Methionyl Soluble Ribonucleic Acid Transformylase

I. PURIFICATION AND PARTIAL CHARACTERIZATION

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SUMMARY

The N\textsuperscript{\textalpha}-formyltetrahydrofolate: methionyl soluble ribonucleic acid transformylase has been purified over 1500-fold from Escherichia coli B. Although not homogeneous at this stage, the enzyme was of sufficient purity to determine some physical characteristics. The sedimentation constant was 2.02 which would indicate a molecular weight of approximately 25,000. In addition to the substrates of the reaction a partial dependency on magnesium ion was observed. The identity of the formyl donor was elucidated and found to be N\textsuperscript{\textbeta}-formyltetrahydrofolate.

The present report describes the purification and properties of the transformylase from E. coli B. A definition of the folate donor specificity as well as a description of some of the characteristics of the transformylase reaction are included in this report.

EXPERIMENTAL PROCEDURE

Materials and Methods

E. coli B, harvested in mid-logarithmic phase, was purchased from Grain Processing Corporation, Muscatine, Iowa. E. coli sRNA was purchased from General Biochemicals. Crystalline N\textsuperscript{\textalpha}-formyl-H\textsubscript{4}-folate synthetase was a generous gift of Dr. T. Stadtman. Radioactive sodium formate (50 m\textsuperscript{c} per mmole) was purchased from New England Nuclear, and H\textsubscript{4}-folate (90\% purity) was obtained from Sigma. Methionyl-sRNA (F) (10-fold enriched) was a generous gift of Dr. B. P. Doctor, Department of Biochemistry, Walter Reed Army Medical Center, Washington, D.C.

Preparation of N\textsuperscript{\textalpha}-formyl-H\textsubscript{4}-folate—The enzymatic production of labeled \textsuperscript{14}C-N\textsuperscript{\textalpha}-formyl-H\textsubscript{4}-folate was in accord with the procedure of Rabinowitz and Pricer (11). As the N\textsuperscript{\textalpha}-formyl derivative is labile because of oxidation, the reaction product was acidified and converted to the more stable N\textsuperscript{\textalpha}-\textsuperscript{14}C-methenyl-H\textsubscript{4}-folate. The latter compound was purified by cellulose chromatography according to the procedure of Huennekens, Ho, and Scrimgeour (12) and stored at \textdegree C in the eluting solvent (0.1 m formic acid-0.01 m 2-mercaptoethanol).

Preparation of Methionyl-sRNA—Aminoacyl-sRNA synthetases were prepared by the method of Kellogg et al. (9).

sRNA was decylated by incubating at p\textsuperscript{H} 10.5 for 30 min at 37\%. After precipitation with cold absolute ethanol, the decylated sRNA was dialyzed for 16 hours in distilled water with two changes.

Three grams of decylated sRNA (A\textsubscript{260} = 950 units per ml) were added to a reaction mixture containing sodium cacodylate, 0.1 m, p\textsuperscript{H} 7.0; magnesium acetate, 0.01 m; potassium chloride, 0.01 m; ATP, 0.005 m; L-methionine, 2 \times 10\textsuperscript{-3} m; and E. coli B 100,000 \times g supernatant, 392 mg of protein, in a final volume of 300 ml. Incubations were for 20 min at 37\%. The percentage charging of the sRNA was checked by the determination of radioactivity in a parallel incubation containing \textsuperscript{14}C-L-methionine (198 m\textsuperscript{c} per mmole). After incubation the reaction mixture was extracted with water-saturated phenol and the nucleic acids were precipitated with 2 volumes of cold ethanol. The precipitate
was dissolved in distilled water and dialyzed against distilled water, with two changes, for 10 hours. The preparation of methionyl-sRNA (F) was essentially the same as with the less purified sRNA fraction, with the exception that the ethanol precipitation was omitted and the aqueous phase of the phenol extraction was passed through a Sephadex G-25 column (2 × 15 cm) with the use of potassium cacodylate, 10^{-4} M, pH 5.5, as the eluting solution. The percentage charging obtained with heterogeneous sRNA was 2% and with methionyl-sRNA (F) was 15 to 18%. Of the 2% charged material, it was assumed from the data of Marcker (6) that 70% of the heterogeneous methionyl-sRNA was capable of accepting formyl groups, while of the 15 to 18% charged material, all of the methionyl-sRNA was capable of accepting formyl groups.

