and -4) and of a Tyr in position -2 (Figure 4B). The C-terminal positions (+ 1 to +5) were mainly enriched in charged amino acids (Gln, Lys, and Glu), similarly to efficiently reduced MetO. This apparent contradiction may indicate that the amino acids in C-terminal position of the considered MetO did not really influence the efficiency of RsMsrP, but were observed simply because of the inherent composition of the overall identified peptides. We then compared the variation of amino acids composition of the

MetO-containing peptides between both datasets, using the inefficiently reduced MetO as negative dataset (Figure 4C). The results resembled those obtained by comparison with the entire theoretical proteome of the bacterium, *i.e*. most enriched amino acids were polar (Glu, Gln, Asp and Lys) at most extreme positions (-5, -4 and + 2 to + 5). Of note, the conserved presence of a Gly in position – 1, and the presence of several other Met around the central Met. This potential enrichment of Met around an oxidation site is consistent with the result found for the sensibility of oxidation (Figure 3B), and indicates that potential clusters of oxidized Met could be preferred substrates for RsMsrP. We found 16 peptides containing 2 or 3 MetO, reduced at more than 25 % by RsMsrP (Table S2). This was illustrated, for example, by the cell division coordinator CpoB (YP_353742.1) which possesses two close Met residues (66 and 69) highly reduced by the RsMsrP, or by the uncharacterized protein (YP_353998.1) which possesses 4 clusters of MetO reduced by the RsMsrP (Table S2).

From this analysis, the only depleted amino acids appeared to be Thr and Pro in positions -4 and -3 (Figure 4C). To our knowledge, the presence of a Thr close to a MetO was not previously shown to influence the activity of any MSR enzyme, but the presence of a Pro was shown to decrease or totally inhibit the reduction of a MetO by the human MsrA and MsrB3, depending on its position (Ghesquière et al., 2011).

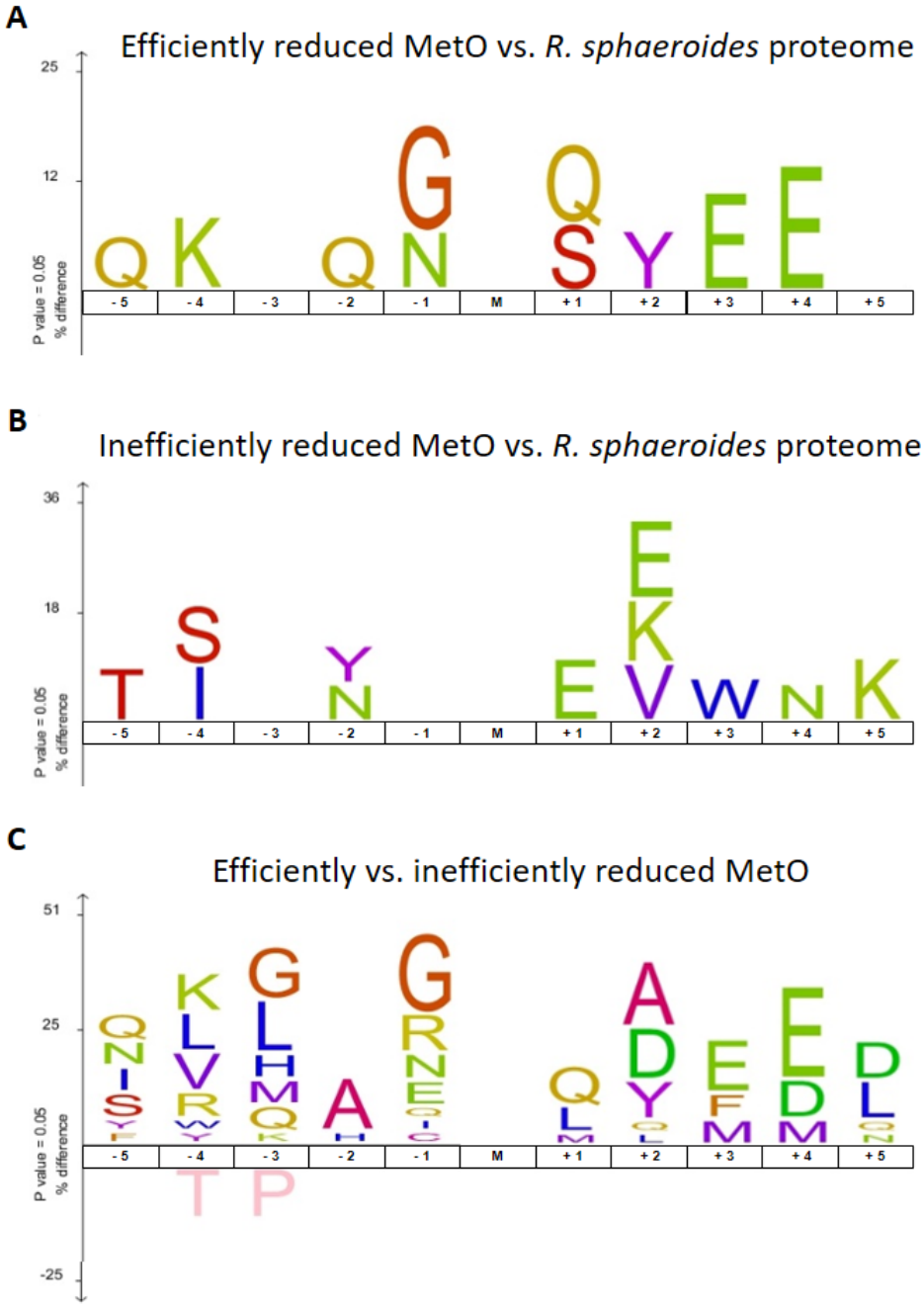

Figure 4. IceLogo representation of enriched and depleted amino acids around site of MetO reduction by RsMsrP. A) Enrichment of amino acids in peptides centered on the MetO for which the percentage of reduction and the decrease in percentage of oxidation were both superior to 50 % by comparison with the theoretical proteome of *R. sphaeroides*. B) Enrichment of amino acids from peptides centered on the MetO for which the percentage of reduction and the decrease in percentage were inferior to 10 % by comparison with the theoretical proteome of *R. sphaeroides*. C) Enrichment and depletion of amino acids from efficiently reduced MetO-containing peptides (dataset used in A)) by comparison with inefficiently reduced MetO-containing peptides (dataset used in B)). Analysis were performed using the IceLogo server (http://iomics.ugent.be/icelogoserver/index.html) (Colaert et al., 2009).

