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Kinetic Studies of *Escherichia coli* Transfer RNA (Uracil-5-)-methyltransferase†

Lee Shugart

ABSTRACT: The kinetic mechanism of a semipurified tRNA (uracil-5-)-methyltransferase (EC 2.1.1.35) preparation obtained from *Escherichia coli* has been studied at pH 9.0 in the presence and absence of products. The initial velocity and product inhibition patterns are consistent with a random order of addition of adenosylmethionine and transfer RNA to separate and independent binding sites on the enzyme. Values have been determined for the Michaelis and product inhibitor constants.

An important step(s) in the maturation of transfer RNA[1] (Smith, 1976; Altman, 1975) in prokaryotic organisms such as *Escherichia coli* involves a class of enzymes (Fleissner & Borek, 1962) designated tRNA methyltransferases. Their function is to bring about the substitution of methyl groups onto specific nucleotides (Kerr & Borek, 1973), utilizing as substrates AdoMet and precursor tRNA of the same size as mature tRNA (Davis & Nierlich, 1974).

A considerable body of experimental evidence suggests that methylation of tRNA results in a large number of diverse structural entities which can modulate various biochemical activities of the cell (Starr & Sell, 1969; Borek, 1970; Söll, 1971; Hall, 1971; Kerr & Borek, 1972, 1973; Nau, 1976; Salvatore et al., 1977).

Although the properties of several tRNA methyltransferases isolated from *E. coli* have been reported (Hurwitz et al., 1969a,b; Salvatore et al., 1977), information about the kinetic mechanisms of these enzymes is limited. In this paper, initial velocity measurements, both in the presence and in the absence of products (Cleland, 1970), have been determined in an effort to understand the kinetic mechanism of the enzyme rTase (EC 2.1.1.35) from *E. coli*.

Experimental Procedure

Materials

All chemicals were reagent grade. [^{14}C]CH_3AdoMet (specific activity, 56 Ci/mol) was obtained from Amersham/Searle, and AdoHcy was from Sigma Chemical Co. Aminex A-6, Cellex-D, and Bio-Gel HTP were obtained from Bio-Rad Laboratories. Sephadex G-200 was obtained from Pharmacia Fine Chemicals. An ultrafiltration apparatus, Model 202, was purchased from Amicon Corp.

† From the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830. *Received October 7, 1977.* Research sponsored by the Department of Energy under contract with the Union Carbide Corporation.

[1] Abbreviations used: $tRNA_n$, normal transfer RNA; $tRNA_{md}$, tRNA isolated from *rel*$^-$ mutant of *E. coli* after methionine starvation, a mixture of normal and methyl-deficient species [since relative proportions of two species can vary, see Shugart (1973) for discussion]; AdoMet, adenosylmethionine; AdoHcy, adenosylhomocysteine; A_{260} unit, that amount of tRNA in 1 mL which possesses an absorbance of 1 when measured with a 1-cm optical path at a wavelength of 260 nm; rTase, tRNA (uracil-5-)-methyltransferase; AdoHcyase, adenosylhomocysteine nucleosidase; DEAE-cellulose, *O*-(diethylaminoethyl)cellulose.

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Methods

Cell Extract. E. coli strain K_{12}MO7 was cultured and harvested as previously described (Kelmers et al., 1971). Cell rupture and preparation of 36 000*g* supernatant solution were as detailed by Stulberg (1967).

Column Chromatography. (a) DEAE-cellulose. An enzymatic assay mixture containing methylated tRNA was made 0.1 M with respect to sodium acetate buffer (pH 4.5) and applied directly to a DEAE-cellulose column (0.9 × 6 cm) at 6 °C that had been equilibrated in 10 mM sodium acetate buffer (pH 4.5) containing 50 mM NaCl, 10 mM magnesium acetate, and 1 mM ethylenediaminetetraacetic acid. The column was washed with 25 mL of equilibrating buffer in 0.25 M NaCl, and finally with equilibrating buffer in 0.7 M NaCl. The methylated tRNA eluted in the final column wash and was concentrated by ethanol precipitation.

