Direct evidence for enzyme persulfide and disulfide intermediates during 4-thiouridine biosynthesis†

Chapman M. Wright, Glenn D. Christman, Ann M. Snellinger, Murray V. Johnston and Eugene G. Mueller*

Received (in Cambridge, MA, USA) 20th March 2006, Accepted 31st May 2006
First published as an Advance Article on the web 14th June 2006
DOI: 10.1039/b604040c

Two proposed mechanisms for 4-thiouridine generation share key cysteine persulfide and disulfide intermediates, and indirect evidence of their existence has been previously reported; chemical trapping and mass spectrometry have now provided direct and definitive evidence of these key intermediates.

Over 100 post-transcriptional modifications are made to nucleosides in rRNA and tRNA,[1] including 4-thiouridine (s^4U). This thionucleoside occurs at position 8 of bacterial tRNAs and undergoes a 2 + 2 cycloaddition reaction with cytidine-13 when exposed to near-UV light; the resulting cross-link leads to an accumulation of uncharged tRNAs in the bacterium and thereby causes a growth arrest, which protects the cell from the effects of UV exposure.[2] A screen based on near-UV light exposure revealed that ThiI, an enzyme shared with *thi*amin biosynthesis,[3] is essential for s^4U biosynthesis.[4] The PLP-dependent cysteine desulfurase IscS is also required for the biosynthesis of s^4U.[5] IscS forms a persulfide (*R*–S–SH) group on an active site Cys‡ at the expense of free cysteine[6] and then transfers the terminal sulfur to ThiI, which in turn transfers it to tRNA.[7]

Cys-456 and Cys-344 of ThiI are conserved and critical for s^4U production.[8,9] Cys-456 lies in a C-terminal domain that is homologous to the sulfur transferase rhodanese, which operates by forming a transient persulfide group on the active site Cys that aligns with Cys-456 of ThiI.[8] When [^{35}S]cysteine is the substrate, Cys-456 of ThiI becomes radiolabeled, and the label is lost upon treatment with a reductant, as expected for persulfide formation.[10] These pieces of evidence allowed us to propose two chemically reasonable mechanisms for s^4U generation (Fig. 1). However, Cys trisulfide (*R*–S–S–SH) or higher order polysulfide groups have been generated on IscU, a "scaffold protein" for Fe–S cluster biosynthesis, upon incubation with IscS and cysteine,[11] so we sought direct evidence for the formation of a persulfide rather than a higher order polysulfide group using MALDI-TOF mass spectrometry (MALDI-MS).

A ThiI variant in which Cys-344 is replaced with Ala was chosen for these experiments to maximize the stability of the persulfide (or trisulfide) group on Cys-456. To avoid side reactions between it and the other three Cys of ThiI, they were also replaced with Ala to generate C108/202/207/344A ThiI ("C456only ThiI" for simplicity). This enzyme is 2,900-fold less active than wild-type ThiI, which is essentially identical to the activity of C344A ThiI, as expected because the separate substitution of Cys-207 had only a 4-fold effect on the rate of s^4U generation and substitution of Cys-108 and Cys-202 had essentially no effect.[9] C456only ThiI was incubated with cysteine and IscS to form a persulfide (or trisulfide) group at Cys-456. After removal of free cysteine by spin size-exclusion chromatography, the nucleophilic group at Cys-456 was trapped by treatment with *I*-AEDANS, a fluorescent derivative of iodoacetamide. The treatment labeled ThiI, and the fluorophore was lost upon the addition of a reductant (Fig. 2), which verifies that S–S bonds connect the protein and label, as expected for a persulfide or trisulfide group on Cys-456. To establish the identity of that group definitively, a sample of the fluorescently labeled ThiI was separated from IscS and digested with trypsin; the resulting peptides were analyzed by MALDI-MS. A peptide corresponding to a Cys-456 persulfide group alkylated by *I*-AEDANS was detected (1444.79 *m*/*z*, 1444.62 m/z_{pred}), and as expected, reduction replaced that peptide with one bearing a thiol group at Cys-456 (Fig. 2). No peptides corresponding to a

