Evidence for the Transfer of Sulfane Sulfur from IscS to ThiI during the in Vitro Biosynthesis of 4-Thiouridine in Escherichia coli tRNA*

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IscS from Escherichia coli is a cysteine desulfurase that has been shown to be involved in Fe-S cluster formation. The enzyme converts L-cysteine to L-alanine and sulfane sulfur (S0) in the form of a cysteine persulfide in its active site. Recently, we reported that IscS can donate sulfur for the in vitro biosynthesis of 4-thiouridine (s4U), a modified nucleotide in tRNA. In addition to IscS, s4U synthesis in E. coli also requires the thiamin biosynthetic enzyme ThiI, Mg-ATP, and L-cysteine as the sulfur donor. We now report evidence that the sulfane sulfur generated by IscS is transferred sequentially to ThiI and then to tRNA during the in vitro synthesis of s4U. Treatment of ThiI with 5-(2-iodoacetamido)ethyl)-1-aminoanthalene sulfonic acid (I-AEDANS) results in irreversible inhibition, suggesting the presence of a reactive cysteine that is required for binding and/or catalysis. Both ATP and tRNA can protect ThiI from I-AEDANS inhibition. Finally, using gel shift and protease protection assays, we show that ThiI binds to unmodified E. coli tRNA in vitro. Together, these results suggest that ThiI is a recipient of S0 from IscS and catalyzes the ultimate sulfur transfer step in the biosynthesis of s4U.

Sulfur trafficking within the cell remains a poorly understood area. The biochemical steps for sulfur incorporation into biotin, lipoic acid, and thiamin, as well as macromolecules such as protein Fe-S clusters and thionucleosides in tRNA, have not been fully elucidated. The biosynthesis of thiamin and 4-thiouridine (s4U)1 in tRNA has been linked by the observation that some mutants lacking s4U in their tRNA were also auxotrophic for thiamin (1). In two independent studies, the thiI gene was isolated and found to complement both s4U (2) and thiamin (3) deficient mutants in Escherichia coli and Salmonella typhi-
min in buffer A minus PMSF, and then reaction mixtures were digested as above. Aliquots were removed at various time intervals and analyzed by SDS-PAGE. Gels were quantitated using a densitometer and ImageQuant software (Molecular Dynamics). The intensity of the bands was quantitatively analyzed by the volume quantitation method, and the object average option was used for background correction.

Analysis of Interaction between ThiI and IscS during sU Synthesis—

IscS (0.9 μM) was incubated with increasing amounts of ThiI (0–1.4 μM) as indicated in the figure legends. Reactions were performed either in the presence or absence of 1 mM ATP in 25 μl of buffer A containing 10 mM Tris-HCl, pH 8.0, 50 mM KCl was included in the incubation mixture in lane 5; lanes 6–11, tRNA incubated with IscS in the molar ratios of 1:2.5, 1:0.5, 1:1, and 1:2, respectively.

RESULTS AND DISCUSSION

tRNA Binding Studies—We first looked for tRNA binding by each of the protein factors involved in the biosynthesis of sU using a gel mobility shift assay. Fig. 1 shows the effect of increasing concentrations of either ThiI (lanes 2–5) or IscS (lanes 8–11) on the mobility of 32P-labeled unmodified E. coli tRNA Phe transcript. Analysis by native PAGE indicates that only ThiI is able to shift the mobility of the tRNA substrate. The addition of an excess of unlabeled tRNA (25–50-fold) could block the observed mobility shift by ThiI (lanes 6–7). To corroborate the gel shift results, we performed protease protection experiments on ThiI (Fig. 2). TPCK-trypsin degrades ThiI into a major fragment with an approximate molecular mass of 45 kDa (designated L in Fig. 2); tRNA protects ThiI from proteolysis (lower left panel). Neither cysteine nor ATP protects ThiI from tryptic degradation at this site. However, ATP lowers the rate of proteolysis of both ThiI and the 45-kDa fragment (Fig. 2, lower right panel). Quantitative analysis of the protein bands in Fig. 2 revealed that ThiI proteolysis was slowed by 2-fold in the presence of ATP. ThiI tryptic digests containing primarily the 45-kDa fragment are devoid of tRNA sulfurtransferase activity (data not shown). Both the gel shift analysis and protease protection experiments clearly show that ThiI binds to tRNA.

