Electronic supplementary material for

A paradigm for biological sulfur transfers *via* persulfide groups: a persulfide-disulfide-thiol cycle in 4-thiouridine biosynthesis

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Materials and methods

Materials. Unless otherwise specified, all materials were purchased from Fisher Scientific (Pittsburgh, Pennsylvania, USA) or its Acros Organics division and used as provided. Sephadex G-25 (DNA grade) and L-[^{35}S]cysteine were purchased from Amersham Biosciences (Piscataway New Jersey, USA). Nuclease P1, dithiothreitol (DTT), ATP, and Tris were purchased from Roche Molecular Biochemicals (Indianapolis, Indiana, USA). A Higgins Analytical CLIPEUS C_{18} 5 μM column (50 x 4.6 mm) was purchased from Bodman Industries (Aston, Pennsylvania, USA). The tRNA substrate was the *in vitro* transcript of *E. coli* $tRNA^{Phe}$, the preparation of which we have described elsewhere[1]. The overexpression, purification, and storage of IscS and ThiI were accomplished using methods that we have previously described in detail[2]. The IscS used in the described experiments bears the C-terminal His_6 tag encoded by pET29b (Novagen, Madison, Wisconsin, USA). All of the ThiI variants used in the described experiments contain the 20-amino acid N-terminal His_6 tag encoded by pET15b (Novagen); however, all amino acid positions are numbered in terms of native ThiI (with no N-terminal His_6 tag).

SDS-PAGE analysis of sulfur transfer. A typical assay (50 μL) was 50 mM Tris•HCl buffer, pH 7.6, containing KCl (50 mM), $MgCl_2$ (10 mM), PLP (20 μM), [^{35}S]cysteine (10 μM, 40,000 Ci/mol), IscS (5.6 μM), and ThiI (1.4 μM). Reactions were initiated by the addition of

recombinant ThiI and incubated at 37 °C for 30 minutes. Aliquots (20 μL) were removed and mixed with non-reducing sample loading buffer (20 μL), which is 125 mM Tris•HCl buffer, pH 6.8, containing glycerol (20% vol/vol), SDS (4% wt/vol), and bromophenol blue. Samples were incubated at 70 °C for 2-5 min and then loaded onto a 10% polyacrylamide gel (7.4 cm x 8.0 cm with a 4% stacking gel) containing SDS (1% wt/vol). After 70 min at 150 V, the gel was placed in a Phosphor Screen cassette (35 x 43 cm); after 2-3 days, the gel was visualized using a PhosphorImager (Molecular Dynamics, now Amersham Biosciences) and ImageQuant software, version 5.0, and then stained with Coomassie Blue to visualize total protein and confirm loading levels (data not shown). Four replicates using different protein preparations gave qualitatively consistent results.

Pre-loading IscS. For single turnover s^4U generation assays (see below), the recombinant IscS was first "pre-loaded" with ^{35}S to generate the persulfide form of the enzyme. A pre-loading reaction (150 μL) was 50 mM Tris•HCl buffer, pH 8.5, containing $MgCl_2$ (5 mM), PLP (40 μM), [^{35}S]cysteine (300 μM, 667 Ci/mol), and IscS (25 μM). After 20 min at 37 °C, the reaction mixture was freed of [^{35}S]cysteine and other small molecules by spin size exclusion chromatography over a column (0.8 x 1.6 cm) of Sephadex G-25. The eluate, which contains the pre-loaded IscS, was then used as described below.

Single-turnover s^4U generation assays. Single-turnover assays were run in the absence of exogenous reductants. A typical single-turnover assay (150 μL) was 50 mM potassium phosphate buffer, pH 8.5, containing $MgCl_2$ (5 mM), ATP (4 mM), PLP (120 μM), tRNA (5 μM), pre-loaded IscS (1 or 2 μM total IscS polypeptide), and ThiI (10-300 nM). The spin column eluate containing pre-loaded IscS was added as quickly as possible to the assay mixture, which was complete except that the ThiI was contained in a droplet on the side of the tube. To

initiate the reaction, the tubes were spun and then mixed by vortexing immediately after the addition of eluate. After 3 h at 37 °C, the reaction mixture was quenched by spin size exclusion chromatography over a column (0.8 x 1.6 cm) of fresh† Sephadex G-25 resin to remove small molecules. The [^{35}S]s^{4}U in tRNA was quantified by subjecting the eluate to the total digestion/HPLC method detailed elsewhere[3]. Briefly, the tRNA was digested by incubation with nuclease P1 and alkaline phosphatase[4]; the resulting nucleosides were resolved by HPLC using a C_{18} column and a gradient of acetonitrile in aqueous buffer[5], and [^{35}S]s^{4}U was quantitated by in-line scintillation counting.

Aging of Pre-Loaded IscS. IscS was pre-loaded as described above, but the spin column eluate was incubated at 37 °C for various times (0-120 min) before use. To assess the rate of decay of the IscS persulfide group, the aged IscS was used in single turnover assays as described above except that the concentration of ThiI (100 nM) was held constant. Production of s^{4}U fell with time in a first-order process (Figure S1).

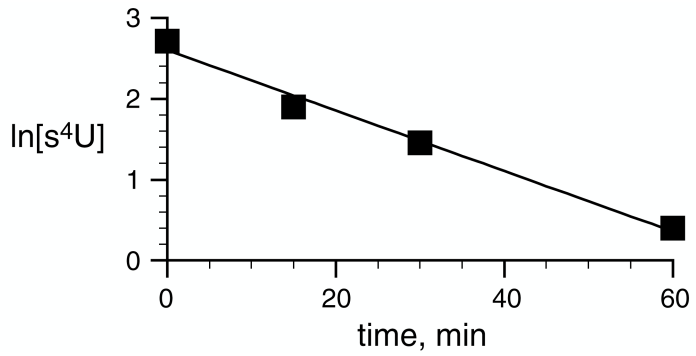

Fig. S1 The decay of the ability of pre-loaded IscS to support s^{4}U generation. The linear fit yields k_{decay} = 0.037 min^{-1}. The fit for first order decay (R^2 = 0.99) is better than the fits for zero order decay (R^2 = 0.81) and second order decay (R^2 = 0.94).

Recovery of Activity from Aged IscS. To assess the effect of aging on IscS activity, the pre-loaded IscS was incubated for 3 h at 37 °C. It was then added (to a final concentration of 4 nM) to a standard[2] s^4U generation assay (100 μL), which was 50 mM Tris•HCl buffer, pH 8.5, containing $MgCl_2$ (5 mM), ATP (4 mM), PLP (40 μM), [^{35}S] cysteine (500 μM, 150 Ci/mol), DTT (1 mM), tRNA (1 μM), IscS (4 nM), and ThiI (1 nM). After various times (10 - 60 min) at 37 °C, reaction mixtures were passed over a Sephadex G-25 spin column to remove small molecules, thereby quenching the reaction. [^{35}S]s^4U was quantitated by total digestion of tRNA and HPLC of the resulting nucleosides as described above[3].

Note and references

†Irreproducible results are obtained if the resin has been previously used in s^4U generation assays, even after the resin has been exhaustively washed with buffer.

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