Evidence against the Folate-mediated Formylation of Formyl-accepting Methionyl Transfer Ribonucleic Acid in Streptococcus faecalis R*

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SUMMARY

The formylation of formyl-accepting methionyl transfer ribonucleic acid (tRNA^fMet) was investigated in *Streptococcus faecalis* R, an organism incapable of synthesizing folic acid but whose growth does not require the vitamin under defined conditions of culture. These studies were carried out because it was not clear how an organism with no folate could initiate protein biosynthesis. Growth of *S. faecalis* R on media free of folate but containing serine, methionine, thymine, adenine, and guanine was not affected by the presence of the folate antagonists, trimethoprim and aminopterin. However, growth of the organism on media lacking thymine and dependent upon the presence of either folic acid or 5-formyltetrahydrofolate was severely inhibited by the analogues. It was demonstrated that extracts prepared from *S. faecalis* R grown on a synthetic medium containing folate acid catalyzed the formylation of methionyl-tRNA^fMet^ only when 10-formyltetrahydrofolate but not when formate was used as the source of the formyl group. Extracts of these folate-free cells catalyzed the formylation of methionyl-tRNA^fMet^ by formate if tetrahydrofolate was added to the assay mixture. These results indicate that *S. faecalis* R grown in the absence of folate but in the presence of the supplements does not contain tetrahydrofolate and therefore formylated methionyl-tRNA^fMet^ may not be involved in initiation of protein synthesis in *S. faecalis* R, or the formylmethionyl ester of tRNA^fMet^ (fMet-tRNA^fMet^) may be formed by some as yet unknown enzyme system.

The discovery by Mareker and Sanger (1) of the presence of N-formylmethionyl transfer ribonucleic acid in extracts of *Esche-

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folic acid, but in the presence of the supplements mentioned above might occur by a mechanism different from that now accepted for other prokaryotic organisms. This investigation reports our results concerning the formyl donor dependence of the formylation in vitro of Met-tRNA\textsuperscript{Met} catalyzed by enzymes present in the S-100 supernatant fractions prepared from \textit{S. faecalis} R grown in medium containing folic acid, or in a medium free of added folate but containing serine, methionine, thymine, adenine, and guanine. The results obtained with \textit{S. faecalis} R are compared to those obtained from parallel control experiments with \textit{E. coli} A19.

**EXPERIMENTAL PROCEDURE**

**Materials**—\textsuperscript{14}C-Labeled sodium formate (specific activity 53.5 mCi per mmole) was purchased from Schwarz BioResearch. Trimethoprim and aminopterin were obtained from Calbiochem. \textit{E. coli} B tRNAs (stripped) was purchased from General Biochemicals. Bacto-folic acid assay medium was purchased from Difco, folic acid was purchased from Sigma, and (±)-\textsuperscript{5}-formyl-tetrahydrofolate (leucovorin, calcium salt) was kindly donated by the American Cyanamid Company. Alumina A-305 was obtained from Alcon. A stock culture of \textit{S. faecalis} R was kindly provided by Dr. H. Fraenkel-Conrat (Department of Molecular Biology and Virus Laboratory, University of California, Berkeley, California) and a culture of \textit{E. coli} mutant RNase 119 (34) was provided by Dr. H. Fraenkel-Conrat (Department of Molecular Biology and Virus Laboratory, University of California, Berkeley, California).

**Preparation of Tetrahydrofolate**—A modification of the method described by Blakley (35) was used to prepare (±)-\textsuperscript{5}-tetrahydrofolate. After reduction, the material was purified on diethylaminoethylcellulose with 0.2 M Tris-chloride, pH 7.0, containing 0.5 M 2-mercaptoethanol as the eluting buffer.

**Preparation of 10-\textsuperscript{14}C Formyltetrahydrofolate**—Formyltetrahydrofolate was prepared enzymatically from \textsuperscript{14}C formate and (±)-\textsuperscript{5}-tetrahydrofolate with crystalline \textit{Clostridium cylinder} formyltetrahydrofolate synthetase described by Rabinowitz and Prieue (36). The 10-\textsuperscript{14}C formyltetrahydrofolate was purified on diethylaminoethylcellulose by a modification of the method described by Ho and Jones (37). A linear ammonium acetate gradient, 0.05 to 0.30 M containing 0.1 M 2-mercaptoethanol, was used to elute the 10-\textsuperscript{14}C formyltetrahydrofolate which was subsequently converted to and stored as 5,10-\textsuperscript{14}C methylenetetrahydrofolate. 10-\textsuperscript{14}C formyltetrahydrofolate could be generated by adjusting solutions of the methenyl derivative to neutral pH.