Inhibition of ThiI Catalyzed sU Formation by I-AEDANS—ThiI (3.0 μg, 54 pmol) in 30 μl of 50 mM Tris-HCl, pH 8.0, 50 mM KCl was incubated with 500 pmol of I-AEDANS (2.3-fold molar excess of cysteine) in ThiI at 25 °C for 30 min in dark. In the experiments in which the protective effect of substrates was assessed, 1.6 mM ATP, 1.9 mM GTP, 16.7 μM [35S]cysteine, or 5.0 μg of unmodified tRNA Phe were included in the incubation mixture. Reactions were quenched with DTT (8.2 mM final concentration) and diluted to 50 μl with sulfurtransferase assay components. The standard assay mixture is composed of buffer A containing 1 mM ATP, 10 μM [35S]cysteine (1338 CPM/pmol), 5.0 μg of unmodified tRNA Phe, 20 μg PLP, and 0.2 μg of IscS. For maximal sulfur mobilization, we used saturating amounts of IscS in the assays. The concentrations of ThiI used in the inhibition studies were within the linear range of the assay. The sulfurtransferase assay was carried out at 37 °C for 30 min, and samples were applied to DEAE-81 filters (2.4 cm, Whatman). The filters were washed once with 0.5 M Tris-HCl, pH 9.0, for 30 min and three times with 0.3 M KCl, 20 mM cysteine for 5 min. The filters were finally washed three times with H2O in a Buchner funnel and counted in a liquid scintillation counter.

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Observation of Transfer of 35S from IscS to ThiI during sU Formation—We used 35S-labeled cysteine in an attempt to follow the transfer of sulfur during sU synthesis in vitro. Reactions containing an increasing amount of ThiI, with or without ATP and tRNA, were incubated for 10 min and then quenched with SDS buffer and subjected to electrophoresis (Fig. 3). Each lane in Fig. 3 corresponds to a separate reaction mixture. 35S-labeled bands were visualized by PhosphorImager analysis (Fig. 3, B and C). As expected, only IscS can be labeled with [35S]cysteine alone. In reactions with increasing concentrations of ThiI (Fig. 3B, lanes 3–6), we observed a 35S-labeled band of increasing intensity with mobility identical to ThiI. The proportion of 35S label comigrating with IscS was observed to decrease with an increasing ThiI concentration (Fig. 3B, lanes 4–6). However, 35S label on IscS increased 47% in the presence of small amounts of ThiI (Fig. 3B, lane 3). Interestingly, this increase was not observed in the absence of ATP (Fig. 3C, lane 3).

When the tRNA substrate was included in the reaction mixture (Fig. 3B, lane 7), the label corresponding to ThiI and IscS was greatly diminished, and a high intensity band that correlates with the position of the tRNA was observed. Quantitation of the labeled bands shown in Fig. 3B reveals that the amount of label on ThiI and IscS decreased by 81 and 98%, respectively, in the presence of tRNA (Fig. 3D). Together, these labeling patterns suggest that ThiI is covalently modified with sulfur mobilized by IscS and catalyzes the ultimate sulfur transfer in the synthesis of sU. Under previously reported sulfurtransferase assay conditions (4), ThiI and IscS catalyze the transfer of 0.2 mol of 35S to 1 mol of tRNA. HPLC analysis of 35S-labeled tRNA nuclease digests confirms that sU accounts for >80% of the 35S label. In the present experiment...
Panels C and D show the PhosphorImager scans of reactions performed in the presence and absence of ATP, respectively. Panels D and E show the quantitation of $^{35}$S label relative to IscS in the protein bands assigned to IscS and ThiI, as a function of increasing ThiI concentration. The last column in panels D and E shows the levels of $^{35}$S label in the protein bands after the addition of tRNA.