The RsMsrP preferentially reduces unfolded oxidized proteins.

Previous biochemical characterization of MsrA and MsrB showed that these thiol-oxidoreductases preferentially reduce unfolded oxidized proteins. When treated with the chaotropic agent urea, the tested oxidized proteins were more efficiently reduced by the MSRs (Tarrago et al., 2012). In the case of MsrB, this was due to a better access to the MetO included in the hydrophobic core of the proteins, but for MsrA, this increased activity using unfolded protein was independent of the number of MetO that could be reduced (Tarrago et al., 2012). To test whether RsMsrP preferentially reduces unfolded oxidized protein or acts with equal efficiency whatever the folding state of the protein, we used the lysozyme as model protein. Beside the fact that it is available commercially at gram-scale, and thus easily prepared at high concentration, it is also a very well folded protein highly stabilized with four disulfide bonds (Ray et al., 2001). Moreover, the lysozyme was used for the preparation of the periplasmic proteins and was found to have its two Met oxidized in our proteomic analysis (Table S2). We oxidized the lysozyme with H_2O_2 and checked its oxidation state by mass spectrometry (Figure S2). Surprisingly, using a protocol similar to the one allowing the complete oxidation of the 6 Met of β-casein, we observed only a weak and incomplete oxidation of the protein. The major peak corresponded to the non-oxidized form and a small fraction had an increase of mass of 16 Da, likely corresponding to the oxidation of one Met. This indicates that the burying of the Met in the core of the protein protected them from oxidation, similarly to observed for model proteins (Tarrago et al., 2012). Nevertheless, we prepared from this oxidized sample, an unfolded oxidized lysozyme by reduction with dithiothreitol in 4M urea followed by iodoacetamide alkylation of cysteines, and both samples (oxidized and unfolded oxidized), were used as substrates for RsMsrP (Figure 5). We also used glutathione-*S*-transferase (GST) which possesses 9 Met and is highly structured. After oxidation with hydrogen peroxide, GST was incubated with 4 M of the chaotropic agent urea,

a concentration sufficient to induce complete unfolding of the protein (Tarrago et al., 2012). For both oxidized proteins, we observed a dramatic increase in activity after unfolding. Indeed, the RsMsrP activity increased 7-fold with the unfolded oxidized lysozyme compared to the folded one, and 6-fold in the case of the unfolded oxidized GST compared to the folded oxidized GST (Figure 5). As the unfolded oxidized protein solutions of lysozyme or GST contained a substantial amount of urea, we made controls in which the urea was added extemporaneously in the cuvette during the measurements, showing that urea did not influence the RsMsrP activity (Figure S3).

Mass spectrometry analysis showed that the RsMsrP was able to completely reduce the oxidized lysozyme in these conditions (Figure S3), suggesting that observed differences of repair between the folded- and unfolded-oxidized lysozyme were not due to the incapacity of the RsMSRP to reduce some MetO, but were due to kinetics parameters. We thus determined the kinetic parameters of the RsMsrP activity using these proteins as substrate (Table 1, Figure S3). With the oxidized lysozyme, the k_{cat} and the K_M values were ~ 4 s^{-1} and ~ 900 µM, respectively. Using the unfolded oxidized lysozyme, the k_{cat} increased to ~ 7 s^{-1} and the K_M decreased to ~ 100 µM. The catalytic efficiency determined with the unfolded oxidized lysozyme was thus ~ 18-fold higher than the one determined using the oxidized lysozyme before unfolding (70,200 vs. 4,000 $M^{-1}.\ s^{-1}$). Similar results were obtained with the GST. Indeed, with the oxidized GST, we recorded k_{cat} and K_M values of ~ 8 s^{-1} and ~ 640 µM, respectively (Table 1). In the case of the unfolded oxidized GST, the k_{cat} was slightly higher (~ 12 s^{-1}), and the K_M was ~ 6-fold lower (~ 100 µM) (Table 1). The catalytic efficiencies were thus ~ 12,400 $M^{-1}.s^{-1}$ and 120,000 $M^{-1}.s^{-1}$ for the oxidized GST and the unfolded oxidized GST, respectively. These results indicated that the RsMsrP was 10-fold more efficient for the reduction of the unfolded oxidized GST than for its folded counterpart.

Altogether, these results showed that the RsMsrP is more efficient in reducing MetO in unfolded oxidized proteins than in folded oxidized proteins. Moreover, as evidenced with lysozyme that contained only one MetO in our conditions, the increase in activity using unfolded substrate is not dependent of the number of MetO reduced.

