Aminoacyl Formylmethionyl Transfer Ribonucleic Acid Transformylase. Inhibitor Studies and the Reversal of the Reaction*

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ABSTRACT: A study was undertaken of the inhibition of N^{10}-formyl-H_4-folate–methionyl-$tRNA^{fMet}$ transformylation by a series of pteridine compounds. Those which were inhibitors are in order of decreasing effectiveness: H_4-folate, H_4-pteroate, H_4-folate triglutamate, N^5-methyl-H_4-folate, N^5-formyl-H_4-folate, H_4-homofolate, H_4-homopteroate, H_4-pteridine. Of these inhibitors, all were competitive with N^{10}-formyl-H_4-folate except N^5-methyl-H_4-folate which was noncompetitive. A model of the pteroyl molecule which binds to the enzyme is suggested from the observed inhibitions. Under appropriate conditions, a reduced pteroyl-dependent deformylation of N-[^{14}C]formylmethionyl-$tRNA^{fMet}$ was observed. Of the various pteridine derivatives studied, only H_4-folate and H_4-pteroate were effective as formyl acceptors. The product of the reaction was identified as $N^{5,10}$-methenyl-H_4-folate when H_4-folate was used as the acceptor. H_4-Homofolate was an effective inhibitor of the deformylation reaction. The deformylation reaction was compared with previously described deacylations of other acylaminoacyl transfer ribonucleic acid molecules and acyl polypeptides.

The identification of fMet-$tRNA^{fMet}$ [1] (Marcker and Sanger, 1964) has been followed by a great deal of information regarding the structure and function of this species of transfer RNA in *Escherichia coli*. In contrast, little attention has been directed to the mechanism of the formylation reaction in which Met-$tRNA^{fMet}$ is the formyl group acceptor. This reaction is similar to the aminoacyl-tRNA synthetase reactions in which the recognition of a specific tRNA molecule by an enzyme is a component step in the overall reaction. Previous studies from this laboratory have provided indirect evidence that the transformylase and Met-$tRNA^{fMet}$ form a complex, while Met-$tRNA^{Met}$ and the nonacylated $tRNA^{fMet}$ do not interact with purified enzyme (Dickerman and Smith, 1967). This suggested that a recognition step between the enzyme and the aminoacyl-tRNA substrate preceded the formylation reaction.

As regards the folate participation in the reaction, N^{10}-formyl-H_4-folate was identified as the formyl donor (Marcker, 1965; Dickerman *et al.*, 1967). At present, the Met-$tRNA^{fMet}$ transformylase is the fourth transferase in which a folate derivative has been identified as the required formyl donor. The other reactions are (1) N^5,N^{10}-methenyl-H_4-folate–glycinamide ribotide transferase, (2) N^{10}-formyl-H_4-folate–4-amino-5-carboxamide ribotide transferase (Hartman and Buchanan, 1959), and (3) N^5-formyl-H_4-folate–L-glutamate transferase (Silverman *et al.*, 1957). Aside from the specificity of the donors, little is known about the mechanism of these reactions.

In the present study, the mechanism of Met-$tRNA^{fMet}$ formylation was approached in two ways. First, the effect of different pteridine derivatives on the rate of formylation was determined. The objective was to determine if a competitive inhibition was present between these derivatives and N^{10}-formyl-H_4-folate. The structural specificities of this inhibition would yield information on the aspects of the formyl donor which are required for binding to the transformylase.

The second aspect of the mechanism of Met-$tRNA^{fMet}$ formylation which was investigated was the reversal of the reaction. This reversal was assayed as a H_4-pteroyl-dependent release of radioactivity from N-[*formyl*-^{14}C]fMet-tRNA. The characteristics of the reverse reaction and their implications as regards the mechanism of the N^{10}-formyl-H_4-folate–Met-$tRNA^{fMet}$ transformylation are examined in the present study.

Materials and Methods

E. coli B cells which had been grown in minimal medium, harvested at the midlogarithmic phase, and frozen were purchased from General Biochemicals, Inc., Chagrin Falls, Ohio. These cells were used as the source for N^{10}-formyl-H_4-folate–Met-$tRNA^{fMet}$ transformylase which was purified as described in a prior publication (Dickerman *et al.*, 1967). *E. coli* B tRNA was purchased from Schwarz BioResearch, Inc., Orangesburg, N. Y. [^{3}H]Methyl-labeled L-methionine (110 mCi/mmole) and [^{14}C]sodium formate (50 mCi/mmole) were purchased from the New England Nuclear Corporation,

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[1] The following abbrevations are used in the text: Met-tRNA, Phe-tRNA, etc., heterogenous *Escherichia coli* B tRNA esterified with L-methionine, L-phenylalanine, etc.; fMet-tRNA, heterogenous *E. coli* B tRNA esterified with *N*-formyl-L-methionine; $tRNA^{fMet}$, a molecule of methionine accepting tRNA which can be enzymatically formylated; $tRNA^{Met}$, a molecule of methionine accepting tRNA which cannot be enzymatically formylated; Met-$tRNA^{fMet}$, Met-$tRNA^{Met}$, the aminoacyl forms of $tRNA^{fMet}$, $tRNA^{Met}$; fMet-$tRNA^{fMet}$, the formylmethionyl ester of $tRNA^{fMet}$. In the reciprocal plots, the abbreviation, acylaminoacyl $tRNA_{fMet}$ is used to designate the mole fraction of heterogenous *E. coli* B tRNA present as the $tRNA^{fMet}$ species.

