Isolation and Some Properties of Methionine Transfer Ribonucleic Acid from *Escherichia coli**

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ABSTRACT: At least two distinct species of $tRNA_f^{Met}$ in *Escherichia coli* B can be demonstrated by chromatography on either hydroxylapatite or reverse-phase columns. The latter columns will also separate two $tRNA_m^{Met}$ species. Sequence differences between the two $tRNA_f^{Met}$ and between the two $tRNA_m^{Met}$ species can be demonstrated by two-dimensional thin-layer chromatography of the complete ribonuclease T1 digestion products on poly(ethyleneimine)-cellulose. Both fMet-$tRNA_f^{Met}$ species show the same relative ability to bind to ribosomes and to react with puromycin in the presence of the trinucleotides AUG and GUG. The kinetics of charging and transformylation of these various methionine tRNAs were examined. One of the two $tRNA_f^{Met}$ species has a lower K_m than the other for both reactions and the kinetic data are also consistent with the recognition of both $tRNA_f^{Met}$ and $tRNA_m^{Met}$ by a single activating enzyme. All of these various tRNAs contain a single 4-thiouridine-like residue and triiodide oxidation of this residue affects different $tRNA^{Met}$ subspecies in different ways. For one of the $tRNA_f^{Met}$ species, the K_m for methionine charging is unchanged while V_{max} is approximately halved, and for the other, both V_{max} and the apparent K_m are lowered. The most striking change occurs with one of the $tRNA_m^{Met}$ species, the acceptance capacity of which is abolished by triiodide oxidation. It was also found that uncharged $tRNA_f^{Met}$ did not inhibit the transformylation of Met-$tRNA_f^{Met}$, even when present in large excess. This observation suggests the possibility that the transformylase recognition site on the $tRNA_f^{Met}$ may undergo a structural reorganization when the tRNA is aminoacylated.

The role of fMet-$tRNA_f^{Met}$ as a peptide chain initiator in *Escherichia coli* and other bacterial protein synthetic systems is well documented (for a review, see Lengyel, 1967), and primary sequence data for the formylatable ($tRNA_f^{Met}$) and nonformylatable ($tRNA_m^{Met}$) subspecies of this tRNA have been published (Dube *et al.*, 1968; Cory *et al.*, 1968). More recently evidence for multiple forms of $tRNA_f^{Met}$ (Dube *et al.*, 1968; Doctor *et al.*, 1969; Weiss *et al.*, 1968) and $tRNA_m^{Met}$ (Doctor *et al.*, 1969; Seno *et al.*, 1968) has been presented. In this report we present further evidence for such multiple subspecies together with a comparison of their biochemical characteristics in the methionine-charging, formylation, and ribosomal binding reactions. In addition, the effect of triiodide oxidation of $tRNA^{Met}$ on certain of its biochemical properties is described.

Materials and Methods

Materials. E. coli B tRNA (lot no. 6704) was purchased from Schwarz BioResearch, Orangeburg, N. Y., and was used as received. Methionine (52 mCi/mmole) was obtained from the same source. DEAE-Sephadex A-50 was supplied by Pharmacia, Piscataway, N. J. Folinic acid (leucovorin) was purchased from Lederle Laboratories, Pearl River, N. Y. Ribonuclease T1 (RNase T1) was obtained from the Worthington Biochemical Corp., Freehold, N. J.

Column Chromatography. All columns were operated at room temperature. DEAE-Sephadex columns were prepared and operated essentially as described by Nishimura *et al.* (1967) except that the flow rate was reduced to about one-half that used by these authors. Hydroxylapatite was prepared as described by Levin (1962). In order to retard bacterial growth on the hydroxylapatite columns, 1% methanol and 0.02% sodium azide[1] was added to the eluting buffer which was also passed through a solvent-resistent filter (Millipore Solvinert, 0.25 μ pore size) before entering the column. Reverse-phase column packing (RPC 3) was prepared as described by Weiss *et al.* (1968). Relatively short variable bed height columns (1.8 × 40 cm) were packed with this material by compacting the Chromosorb mechanically to such a density that a positive pressure of about 20 psi was needed to maintain a flow rate of 1 ml/min. Concave salt gradients were prepared with a Varigrad nine-chamber unit according to the directions of Weiss and Kelmers (1967).

Ultraviolet absorption spectra were measured point-by-point with a Zeiss PMQ II spectrophotometer.

