Sub-unit Structure and Specificity of Methionyl-Transfer-Ribonucleic Acid Synthetase from *Escherichia coli*

By C. J. BRUTON AND B. S. HARTLEY

Medical Research Council Laboratory of Molecular Biology, Cambridge

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1. The purification of methionyl-transfer-RNA synthetase from *Escherichia coli* by a modified technique gives a 16% yield of a protein that appears homogeneous by the criteria of disc gel electrophoresis, ultracentrifugation and end-group analysis. 2. The molecular weight is 96 000 and the protein consists of two sub-units of 48 000, which appear to be identical. The amino acid composition and thiol content are reported. 3. Kinetic data are reported for analogues of methionine and for pure t-RNA_F and t-RNA_M, which are respectively the methionine transfer RNA that can exist in the formylmethionyl form and the one that can exist only in the methionyl form. The enzyme binds and acylates both species of transfer RNA identically.

Aminoacyl-t-RNA* synthetases occupy a central position in protein biosynthesis. They possess an extremely high degree of specificity towards both amino acids and t-RNA species. In translation of the genetic message, the selection of an amino acid rests entirely on this group of enzymes, and after its attachment to its specific t-RNA no further selection takes place (Chapeville *et al.* 1962). Several of the aminoacyl-t-RNA synthetases of *Escherichia coli* have now been purified: arginyl-t-RNA synthetase (Mitra & Mehler, 1967), isoleucyl-t-RNA synthetase (Baldwin & Berg, 1966), lysyl-t-RNA synthetase (Stern & Mehler, 1965), phenylalanyl-t-RNA synthetase (Stulberg, 1967) and tyrosyl-t-RNA synthetase (Calendar & Berg, 1966a). However, very little is known about the interactions between polynucleotides and proteins.

The role of methionine in the initiation of protein biosynthesis (Clark & Marcker, 1965; Adams & Capecchi, 1966; Webster, Engelhardt & Zinder, 1966) and the presence in *E. coli* of two different t-RNA species that accept methionine, namely t-RNA_F and t-RNA_M (Marcker, 1965; Clark & Marcker, 1966), makes the methionyl-t-RNA synthetase an obvious choice for us to study, particularly since the two t-RNA species are available in purified form in this Laboratory. It has been suggested (Cerhova & Rychlik, 1967) that two distinct methionyl-t-RNA synthetases exist in *E. coli*, each charging a different t-RNA species.

* Abbreviations: t-RNA, transfer RNA; t-RNA_F, the methionine t-RNA that can exist in the formylmethionyl form; t-RNA_M, the methionine t-RNA that can exist only in the methionyl form.

However, Heinrikson & Hartley (1967) reported a method for the purification of the methionyl-t-RNA synthetase from *E. coli* to apparent homogeneity, and gave evidence that their enzyme charged both t-RNA_F and t-RNA_M. We have confirmed the latter results with pure enzyme and pure t-RNA_F and t-RNA_M, and find almost identical kinetics for the charging of both t-RNA species. We also report studies on the specificity, amino acid composition, molecular weight and sub-unit structure of methionyl-t-RNA synthetase.

MATERIALS

A strain of *E. coli* (M.R.E. 244) was grown by the Microbiological Research Establishment, Porton, Wilts. The cells were stored as a frozen paste at -20° until required. Calcium phosphate gel was obtained from British Drug Houses Ltd., Poole, Dorset. Hydroxyapatite was prepared by the method of Tiselius, Hjerten & Levin (1956).

Yeast alcohol dehydrogenase was a gift from Dr P. J. G. Butler. Bovine pancreatic ribonuclease (five times recrystallized) and bovine pancreatic deoxyribonuclease I were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.

L-[¹⁴C]Methionine, [¹⁴C]maleic anhydride and Na₄³²P₂O₇ were purchased from The Radiochemical Centre, Amersham, Bucks. Crude *E. coli* B t-RNA was purchased from General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A. Purified t-RNA_F from a similar strain of *E. coli* (M.R.E. 265) was a gift from Dr B. F. C. Clark, and purified t-RNA_M from the same source was a gift from Miss S. Cory.

