Biosynthesis of Pseudouridine in Transfer Ribonucleic Acid*

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SUMMARY

An enzyme that catalyzes the formation of specific pseudouridine residues in the anticodon region of tRNA has been characterized in extracts of Salmonella typhimurium and Escherichia coli. It has previously been shown that histidine regulatory mutants of S. typhimurium lack the pseudouridine modifications in the anticodon region of histidine and several other tRNA species. This has permitted the development of a simple radiochemical assay for the modification enzyme, based on the release of tritium from histidine tRNA isolated from cells grown in [5-3H]uridine. This enzyme activity is absent from histidine mutants and is relatively heat-labile in extracts from temperature-sensitive histidine mutants, which suggests that the modification enzyme itself is the primary gene product of the histidine gene.

Among the various minor nucleosides present in tRNA, pseudouridine is the most common. All known tRNA sequences contain a pseudouridine (ψ) residue in the "TYCPu" side-arm loop (1) except for yeast tRNA^Met (2) and a tRNA^Ala not used in protein synthesis (3). Pseudouridine residues have been located in other regions of the primary structure of tRNA. In many tRNA species from Escherichia coli and Salmonella typhimurium 1 or 2 pseudouridine residues have been found in the stem and loop of the anticodon region (4–10).

During the study of the regulation of histidine biosynthesis in S. typhimurium, we discovered that histidine mutants (11), one of the six classes of mutants derepressed for the histidine operon (12), contained a tRNA^His lacking 2 pseudouridine residues which are normally present in the anticodon region of wild type tRNA^His (13). In contrast, the pseudouridine in the TYCPu loop was present in both the wild type and mutant tRNA^His (12). We have also presented evidence that, as a consequence of the histidine mutation, the chromatographic properties of several other species of tRNA are altered without greatly affecting the function of these tRNAs in protein synthesis (13).

Such findings strongly implied that the histidine gene product might be an enzyme that catalyzes the formation of pseudouridine in the anticodon region of specific tRNA species. If this were true, then bulk tRNA from a histidine mutant should contain a significant number of residues specifically capable of modification to pseudouridine by enzymes from wild type strains. This situation is analogous to the ability of wild type extracts to modify the submethylated species of tRNA that are synthesized by mutants with defective methylation enzymes (14), or the submethylated and subthiolated tRNA produced by relaxed amino acid auxotrophs (15, 16).

In this paper, we identify an enzymatic activity in wild type strains of S. typhimurium and E. coli which can modify specific uridine residues of bulk histidine tRNA. This activity is not detectable in extracts derived from histidine strains and is heat-labile in temperature-sensitive histidine strains. These findings offer further evidence that this pseudouridylation enzyme represents the histidine gene product.

MATERIALS AND METHODS

Chemicals—Chemicals used for the growth of the cells and for the extraction and analysis of nucleic acid were of reagent grade or otherwise of the highest available purity. [5-3H]Uridine (20,000 mCi per mmole), [6-3H]uridine (20,000 mCi per mmole), and a^P1 (carrier-free) were purchased from Schwarz-Mann. DEAE-cellulose paper (Machery, Nagel and Co.), T1 ribonuclease (Calbiochem), and bovine pancreatic RNase A (Worthington) were purchased from commercial sources.

Bacterial Strains—Isogenic histidine (TA253) and wild type S. typhimurium LT-2 (TA265) were constructed by George Chang (Univ. of California, Berkeley) by transducing hisT1604 and hisT+ into an aroD purF double auxotroph (11).

Growth of Cells and Preparation of Extracts—Cells were grown in Vogel-Bonner minimal E medium with 0.2% glucose at 37°C, harvested in late log phase (A = 1.0 at 650 nm), and kept frozen for several months. Cells were suspended in 2 volumes of buffer containing 10 mM Tris-HCl buffer, pH 7.8, 1 mM EDTA, 1 mM 2-mercaptoethanol, 10 mM MgCl2, and 10% glycerol (v/v), and were sonicated three times for 30 s each. After an initial centrifugation at 30,000 × g for 20 min, the supernatant was spun at 300,000 × g for 90 min. Such extracts were used directly for all of the work reported here.