Thin-Layer Chromatography. Poly(ethyleneimine)-cellulose thin-layer sheets were prepared as described by Randerath and Randerath (1967). Equal volumes of tRNA (2 mg/ml in 0.2 M Tris-HCl–5 mM EDTA, pH 7.6) and RNase T1 (0.2 mg/ml, dialyzed *vs.* distilled water) were mixed and incubated at 37° for 30 min, and were then applied directly

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[1] The possibility of an interaction between azide and 4-thiouridine residues has been pointed out to us. However, prolonged incubation of *E. coli* tRNA with 0.02% sodium azide did not lead to any detectable changes in the ultraviolet absorption characteristics of the 4-thiouridine residues in tRNA.

to the PEI[2] layer. Chromatography in the first dimension was carried out with 0.27 M ammonium carbonate (freshly prepared) for 3 hr, and the sheet was then dried and washed in methanol containing 0.5% acetic acid. Chromatography in the second dimension was carried out with 0.1 M phosphate buffer (pH 6.5) for 4 hr. The effective height of the chromatographic strips in both dimensions was increased by sewing Whatman No. 3MM paper wicks to the top of the strip. Ultraviolet-absorbing spots on the chromatogram were detected visually and the chromatogram was also examined by the highly sensitive low-temperature phosphorescence technique of Randerath (1967). Some spots were revealed by this method that were not visible under ultraviolet illumination and these are marked with a dashed line in Figure 4.

Methionine Acceptance and Formylation Assays. The source of $tRNA^{Met}$ synthetase and transformylase enzymes was a crude 100,000*g* supernatant fraction from *E. coli* A 19 (an RNase$^-$ strain) which had been treated with streptomycin and dialyzed. The mixture for direct assay of column fractions contained, per 0.1 ml, 0.01–0.1 ODU of tRNA, 10 μmoles of Tris-HCl (pH 7.4), 2.5 mμmoles of $MgCl_2$, 1 μmole of mercaptoethanol, 5 μmoles of [^{14}C]methionine (10 mCi/mmole), 0.05 μmole of ATP, and 5 μl of crude synthetase. This mixture was incubated for 20 min at 37°, then 4 ml of ice-cold trichloroacetic acid was added and the acid-insoluble radioactivity, collected on Millipore filters, was counted in a gas-flow counter with an efficiency of 35%. The addition of the other 19 [^{12}C]amino acids to the assay mixture did not alter the results and these amino acids were omitted in routine assays. In all cases the radioactivity incorporated could be specifically diluted by addition of [^{12}C]methionine to the assay mixture. In the formylation assays, 25 μmoles of folinic acid was included in the assay mixture and, after 20-min incubation, 1.5 ml of 0.025 M $CuSO_4$ in 0.2 M Tris (pH 7.5) was added and the incubation was continued for 5 min at 37° before precipitation with trichloroacetic acid. This procedure hydrolyzes almost completely any Met-tRNA but leaves fMet-tRNA intact (Schofield and Zamecnik, 1968). For determination of the absolute acceptance capacity, the assay conditions were optimized according to the procedure of Rubin *et al.* (1967), and, for these assays, the incubation mixture contained, per 0.1 ml, *ca.* 0.05 ODU of tRNA, 10 μmoles of sodium cacodylate (pH 8.6), 1 μmole of mercaptoethanol, 10 μmoles of KCl, 5 mμmoles of [^{14}C]methionine, 0.05 μmole of ATP, 1 μmole of $MgCl_2$, and 5 μl of crude enzyme preparation. Incubation was for 20 min at 37°.

Kinetic Studies. For the charging reaction the crude enzyme preparation was diluted 1:51 with 1 mg/ml of bovine serum albumin and assays were performed under the optimized conditions noted above except that higher specific activity methionine (52 mCi/mmole) was used. Under these conditions, [^{14}C]methionine incorporation was linear for at least 10 min and aliquots at several time points between 0 and 10 min were always taken. The substrate concentration was calculated from the maximum [^{14}C]methionine acceptance under the same assay conditions but with a 50-fold greater enzyme concentration.

For the formylation reaction, [^{14}C]Met-$tRNA_{f1}^{Met}$ and -$tRNA_{f2}^{Met}$ was prepared under the optimum loading conditions and, after deproteinization with 1% isoamyl alcohol in chloroform, the tRNA was precipitated with ethanol, washed by reprecipitation, and stored in 0.1 M sodium acetate buffer (pH 5). As a source of transformylase the crude enzyme preparation was diluted 1:10 with 1 mg/ml of bovine serum albumin. In the absence of added AMP and PP_i, this enzyme preparation caused no significant discharge of the Met-$tRNA_f^{Met}$ under the assay conditions used. In addition to [^{14}C]Met-$tRNA_f^{Met}$, the assay mixture contained 20 μmoles of HEPES buffer (pH 7.0), 2 μmoles of $MgCl_2$, 1 μmole of mercaptoethanol, 0.05 μmole of folinic acid, and 10 μl of enzyme in a total volume of 0.1 ml. After incubation for the required period, aliquots of the mixture were transferred to 1.5 ml of 0.025 M $CuSO_4$–0.2 M Tris (pH 7.5), and the incubation was continued for 5 min before trichloroacetic acid was added. All kinetic studies were carried out at 25°. In the case of the triiodide-oxidized samples, it was shown that the presence of mercaptoethanol in the assay mixture did not lead to any significant regeneration of the 4-thiouridine absorption band over the brief (0–10 min) incubation time used.