(b) Sephadex G-200. The 36 000*g* cell extract from *E. coli* K_{12}MO7 was recentrifuged at 150 000*g* for 2 h, and a 25-mL aliquot was applied to a Sephadex G-200 glass bead column (2 cm × 11 ft) at 6 °C (Sachs & Painter, 1972). The column had been equilibrated and the sample was eluted in 5 mM potassium phosphate buffer (pH 7.6) containing 1 mM $MgCl_2$ and 1 mM diethiothreitol.

(c) Hydroxyl apatite. A 5-mL aliquot of concentrated methylase preparation from Sephadex G-200 column chromatography was applied to a hydroxyl apatite column (1.2 × 29 cm) at 6 °C. The column had been equilibrated in 5 mM potassium phosphate buffer (pH 7.6) containing 1 mM dithiothreitol and 10% glycerol (v/v). The sample was washed with 100 mL of equilibrating buffer and eluted with a 200-mL linear potassium phosphate gradient from 5 mM to 0.2 M at pH 7.6 in 1 mM dithiothreitol and 10% glycerol (v/v).

tRNA. The preparation of unfractionated $tRNA_n$ and $tRNA_{md}$ through the DEAE-cellulose step was as outlined by Shugart & Stulberg (1974). The *E. coli* cells for preparing the $tRNA_{md}$ preparation had been starved for methionine for 9 h (Shugart et al., 1968), and polyacrylamide gel electrophoretic analysis (data not shown) indicated that the $tRNA_{md}$ contained less than 5% ribosomal RNA as a contaminant.

In Vitro Transmethylation. Enzymic methylation of methyl-deficient tRNA with [^{14}C]CH_3AdoMet was performed as previously described by Shugart & Stulberg (1974) and quantitated by the filter paper disk method (Kelmers et al., 1965). One unit of enzyme is defined as the amount that transfers 1 nmol of methyl groups to $tRNA_{md}$ in 1 h. To determine the position within the tRNA of the enzymatically incorporated methyl group(s), the methylated tRNA was recovered by DEAE-cellulose column chromatography and then enzymatically hydrolyzed to the nucleoside level (Rogg et al., 1976); the distribution of methyl groups incorporated into nucleosides was determined after separation and detection by cation-exchange column chromatography (Shugart & Stulberg, 1974).

AdoHcyase Analyses. The determination of AdoHcyase activity was a modification of the procedure of Duerre (1962). To a 250-μL microcentrifuge tube were added 10 μL of 11 mM AdoHcy, 10 μL of 0.1 M potassium phosphate buffer (pH 6.2), and 10 μL of enzyme solution. The contents were capped, mixed, and allowed to incubate at 30 °C for 30 min, at which time the assay was terminated by the addition of 10 μL of 4 M formic acid. The enzymatic conversion of AdoHcy to Ade was quantitatively measured in the following manner: 30 μL of the assay mixture was applied to an A-6 cation-exchange column (0.6 × 24 cm) maintained at 48 °C. The sample was eluted with 0.85 M ammonium formate buffer (pH 5.65) in 5% ethanol at a flow rate of 1.07 cm/min. The eluate was monitored at 254 and 280 nm. Under these conditions Ado, AdoHcy, and Ade eluted at 15, 19, and 28 min, respectively. One unit of enzyme is defined as the amount that hydrolyzed 1 μmol of AdoHcy in 1 h.

TABLE I: Purification of *E. coli* rTase.[a]

Fraction	Specific activity: rTase (nmol h^{-1} mg^{-1})	Specific activity: AdoHcyase (μmol h^{-1} mg^{-1})
150 000*g* supernatant solution	1.1	0.5
After Sephadex G-200 column chromatography	3.8	1.7
After hydroxyl apatite column chromatography[b]	10.0	0.0

[a] See Experimental Procedure for details. [b] Protein concentration: 11 mg/mL.

Miscellaneous. The protein concentration of enzyme preparations was estimated by the method of Lowry et al. (1951).

Column fractions from Sephadex G-200 or hydroxyl apatite columns were concentrated, suspended in appropriate buffers, and reconcentrated three times by use of an Amicon Model 202 ultrafiltration apparatus at 6 °C with a type UM-10 membrane.