L-Methionine methyl ester hydrochloride, L-methionine ethyl ester hydrochloride, L-ethionine, L-norleucine, seleno-DL-methionine, DL-homocysteine and 2,2'-dithiobis-(5-nitrobenzoic acid) were purchased from Sigma Chemical

Co. *S*-Methyl-L-cysteine was purchased from the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. L-Methionine amide hydrochloride was purchased from the Cyclo Chemical Corp., Los Angeles, Calif., U.S.A.

DEAE-Sephadex (lot no. 9699, capacity 3.5 ± 0.5 m-equiv./g.), Sephadex G-200 (lot no. 8987) and Sephadex G-25 (coarse grade) were purchased from Pharmacia, Uppsala, Sweden. All other reagents were A.R. grade where possible.

METHODS

Preparation of enzyme. The method of Heinrikson & Hartley (1967) was modified to give a better yield and to permit working on a larger scale. All manipulations were carried out at 0–4°. The potassium salts of the phosphate buffers were used exclusively, and all buffers contained 0.1 M-2-mercaptoethanol.

(a) *Extraction.* Frozen cell paste (1 kg.) was thawed and blended in a stainless-steel Waring Blender with 2 kg. of Ballotini 11 glass beads and 500 ml. of 0.1 M-phosphate buffer, pH 7.0, containing 1.0 mM-EDTA. Blending was continued for a total of 10 min. with intervals for cooling. The temperature was not allowed to rise above 20°. Extract buffer (11.) was added, and the beads were removed by mild centrifugation (1000 rev./min.). The beads were washed twice with 200 ml. volumes of extract buffer. The combined supernatants were centrifuged at 23000g for 45 min.

(b) *Autolysis.* Ribonuclease (10 mg.) and deoxyribonuclease (10 mg.) were added to the supernatant from the extraction, which was then incubated at 37°. After 3 hr. the acid-soluble E_{260} value rose to 75% of the total E_{260} . The material was cooled to 5°, and the precipitate was removed by centrifugation at 23000g for 45 min.

(c) *Ammonium sulphate fractionation.* Solid $(\text{NH}_4)_2\text{SO}_4$ (219 g./l. of soln.) was added to the autolysate and this was stirred for 15 min. The precipitate was removed by centrifugation. Additional $(\text{NH}_4)_2\text{SO}_4$ (94 g./l. of soln.) was added to the supernatant, and this was stirred for 15 min. The precipitate was collected by centrifugation and dissolved in 5 mM-phosphate buffer, pH 7.0. The solution was dialysed against the same buffer. Any precipitate formed was removed by centrifugation.

(d) *Calcium phosphate gel.* Calcium phosphate gel (1.5 mg. dry wt. of gel/mg. of protein) was added to the 35–50%-saturated $(\text{NH}_4)_2\text{SO}_4$ fraction and the mixture was stirred for 30 min. The gel was collected by centrifugation, washed with 300 ml. of 5 mM-phosphate buffer, pH 7.0, and eluted with 300 ml. of 10 mM-phosphate buffer, pH 7.8, and 300 ml. of 0.1 M-phosphate buffer, pH 7.8.

(e) *DEAE-Sephadex chromatography.* The combined eluates from the calcium phosphate gel were brought to 70% saturation with $(\text{NH}_4)_2\text{SO}_4$. The precipitated protein was spun down and dissolved in 10 mM-phosphate buffer, pH 7.0. Residual $(\text{NH}_4)_2\text{SO}_4$ was removed by passage through a column (65 cm. $\times$ 3.0 cm.) of Sephadex G-25 (coarse grade) equilibrated with the same buffer. The material eluted at the breakthrough volume from this column was applied to a column (40 cm. $\times$ 4.4 cm.) of DEAE-Sephadex equilibrated with 10 mM-phosphate buffer, pH 7.0. The column was eluted with a linear phosphate gradient from 0.05 M to 0.25 M, pH 7.0. The total volume of the gradient was 21., the flow rate was 33 ml./hr., and fractions (11 ml.) were collected. Fractions containing

enzyme with a specific activity at least twice that of the sample applied to the column were pooled.