Ribosome Binding Studies. For the determination of relative binding levels the incubation mixture contained, per 50 μl, 2.5 μmoles of Tris-acetate (pH 7.5), 5 μmoles of NH_4Cl, 0.25 μmole of $Mg(OAc)_2$, 5 μmoles of GTP, 10 mμmoles of trinucleotide, 30 $\mu\mu$moles of ribosomes, 20 $\mu\mu$moles of [^{14}C]Met-tRNA (52 mCi/mmole), and 60 μg of crude initiation factors (1 M ammonium chloride ribosomal wash). Incubation was for 20 min at room temperature and the solution was then filtered (Millipore HAWP filters), washed, and the dried filter was counted in a liquid scintillation counter. In Figure 5, blank values (binding in the absence of added trinucleotide) have been subtracted. These values were always less than 20% of the maximum binding observed. For measurement of the rate of formation of fMet-puromycin, the incubation mixture contained, in a final volume of 150 μl, 7.5 μmoles of Tris-acetate (pH 7.5), 1.5 μmoles of NH_4Cl, 0.75 μmole of $Mg(OAc)_2$, 15 mμmoles of GTP, 30 mμmoles of trinucleotide, 30 $\mu\mu$moles of ribosomes, 120 μg of initiation factors, 150 mμmoles of puromycin, and 60 $\mu\mu$moles of [^{3}H]Met-tRNA (3000 mCi/mmole). After incubation for 1–5 min at room temperature, 30-μl aliquots were transferred into 2 ml of a 1:1 mixture of sodium acetate buffer (pH 5.5) and ethyl acetate. After thorough mixing, the ethyl acetate layer was removed and counted in a scintillation counter. The data were corrected on the assumption that the ethyl acetate extraction removes only 50% of the fMet-puromycin (Leder and Bursztyn, 1966).

Results

A preliminary fractionation of *E. coli* B tRNA on DEAE-Sephadex (*cf.* Nishimura *et al.*, 1967) yielded a highly enriched (approximately 80% pure with respect to methionine acceptance) $tRNA^{Met}$ fraction which was then rechromatographed on hydroxylapatite (Pearson and Kelmers, 1966). The pattern of absorbance and of methionine and formyl group acceptance from this column is shown in Figure 1. The methionine acceptance capacities of the various pooled fractions together

[2] Abbreviations used are: PEI, poly(ethyleneimine); ODU, amount of tRNA in 1 ml of 0.15 M NaCl–0.015 M sodium citrate buffer (pH 7) corresponding to an $A_{260\,m\mu}$ of 1.00; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TU, thiouridine.

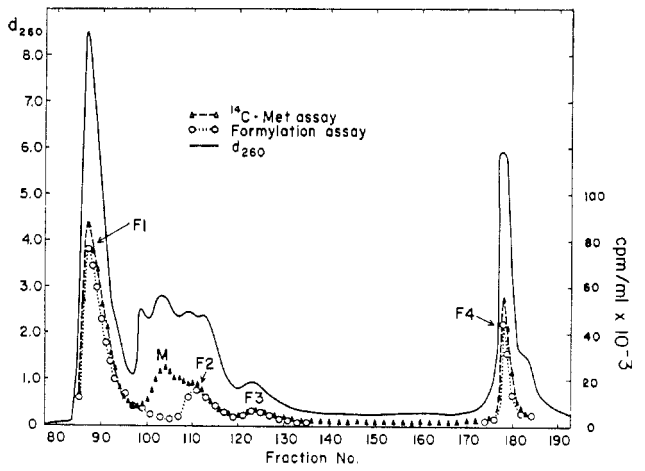

FIGURE 1: Chromatography of $tRNA^{Met}$ on hydroxylapatite. $tRNA^{Met}$ (2080 ODU) in 0.08 M sodium phosphate buffer (pH 6.8) containing 1% methanol and 0.02% sodium azide was loaded on to a 2.5 × 70 cm column of hydroxylapatite preequilibrated with the same buffer and the column was washed with this same buffer for 24 hr before the tRNA was eluted with a linear gradient (1.5 + 1.5 l.) of 0.08–0.16 M sodium phosphate (pH 6.8) at a flow rate of 20.6 ml/hr. The fraction volume was 13.7 ml. The recovery of absorbance (260 mμ) was 99%.