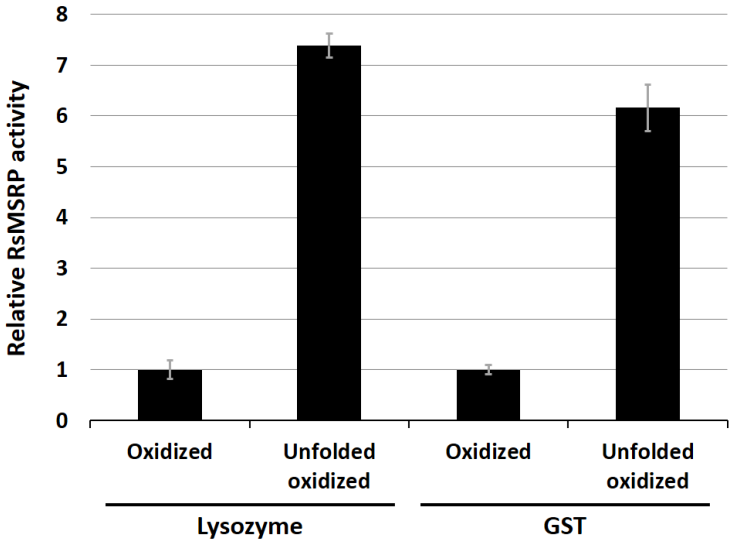

Figure 5. Relative RsMsrP activity using unfolded oxidized proteins. The RsMsrP activity was determined using benzyl viologen (0.2mM) as electron provider under nitrogen in a glove box. Benzyl viologen was initially reduced with sodium dithionite and oxidation was followed at 600 nm after addition of the enzyme (300 nM). Oxidized and unfolded oxidized lysozyme were incubated at 100 µM in 50 mM MES, pH 6.0. Initial turnover numbers were 0.65 ± 0.12 s^{-1} and 7.38 ± 0.23 s^{-1} with oxidized and unfolded oxidized lysozyme, respectively. Activity with oxidized and unfolded oxidized GST (75 µM) was determined similarly except that reaction buffer was 30 mM Tris-HCl, pH 8.0 because unfolded oxidized GST precipitated in 50 mM MES, pH 6.0. Initial turnover numbers were 0.86 ± 0.08 s^{-1} and 5.31 ± 0.39 s^{-1} with oxidized and unfolded oxidized GST, respectively. Data presented are average of three replicates. ± S.D.

RsMsrP reduces both Met-R-*O and Met*-S-*O of an oxidized model protein.*

The results obtained by Gennaris and coworkers (Gennaris et al., 2015), showing a lack of stereospecificity of the EcMsrP, prompted us to evaluate the stererospecificity of the RsMsrP. As the EcMsrP was determined to be 5-fold less efficient to reduce the Met-*S*-O than Met-*R*-O (Gennaris et al., 2015), and knowing that all previously identified enzymes able to reduce MetO, or related sulfoxides, were absolutely stereospecific toward one enantiomer, we thought that it cannot be excluded that a protein contamination might explain the apparent ability of the EcMsrP to reduce the Met-*S*-O (Gennaris et al., 2015). Such potential Met-*S*-O reductase contaminant should be able to use benzyl viologen as electron provider and one good candidate is the periplasmic DMSO reductase, which possesses a MetO reductase activity and presents an absolute stereospecificity toward the *S*-diastereoisomer (Abo et al., 1995; Weiner et al., 1988). To avoid this potential contaminant, we prepared the recombinant RsMsrP from a *R. sphaeroides* strain mutated for the gene *dorA* encoding the catalytic subunit of the DMSO reductase (Sabaty et al., 2013). After purification on a nickel affinity column and removal of the polyhistidine tag by TEV cleavage, the mature enzyme was further purified by gel filtration, then by strong anion exchange. Main fractions were pooled and concentrated, yielding a highly pure enzyme (Figure S4), which was used in all the following enzymatic assays.

To determine whether the RsMsrP can reduce both MetO diastereomers in an oxidized protein, we chose the bovine β-casein as model substrate because it was shown as an efficient substrate for the yeast MsrA and MsrB, after oxidation, indicating the presence of *R* and *S* diastereomers of MetO (Tarrago et al., 2012). We oxidized the β-casein with hydrogen peroxide to obtain a form containing both diastereomers of MetO, then took advantage of stereospecificity of the MsrA and MsrB to obtain two forms of oxidized β-casein containing a single diastereomer of MetO. The oxidized β-casein was treated with the yeast MsrA yielding

441 a protein containing only Met-*R*-O (*"β-casein*-R-*O"*), or by the yeast MsrA to obtain a form
442 in which only Met-*S*-O was present (*"β-casein*-S-*O"*). The absence of one or the other
443 diastereomer of MetO was validated by the absence of MSR activity (Figure S5). These three
444 forms containing 2 (the oxidized β-casein) or only one diastereomer of MetO were tested as
445 substrate using the previously "highly purified" RsMsrP. At 100 µM oxidized protein as
446 substrate we measured a k_{cat} of ~ 45 s^{-1} with the oxidized β-casein, which decreased to ~ 30
447 and to 5 s^{-1} for the β-casein containing the *R* or the *S* sulfoxide, respectively (Figure 6). This
448 result showed that the RsMsrP can reduce both diastereomers of MetO, but appeared 6-fold
449 less efficient to reduce the Met-*S*-O than the Met-*R*-O, similarly to as reported for the EcMsrP
450 (Gennaris et al., 2015).

451 From this result, we postulated that the RsMsrP should be able to reduce all MetO in
452 the oxidized β-casein, as this protein was intrinsically disordered and thus all MetO were very
453 likely accessible. We evaluated this hypothesis by mass spectrometry analysis. When
454 incubated with the RsMsrP, in presence of benzyl viologen, the mass of the oxidized protein
455 decreased of 96 Da, showing that all MetO were reduced (Figure 1). Altogether, these results
456 clearly showed that the RsMsrP was able to reduce both *R*- and *S*-diastereomers of MetO
457 contained in the oxidized β-casein, and thus lacked stereospecificity.