(f) *Hydroxyapatite chromatography.* The pooled DEAE-Sephadex eluate was dialysed against 20 mM-phosphate buffer, pH 7.0. The non-diffusible material was applied to a column (35 cm. $\times$ 2.2 cm.) of hydroxyapatite equilibrated with the same buffer. The column was eluted with a linear phosphate gradient from 0.05 M to 0.20 M, pH 7.8. The total volume of the gradient was 21., the flow rate was 30 ml./hr., and fractions (10 ml.) were collected. The fractions with the highest specific activity were pooled and immediately adjusted to pH 7.0 with phosphoric acid. For storage this fraction was concentrated to approx. 5 ml. by ultrafiltration as described by Crestfield, Stein & Moore (1963).

Polyacrylamide gel electrophoresis. The homogeneity of enzyme fractions was assessed by polyacrylamide-gel electrophoresis as described by Davis (1964), with a 7% acrylamide gel with or without 8 M-urea. Proteins were fixed and stained with a 1% solution of Amido Schwartz in 7% (v/v) acetic acid. The gels were destained electrophoretically.

Enzyme assays. Methionyl-t-RNA synthetase assays were carried out as described by Heinrikson & Hartley (1967). Yeast alcohol dehydrogenase was assayed as described by Racker (1955).

Protein determinations. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) and spectrophotometrically (Layne, 1957).

Amino acid analysis. Concentrated enzyme samples (0.5 mg.) were dialysed against 10 mM-sodium acetate to remove thiol and phosphate. After being freeze-dried, the protein was hydrolysed *in vacuo* at 105° for 36 hr. with 6 N-HCl to which a crystal of phenol had been added to protect the tyrosine residues against oxidation. The hydrolysates were analysed on a Beckman amino acid analyser model 120C (Spackman, Stein & Moore, 1958). Half-cystine was determined as cysteic acid after performic acid oxidation (Hirs, 1956) followed by hydrolysis for 16 hr. or 20 hr. Tryptophan was determined spectrophotometrically (Beaven & Holiday, 1952).

Molecular weight studies. Sedimentation-equilibrium studies were carried out in a Beckman-Spinco model E ultracentrifuge equipped with temperature control and schlieren and interference optics. The molecular weight was calculated from the sedimentation-equilibrium results by method I and method II of Van Holde & Baldwin (1958). A rotor speed of 12590 rev./min. was used, with the Beckman-Spinco type AN-D rotor. The solution contained 0.1 M-phosphate buffer, pH 7.0, and, in the case of the maleylated protein, 0.4 M-NaCl also. The temperature was maintained at 20° throughout. Ultracentrifuge plates were measured on a Nikon profile projector model 6C.

Maleylation of methionyl-t-RNA synthetase. Redistilled maleic anhydride (2.5 mg.) (30-fold excess over all free amino groups) was added to 1.5 mg. of protein in 200 μ l. of 0.5 M-sodium borate buffer, pH 9.5 (Butler, Harris, Hartley & Leberman, 1967), and incubated at room temperature for 1 hr. It was then dialysed against 0.1 M-phosphate buffer, pH 7.0, containing 0.4 M-NaCl. To prepare [^{14}C]maleyl-protein, 300 μ l. of [^{14}C]maleic anhydride in benzene (16 μ moles/ml.; 3.14 $\mu\text{C}/\mu\text{mole}$) was added to 0.2 mg. of protein in 25 μ l. of 0.5 M-sodium borate buffer, pH 9.5, and the mixture was incubated at room temperature for 1 hr.

