Selenite reduction by the thioredoxin system: kinetics and identification of protein-bound selenide

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Note

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Selenite (SeO$_3^{2-}$) assimilation into a bacterial selenoprotein depends on thioredoxin (trx) reductase in *Escherichia coli*, but the molecular mechanism has not been elucidated. The mineral-oil overlay method made it possible to carry out anaerobic enzyme assay, which demonstrated an initial lag-phase followed by time-dependent steady NADPH consumption with a positive cooperativity toward selenite and trx. SDS-PAGE/autoradiography using $^{75}$Se-labeled selenite as substrate revealed the formation of trx-bound selenide in the reaction mixture. The protein-bound selenium has metabolic significance in being stabilized in the divalent state, and it also produced the selenopersulphide (-S-SeH) form by the catalysis of *E. coli* trx reductase (TrxB).

Key words: thioredoxin reductase; selenite; *Escherichia coli*; anaerobic enzyme assay; selenopersulphide

Formate dehydrogenase H (FDH-H) is a bacterial selenoprotein that constitutes the anaerobic nitrate respiration system in *E. coli*. It has a catalytically essential selenocysteine (Sec) residue in its active site, which is incorporated into the growing polypeptide chains at in-frame opal codon UGA.$^{2,3}$ Although the mechanism of co-translational Sec insertion differs in several respects in the three domains of life forms, bacteria, eukaryotes, and archaea, it is universally conserved in that Sec is directed by the UGA codon and is synthesized on a specific tRNA$_{UGA}$ that is initially charged with L-serine.$^{4,5}$ It is also common to the three domains that the synthesis of Sec-tRNA$_{UGA}$ requires a highly reactive selenium donor compound, monoselphosphate, which is synthesized from ATP and selenide by selenophosphate synthetase (SPS), the selD product in the case of *E. coli*.$^{4,6}$

Uptake and facile assimilation of extracellular inorganic selenite (SeO$_3^{2-}$) into FDH-H served as a model system for studying the selenium uptake mechanism in the past decade. Because the concentration of selenium is normally very low in bacterial cells, SeO$_3^{2-}$ must be incorporated into selenoproteins through a specific pathway.$^{7,8}$ The metabolic pathway by which inorganic tetravalent selenium (IV) is reduced to divalent selenide in *E. coli* is perhaps distinct from the sulfate-reducing pathway.$^{9}$ An initial attempt to elucidate the reduction product, presumably selenide, was reported in several following studies that selenite reduction by GSH, the most abundant thiol in the cytoplasm.$^{9,11-13}$ However, at physiological pH levels, the GS-Se-SG intermediate can be very unstable, and it is rapidly decomposed to produce elemental selenium as end product.

Our preceding study indicated the physiological significance of the thioredoxin-dependent reducing system in bacterial selenite assimilation. The wild-type MC4100 strain and a glutathione reductase knockout (gor::tet) mutant produced FDH-H activity and incorporated $^{75}$Se-labeled selenite into the polypeptide of FDH-H, whereas the thioredoxin reductase knockout (trxB:::kan) strain failed to incorporate it into the bacterial selenoprotein.$^{14}$ This suggests that reduction of selenite in *E. coli* depends on the thioredoxin-reducing system, but the mechanism of trxB-dependent selenite assimilation is not clearly understood.

Kumar et al. first reported that sodium selenite is a good substrate for the *E. coli* trx system, causing oxygen-dependent, non-stoichiometric NADPH consumption under aerobic conditions and stoichiometric oxidation of 3 mol NADPH/mol selenite under anaerobic conditions.$^{15}$ It was also reported that the initial rates under aerobic and anaerobic conditions were nearly identical, suggesting that the non-stoichiometric NADPH-consumption resulted from a continued reaction by the reduction product, presumably selenide, which is highly sensitive to auto-oxidation. Although it was reported in several following studies that selenite can be bound to a pair of cysteine residues such as protein disulfide isomerase,$^{16,17}$ Kumar et al. concluded that the product was a free selenide, which mediates the reoxidation of trx by dissolved oxygen in the solution. Here we report the kinetics of *E. coli* trx-mediated selenite reduction with a novel positive cooperativity...
with respect to trx and selenite. We also succeeded in inidentifying stable selenium-bound trx in the reaction mixture. The formation of Se-bound trx requires catalytic turnover driven by NADPH and thioredoxin reductase (TrxB). Our findings strongly suggest that bacterial selenite assimilation involves a Se-bound trx, which functions as a specific Se-delivery protein.

