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LYSYL tRNA SYNTHETASE OF *ESCHERICHIA COLI* B: FORMATION AND REACTIONS OF ATP–ENZYME AND LYSYL-AMP–ENZYME COMPLEXES

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SUMMARY

1. Lysyl-tRNA synthetase (L-lysine: tRNA ligase (AMP), EC 6.1.1.6), purified from *Escherichia coli* B, bound ATP in a stable complex, in the ratio 30–60 pmoles/100 pmoles enzyme. This ATP–enzyme complex gave rise to lysyl-AMP–enzyme complex or to lysyl-tRNA when incubated with the appropriate substrates.

2. Monovalent cations, in particular NH_4^+, inhibited the formation of lysyl-tRNA from lysine, tRNA, and ATP–enzyme complex or ATP. The synthesis was dependent upon the presence of Mg^{2+}. In contrast the synthesis of lysyl-tRNA from lysyl-AMP–enzyme was dependent upon the presence of NH_4^+ or other monovalent ions, and was inhibited by Mg^{2+}. The enzyme synthesizes lysyl-tRNA by two different mechanisms, one avoiding the adenylate pathway.

3. The enzyme (estimated mol. wt. 150 000 using gel filtration on Sephadex G-200) appears to be an aggregate, perhaps a dimeric protein with unequal subunits.

INTRODUCTION

The formation of aminoacyladenylate–enzyme complexes, following incubation of aminoacyl-tRNA synthetases (amino acid: tRNA ligases (AMP), EC 6.1.1) with ATP, Mg^{2+} and amino acid has been known for some years[1], and the function of these enzyme bound intermediates in the following reaction mechanism well established.

$$\text{Amino acid} + \text{ATP} + \text{enzyme} \overset{Mg^{2+}}{\rightleftarrows} \text{aminoacyl-AMP–enzyme} + PP_i \quad (1)$$

$$\text{Aminoacyl-AMP–enzyme} + \text{tRNA} \rightleftharpoons \text{aminoacyl-tRNA} + \text{AMP} + \text{enzyme} \quad (2)$$

Less is known concerning the formation and reactions of ATP–enzyme complex[2–4]. This intermediate can also participate in the overall reaction mechanism lead-

Abbreviations: PP_i–ATP exchange, isotope exchange between [^{32}P]pyrophosphate and ATP; Lysyl-AMP–enzyme complex, lysyladenylate enzyme complex.

ing to the synthesis of aminoacyl tRNA. In studies on the formation of lysyl-AMP–enzyme complex by *Escherichia coli* B lysyl-tRNA synthetase (L-lysine: tRNA ligase, EC 6.1.1.6) we discovered that only [^{3}H]adenine label was bound to the enzyme when the enzyme was incubated with [^{3}H]ATP and [^{14}C]lysine at a Mg^{2+} concentration above 10 mM. This suggested that this synthetase could also form an ATP–enzyme complex, although a similar lysyl-tRNA synthetase, isolated by WALDENSTRÖM[5] from *E. coli* B was apparently unable to do so.

We used this property of our synthetase to examine further the part played by monovalent cations and Mg^{2+} in the synthesis of lysyl-tRNA both from lysyl-AMP–enzyme complex and *via* Reactions 1 and 2. It has already been reported that these ions exert very different effects upon these two reactions[5,6]. Some of these observations have received preliminary publication[7].

METHODS

Enzyme preparation

All procedures were carried out at 4°. Extracts of frozen *E. coli* B cells were prepared according to BALDWIN AND BERG[8]. Nucleic acids were precipitated from the 10 000 × *g* supernatant by the addition of streptomycin sulfate (0.5 vol. of a 5% solution) followed by centrifugation. Proteins were precipitated by the addition of 45.5 g $(NH_4)_2SO_4$ per 100 ml ("65% saturation") followed by centrifugation, then redissolved, and dialyzed overnight against 0.01 M potassium phosphate (pH 6.0) containing 1 mM EDTA. The protein was adsorbed onto calcium triphosphate gel (1 mg dry weight gel per mg protein) and the enzyme activity eluted with a freshly prepared solution consisting of equal vol. of 1.0 M Tris–HCl (pH 8) and 0.2 M sodium phosphate (pH 8), containing 17.5 g $(NH_4)_2SO_4$ per 100 ml. The eluate was immediately brought to neutrality with 10 M acetic acid, and fractionated with $(NH_4)_2SO_4$. 10.5 g/100 ml were added to give "40% saturation", and the first precipitate removed by centrifugation, then a second precipitate obtained by the addition of 17.5 g/100 ml ("65% saturation") followed by centrifugation. The protein was dialyzed overnight against 0.05 M potassium phosphate (pH 7) containing 1 mM EDTA. The enzyme present in the "40–65 saturation" fraction was further purified by column chromatography.

Column I DEAE-cellulose (Whatman DE 23) (2.2 cm × 30 cm) was equilibrated with 0.05 M potassium phosphate buffer (pH 7.0). A linear gradient of increasing ionic strength and decreasing pH was used; the mixing vessel contained 1000 ml of 0.05 M potassium phosphate buffer (pH 6.5). A flow rate of 60 ml/h was used, and fractions of 10 ml were collected. The fractions with the highest specific enzyme activity were pooled, concentrated in dialysis bags against Carbowax 6000 flakes, and then dialyzed against 0.05 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA.