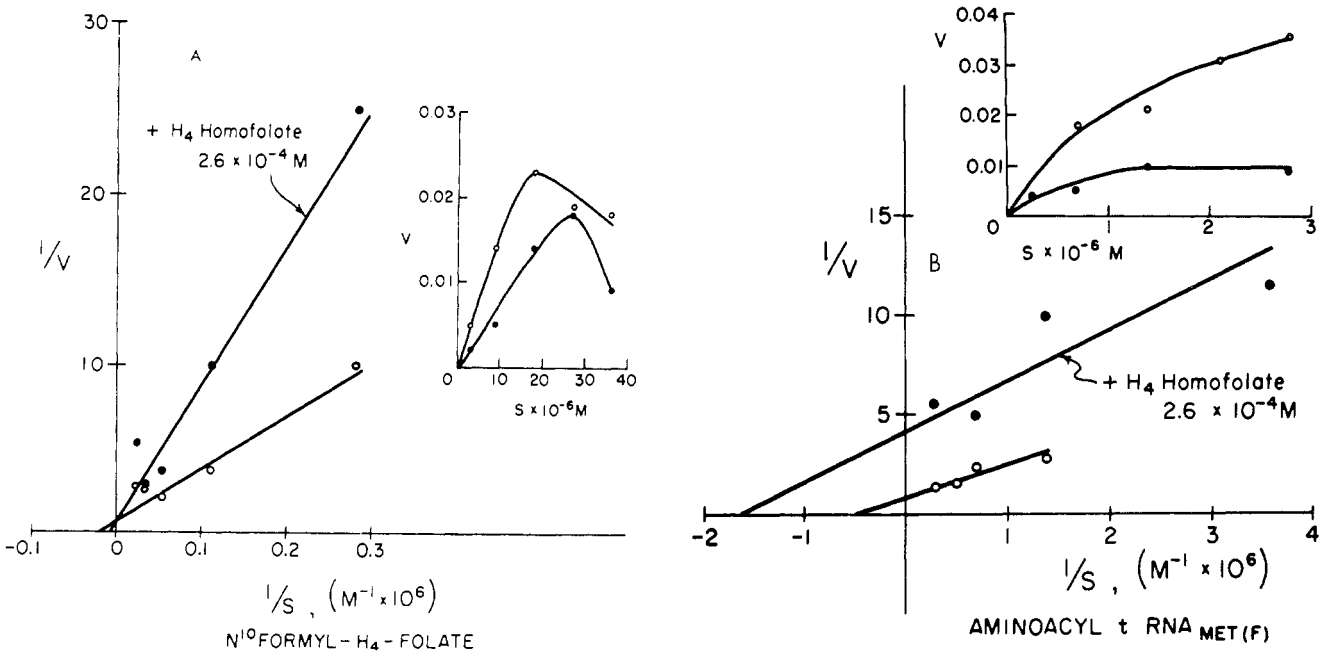

FIGURE 1: The effect of substrate concentrations on *dl*-L-H_4-homofolate inhibition of Met-tRNA formylation: (A) variation of N^{10}-formyl-H_4-folate concentration; (B) variation of Met-tRNA concentration. In these kinetic experiments as well as subsequent ones, the conditions of enzyme dilute and time of incubation were chosen to ensure that the observed product formation represented the initial rate of reaction. The assay procedure was as described previously. The reciprocal velocity determinations were corrected for the volumes of the reaction mixtures.

Boston, Mass. [^{3}H]4-ring-labeled phenylalanine (1 Ci/mmole) and [^{14}C]sodium acetate (52.9 mCi/mmole) were obtained from the Amersham-Searle Corporation, Des Plaines, Ill.

The H_4-folate derivatives were prepared by the dithionite method of Futterman. H_4-Folate, H_4-aminopterin, H_4-pteridine, H_4-pteroate, H_4-homofolate, and H_4-homopteroate were formed from their oxidized analogs according to the procedure described by Kisliuk (1957). H_4-Folate triglutamate was prepared from folate triglutamate and reduced by the method of Scrimgeour and Vitols (1966). N^5-Methyl-H_4-folate was prepared by the method of Keresztesy and Donaldson (1961). The identity of the above compounds were determined by their agreement with spectral values reported in the literature.

The details for the preparation of [^{14}C]N^5,N^{10}-methenyl-H_4-folate, partially purified *E. coli* B aminoacyl-tRNA synthetases, [^{3}H]Met-tRNA, and the assay for the formylation of Met-tRNA^{fMet} have all been described in prior publications (Dickerman *et al.*, 1967; Dickerman and Smith, 1967). [^{14}C]-Acetyl-[^{3}H]Met-tRNA and [^{14}C]acetyl-[^{3}H]Phe-tRNA were prepared by the methods of Lapidot *et al.* (1967). The H_4-pteroyl-dependent deformylation of fMet-tRNA was assayed by the following method. The reaction mixture included potassium phosphate (pH 5.8), 10 μmoles; [^{14}C]formyl-[^{3}H]Met-tRNA (the specific fraction of the total tRNA as fMet-tRNA was 0.008–0.01 and the specific activity was 56,000 cpm/mμmole), 0.14–0.20 mμmole; H_4-folate or H_4-pteroate, 25 mμmoles; and Met-tRNA^{fMet} transformylase in a final volume of 0.05 ml. The mixture was incubated at 37° for 2 min.

At this point, three variations of the assay were employed.

Procedure A. The incubation was terminated by the addition of 1 ml of 10% trichloroacetic acid and the mixture was kept in an ice bath for 10 min. After this the tubes were centrifuged at 27,000*g* for 5 min and 0.5 ml of the supernatant was transferred to a counting vial. The aliquot was neutralized with 1 N NH_4OH (0.2–0.4 ml) and 10 ml of a naphthalene–dioxane solution (Bray, 1960) was added. The radioactivity was determined by liquid scintillation detection.

Procedure B. The incubation was terminated by the addition of 1 ml of ice-cold ethanol and 0.1 ml of 20% potassium acetate (pH 5.2). Deacylated *E. coli* B tRNA (1 mg) was added prior to the ethanol to ensure uniform conditions of precipitation. The remaining steps were as in procedure A with the exception that the addition of NH_4OH was omitted.

Procedure C. The assay was the same as for the forward reaction. In this case, a disappearance of acid-precipitable counts was followed as an index of deformylation.

The concentration of tRNA was estimated on the basis of E^{M}_{cm} 6.4 $\times$ 10^5 derived from the data of Tissieres (1959). Protein was determined by the procedure of Lowry *et al.* (1951).

The kinetic constants were determined from data on the initial rates of formylation in the presence and absence of appropriate concentrations of the H_4-pteridine derivatives. The apparent inhibitor constant for competitive inhibitors were calculated from the equation $K_p = K_m(1 + i/K_i)$, where K_p = substrate concentration giving half-maximal velocity in the presence of the inhibitor, K_m = the Michaelis constant, K_i = inhibitor constant, and i = inhibitor concentration. The constant for noncompetitive inhibition was calculated from the equation $V_p = V(1 + i/K_i)$, where V_p = maximum velocity in the presence of inhibitor, V = maximum velocity in absence of the inhibitor, K_i = inhibitor constant, and i = inhibitor concentration (Dixon and Webb, 1958).