For our in vitro kinetic study, approximately 6 mg of C-terminally His-tagged trx was purified from a 100-mL culture of E. coli BL21 (DE3) (Novagen, Madison, WI) harboring pET32a-trx-6xHis, which was constructed by introducing a TAA codon at the end of trx-tag in pET-32a plasmid (Novagen). The genes of introducing a TAA codon at the end of trx-tag in pET-harboring pET32a-trx-6xHis, which was constructed by culture of C-terminally His-tagged trx was purified from a 100-mL mixture. The formation of Se-bound trx requires with respect to trx and selenite. We also succeeded in inidentifying stable selenium-bound trx in the reaction mixture. The formation of Se-bound trx requires catalytic turnover driven by NADPH and thioredoxin reductase (TrxB). Our findings strongly suggest that bacterial selenite assimilation involves a Se-bound trx, which functions as a specific Se-delivery protein.

...and N-terminally 6xHis-tagged TrxB by DTNB-depend-state kinetic studies using C-terminally 6xHis-tagged trx and N-terminally 6xHis-tagged TrxB by DTNB-dependent spectrophotometric assay revealed that K_m and k_cat values for the recombinant trx were 1.26 ± 0.11 µM and 1,520 min⁻¹, close to the reported values of 1.25 µM and 1,320 min⁻¹ for the DTNB-dependent E. coli trx assay, suggesting that the 6xHis tags on the recombinant proteins did not disturb their catalytic functions significantly.

...A convenient, effective technique was developed to measure the rate of selenium reduction under anaerobic conditions simply by overlaying 50 µL of mineral oil (ABI, Tokyo) over the enzyme reaction mixture (1 mL) in the quartz cuvette. The components of solutions such as the buffer and substrates were thoroughly purged with argon gas before they were mixed, and the solutions were mixed beside a UV spectrometer in the open air. Additional purging may be required to obtain a reproducible NADPH consumption rate after several measurements due to gradual air-oxidation of the reserved solutions. Accordingly, selenium reduction was performed in 1 mL of reaction mixture containing 40 mM HEPES pH 7.2, 20 mM KCl, 3 mM MgCl₂, 0.1 mM EDTA, 0.2 mM NADPH, 10 units of SOD, and 10 units of catalase. NADPH-dependent selenium reduction was carried out using the recombinant E. coli TrxB at a concentration of 1.2 µM in the presence of various concentrations of recombinant trx in a range of 0–1.2 µM. The concentrations of sodium selenite were 50, 125, and 250 µM.