**Assay of Transformylase**—The reaction mixture included Tris-Cl, pH 7.4, 5 μmoles; magnesium chloride, 1.5 μmoles; 2-mercaptoethanol, 1 μmole; crystalline bovine serum albumin, 45 μg; N^{5,10}-methylene-H_{4}-folate, 0.37 μmole (82,000 cpm per μmole; methionyl-sRNA, 0.2 μmole; and varying amounts of the enzyme fractions. The final reaction mixture was 50 μl, and the standard incubation was at 97° for 5 min. The reaction was stopped by the addition of cold 5% trichloroacetic acid, vigorously mixed, and decanted onto a cellulose nitrate (Millipore Company) membrane. The reaction tube was washed twice with 5% cold trichloroacetic acid and the washes were decanted onto the membrane. After filtration, the membrane containing precipitated nucleic acid and protein was transferred to counting vials, 10 ml of a naphthalene-di-oxane (13) counting fluid were added, and the membrane was dissolved in the counting fluid prior to the determination of radioactivity by liquid scintillation counting. The assay was linear with the enzyme concentrations used and with time for periods up to 5 min. A unit of transformylase activity catalyzed the formation of a millimicromole of formylnmethionyl-sRNA per hour. The percentage charging obtained with heterogeneous sRNA was 2% and with methionyl-sRNA (F) was 15 to 18%. Of the 2% charged material, it was assumed from the data of Marcker (6) that 70% of the heterogeneous methionyl-sRNA was capable of accepting formyl groups, while of the 15 to 18% charged material, all of the methionyl-sRNA was capable of accepting formyl groups.

**Purification of Methionyl-sRNA Transformylase**—All the following operations were conducted at 0–4°.

The starting material was a neutralized pH 4.5 supernatant fraction which had been obtained by Dr. Robert Taylor during the preparation of the N^{5}-methyl-H_{4}-folate-homocysteine transform-methylase from *E. coli* B. The previous steps employed by Dr. Taylor after preparation of a cell-free extract included (a) treatment with manganese chloride to remove nucleic acids, (b) precipitation of the proteins with ammonium sulfate (0 to 60% saturation), (c) retreatment with protamine sulfate and manganese chloride to remove nucleic acids, (d) a second precipitation with ammonium sulfate, and (e) adjustment of the ammonium sulfate fraction with 1 N acetic acid to pH 4.5, and retention of the supernatant fraction. The details are presented in another report (14).

The neutralized supernatant fraction was dialyzed in 20 liters of 0.01 M potassium phosphate, pH 6.4. The enzyme fraction was dialyzed overnight with one change of buffer. An IRC-50 column (2.5 × 21 cm) was equilibrated with potassium phosphate, 0.01 M (pH 6.4) (15) and the dialyzed fraction was passed onto the column at a rate of 100 ml per hour. After adsorption of the protein, the column was eluted with 1.5 column volumes of potassium phosphate, 0.01 M (pH 6.4), and then 1.5 column volumes of sodium chloride (0.05 M)-potassium phosphate (0.01 M, pH 6.4). The enzyme was then eluted from the resin with 3 column volumes of sodium chloride (0.5 M)-potassium phosphate (0.01 M, pH 6.4). Fractions of 20 ml were collected and those with a specific activity exceeding 720 units per mg of protein were further purified. These fractions were diluted 10-fold with potassium phosphate (0.01 M, pH 6.4) and applied to an IRC 50 column (2.5 × 21 cm) which was washed with 2 column volumes of potassium phosphate (0.01 M, pH 6.4). A 500 ml gradient of 0.05 M to 0.5 M sodium chloride-potassium phosphate (0.01 M, pH 6.4) was then started and 10-ml fractions were collected at the rate of 70 ml per hour. The fractions containing a specific activity exceeding 5200 units per mg of protein were dialyzed against Tris-Cl (0.01 M, pH 7.4) and lyophilized. There was a 30% decrease in activity in the most purified fractions when stored at −20° for 1 month.

All ultracentrifugal studies were carried out on a Spinco model E ultracentrifuge equipped with the schlieren and Raleigh optical systems. Temperatures were maintained at 20° with the RTIC control unit. Sedimentation values were calculated as described by Schachman (16). High speed equilibrium centrifugation was carried out by the technique of Yphantis (17). A partial specific volume of 0 = 0.725 ml per g was assumed for the molecular weight calculations.

Polycracyamide disc gel electrophoresis was performed by the method of Davis (18). The gels were run in duplicate; one gel was stained for protein while the other was cut into sections of 0.5-cm length. The gel fragments were suspended in 1.0 ml of 0.5 M NaCl-0.01 M potassium phosphate (pH 6.4) for 1 hour at 0° and the supernatant was assayed for transformylase activity.