End-group analysis. The protein solution, containing 5 μ moles of protein, was dialysed against 0.5 M-NaHCO₃

to remove thiol, and freeze-dried. It was dissolved in 250 μ l. of m -NaHCO₃ in 8*M*-urea that had been deionized free from ammonia and cyanate. To this was added 250 μ l. of 1-dimethylaminonaphthalene-5-sulphonyl chloride in acetone (20 mg./ml.), and the mixture was incubated for 5 hr. at 37°. Excess of 1-dimethylaminonaphthalene-5-sulphonic acid and sulphonamide were removed by dialysis against water. The non-diffusible material was dried *in vacuo* and hydrolysed at 105° with 6*N*-HCl *in vacuo*. The hydrolysate was dried *in vacuo* and dissolved in 10 μ l. of 95% (v/v) ethanol. The 1-dimethylaminonaphthalene-5-sulphonyl-amino acids were separated by two-dimensional chromatography on polyamide thin layers (Woods & Wang, 1967).

Titration of thiol groups. The reaction of thiol groups with 2,2'-dithiobis-(5-nitrobenzoic acid) was followed by the extinction at 412 $m\mu$ (Ellman, 1959). The reaction was carried out in 0.1 *M*-tris-HCl, pH 8.0, at room temperature. Before the incubation, the protein solution was passed through a column (60 cm. $\times$ 1.1 cm.) of Sephadex G-25 (coarse grade) in 0.1 *M*-tris-HCl, pH 8.0, to remove thiol.

RESULTS

Enzyme purification. The purification of methionyl-t-RNA synthetase from 1 kg. of *E. coli* is summarized in Table 1. The main modification to the procedure described by Heinrikson & Hartley (1967) was that the autolysis step allowed us to avoid the rather laborious 100 000 *g* centrifugation without loss of purity or yield. By omitting a pH 5 precipitation step we obtained a higher yield and equivalent purification after ammonium sulphate precipitation. The gel adsorption, DEAE-Sephadex chromatography and hydroxyapatite chromatography were similar to those previously described. The enzyme eluted from hydroxyapatite was homogeneous, as judged by polyacrylamide-gel electrophoresis and ultracentrifugation (Fig. 2), so further purification on Sephadex G-200 was unnecessary. The specific activity of different purified preparations ranged from 360 to 480 units/mg. During the purification no fraction other than those mentioned in Table 1 had more than 10% of the activity of the fraction retained, and there was no indication during DEAE-Sephadex chromato-

graphy of a second peak of enzyme activity. We therefore believe that all the methionyl-t-RNA synthetase activity of our extract is accounted for by this one enzyme.

Molecular weight. The molecular weight of the purified enzyme was determined by both gel filtration on Sephadex G-200 and ultracentrifugation. The elution profile obtained from the gel filtration of a mixture of purified enzyme, yeast alcohol dehydrogenase, bovine serum albumin, blue dextran and ϵ -DNP-lysine is presented in Fig. 1.

Fig. 1. Determination of the molecular weight of methionyl-t-RNA synthetase by gel filtration on Sephadex G-200. A sample containing 1 mg. of yeast alcohol dehydrogenase (YADH), 5 mg. of bovine serum albumin (BSA), 0.1 mg. of purified methionyl-t-RNA synthetase and 1 drop each of blue dextran solution and ϵ -DNP-lysine soln. was pumped on to a column (95 cm. $\times$ 1.0 cm.) of Sephadex G-200 equilibrated with 10 mM-phosphate buffer, pH 7.0, containing 10 mM-mercaptoethanol. Elution was continued with the same buffer. The column was pumped upwards at 2 ml./hr., and fractions (1 ml.) were collected. Fractions were assayed for methionyl-t-RNA synthetase and YADH activities, as described in the Methods section. Enzyme activities are expressed in arbitrary units for clarity of presentation. —, YADH activity; ·····, methionyl-t-RNA synthetase activity; —, E_{280} .

Table 1. *Purification of methionyl-t-RNA synthetase from E. coli*

Details of the purification and assay procedures are presented in the Methods section. Specific activities are expressed as μ moles of PP₁ exchanged/15 min./mg. of protein.