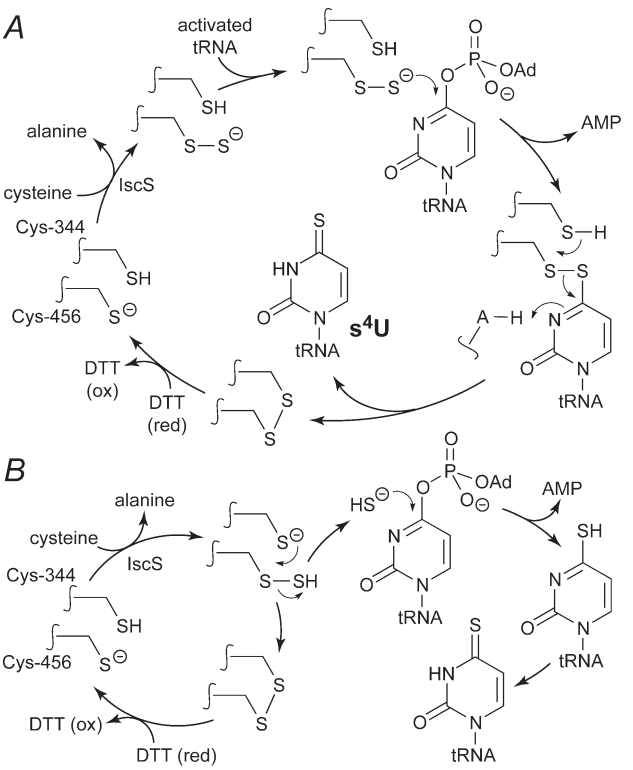

Fig. 1 Proposed mechanisms of 4-thiouridine biosynthesis. Uridine-8 in tRNA is depicted after adenylation at the expense of ATP. The two mechanisms differ mainly in which sulfur species attacks uridine.

Department of Chemistry and Biochemistry, University of Delaware, Newark, DE 19716. E-mail: emueller@udel.edu; Fax: +1 302 831 6335
† Electronic supplementary information (ESI) available: Experimental procedures and additional data. See DOI: 10.1039/b604040c