Column II DEAE-Sephadex A50 (0.9 cm × 60 cm) equilibrated with 0.05 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA. The linear gradient used was identical to that used with Column I. The flow rate was 8 ml/h and fractions of 5 ml were collected. The enzyme activity emerged after about 100 h. The fractions with the highest specific activity were pooled and concentrated in dialysis bags

against powdered Sephadex, followed by dialysis against 0.05 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA.

Column III Sephadex G-200 (0.9 cm × 210 cm) equilibrated and developed with 0.02 M potassium phosphate buffer (pH 7.2). The flow rate was 1.2 ml/h, and fractions of about 1.5 ml were collected. About 5% of the total protein separated as a high molecular weight contaminant from the single peak of protein and enzyme activity. No significant increase in specific enzyme activity was observed and essentially all the enzyme activity was recovered.

TABLE I

PURIFICATION OF LYSYL-tRNA SYNTHETASE

1 enzyme unit = 1 nmole [^{32}P]pyrophosphate exchanged into ATP per min per ml reaction mixture

	Total protein (mg)	*Specific enzyme activity (units/mg)*	*Total enzyme units (× 10^6)*	*Recovery (%)*
10 000 × *g* supernatant of initial extract	16 600*	90	1.49	
"0–65%" $(NH_4)_2SO_4$	5 920	214	1.26	85
"40–65%" $(NH_4)_2SO_4$ after gel	795	667	0.54	36
DEAE-cellulose	54	5 960	0.52	22
DEAE-Sephadex	16	19 600	0.31	21

* Obtained from 330 g of frozen *E. coli* B paste.

The steps in the purification are shown in Table I. The purification obtained was about 220 fold. Similar purification procedures for this enzyme have been devised by other investigators[3,5,6]. The preparations catalysed a variable level of histidine dependent PP_i–ATP exchange, 1–3% of the rate observed with lysine but no significant levels of other synthetases were detected. The preparation did not synthesize histidyl-tRNA. The ratio of absorbances of the enzyme preparation measured at 280 nm/260 nm was about 1.6 and was measured in 0.05 M potassium phosphate buffer, (pH 7). The enzyme could be stored in phosphate buffer at least eight months at 4°, and at least 2 years at −15°, without loss of activity. PP_i–ATP exchange activity was rapidly lost on storage in $1 \cdot 10^{-4}$ M mercaptoethanol.

Enzyme assays

PP_i–ATP exchange. The assay system contained 2 mM sodium ATP, 7.5 mM magnesium acetate, 2 mM sodium [^{32}P]pyrophosphate, 100 mM Tris–HCl buffer (pH 8), 1 mM lysine and 0.5 mg serum albumin per ml. Incubation was for 5 min at 37°. The reaction was terminated by adding 1.0 ml of a suspension of acid washed charcoal (5 mg/ml in 1.0 M $HClO_4$, containing 0.1 M sodium pyrophosphate) to 0.1-ml aliquots of the reaction mixture. The charcoal was filtered onto glass fiber discs (Whatman GF/A) held on a Millipore filter holder, washed with water, dried, and counted by liquid scintillation counting.

Aminoacylation of tRNA. The assay system contained 2 mM sodium ATP, 10 mM magnesium acetate, 50 mM Tris–HCl buffer (pH 8). 0.1 mM [^{14}C]lysine 25 μC/

mole, and 20–50 $A_{260\ nm}$ units of tRNA per ml. Incubation was at 25°. Aliquots of 0.05 ml were removed at designated intervals of time, placed on GF/A discs, dipped in cold 10% trichloroacetic acid, then placed in a Gooch crucible and washed with cold 10% and 5% trichloroacetic acid, dried, and counted by liquid scintillation counting. The aminoacylation reaction showed non-linear kinetics similar to those described by MARSHALL AND ZAMECNIK[6]. Enzyme activity was assessed by taking tangents to time curve measured over 10 min.

Gel electrophoresis. The procedure of DAVIS[9] was used for analytical gel electrophoresis. Molecular weight determinations in 0.2% sodium dodecyl sulfate employed the gel electrophoresis system of WEBER AND OSBORN[10], using gels containing half the normal amount of cross linker. The 0.1% sodium dodecyl sulfate system of MARSHALL AND ZAMECNIK[11] was also employed.

Liquid scintillation counting was performed in a Nuclear Chicago Unilux I ambient temperature liquid scintillation counter. The counting efficiency for ^{14}C was 80%. The following toluene based scintillation mixture was used with isotopes prepared on solid media: [4.0 g 2,5-diphenyloxazole, 100 mg *p*-bis-[2-(5-phenyloxazolyl)-1-benzene per l]. Enzyme–substrate complexes were assayed in BRAY'S[12] solution.

Materials. Frozen cells of *E. coli* B, and *E. coli* B tRNA (stripped) were obtained from General Biochemicals Corporation. ^{3}H and ^{14}C isotopically labelled compounds were obtained from New England Nuclear Corporation; and [β,γ-^{32}P]ATP from Schwartz Bioresearch Inc. Dialysis tubing was cleaned by heating at 80° for 1 h in 50 mM sodium EDTA, followed by thorough rinsing with distilled water. Protein concentration was determined by the reaction of LOWRY *et al.*[13] by ultraviolet absorption[14] or by a nephelometric method[15].