(Fig. 3), the radioactivity observed in ThiI is 1% that of the label on tRNA. The higher intensity of the label in the tRNA results from the greater chemical stability of s4U relative to the putative protein persulfides. An additional unidentified labeled band with electrophoretic mobility in between IscS and tRNA was also observed (Fig. 3), the radioactivity observed in ThiI is 1% that of the label in tRNA. Because we do not see this band in the Coomassie-stained gel. Panels B and C show the PhosphorImager scans of reactions performed in the presence and absence of ATP, respectively. An alignment of amino acid sequences of ThiI from E. coli and homologs from a range of other organisms.
shows that Cys$^{344}$ is present in all of the analyzed sequences (data not shown). These results support the possibility that ThiI possesses a reactive cysteine that can accept S$^0$ for the synthesis of s$^4$U. We are currently attempting to identify the modified cysteine residue(s), as well as prepare site-directed mutants for analysis both in vitro and in vivo.

**General Scheme for Sulfur Trafficking in the Synthesis of s$^4$U**—Based on the model proposed by Flint (8) and the evidence presented in our current work, we propose a scheme for sulfur mobilization and transfer in the biosynthesis of s$^4$U in *E. coli* (Fig. 5). The initial mobilization of sulfur from cysteine results in the formation of S$^0$ covalently bound to a cysteine in the active site of IscS as a persulfide (IscS-SSH). This persulfide sulfur is then transferred, presumably to a cysteine residue on ThiI, to give the ThiI persulfide (ThiI-SSH). Finally, in the presence of ATP, the persulfide sulfur from ThiI-SSH is mobilized and transferred to tRNA. The exact mechanism of the mobilization in vivo is not yet clear, but it could involve oxidation of the protein via internal reduction of the persulfide by a cysteine residue to give a disulfide that is reduced later. Alternatively, an external reducing agent could be required to directly mobilize the sulfur from the persulfide. Because native ThiI has not yet been isolated, it is not possible to ascertain the oxidation state of the naturally isolated enzyme. It is interesting to note that in Flint’s (8) original isolation of IscS in the absence of reducing agents, the enzyme was found to be part of a mixed disulfide with the phosphopantetheine moiety of acyl carrier protein.

The role of ATP in s$^4$U synthesis has not yet been elucidated. Lipsett and colleagues (5) proposed that Factor A utilized Mg-ATP to activate the oxygen at the 4-position of uridine for attack by a sulfur nucleophile (4). Our observation that ATP protects ThiI from inhibition by I-AEDANS and lowers the rate of ThiI trypsinolysis is consistent with this suggestion. The observed in vitro shuttling of S$^0$ from IscS to ThiI may represent a general strategy in vivo for efficient sulfur transfer in a number of metabolic pathways (10). It is possible that IscS or other NifS homologs in *E. coli* transfer S$^0$ to a variety of other acceptors, including Fe-S cluster-containing enzymes and other sulfurtransferases. We have recently found that *E. coli* *iscS* mutants are viable but are unable to synthesize s$^4$U or thiamin.$^3$ It is thus likely that IscS initiates sulfur mobilization for the synthesis of both s$^4$U and thiazole in vivo. Current efforts are focused on a full characterization of the *iscS* phenotype in *E. coli* and further elucidation of the scope of IscS-initiated sulfur transfer in thionucleotide biosynthesis.

**Addendum**—While this paper was in review, a paper was published by Mueller and Palenchar (11) showing that ThiI contains a unique P-loop motif (185SGGXDS$^{190}$) that is common to the pyrophosphate synthetase family of enzymes that catalyze the adenylation and substitution of carbonyl oxygens. We note that ThiI contains significant ATPase activity independent of RNA,$^3$ as was earlier reported for Factor A (5). Thus far, we have only found evidence of ADP formation. However, it is possible that the tRNA-independent ATP hydrolysis is obscuring our ability to observe AMP formation.

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$^3$ C. T. Lauhon and R. Kambampati, manuscript in preparation.
