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Thioredoxin 2 from *Escherichia coli* is not involved *in vivo* in the recycling process of methionine sulfoxide reductase activities

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ABSTRACT

Thioredoxins (Trx) 1 and 2, and three methionine sulfoxide reductases (Msr) whose activities are Trx-dependent, are expressed in *Escherichia coli*. A *metB*$_1$ *trxA* mutant was shown to be unable to grow on methionine sulfoxide (Met-O) suggesting that Trx2 is not essential in the Msr-recycling process. In the present study, we have determined the kinetic parameters of the recycling process of the three Msrs by Trx2 and the *in vivo* expression of Trx2 in a *metB*$_1$ *trxA* mutant. The data demonstrate that the lack of growth of the *metB*$_1$ *trxA* mutant on Met-O is due to low *in vivo* expression of Trx2 and not to the lower catalytic efficiency of Msrs for Trx2.

1. Introduction

Thioredoxins (Trx) are conserved in all living organisms from archaea to humans. They are small redox proteins capable of catalyzing thiol-disulfide redox reactions. All Trxs share a similar three-dimensional structure and possess a conserved WCGPC catalytic motif. Two Trxs[2] were identified in *Escherichia coli*. The first, Trx1, is encoded by the gene *trxA*, and was first identified *in vitro* as an electron donor for ribonucleotide reductase in 1964 [1]. Further characterization of Trx1 *in vitro* has shown, however, that it can function as an electron donor for a range of enzymes, including 3′-phosphoadenylylsulfate reductase [2], methionine sulfoxide reductase (Msr) [3,4] and the periplasmic isomerase DsbC *via* the membrane protein DsbD [5]. *In vivo* experiments with *E. coli* mutant strains have confirmed that Trx1 can serve as an electron donor for ribonucleotide reductase, 3′-phosphoadenylylsulfate reductase, Msr and DsbC *via* DsbD [6], and that Trx1 is also required for the assembly of the filamentous *E. coli* phages f1, T7 and M13, though this function is not related to its redox properties [7].

More recently, a second Trx, Trx2 (*trxC* gene product), was identified in *E. coli*. Trx2 contains two distinct domains: a C-terminal domain with the conserved WCGPC active site signature and an additional N-terminal domain of 32 residues which includes two conserved CXXC motifs [8] responsible for the tight binding of a zinc atom [9]. Recently, the crystal structure of Trx2 from *Rhodobacter capsulatus* was determined, confirming that the fold of the C-terminal domain is similar to that of Trx1 [10]. *In vitro* studies showed that *E. coli* Trx2 is a functional Trx, as it is capable of reducing insulin and ribonucleotide reductase and is an electron donor for 3′-phosphoadenylylsulfate reductase [8,11]. *In vivo*, Trx2 can substitute for Trx1 function, but only when Trx2 is overexpressed. It is the case for reduction of DsbC *via* DsbD [12]. This is in accord with the fact that low expression levels of Trx2 are insufficient to enable Trx2 to substitute for Trx1 in the reducing pathway of sulfate assimilation [11]. Taken together, these results suggest that, despite its structurally distinct features, Trx2 is able to fulfil most of the roles of Trx1 as a disulfide oxidoreductase, provided that it is expressed at adequate levels.

Methionine (Met) in proteins is easily oxidized to methionine sulfoxide (Met-O) under both normal and oxidative stress conditions, a modification which can affect the function of the oxi-

Abbreviations: Met-O, methionine sulfoxide; Msr, methionine sulfoxide reductase; Trx, thioredoxin

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[2] An *E. coli* Trx homolog YbbN/Trxsc was recently described [26]. It includes a SXXC active site signature and is reported to display a weak disulfide oxidoreductase activity. In fact, it is probable that this Trx homolog displays no disulfide oxidoreductase activity because the catalytic Cys is substituted by a Ser residue.