RESULTS

Analytical gel electrophoresis and determination of molecular weight

The enzyme preparation obtained from Column II or Column III showed two major bands on analytical gel electrophoresis[9]. Lysyl-tRNA synthetase activity was only associated with the protein eluted from the faster moving of the two bands. The protein eluted from the slower moving of the two bands showed no lysyl-tRNA synthetase or other synthetase activity. Both proteins bound [^{14}C]ATP to the same extent. The slower moving band stained poorly with coomassie blue, and the dye underestimated the amount of protein that could be eluted from the gel. Phenol red stained the two bands equally. Only one band was observed upon gel electrophoresis in a continuous system[16] (0.1 M Tris–HCl, pH 8.5) for 6 h.

Gel filtration on Sephadex G-200 showed that a single symmetrical peak of synthetase activity eluted close to or with the peak of yeast alcohol dehydrogenase activity, suggesting a molecular weight of about 150 000 (Fig. 1a). The protein peak always eluted in the same exclusion volume as the alcohol dehydrogenase marker. This was also true of gel filtration on Column III, where samples taken at different points from the protein peak and submitted to analytical gel electrophoresis showed that no separation of the two major proteins present in the preparation was achieved. We have assumed a molecular of 150 000 in calculating the binding of substrate to enzyme.

Molecular weights for the two proteins were determined by gel electrophoresis

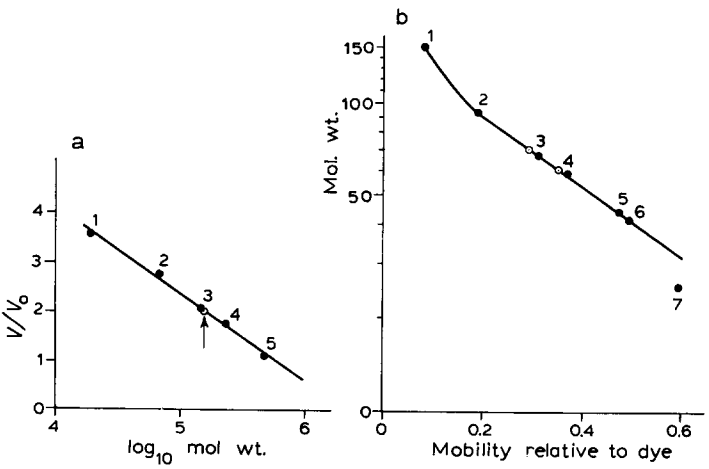

Fig. 1. Determinations of molecular weight for lysyl-tRNA synthetase. (a) Gel filtration on Sephadex G-200 by the procedure of ANDREWS[17]. The column (2.5 cm × 100 cm) was equilibrated at 4° with 0.05 M potassium phosphate buffer (pH 7), containing 1 mM EDTA. The flow rate was 12.5 ml/h, and 5-ml fractions were collected. The marker proteins were 1, myoglobin (mol. wt. 17 500); 2, hemoglobin (68 000); 3, yeast alcohol dehydrogenase (150 000); 4, catalase (244 000); 5, apoferritin (450 000); ○, synthetase assayed by lysine-dependent PP_i–ATP exchange. 5 mg of marker proteins and 1 mg of synthetase proteins were used. The enzyme was run simultaneously with the markers. (b) Gel electrophoresis by the procedure of WEBER AND OSBORN[10]. The marker proteins were 1, thyroglobulin (150 000); 2, phosphorylase a (94 000); 3, bovine serum albumin (68 000); 4, catalase (60 000); 5, ovalbumin (43 000); 6, yeast alcohol dehydrogenase (41 000); 7, chymotrypsin (25 700); ○, synthetase. About 10 μg of the marker proteins and 25 μg of the synthetase protein were used.

in sodium dodecyl sulfate. Values of 72 000 and 62 000 were obtained by the procedure of WEBER AND OSBORNE[10] (Fig. 1b) and 106 000 and 80 000 by the procedure of MARSHALL AND ZAMECNIK[11]. These latter values were essentially the same as those obtained by these investigators for the proteins present in their Fraction II preparation of lysyl-tRNA synthetase, and suggested that we were investigating the same, or a very similar preparation.

Formation and some properties of ATP–enzyme and lysyl-AMP–enzyme complexes

The enzyme preparations bound about 30–60 pmoles of ATP per 100 pmoles enzyme as ATP–enzyme (Figs. 2a, 2b). High concentrations of Mg^{2+} in the incubation mixture prevented the formation of lysyl-AMP–enzyme (Fig. 2a).

Essentially all the added enzyme units were recovered with the ATP–enzyme (Fig. 2a) or with lysyl-AMP–enzyme (unpublished results).

The formation of ATP–enzyme and lysyl-AMP–enzyme was entirely dependent upon the addition of Mg^{2+}. Small amounts of [^{3}H]AMP were bound to the enzyme; equivalent to about 5% of the ATP bound. At pH 7 (100 mM Tris–HCl) the amount of both complexes formed was 75% of that formed at pH 8. 120 mM NH_4Cl depressed the synthesis of ATP–enzyme to about 70% of the control value.