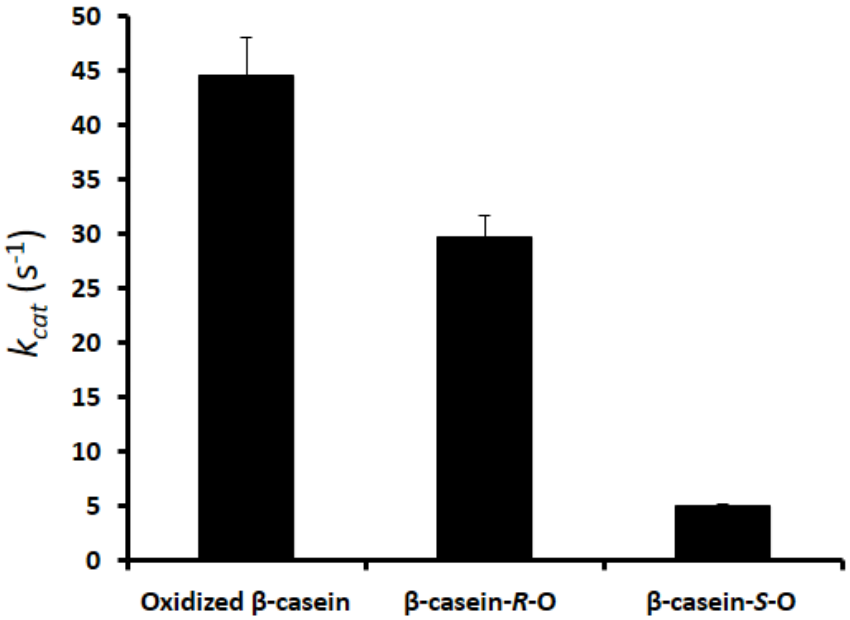

Figure 6. RsMsrP activity using oxidized β-casein, β-casein-*R*-O and β-casein-*S*-O as substrates. The oxidized β-casein containing both diastereomers of MetO, only the *R* one ("*β-casein*-R-*O"*), or only the *S* one ("*β-casein*-S-*O*") were assayed as substrate with the highly purified RsMsrP as described in figure 4. The oxidized β-casein concentrations were 100 µM. Data presented are average of three replicates. ± S.D.

The RsMsrP preferentially reduces Met-R-*O but acts effectively on Met*-S-*O too.*

To gain insight into the preference of substrate of RsMsrP toward one of the diastereomer of MetO, we performed kinetics analysis using the oxidized β-casein containing the *R* or *S* diastereomers of MetO (Table 1). When using the oxidized β-casein containing only the *R*-diastereomer of MetO (*"β-casein*-R-*O"*), we determined a k_{cat} of ~ 50 s^{-1}, a K_M of ~ 50 µM and thus a catalytic efficiency of ~ 950,000 $M^{-1}.s^{-1}$, similarly to as obtained with the β-casein-*R,S*-O (Table 1). In the case of the oxidized β-casein containing only the *S*-diastereomer of MetO (*"β-casein*-S-*O"*), the k_{cat} and K_M were ~ 8 s^{-1} and of ~ 50 µM, respectively. This yielded a catalytic efficiency of 142,000 $M^{-1}.s^{-1}$. This value, ~ 7 fold lower than the one obtained with the β-casein-*R*-O, was due to the lower k_{cat} as the K_M was not changed (Table 1). These values seem to indicate that the RsMsrP preferentially reduced the *R* than the *S* diastereomer of MetO in the oxidized β-casein. However, as we could not exclude that the proportion of Met-*R*-O was higher than the proportion of Met-*S*-O in the protein, we developed an assay to estimate the number of MetO reduced by the RsMsrP in the three forms of oxidized β-casein. We measured the total moles of benzyl viologen consumed for the reduction of all MetO using subsaturating concentrations of the oxidized protein. Practically, the absorbance at 600 nm was measured before and 90 min after the addition of the substrate. As two moles of benzyl viologen are consumed per mole of MetO reduced, we obtained the apparent stoichiometry of RsMsrP toward the oxidized protein by determining the slope of the linear regression of the straight defined by the amount of MetO reduced as a function of substrate concentration (Figure S7). The values determined were ~ 4.6, ~ 3.2 and ~ 1.8 for the oxidized β-casein, the β-casein-*R*-O and the β-casein-*S*-O, respectively. In the case of the oxidized β-casein, we expected a value of 6 based on the data obtained by mass spectrometry (Figure 1). This may have been due to the heterogeneity of the oxidized β-casein (all Met were not fully oxidized initially) and/or to a too short time of incubation (all MetO were not

fully reduced, as indicated by the presence of a peak corresponding to a portion of β-casein not fully reduced in Figure 1C). In order to compare the catalytic parameters, the data were normalized by multiplying the K_M by these apparent stoichiometries, yielding values per MetO reduced and thus allowing the removal of variation due to the different numbers of Met-*R*-O or Met-*S*-O reduced. The catalytic efficiencies were thus 230,000, 300,000 and 80,000 $M^{-1}.s^{-1}$ for the oxidized β-casein, the β-casein-*R*-O and the β-casein-*S*-O, respectively (Table 1). The highest value was thus those obtained for the β-casein containing only the *R* form of MetO, indicating that this diastereomer was the preferred substrate for the RsMsrP. However, the value obtained with the β-casein-*S*-O was only less than 4-fold lower, showing that the RsMsrP can also act effectively on the Met-*S*-O.

The RsMsrP does not reduce Met sulfone.

Finally, as the active site seemed to be able to accommodate both diastereomers of MetO, we hypothesized that it could accommodate the Met sulfone, the oxidized form of Met in which two atoms of oxygen are linked to its sulfur atom, somehow mimicking both *R* and *S* diastereomers. However, no activity was detected using low (100 µM) or high concentration (1 mM) of Met sulfone as substrate. Moreover, the presence of Met sulfone did not decrease the k_{cat} measured using MetO (Figure S8). These results showed that the Met sulfone cannot be reduced by the RsMsrP, nor act as efficient inhibitor.

Discussion

All organisms can cope with the harmful oxidation of proteins and almost all possess canonical Msr that protect proteins by reducing MetO. Bacteria differ from other organisms by the presence of molybdoenzymes able to reduce MetO, as a free amino acid for the DMSO reductase (Weiner et al., 1988) or biotin sulfoxide reductase BisC/Z (Dhouib et al., 2016; Ezraty et al., 2005), but also included in proteins in the case of the MsrP (Gennaris et al., 2015; Melnyk et al., 2015). Particularly, the MsrP seems dedicated to the protection of periplasmic proteins and was shown to have a broad spectrum of substrates in *E. coli* (Gennaris et al., 2015) and to reduce a methionine-rich protein apparently dedicated to the scavenging of reactive oxygen species in *A. suillum* (Melnyk et al., 2015). Because of its conservation in most gram-negative bacteria (Gennaris et al., 2015), MsrP is very likely a key player in the protection of bacteria against oxidative stress. The purple bacteria *R. sphaeroides* is a model organism to study photosynthesis and the effect of singlet oxygen produced during light harvesting. This strong oxidant can have a deleterious effect on proteins and the RsMsrP could play an important role in the protein protection, as suggested by the induction of its expression in response to photooxidative treatment (Glaeser et al., 2007). The enzyme, formerly known as YedY, was used as model molybdoenzyme but the nature of the RsMsrP substrate was unknown. Moreover, the genetic studies of *E. coli* (Gennaris et al., 2015) and *A. suillum* MsrP (Melnyk et al., 2015) undoubtedly showed the ability of the enzyme to reduce MetO in proteins, although an in-depth characterization of its substrate specificity was still lacking. In this work, we tested the ability of the RsMsrP to reduce protein-bound MetO and investigated its substrate specificity using a combination of proteomics and enzymologic approaches. Using oxidized proteins and peptides as model, we showed that RsMsrP is a very efficient protein-containing MetO reductase, with apparent affinities (K_M) for oxidized proteins 10 to 100-fold lower than for the tripeptide Ser-MetO-Ser or the free MetO (Table 1).