RESULTS

Table I presents a summary of the purification of methionyl-sRNA transformylase from *E. coli* B. After the gradient elution on IRC-50, the enzyme was purified 1500-fold in comparison to the initial extract. Suitable column chromatography steps were possible only when the extracts had a A_{260}:A_{280} exceeding 1.1 which was obtained following the acid precipitation step. The peak activity tubes of the IRC-50 gradient were concentrated by lyophilization and examined by various physical techniques.

Analytical ultracentrifugation of the fraction obtained following chromatography on IRC-50 revealed a single, rather diffuse component with an s_{20,w} of 2.02. The high speed equilibrium technique of Yphantis indicated heterogeneity for the same fraction with a number average molecular weight for the lightest component equal to 22,000 g per mole, while the Z average molecular weight was calculated to be 31,000 g per hole. Polycracyamide disc gel electrophoresis showed two major components. Transformylase activity was associated with the less dense band.
TABLE II
Requirements for formylation of methionyl-sRNA

The reaction conditions were as described in "Materials and Methods." In the cases where deacylated sRNA was used instead of methionyl-sRNA, the same concentration was used with the assumption that the methionine-accepting fraction represented 2% of the total uncharged sRNA. The methionyl-sRNA used in these experiments was charged with L-methionine to 2% of the total sRNA concentration. The concentrations of L-methionine and ATP which were used were the same as those used in the method of preparation of methionyl-sRNA described in "Materials and Methods."

<table>
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<th>Experiment</th>
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<td></td>
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FIG. 1. The effect of magnesium ion concentration on the transformylase reaction. The standard incubation mixture was used as described in "Materials and Methods" with the exception of the variance in magnesium chloride concentration.

FIG. 2. Lineweaver-Burk plot of effect of methionyl-sRNA (F) concentration (A); N¹⁹-formyl-H₄-folate concentration (B).

FIG. 3. The effect of pH on the transformylase reaction. Standard incubation mixture was used as described in "Materials and Methods" with the exception that the buffers were changed for the desired pH.
**TABLE III**

Substrate specificity of methionyl-sRNA transformylase

A modified incubation was used for these experiments. The N10-formyl-H4-folate was prepared from the methenyl derivative by adjustment to pH 10 with 1 N NH4OH (18) and then both derivatives were kept at room temperature under anaerobic conditions. A disappearance of the 358 nm maxima characteristic of the N3,10-methenyl derivative was observed in the alkaline-treated solution. The derivatives were added to the incubation mixtures which contained potassium maleate, pH 7.0, at same ionic strength as the Tris-Cl of the standard mixture. The reaction steps of purine biosynthesis de novo. Since the purified methionyl-sRNA transformylase was found to be devoid of cyclohydrolase activity, the specificity of the formyl donor in the reaction was rapid in Tris-Cl or potassium phosphate buffer at the same pH. These authors used the inhibitory effect of 5,10-methenyl-H4-folate cyclohydrolase, an enzyme which contrasts with the irreversible conversion of N10-formyl-H4-folate to the imidazolinium derivative, N10-methenyl-H4-folate. There is evidence by polyacrylamide electrophoresis and equilibrium centrifugation, the major component appears to represent 90% of the total fraction. The sedimentation value of s20,w = 4.02, together with the Yphantis data, is compatible with a molecular weight in the range of 25,000 g per mole. This is of interest in that the enzyme is required to recognize a unique sRNA molecule as do the aminomethyl-sRNA synthetases. However, unlike the synthetases of known molecular weight (20), the transformylase is of approximately the same molecular weight as its substrate, methionyl-sRNA (F). The smaller size of the transformylase may facilitate the study of enzyme protein-sRNA interactions.

The purified transformylase preparation may provide a means of approach to the study of the recognition of a unique sRNA species by an enzyme. Preliminary results in our laboratory have shown that 100,000 × g supernatants derived from *E. coli* K12, and *Clostridium tetanomorphum* were capable of formylating *E. coli* B methionyl-sRNA. This suggests that in these species, the nucleotide sequence of methionyl-sRNA (F) determining the specificity for formylation is similar.

Other aspects of the methionyl-sRNA (F) transformylation which were elucidated during the purification were the partial dependency on magnesium ions and the identity of the formyl donor as N10-formyl-H4-folate. The optimal concentration of magnesium ions was 0.03 M which contrasts with the value of 0.006 M magnesium ions needed to show the dependency on N-formyl-methionyl-sRNA for initiation of peptide synthesis (8).

**REFERENCES**

2. **Clark, B. F. C., and Marker, K., Nature, 207, 1038 (1965).**
17. **Yphantis, D. A., Biochemistry, 3, 297 (1964).**
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