Incubation of enzyme with Mg^{2+} and ATP labelled in the adenine and pyrophosphate moieties with ^{3}H and ^{32}P gave complexes in which both isotopes were present in equimolar amounts. Both isotopes remained with the enzyme in constant ratio throughout the gel filtration procedure (Fig. 2b). That the bound nucleotide

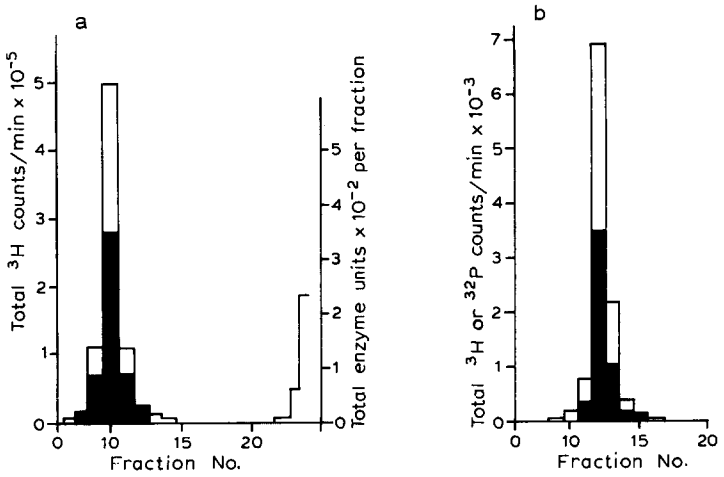

Fig. 2. (a) Formation of ATP–enzyme complex at high Mg^{2+} concentration. 1320 pmoles of enzyme were incubated with 1.4 nmoles [^{3}H]ATP (12.2 C/mmole), 19.6 nmoles [^{14}C]lysine (250 μC/μmole) 21 μmoles magnesium acetate and 70 μmoles Tris–HCl (pH 8) in a volume of 0.7 for 15 min at 4°. The complex was isolated at 4° by gel filtration on a column of Sephadex G-50 (Medium) 2.2 cm × 30 cm equilibrated with 0.05 M potassium phosphate buffer (pH 7), containing 1 mM EDTA. The separation took about 30 min to complete. 4-ml fractions were collected and the radioactivity of 2-ml aliquots was measured in Bray's solution. The few counts observed in the ^{14}C channel could be accounted for by the ^{3}H radioactivity. 53 pmoles of [^{3}H]ATP were bound per 100 pmoles enzyme. 590 enzyme units were added to the incubation mixture and 535 were recovered. Enzyme activity was measured by aminoacylation of tRNA (1 enzyme unit = 1 nmole lysyl-tRNA synthesized per min). Open blocks: total ^{3}H counts/min per fraction, black blocks, total enzyme units per fraction. (b) Formation of [^{3}H] [β,γ-^{32}P]ATP–enzyme complex. 165 pmoles of enzyme were incubated at 4° for 20 min in a volume of 0.1 ml with 0.2 μmole magnesium acetate 10 μmoles Tris–HCl (pH 8) and 1.98 nmoles [^{3}H] [β,γ-^{32}P]ATP magnesium acetate, (2 C/mmole of ^{3}H, 82 700 counts/min of $^{32}PP_i$ nmole). The complex was isolated and the radioactivity counted as for (a). Total counts/min isolated were: $^{32}PP_i$ 5174 (62 pmoles) and [^{3}H]adenine 10 320 (64 pmoles). Open blocks, total counts/min of ^{3}H, black blocks total counts/min of ^{32}P.

was predominantly ATP was demonstrated by adsorption of at least 80% of the ^{32}P label on to acid washed charcoal and by chromatography on a column of DEAE-cellulose.

When the enzyme was incubated with Mg^{2+}, [β,γ^{32}P]ATP and [^{3}H]lysine, ^{32}P label was bound to the enzyme concomitantly with [^{3}H]lysine. About 70% of this bound ^{32}P label was identified as ATP by chromatography on a column of DEAE-cellulose; the remainder of the ^{32}P label was probably present as PP_i.

After storage for over 3 months the capacity of the enzyme preparations to form complexes increased, and the ratio of binding of substrates to enzyme increased to, and exceeded unity. The increases varied considerably, but ratios of up to 2 moles lysine or 4 moles of adenine bound per mole of enzyme were observed.

Synthesis of ATP–enzyme was maximal at a Mg^{2+} concentration of 2 mM and an ATP concentration of 2 μM (Figs. 3a, 4a). At these concentrations the synthesis of lysyl-AMP–enzyme was less than 20% of the maximum (Fig. 4a), and the synthesis of this small fraction of lysyl-AMP–enzyme was relatively uninhibited by Mg^{2+} concentrations up to 10 mM (Fig. 3b). The synthesis of the remaining 80% of lysyl-AMP–enzyme required an ATP concentration of at least 20 μM (Fig. 4a). The synthesis of this fraction of lysyl-AMP–enzyme was inhibited by increasing the concen-