Fraction	Protein (mg.)	Specific activity	Relative purification	Yield (%)
23000 <i>g</i> supernatant	47900	0.50	1	100
Autolysate	20900	1.15	2	100
35–50%-satd. (NH ₄) ₂ SO ₄ fraction	7160	2.79	5.5	83
Calcium phosphate gel eluate	1800	5.90	12	44
DEAE-Sephadex eluate	132	40	80	22
Hydroxyapatite eluate	9.8	390	780	16

The enzymic activity emerged considerably after the yeast alcohol dehydrogenase, but before the bovine serum albumin. By plotting the elution volume against the logarithm of the molecular weight of the markers (Andrews, 1965), a value of 95 000 was determined for methionyl-t-RNA synthetase. No activity was detected in any other region by an assay technique that would have detected 2% of the activity observed.

Sedimentation-equilibrium studies were carried out with short columns (approx. 3 mm.). Plots of

Fig. 2. Plot of $(1/r).dc/dr$ versus $(c - c_0)$ of sedimentation-equilibrium data for purified methionyl-t-RNA synthetase (method II of Van Holde & Baldwin, 1958). The protein concentration was 1.5 mg./ml., and the rotor speed of the Beckman-Spinco model E ultracentrifuge with the type AN-D rotor was 12590 rev./min. at 20°. Arbitrary units are used for both axes.

Table 2. *Molecular weight of methionyl-t-RNA synthetase*

Details of the ultracentrifugation are presented in the Methods section. The rotor speed of the Beckman-Spinco model E ultracentrifuge with the type AN-D rotor was 12590 rev./min. at 20° in all cases. The weight-average molecular weight $(M_w = \frac{\sum n_i M_i^2}{\sum n_i M_i})$ and the Z-average molecular weight $(M_z = \frac{\sum n_i M_i^3}{\sum n_i M_i^2})$ were calculated by method I and method II of Van Holde & Baldwin (1958).

Concn. of protein (mg./ml.)	M_w	M_z
6.0	96 000	94 500
1.5	96 000	97 000

$(1/r).dc/dr$ versus $(c - c_0)$ obtained from measurements of the schlieren and interference photographs are shown in Fig. 2. The values determined for the molecular weight at two different protein concentrations are shown in Table 2. The partial specific volume was estimated from the amino acid composition by the method of Cohn & Edsall (1943).

Measurement of the sedimentation coefficient was carried out at two different protein concentrations by using schlieren optics. A rotor speed of 59 780 rev./min. was used and the temperature was 20°. Extrapolation to zero protein concentration gave $S_{20,w}^0$ of 4.7 s.

Sub-units. The apparent molecular weight of the maleylated enzyme was also determined by gel filtration on Sephadex G-200 and ultracentrifugation. The elution profile of a sample of [^{14} C]maleyl-enzyme and blue dextran is shown in Fig. 3. The same column was used for the native (Fig. 1) and maleyl-enzyme. The void volume (blue dextran) and total volume (ϵ -DNP-lysine or [^{14} C]maleic acid) were the same in each case, so it was reasonable to use the calibration curve determined in the first experiment. From the elution volume an apparent molecular weight of 42 000 was estimated for the maleyl-enzyme.

Sedimentation-equilibrium studies were carried

Fig. 3. Apparent molecular weight of maleyl-methionyl-t-RNA synthetase by gel filtration on Sephadex G-200. A sample containing 0.2 mg. of [^{14} C]maleyl-methionyl-t-RNA synthetase and 1 drop of blue dextran solution was pumped on to a column (95 cm. $\times$ 1.0 cm.) of Sephadex G-200. The column used was the same as that used for the experiment described in Fig. 1. Elution was carried out with 10 mM-phosphate buffer, pH 7.0, containing 10 mM-mercapto-ethanol. The column was pumped upwards at 2 ml./hr., and fractions (1 ml.) were collected. Samples (100 μ l.) of each tube were dried down on a planchet and counted in a Nuclear-Chicago gas-flow counter (model 186A). ----, Radioactivity (counts/min./ μ l.); —, E_{280} . a, b and c represent the elution positions of yeast alcohol dehydrogenase, native methionyl-t-RNA synthetase and bovine serum albumin respectively on this column.