**Growth of Cells—\textit{S. faecalis} R** (ATCC 8043) was grown in Bacto-folic acid assay medium (38) supplemented with either folic acid, 0.001 mg per ml (final concentration), or L-serine, 0.50 mg per ml; L-methionine, 0.20 mg per ml; thymine, 0.05 mg per ml; adenine, 0.01 mg per ml; and guanine, 0.01 mg per ml. The \textit{S. faecalis} R inoculum culture used in growth experiments was prepared by a series of four transfers on the Bacto-folic acid assay medium supplemented with L-serine, L-methionine, thymine, guanine, and adenine. The inoculum was prepared from cells transferred four times on this medium. They were then collected by centrifugation and suspended in an equal volume of medium deficient in folate and thymine. A 1% inoculum of the above suspension was used. Cultures were grown for 12 hours at 37° and the absorbance at 660 nm was measured on a Bausch and Lomb Spectronic 20 in 18-mm diameter tubes. Large scale growth of \textit{S. faecalis} R yielded 2.0 g of cells from 2.5 liters of either medium. The cells were harvested by centrifugation, washed twice with 0.9% sodium chloride, and stored at −90° for less than 1 week. \textit{E. coli} A19 was grown in medium containing the following: KH₂PO₄ (anhydrous), 2.0 g per liter; KH₂PO₄·3H₂O, 11.0 g per liter; (NH₄)₂SO₄, 4.0 g per liter; Casamino acids, 5.0 g per liter; MgSO₄, 0.24 g per liter; FeCl₃, 0.27 mg per liter; glucose, 12.0 g per liter. The cells were harvested by centrifugation, washed, and stored at −90°.

**Preparation of S-100 Supernatant Fractions**—Supernatant fractions, referred to as S-100 fractions, containing methionyl-tRNA synthetase activity, transformylase activity, and the enzymes necessary for the formation of 10-formyltetrahydrofolate were prepared by alumina grinding as described by Nirenberg (39), modified as follows. The standard buffer contained 30 mM 2-mercaptoethanol in order to help stabilize any endogenous tetrahydrofolate present. After centrifugation at 30,000 x g, the supernatant solution was centrifuged at 105,000 x g for 120 min. A portion of the upper four-fifths of the resulting S-100 solution was then dialyzed 8 hours against 120 volumes of standard buffer with one change of the dialyzing medium. The dialyzed and nondialyzed S-100 fractions were then frozen quickly, stored at −90°, and used within 2 weeks.

**Protein Determinations**—Protein concentrations were determined by a modification of the phenol reagent method with crystalline bovine serum albumin as the reference standard. The reagents used were twice as concentrated as those described by Lowry et al. (40) and the color development was allowed to proceed for 10 min instead of 30 min.

**Assay for Transformylation**—Transformylase activity was determined using either 10-\textsuperscript{14}C formyltetrahydrofolate or [\textsuperscript{5}]formate as the formyl donor by a method based on that described by Dieckerman et al. (14) and Marek and Sanger (1). The standard reaction mixture (50 μl) used when 10-formyltetrahydrofolate was the formyl donor included Tris-chloride, pH 7.4, 0.10 M; magnesium chloride, 15 mM; ammonium acetate, 10 mM; 2-mercaptoethanol, 20 mM; ATP, 5 mM; L-methionine, 0.20 mM; heterogeneous \textit{E. coli} B tRNA, 100 μg, varying amounts of S-100 enzyme fraction; and 10-\textsuperscript{14}C formyltetrahydrofolate, 0.022 mM. When formate was used as the source of the formyl group, the standard reaction mixture (100 μl) contained the same concentrations of reaction components, with 0.126 mM [\textsuperscript{5}]formate instead of the 10-\textsuperscript{14}C formyltetrahydrofolate. After incubation at 37° for the amount of time indicated, the reaction was stopped by the addition of cold 10% trichloroacetic acid, mixed vigorously, and the precipitate was collected on Whatman glass fiber filters which had been prewashed with 1 mM sodium formate. The reaction tube was washed 5 times with cold 5% trichloroacetic acid and the washes were decanted onto the glass fiber filter. The filters were then counted for radioactivity in plastic scintillation vials containing 10 ml of Bray’s solution in a Nuclear-Chicago Mark I liquid scintillation counter to give a standard error of counting rate of less than 0.5%. All samples were checked for quenching by the channel ratio method but no correction was required.

