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

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In support of the key features of sulfur transfer in the proposed mechanisms of 4-thiouridine generation, the enzyme ThiI can turn over only once in the absence of reductants of disulfide bonds, and Cys-456 of ThiI receives the sulfur transferred from the persulfide group of the sulfurtransferase IscS.

Many bacteria convert uridine-8 in tRNA to 4-thiouridine, s^{4}U, which serves as a photosensor. In tRNA, the s^{4}U lies next to cytidine-13, and the two bases become cross-linked by a photo-induced 2 + 2 cycloaddition when the bacterium is exposed to near-UV light.[1,2] Such cross-linking makes tRNAs poor substrates for aminoacylation,[3] so protein synthesis stops. The cells enter growth arrest until the near-UV exposure ends and newly made tRNA (not cross-linked) can support renewed protein synthesis. The free amino acid cysteine serves as the overall sulfur donor for s^{4}U biosynthesis,[4] and the reaction requires ATP.[5] The two enzymes responsible for converting uridine to s^{4}U are ThiI[6] and IscS,[7] named for their roles in the biosynthesis of thiamin[8,9] and iron–sulfur clusters,[10] two physiologically critical cofactors that contain sulfur. Elucidation of the mechanism of s^{4}U generation, then, provides insight into other biological sulfur transfer processes.

IscS uses the cofactor pyridoxal 5′-phosphate to transfer sulfur from substrate cysteine to an active site Cys§ to generate an enzymic persulfide group and free alanine.[10,11] We reasoned that the terminal sulfur of the IscS persulfide group would be transferred to ThiI to make a new persulfide group and then on to tRNA. We noted that Cys-456 of ThiI aligns with the active site Cys of sulfur transferases that proceed through a persulfide intermediate, and we used site-directed mutagenesis to demonstrate that Cys-456 is essential for s^{4}U generation.[12] We proposed two alternate mechanisms based on these results (Fig. 1), and both invoke the formation of a persulfide group on Cys-456 of ThiI at the expense of the IscS persulfide group. Consistent with the proposed mechanisms, Kambampati and Lauhon demonstrated that sulfur flows from cysteine to IscS to ThiI to tRNA.[13] Both proposed mechanisms also postulate formation of a disulfide bond between Cys-456 and a second Cys. Sequence analysis implicated Cys-344 for that role, and we found that C344A ThiI is drastically impaired *in vitro*, so much so that it does not function *in vivo*.[14] If the Cys-456–Cys-344 disulfide bond forms during catalysis, then the exclusion of reductant will prevent more than a single turnover of ThiI. When the reaction was run without exogenous reductant and a minimal cysteine concentration, 0.7 equivalent of s^{4}U was produced relative to ThiI.[14] However, only a single concentration of ThiI (6 μM) was examined, which is insufficient to establish the dependence of s^{4}U production on the concentration of ThiI. After the reaction, the number of free thiol groups in ThiI had decreased significantly but not to the full extent predicted for the amount of s^{4}U produced, likely because the resulting disulfide bonds were partially reduced by the substrate cysteine.[14] It remained possible, however, that nonspecific oxidation of ThiI both decreased the number of free thiol groups and inactivated the enzyme. We now report a direct probe of the chemical events at Cys-456 and single turnover experiments over a range of ThiI concentrations that strongly support the proposed persulfide–disulfide–thiol cycle at Cys-456 of ThiI.

To achieve true single turnover conditions, IscS was 'pre-loaded' in the persulfide form by incubation with [^{35}S]cysteine followed by spin size-exclusion chromatography to remove small molecules. Aliquots of the eluate were immediately added to s^{4}U generation assays containing $MgCl_2$, ATP, tRNA, and a variable concentration of ThiI (5–300 nM). After 3 h,¶ [^{35}S]s^{4}U was quantitated by total enzymatic digestion of tRNA, separation of the resulting nucleosides by reverse phase HPLC, and in-line scintillation counting as we have previously described.[12] Production of s^{4}U generation closely approaches one equivalent at lower concentrations of ThiI then plateaus at higher concentrations of ThiI (Fig. 2). The level of the plateau is twice as high when the concentration of IscS is doubled, strongly suggesting that the plateau reflects the amount of competent IscS persulfide that can be generated and transferred to the assay. To test this hypothesis more fully, several controls were performed. First, when IscS is omitted from the pre-loading reaction, the spin column eluate cannot support s^{4}U generation