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dized proteins. Two classes of Msr proteins, MsrA and MsrB [13], are capable of restoring the function of such oxidized proteins, *via* the reduction of the (*S*)- and (*R*)-isomers, respectively, of Met-O. A new class of Msrs, called fRMsr, was recently discovered, which shows strict specificity for free Met-*R*-O [14]. All three classes share the same catalytic mechanism [15,16] which involves a sulfenic acid intermediate, followed by formation of at least one intra-disulfide bond between the catalytic Cys and a recycling Cys, when this latter is present. In the absence of recycling Cys, the reducer is not Trx but a small reducer molecule whose nature *in vivo* remains to be characterized. In the case of Msrs from *E. coli*, the recycling Cys is present and the reduction of the Msr intra-disulfide bond, referred to as the 'recycling process', is accomplished by the Trx/Trx reductase system. Recent kinetic studies showed that the Trx-recycling is the rate-limiting step for both MsrA and MsrB [13], whereas the rate-limiting step remains to be determined for fRMsr. It should be noted that there exists a fourth class of methionine sulfoxide reductase, named BisC, whose physiological substrate is dimethyl sulfoxide and which also accepts Met-*S*-O as substrate [17]. But no BisC activity was observed *in vitro* in the presence of NADPH and the Trx reductase/Trx couple although a *trxA* mutation prevented use of Met-*S*-O as substrate. Ezraty et al. proposed that a supplementary cofactor is required to transfer electrons from the Trx reductase/Trx couple [17]. In the paper, Msr will refer to MsrA, MsrB or fRMsr whose recycling activities are directly dependent on Trx.

A Met auxotrophic *E. coli* strain in which the *trxA* gene is inactivated, was found to be unable to grow in the presence of D, L-Met-*R*, *S*-O ('Met-O') as the sole Met source [7,12,18]. This observation suggested that only Trx1 is capable of the *in vivo* recycling of Msrs. However, there are two possible explanations for these data: (i) Trx2 is not catalytically efficient in the recycling of the Msr activities including fRMsr; and/or (ii) Trx2 is so poorly expressed in *E. coli* that the level of Trx2, regardless of its catalytic efficiency, is too low to complement growth defects whenTrx1 is not expressed.

In the present study, the kinetic parameters of MsrA, MsrB and fRMsr from *E. coli* were determined under steady-state conditions with Met-O as substrate and Trx2 as the recycling reducer and compared to those obtained with Trx1. Trx2 was also overexpressed in an *E. coli metB$_1$ trxA*::Kan^r mutant containing a plasmid harboring *trxC* under the control of P_{TRC} promoter, and the ability of the transformed strain to grow on Met-O evaluated. Using antibodies and purified Trxs to quantify Trx1 and Trx2, we show that the lack of growth of the *metB$_1$ trxA E. coli* mutant on Met-O is due to the low expression of Trx2 and not to the lower catalytic efficiency of the three Msrs for Trx2.

2. Materials and methods

2.1. Bacterial strains and culture media

The *E. coli* strains DH5α (*supE44,* Δ*lac*U169 (Φ80 *lac*ZΔM15), *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *rel*A1), and *metB$_1$trxA*::Kan^r JB11 [17] were maintained on Luria–Bertani (10 g l^{-1} bactotryptone, 5 g l^{-1} yeast extract and 10 g l^{-1} NaCl) agar medium supplemented with either 50 μg ml^{-1} kanamycin and/or 50 μg ml^{-1} ampicillin, when needed. The ability to utilize Met-O as the sole Met source was tested on M9 plates, with Met-O added to 20 μg ml^{-1}. The strains LCB303 (a *metB$_1$* mutant) and JB11 (a *metB$_1$trxA*::Kan^r mutant), which are unable to synthesize Met *de novo*, were kindly provided by Pr. F. Barras (Laboratoire de Chimie Bactérienne, UPR9043, IFR88 – Institut de Microbiologie de la Méditerranée, CNRS, Marseille, France).

2.2. Plasmid constructions, production and purification of Wild-type E. coli Trx1, Trx2, MsrA, MsrB, fRMsr and Trx reductase

Trx1, Trx reductase, MsrA and MsrB from *E. coli* were prepared as previously described [19–21]. The plasmid pETfRMsr encoding an N-terminal His_6-tag fusion of fRMsr, was obtained by subcloning the synthetic *yebR* open reading frame optimized for expression in *E. coli* (GeneArt AG, Regensburg, Germany) into the pET28b(+) plasmid (Novagen, Merk Chemicals, Darmstadt, Germany) between the *Nde*I and *Sac*I sites.

Production and purification of fRMsr were carried out as previously described [14], except that the final Superdex 75 and Q-Sepharose columns were omitted.

For the production of untagged Trx2, the *trxC* open reading frame of the pET*trxC* vector [8] was subcloned into a pET20b(+) vector (Novagen, Merk Chemicals, Darmstadt, Germany). The purification was performed as previously described [9], except that the final Superdex 75 column was omitted. A yield of 100 mg of pure Trx2 per liter of culture was obtained.