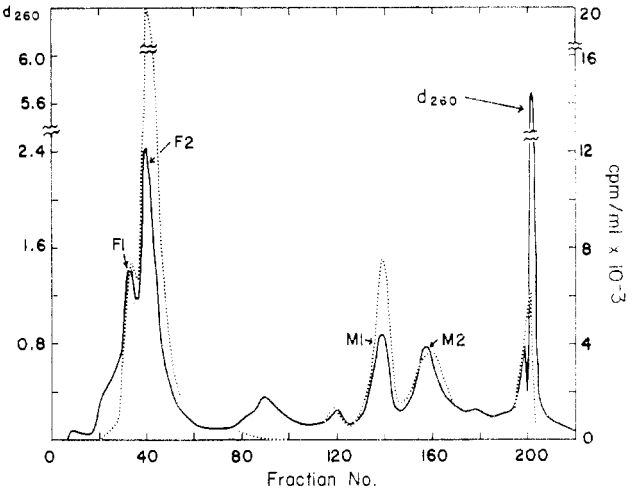

FIGURE 2: Rechromatography of $tRNA^{Met}$ on a reverse-phase column. $tRNA^{Met}$ (1260 ODU) (pooled from several hydroxylapatite columns, see text) was applied to a 1.8 × 40 cm RPC 3 reverse-phase column and was eluted with a nine-chamber (9 × 325 ml) concave salt gradient from 0.375 M NaCl–0.01 M Tris (pH 7) to 0.500 M NaCl–0.01 M Tris (pH 7) (Weiss and Kelmers, 1967) at a flow rate of 86 ml/hr. The fraction volume was 14.3 ml.

with their $tRNA^{Met}$ content are shown in Table I. The recovery of $tRNA_f^{Met}$ acceptance from this column was quantitative (103%) whereas that of $tRNA_m^{Met}$ acceptance was low (24%). We return later to a discussion of the causes of this specific inactivation of $tRNA_m^{Met}$.

The ultraviolet absorption spectra of the pooled fractions F1, F2, and F3 (formylatable $tRNA^{Met}$ species) showed a peak at 336 mμ, the magnitude of which was consistent with the presence of one 4-thiouridine (4-TU) residue per molecule (*cf.* Doctor *et al.*, 1969). F4 showed a weak and extremely broad absorption over the range 300–400 mμ and treatment of this pool with 0.05 M thiosulfate-0.01 M Tris (pH 7) at room temperature (Carbon *et al.*, 1965) led to a gradual regeneration of the absorption maximum at 336 mμ. Chromatographic supports other than hydroxylapatite (see below) did not lead to the appearance of a peak of methionine acceptance resembling F4 in ultraviolet absorption and it seems likely, therefore, that F4 is a chromatographic artifact derived from another species by oxidation of a thiopyrimidine, presumably a 4-TU moiety. The pattern of methionine and formyl group acceptance shown in Figure 1 was obtained consistently and reproducibly on four separate columns with the exception that F3 did not reappear and the relative amounts of F1 and F4 were somewhat variable. This finding is consistent with the interpretation that F4 is a chromatographic artifact and suggests that F3 might have a similar origin.

TABLE I: Characteristics of Harvested tRNA Pools from the Hydroxylapatite Column.

Fraction No.	Met Acceptance (μμmoles/ODU)	% Formylation
F1, 85–94	1530	99
F2, 107–119	1060	84
F3, 120–130	910	90
F4, 177–180	1120	95
M, 102–106	1220	14

Since the F2 and M species were not well resolved by the hydroxylapatite column we attempted to separate these components by reverse-phase chromatography with the system (RPC 3) described by Weiss *et al.* (1968). Pooled fractions from three hydroxylapatite columns corresponding to fractions 95–120 in Figure 1 were rechromatographed with the RPC 3 system and four distinct peaks of methionine acceptance were detected (Figure 2). Peaks F1 and F2 were completely formylatable and, as judged by their relative abundance, correspond to the peaks with the same numbering from the hydroxylapatite column. Peaks M1 and M2 did not accept formyl residues and therefore represent isoaccepting subspecies of $tRNA_m^{Met}$. The acceptor activity of the material in tube 140 (peak M1) was 1670 μμmoles/ODU, close to that predicted for a pure $tRNA^{Met}$ species (*ca.* 1800 μμmoles/ODU), whereas that of the tRNA in tube 160 (peak M2) was somewhat less (1220 μμmoles/ODU).

It is known that chromatography of purified tRNA samples on hydroxylapatite can sometimes generate spurious multiple peaks of amino acid acceptor activity (G. D. Novelli, 1968, personal communication) and it was therefore desirable to demonstrate the presence of two major species of $tRNA_f^{Met}$ in *E. coli* B tRNA by a different method. Chromatography of total *E. coli* B tRNA on benzoylated DEAE-cellulose (Gillam *et al.*, 1967) yielded a fraction which was highly enriched in $tRNA_f^{Met}$ and contained no $tRNA_m^{Met}$. This material was a gift from Dr. Tom Yu. $tRNA_f^{Met}$ was isolated from this fraction by rechromatography on DEAE-Sephadex (Figure 3A) and further rechromatography of the pure $tRNA_f^{Met}$ yielded two partially resolved peaks of absorbance, each of which accepted almost the theoretical amount of