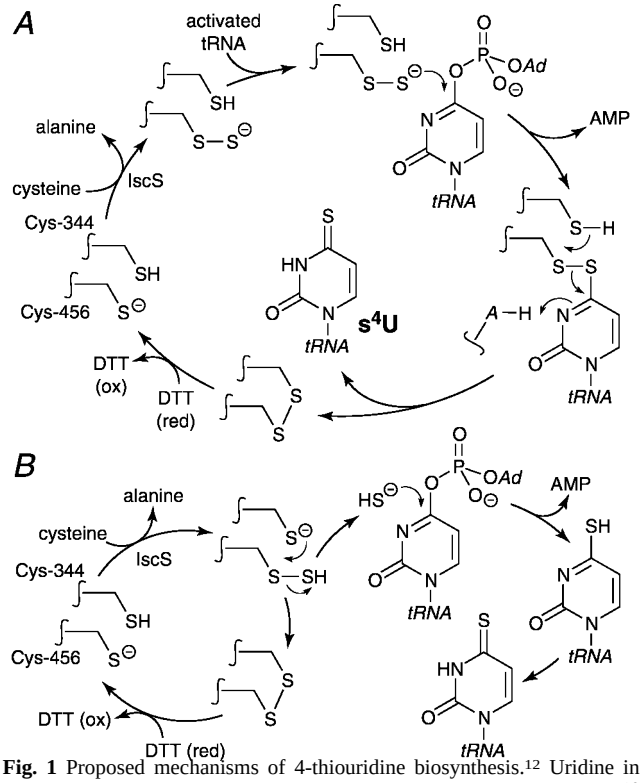

Fig. 1 Proposed mechanisms of 4-thiouridine biosynthesis.[12] Uridine in tRNA is depicted after its activation by adenylation at the expense of ATP.[20] The typical *in vitro* reductant dithiothreitol (DTT) is shown since the physiological reductant remains unknown. *A*, the persulfide group on Cys-456 nucleophilically attacks the activated uridine. *B*, disulfide bond formation generates bisulfide, which attacks the uridine adenylate. *Ad*, adenosine; AMP, adenosine 5′-monophosphate.

† Electronic supplementary information (ESI) available: complete experimental details. See http://www.rsc.org/suppdata/cc/b2/b208626c/

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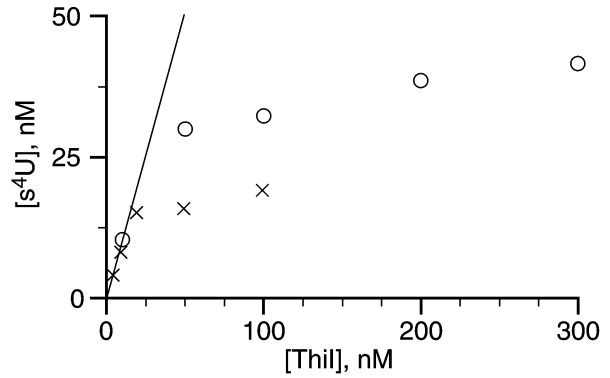

Fig. 2 Single turnover experiments. The line shows the expected results for a single turnover of ThiI. (×) $[IscS]_{total}$ = 1 μM; (○) $[IscS]_{total}$ = 2 μM.

when it and IscS (12.5 μM) are added to the reaction mixture with ThiI (100 nM), which demonstrates that the plateaus do not arise from [^{35}S]cysteine that makes it through the spin column. Second, quantitation of the ^{35}S in spin column eluates indicates that at most 3.8% of the IscS is in the persulfide form, so that the plateau concentrations of s^{4}U (20 nM; 40 nM) are roughly half of the maximum amount of IscS persulfide (38 nM; 76 nM). Third, addition of DTT to the assay mixtures containing lower concentrations of ThiI increased s^{4}U production to the observed plateau but not higher. Fourth, addition of DTT to the assay mixtures containing higher concentrations of ThiI did not result in greater s^{4}U production, demonstrating that oxidative inactivation of ThiI is not responsible for the plateaus. Fifth, aging the pre-loaded IscS resulted in a decay of s^{4}U production that followed first-order kinetics ($t_{1/2}$ = 18.6 min); this result is consistent with the reported decomposition of the persulfide group to yield colloidal S^0 and regenerate the thiol form of IscS.[10] To examine the effect of the aging on IscS activity, small molecules were removed from aged samples of pre-loaded IscS by spin size-exclusion chromatography, and the enzyme (40 nM) was then checked for its ability to support s^{4}U generation in a standard assay[14] containing ThiI (10 nM) and DTT (1 mM). The pre-loaded and aged IscS behaved identically to untreated IscS, showing that fully active IscS is regenerated as expected for the proposed decomposition route. The single turnover experiments, then, demonstrate that ThiI can turn over only once in the absence of a reductant and that the amount of s^{4}U generated depends on the level of competent IscS persulfide, which is precisely the behavior predicted by the proposed mechanisms (Fig. 1).