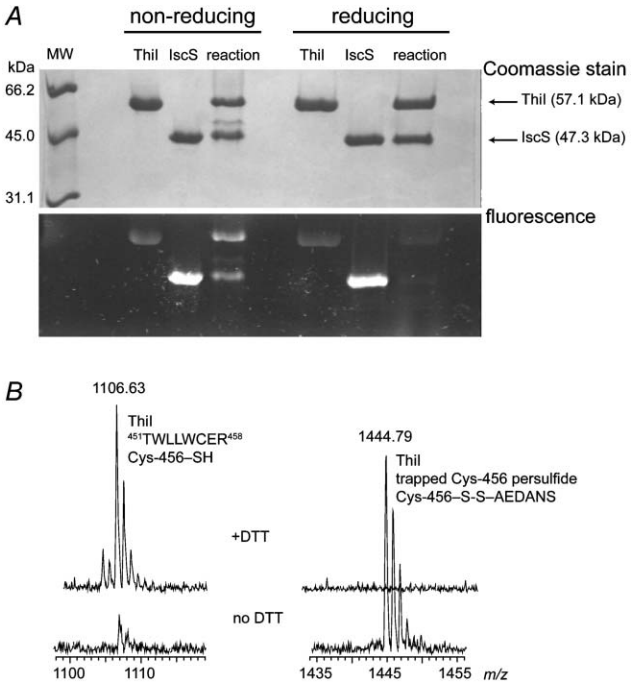

Fig. 2 Detection of a persulfide group at Cys-456 of ThiI. A, SDS-PAGE analysis of C456only ThiI, IscS, and the two proteins after they were incubated together in the presence of cysteine. Under nonreducing conditions, IscS and C456only ThiI are fluorescently labeled in all samples; reduction removes the label in the reaction lane (denoting disulfide linkages), but the label remains in mixtures containing IscS or C456only ThiI alone (denoting thioether linkages). B, Partial MALDI-MS spectra of tryptic peptides from C456only ThiI after incubation with IscS and cysteine and treatment with *I*-AEDANS. The alkylated form of the Cys-456 persulfide group is detected (bottom spectrum); upon addition of DTT, the peptide shifts to lower mass reflecting a free thiol group on Cys-456 (top spectrum).

trisulfide or higher order polysulfide group at Cys-456 were detected. These results demonstrate definitively that a persulfide group forms at Cys-456 of ThiI under conditions that support the efficient formation of s^4U.

Another feature common to both mechanisms proposed for ThiI is the formation of a disulfide bond between Cys-456 and Cys-344. Disulfide bond formation was originally postulated because an exogenous reductant was required for efficient s^4U production, and additional indirect evidence strongly supported a catalytic disulfide bond. In the absence of exogenous reductants, ThiI produced at the most one equivalent of s^4U,[10] and fewer thiol groups were found on ThiI after such single turnover reactions.[9] To establish the disulfide bond between Cys-456 and Cys-344 definitively, MALDI-MS was again employed. These experiments could be confounded by disulfide bond migration during sample work-up, so a ThiI variant with only the two active site Cys was constructed (C108/202/207A ThiI). The activity of this "triple mutant" ThiI is down only 4-fold from the wild-type level, which is essentially the sum of the effects of the three individual substitutions.[9] Single turnover conditions were used to investigate disulfide bond formation. Triple mutant ThiI was incubated with IscS, ATP, tRNA, and cysteine, and the mixture was passed over a Sephadex G-25 spin column to remove unreacted cysteine (a reductant). The unreacted Cys of ThiI and the Cys of IscS were blocked with iodoacetamide to prevent disulfide bond migration, and the sample was digested with trypsin and analyzed by MALDI-MS (Fig. 3). The disulfide-linked peptide containing Cys-456 and Cys-344 (2331.12 m/z, 2331.09 m/z_{pred}) was clearly present. After the addition of reductant and then excess iodoacetamide, the disulfide-linked peptide disappeared, and the separate alkylated peptides containing Cys-456 (1163.82 m/z, 1163.57 m/z_{pred}) and Cys-344 (1284.80 m/z, 1284.39 m/z_{pred}) appeared.

These results provide strong evidence that a disulfide bond forms between Cys-456 and Cys-344 during turnover, but the tryptic peptide containing the active site Cys of IscS disulfide-bonded to substrate cysteine (2332.08 m/z_{pred}) would overlap with the expected disulfide-linked peptide from ThiI. The low intensity of the peptide containing Cys-344 (^{339}TMPEYCGVISK^{349}) is not worrisome in itself because MALDI-MS is not rigorously quantitative and the peptide ends in Lys, which lessens its intensity relative to peptides terminated with a more readily ionizable Arg.[12] This phenomenon, however, could have frustrated the detection of a Cys-344–Cys-344 disulfide-linked peptide, even if it were present in a substantial amount. Lys-349 was, therefore, replaced with Arg in the context of triple mutant ThiI to increase the intensity of peptides containing Cys-344 and to shift the disulfide-linkage peptide containing Cys-344 and Cys-456, thus establishing its identity conclusively. The substitution did not affect enzymatic activity, and the single turnover experiment and subsequent analysis were repeated with K349R triple mutant ThiI.