2.3. Determination of k_{cat} and K_M of Msrs for Trx1 and Trx2 under steady-state conditions

Initial rate measurements were carried out at 25 °C by following the decrease of the absorbance of NADPH at 340 nm. The experimental conditions were the following: *E. coli* Trx reductase (4.8 μM), NADPH (0.3 mM), Met-O under saturating concentrations (150 mM for MsrA and MsrB and 10 mM for fRMsr), MsrA (0.250 μM), MsrB (1 μM), fRMsr (0.1 μM with Trx1 and 1 μM with Trx2) and variable concentrations of *E. coli* Trx1 (from 1 to up 250 μM, depending on the Msrs) or Trx2 (from 2 to up 500 μM, depending on the Msrs), in buffer A (50 mM Tris-HCl, 2 mM EDTA) at pH 8. When saturating concentrations of Trxs were observed, the initial rate data were fitted to the Michaelis–Menten equation using least square analysis to determine k_{cat} and K_M [20]. For fRMsr with Trx2, the value of the pseudo-second-order rate constant (k_2) was obtained by linear fitting of initial rate data at sub-saturating concentrations of Trx2.

2.4. Functional complementation in E. coli JB11 Trx1 deficient cells

The open reading frames coding for Trxs were amplified by PCR and cloned into pTrc99A vector under the P_{TRC} promoter. *E. coli* JB11 cells were transformed with pTrc99A, pTrc*trxA*, or pTrc*trxC* and grown in Luria–Bertani medium with antibiotic. When an A_{600} of 0.5 was reached, 1 mM IPTG was added to induce cultures and growth was continued for 6 h. To test the ability of the transformants to grow on Met-O, drop assays were performed on M9 plates supplemented with antibiotic, 1 mM IPTG, and 20 μg ml^{-1} Met-O, and then incubated for two days at 37 °C.

2.5. Quantification of protein levels

Proteins were immunodetected in total protein extracts. The protein concentrations of the cell lysates were measured with a protein assay kit (Bio-Rad) according to the manufacturer's protocol. Purified Trx1 and Trx2 were used in order to provide a standard for the conversion of signals in the Western to absolute protein levels. The amounts of cell extract used for quantification were varied to obtain values within the range of the Trx standards. Portions of the cell extracts were subjected to electrophoresis using a 15% SDS–polyacrylamide gel, together with increasing amounts of purified Trx1 or Trx2 as standard. The concentrations of the Trx1 and Trx2 proteins were determined by measuring the absorbance at 280 nm, using the extinction coefficients $\varepsilon_{280\ nm}$ = 14,500 M^{-1} cm^{-1} for Trx1 and ε_{280nm} = 17,070 M^{-1} cm^{-1}

for Trx2. After transfer to a nitrocellulose membrane, the proteins were probed with polyclonal antibodies raised against Trx1 or Trx2. Primary antibodies were detected with horseradish peroxidase-conjugated anti-rabbit antibodies followed by enhanced chemiluminescence (ECL Plus, Amersham). Immuno-detection signals corresponding to Trx1 and Trx2 were scanned with a Typhoon (9410) Healthcare scanner and quantified with the Image-Quant software (Molecular Dynamics).

To convert the total amounts of Trx1 and Trx2 in cell extracts into intracellular concentrations, it was necessary to determine water and protein contents. For this, the LCB303 strain was grown in LB medium until the $OD_{600\ nm}$ reached 0.5. A portion of the culture was used to determine the number of bacterial cells per unit volume. A defined volume of the culture was centrifuged at 12,000*g* for 30 min. After removal of the supernatant, the pelleted cells were resuspended in a small volume of mineral medium and transferred to a tared tube. After centrifugation and total elimination of the supernatant, the tube was weighed before and after drying at 50 °C during 48 h. Another portion of the culture was disrupted by sonication, centrifuged at 12,000*g* for 30 min, and the supernatant used for measuring the cytoplasmic protein concentration. For the determination of the Trx1 and Trx2 cytoplasmic concentrations, molecular weights of 11 800 and 15 500 g mol^{-1} were used, respectively.