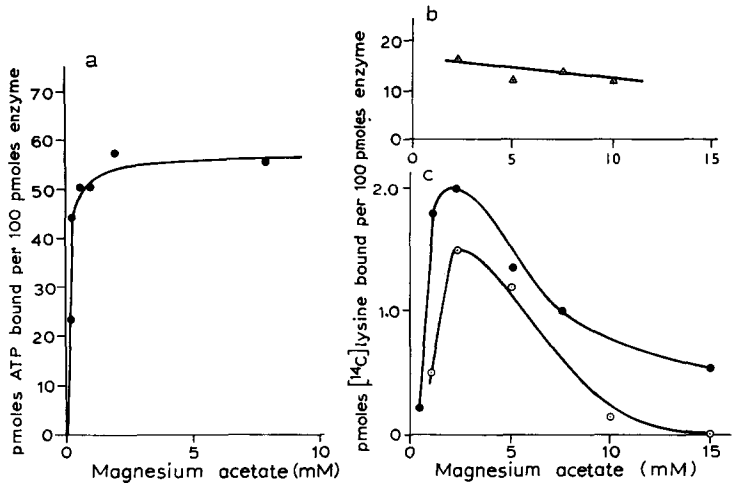

Fig. 3. (a) Effect of Mg^{2+} concentration on the formation of ATP–enzyme. 66 pmoles [^{3}H]ATP (4 C/mmole), 10 μmoles Tris–HCl (pH 8), and varying concentrations of magnesium acetate in a volume of 0.1 ml. The complexes were isolated by gel filtration, and assayed for radioactivity as described for Fig. 2a. (b) Effect of Mg^{2+} concentration on the formation of lysyl-AMP–enzyme at 2 μM ATP. 66 pmoles of enzyme were incubated at 4° for 20 min with 0.2 nmole [^{3}H]ATP (4 C/mmole) 10 μmoles Tris–HCl (pH 8) and varying concentrations of magnesium acetate in a volume of 0.1 ml. The complexes were isolated and counted as described for Fig. 2a. (c) Effect of Mg^{2+} concentration on the formation of lysyl–AMP–enzyme at higher ATP concentrations. 132 pmoles of enzyme were incubated at 4° for 20 min in a volume of 0.1 ml with 2 nmoles [^{14}C]-lysine (250 μC/μmole), 10 μmoles Tris–HCl (pH 8), either 0.4 μmole (●) or 2 nmoles (○) non-labeled ATP, and varying concentrations of magnesium acetate. The complexes were isolated and counted as described for Fig. 2a.

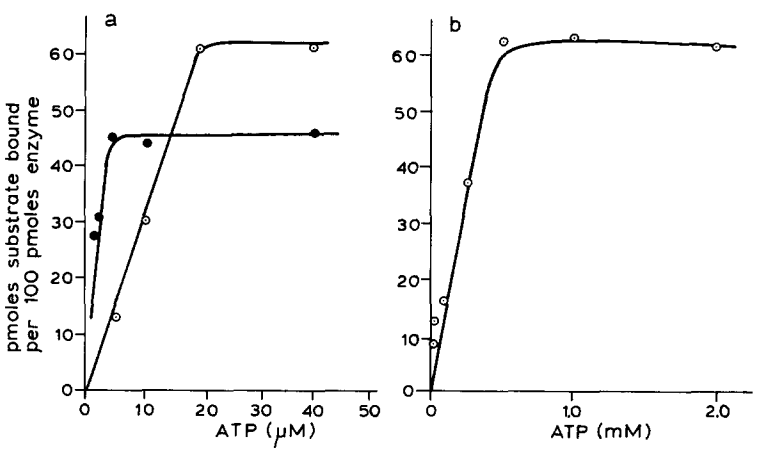

Fig. 4. (a) The effect of ATP concentration on the formation of ATP–enzyme and lysyl-AMP–enzyme at 2 mM Mg^{2+}. 66 pmoles of enzyme were incubated at 4° for 20 min in a volume of 0.1 ml with 0.2 μmole magnesium acetate, 10 μmoles Tris–HCl (pH 8) and varying amounts of [^{3}H]ATP (12.1 C/mmole), or with non-labeled ATP and 2.5 nmoles [^{14}C]lysine (255 μC/μmole). The complexes were isolated by gel filtration and assayed for radioactivity as described for Fig. 2a. The symbols are designated as follows: ●, synthesis of [^{3}H]ATP–enzyme; ○, synthesis of [^{14}C]lysyl-AMP–enzyme. (b) Effect of ATP concentration on the formation of lysyl-AMP–enzyme at 10 mM Mg^{2+}. 660 pmoles enzyme were incubated at 4° for 20 min in a volume of 0.4 ml with 4 μmoles magnesium acetate, 40 μmoles Tris–HCl (pH 8), 10 nmoles [^{14}C]lysine (250 μC/mole) and varying amounts of non-labeled ATP. The complexes were isolated and counted as described for Fig. 2a.

tration of Mg^{2+} above 2 mM. This inhibition was partially reversed by further raising the ATP concentration (Figs. 3c, 4b).

Lysyl-AMP–enzyme was formed at about twice the rate of ATP–enzyme when the synthetase was incubated at 4° with or without the addition of lysine, at the same concentrations of Mg^{2+} and ATP. Maximum synthesis of lysyl-AMP–enzyme at 4° was obtained at concentrations at or above 20 μM lysine.

The stability of the ATP–enzyme complex was determined by re-filtering preformed complex through a column of Sephadex G-50, and re-assaying the radioactivity emerging in the exclusion volume. The complex was stable at 4° for about 2 h and the addition of 2 mM Mg^{2+} did not alter the stability. All the complex disappeared after 8 h at 4°. In the presence of 50 μM nonlabeled lysine about half the bound ATP disassociated from the enzyme in 15 min at 4°.