Fig. 4. Plot of $(1/r).dc/dr$ versus $(c-c_0)$ of sedimentation-equilibrium data for maleyl-methionyl-t-RNA synthetase (method II of Van Holde & Baldwin, 1958). The protein concentration was approx. 2mg./ml., and the rotor speed of the Beckman-Spinco model E ultracentrifuge with the type AN-D rotor was 14290 rev./min. at 20°. Arbitrary units are used for both axes.

out on the unlabelled maleyl-enzyme after dialysis against 0.1M-phosphate buffer, pH 7.0, containing 0.4M-sodium chloride for 2 weeks to ensure complete equilibration. A plot of $(1/r).dc/dr$ versus $(c-c_0)$ is shown in Fig. 4. The molecular weight calculated from this run was 48700. The linearity of the $(1/r).dc/dr$ versus $(c-c_0)$ plot suggests that the maleyl protein is homogeneous, in size at least.

A sample of performic acid-oxidized enzyme was subjected to electrophoresis on polyacrylamide gel in 8M-urea. Staining was carried out in the usual way and revealed a single protein band.

Amino acid composition. The results obtained from analyses of different preparations of methionyl-t-RNA synthetase subjected to acid hydrolysis are presented in Table 3. The number of residues/mol. was calculated for a molecular weight of 96000. Spectrophotometric analysis revealed that the ratio of tyrosine to tryptophan was 2.88. The value of 45 previously reported by Henrikson & Hartley (1967) was an arithmetical error and should have been 2.75.

The amino acid analysis indicated 10 half-cystine residues/mol. Titration of the native protein with 2,2'-dithiobis-(5-nitrobenzoic acid) showed that 10.1 thiol groups/mol. reacted in 4hr. Kinetic analysis of the reaction suggested that 8 of these groups reacted rapidly with a first-order rate constant of about 0.0025sec^{-1} , but 2 of them reacted much more slowly, with a first-order rate constant of 0.00005sec^{-1} (see Fig. 5). In 8M-urea, 10.2 groups reacted completely in 4min. and the

Table 3. *Amino acid analysis of methionyl-t-RNA synthetase*

Details of the analyses are presented in the Methods section. The values are the mean of three analyses, except that the tyrosine value is a single analysis of the native protein and the cysteine acid and methionine values are duplicate analyses on oxidized protein. The serine and threonine values are extrapolated to zero hydrolysis time, and the valine and isoleucine values are extrapolated to infinite hydrolysis time. Tryptophan was estimated spectrophotometrically. The values in parentheses are those of Henrikson & Hartley (1967).

Amino acid	Residues/mol. (mol. wt. 96000)
Lysine	44.3 (41.6)
Histidine	19.0 (21.1)
Arginine	42.6 (34.8)
Cysteic acid	10.1 (10.1)
Aspartic acid and asparagine	91.7 (94.2)
Methionine sulphone	18.6 (17.5)
Threonine	43.8 (42.2)
Serine	49.6 (52.1)
Glutamic acid and glutamine	100.8 (97.9)
Proline	33.8 (39.3)
Glycine	71.3 (67.5)
Alanine	82.5 (78.5)
Valine	57.6 (55.2)
Isoleucine	46.1 (46.1)
Leucine	80.4 (69.9)
Tyrosine	25.1 ( )
Phenylalanine	37.1 (41.2)
Tryptophan	8.7 ( )
Total	863.1 (807)

Fig. 5. First-order plot for the reaction of native methionyl-t-RNA synthetase with 2,2'-dithiobis-(5-nitrobenzoic acid). The conditions were as described in the Methods section. a , the total number of thiol groups per mol. of protein; x , the number that have reacted at any given time. The upper linear portion of the graph represents the slowly reacting thiol groups (1.98 groups/mol.).

reaction was again first-order ($k = 0.031 \text{ sec.}^{-1}$). The enzyme was inhibited by reaction with 0.1 mM-*p*-hydroxymercuribenzoate, and required mercapto-ethanol or other thiol for stability on storage, in contrast with the lysyl-t-RNA synthetase from *E. coli* (Stern, DeLuca, Mehler & McElroy, 1966).