Selenium served as an efficient oxidant for the recombinant trx system, as has been reported for native proteins, and the absorption at 340 nm did not decrease when a component of the thioredoxin system, trx, TrxB, or NADPH, was omitted from the reaction mixture. A redox-inert trx protein bearing a Cys32Ser point mutation did not show selenite-dependent NADPH consumption, suggesting that selenium reduction occurs at the active site of trx. Catalysis was initiated by the addition of trx, the time course of the enzyme reaction showed a clear lag in the initial stage of the enzyme reaction, and the steady-state rate was obtained from the linear decrease in absorption at 340 nm after the lag phase (Fig. 1A). The initial reaction rate reached maximum velocity, V_max, when the concentration of trx was increased to 1.2 µM (Fig. 1B), and V_max varied significantly as a function of the selenium concentration (Table 1). The maximum velocity for NADPH consumption increased as the concentration of Na₂SeO₃ increased from 50 to 250 µM (Fig. 1C). The plot of the trx concentration versus the NADPH oxidation rate in the presence of 250 µM and 50 µM sodium selenite. C. Effects of sodium selenite concentrations on the NADPH oxidation rate in the presence of 0.04 (○), 0.12 (△), 0.48 (▲), and 1.2 µM (■) trx. The V_max values for varioustrx concentrations are summarized in Table 1.
Our kinetic studies using a recombinant trx system indicated for the first time that enzymatic selenite reduction shows remarkable sigmoid kinetics with respect to selenite and trx concentrations. Because TrxB did not show such cooperativity when insulin or GSSG was used as the terminal oxidant for trx turnover, the substrate in the present NADPH consumption is not likely to be oxidized trx. To identify the substrate compound generated in the reaction mixture, $^{75}\text{Se}$-labeled selenite was added to the reaction mixture, and the proteins in the solution were developed on SDS–PAGE in the absence of $\beta$-mercaptoethanol. $^{75}\text{Se}$-Labeled proteins subsequently visualized by autoradiography were identified by their molecular masses after Coomassie Blue staining. Most strikingly, trx was efficiently labeled with radioactive selenium in the complete mixture (Fig. 2, lane 1). The solution in which TrxB was omitted also allowed trx to be labeled with $^{75}\text{Se}$, but much less efficiently (lane 2). The selenium-bound trx accumulated without TrxB presumably indicated the formation of $\text{Trx-S-SeO}_3^-$ adduct form. A very small amount of selenium was also bound to TrxB when trx or NADPH was absent from the solution (lanes 3–4). In the case of the absence of trx, TrxB was labeled with a very small amount of selenium during incubation. In contrast, the absence of reducing equivalent NADPH, resulted in poor labeling on trx and TrxB, suggesting that the formation of $^{75}\text{Se}$-labeled trx and TrxB to lesser extent depends on the enzymatic reaction.

Sulfur transferases and sulfur-specific delivery proteins have been identified and characterized in detail in sulfur metabolic pathways.²¹,²² On this analogy, the involvement of specific selenium-delivery proteins was proposed based on two major biochemical factors. First, in vitro assay of bacterial SPS normally requires high levels of selenide and diithiothreitol, and yet the apparent $K_m$ value for the selenide anion is reportedly as high as 20 mM, far above the optimal concentrations of selenium for the growth of various bacterial species.²³ In fact, levels above 10 mM selenium are highly toxic to many bacterial species. Secondly, effective selenium assimilation requires a specific gateway mechanism that discriminates selenium from sulfur, which is 1,000-fold more abundant in cells. Also, a hypothetical selenium-transport protein must fulfill conflicting requirements: it must produce a stable complex with selenium so as to discriminate selenium from sulfur, but at the same time the delivery protein must liberate divalent selenide for SPS. Rhodanese-like proteins have been postulated to be selenium-carrier proteins, which perhaps form the selenopersulfide intermediate state at the active site (Protein-S-SeH)²⁴,²⁵ Ambiguity remains, however, about this selenopersulfide intermediate due to the lack of chemical evidence for the selenopersulfide compounds (-S-SeH). It has been found experimentally that protein-bound selenium is unexpectedly stable. It even required a dithiol reductant such as 2 mM reduced lipoic acid, when the bound selenide was released from glycerlaldehyde-3-phosphate dehydrogenase (GAPDH).²⁴,²⁵ Although selenopersulfide is one of the chemical forms most likely to be generated, the protein-bound selenium might yet be a selenotrisulfide form with one equivalent of glutathione retained on the protein-bound selenide, GAPDH-S-Se-SG. In fact, it has been reported that binding of the selenotrisulfide form of penicillamine (Pen-S-Se-S-Pen) to the Cys residue of hemoglobin depends essentially on the thiol exchange reaction.²⁶,²⁷ In contrast to the low efficiency of mono-Cys type carrier proteins, the trx system has the advantage of being implemented with NADPH-dependent reducing machinery. The non-stoichiometric oxidative burst in the presence of oxygen strongly suggests that a transitory selenopersulfide is generated at the active site of trx. It remains to be determined whether the catalytic formation of selenopersulfide on trx can serve as an efficient substrate for bacterial SPS.

Acknowledgments

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References