Lysyl-AMP–enzyme was completely stable for up to 48 h at 4°, as measured by the transfer of [^{14}C]lysine to tRNA.

Reactions of the complexes to give lysyl-tRNA or lysyl-AMP–enzyme

Incubation of ATP–enzyme with tRNA and radioactive lysine led to the synthesis of aminoacyl-tRNA (Table II). The reaction was dependent upon quite high

TABLE II

FORMATION OF LYSYL-tRNA FROM ATP–ENZYME

Expt. 1. ATP–enzyme was prepared by incubating 450 pmoles enzyme with 5.9 nmoles [^{14}C]ATP (357 μC/μmole) and 0.88 μmole magnesium acetate in a volume of 0.22 ml at 4° for 30 min. 4.75 ml containing 125 pmoles of bound ATP was isolated by gel filtration. 0.5-ml samples (13.2 pmoles ATP) were incubated at 37° for 5 min with 10 $A_{260\ nm}$ units of tRNA, 1.25 nmoles of [^{3}H]lysine (1.95 C/nmole) and 10 μmoles magnesium acetate. The incubation with tRNA was started 20 min after the separation of the ATP–enzyme by gel filtration. Incubation was terminated by adding 10 ml cold 10% trichloroacetic acid to precipitate the tRNA. After standing for 15–30 min on ice the precipitate was filtered onto a glass fiber disc (Whatman GF/C) held in a Millipore filter holder, washed with cold trichloroacetic acid, dried, and counted by liquid scintillation counting. *Expt. 2* ATP–enzyme was prepared by incubating 660 pmoles of enzyme with 5.1 nmoles [^{3}H]ATP (2.5 C/mmole) and 4.8 μmoles magnesium acetate in a volume of 0.32 ml at 4° for 25 min. 384 pmoles of bound ATP were isolated in 4 ml by gel filtration. Aliquots of 0.25 ml (24 pmoles ATP) were incubated under conditions similar to that for Expt. 1, using [^{14}C]lysine (50 μC/μmole). The incubation was started 60 min after the separation of the ATP–enzyme by gel filtration. The procedure used for isolating radioactive acid insoluble material was as described for Expt. 1.

	Counts/min in lysyl-tRNA	
	Expt. 1	*Expt. 2*
Complete system (20 mM Mg^{2+})	1530 (3.1 pmoles)	1222 (13.8 pmoles)
10 mM Mg^{2+}	1471	984
5 mM Mg^{2+}	447	
2.5 mM Mg^{2+}	219	
No Mg^{2+}	202	250
Complete system		
with 120 mM NH_4Cl	253	244
with 40 mM NH_4Cl	408	
with 120 mM NaCl	337	
no tRNA	156	190
no ATP–enzyme	202	184
Control, $1 \cdot 10^{-7}$ M ATP	299	

TABLE III

FORMATION OF LYSYL-AMP–ENZYME FROM ATP–ENZYME

Expt. 1. ATP–enzyme was formed by incubating 250 pmoles enzyme with 5 nmoles [^{3}H]ATP (4.0 C/mmole) and 0.5 μmole magnesium acetate in a volume of 0.25 ml at 4° for 20 min. The complex was isolated by gel filtration, and 3.5 ml of the fraction containing the highest level of radioactivity incubated at 4° for 20 min with 70 nmoles [^{14}C]lysine (250 μC/mole) without added magnesium acetate. The lysyl-AMP–enzyme was isolated by gel filtration. Fractions of 4 ml were taken, and 2-ml aliquots counted in Bray's solution. Total radioactivity recovered is given as the sum of the radioactivity in the four fractions with the highest radioactivity. *Expt. 2.* ATP–enzyme was formed by incubating 250 pmoles enzyme with 5 nmoles [^{14}C]ATP (375 μC/μmole) and 0.5 μmole magnesium acetate in a volume of 0.25 ml at 4° for 20 min. The complex was isolated by gel filtration and 3.0 ml of the fraction containing the highest level of radioactivity incubated at 4° for 20 min with 66 nmoles [^{3}H]lysine (2.4 C/mmole) without added magnesium acetate. The lysyl-AMP–enzyme was isolated and estimated as for Expt. 1.

	Expt. 1		*Expt. 2*	
	Counts/min	*pmoles*	*Counts/min*	*pmoles*
Bound adenine added to incubation with lysine	16 850	55	4 384	45
Bound adenine recovered	14 716	29	2 842	30
Bound lysine recovered	2 592	29	11 058	28

concentrations of Mg^{2+}, and was extremely sensitive to inhibition by NH_4^+ and Na^+. The highest concentration of radioactive ATP bound to the enzyme was less than $1 \cdot 10^{-7}$ M. A control experiment in which $1 \cdot 10^{-7}$ M free ATP was used gave no significant synthesis of lysyl-tRNA.