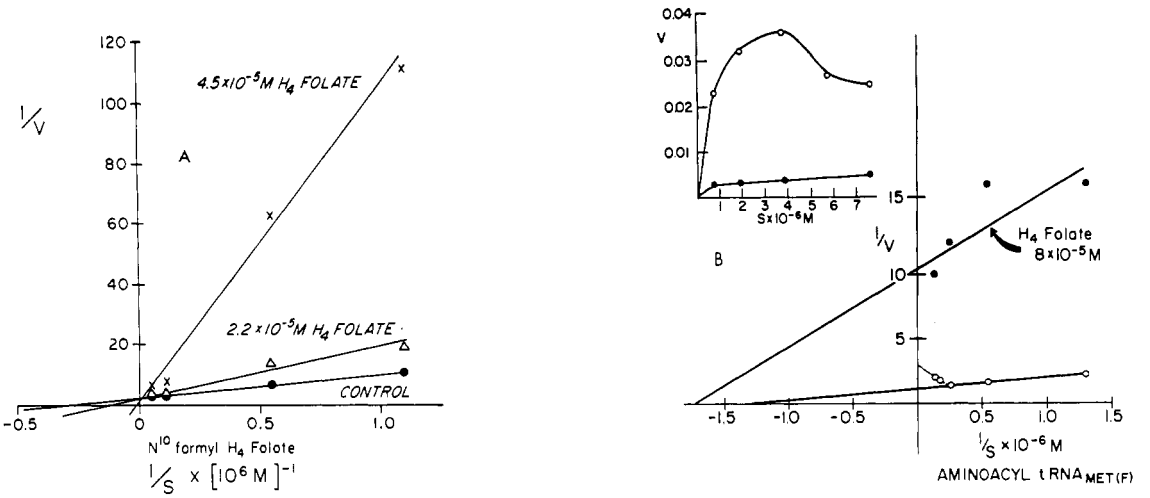

FIGURE 2: The effect of substrate concentration on *dl*-L-H_4-folate inhibition of Met-tRNA formylation: (A) variation of N^{10}-formyl-H_4-folate concentration; (B) variation of Met-tRNA concentration.

Results

The identification of N^{10}-formyl-H_4-folate as the formyl donor in the formylation of Met-tRNA^{tMet} suggested that other pteridine derivatives might affect this reaction. A preliminary survey of such compounds demonstrated that dl-L-H_4-homofolate was an inhibitor of the reaction at concentrations about tenfold greater than *l*-L-N^{10}-formyl-H_4-folate. H_4-Homofolate differs from H_4-folate by an additional methylene group between carbon 9 and the amino group of the *p*-aminobenzoyl moiety of the reduced pteroyl derivative. A double-reciprocal plot demonstrated that H_4-homofolate was a competitive inhibitor of N^{10}-formyl-H_4-folate and uncompetitive with Met-tRNA (Figure 1). The decrease in velocities at the highest substrate concentration in Figure 1A reflect either substrate or product inhibition. It is difficult to dissociate these inhibitory mechanisms. However the subsequent demonstration (see Figure 2A) that H_4-folate is an inhibitor of formylation suggests that product inhibition is the cause of the observed decrease in velocities. The K_i of H_4-folate (Table I) is 2×10^{-5} M and this is consistent with the inhibition seen in Figure 1A.

H_4-Folate, a presumed product of the formylation reaction, was found to be inhibitory at concentrations approximately equal to the substrate, N^{10}-formyl-H_4-folate. An analysis of the inhibition indicated that *dl*-L-H_4-folate was competitive with N^{10}-formyl-H_4-folate and uncompetitive with Met-tRNA (Figure 2). The inhibition was reversible with time of incubation even when *dl*-L-H_4-folate was present at a concentration tenfold greater than N^{10}-formyl-H_4-folate (Figure 3). The

TABLE I: Inhibition of Met-tRNA Formylation by Pteridine Derivatives.

Pteridine Derivative	Inhibitor Const (M)	Type of Inhibition
dl-L-H_4-Folate	2×10^{-5}	Competitive
dl-L-H_4-Pteroate	3×10^{-5}	Competitive
dl-L-H_4-Folate triglutamate	4×10^{-5}	Competitive
N^5-Formyl-*dl*-L-H_4-folate	5×10^{-5}	Competitive
N^5-Methyl-*dl*-L-H_4-folate	5×10^{-5}	Noncompetitive
dl-L-H_4-Homofolate	1×10^{-4}	Competitive
dl-L-H_4-Homopteroate	2×10^{-4}	Competitive
2-Amino-4-hydroxyl-H_4-pteridine	2×10^{-4}	Competitive
dl-L-H_4-Aminopterin		Noninhibitory[a]
L-H_2-Folate		Noninhibitory[a]
L-Folate		Noninhibitory[a]
L-N^{10}-Methylfolate		Noninhibitory[a]

[a] A compound was evaluated as noninhibitory if it was less than 10% inhibition at concentrations of 1×10^{-3} M under standard assay conditions.

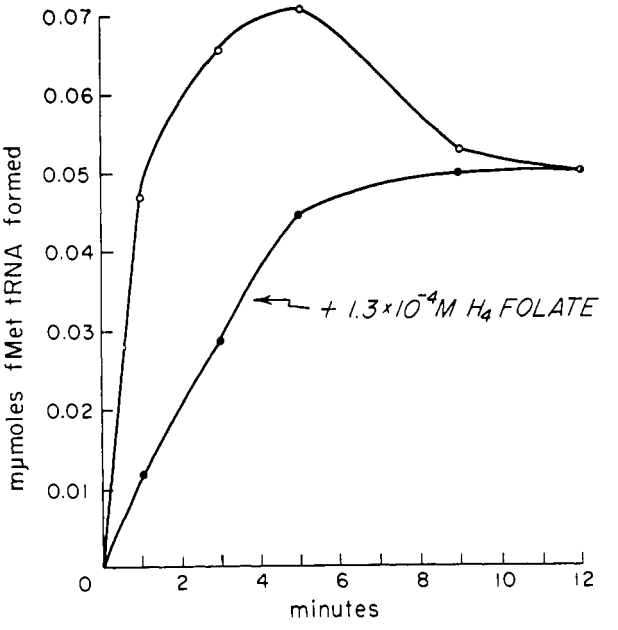

FIGURE 3: The effect of the duration of incubation on *dl*-L-H_4-folate inhibition of Met-tRNA formylation.

