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Selenium utilization in thioredoxin and catalytic advantage provided by selenocysteine

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ABSTRACT

Thioredoxin (Trx) is a major thiol-disulfide reductase that plays a role in many biological processes, including DNA replication and redox signaling. Although selenocysteine (Sec)-containing Trxs have been identified in certain bacteria, their enzymatic properties have not been characterized. In this study, we expressed a selenoprotein Trx from *Treponema denticola*, an oral spirochete, in *Escherichia coli* and characterized this selenoenzyme and its natural cysteine (Cys) homologue using *E. coli* Trx1 as a positive control. ^{75}Se metabolic labeling and mutation analyses showed that the SECIS (Sec insertion sequence) of *T. denticola* selenoprotein Trx is functional in the *E. coli* Sec insertion system with specific selenium incorporation into the Sec residue. The selenoprotein Trx exhibited approximately 10-fold higher catalytic activity than the Sec-to-Cys version and natural Cys homologue and *E. coli* Trx1, suggesting that Sec confers higher catalytic activity on this thiol-disulfide reductase. Kinetic analysis also showed that the selenoprotein Trx had a 30-fold higher K_m than Cys-containing homologues, suggesting that this selenoenzyme is adapted to work efficiently with high concentrations of substrate. Collectively, the results of this study support the hypothesis that selenium utilization in oxidoreductase systems is primarily due to the catalytic advantage provided by the rare amino acid, Sec.

1. Introduction

Selenium is an essential trace element in mammals that is incorporated into proteins in the form of the rare amino acid selenocysteine (Sec) during protein translation [1]. Sec-containing proteins, which are known as selenoproteins, are found in all three kingdoms of life. Oxidoreductases, including formate dehydrogenase H, glutathione peroxidase, and methionine sulfoxide reductase, are among the most well characterized selenoproteins [2–4]. In selenoprotein forms of oxidoreductases, Sec replaces cysteine (Cys) residue in catalytic sites of their orthologue proteins. Sec is translationally inserted into protein using an UGA codon, which is normally a stop codon. A stem-loop structure known as the SECIS (Sec insertion sequence) element on the selenoprotein mRNA is necessary to recode the UGA codon for the Sec insertion [1,5]. SECIS elements are located immediately downstream of the Sec UGA codons in bacteria, whereas they are present in the 3′-untranslational regions in archaea and eukaryotes. A consensus bacterial SECIS structural model has been suggested in which a conserved G nucleotide in the apical loop of SECIS is critical to the function of SECIS [6].

The thioredoxin (Trx) system is a major disulfide reductase system that controls cellular redox status [7]. The Trx system is composed of NADPH, Trx reductase, and Trx, wherein electrons are transferred from NADPH to Trx via Trx reductase. Trxs, typically 12 kDa small thiol-disulfide oxidoreductases, were originally discovered as a hydrogen donor of *Escherichia coli* ribonucleotide reductase [8], and are now a well-known multifunctional protein involved in a variety of physiological processes, including DNA replication, transcription, antioxidant defense, and redox signaling [9]. Trxs involve two Cys residues in catalysis and contain a characteristic CGPC motif in the active site, in which the N-terminal Cys residue acts as a catalytic residue and the C-terminal Cys residue as a resolving Cys.

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Sec-containing forms of Trx have been found in bacteria such as *Treponema denticola* [10], but have not been previously characterized. *T. denticola* is a Gram-negative, obligate anaerobic bacterium associated with periodontitis [11]. *T. denticola* growth is dependent on selenium in medium [12], and this organism contains at least seven selenoproteins, including Trx [10]. In this study, we expressed the *T. denticola* selenoprotein Trx in *E. coli* and characterized this selenoenzyme and its natural Cys homologue using *E. coli* Trx1 as a positive control.

2. Materials and methods

2.1. Cloning of the T. denticola Sec- and Cys-containing Trxs

A coding region of the *T. denticola* selenoprotein Trx gene, *trx1*, was amplified by PCR using genomic DNA from *T. denticola* ATCC 33521 and forward (5′-GCGCCATATGATTATGGCAGTATTGG-3′) and reverse (5′-GCGCCTCGAGGATATGCTTTGAAACAAAG-3′) primers. The amplified DNA fragment digested with *Nde*I and *Xho*I was cloned into pET21b (Novagen). The resulting construct, designated pET21-Td-Trx1, encoded full-length selenoprotein Trx1 (tTrx1) with a C-terminal His-tag (LEHHHHHH). The Cys-containing homologue Trx gene, *trx2*, was also PCR-cloned into *Nde*I/*Xho*I sites of pET21b using forward (5′-GCGCCATATGATTGAATTGACAAAAG-3′) and reverse (5′-GCGCCTCGAGGATGTATTTTTTTACCATC-3′) primers. The resulting plasmid, pET21-Td-Trx2, encoded full-length Trx2 (tTrx2) with a C-terminal His-tag (LEHHHHHH). We also generated Sec-to-Cys version (tTrx1/U32C) and Trp mutant (tTrx1/U32W) of selenoprotein tTrx1 in which Sec32 was replaced with Cys and Trp, respectively, by site-directed mutagenesis.