Incubation of ATP–enzyme with radioactive lysine led to the binding of amino acid in amounts equimolar with the bound radioactive adenine (Table II). Only about half the added bound ATP was recovered by recycling on a gel filtration co-

TABLE IV

THE INHIBITION BY Mg^{2+} OF THE FORMATION OF LYSYL-tRNA FROM LYSYL-AMP–ENZYME

Lysyl-AMP–enzyme was prepared by incubating 165 pmoles enzyme with 0.5 μmole non-labeled ATP, 6.5 μmoles [^{14}C]lysine (250 μC/μmole) 25 μmoles Tris–HCl (pH 8) and 0.5 μmole magnesium acetate in a volume of 0.25 ml at 25° for 15 min. The complex was separated by gel filtration, and a 4-ml fraction containing 126 pmoles bound [^{14}C]lysine used in the following experiments. 0.2 ml of lysyl-AMP–enzyme containing 2360 counts/min of [^{14}C]lysine (6.3 pmoles) was incubated at 37° for 4 min in a volume of 0.25 ml with 5 $A_{260\ nm}$ units of tRNA and 40 μmoles NH_4Cl, with or without magnesium acetate as indicated. Incubation was terminated by adding 0.25 mg albumin in 0.05 ml phosphate buffer and then rapidly withdrawing an aliquot of 0.25 ml which was placed on a glass fiber disc (Whatman GF/B). This was soaked in cold 10% trichloroacetic acid, held in a Gooch crucible and washed with cold trichloroacetic acid, dried, and counted by liquid scintillation counting.

	Counts/min in lysyl-tRNA
Complete system, no Mg^{2+}	1450
with 2.5 mM Mg^{2+}	487
with 5.0 mM Mg^{2+}	290
with 20.0 mM Mg^{2+}	190
Omit NH_4Cl, no Mg^{2+}	247
Omit tRNA	102

lumn after incubation of ATP–enzyme with amino acid. Increasing the incubation time to 45 min did not increase the amount of lysine taken up by the enzyme. Both complexes were completely stable during the period of time in which the experiment was performed.

The formation of lysyl-AMP–enzyme did not require the addition of Mg^{2+}. The binding of labeled amino acid was inhibited by 15 mM Mg^{2+}, but was unaltered by 1.5 mM Mg^{2+} or by 120 mM NH_4Cl. When lysyl-AMP–enzyme formed from ATP–enzyme was incubated with tRNA and NH_4Cl very small amounts of radioactive trichloroacetic acid insoluble material were formed, presumably lysyl-tRNA.

Maximum synthesis of lysyl-tRNA from lysyl-AMP–enzyme was dependent upon the addition of NH_4^+, and was inhibited by Mg^{2+} (Table IV) as previously demonstrated by WALDENSTRÖM[5].

The different cations exerted effects upon the rates of Reactions 1 and 2 catalysed by the enzyme in a manner similar to that reported by MARSHALL AND ZAMECNIK[6]. The overall reaction was markedly inhibited by 100 mM NH_4^+ and Na^+. Lysine dependent PP_i–ATP exchange was slightly stimulated or depressed at this concentration of NH_4^+ and Na^+. Control experiments carried out in 0.05 M potassium phosphate buffer (pH 7), showed that none of these effects of monovalent cations could be attributed to the use of Tris–HCl buffer in some of the assay systems.

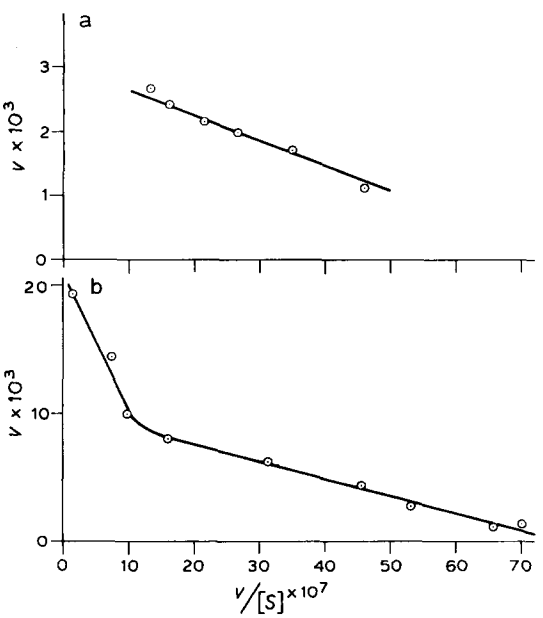

Fig. 5. (a) Relationship between the concentration of lysine and the initial rate of formation of lysyl-tRNA. v = nmoles lysyl-tRNA formed per mg protein per 10 min. The 10-min values were obtained by extrapolation of tangents to time curves as described under METHODS. The assay was performed at a temperature of 25°, with 50 $A_{260\ nm}$ units/ml. (b) Relationship between the concentration of lysine, and the initial rate of exchange of $^{32}PP_i$ into ATP. v = nmoles $^{32}PP_i$ exchanged per mg protein per min. The assay described under METHODS was employed, at a temperature of 37°.

The effect of lysine concentration upon reaction rates

Our estimate for the K_m for lysine measured in the aminoacylation reaction at 25° over the range $2.5 \cdot 10^{-6}$–$20 \cdot 10^{-6}$ M (Fig. 5a) was $4 \cdot 10^{-6}$ M. This is in reasonable agreement with the lower of two values reported by MARSHALL AND ZAMECNIK[6], $2.4 \cdot 10^{-6}$ M, measured at 25° over the range $1.5 \cdot 10^{-6}$–$15 \cdot 10^{-6}$ M lysine. LANSFORD *et al.*[18], using the range $4 \cdot 10^{-6}$–$25 \cdot 10^{-6}$ M lysine, obtained a value of $2 \cdot 10^{-5}$ M at 37° and WALDENSTRÖM[19], working over an undisclosed range at 37°, obtained a value of $1.8 \cdot 10^{-5}$ M.