Preparation of 3H-labeled tRNA—Cells were grown in 500 ml of the minimal glucose medium (17) containing 10 mCi of [5-3H]uridine. When they reached the late log phase, they were centrifuged at low speed, suspended in 4 ml of 10 mM Tris-HCl, pH
7.5, and 10 mM MgCl₂ (Buffer A), disrupted by sonication, then centrifuged at 30,000 × g for 30 min. The supernatant was resuspended at 100,000 × g in a Spincow SW 39 rotor for 4 hr. The supernatant was extracted with distilled phenol saturated with Buffer A, and the tRNA was further purified as previously described (18). The specific activity averaged 10 µCi per A₂₆₀ unit of tRNA. Of the incorporated radioactivity, 63% was found in CMP and 37% in UMP and UMP derivatives, on the basis of alkaline and enzymatic hydrolysis of the product. Both methods of hydrolysis gave identical results.

Preparation of [³²P]tRNA and Nucleotide Analysis—Preparation of [³²P]tRNA was done according to the procedure described by Singer and Smith (10). After enzymatic modification of the tRNA (see below), the reaction was stopped by the addition of 1 volume of distilled phenol, neutralized with Buffer A. Carrier yeast tRNA (50 µg) was added and the samples were shaken for 1 hr at 0°. Approximately 80% of the aqueous phase was carefully removed with a micropipette and the operation was repeated after addition of 0.1 ml of water to the remaining sample. The aqueous phases were combined and NaCl was added (final concentration, 0.5 M), followed by 2.5 volumes of ethanol. The samples were left at -20° overnight and were centrifuged in a clinical centrifuge. The small pellets were resuspended in 10 µl of water and subjected to alkaline hydrolysis, as described earlier (10). The hydrolysates were resolved initially by electrophoresis on DEAE-cellulose paper in pyridine-acetate buffer, pH 3.5, for 3 hours at 2500 volts. UMP and 2MP migrate together in this system. After radioautography, the spot containing the two nucleotides was eluted, taken to dryness and chromatographed on Whatman No. 1 paper, using the solvent, isopropyl alcohol-HCl-water (75:17:1, v/v/v). The UMP and 2MP spots were well separated in this system (10) and after radioautographic identification, they were cut from the paper and counted by Cerenkov radiation in a Nuclear-Chicago Mark II scintillation spectrometer.

Enzyme Assays—The tritium release assay for pseudouridylate activity was carried out as follows. The incubation mixtures, 0.1-ml total volume, contained 100 mM glycine-NaOH buffer, pH 9.0; 20 mM MgCl₂; 0.02 A₂₆₀ unit of tritiated tRNA; and enzyme. The reaction was started by addition of enzyme, and the samples were incubated at 37°. The reaction was terminated by addition of 1 ml of 12% activated charcoal (Norit A) in 0.1 N HCl (w/v). The samples were left at room temperature for 20 min, with occasional stirring, and then were filtered onto membrane filters (0.45-µm pore size). The filtrate was collected directly into scintillation vials and, after addition of 10 ml of Bray’s solution, was counted in a scintillation spectrometer with an over-all efficiency of 15%. Control incubation mixtures from which the enzyme had been omitted (Table I) or which were sampled at zero time gave blanks in the range of 40 to 60 cpm. The data in most of the figures shown have been corrected for these blank values.

Pseudouridylate synthetase was assayed according to Breitman (20).

**RESULTS**

Identification and Specificity of Enzyme Activity—During the synthesis of pseudouridine, the formation of the C—C bond between C₃ of the pyrimidine ring and C₁ of the ribose must be accompanied by the release of a proton into the medium (Fig. 1). The availability of pyrimidine derivatives, tritiated at C₃ of the ring, has permitted the development of highly sensitive tritium release assays for a number of enzyme reactions involving a similar proton release (20–24). We have used such a tritium release procedure, starting with a labeled tRNA substrate from a hisT mutant for the detection of the pseudouridylation activity.