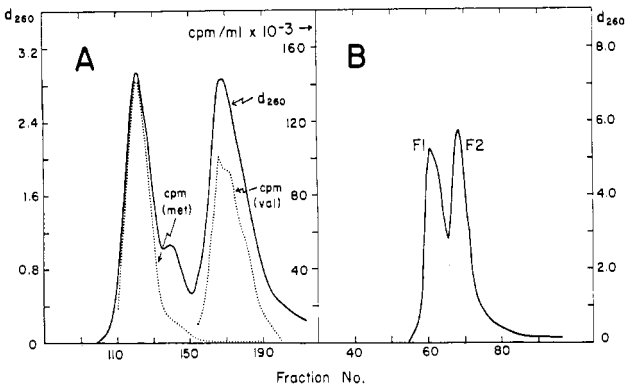

FIGURE 3: Rechromatography studies. (A) Of enriched tRNA$_f^{Met}$ on DEAE-Sephadex. tRNA (13,500 ODU) was chromatographed on a 4 × 74 cm DEAE-Sephadex A-50 column. The conditions for chromatography are as given in the legend to Figure 1 except that a 5 l. + 5 l. gradient was used at a flow rate of 44.4 ml/hr. The fraction volume was 14.8 ml. (B) Of the tRNA$_f^{Met}$ peak from the previous DEAE-Sephadex column (Figure 3A, fractions 115–130) on a reverse-phase column. tRNA$_f^{Met}$ (2070 ODU) was chromatographed as described in the legend to Figure 2 except that a 0.370–0.500 M NaCl–0.01 M Tris (pH 7) concave gradient was used at a flow rate of 82.8 ml/hr. The fraction volume was 13.8 ml.

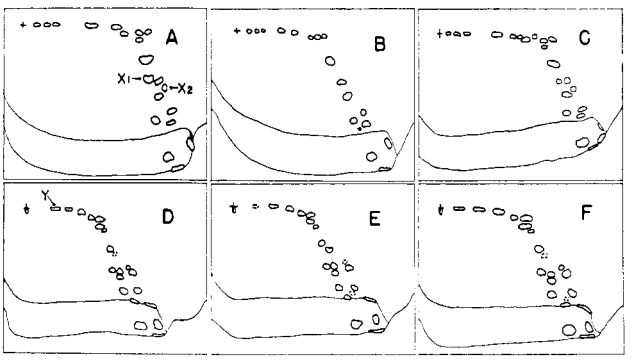

FIGURE 4: Two-dimensional thin-layer chromatography of complete T1 RNase digests of tRNA$_f^{Met}$ and tRNA$_m^{Met}$. Panel A, species F1; panel B, species F2; panel C, an equimolar mixture of F1 and F2; panel D, species, M1; panel E, species M2; panel F, an equimolar mixture of M1 and M2. The species numbering is as illustrated in Figure 2. A total of 50 μg of tRNA was digested and chromatographed in each case.

methionine and was completely formylatable (Figure 3B). The identification of these two peaks as F1 and F2 was made on the basis of their characteristic and distinct circular dichroism spectra in the region 300–400 mμ (Scott and Schofield, 1969).

Comparison of the RNase T1 Digest Patterns of tRNA^{Met} Species by Thin-Layer Chromatography. The technique of two-dimensional chromatography on poly(ethyleneimine)-cellulose (PEI-cellulose) thin layers has been used by Randerath and Randerath (1967) to separate a model mixture of small oligonucleotides. With the appropriate solvent systems, the relatively more complex mixture of oligonucleotides present in a complete RNase T1 digest of a pure tRNA species can also be resolved by this method. In Figure 4A–C are shown the oligonucleotide patterns obtained from F1 (Figure 1, pooled fractions 106-115), F2 (Figure 2, fractions 39–50), and an equimolar mixture of these two tRNAs, respectively. The chromatographic patterns show clear differences between the two tRNA$_f^{Met}$ species. Spots X1 and X2 from F1 (Figure 4A) are absent from the pattern given by F2 (Figure 4B) and there are also differences in the region corresponding to the larger oligonucleotides. The major difference between the two tRNA$_m^{Met}$ species is the spot Y which is present in the M1 pattern (Figure 4D) but absent from the M2 pattern (Figure 4E). Some material remained at the origin in all three digest patterns (Figure 4D–F) and it is therefore possible that further differences exist between these species that were not resolved by this chromatographic technique. A comparison of Figure 4A,D demonstrates the extensive sequence differences between tRNA$_f^{Met}$ and tRNA$_m^{Met}$ previously found by Cory *et al.* (1968).

Coding Response of the Multiple tRNA^{Met} Species. In Figure 5 are shown both the relative levels of binding of fMet-tRNA$_f^{Met}$ to ribosomes in the presence of various trinucleotides and the rates of reaction of fMet-tRNA$_f^{Met}$ with puromycin in the presence of ribosomes, initiation factors, and the same trinucleotides. These two different kinds of measurement of codon response, one a steady state, the other a kinetic measurement, gave essentially the same results and suggest that the four tRNA$_f^{Met}$ species differ little, if at all, in their coding properties. The typical strong response of fMet-tRNA to both AUG and GUG trinucleotides was found in all cases. Measurement of the relative levels of binding of these fMet-tRNAs to the natural R17 mRNA also failed to reveal differences between them. Similar binding properties for purified multiple tRNA^{Met} species were reported by Doctor *et al.* (1969). The binding studies reported above were carried out by Dr. J. C. Brown (Harvard University).