3. Results

3.1. Kinetic parameters of MsrA, MsrB and fRMsr for Trx1 and Trx2 with Met-O as a substrate

The catalytic constants of MsrA, MsrB and fRMsr from *E. coli* with Trx2 as the recycling reducer, were determined under steady-state conditions at saturating concentration of Met-O. The k_{cat} values for MsrA and MsrB are $2.7 \pm 0.2\ s^{-1}$ and $0.16 \pm 0.01\ s^{-1}$, respectively, while the respective K_M values for Trx2 are 37 ± 11 μM and 26 ± 8 μM. This corresponds to a catalytic efficiency of $70 \times 10^3\ M^{-1}s^{-1}$ and $6 \times 10^3\ M^{-1}s^{-1}$, respectively. In contrast, no saturating effect for fRMsr was observed up to 200 μM of Trx2. The k_2 value, which represents the k_{cat}/K_M value, was deduced from the slope of the curve k_{obs} versus Trx2 concentration. The k_2 value is $2.0 \pm 0.1 \times 10^3\ M^{-1}s^{-1}$; and at 500 μM of Trx2, the rate constant is $0.7\ s^{-1}$ (Fig. S1).

The k_{cat} values of MsrA, MsrB and fRMsr with Trx1 as the recycling reducer, are $3.1 \pm 0.1\ s^{-1}$, $0.14 \pm 0.01\ s^{-1}$, and $6.6 \pm 0.2\ s^{-1}$, respectively, while the respective K_M values for Trx1 are 6 ± 1 μM, 7 ± 3 μM and 16 ± 2 μM. This corresponds to a catalytic efficiency of $500 \times 10^3\ M^{-1}s^{-1}$, $20 \times 10^3\ M^{-1}s^{-1}$ and $400 \times 10^3\ M^{-1}s^{-1}$, respectively.

3.2. Trx2 can substitute for Trx1 as an electron donor for Msrs in vivo when Trx2 is overproduced

The *E. coli metB$_1$ trxA*::Kan^r strain JB11 is auxotrophic for Met and lacks a functional *trxA* gene [17]. As described previously [17], and confirmed here, JB11 strain grows on minimal medium containing Met but not Met-O (Fig. 1, lane 2), in contrast to the parental *metB$_1$* strain LCB303 which grows on a minimal medium supplemented not only with Met but also with Met-O (Fig. 1, lane 1). These results indicate that Msrs, which provide Met to the strain *via* reduction of Met-O, are recycled *in vivo* by Trx1. This finding suggested that Trx2 is not involved in Msr recycling. In an attempt to clarify its role *in vivo*, Trx2 was overproduced in JB11 mutant strain using the IPTG inducible promoter of the pTrc99A vector. As a control, Trx1 was overproduced using the same method. As shown in Fig. 1, the phenotype of the JB11 mutant strain was fully complemented by Trx2 (lane 4) and, as expected, by Trx1 (lane 3). These results indicate that in the experimental conditions used for its overexpression, Trx2 can functionally substitute for the *E. coli* Trx1 as an electron donor for Msrs *in vivo*.

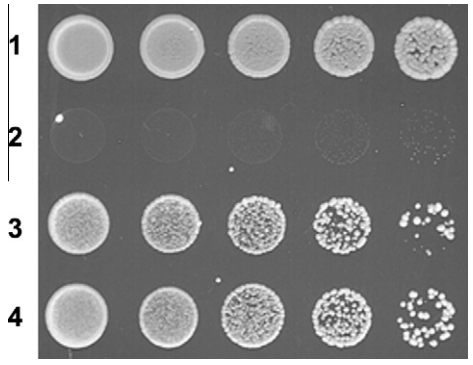

Fig. 1. Trx2 restores the growth phenotype of a *metB$_1$ trxA E. coli* strain on Met-O when overexpressed. The *metB$_1$ trxA* (JB11) *E. coli* strain was transformed with the empty vector pTrc99A (lane 2), pTrc*trxA* (lane 3), or pTrc*trxC* (lane 4). *metB$_1$* mutant cells (strain LCB303) were also transformed with pTrc99A as a control (lane 1). Induced cultures were adjusted to $OD_{600} = 0.5$, and 5 μl of serial dilutions (from left to right) were spotted on M9 minimal medium supplemented with antibiotic, 1 mM IPTG, 20 μg ml^{-1} Met-O. Plates were incubated for two days at 37 °C.