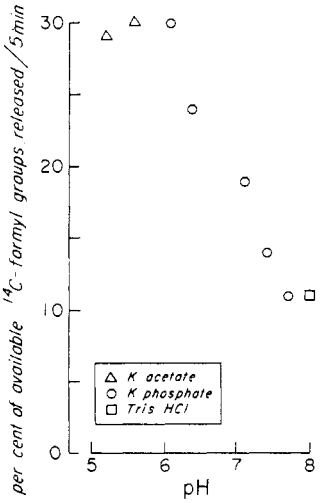

FIGURE 4: The effect of pH on the H_4-folate dependent release of acid-soluble radioactivity from *N*-[^{14}C]formyl-Met-tRNA. The conditions of the assay are described in the section on Methods (procedure A).

TABLE II: Requirements for the Deformylation of fMet-tRNA.[a]

Omissions	mμmoles of Acid Soluble/2 min	
	H_4-Folate	H_4-Pteroate
None	0.025	0.043
Enzyme	0.014	0.009
KPO_4, pH 5.8	0.009	0.029
H_4-Folate	0.012	
H_4-Pteroate		0.009

[a] The reaction mixture included K-PO_4 (pH 5.8), 10 μmoles; $MgCl_2$, 1.5 μmoles; H_4-folate, 0.1 μmole or H_4-pteroate, 0.035 μmole; [^{14}C]formyl-[^{3}H]Met-RNA, 0.144 mμmole (formyl-Met-tRNA represents 1% of added tRNA); and Met-tRNA transformylase, 10 μg. H_4-Folate and H_4-pteroate were acid soluble and incubated for 2 min at 37°. The assay was performed as described in the section on Methods (procedure B).

decrease in the product in the control incubation is due to the lability of fMet-tRNA. In the assay of formylation, 0.2 M sodium cacodylate (pH 7.8) was added to facilitate the non-enzymatic conversion of N^5,N^{10}-methenyl-H_4-folate into N^{10}-formyl-H_4-folate. However, under these conditions, there was a decrease in the product such that 10–15% of [^{14}C]-fMet-tRNA is no longer acid precipitable at 5 min and 20–25% at 12 min. This loss of the product limits the linearity of the assay of formylation to very short durations of incubation. The lability is pH dependent as there is but a 5% loss in acid-precipitable fMet-tRNA following a 12-min incubation at pH 5.8. The lability of the [^{14}C]fMet-tRNA is probably a release of [^{14}C]formylmethionine from the tRNA at the pH and ionic strength of the assay conditions. The decrease of the inhibition with duration of incubation suggests that an inhibited complex dissociates to H_4-folate and free enzyme; the latter then becomes available for interaction with the substrate.

A summary of the effects of the pteridine derivatives is presented in Table I. With a single exception, all those compounds which were inhibitory were competitive with N^{10}-formyl-H_4-folate. The exception was N^5-methyl-*dl*-L-H_4-folate which although an effective inhibitor, was found to be noncompetitive with the formyl donor. The derivatives could be assorted on the basis of decreasing affinity for Met-tRNA^{fMet} transformylase as follows: H_4-folate monoglutamate > H_4-pteroate, H_4-folate triglutamate, N^5-formyl-H_4-folate > H_4-homofolate, H_4-homopteroate, H_4-pteridine. Folic acid, H_2-folate, N^{10}-methylfolate, and H_4-aminopterin were not significant inhibitors at concentrations $<1 \times 10^{-3}$ M. The structural aspects of these inhibitors which related to binding of N^{10}-formyl-H_4-folate to the transformylase will be reviewed in the discussion.

The Reversal of the Reaction

Recently, other investigators (Cuzin *et al.*, 1967; Raj-Bhandary and Kossel, 1968) have reported the presence of bacterial enzymes which cleave the acylaminoacyl groups from acylaminoacyl-tRNAs. Paulin and coworkers (1968) have found that a purified enzyme from *E. coli* MRE-600 cleaves formyl as well as acetylamino acids from chemically acylated tRNA. Another form of a deacylase would be the reversal of Met-tRNA^{fMet} formylation in which the formyl group is released from fMet-tRNA^{fMet} and a reduced pteroyl derivative, presumably H_4-folate, would serve as the acyl acceptor instead of water as is the case with the other deacylases. Attempts to demonstrate such a reversal of the transformylase reaction were inconsistent until advantage was taken of the nonenzymatic interconversion of N^{10}-formyl-H_4-folate and N^5,N^{10}-methenyl-H_4-folate. The former is the formyl donor in the forward reaction while the latter is not (Dickerman *et al.*, 1967). The two folate derivatives are readily interconverted and the predominant equilibrium form is determined by the hydrogen ion concentration. At neutral or slightly alkaline pH, N^{10}-formyl-H_4-folate is the predominant form, while N^5,N^{10}-methenyl-H_4-folate is favored at acidic pH (Rabinowitz, 1960). Due to this effect of the hydrogen ion concentration, the immediate product of fMet-tRNA^{fMet} deformylation, N^{10}-formyl-H_4-folate could be trapped as the nonutilizable derivative, N^5,N^{10}-methenyl-H_4-folate. This is described as follows.

$$N\text{-}[^{14}C]\text{formyl-Met-tRNA}^{fMet} + H_4\text{-folate} \longrightarrow \text{Met-tRNA}^{fMet} + N^{10}\text{-}[^{14}C]\text{formyl-}H_4\text{-folate} \quad (1)$$

$$N^{10}\text{-}[^{14}C]\text{formyl-}H_4\text{-folate} \underset{H^-}{\rightleftharpoons} N^5,N^{10}\text{-}[^{14}C]\text{methenyl-}H_4\text{-folate} \quad (2)$$

If the above scheme was valid, then an increase in the extent of H_4-folate-dependent enzymatic deformylation of fMet-tRNA^{fMet} would be anticipated at increased hydrogen ion concentrations. This is borne out by the data shown in Figure 4: the optimum of the deformylation reaction occurred between pH 5.2 and 6.1. This is in contrast to the optimal pH for the forward reaction where there was an abrupt decrease in the rate below pH 7.0 (Dickerman *et al.*, 1967).