As reported for canonical MsrA and MsrB (Tarrago et al., 2012), we observed important variations in the k_{cat} of reduction of different oxidized proteins, the lowest value was observed with the oxidized lysozyme (4 s^{-1}) and the highest with the oxidized β-casein (100 s^{-1}) (Table 1).

To find potential physiological substrates of RsMsrP, we used a proteomic approach aiming to compare the oxidation state of periplasmic proteins after treatment with the strong oxidant NaOCl, then followed by RsMsrP reduction. We found 202 unique Met, belonging to 70 proteins, for which the sensitivity of the oxidative treatment and the ability to serve as RsMsrP substrate after oxidation varied greatly (Figure 2, Table S2). Numerous proteins, such as SurA, PpiA, DsbA, PotD and ProX, were previously proposed as substrate of the *E. coli* MsrP homolog (Gennaris et al., 2015). Efficiently reduced MetO by RsMsrP belong to structurally and functionally unrelated proteins, indicating that RsMsrP very likely does not possess specific substrates and act as a global protector of protein integrity in the periplasm. Interestingly, we observed from our IceLogo analysis that Met sensitive to oxidation are generally presented in a polar amino acid environment and can be found in cluster (Figure 3). These properties might be common to all Met in proteins as similar results were found in human cells (Ghesquière et al., 2011; Hsieh et al., 2017) and plants (Jacques et al., 2015). Moreover, MetO efficiently reduced by the RsMsrP show similar characteristics (Figure 4). The presence of oxidation sensitive Met efficiently reduced by the RsMsrP in clusters on polar parts of proteins should facilitate the oxidation/reduction cycle aiming to scavenge reactive oxygen species as previously proposed for canonical Msrs (Luo and Levine, 2009). This is also illustrated by the methionine-rich protein MrpX identified as main substrate of the *A. suillum* MsrP, which is almost only composed of Met, Lys, Glu and Asp (Melnyk et al., 2015). The presence of numerous MetO on a single molecule of protein substrate should

564 increase the RsMsrP efficiency as one molecule of substrate allows several catalytic cycles,
565 potentially without breaking physical contact between the enzyme and its substrate.

566 Comparison of the RsMsrP activity using folded or unfolded protein substrates
567 (lysozyme and GST) showed that it is far more efficient to reduce unfolded oxidized proteins
568 (Figure 5). Similar results were found for canonical Msrs using GST as model (Tarrago et al.,
569 2012). In the case of the MsrB it was because more MetO were accessible for reduction in the
570 unfolded GST whereas for MsrA this increase (2.2-fold higher k_{cat}/K_M value for the unfolded
571 compared to the folded oxidized GST) was independent of the number of MetO reduced.
572 Here, the use of the lysozyme containing only one MetO (Figure S2) undoubtedly showed that
573 the increase in activity is not related to the unmasking of additional MetO upon protein
574 denaturation (Table 1; Figure 5). Indeed, the k_{cat}/K_M value obtained with the unfolded
575 oxidized lysozyme was almost 18-fold higher than the value obtained with the fully folded
576 protein. This could indicate that the RsMsrP has a better access to the MetO in the protein or
577 that the MetO is more easily accommodated in the active site of the enzyme because of
578 increased flexibility. This should provide a physiological advantage to the bacteria during
579 oxidative attacks, which could occur other stresses such as acid or heat, hence promoting
580 simultaneous oxidation and unfolding of proteins. Particularly, hypochlorous acid, shown to
581 induce *msrP* expression in *E. coli* (Gennaris et al., 2015) and *A. suillum* (Melnyk et al., 2015)
582 has strong oxidative and unfolding effect on target proteins (Winter et al., 2008).

583 Finally, the work performed by Gennaris and coworker indicated that the *E. coli* MsrP
584 lacks stereospecificity and can reduce both *R*- and *S*-diastereomers of MetO chemically
585 isolated from a racemic mixture of free L-Met-*R,S*-O (Gennaris et al., 2015). This discovery is
586 of fundamental importance as it breaks a paradigm in the knowledge about Met oxidation and
587 reduction, and very likely for all enzymology as non-stereospecific enzymes were very rarely
588 described. Indeed, to our knowledge, all previously characterized enzymes able to reduce Met