2.2. Expression and purification of the Sec- and Cys-containing Trxs

To express tTrx1 in *E. coli*, the plasmid pET21-Td-Trx1 was introduced into BL21(DE3) cells also harboring a plasmid pSUABC [13], which encodes *E. coli* selA, selB, and selC. The transformed cells were grown in LB media containing 2 μM sodium selenite, 100 μg/ml ampicillin, and 25 μg/ml chloramphenicol at 37 °C. IPTG (0.1 mM) was added when the culture reached an optical density at 600 nm of ~0.6–0.8, and the cells were cultured for an additional 4 h at 30 °C. After centrifugation, the cell pellets were resuspended in extraction buffer (50 mM sodium phosphate, pH 7.0, 300 mM NaCl, 10 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride) and lysed by sonication. The supernatant of the lysate was then loaded onto a Talon-metal affinity resin (Clontech), washed with extraction buffer, and eluted with buffer containing 50 mM sodium phosphate (pH 7.0), 50 mM NaCl, and 150 mM imidazole. The eluted proteins were concentrated and dialyzed against 50 mM sodium phosphate (pH 7.5) and 50 mM NaCl. The typical yield of the purified selenoprotein Trx1 was 40–70 μg from 500 ml culture broth based on Western blot assays.

To express the *T. denticola* Cys-containing Trx forms (tTrx1/U32C or tTrx2) or Trp mutant tTrx1/U32W, *E. coli* BL21(DE3) cells transformed with the corresponding plasmids were cultivated in LB media containing 100 μg/ml ampicillin at 37 °C until the optical density at 600 nm reached ~0.6–0.8, at which time 0.1 mM IPTG was added and the cells were cultured for an additional 4 h at 30 °C. The proteins were purified as described above for selenoprotein tTrx1. We also expressed and purified *E. coli* Trx1 (eTrx1) by a Talon-metal affinity chromatography as previously described [14]. The samples were analyzed for purity by SDS–PAGE and found to consist almost exclusively of the ectopic protein.

2.3. ^{75}Se metabolic labeling

To verify expression of *T. denticola* selenoprotein Trx1 in *E. coli*, ^{75}Se metabolic labeling was performed as previously described [15]. Briefly, *E. coli* BL21(DE3) cells transformed with an empty vector, pET21-Td-Trx1, or pET21-Td-Trx1/U32C were grown at 37 °C in 5 ml LB media containing ampicillin until the optical density at 600 nm reached ~0.6. Next, 0.05 mCi of freshly neutralized [^{75}Se] selenous acid and 1 mM IPTG were added to the cell culture, after which the cells were cultured at 37 °C for 4 h. The harvested cells were then washed with phosphate-buffered saline and lysed. A total of 30 μg protein was separated by SDS-PAGE and transferred onto a PVDF membrane. Finally, the ^{75}Se radioactivity pattern on the membrane was visualized using a PhosphorImager (GE Health Care).

2.4. Determination of protein concentration

Due to small amounts of purified recombinant selenoprotein tTrx1, the concentration of this selenoprotein was determined by Western blot analysis using anti-His antibodies, followed by quantification of the blot signals using the ImageJ (National Institutes of Health) program. The tTrx1/U32C protein was used as an internal standard. Concentrations of purified Cys-containing Trxs and Trp mutant tTrx1/U32W were determined by the Bradford method using a BioRad protein assay reagent and bovine serum albumin as a standard.

2.5. Trx activity assay and analysis of kinetics

Trx activity was measured by the insulin disulfide reduction assay as described by Holmgren [16]. The reaction mixture (200 μl) contained 100 mM sodium phosphate (pH 7.0), 2 mM EDTA, 5–100 μM insulin (Sigma–Aldrich), and 0.8–2 μM Trx. The reaction was started by the addition of 0.5 mM dithiothreitol. Reduction of insulin was monitored as an increase in turbidity at 650 nm for 50 min due to insulin precipitation. Non-enzymatic reduction of insulin by dithiothreitol was measured as a negative control. Trx activity was defined as the increase in absorbance per min (ΔA/min) in the interval below 1.0 of optical density. V_{max} and K_m values were determined by non-linear regression using the Prism 5 (GraphPad) software.

3. Results

3.1. Sec- and Cys-containing Trxs from T. denticola

The *T. denticola* genome contains at least seven selenoprotein genes (six known and one predicted) [10]. The known selenoproteins identified include SelD, glutathione peroxidase, glycine reductase A, two glycine reductase Bs, and Trx. Sec-containing Trxs have also been found in other bacteria, including *Geobacter metallireducens* and *Anaeromyxobacter dehalogenans*, but are absent from eukaryotes [10]. In addition, the *T. denticola* genome harbors a separate Cys-containing Trx. We designated the *T. denticola* selenoprotein Trx as tTrx1 and the Cys-containing Trx as tTrx2. tTrx1 consists of 107 amino acids and contains the ^{32}UPGC^{35} (U, Sec) catalytic motif (Fig. 1). There are no other Cys residues in the sequence. tTrx2 protein has 105 amino acids, including the catalytic ^{29}CVPC^{32} motif (Fig. 1). tTrx1 protein shows 29.4% identity with tTrx2. Both selenoprotein tTrx1 and Cys-containing tTrx2 belong to the typical small Trx family. Multiple sequence alignment revealed differences between *T. denticola* Trxs and other known Trxs around the catalytic CxxC motif (Fig. 1).