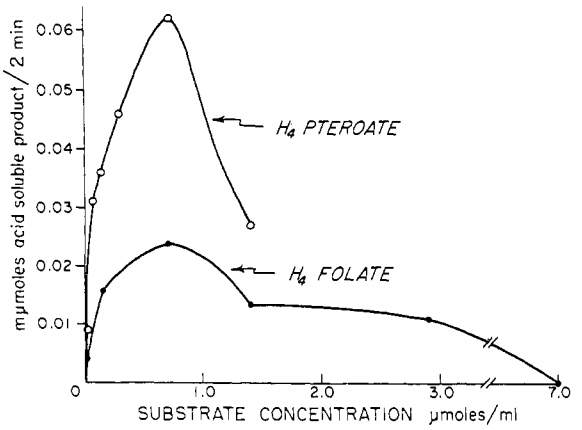

FIGURE 5: The effect of H_4-pteroate–H_4-folate concentration on the H_4-pteroyl-dependent deformylation of fMet-tRNA. The conditions of the assays are described in the section on Methods (procedure B).

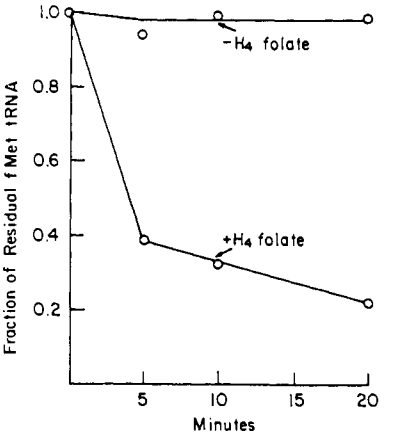

FIGURE 6: The effect of the duration of incubation on the H_4-folate-dependent release of [^{14}C]formyl groups from fMet-tRNA. The incubation conditions are as described in the section on Methods (procedure C).

The decrease was due to the conversion of N^{10}-formyl-H_4-folate into N^5,N^{10}-methenyl-H_4-folate and is consistent with the suggestion that the reverse reaction is amplified by the trapping of the formyl product as N^5, N^{10}-methenyl-H_4-folate.

The requirements for the deformylation of fMet-tRNA are described in Table II. H_4-Pteroate or H_4-folate and the transformylase were required for the reaction as well as the formyl donor, N-[^{14}C]formyl-Met-tRNA.[2] In the data shown in Table II, there is but a moderate increase in deformylation by addition of either H_4-folate or enzyme when H_4-folate is the acceptor. The final concentration of H_4-folate is 2×10^{-3} M. This is consistent with the data of Figure 5 in which a concentration optimum for H_4-folate wax found to exist with inhibition occurring at values exceeding 7×10^{-4} M. In addition, the buffer was a requirement for the reaction as would be anticipated from the effect of pH on the release of formyl groups as seen in Figure 4. At optimal concentrations, H_4-pteroate was three times as active as H_4-folate as a formyl acceptor in the reaction. Other reduced pteroyl derivatives were tested and none were active formyl acceptors. These included H_2-folate, H_4-homofolate, H_4-aminopterin, and H_4-pteridine. The response of the deformylation reaction to variation in concentration of H_4-pteroate or H_4-folate is shown in Figure 5. With both derivatives, the reaction was saturated at 7×10^{-4} M and a significant inhibition was observed at higher concentrations. The dependence on H_4-folate is also demonstrated in Figure 6. While there was essentially no loss in acid-precipitable radioactivity in the absence of H_4-folate, there was a marked decrease when H_4-folate was present. A linear relationship of reaction rate with time up until 8 min was observed in other experiments in which dilute enzyme solutions were used.

The effect of the fMet-tRNA concentration on the deformylation reaction is shown in Figure 7. The reaction was saturated with fMet-tRNA at 8×10^{-6} M when H_4-folate was the acceptor. The results were essentially the same when H_4-pteroate was used as the formyl acceptor. The experimental determination of the specificity of the acyl donor is shown in Figure 8. N^{10}-[^{14}C]Formyl-Met-tRNA and N^{10}-[^{14}C]acetyl-Met-tRNA were compared at equivalent acylaminoacyl-tRNA concentrations as substrates for the H_4-folate-dependent deacylation. Although 55–65% of the available formyl groups were released under conditions of excess enzyme, less than 5% of the available acetyl groups were removed. There was little if any hydrolase activity present when the purified transformylase was used with either acylaminoacyl-tRNAs.

Figure 9 demonstrates the variation of the rate of the H_4-folate-dependent deformylase with the amount of purified transformylase. The maximum extent of the reaction was observed with 1.5 μg or more of the enzyme per reaction tube. A direct comparison of the deformylation reaction rate with that of the formylation of Met-tRNA was not possible because of the different hydrogen ion concentrations of the reaction mixtures.

The identity of the soluble product of the H_4-folate-dependent deformylation of N^{10}-[^{14}C]formyl-Met-tRNA was determined in order to clarify the mechanism of the reaction. Following the procedure described in the legend of Figure 10 the peak of the applied radioactivity was found to coincide with the fluorescent area of N^5,N^{10}-methenyl-H_4-folate. This

TABLE III: Effect of H_4-Homofolate on Reverse Reaction.[a]

H_4-Homofolate (M)	mμmoles of Product/2 min	% Inhibition
	0.045	
2.4×10^{-4}	0.034	24
1.2×10^{-3}	0.017	62
2.4×10^{-3}	0.009	80

[a] H_4-Folate concentration was 8×10^{-4} M.

[2] Addition of magnesium chloride had a stimulatory effect on the H_4-folate dependent deformylation of fMet-tRNA. Under the assay conditions including procedure C, 0.013 mμmole of the substrate was deformylated in the absence of added magnesium chloride, while 0.0175 mμmole was deformylated when the final concentration of the salt was 3×10^{-2} M.