Kinetic Properties of tRNA^{Met} in the Charging and Transformylation Reactions. The kinetics of charging of F1, F2, and M1 (*cf.* Figure 2) were examined using a crude methionine-tRNA synthetase and transformylase preparation (Materials and Methods). Lineweaver–Burk plots of these data are shown in Figure 6 and the corresponding K_m values are collected in Table II. The responses of the F1 and F2 species in this assay are somewhat different, the K_m for F2 being greater than that for F1. M1 has a K_m similar to F1 and the V_{max} values for all three species are very similar. At the time these assays were done the M2 sample had completely lost all methionine acceptance capacity and attempts to renature

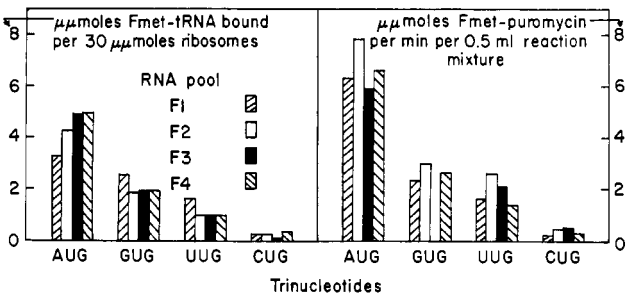

FIGURE 5: Binding to ribosomes and rate of reaction with puromycin of fMet-tRNA$_f^{Met}$ species F1-F4 (from the hydroxylapatite fractionation) in the presence of various trinucleotides.

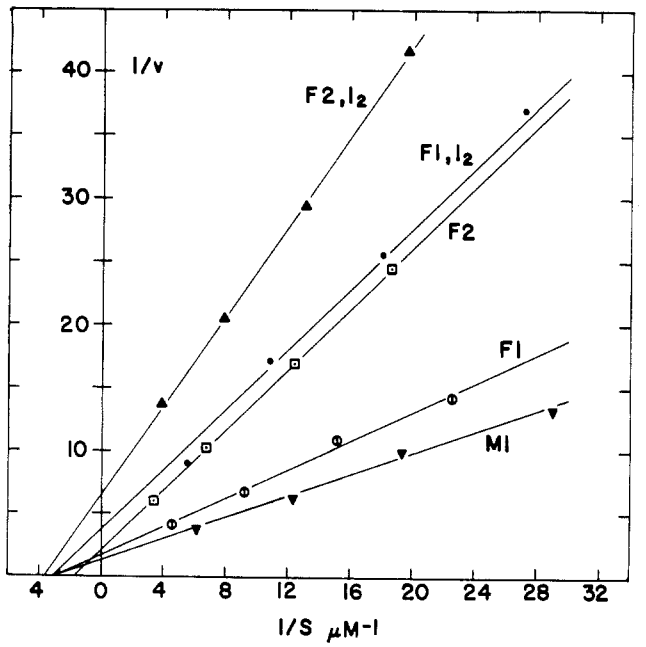

FIGURE 6: Kinetics of charging of various $tRNA^{Met}$ species. The species numbering is as shown in Figure 2. F1, I_2 and F2, I_2 represent the triiodide-oxidized species. The units of $1/v$ are arbitrary and identical for all species. The solid lines represent a least-squares best fit of the experimental data.

it by the methods of Lindahl *et al.* (1966) were unsuccessful. K_m values of the transformylation of F1 and F2 were close to the corresponding values for the charging reaction and again demonstrated the different kinetic properties of these two isoacceptors. In our hands the transformylase enzyme was much less stable than the $tRNA^{Met}$ synthetase and it was not possible to compare V_{max} values between one series of experiments and a different series.

The effect of iodine oxidation on these tRNAs was also tested since the ultraviolet absorption spectra suggested that all contained a single 4-thiouridine-like residue. The highly specific attack of triiodide on thiobases (Carbon *et al.*, 1965) and on isopentenyl side chains (Fittler and Hall, 1966) has been well documented although the nature of the oxidation product obtained from a tRNA containing a single 4-TU residue has not been elucidated. Triiodide treatment of F1 and M1 resulted in the abolition of the ultraviolet absorption band at 336 mμ and the appearance of a new band at *ca.* 315–320 mμ. In contrast, the long-wavelength ultraviolet spectrum of F2 was unaffected by triiodide; the possible significance of this observation has been discussed elsewhere (Scott and Schofield, 1969). The effect of this chemical modification on the kinetics of charging of F1 and F2 is shown in Figure 6. The K_m for charging of F1 is unchanged while V_{max} is reduced. F2 is affected somewhat differently and triiodide treatment results in a change both in V_{max} and in the apparent K_m. The acceptance capacity of these two $tRNA_f^{Met}$ species was unaffected by the triiodide treatment (Table II) whereas that of M1 was completely abolished. K_m values for the transformylation of F1 and F2 were not changed by the triiodide treatment.