The tRNA, tritiated at C₃ of the pyrimidine ring, was prepared from a hisT mutant of S. typhimurium grown in the presence of [³-H]uridine (see “Materials and Methods”). In the presence of a pseudouridylation activity, tritium should be released into the medium at a rate proportional to the enzyme activity. The data shown in Table I demonstrate the presence of such an activity in extracts from wild type S. typhimurium. Under the same conditions, no tritium release occurred with labeled wild type tRNA, which indicates that this tRNA lacked modifiable pyrimidine residues. It was also possible to detect this activity in extracts from E. coli B which had about the same activity as found in S. typhimurium.

The tritium release activity was also tested with extracts from two independently isolated hisT mutants: hisT1504, the source organism for the labeled tRNA and the strain used for determination of the tRNAH₁ sequence (13); and hisT1509, an amber mutant in the same gene (11). Tritium release activity was undetectable in extracts from both hisT strains.

With hisT tRNA labeled at C₃, the rate of tritium release was linear for 5 or 10 min and continued for 30 min or more under the conditions shown in Fig. 2. On the other hand, tRNA from the same mutant, but tritiated at the C₄ position of the pyrimidine residues, did not release any tritium, even after extended
reaction of about 0.4 mole of uridine per mole of tRNA.

In Fig. 5, the initial rates of tritium release are plotted as a function of tRNA concentration. From the Lineweaver-Burk plot (see inset), an apparent \( K_m \) of approximately \( 10^{-4} \) M is obtained. This value is a maximum estimate, since it is based on the assumption that every tRNA molecule is a substrate for the enzyme. However, since not all molecules of tRNA contain pseudouridine modifications in the anticodon region (1), the affinity of the enzyme for the specific tRNA substrate is probably even greater than the apparent \( K_m \) would indicate.

At high concentrations of tRNA, there appears to be a small divergence from ideal Lineweaver-Burk kinetics (see inset, Fig. 5). This could indicate that the substrate tRNA mixture contained nonmodifiable sequences or configurations which competed for the enzyme site or which interfered with the enzyme activity. This feature deserves further examination.

Other reaction requirements are given in Figs. 6 and 7. The \( \text{pH} \) dependence of the reaction (Fig. 6) showed a sharp optimum at 9.0. The reaction also showed an absolute requirement for Mg\(^{2+}\) (data not shown) and was optimal above a Mg\(^{2+}\) concentration of 1.0 mM.

Kinetic Features of the Enzyme Reaction—Fig. 3 depicts the tritium release activity as a function of the enzyme concentration. With hisT tRNA at 0.4 n mole per ml, the rate of tritium release was a linear function of the concentration of enzyme, up to a saturating level of 3 mg per ml of protein.

The maximum extent of tritium release was determined by prolonged incubation with subsaturating levels of enzyme and was directly proportional to the initial concentration of tRNA (Fig. 4). From the specific activity of the tRNA and its uridine content, we estimate that the plateau levels correspond to the reaction of about 0.4 mole of uridine per mole of tRNA.

The activity was highly sensitive to increased salt concentration (Fig. 7). At 65 mM NaCl, the activity was only 50% of the optimum rate. However, full enzyme activity was maintained.

This value has been determined as follows. The concentration of tRNA was calculated from an assumed absorbance of 20 \( A_{260} \) per mg, and an average molecular weight of 35,000. Accordingly, 0.02 \( A_{260} \) unit was equivalent to 1 \( \mu \)g or 40 pmoles of tRNA. Each picomole of hisT tRNA was assumed to contain 14 pmoles of uridine (20), which accounted for 37% of the \(^3\)H, as determined by direct experiment (see “Materials and Methods”). With a specific activity of 1,200 cpm per pmole of tRNA, the modification of 1 pmole of uridine would correspond to the release of 1,200 \times 0.37/14 = 31.7 cpm of \(^3\)H.
when the salt concentration was restricted to 20 mM or less. The inhibition by salts appeared to be nonspecific, since (NH₄)₂SO₄, NH₄Cl, KCl, and NaCl were equally inhibitory at a concentration of 100 mM.

The extract was readily desalted by passage through Sephadex G-50, but the activity was sometimes lost after dialysis. The preparation could be stored for at least 10 days in 10% glycerol with no loss of activity, but freezing and thawing led to severe losses.