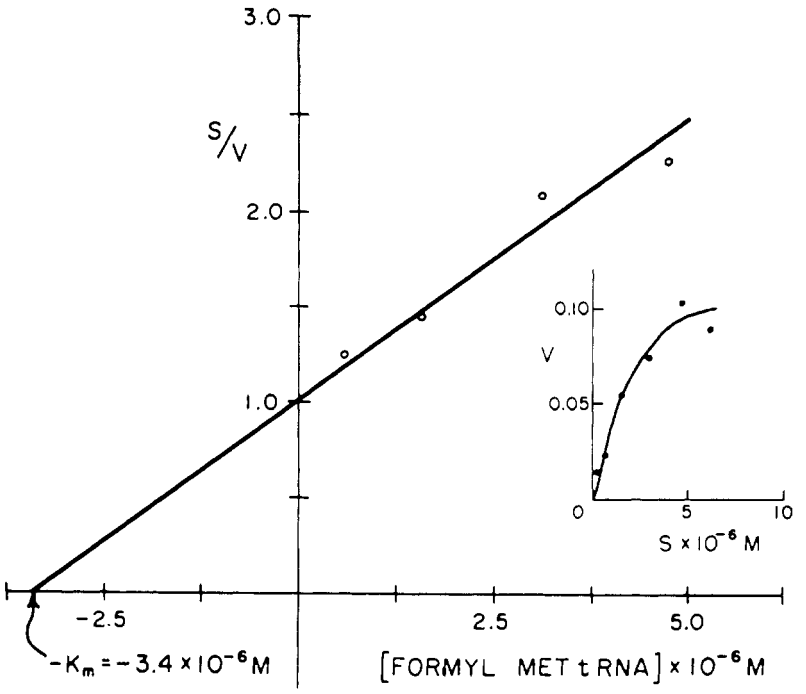

FIGURE 7: The effect of fMet-tRNA concentration on the H_4-pteroyl-dependent deformylation of fMet-tRNA. In these experiments, the fMet-tRNA content represented 1% of the added tRNA. The conditions of the assay are described in the section on Methods (procedure A).

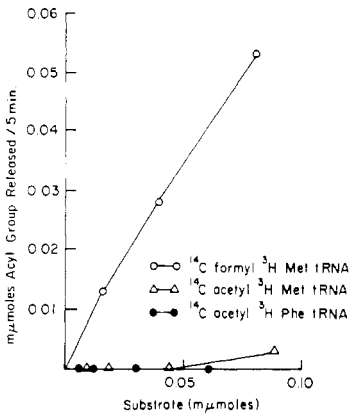

FIGURE 8: The effectiveness of formyl-Met-tRNA, acetyl-Met-tRNA' and acetyl-Phe-tRNA as substrates in the H_4-folate-dependent deacylation reaction. The reaction mixtures were 50 μl in volume and included potassium phosphate (pH 5.8), 10 μmoles; magnesium chloride, 1.5 μmoles; H_4-folate, 0.03 μmole; and purified Met-tRNA transformylase, 0.5 μg. The mole fractions of the acylamino-acyl-tRNAs were as follows: [^{14}C]formyl Met-tRNA, 0.007; [^{14}C]-acetyl-Phe-tRNA, 0.005. In the control tubes, 0.02 μmole of mercaptoethanol was substituted for H_4-folate and this amount of the sulfhydryl reagent was equivalent to that in which the H_4-folate was dissolved. The incubation was for 5 min at 37°. The remainder of the assay was according to procedure C in the section on Methods.

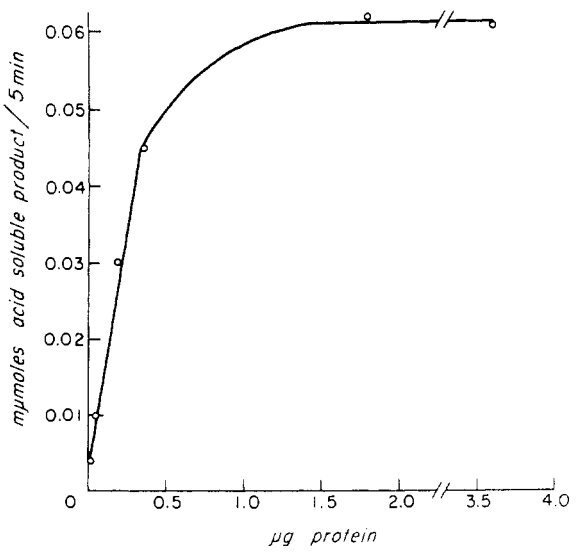

FIGURE 9: The effect of enzyme concentration on the H_4-folate-dependent release of [^{14}C]formyl groups from fMet-tRNA. The incubation conditions are as described in the section on Methods (procedure A).

radioactivity represented a 35% recovery while, under the same conditions, 43% of the standard marker was recovered (Figure 10A). An alternate identification was executed by chromatography on a 1 × 15 cm microcrystalline cellulose column (W. and R. Balston Ltd., England) (Figure 10B). A correspondence occurred again between the spectral peak of 355 mμ and the observed radioactivity. However, under these conditions, a minor peak of radioactivity preceded the peak identified with N^5,N^{10}-methenyl-H^4-folate. This peak was also observed in incubations in which the transformylase was omitted. The identification of labeled N^5,N^{10}-methenyl-H_4-folate[3] as the product of the reaction as well as the requirement for the acyl donor and acceptor indicate that the sequence outlined in eq 1 and 2 is probable. It is unlikely that the

[3] The reactivity of the reversal product in the forward reaction, the formylation of Met-tRNA, was tested under standard conditions. The reversal product was isolated by the method described in the legend of Figure 10B and concentrated by lyophilization. Following a 10-min incubation at 37°, 0.12 mμmole of an acid-precipitable product was