End-group analysis. The *N*-terminal residue, determined as described in the Methods section, was alanine. Large quantities of ϵ -l-dimethylaminonaphthalene-5-sulphonyl-lysine and *O*-l-dimethyl-

aminonaphthalene-5-sulphonyltyrosine were also present on the chromatogram, but no other l-dimethylaminonaphthalene-5-sulphonyl amino acid. A second extraction of the hydrolysate with 1.0M-ammonia, to ensure complete extraction of acidic amino acids, showed no new spots.

Acylation of methionine t-RNA. The acylation of highly purified t-RNA_F and t-RNA_M was studied by the standard acylation assay method of Henrikson & Hartley (1967), except that the crude t-RNA was replaced by t-RNA_F or t-RNA_M. Each of these preparations was at least 95% pure as judged by their capacity to accept methionine—a significant improvement on those used by Henrikson & Hartley (1967). The purified enzyme catalysed the acylation of both species with [¹⁴C]methionine. The Michaelis constant for t-RNA_F was 0.5 μM , and that for t-RNA_M was 0.3 μM . The maximum velocities for each species were the same within the limit of experimental error (see Fig. 6).

Other properties of methionyl-t-RNA synthetase. Several methionine analogues were tested in the ATP-PP_i exchange assay. Of these, D-methionine, DL-homocysteine, S-methyl-L-cysteine, DL-methionine sulphone and DL-methionine sulfoxide were inactive (at a concentration of 10mM the rate was less than 1% of the rate with L-methionine). Only L-ethionine, L-norleucine and seleno-DL-methionine were active. In each case a sample of the analogue was analysed on the Beckman-Spinco amino acid analyser in parallel with a sample of the analogue plus 0.1% of methionine to show that this quantity of impurity could be detected. All samples contained less than 1% of methionine, and hence must be true activators of PP_i exchange. L-Methionine esters and amide were also tested as inhibitors of the exchange and acylation reactions.

Fig. 6. Lineweaver & Burk (1934) plots of the acylation of t-RNA_F and t-RNA_M. Conditions were as described in the Methods section except that purified t-RNA_F (○) or t-RNA_M (●) replaced the crude t-RNA. Each point represents the initial velocity of the reaction under the conditions used. The velocity, *v*, is defined as the number of μmoles of L-methionine incorporated into t-RNA/min./mg. of protein. [S], Concn. of substrate (M).

Table 4. *Michaelis constants for substrates and inhibitors*

Details of the assay procedures are presented in the Methods section. Michaelis constants and maximum velocities were determined by the reciprocal-plot method of Lineweaver & Burk (1934). For the exchange assay, velocities are expressed as μmoles of PP_i exchanged/15 min./mg. of protein. For the acylation assay, velocities are expressed as μmoles of L-methionine incorporated into t-RNA/min./mg. of protein.

	ATP-PP _i exchange			Acylation		
	<i>K_m</i> (mM)	<i>V_{max.}</i>	<i>K_t</i> (mM)	<i>K_m</i> (mM)	<i>V_{max.}</i>	<i>K_t</i> (mM)
L-Methionine	0.07	400		0.02	0.80	
L-Methionine methyl ester			2.5			0.16
L-Methionine ethyl ester			3.5			0.27
L-Methionine amide			0.76			0.10
L-Ethionine	12	120				
L-Norleucine	10	200				
Seleno-DL-methionine	0.11	400				
ATP	0.52	420				
t-RNA _M				0.22	0.84	
t-RNA _F				0.0003	0.85	
				0.0005	0.83	

The results of these experiments are summarized in Table 4. *N*-Acetyl-L-methionine, glycyl-L-methionine and L-methionyl-L-alanine did not inhibit the exchange reaction at concentrations of 10 mM.