3.3. Protein levels of Trx1 and Trx2 in LCB303 and overexpressing strains

To quantify the *in vivo* concentrations of Trxs, it was necessary to determine the intracellular water content and the level of Trxs

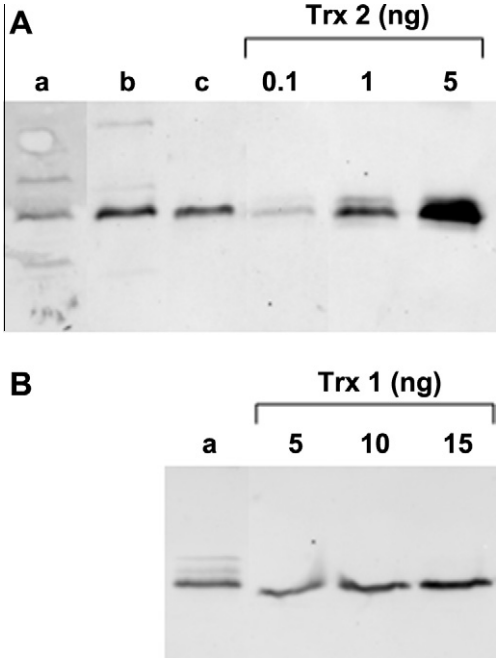

Fig. 2. Quantitative Western blots. The right hand three lanes contain recombinant Trx1 or Trx2 used to standardize measurements of cell extracts. The amounts of cell extract used for quantification were varied to obtain values within the range of the standard curves. (A) Quantification of Trx2 level in (a) 15 μg of LCB303 cell extract; (b) 2 μg of cell extract from *metB$_1$ trxA* strain transformed with pEJS33; and (c) 0.1 μg cell extract from *metB$_1$ trxA* strain transformed with pTr*trxC*. For the standard curve, we used 0.1, 1 and 5 ng of purified Trx2 protein. (B) Quantification of Trx1 level in (a) 1 μg of cell extract of LCB303. For the standard curve we used 5, 10 and 15 ng of purified Trx1 protein.

by immunological technique using purified Trx1 and Trx2 as standards.

We found an intracellular water content of 1.5 ml per g dry weight for the LCB303 strain which is in good agreement with data from Epstein and Schultz [22], and slightly lower than that reported by Zwaig et al. [23]. We further found (1) that 25% of the dry weight of the cells is cytoplasmic proteins; and (2) a water content of 6 μl mg^{-1} of cytoplasmic protein. Next, we measured the amounts of Trx1 and Trx2 in cell extracts. Trx1 and Trx2 proteins were detected immunologically and quantified using highly purified Trx1 and Trx2 proteins as standards (Fig. 2). In the LCB303 strain, the Trx2 level is very low (37 ng mg^{-1} of protein; ≈0.4 μM) (Fig. 2A, lane a). In contrast, the Trx1 level is high (5400 ng mg^{-1}; ≈75 μM) (Fig. 2B, lane a). Similar values were obtained with the DHB4 strain (data not shown) which was used by Potamitou et al. to quantify the expression of Trx1 and Trx2 [24]. The fact that the concentration of Trx2 in LCB303 and DHB4 strains is 5-fold lower compared to that determined by Potamitou et al. is likely due to the different experimental conditions used. In the JB11 strain containing the pTrc*trxC* plasmid, the Trx2 overexpression level is high (7500 ng mg^{-1}; 80 μM) (Fig. 2A, lane c). In view of this result, we were interested to determine why only a partial growth complementation was observed when *trxC* was overexpressed under the control of the arabinose promoter P_{BAD} [12,18]. Using the JB11 strain containing the pEJS33 plasmid harboring the *trxC* gene under arabinose promoter, we quantified the Trx2 level as 400 ng mg^{-1} (4.2 μM) (Fig. 2A, lane b) that is much lower than from the JB11 strain containing the pTrc*trxC* plasmid.

4. Discussion

Under normal growth conditions, *E. coli* expresses two Trx and three Msrs. The previous observation that a Met auxotrophic *E. coli* strain, in which the *trxA* gene is inactivated, was unable to grow on a media with Met-O as the sole Met source, suggested that Trx1 may be the sole thioredoxin involved in recycling the Msrs activities. Moreover, when Trx2 was overexpressed under the control of the arabinose promoter, only partial growth complementation was observed [6,12]. These results could be the consequence of either an insufficient overexpression levels of Trx2 or/and to a low catalytic efficiency of the recycling process of Msr activities by Trx2 in the cells under standard growth conditions. Indeed, as pEJS33 and pTrc*trxC* vectors contain a pBR322 origin of replication, it was reasonable to assume that cells transformed with each of these vectors would contain the same plasmid copy number. As a consequence, results obtained in complementation tests could not be explained by a difference in plasmid copy number.