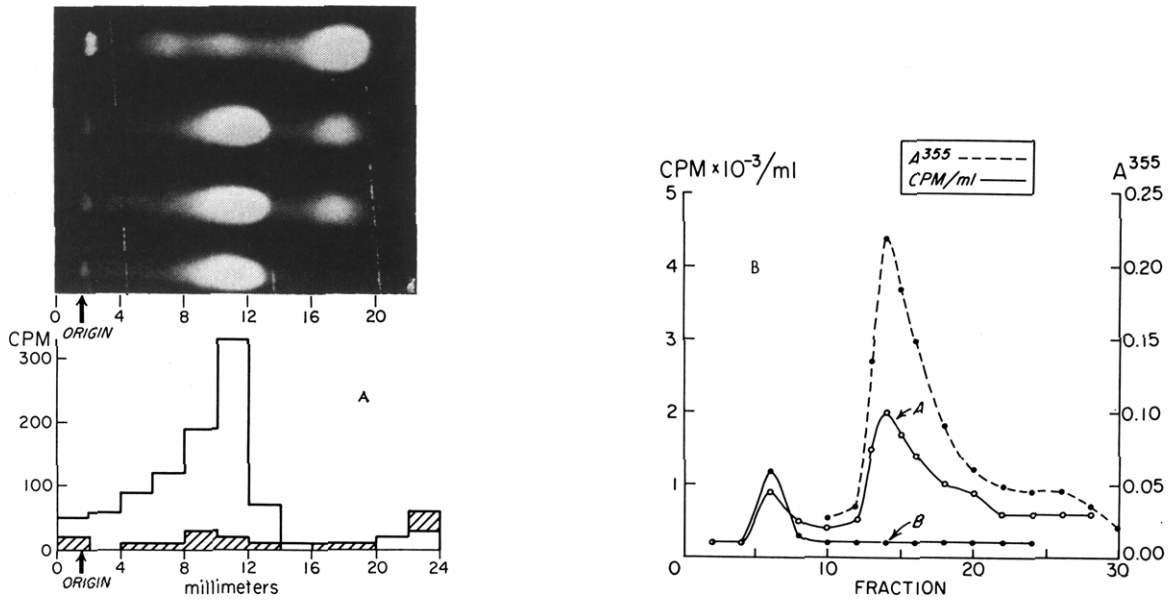

FIGURE 10: The standard assay reaction mixture was increased in the following manner. Two reaction mixtures, 0.5 ml of final volume, contained potassium phosphate (pH 5.8), 100 μmoles; magnesium chloride, 15 μmoles; H_4-folate, 1.7 μmoles; fMet-tRNA, 2.2 mμmoles and water to adjust to final volume. To one mixture, 50 μg of purified transformylase was added while the other mixture was devoid of enzyme. The mixtures were incubated at 37° for 20 min. At the end of the incubation, 0.55 μmole of N^5,N^{10}-methenyl-H_4-folate were added to both reaction mixtures. Following this addition, 1 ml of 10% trichloroacetic acid–0.1 M mercaptoethanol was added and the tubes were kept at 0° for 5 min. After centrifugation, the supernatants were removed and these were extracted three times with cold ether. At this step, there were 101,500 cpm in the enzymatic reaction supernatant and 17,500 in that derived from the nonenzymatic mixture. (A) Aliquots (50 μl) were taken from the supernatants and applied to Whatman No. 1 paper (15 × 25 cm). The chromatogram was developed by an ascending technique with 1 N formic acid–0.1 M mercaptoethanol. Standards for H_4-folate and N^5,N^{10}-methyl-H_4-folate are run in the upper and lower tracts, respectively. The fluorescent spots were visualized in ultraviolet light. The developed chromatograph was cut into 1-cm strips and transferred to 10 ml of the naphthalene–dioxane scintillation solution and the radioactivity is recorded in the lower aspect of the figure. (B) The reaction mixtures (1 ml) were applied sequentially to a microcrystalline cellulose column (Whatman CC31, W and R Balston, Ltd.) (1 × 15 cm) and eluted with 0.1 N formic acid–0.01 M mercaptoethanol. Both the radioactivity (cpm/ml) and the absorbance at 355 mμ of the fractions are recorded. Each fraction was 3.5 ml and the rate of elution was 3 fractions/hr.

N^5,N^{10}-methenyl-H^4-folate resulted from formylmethionine following deacylation, as the addition of nonradioactive DL-formylmethionine during the reaction did not lead to a dilution of radioactivity in the product. In addition, there was no detectable N^{10}-formyl-H_4-folate synthetase activity in the enzyme preparation which was used.

Although only H_4-pteroate and H_4-folate were found to be active formyl acceptors, H_4-homofolate was an effective inhibitor of the deformylation reaction as shown in Table III. H_4-Homopteroate, H_4-pteridine, H_4-aminopterin, N^5,N^{10}-methenyl-H_4-folate, and H_2-folate did not lead to significant inhibitions when present at equimolar concentrations with H_4-folate.

Discussion

Although at least three enzymatic transformylation reactions have been studied in which the formyl donor is a derivative of H_4-folate, there is little information about the requirements for binding of the donor molecule to the respective enzymes. N^{10}-Formyl-H_4-folate–Met-tRNA^{fMet} transformylase is an addition to this class of enzymes with the peculiarity that it catalyzes the formylation of a macromolecular derivative. In the present study, analogs of the formyl donor were tested for their inhibitory effect and, with one exception, all those compounds which were inhibitory were competitive with N^{10}-formyl-H_4-folate. On the basis of these studies, a model of the groups on the donor molecule which effect binding to the enzyme can be presented.

Reiner has commented that competitive kinetics does not prove that the inhibitor binds at the active site but only that the enzyme cannot further complex with the substrate, regardless of where the inhibitor is bound to the enzyme (Reiner, 1959). While aware of this criticism, the authors suggest that the close structural similarities of the inhibitors to the substrate, as well as the decrease of inhibition with the duration of the reaction, make binding at a common site the most likely possibility.

On this basis, the contribution to the binding of the various parts of the N^{10}-formyl-H_4-folate molecule (Figure 11) are interpreted as follows.

(1) There is a clear preference for a 4-hydroxyl substituent on the pyrimidine ring rather than a 4-amino group for binding to the enzyme. This is borne out by the effective inhibition of H_4-folate, H_4-pteroate, and H_4-folate triglutamate in contrast to the apparent absence of inhibition by H_4-aminopterin. The tautomer of the hydroxyl group at position 4 of the pyrimidine ring which is stable is the keto rather

formed with the reversal product (specific activity, 560 cpm/mμmole) and 0.10 mμmole of product with the standard donor, [^{14}C]N^5,N^{10}-methenyl-H_4-folate (specific activity 53,000 cpm/mμmole). The decreased specific activity of the reversal product was due to the carrier N^5,N^{10}-methenyl-H_4-folate added prior to the isolation.

TABLE IV: Macromolecular Deacylation Reactions.

	H_4-Pteroyl-Dependent Deformylation	Acylaminoacyl-tRNA Hydrolysis	Peptidyl Deacylation
Substrate	Formyl-Met-tRNA^{fMet}	All acylaminoacyl-tRNAs; oligopeptidyl-tRNA	Formyl peptides, acetyl peptides, formyl polypeptides
Released Group	Formyl	Acylamino acid	Formyl, acetyl
Acceptor	H_4-Pteroate H_4-Folate	Water	Water
Location	Supernatant	Ribosome	Supernatant
Effect of mercaptoethanol	None	?	Inhibitory
Stability of enzyme	Stable	Stable	Labile

than the enol form (Collin and Pullman, 1964; Baker and Ho, 1964). This structural determinant coupled with loss of inhibition by an amino group which cannot exist in a keto form suggests that a keto group at position 4 may form a bond with an enzyme-bound hydrogen.