DISCUSSION

The disaggregating effect of succinylation on proteins is well known (Habeeb, Cassidy & Singer, 1958; Klotz & Keresztes-Nagy, 1963), and maleylation almost certainly has a similar effect (Butler *et al.* 1967). This is of particular advantage in investigating dissociation into sub-units by Sephadex elution, since the products are soluble at alkaline pH and can be run on the same calibrated column as the native enzyme, without the use of urea or guanidine, which affect the behaviour of the column. In this way, using only 0.2 mg. of protein we found a molecular weight of 95 000 for the native enzyme and a molecular weight of 42 000 for the enzyme after reaction with [^{14}C]maleic anhydride. These approximate results were confirmed by sedimentation-equilibrium studies, which gave a molecular weight of 96 000 for the enzyme and one of 48 700 for the sub-unit. The linearity of the plots shown in Figs. 2 and 4, which is a good criterion for homogeneity, together with the agreement with the gel-elution results give us confidence in these values. The sub-units are probably identical, since the oxidized enzyme still gave a single band when subjected to electrophoresis on polyacrylamide gel in 8 M-urea. It is extremely unlikely that two dissimilar chains would not separate under these conditions. The results of the end-group analysis also support this conclusion. Only one *N*-terminal amino acid was found by the procedure used, but this experiment was not done quantitatively. In view of the preponderance of methionine, serine and alanine as *N*-terminal residues in *E. coli* proteins (Waller, 1963), this can not be regarded as conclusive since two different chains may very easily have the same *N*-terminal residue. However, this result fits in with the other indications that the chains are identical.

The amino acid analysis shown in Table 3 is calculated as residues/mol. of molecular weight 96 000. It closely resembles that reported by Henrikson & Hartley (1967) and the few differences may not be significant. If, however, the sub-units are identical they would contain about 430 residues including 5 cysteine, 9 methionine and 4 tryptophan residues.

Our results conflict, in some degree, with previous reports of the molecular weight of this enzyme. By gel filtration on Sephadex G-200 of enzyme purified from *E. coli* (M.R.E. 600) by a slightly different procedure, Henrikson & Hartley (1967) found a

single peak of activity corresponding to a molecular weight of 190 000. This probably represented a genuine difference, since we have used similar columns and similar protein markers. Their enzyme was eluted before yeast alcohol dehydrogenase, whereas ours was eluted well after. They found no activity in the region corresponding to a molecular weight of 100 000 even in the crude extract, whereas we found no activity in the higher region. Nass & Stoffer (1967) reported a molecular weight of 145 000 for the activity in a crude extract of *E. coli* B by Sephadex G-200 gel filtration. In all other respects the enzyme purified by Henrikson & Hartley (1967) appears identical with that described in the present paper. One is left with the unsatisfactory conclusion that small strain differences may account for the discrepancies, or that autolysis of the extracts may cause dissociation of an active tetramer into an active dimer.

Our studies with methionine analogues help to define the specificity of the enzyme. The activation of ethionine has been reported by Glenn (1961), using a crude rat liver enzyme. We find that the pure enzyme has considerable activity towards both ethionine and norleucine, though the K_m for these analogues is much higher than that for methionine. With seleno-DL-methionine both the K_m and the maximum velocity are almost identical with those for methionine in the PP_i -exchange assay, which is rather surprising in view of the greater electronegativity and larger covalent radius of selenium (Rosenfeld & Beath, 1964). One can implicate the amino group of the substrate in binding to the enzyme, since methionine esters and amide are reasonable competitive inhibitors, whereas *N*-acyl derivatives are quite ineffective. Unlike the tyrosyl-t-RNA synthetase (Calendar & Berg, 1966*b*) the methionine enzyme is quite specific for the L-amino acid.

The more interesting aspect of the specificity of this enzyme is that towards its t-RNA. The enzyme has equally great affinity for both t-RNA_F and t-RNA_M and aminoacylates both at equal rates. It thus appears unable to distinguish between these substrates—a task that the methionyl-t-RNA transformylase (Marcker, 1965) accomplishes with complete specificity. Moreover, the ribosome accepts only the formylmethionyl-t-RNA_F at the 'peptidyl' binding site (Bretscher & Marcker, 1966; Anderson, Bretscher, Clark & Marcker, 1967; Ghosh & Khorana, 1967). This must mean that only structures that are common to both t-RNA_F and t-RNA_M are involved in the acylation reaction, whereas formylation and binding to the ribosome involve other parts of the molecule.

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