As expected from the substitution, the disulfide-linked peptide was mass-shifted (2359.54 m/z, 2359.10 m/z_{pred}) and replaced upon reduction and alkylation by the unshifted peptide containing Cys-456 and a peptide containing Cys-344 (1312.92 m/z, 1312.57 m/z_{pred}) that was more intense and mass-shifted by the change of Lys to Arg (see ESI†, Fig. S1). No other disulfide-linked peptides were observed. Furthermore, nonreducing SDS-PAGE analysis has revealed that ThiI migrates as a monomer after a single turnover (see ESI†, Fig. S2), showing that the disulfide bond is intra- rather than intermolecular. Together, these results confirm that a disulfide bond forms between Cys-456 and Cys-344 on the same ThiI polypeptide during the generation of s^4U.

The work presented here definitively establishes key persulfide and disulfide intermediates in the biosynthesis of s^4U but leaves

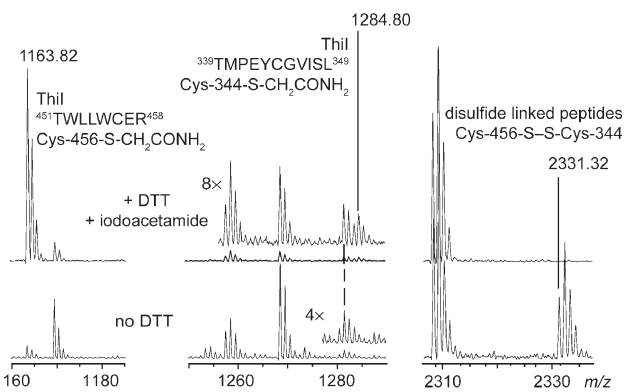

Fig. 3 Partial MALDI-MS spectra of tryptic peptides from triple mutant ThiI after a single turnover. Initially, Cys-344 and Cys-456 are linked by a disulfide bond (bottom spectrum). When DTT is added, the disulfide-linked peptide is replaced with the separate peptides containing Cys-344 and Cys-456 (top spectrum).

some mechanistic questions unanswered. Recently, intermediate sulfur carrier proteins have been discovered in the chemically similar biosynthesis of 2-thiouridine in the anticodon of certain tRNA.[13] The discovery of the sulfur carrier proteins in a related biosynthetic pathways raises the question of whether similar proteins function in s^4U biosynthesis. The sulfur carrier proteins conduct sulfur in the form of persulfide groups from IscS to tRNA bound to MnmA,[13] the enzyme that catalyzes the thiolation of uridine derivatives to make 2-thiouridine in tRNA.[14] Previous work had demonstrated that IscS and MnmA alone were capable of generating 2-thiouridine *in vitro* but at a puzzlingly sluggish rate, some two orders of magnitude slower than s^4U production by ThiI and IscS.[14] The slow rate of 2-thiouridine formation was a major impetus to search for other proteins that may be involved, and inclusion of the sulfur carrier proteins enhances the rate of *in vitro* 2-thiouridine formation by two orders of magnitude over the rate with IscS and MnmA alone.[13] The rate of s^4U synthesis by ThiI and IscS matches the rate of 2-thiouridine formation by the complete system (including the sulfur carrier proteins),[13] which argues for the sufficiency of ThiI and IscS alone to support s^4U biosynthesis at a reasonable rate. Furthermore, ^{35}S-labeling studies have provided evidence for persulfide group formation on the carrier proteins,[13] but no such evidence for a persulfide group on MnmA has been offered despite explicit attempts to detect it.[13,14] As we have now demonstrated unequivocally that Cys-456 bears a persulfide group upon incubation with IscS and cysteine, no intermediate sulfur carrier protein is chemically required. Due to these kinetic and chemical considerations, we consider it unlikely that proteins other than ThiI and IscS are responsible for s^4U biosynthesis.