Results

Before initial rate kinetic analysis could be performed, two problems had to be overcome. First, a procedure for the isolation of a semipurified enzyme had to be devised which would produce a stable preparation sufficiently free of contaminating activities—in general RNase, and specifically AdoHcyase (Duerre, 1962). Second, the most appropriate conditions had to be selected for the initial rate kinetic experiment.

rTase Isolation. Table I summarizes the purification scheme used to obtain a stable enzyme preparation free of contaminating activities. First, the 36 000*g* cell extract from *E. coli* K_{12}MO7 cells was recentrifuged at 150 000*g* and applied to a Sephadex G-200 column. The column fractions were assayed for tRNA methyltransferase activity (Shugart & Stulberg, 1974), pooled, concentrated by ultrafiltration, and applied to a hydroxyl apatite column. AdoHcyase activity, which chromatographs near the major tRNA methyltransferase activity on the Sephadex G-200 column, elutes in the 5 mM potassium phosphate wash of the hydroxyl apatite column, while the rTase activity elutes at about 0.1 M potassium phosphate in the linear gradient. No RNase activity was found associated with the concentrated rTase fraction from the hydroxyl apatite column (data not shown). The rTase material purified through this stage could be stored at −85 °C for several months without loss of activity.

rTase Assay Conditions. Previous studies (Shugart et al., 1968; Shugart & Stulberg, 1974) with crude tRNA methyltransferase preparations from *E. coli* have shown that pH, [NH_4^+], and [Mg^{2+}] are important considerations when the in vitro assay conditions are chosen. These parameters were reinvestigated with the rTase material purified through hydroxyl apatite column chromatography, and it was found that the optimum pH for the enzymatic assay was still 9.0. Further, at this pH, AdoHcyase activity was greatly reduced (less than 10% of that found at its optimum pH). The results on the effect of cations in the in vitro assay with the semipurified rTase showed that the enzymatic reaction was no longer stimulated by NH_4^+ as it was with crude preparations (Shugart &

