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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}