**RESULTS**

**Folic Acid Requirement for Growth of \textit{S. faecalis} R**—Growth of \textit{S. faecalis} R is dependent upon the addition of folic acid or a combination of the metabolites serine, methionine, a purine, and thymine. These metabolites are now recognized to be products...
of biosynthetic reactions that require the participation of a tetrahydrofolate derivative. Since the Bacto-folic acid assay medium is a synthetic medium that contains hydrolyzed casein, adenine, guanine, and p-aminobenzoic acid, growth of \textit{S. faecalis R} on this medium was found to be entirely dependent upon the addition of folic acid or thymine. The relative rates and extents of growth of the organism in the presence of optimal quantities of folic acid or thymine, and additional serine, methionine, adenine, and guanine were compared. The inoculum used was grown through four successive transfers on folic acid-free medium with supplements in order to assure the absence of any folic acid in the inoculum. The growth rate on the medium containing folic acid was somewhat faster than on the non-folate medium, with doubling times of 34 and 48 min, respectively. The yield of cells after 24 hours growth was the same for both media.

Effect of Folate Antagonists—Although the results of the experiment described above suggest very strongly that \textit{S. faecalis R} can be grown in the absence of any folate when it is supplied with various products of folate-mediated reactions, we considered the possibility that the organism is capable of very limited synthesis of folate, and that the limited amount that is made is used exclusively for the transfer of formyl groups to Met-tRNA\textsubscript{Met} for initiation of protein synthesis. The effect of the folate antagonists trimethoprim and aminopterin on the growth of \textit{S. faecalis R} under the two conditions of culture was therefore determined. Neither trimethoprim (Fig. 1) nor aminopterin (Fig. 2) inhibited growth of \textit{S. faecalis R} grown in a folate-free medium supplemented with thymine, whereas both analogues were effective inhibitors of growth in the presence of added folic acid or 5-formyltetrahydrofolate. Aminopterin was active as an inhibitor at much lower concentrations than was trimethoprim, and was much more effective in the presence of folate than it was in the presence of 5-formyltetrahydrofolate. These results provide additional evidence that \textit{S. faecalis R} can grow at a relatively normal rate in the absence of added folic acid and that the cell does not contain any functional folic acid.

Transamination with \textit{L-5-Formyltetrahydrofolate}—Extracts of \textit{S. faecalis R} cells grown on folic acid and grown in the absence of folic acid were examined for the presence of \textit{L-5-Formyltetrahydrofolate}·methionyl-tRNA\textsubscript{Met} transaminase essentially as described by Marcker and Sanger (1) and by Dickerman \textit{et al.} (14) with minor modifications described under “Experimental Procedure.” The product of the enzymatic reaction resulting from the transfer of the \textit{[14C]formyl} group of \textit{10-[14C]formyltetrahydrofolate} into trichloroacetic acid-precipitable counts was characterized as \textit{[14C]formyl-Met}-tRNA in the following manner. Treatment of the reaction mixture of a complete system with pancreatic RNase (10 \textmu g per ml) after incubation under standard conditions solubilized 94 to 99% of the trichloroacetic acid-precipitable counts, whereas the counts were not solubilized when the mixture was treated with Pronase (50 \textmu g per ml) for 60 min.

![Fig. 1: Effect of trimethoprim on the growth of \textit{S. faecalis R}](image)

![Fig. 2: Effect of aminopterin on the growth of \textit{S. faecalis R}](image)

![Fig. 3: Kinetics of fMet-tRNA formation catalyzed by S-100](image)
Met-tRNA_fM Formylation in S. faecalis R

Fig. 4. Kinetics of fMet-tRNA formation catalyzed by S-100 from S. faecalis R grown on a medium lacking folic acid but supplemented with L-serine, L-methionine, thymine, guanine and adenine. A, B, and C as in Fig. 3. Incubation mixture contained 13 μg of S-100 protein in each case.

Fig. 5. Kinetics of fMet-tRNA formation catalyzed by S-100 from E. coli A19. A, B, and C as in Fig. 3. Incubation mixture contained 4 μg of S-100 protein in each case.

Fig. 6. Kinetics of fMet-tRNA formation with [14C]formate as donor. The standard incubation mixture was used as described under "Experimental Procedure." A (O O), S-100 (68 μg of protein) prepared from S. faecalis R grown in medium containing folic acid; B (Δ—Δ), S-100 (104 μg of protein) from S. faecalis R grown in medium supplemented with L-serine, L-methionine, thymine, guanine, and adenine.