Characterisation of Pseudouridylation Formation by Enzyme Extracts—In order to verify the occurrence of the pseudouridylation modification, it was necessary to demonstrate that the action of the enzyme led to the disappearance of uridy late or cytidylate residues, with the concurrent appearance of pseudouridy late. This was carried out by incubation of [32P]tRNA from the hisT mutant with wild type enzyme extract. As a control, the hisT tRNA was treated with homologous enzyme from the same hisT strain. The results are shown in Fig. 8. In order to normalize the results for variable losses during the analytical steps, the activity is expressed in terms of the ratios of UMP:IMP during the course of the reaction. For bulk hisT tRNA, this ratio is equal to 10, whereas it is equal to 5 for wild type tRNA. When the hisT tRNA was treated with enzyme from wild type cells, this ratio decreased to 5 within a 15-min incubation period (Fig. 8). In the control incubation, the ratio changed only slightly, from 10 to approximately 9, during the same time course.

It is doubtful whether the small change in UMP:IMP ratio in the control incubation has any significance, particularly since such homologous reaction mixtures were inactive in the tritium release assay (Table 1). In other experiments (not shown) we have found no change in the IMP content of hisT tRNA after incubation with homologous extracts. We suspect that the changes in the control incubation were a result of the selective cleavage of UMP residues by the mixture of nuclease activities in the crude cell extracts.

Defective Enzyme Activity in a Temperature-sensitive Mutant—Further evidence that hisT mutants are altered in a protein responsible for the formation of pseudouridine residues has been obtained from the study of a temperature-sensitive hisT mutant. This mutant, TA263, is subject to normal regulation of the histidine operon when it is grown at 30°, but is derepressed 2- to 3-fold at 37° and 8-fold at 42° (11). If the hisT gene product is an enzyme that catalyzes formation of tRNA pseudouridine residues, then the enzyme activity itself should be temperature-sensitive in extracts from strain TA263.

This has proven to be the case when the activity is measured by the tritium release assay (Fig. 9). When the extracts were heated at 60°, the activity from strain 1A263 was inactivated 6 to 7 times as rapidly as the activity in wild type extracts.

DISCUSSION

Despite a variety of early conflicting evidence (reviewed in Ref. 26), it is now abundantly clear that the pseudouridine modifications in tRNA occur after assembly of the tRNA chain (27, 28), as is the case with all other known tRNA modification reactions.

The tRNA species which accumulate in hisT mutants of S. typhimurium provide a natural source of unmodified loci for a specific group of pseudouridine modifications. By the use of hisT tRNA, tritiated specifically at the C₄ pyrimidine residues, the tritium release assay provides a convenient and rapid measure of at least a partial reaction involved in pseudouridine formation. The specificity of this assay is implicit from the following considerations. (a) Tritium release occurs only when the tRNA from hisT cells is treated with wild type extracts (Fig. 2). No release is found when wild type tRNA is used, since all potential sites are already modified. No release is obtained with extracts

FIG. 6 (left). pH Dependence of tritium release. The standard assay procedure was used, but the buffer pH was varied as shown, at a concentration of 100 mM. The assay mixtures contained 20 μg of enzyme protein and were incubated for 5 min.

FIG. 7 (center). Effect of salt concentration on rate of tritium release. The reaction conditions were those given in Table 1, except that 20 μg of enzyme protein were used, and NaCl was added to the final concentrations shown. The incubation was for 5 min at 37°.