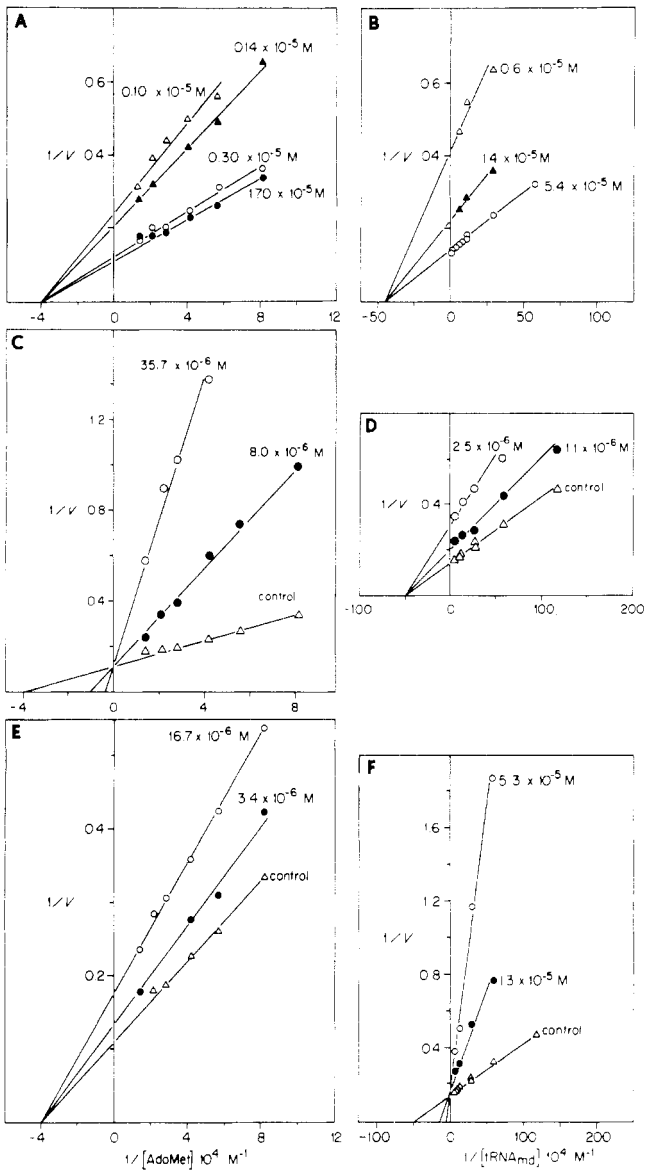

FIGURE 1: Double-reciprocal plots. Initial velocity patterns: (A) with AdoMet as the varied substrate and $tRNA_{md}$ at concentrations shown; and (B) with $tRNA_{md}$ as the varied substrate and AdoMet at concentrations shown. Inhibition patterns: with AdoHcy as the inhibitor at concentrations shown and (C) AdoMet or (D) $tRNA_{md}$ as the varied substrate; and with $tRNA_n$ as the inhibitor at concentrations shown and (E) AdoMet or (F) $tRNA_{md}$ as the varied substrate. Kinetic analyses of the enzymatic formation of CH_3-tRNA were performed as detailed in Experimental Procedure. Initial velocities are expressed as pmol of [^{14}C]-CH_3-tRNA per min. Protein concentration was 1.29 mg/mL. In C and E $tRNA_{md}$ concentration was 1.7×10^{-4} M, and in D and F AdoMet concentration was 3.3×10^{-5} M.

Stulberg, 1974). However, there appeared to be a slight stimulation with Mg^{2+} (10%). The effect of the reducing agent was not investigated since the enzyme as routinely prepared is in 1 mM dithiothreitol.

Based on these observations, in vitro methylation assays for obtaining mechanistic information about the semipurified rTase were performed in 1 mM Mg^{2+}, 11 mM Tris buffer (pH 9.0), with no NH_4^+ present. Using these specified conditions the time course of CH_3-tRNA formation with the semipurified rTase preparation was followed in a 280-μL reaction mixture containing 48 nmol of [^{14}C]CH_3-AdoMet, 0.055 A_{260} unit of $tRNA_{md}$, and 0.36 mg of protein. The data showed that CH_3-tRNA formation was linear for the first 5 min of the reaction and remained at a constant value of 1.5 nmol of methyl groups incorporated per A_{260} unit of tRNA thereafter for an additional 10 min. The enzymatic specificity of the rTase preparation was verified by nucleoside analysis of $tRNA_{md}$ after methylation for 15 min. It was found that, upon digestion of the tRNA to the nucleoside level, all the enzymatically incorporated [^{14}C]CH_3 groups cochromatographed on an A-6 cation-exchange column with authentic rThd. Thus, the degree of methyl deficiency of the nucleoside ribothymidine in $tRNA_{md}$ was taken to be 1.5 nmol/A_{260} unit of $tRNA_{md}$.

Initial Velocity Patterns. Equation 1 shows that the transmethylation of $tRNA_{md}$ by rTase is a bisubstrate, biproduct reaction;[2] therefore, it should be possible to obtain some mechanistic information about the enzyme by performing initial rate kinetic analysis.

$$tRNA_{md} + AdoMet \xrightarrow{rTase} CH_3\text{-}tRNA + AdoHcy \quad (1)$$

Initial velocity measurements were conducted by varying independently the concentration of either one of the two substrates (AdoMet and $tRNA_{md}$) while keeping a fixed concentration of the other. As shown in Figure 1A, four straight lines were obtained in a double-reciprocal plot when the initial rate of CH_3-tRNA formation was determined by varying linearly the concentration of AdoMet while keeping four fixed concentrations of $tRNA_{md}$. Since the $1/v$ vs. $1/S$ plots all intercept on the x axis and since V_{max} increases as the concentration of the fixed substrate ($tRNA_{md}$) is increased, the same K_m but different V_{max} are obtained. Similar (symmetrical) results are obtained when the concentration of $tRNA_{md}$ is varied for three fixed concentrations of AdoMet (Figure 1B). The internal consistency of these kinetic data is reinforced by an intercept replot of the primary plots (Figures 1A and 1B). Two straight lines are obtained which yield the same V_{max} and two K_ms identical with those obtained by the primary plots (data not shown).