589 sulfoxide or related substrates were shown as absolutely stereospecific. This was the case for
590 the canonical MsrA and MsrB, which reduce only the *S*-diastereomer and the *R*-diastereomer,
591 respectively (Ejiri et al., 1979; Grimaud et al., 2001; Kumar et al., 2002; Lowther et al., 2002;
592 Moskovitz et al., 2002; Sharov et al., 1999; Vieira Dos Santos et al., 2005), as well as for the
593 free Met-*R*-O reductase (Le et al., 2009; Lin et al., 2007) and for the molybdoenzymes DMSO
594 reductase (Abo et al., 1995; Weiner et al., 1988) and BisC/Z (Dhouib et al., 2016; Ezraty et
595 al., 2005). To evaluate the potential lack of stereospecificity of the RsMsrP, we chose to use a
596 strategy different than the one used for *E. coli* MsrP, based on chemically isolated *R*- and *S*-
597 diastereomers from a mixture of free Met-*R,S*-O (Gennaris et al., 2015). We prepared
598 oxidized β-casein containing only one or the other diastereomer of MetO using yeast MsrA
599 and MsrB to eliminate the *S*- and the *R*-diastereomers, respectively. Activity assays and
600 kinetics experiments using a highly purified RsMsrP demonstrated that it can efficiently
601 reduce the β-casein containing only the *R*- or the *S*-diastereomer (Table 1; Figure 6 and S6).
602 Moreover, this lack of stereospecificity was undoubtedly confirmed by the ability of the
603 RsMsrP to reduce all 6 MetO formed on the oxidized β-casein (Figure 1). These results,
604 consistent with Gennaris and coworkers finding, indicate that this lack of stereospecificity is
605 very likely common to all MsrP homologs. Together with the apparent ability of the enzyme
606 to repair numerous unrelated oxidized proteins, the capacity to reduce both diastereomers of
607 MetO, argues for a role of the MsrP in the general protection of envelope integrity in Gram
608 negative bacteria. However, it raises questions regarding the structure of its active site as the
609 enzyme should be able to accommodate both diastereomers. From this, we wondered whether
610 the RsMsrP could reduce the Met sulfone, which can be imagined as a form of oxidized Met
611 containing both *R*- and *S*-diastereomers, but we did not detect any activity (Figure S8).
612 Although it could be because of an incompatibility in redox potential, it may indicate that this
613 form of oxidized Met cannot reach the catalytic atom. The three-dimensional structure of the

oxidized form of *E. coli* MsrP indicated that the molybdenum atom, which is supposed to be the catalytic center of the enzyme, is buried 16 Å from the surface of the protein (Loschi et al., 2004). The next challenge will be to understand the MsrP reaction mechanism and will require the determination of the enzyme structure in its oxidized and reduced forms bound to its MetO-containing substrates.

Experimental procedures

Production and purification of recombinant proteins

Recombinant MsrP was produced similarly as described in Sabaty et al., 2013. Briefly, *R. sphaeroides* f sp. *denitrificans* IL106 *dmsA*$^{-}$ strain carrying pSM189 plasmid allowing the production of a periplasmic MsrP with a 6-His N-terminal tag was grown in 6-liter culture under semi-aerobic condition in Hutner medium until later exponential phase. Periplasmic fraction was extracted and load in HisTrap column (GE Healthcare) then MsrP was eluted by an imidazole step gradient. MsrP solution was concentrated using 15-ml Amicon® Ultra concentrators with 10-kDa cutoff (Millipore), desalted with Sephadex G-25 in PD-10 Desalting Columns (GE Healthcare). The protein concentration was adjusted to 1 mg.ml^{-1} in Tris 30 mM, 500 mM NaCl, pH 7.5 and the Tobacco Etch Virus (TEV) protease was added (1:80 TEV:RsMsrP mass ratio) and the solution incubated overnight at room temperature to remove the polyhistidine tag. Untagged RsMsrP was purified on a second HisTrap column, then concentrated and desalted in 50 mM HEPES, pH 8.0. Unless specified in the text, the protein was used without further purification. For the evaluation of RsMsrP stereospecificity toward *R*- and *S*-diastereomer of MetO, untagged RsMsrP was then purified by gel filtration in Superdex^{TM} 200 10/30 column equilibrated with Tris 30 mM, pH 7.5. Main fractions were pooled and applied to a MonoQ^{TM} 4.6/100 PE (GE Healthcare). RsMsrP was then eluted using a linear NaCl gradient (0 to 500 mM). Fractions were analyzed on SDS-PAGE using NuPAGE^{TM}, 10 % Bis-Tris gels with MES-SDS buffer (ThermoFisher). Recombinant MsrA, MsrB, Thioredoxin Reductase (TR) 1, Thioredoxin 1 (Trx1) from *Saccharomyces cerevisiae* and containing a polyhistidine tag, as well as the glutathione-S-transferase (GST) from *Schistosoma japonicum*, were produced and purified as previously described (Tarrago et al., 2012). Protein concentrations were determined spectrophotometrically using specific molar extinction coefficients at 280 nm: 6-His-RsMSRP, 56,380 M^{-1}.cm^{-1}; untagged RsMsrP,

644 54,890 $M^{-1}.cm^{-1}$; MsrA, 34,630 $M^{-1}.cm^{-1}$; MsrB, 24,325 $M^{-1}.cm^{-1}$; TR1, 24,410 $M^{-1}.cm^{-1}$;
645 Trx1, 9,970 $M^{-1}.cm^{-1}$; GST, 42,860 $M^{-1}.cm^{-1}$, bovine β-casein (Sigma Aldrich), 11,460
646 $M^{-1}.cm^{-1}$ and chicken lysozyme (Sigma-Aldrich), 32,300 $M^{-1}.cm^{-1}$. Protein solutions were
647 stored at -20°C until further use.

648

649 *Preparation of oxidized bovine β-casein and its Met*-R-*O and Met*-S-*O containing*
650 *counterparts*

651 For oxidation, bovine β-casein was incubated in PBS at 1 $mg.ml^{-1}$ in presence of 200
652 mM H_2O_2 and incubated overnight at room temperature. H_2O_2 was removed by desalting
653 using PD-10 column and the protein solution was concentrated with 10-kDa cutoff Amicon®
654 Ultra concentrator. Oxidized GST was prepared similarly using 100 mM H_2O_2. To prepare
655 Met-*R*-O containing β-casein, a solution of oxidized β-casein was incubated in Tris 30 mM,
656 pH 8 at a final concentration of 6.5 $mg.ml^{-1}$ (260 µM) in presence of 25 mM DTT and 10 µM
657 MsrA and incubated overnight at room temperature. The solution was 10-fold diluted in Tris
658 30 mM, pH 8 and passed on HisTrap column to remove the his-tagged MsrA. After
659 concentration, the DTT was removed by desalting using PD-10 column. Met-*S*-O containing
660 β-casein was prepared similarly replacing the MsrA by the MsrB (14 µM). The protein
661 solutions were concentrated with 10-kDa cutoff Amicon® Ultra concentrator and the final
662 concentration was determined spectrophotometrically. Protein solutions were stored at -20°C
663 until further use.