FIG. 8 (right). Formation of tRNA pseudouridylation during incubation of hisT tRNA with enzyme extracts. The incubation mixtures contained 100 mM glycine buffer, pH 9.0; 20 mM MgCl₂; 100 pmoles per ml of [32P]-labeled hisT tRNA and 500 μg per ml of enzyme protein from either the wild type strain or from strain hisT1694. At the designated time points, 0.1-ml samples were removed and mixed with an equal volume of neutralized phenol. Carrier yeast tRNA was added and the nucleic acids were isolated, hydrolyzed, and subjected to nucleotide analysis as described under “Materials and Methods.”
from hisT cells, since they lack the enzyme. (b) The replacement of pseudouridine residues by uridine residues in the anticodon region of tRNA\textsuperscript{His} (13) and tRNA\textsuperscript{Aeu} (20) of hisT mutants has been established by sequencing. In addition, the chromatographic properties of other species of tRNA are altered in hisT mutants, notably those of tRNA\textsuperscript{787} (13) and tRNA\textsuperscript{His} (Footnote 2) and the major (13) and minor species of tRNA\textsuperscript{Aeu}.\textsuperscript{3} This pattern is consistent with the known presence of pseudouridine in this position in these tRNA species. (c) Although other modification reactions would be measured by the tritium release assay, e.g. the formation of 5-methylated or other 5-substituted pyrimidine derivatives (30), it is unlikely that such reactions contribute to the tritium release to any significant extent. This conclusion is based on several facts. First, no additional cofactors were necessary for tritium release, as might be expected for other modification reactions. Second, the release of tritium by wild type extracts took place only with hisT tRNA as substrate; wild type tRNA, labeled in the same manner, did not release tritium upon treatment with wild type extract. Finally, there is no evidence that hisT tRNA has defects in modification other than the absence of certain pseudouridine residues (13).

This assay system may also be suitable for characterizing the pseudouridine modification enzymes of other organisms, particularly by employing heterologous reactions, in which the labeled tRNA is derived from organisms of low pseudouridine content. Such heterologous reactions are readily demonstrable with the tRNA methylation enzymes (31, 32).

Several insights into the enzyme mechanism can be derived, even with the limited information available at this time. (a) The enzyme described here is not responsible for the pseudouridine modification in all regions of the tRNA molecule. This is evident from the fact that hisT mutants, which lack this activity, still produce tRNA with pseudouridine in the T\textsuperscript{CPu} loop (13). This residue therefore must be modified independently, presumably by a second enzyme which may act on a tRNA precursor species (33). It is not known whether the pseudouridine residues of ribosomal RNA (34, 35) are formed by still another enzyme or not. For these reasons, we propose the designation tRNA pseudouridylate synthetase I for the enzyme described here, which is absent or defective in hisT mutants, and tRNA pseudouridylate synthetase II for the activity implicated in the T\textsuperscript{CPu} sequence. (b) Although there has been some speculation that the formation of pseudouridine could derive from a cytidine residue (see Ref. 24, p. 310), the most likely precursor would appear to be an unmodified uridine residue. This likelihood is implicit in the normal base-pairing relationships of the pseudouridine residues in the anticodon stem (10, 13), and in the fact that hisT tRNA contains uridine, rather than cytidine, residues, in place of the missing pseudouridine residues of wild type tRNA (13). (c) The reaction mechanism probably does not involve loss of a hydrogen atom from carbon 6, since hisT tRNA tritiated at carbon 6 does not react with wild type enzyme (Fig. 2).

Breitin (20) has used this type of tritium release assay to characterize the enzyme, pseudouridylate synthetase, which catalyzes the formation of \textsuperscript{3}RMP from uracil and ribose-5-P, in extracts from various strains of E. coli. We have been unable to detect this activity in our wild type strain of S. typhimurium (LT-2) by direct assay. In addition, strain LT-2 is unable to grow on pseudouridine as a carbon source, nor can the growth of pyr auxotrophs of LT-2 be supported by pseudouridine. These findings, and the lack of a specific tRNA requirement in the pseudouridylate synthetase reaction, clearly show that it is a distinct enzyme from the tRNA pseudouridylate synthetase described here.

The biological effects of the hisT mutation are uniquely pleiotropic. The lack of the pseudouridine residues in the anticodon region of tRNA\textsuperscript{His} in some manner affects the production of the histidine biosynthetic enzymes. The same hisT mutant is also altered in the regulation of the leu and ile operons.\textsuperscript{2} HisT mutants also possess an altered tRNA\textsuperscript{787} and are resistant to inhibition by the lysine analog, 2-S-aminomethylcysteine.\textsuperscript{2} The manner in which the lack of pseudouridine modifications can lead to these regulatory defects remains unclear, but is obviously of great interest.

Finally, the analysis of a temperature-sensitive hisT mutant suggests that the hisT gene codes for the modification enzyme rather than for a protein which regulates the activity or synthesis of the modification enzyme.

**REFERENCES**

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