TABLE II: Effect of Triiodide Treatment on Met Acceptance and K_m Values for Charging and Transformylation of Various $tRNA^{Met}$ Subspecies.

tRNA	Met Acceptance (μμmoles/ODU)	$K_m \times 10^7$ M	
		Charging	Transformylation
F1	945	3.6	2.1
F1, I_2 treated	749	3.3	2.8
F2	1140	5.9	8.3
F2, I_2 treated	1040	2.8	8.3
M1	680	3.3	
M1, I_2 treated	<15		

TABLE III: Failure of Uncharged $tRNA_{f2}^{Met}$ to Inhibit Transformylation of Met-$tRNA_{f2}^{Met}$.[a]

$tRNA_{f2}^{Met}$ Added (μμmoles)	μμmoles of fMet-$tRNA_{f2}^{Met}$ Formed/min
0	0.38
34	0.39
68	0.40
136	0.40

[a] In addition to the standard components for the transformylation reaction (Materials and Methods), each tube contained 31 μμmoles of Met-$tRNA_{f2}^{Met}$ and varying amounts of uncharged $tRNA_{f2}^{Met}$. The reaction was allowed to proceed for 8 min before the mixture was analyzed for its fMet-$tRNA_{f2}^{Met}$ content. Under these conditions, incorporation of formyl groups is linear for at least 15 min. Rate values quoted are the mean of two determinations.

The substrate in the transformylation reaction is Met-$tRNA_f^{Met}$ and it was of interest to test whether *uncharged* $tRNA_f^{Met}$ could inhibit this transformylation. The results of such an experiment are shown in Table III. The addition of a fourfold excess of uncharged $tRNA_f^{Met}$ has no effect on the rate of the reaction.

Discussion

Multiple species of isoaccepting tRNA have frequently been observed. It is also clear, however, that such multiple isoacceptors do not necessarily represent distinct molecular entities. A critical test for the presence of true multiple isoaccepting tRNA species is provided by the technique of two-dimensional mapping of enzymatic digests of the species in question. Mapping studies on highly purified species of $tRNA_f^{Met}$ and $tRNA_m^{Met}$ clearly show the presence of two of each of these tRNAs in *E. coli* B. The extent of sequence difference between the two $tRNA_f^{Met}$ isoacceptors is greater than the 7-MeG–A substitution found by Dube *et al.* (1968) for $tRNA_f^{Met}$. In addition, both the two $tRNA_f^{Met}$ and the two $tRNA_m^{Met}$ species are distinguished by the different

chemical and optical properties of the residues having an absorption maximum in the 330–340-mμ region (Scott and Schofield, 1969). Further sequence studies on these various isoacceptors are currently in progress.

Binding studies with the trinucleotides AUG, GUG, CUG, and UUG support and extend the similar studies of Doctor *et al.* (1969), and it is clear that the unusual AUG–GUG ambiguity found for $tRNA_f^{Met}$ *in vitro* cannot be explained by the existence of two molecular species of $tRNA_f^{Met}$, one responding to AUG, the other to GUG. It is interesting to note that F4, thought to be a chromatographic artifact derived from oxidation of a 4-TU residue in the tRNA, showed no difference in either binding or methionine-acceptance capacity from F1 in which the 4-TU residue is intact. This observation prompted us to examine the effect of triiodide oxidation on the charging and transformylation reactions. In both cases the kinetics of the reaction were studied rather than the plateau loading level in the hope that differences obscured in the latter might be revealed by the former assay. One reservation concerning these data derives from the fact that none of the $tRNA^{Met}$ species showed 100% of the theoretical maximum acceptor activity and the K_m values quoted were calculated from the observed acceptance. It is possible that the $tRNA^{Met}$ which is incompetent as a methionine acceptor might act as a competitive inhibitor in the charging reaction. However, the K_m values found in this study agree well with those previously reported for *E. coli* $tRNA_f^{Met}$ and $tRNA_m^{Met}$ (Bruton and Hartley, 1968), and are also of the same order of magnitude as those found for other tRNAs (Calendar and Berg, 1966; Makman, 1966).

These studies revealed a kinetic distinction between the F1 and F2 species in the charging reaction. This distinction, although relatively slight, also appears in the formylation reaction. Both the F1 and F2 species have been crystallized (Kim and Rich, 1968; Kim *et al.*, 1969) and it is therefore possible that this difference in kinetic properties might eventually be related to differences in three-dimensional structure. The very similar values of V_{max} found for F1, F2, and M1 support the contention that the charging of $tRNA_f^{Met}$ and $tRNA_m^{Met}$ is carried out by a single enzyme (Bruton and Hartley, 1968).