These results [(a) the common intercept of the curves on the $1/S$ axis, and (b) the influence of concentration of one substrate on the V_{max} obtained by varying the concentration of the other] represent the distinguishing kinetic features of a sequential mechanism in which both substrates must add to the enzyme at separate and independent sites before either product is released.

These results do not tell, however, whether or not there is an obligatory or random order of addition or release of reactants.

Product Inhibition Patterns. A product inhibition study was performed in which initial velocity measurements were made with each product as an inhibitor vs. each substrate as a variable.

AdoHcy gave linear competitive inhibition kinetics with AdoMet as the variable substrate (Figure 1C) and linear noncompetitive inhibition kinetics with $tRNA_{md}$ (Figure 1D).

$tRNA_n$ gave linear noncompetitive inhibition kinetics with AdoMet as the variable substrate (Figure 1E) and linear competitive inhibition kinetics with $tRNA_{md}$ (Figure 1F).

These are the predicted product inhibition patterns for the combination of reactants in a random manner (Cleland, 1970).

A summary of the patterns obtained from the initial velocity

[2] The relatively high energy content of the sulfonium compound (AdoMet) provides the driving force for the largely exergonic and essentially irreversible transmethylation reactions (Shapiro & Schlenk, 1965).

TABLE II: Summary of Kinetic Data from Initial Velocity and Product Inhibitor Studies with *E. coli* rTase.

Substrate			Patterns[a]		Constant[b]	
Fixed	Variable	Product	Initial velocity	Inhibitor	K_m ($\times 10^{-5}$ M)	K_i ($\times 10^{-4}$ M)
$tRNA_{md}$	AdoMet		NC		2.5	
AdoMet	$tRNA_{md}$		NC		0.2	
	AdoMet	AdoHcy		C		0.04
	$tRNA_{md}$	AdoHcy		NC		0.024
	AdoMet	$tRNA_n$		NC		0.3
	$tRNA_{md}$	$tRNA_n$		C		1.0

[a] C, competitive; NC, noncompetitive (Cleland, 1970). [b] K_m data from Figures 1A and 1B; K_i data obtained from Figures 1C, 1D, 1E, and 1F by replotting slopes vs. inhibitor concentrations.

and inhibitor studies is presented in Table II along with the derived K_ms and K_is.

Discussion

rTase. $tRNA_{md}$ as a Homologous Substrate. Of those mature tRNAs which have been isolated from *E. coli* and subsequently sequenced, all have been found to contain one rThd per molecule of tRNA, always situated at the 23rd nucleotide from the 3′ end of the chain. This modification occurs during the processing of precursor tRNA molecules to mature ones; it is the result of an enzymatic methylation of an existing tRNA structure within the precursor molecule that contains a uridine at the position to be methylated. This is only one of several maturation events in the total synthesis of tRNA in prokaryotic organisms (Smith, 1976; Altman, 1975). When the methylation occurs, in relation to the overall synthesis and processing of tRNA, is not known, but it is probably when the precursor molecule is approximately the same size as mature tRNA (Davis & Nierlich, 1974).

The "tight" tRNA substrate specificity of the tRNA methyltransferases (Fleissner & Borek, 1963) has made it difficult to find a suitable tRNA as substrate for in vitro methylation studies. The use of tRNA from other organisms (heterologous tRNA) for such studies makes an interpretation of the physiological significance suspect. One of the better substrates suitable for measuring homologous methylation of *E. coli* tRNA methyltransferases, and the one employed in this investigation, is the methyl-deficient tRNA extracted from *rel*$^-$ mutants of the organism after they were cultured under methionine deprivation conditions (Söll, 1971; Salvatore et al., 1977). Previous studies by Shugart (1973, 1976) have shown that all the tRNAs synthesized by the *rel*$^-$ mutant during methionine deprivation are deficient in rThd. In agreement with this finding, individual methyl-deficient tRNA species purified from the total mixed population (Biezunski et al., 1970; Marmor et al., 1971; Isham & Stulberg, 1974) have been shown to be devoid of rThd. Further, the methionine starvation time of 9 h employed for the preparation of methyl-deficient tRNAs used for this investigation (see Experimental Procedure) ensures that more than 75% of the total tRNAs present are methyl-deficient species. This was substantiated by the quantitative determination of the rThd deficiency of the preparation, which was shown to about 1.5 nmol/A_{260} unit of $tRNA_{md}$.