Finally, the key persulfide and disulfide intermediates in the biosynthesis of s^4U are shared by the two proposed mechanisms (Fig. 1) but do not distinguish between them. Essentially, they vary in the identity of the sulfur nucleophile—either the Cys-456 persulfide group or bisulfide. Lauhon and co-workers have reported efficient s^4U production using bisulfide in place of cysteine and IscS,[15] which might seem to settle the issue. However, the required bisulfide concentration (50 mM) is well above a physiologically reasonable level.[15] Bisulfide, then, is either an opportunistic participant in s^4U generation *in vitro* or a *bona fide* intermediate that ThiI generates and tightly sequesters *in vivo* so that exogenous bisulfide is only poorly used as a substrate. Such sequestration is precedented by enzymes that hydrolyze glutamine and use the generated ammonia in a second reaction.[16] The key feature of the mechanism featuring a nucleophilic persulfide group is a disulfide-linked ThiI-tRNA adduct, and its detection and the demonstration of its chemical and kinetic competence would essentially identify the Cys-456 persulfide group as the nucleophile in s^4U biosynthesis. The search for the disulfide-linked ThiI-tRNA adduct is already underway using gel and mass-spectrometric methods similar to those described here, and the resolution of which of the two proposed mechanisms (Fig. 1) operates in s^4U biosynthesis awaits the outcome of those experiments. However, under the conditions that support efficient s^4U generation, the formation of a persulfide group at Cys-456 is now established, as is the formation of an intramolecular disulfide bond between Cys-456 and Cys-344.

This work was supported by the National Institutes of Health Grant GM59636. G. D. C. was supported by HHMI Undergraduate Science Education Grant 52002062 to the University of Delaware. A. M. S. was supported by National Science Foundation grant number DBI-0096578.

Notes and references

‡ In the text, "cysteine" denotes the free amino acid, and "Cys" denotes a cysteine residue in a protein.

1 H. Grosjean and R. Benne, *Modification and Editing of RNA*, ASM Press, Washington, DC, 1998.
2 A. Favre, in *Bioorganic Photochemistry: Photochemistry and the Nucleic Acids*, ed. H. Morrison, John Wiley & Sons, New York, 1990, pp. 380–411.
3 E. Webb, K. Claas and D. M. Downs, *J. Bacteriol.*, 1997, **179**, 4399–4402.
4 E. G. Mueller, C. J. Buck, P. M. Palenchar, L. E. Barnhart and J. L. Paulson, *Nucleic Acids Res.*, 1998, **26**, 2606–2610.
5 R. Kambampati and C. T. Lauhon, *Biochemistry*, 1999, **38**, 16561–16568.
6 D. H. Flint, *J. Biol. Chem.*, 1996, **271**, 16068–16074.
7 R. Kambampati and C. T. Lauhon, *J. Biol. Chem.*, 2000, **275**, 10727–10730.
8 P. M. Palenchar, C. J. Buck, H. Cheng, T. J. Larson and E. G. Mueller, *J. Biol. Chem.*, 2000, **275**, 8283–8286.
9 E. G. Mueller, P. M. Palenchar and C. J. Buck, *J. Biol. Chem.*, 2001, **276**, 33588–33595.
10 C. M. Wright, P. M. Palenchar and E. G. Mueller, *Chem. Commun.*, 2002, **2002**, 2708–2709.
11 A. D. Smith, J. Frazzon, D. R. Dean and M. K. Johnson, *FEBS Lett.*, 2005, **579**, 5236–5240.
12 E. Krause, H. Wenschuh and P. R. Jungblut, *Anal. Chem.*, 1999, **71**, 4160–4165.
13 Y. Ikeuchi, N. Shigi, J.-I. Kato, A. Nishimura and T. Suzuki, *Mol. Cell*, 2006, **21**, 97–108.
14 R. Kambampati and C. T. Lauhon, *Biochemistry*, 2003, **42**, 1109–1117.
15 C. T. Lauhon, W. M. Erwin and G. N. Ton, *J. Biol. Chem.*, 2004, **279**, 23022–23029.
16 F. Massière and M.-A. Badet-Denisot, *Cell. Mol. Life Sci.*, 1998, **54**, 205–222.