(2) There was an absolute requirement, among the inhibitors, for a fully reduced pyrazine ring. The structural factors that might influence binding of the derivatives to the enzyme are as follows; (a) the stereospecificity of the substrate and of those compounds which are inhibitors by the introduction of an asymmetric carbon atom at position 6 on full reduction of the pyrazine ring; (b) a marked rearrangement of the pteridine rings in reference to the *p*-aminobenzoylglutamyl portion of the molecule occurs on conversion of the trigonal carbon at position 6 into a tetrahydral one; (c) the effect that the quaternary nitrogen of the reduced pyrazine ring may exert on the hydroxyl group attached to carbon 4 of the pyrimidine ring.

It is unclear, at this time, which of the above, alone or in combination, explain the sharp distinction in inhibition between tetrahydropteridine derivatives and their more oxidized analogs.

(3) There was little change in the inhibition by the introduction of substituents at the nitrogen 5 position. Both N^5-methyl-H_4-folate and N^5-formyl-H_4-folate had inhibition constants in the same range as H_4-folate. In the case of the methyl derivative, the inhibition was noncompetitive. The mechanism of this difference is unknown at this time.

(4) There was a significant decrease in inhibitory effectiveness with enlargement of the bridge between the pteridine and the *p*-aminobenzoylglutamyl portions of the molecule

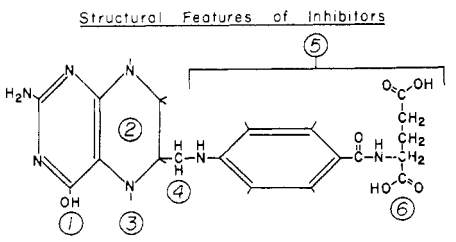

FIGURE 11: Structural features of the inhibitors.

by an additional methylene group. This was demonstrated by an approximately tenfold increase in the inhibition constants of H_4-homopteroate and H_4-homofolate as compared with their homologs. These results contrast with the studies on *E. coli* thymidylate synthetase. Plante and his coworkers (1967) found that the presence of an extra methylene group was sufficient to convert a derivative from a substrate for the synthetase into an inhibitor. In the case of H_4-homofolate, the inhibition occurred at concentrations of the inhibitor significantly below those of the substrate, N^5,N^{10}-methylene-H_4-folate.

(5) There was not an absolute dependence on the *p*-aminobenzoylglutamyl residue for a derivative to be an inhibitor of the enzyme. Although only one reduced pteridine compound was examined, it was found to be inhibitory in the same range as was H_4-homofolate and H_4-homopteroate. The failure of H_4-aminopterin to inhibit also diminishes the importance of binding by the *p*-aminobenzoylglutamyl residue as there are no differences between H_4-folate and H_4-aminopterin beyond the pyrimidine ring.

(6) A derivative did not require L-glutamic acid as part of its structure in order to be an inhibitor. H_4-Pteroate was almost as effective an inhibitor as H_4-folate and there was little difference between H_4-homofolate and H_4-homopteroate. H_4-Folate triglutamate was a slightly less effective inhibitor which may be a result of steric hindrance by the additional diglutamyl group. This contrasts with the results with *E. coli* thymidylate synthetase where a H_4-pteroyl group containing a dicarboxylic acid moiety with properly oriented alpha amino and alpha hydrogen groups was a requirement for substrate activity (Plante *et al.*, 1967).

The presence of the H_4-pteroyl-dependent deformylation of fMet-tRNA^{fMet} indicates that the formylase is reversible under appropriate conditions. An aspect of the reversal is that the enzyme must recognize fMet-tRNA^{fMet} as well as Met-tRNA^{fMet} and, thereupon, catalyze the removal of a formyl group from the amino group of methionine esterified to tRNA. In this respect, the transformylase may be compared with other enzymes which catalyze deacylation of macromolecules (Table IV). Although the transformylase and the peptidyl deacylase catalyze the release of an acyl group, the transformylase specifically catalyzes the release of a formyl group while the peptidyl deacylase releases both formyl and acetyl groups. In addition, the transformylase is

specific for an acylaminoacyl-tRNA in contrast to that of an acyl peptide sequence. In regard to the enzyme described originally by Cuzin and coworkers (1967), the specificity is for acylaminoacyl-tRNAs with one significant exception, *N*-formyl Met-tRNA^{fMet}. Two groups (RajBhandary and Kossel, 1968; Vogel *et al.*, 1968) have explicitly stated that this molecule is not a substrate for the hydrolase while Paulin and coworkers (1968) report that it is an active substrate. The ribonucleic acid specificity of the transformylase reaction has not been definitively determined. A contrast between the two enzymes is that the hydrolase catalyzes the release of an acylamino acid regardless of the structure of the acyl group (acetyl, formyl, or carbobenzoxy derivatives) while the transformylase effects the release of a formyl, not an acetyl group.

Another distinction between the Met-tRNA transformylase and the other enzymes is that in the former, a reduced pteroyl compound, either H_4-folate or H_4-pteroate, is the acyl acceptor while in the other reactions, water is the acceptor. H_4-Pteroate was a more effective substrate than H_4-folate which indicates that the L-glutamyl group is not a requirement. This was also the case as regards the effectiveness of H_4-pteroyl derivatives as inhibitors of the forward reaction. Unlike the forward reaction, only H_4-homofolate was a significant inhibitor of the deformylation of *N*-formyl-Met-tRNA. This may be spurious as the reverse reaction required higher concentrations of H_4-folate or H_4-pteroate and effective inhibitor concentrations may not have been obtained.

Finally, the Met-tRNA transformylase and the peptidyl deacylase are found in the cell supernatant while the *N*-acylaminoacyl-tRNA hydrolase is ribosomal bound. It is unclear if this spatial localization has any bearing on polypeptide synthesis or whether the H_4-folate-dependent deformylation of *N*-formyl-Met-tRNA^{fMet} is of physiological significance.

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