Suitability of Enzyme Preparation. Nau (1976) has reviewed the numerous difficulties associated with any effort to purify to homogeneity the tRNA methyltransferases. Several of these problems have been experienced by this investigator during tRNA methyltransferase purification in the past (Shugart & Stulberg, 1977). Therefore, in the work reported here, no attempt was made to obtain a homogeneous enzyme preparation. Rather, an enzyme purification scheme was devised that results in a semipurified product which contains only one tRNA methyltransferase activity (rTase). It should be noted that the enzyme material is easily prepared, is stable for a relatively long period of time under the conditions specified, and is sufficiently free of contaminating activities so that a valid interpretation of the kinetic data could be made. Further, the assay conditions for the rTase are less stringent with respect to cation requirements than those found for crude enzyme preparations.

Kinetic Mechanism. The intersecting initial velocity patterns shown in Figures 1A and 1B demonstrate that rTase from *E. coli* has a sequential mechanism in which both substrates must add to the enzyme before either product is released. The inhibition studies confirm this, in that the inhibitor patterns (Figure 1C through 1F) show each product to act (a) as a competitive inhibitor with its cognate substrate, and (b) as a noncompetitive inhibitor with the other. In the latter case, this type of inhibition is demonstrated because of the apparent formation of dead-end complexes with the enzyme (i.e., the substrate and product react with the enzyme at different sites, forming a catalytically inactive complex). Thus the plots of initial velocity measurements, both in the presence and in the absence of products, result in patterns that typify the combination of reactants in a random mechanism.

Conclusion

The fact that the kinetic mechanism by which rTase effects the methylation of $tRNA_{md}$ is random implies that the binding of each substrate ($tRNA_{md}$ and AdoMet) to the enzyme (a) occurs at a different site for each substrate, (b) is independent of the binding of the other substrate, and (c) is freely reversible prior to a reaction involving the triple complex of enzyme, $tRNA_{md}$, and AdoMet (see Cleland, 1970, for a scheme that depicts a sequential mechanism in which the combination of reactants is random). Under normal growth conditions in *E. coli,* no demonstrable quantities of precursor tRNAs are found. This implies that the processes of synthesis and maturation for tRNA occur rapidly. Although the methylation of Urd to rThd is but one of several maturation steps involved in these processes, the random kinetic mechanism exhibited by rTase is amenable to a facile conversion of precursor to mature tRNA. That is, the pathway taken in vivo toward the formation of the triple complex, and ultimately the products, by rTase will depend mainly upon the availability of either substrate and not, for example, upon the prior formation of a particular enzyme–substrate complex, as would be required if the mechanism were ordered. The apparent concentration of products in the cell will also affect the activity of the rTase. The relatively strong inhibitory capacity of AdoHcy toward rTase (Table II) is eliminated by the action of the enzyme

AdoHcyase (Shugart, 1976), while the relatively weak inhibitory effect of mature tRNAs[3] is probably offset by the stabilizing effect they exhibit when complexed with the tRNA methyltransferases (Shugart et al., 1971; Shugart & Chastain, 1977).

rThd is the most common methylated nucleoside found in tRNA. It occurs in all the tRNAs of *E. coli* that have been sequenced to date and is totally absent in certain species of tRNAs from eukaryotic organisms (Marcu et al., 1973; Reszelback et al., 1977). It should be noted that, in the tissue of higher organisms, significant levels of both AdoMet and AdoHcy are present (Lombardini & Talalay, 1971), as opposed to low levels found in rapidly growing tissue and prokaryotic organisms. No physiologically significant event has been established for which the rThd content of *E. coli* tRNA is important (Björk & Neidhardt, 1971; Salvatore et al., 1977). In Bacillus subtilis, rThd formation occurs via a tetrahydrofolate-type substrate rather than AdoMet (Arnold et al., 1976).

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[3] Calculations based on quantitative data (Shugart, 1973, and unpublished) place the cellular concentration of $tRNA_n$ in *E. coli rel*$^-$ mutant during exponential growth at 1×10^{-4} M.