Triiodide treatment of F1 leaves K_m unchanged but reduces V_{max} to approximately 50% of the control value, whereas F2, after triiodide treatment, behaves as though an uncompetitive inhibitor had been introduced into the system. This effect is particularly puzzling since F2, as judged by its long-wavelength absorption, is resistent to the triiodide treatment (Scott and Schofield, 1969). The total abolition of acceptor activity of M1 after triiodide treatment may explain the poor recovery of $tRNA_m^{Met}$ acceptance after chromatography on hydroxylapatite since such columns can apparently catalyze the oxidation of thiobases in $tRNA_f^{Met}$. However, $tRNA_m^{Met}$ also contains a number of unidentified bases (Cory *et al.*, 1968) and, until their structures are elucidated, it would be premature to conclude that oxidation of the 4-TU residue in M1 is responsible for the observed loss in acceptor activity. As far as the functional significance of 4-TU in $tRNA_f^{Met}$ is concerned, the results presented here together with those of Doctor *et al.* (1969) indicate that this residue does not play an essential role in either the charging or transformylation reactions.

Uncharged $tRNA_{f2}^{Met}$ does not inhibit the formylation of Met-$tRNA_{f2}^{Met}$ even when present in fourfold excess over the latter. This is somewhat surprising since some form of transformylase–tRNA recognition is clearly indicated by this enzyme's ability to discriminate between Met-$tRNA_f^{Met}$ and Met-$tRNA_m^{Met}$, and by the observation of Trupin *et al.* (1966) that ethionyl- and norleucyl-$tRNA_f^{Met}$ can be enzymatically formylated. One possible explanation is that some feature of the tertiary and/or secondary structure of $tRNA_f^{Met}$ is altered upon charging with methionine and it is this altered structure which is recognized by the transformylase. Suggestive evidence for structural differences between uncharged and AA-tRNA has previously been found (Sarin and Zamecnik, 1965) and, more recently, chromatographic evidence for this type of structural modification has been obtained by Stern *et al.* (1969). We are currently using optical techniques in an attempt to detect such changes.

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Solubilization and Purification of β-Hydroxy-β-methylglutaryl Coenzyme A Reductase from Rat Liver*

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ABSTRACT: β-Hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) reductase from the rat liver microsomal fraction has been highly purified. This is the first such preparation of HMGCoA reductase obtained from mammalian sources. Solubilization of the enzyme was attained by treatment of the microsomal fraction with sodium deoxycholate and subsequent purification proceeded by standard methods. The molecular weight determined by gel filtration and centrifugation techniques ranged from 217,000 to 226,000. The enzyme showed many properties similar to the HMGCoA reductase of yeast (Kirtley, M. E., and Rudney, H. (1967), *Biochemistry 6*, 230). These were Michaelis constants, inhibition by acyl-CoA derivatives and CoA, a strong requirement for thiols, and inhibition by *o*-phenanthroline. It was also very sensitive to sulfhydryl reagents. Deoxycholate and digitonin inhibited the enzyme while cholesterol had no effect. Some metal ions, *e.g.*, Fe^{2+}, and Fe^{3+}, were strongly inhibitory.

Many agents, *e.g.*, dithiothreitol, deoxycholate, and ethylene diaminetetraacetate, showed a differential effect on the crude and purified preparations. These are discussed in relation to possible control mechanisms.

The incorporation of acetate into cholesterol in the liver can be drastically reduced by cholesterol feeding and by fasting (Gould, 1951; Langdon and Bloch, 1953; Tomkins *et al.*, 1953; Bucher and McGarrahan, 1956) while extrahepatic tissues are relatively unaffected by these regimes (Dietschy and Siperstein, 1967).

The inhibition of hepatic cholesterogenesis observed with mevalonate as substrate was minor compared with the inhibition from acetate (Bucher *et al.*, 1959). These observations led to the conclusion that the major part of the physiological regulation of cholesterol biosynthesis lies at the step between HMGCoA and mevalonate (Bucher, 1959; Siperstein and Guest, 1959; Bucher *et al.*, 1960; Siperstein and Fagan, 1966).

The enzyme catalyzing the reduction of HMGCoA to mevalonate (HMGCoA reductase–mevalonate:NADP-oxidoreductase (acylating-CoA), EC 1.1.134) has been purified by Durr and Rudney (1960), and Kirtley and Rudney (1967), and by Knappe *et al.* (1959) from yeast. Recently a report by Linn (1967) on the solubilization of HMGCoA reductase from extracts of acetone powders of rat liver microsomes appeared. Even without further purification, Linn was able to show clearly that the effect of cholesterol feeding appeared to be directly expressed as a decrease in the activity of the HMG-CoA reductase in these preparations. We attempted to use the acetone powder extracts of Linn as a basis for further purification, but although these extracts were active we were unable to obtain truly soluble preparations of the reductase using his procedure. In this paper we describe a method for solubilizing the HMGCoA reductase using deoxycholate. From this preparation we were able to purify the enzyme to near homogeneity and examine several of the properties of this system. Siperstein (1965) has indicated that deoxycholate could solubilize preparations of HMGCoA from rat liver microsomes, but did not describe any subsequent purification.

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[1] Abbreviations used are: HMGCoA, β-hydroxy-β-methylglutaryl coenzyme A; MVA, mevalonic acid.