As shown in the present study, the Trx2 expression level in the LCB303 strain is ~150-fold lower than Trx1. The catalytic efficiency (k_{cat}/K_M) of the Msr-recycling process with Trx2 as the reducer is also lower when compared to Trx1,but only by a factor of ~3 for MsrB and ~7 for MsrA. For fRMsr, the catalytic efficiency is, however, significantly lower (~200-fold). The fact that similar kinetic data were obtained with fRMsr from *Neisseria meningitidis* (data not shown) is an indication that it is a common property of all fRMsrs. In the case of MsrA and MsrB, the lower catalytic efficiency is due to a K_M effect. Although for fRMsr it is not possible to reach the K_M value for Trx2, its value is likely around 1000 μM that is 60-fold higher compared to the K_M value for Trx1. The origin of this weaker binding remains to be explained at the structural/molecular level. One possibility, however, is that the first domain of Trx2, which contains the Zn^{2+} binding site, perturbs the interactions with fRMsr and as a consequence, decreases the apparent affinity of fRMsr for Trx2.

Complete growth complementation is observed when the Trx2 overexpression level is in the range of the expression of Trx1 (i.e. 80 μM vs. 75 μM, respectively) while only a partial complementation occurs when Trx2 overexpression is 4.2 μM. We conclude, therefore, that the lack of growth of the *metB$_1$ trxA* mutant on Met-O is only due to the low expression of Trx2.

As already mentioned, there exist three Msrs in *E. coli* whose activity is Trx-dependent; and as demonstrated in the present study, the recycling of the Msr activities involves only Trx1 under normal growth conditions[3]. The *metB$_1$* LCB303 strain can grow on a medium that contains either DL-Met-*S*-O or DL-Met-*R*-O (data not shown). This finding means that the *S* isomer is reduced by MsrA which displays a high catalytic efficiency, while the *R* isomer is likely reduced by fRMsr, as shown in *Saccharomyces cerevisiae* [25]. Indeed, the catalytic efficiency of the recycling process of fRMsr by Trx1 is 20-fold higher compared to MsrB, due essentially to a k_{cat} effect, and in the range of that of MsrA. Moreover, the fact that the *in vivo* Trx1 concentration is 75 μM means that Trx1 concentration is saturating in the recycling process of both MsrA and fRMsr activities. Or in other words, the concentration of Trx1 is not limiting in the *in vivo* Msr recycling process.

When Met-O is included in a polypeptide chain, however, fRMsr is not able to reduce the *R* isomer. As indicated in the Introduction section, the rate-limiting step of MsrB is associated with the recycling process [21] and is low i.e. 0.14 s^{-1} for the *E. coli* MsrB compared to 3.1 s^{-1} for *E. coli* MsrA. Therefore, we have to assume that although the k_{cat} value of MsrB is 20-fold less compared to that of MsrA with a K_M value of both Msrs for Met-O included in a polypeptide chain of ~1 mM, the catalytic efficiency of MsrB is nonetheless sufficient to reduce the Met-*R*-O in oxidized proteins. Thus together, MrsB and MrsA, which are specific for the *R* and the *S* isomers of the sulfoxide function, respectively, can restore the function of proteins oxidized on their Met residues.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.04.070.

[3] Under oxidative stress conditions, the expression of the *trxC* gene, which is under the control of the oxidative stress transcription factor OxyR, was described to be 20-fold increased [18]. However, such an overexpression was only observed in an *E. coli* strain which lacks both catalase and alkyl hydroperoxidase. In this strain, the intracellular level of H_2O_2 remains high. In contrast, in the presence of both catalase and hydroperoxidase activities, the concentration of the overexpressed Trx2 remains low i.e. twofold increase (data not shown) in a range similar to that described by Collet and coll. (approximately fourfold increase) [27]. Therefore, Trx2 cannot substitute Trx1 in the Msr-recycling process in an *E. coli metB$_1$ trxA* mutant which expresses the *ahpCF* and *katG* genes. Moreover, in our hands, under 100 μM of H_2O_2, no growth of the *E. coli metB$_1$ trxA* strain was observed with Met-O in contrast to that observed with Met.

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