664

665

666

667 *Enzymatic activity and apparent stoichiometry measurements*

668 RsMsrP reductase activity was measured similarly to described in Sabaty et al., 2013
669 with few modifications. Benzyl viologen was used as electron donor and its consumption was
670 followed at 600 nm using at UVmc1® spectrophotometer (SAFAS Monaco) equipped with
671 optic fibers in a glovebox workstation (MBRAUN Labstar) flushed with nitrogen. We
672 determined the specific molar extinction coefficient of benzyl viologen at 8,700 $M^{-1}.cm^{-1}$ in
673 100 mM MES, pH 6.0 buffer. Each reaction mixture (1 ml or 0.5 ml) contained 0.2 mM
674 benzyl viologen reduced with sodium dithionite, and variable concentrations of substrates in
675 50 mM MES, pH 6.0 buffer.

676 Reactions were started by the addition of the RsMsrP enzyme (150, 300 or 460 nM).
677 Reduction of MetO rates were calculated from $\Delta A_{600\ nm}$ slopes respecting a stoichiometry of 2
678 (2 moles of benzyl viologen are oxidized for 1 mole of MetO reduced).

679 The apparent stoichiometry was determined similarly using subsaturating
680 concentrations of substrates: 1–10 µM oxidized β-casein, 1–10 µM Met-*R*-O containing
681 β-casein and 1.5–15 µM Met-S-O containing β-casein. The amount of oxidized benzyl
682 viologen was determined 1 hour after the addition of the RsMsrP (460 nM) by substracting
683 the final A_{600} nm value to the initial one. Controls were made without the RsMsrP enzyme,
684 and without the MetO-containing substrate. Quantities of MetO reduced were plotted as
685 function of substrates quantities of and the apparent stoichiometry was obtained from the
686 slope of the linear regression.

687 MsrA and MsrB activities were measured following the consumption of NADPH
688 spectrophotometrically at 340 nm using the thioredoxin system similarly as previously
689 described (Tarrago et al., 2012). A 500-µl reaction cuvette contained 200 µM NADPH, 2 µM
690 TR1, 25 µM Trx1 and 5 µM MsrA or MsrB and 100 µM oxidized β-casein. Production of

691 Met was calculated respecting a stoichiometry of 1 (1 mole of NADPH is oxidized for 1 mole
692 of Met produced).
693 Analysis and kinetics parameters determination were made using GraphPad Prism 4.0
694 software (La Jolla, CA, USA).
695
696 *Electrospray ionization/Mass spectrometry analysis of purified proteins*
697 For oxidation, bovine β-casein (5 $mg.ml^{-1}$) in 50 mM HEPES, pH 7.0, was incubated
698 overnight at room temperature with H_2O_2 (50 mM). H_2O_2 was removed by desalting using
699 PD-10 column and the protein solution was concentrated with 10-kDa cutoff Amicon® Ultra
700 concentrator. Oxidized β-casein (100 µM) was reduced by addition of 440 nM MSRP in a
701 reaction mixture containing 50 mM HEPES pH 7.0, 0.8 mM benzyl viologen and 2 mM
702 sodium dithionite. After two hours reaction in the glove-box, the repaired β-casein was
703 analyzed by mass spectrometry in comparison to non-oxidized and oxidized β-casein.
704 Mass spectrometry analyses were performed on a MicroTOF-Q Bruker (Wissembourg,
705 France) with an electrospray ionization source. Samples were desalted and concentrated in
706 ammonium acetate buffer (20mM) (Sigma-Aldrich) prior analyses with Centricon Amicon
707 (Millipore) with a cut off of 30kDa. Samples were diluted with CH_3CN/H_2O (1/1-v/v), 0.2%
708 Formic Acid (Sigma). Samples were continuously infused at a flow rate of 3 µL/min. Mass
709 spectra were recorded in the 50-7000 mass-to-charge (m/z) range. MS experiments were
710 carried out with a capillary voltage set at 4.5 kV and an end plate off set voltage at 500 V. The
711 gas nebulizer (N2) pressure was set at 0.4 bars and the dry gas flow (N2) at 4 L/min at a
712 temperature of 190 °C. Data were acquired in the positive mode and calibration was
713 performed using a calibrating solution of ESI Tune Mix (Agilent) in CH_3CN/H_2O (95/5-v/v).
714 The system was controlled with the software package MicrOTOF Control 2.2 and data were
715 processed with DataAnalysis 3.4.

716 *Generation of* R. sphaeroides *2.4.1* msrP$^-$ *mutant*

717 The *msrPQ* operon was amplified from *R. sphaeroides* 2.4.1 genomic DNA with the primers
718 5'-AGATCGACACGCCATTCACC-3' and 5'-TCGGTGAGGCGCTATCTAGG-3'. The 2.2
719 kb PCR product was cloned into pGEMT Easy (Promega). An omega cartridge encoding
720 resistance to streptomycin and spectinomycin (Prentki and Krisch, 1984) was then cloned into
721 the *BamHI* site of *msrP*. The resulting plasmid was digested with *SacI* and the fragment
722 containing the disrupted *msrP* gene was cloned into pJQ200mp18 (Quandt and Hynes, 1993).
723 The obtained plasmid, unable to replicate in *R. sphaeroides*, was transferred from *E. coli* by
724 conjugation. The occurrence of a double-crossing over event was confirmed by PCR and
725 absence of the protein from the SDS-PAGE profile.