Fig. 7. Dependence of fMet-tRNA formation on amount of S-100 present in reaction mixture with [14C]formate as the formyl donor. The standard incubation mixture as described under "Experimental Procedure" was used. The reaction mixture was incubated for 30 min at 37°. A (O O), S-100 prepared from S. faecalis R grown with folic acid; B (Δ—Δ), S-100 prepared from S. faecalis R grown in folate-deficient medium supplemented with L-serine, L-methionine, thymine, guanine, and adenine. The protein concentration of the S-100 preparations used in both cases was 1.7 mg per ml.

at 37°. Treatment of the reaction mixture with Tris-acetate at pH 8.0 under the conditions described by Sarin and Zamecnik (41) for the hydrolysis of the aminoacyl-tRNA bond without simultaneous destruction of the tRNA resulted in the solubilization of 99% of the counts, indicating that the [14C]formyl group was attached to an amino acid residue. Formation of the radioactive product was dependent upon the presence of both L-methionine and tRNA in the standard assay as described under "Experimental Procedure." No labeled product was formed when the S-100 fraction was omitted or boiled before use in the standard assay.

The S-100 fraction prepared from S. faecalis R grown in medium containing folic acid catalyzed the transfer of formyl groups from 10 [14C]formyltetrahydrofolate to Met-tRNA_fMet as shown in Fig. 3. The reaction was not inhibited by [14C]formate but was slightly inhibited by tetrahydrofolate (Fig. 3). Similar re-
Faecalis R grown in the presence and in the absence of folate to faecalti R grown with folate catalyzed the formation of fMet-Met-tRNAfMet with I-[14C]formyltetrahydrofolate as the formyl donor.

Standard incubation mixture containing 34 μg of S-100 protein was used as described under "Experimental Procedure" with [14C]formate as the formyl donor. A (O — O), (±)-L-tetrahydrofolate added to give a final concentration of 1 mM; B (Δ — Δ), no further additions.

Results were obtained with S-100 fractions of S. faecalis R grown in medium lacking folic acid but supplemented with L-serine, L-methionine, thymine, adenine, and guanine (Fig. 4). This reaction was likewise not inhibited by [14C]formate but was slightly inhibited by tetrahydrofolate (Fig. 4). The results obtained with S-100 supernatant preparations of E. coli A19 are shown in Fig. 5. [14C]Formate did not inhibit the formylation of Met-tRNAfMet with [10-3H]formyltetrahydrofolate as the formyl donor, but tetrahydrofolate did strongly inhibit the formation of fMet-tRNAfMet by E. coli A19 S-100 (Fig. 5). The formylation of Met-tRNAfMet with [14C]formyltetrahydrofolate was linear with respect to the amount of S. faecalis R or E. coli A19 S-100 protein present in the reaction mixture.

Transformylation with Formate — The ability of extracts of S. faecalis R grown in the presence and in the absence of folic acid to catalyze the transformylation reaction with [14C]formate as the formyl donor was also determined. The reaction under these conditions is dependent upon the presence of endogenous tetrahydrofolate, formyltetrahydrofolate synthetase, methionyl-tRNA synthetase, ATP, and the 10-formyltetrahydrofolate: methionyl-tRNA<sup>fMet</sup> transformylase for the transformylation reaction. Under these conditions, the S-100 preparation from S. faecalis R grown with folate catalyzed the formation of fMet-tRNA<sup>fMet</sup> from the [14C]formate (Fig. 6). The reaction was dependent upon the amount of S-100 preparation used but exhibited autoinhibitory behavior at low enzyme levels (Fig. 7). The reaction was linear with respect to enzyme concentration over a narrow range of enzyme concentrations.

The S-100 preparation obtained from S. faecalis R grown in the absence of folate in a medium supplemented with L-serine, L-methionine, adenine, guanine, and thymine was not capable of catalyzing the transformylation reaction with [14C]formate under comparable conditions (Figs. 6 and 7). However, upon addition of tetrahydrofolate to the incubation mixture, S-100 extracts prepared from S. faecalis R cells grown on folate-free medium catalyzed the formation of [14C]formyl-Met-tRNA with [14C]formate as the formyl donor (Fig. 8).