To address the roles of Cys-344 and Cys-456, the radiolabeling/SDS-PAGE method of Kambampati and Lauhon[13] was employed. IscS was incubated with [^{35}S]cysteine in the presence of wild-type, C344A, or C456A ThiI. After 30 min, aliquots were subjected to non-reducing SDS-PAGE analysis. C456A ThiI remains unlabeled while wild-type and C344A ThiI become labeled (Fig. 3), as expected if Cys-456 is the site of persulfide group formation in the transfer of sulfur from IscS. If the sample treatment buffer contains β-mercaptoethanol as well as SDS, no label is retained by any of the proteins, consistent with the location of ^{35}S in the terminal position of a persulfide group rather than in some other covalent linkage. Closer examination of the autoradiogram (Fig. 3) reveals that C344A ThiI is labeled to a greater extent than wild-type ThiI (IscS serves as an internal standard). The differential labeling

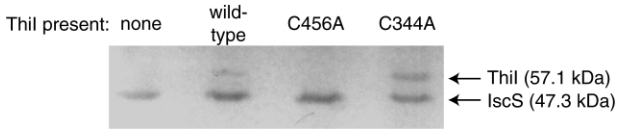

Fig. 3 Labeling of IscS (5.6 μM) and variant ThiI (1.4 μM) by incubation with [^{35}S]cysteine. IscS was present in all reactions. C344A ThiI is labeled more strongly than wild-type ThiI, which indicates the formation of a disulfide bond between Cys-344 and Cys-456 with concomitant loss of [^{35}S]bisulfide.

supports the formation of a disulfide bond between Cys-344 and Cys-456 because that process releases the ^{35}S from ThiI. This result does not, however, distinguish the alternate mechanisms (Fig. 1) since both include the disulfide bond and bisulfide is a sufficiently good leaving group to allow the reaction even if bisulfide is not the normal leaving group in s^{4}U generation.

The experiments reported here, then, establish key features of sulfur transfer in the biosynthesis of s^{4}U. The true single turnover experiments provide a clean demonstration that ThiI can only turnover once in the absence of exogenous reductant as expected for a reaction mechanism with a disulfide intermediate. The results from the radiolabeling experiments are the first direct chemical evidence for the formation of the key persulfide intermediate on Cys-456 of ThiI. The reduced labeling of wild-type relative to C344A ThiI strongly supports the formation of a disulfide bond between Cys-344 and Cys-456 and demonstrates the feasibility of bisulfide generation from the persulfide group on Cys-456. A cognate reaction is a very reasonable mode for the delivery of sulfide to iron–sulfur clusters *via* the IscS persulfide group: after formation of a persulfide group on either an apoenzyme or on a 'scaffold' protein such as IscU[15,16] or NifU,[17] a second Cys would displace sulfide (perhaps already coordinated to iron) for incorporation into a growing cluster. The establishment of the fundamental features of sulfur transfer by ThiI in s^{4}U generation will also inform experiments to elucidate its role in thiamine biosynthesis.[8,9,18,19] The presented evidence for the persulfide–disulfide–thiol cycle at Cys-456 of ThiI, then, confirms the shared features of sulfur transfer in the mechanisms proposed for s^{4}U generation (Fig. 1) and provides a paradigm for the study of sulfur incorporation processes in other metabolic pathways.

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Notes and references

§ In the text, 'cysteine' denotes the free amino acid while 'Cys' denotes a cysteine residue in a protein.

¶ In assays containing ThiI (10 nM), IscS (40 nM) and DTT (1 mM), the incubation time is sufficient for production of 3.2 μM s^{4}U, roughly 1000-fold greater than the plateau concentrations of s^{4}U.

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