726

727 *Preparation of periplasmic samples for proteomics analysis*

728 *R. sphaeroides* 2.4.1 *msrp*$^-$ mutant was grown under semi-aerobic conditions. Periplasmic
729 extract was prepared as previously described (Sabaty et al., 2010) by cells incubation in 50
730 mM HEPES pH 8.0, 0.45 M sucrose, 1.3 mM EDTA and 1 mg.ml^{-1} chicken lysozyme. For
731 Met oxidation, the periplasmic extract (0.7 mg.ml^{-1}) was incubated with 20 mM NEM and 2
732 mM NaOCl (Sigma-Aldrich) in 50 mM HEPES pH 8.0, 50 mM NaCl for 10 min at room
733 temperature. NaOCl was removed by desalting using PD-10 column and buffer was changed
734 for 50 mM MES pH 6.0. The protein solution was concentrated with 3-kDa cutoff Amicon®
735 Ultra concentrator. Three reaction mixtures were prepared in the glove box containing 35 µl
736 periplasmic extract, 1 mM benzyl viologen, 2 mM dithionite in 50 mM MES pH 6.0. The
737 protein concentration in each reaction was 2.5 mg.ml^{-1}. The first reaction contained non-
738 oxidized periplasmic extract, the second and third ones contained oxidized periplasmic

739 extract. For the third reaction (repaired periplasm) 10 µM RsMsrP was added. The reactions
740 were incubated for three hours at room temperature.

741

742 *Trypsin proteolysis and tandem mass spectrometry*

743 Protein extracts were immediately subjected to denaturing PAGE electrophoresis for 5 min
744 onto a 4–12% gradient 10-well NuPAGE (Invitrogen) gel. The proteins were stained with
745 Coomassie Blue Safe solution (Invitrogen). Polyacrylamide bands corresponding to the whole
746 proteomes were sliced and treated with iodoacetamide and then trypsin as previously
747 recommended by Hartmann et al., 2014. Briefly, each band was destained with ultra-pure
748 water, reduced with dithiothreitol, treated with iodoacetamide, and then proteolyzed with
749 Trypsin Gold Mass Spectrometry Grade (Promega) in presence of 0.01% ProteaseMAX
750 surfactant (Promega). Peptides were immediately subjected to tandem mass spectrometry as
751 previously recommended to avoid methionine oxidation (Madeira et al., 2017). The resulting
752 peptide mixtures were analyzed in a data-dependent mode with a Q-Exactive HF tandem mass
753 spectrometer (Thermo) coupled on line to an Ultimate 3000 chromatography system
754 chromatography (Thermo) essentially as previously described (Klein et al., 2016). A volume
755 of 10 µL of each peptide sample was injected, first desalted with a reverse-phase Acclaim
756 PepMap 100 C18 (5 µm, 100 Å, 5 mm x 300 µm i.d., Thermo) precolumn and then separated
757 at a flow rate of 0.2 µL per min with a nanoscale Acclaim PepMap 100 C18 (3 µm, 100 Å,
758 500 mm x 300 µm i.d., Thermo) column using a 150 min gradient from 2.5 % to 25 % of
759 CH_3CN, 0.1% formic acid, followed by a 30 min gradient from 25% to 40% of CH_3CN, 0.1%
760 formic acid. Mass determination of peptides was done at a resolution of 60,000. Peptides were
761 then selected for fragmentation according to a Top20 method with a dynamic exclusion of 10

762 sec. MS/MS mass spectra were acquired with an AGC target set at 1.7 10^5 on peptides with 2
763 or 3 positive charges, an isolation window set at 1.6 *m/z*, and a resolution of 15,000.

764

765 *MS/MS spectrum assignment, peptide validation and protein identification*

766 Peak lists were automatically generated from raw datasets with Proteome Discoverer 1.4.1
767 (Thermo) and an in-house script with the following options: minimum mass (400), maximum
768 mass (5,000), grouping tolerance (0), intermediate scans (0) and threshold (1,000). The
769 resulting .mgf files were queried with the Mascot software version 2.5.1 (Matrix Science)
770 against the *R. sphaeroides* 241 annotated genome database with the following parameters:
771 full-trypsin specificity, up to 2 missed cleavages allowed, static modification of
772 carbamidomethylated cysteine, variable oxidation of methionine, variable deamidation of
773 asparagine and glutamine, mass tolerance of 5 ppm on parent ions and mass tolerance on
774 MS/MS of 0.02 Da. The decoy search option of Mascot was activated for estimating the false
775 discovery rate (FDR) that was below 1%. Peptide matches with a MASCOT peptide score
776 below a p value of 0.05 were considered. Proteins were validated when at least two different
777 peptides were detected. The FDR for proteins was below 1% as estimated with the MASCOT
778 reverse database decoy search option.

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Conflict of interest

The authors declare no conflict of interest

Author contributions

LT, PA, DP and MS designed the study. LT, SG, MIS and MS purified RsMsrP. LT and MS prepared all other proteins. LT, SG, MIS, MS performed biochemical characterization of RsMsrP. LT, MS and DL performed β-casein and lysozyme mass spectrometry analysis and analyzed the data. SG and MS prepared *R. sphaeroides* 2.4.1 *msrP*$^{-}$ mutant and periplasmic proteins samples. BA, GM and JA performed proteomics analysis of periplasmic proteins and LT, MS, GM and JA analyzed the data. LT wrote the manuscript with contribution of DL, PA, DP, JA and MS. All authors approved the final manuscript.

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976

977 **Table 1.** Kinetics parameters of RsMsrP reductase activity towards various MetO-containing
978 substrates.

Substrates	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($M^{-1}.s^{-1}$)
DMSO[a]	28 ± 1	61,000 ± 7,000	465
Free L-Met-*R*,*S*-O	122 ± 20	115,000 ± 27,000	1,000
Ser-MetO-Ser	108 ± 17	13,000 ± 3,400	8,300
Oxidized β-casein	100 ± 5	93 ± 9	1,075,000
β-casein-*R*-O	49 ± 3	51 ± 6	950,000
β-casein-*S*-O	8 ± 1	53 ± 10	142,000
Oxidized lysozyme	4 ± 1	886 ± 349	4,000
Unfolded oxidized lysozyme	7 ± 1	105 ± 17	70,200
Oxidized GST	8 ± 2	643 ± 194	12,400
Unfolded oxidized GST	12 ± 3	99 ± 33	120,000

979 [a] From Sabaty et al., 2013