**DISCUSSION**

Previous work has shown that the folic acid requirement of S. faecalis R may be replaced by supplementing the growth medium with a mixture of substances that are now recognized to be products of biosynthetic reactions in which folic acid functions as a cofactor. Microbiological assay of cells grown in the absence of folic acid, but with these supplements, failed to detect the presence of any folate (28, 32), suggesting that S. faecalis R cannot synthesize folic acid de novo. In experiments reported here, it is shown that the growth rate of the organism on the folate-free medium is somewhat less than on a medium containing folic acid (Table I), thus establishing that this S-100 fraction does not contain an inhibitor that interferes with fMet-tRNA formation by the S-100 fraction prepared from cells grown without folate.
growth of *S. faecalis* R in the absence of the supplements when the addition of folic acid is required. The growth of *E. coli*, under conditions in which it is synthesizing folate, is inhibited by trimethoprim and aminopterin (4). If the transfer of the formyl group from 10-formyltetrahydrofolate to Met-tRNA<sub>Met</sub> was necessary for the initiation of protein biosynthesis in *S. faecalis* R, both trimethoprim and aminopterin would have been expected to cause a decrease and ultimate cessation in growth.

The present investigation indicates that 10-formyltetrahydrofolate: methionyl-tRNA formyltransferase is a constitutive enzyme with respect to folic in *S. faecalis* R, because essentially equal formyltransferase activity was observed with 10-formyltetrahydrofolate as the formyl donor in S-100 preparations of cells grown on media either supplemented with or deficient in folic acid. The observation that folate-free cells of *S. faecalis* R grow at a rate comparable to that of folate-sufficient cells, yet fail to form fMet-tRNA<sub>Met</sub> when formate is utilized as the source of the formyl group, indicates that the formylation of Met-tRNA by the recognized formyltransferase reaction from 10-formyltetrahydrofolate is not required for initiation of polypeptide chain biosynthesis in this organism, thus suggesting that formylation of Met-tRNA may not even be required for initiation of protein biosynthesis under these conditions. The addition of exogenous tetrahydrofolate leads to the formation of fMet-tRNA by these extracts. However, the activity observed upon the addition of tetrahydrofolate to the incubation mixture for the incorporation of [14C]formate into Met-tRNA is somewhat lower than the activity observed utilizing endogenous tetrahydrofolate with *S. faecalis* R S-100 supernatant preparation of cells grown on medium containing folic acid. This difference in activity may be caused by a combination of two effects: slight inhibition of the formyltransferase by the added tetrahydrofolate and a partial repression of the 10-formyltetrahydrofolate synthetase activity by the metabolites used to replace the folic acid in the growth of the *S. faecalis* R cells. The formyltransferase activity of the S-100 preparation of *S. faecalis* R cells grown on medium containing folic acid was not inhibited by the addition of an equal amount of S-100 prepared from cells grown on medium lacking folic acid, thus establishing that the S-100 preparation of non-folate cells does not contain a substance that inhibits its formyltransferase activity.

In contrast to the results reported by Dickerman and Smith (42) showing that tetrahydrofolate is an effective competitive inhibitor of *E. coli* B formyltransferase, which was confirmed in experiments reported here on *E. coli* A19 (formyltransferase, the formyltransferase present in the S-100 preparation derived from *S. faecalis* R grown either with folic acid or with supplements was inhibited only slightly by tetrahydrofolate. The significance of this observation is not completely understood at the present time. The results presented here indicate that fMet-tRNA<sub>Met</sub> may not be involved in the initiation of protein synthesis in *S. faecalis* R when grown in a folate-free medium. However, it is also possible that formylation of the Met-tRNA<sub>Met</sub> may occur with a formyl donor and enzyme other than 10-formyltetrahydrofolate and its specific formyltransferase. The experiments described in this study indicating that [14C]formate is not converted to the formyl group of fMet-tRNA<sub>Met</sub> by extracts of folate-free *S. faecalis* R cells do not altogether eliminate such a possibility, because it is possible that the enzyme catalyzing this alternative formyltransferase reaction is unstable under the experimental conditions used or that the added [14C]formate is not in equilibrium with an endogenous formyl donor.

The possibility that initiation of protein biosynthesis in prokaryotes may not in all cases necessarily involve formylated Met-tRNA<sub>Met</sub> also deserves consideration in view of observations concerning this process in eukaryotes. It has not been possible to demonstrate the presence of formylated aminoacyl-tRNA in eukaryotic cytoplasmic sources such as rat liver (23), guinea pig liver (43), rabbit reticulocytes (25), chicken reticulocytes (44), or HeLa cells (24). It has been suggested that Met-tRNA<sub>Met</sub>, whether formylated or not, may possess a unique structural conformation which facilitates initiation of protein biosynthesis (5, 45, 46). The results presented in this paper indicate that such a mechanism involving a particular conformation of methionyl-tRNA<sub>Met</sub> that allows selective binding to the initiation site on the 30 S ribosomal subunit might also be operative under certain conditions in *S. faecalis* R, a 70 S ribosomal system.

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