Using the PP_i–ATP exchange assay at 37° we obtained a biphasic curve when v was plotted against $v/[S]$ (Fig. 5b) for the range $2 \cdot 10^{-6}$–$1 \cdot 10^{-3}$ M lysine, giving K_m values of $1.4 \cdot 10^{-5}$ and $1.04 \cdot 10^{-4}$ M. Two values for v_{max} were calculated, namely 10 500 and 21 000 nmoles of $^{32}PP_i$ exchanged into ATP per mg protein per min. STERN AND MEHLER[20], and LANSFORD *et al.*[18], working with different concentration ranges of lysine at 37°, obtained values of $5.7 \cdot 10^{-7}$ and $5.9 \cdot 10^{-5}$, respectively. Here it appears that the concentration range studied is important in deciding the value of K_m measured. The K_m for ATP in the aminoacylation reaction at 25° was $2 \cdot 10^{-5}$ M over the range $1 \cdot 10^{-5}$–$2 \cdot 10^{-3}$ M and for tRNA $6 \cdot 10^{-6}$ M over the range $1 \cdot 10^{-6}$–$1 \cdot 10^{-5}$ M (assuming 1 nmole of $tRNA^{Lys}$ per 20 $A_{260\ nm}$ units of tRNA.)

DISCUSSION

Leucyl-tRNA synthetase (L-leucine: tRNA ligase (AMP), EC 6.1.1.4) of *E. coli*, B[4] lysyl-tRNA synthetase of yeast[3] and threonyl-tRNA synthetase (L-threonine: tRNA ligase (AMP), EC 6.1.1.3) of rat liver[2,21] and of *E. coli* B[22] have all been reported to form stable ATP–enzyme complexes. These ATP enzyme complexes[3,4,21] formed aminoacyl-AMP–enzyme complex, and aminoacyl-tRNA, reactions which were also catalyzed by our lysyl-tRNA synthetase preparation. ATP–enzyme complex has been thought to participate in the following reaction mechanism, leading to the synthesis of lysyl-tRNA.

$$\text{ATP} + \text{enzyme} \overset{Mg^{2+}}{\rightleftharpoons} \text{ATP–enzyme}$$

$$\text{ATP–enzyme} + \text{lysine} \rightleftharpoons \text{lysyl-AMP–enzyme} + PP_i$$

$$\text{lysyl-AMP–enzyme} + \text{tRNA} \rightleftharpoons \text{lysyl-tRNA} + \text{AMP} + \text{enzyme}$$

Analysis of the effects of monovalent cations and of Mg^{2+} in our enzyme system suggest that in this case the mechanism cannot proceed as outlined above. When the reaction sequence leading to the synthesis of lysyl-tRNA was initiated in the presence of tRNA, with either ATP–enzyme complex or ATP as the energy source, and Mg^{2+} as an obligatory requirement then the rate of synthesis of lysyl-tRNA was markedly depressed by monovalent cations. If the reaction sequence was initiated in the absence of tRNA by first forming lysyl-AMP–enzyme complex, then the synthesis of lysyl-tRNA from this complex was virtually dependent upon the addition of NH_4^+, and was inhibited by Mg^{2+}. The reactions catalyzed by the enzyme in the absence of tRNA were relatively unaffected by NH_4^+ or Mg^{2+}. These results clearly demonstrated that if the reaction sequence leading to the synthesis of lysyl-tRNA were initiated in the presence of tRNA, then the synthesis did not proceed *via* the adenylate mechanism, a circumstance predicted by LOFTFIELD AND EIGNER[23].

Our estimate for the molecular weight of this enzyme, 150 000, obtained by filtration on Sephadex G-200, may represent that of an aggregate formed between the synthetase protein, and the inactive protein present in the enzyme preparation. The value is double an estimate of 78 000 reported by MARSHALL AND ZAMECNIK[11], also obtained by gel filtration. Lysyl-tRNA synthetase of *E. coli* B may have some tendency to form aggregates, since KALOUSEK AND RYCHLÍK[24] isolated a form of the enzyme with a sedimentation coefficient of 12 S also using a gel filtration procedure. It is possible that the inactive protein in our preparation is a molecule of synthetase. Combination of this inactive protein with a charged molecule or a macromolecule (perhaps of non protein nature, such as a polysaccharide) might account for the lower electrophoretic mobility of this protein in discontinuous buffer systems relative to the active synthetase protein. This synthetase may be a polymeric enzyme with a minimum molecular weight of 78 000. Previous estimates of about 100 000 for the molecular weight of this enzyme[19,25] may be slightly too high, as discussed by MARSHALL AND ZAMECNIK[11].

Our preparation of the synthetase may represent a dimeric form of the enzyme with functionally different subunits, but the evidence for this is not conclusive. Two functionally unequal active sites participate in the synthesis of lysyl-AMP–enzyme, having different requirements for ATP and Mg^{2+}. The occurrence of two values for K_m and v_{max} for lysine might indicate the existence of two active sites with different catalytic properties, although the two values for K_m can probably also be explained in terms of a two to one substrate to enzyme interaction at the higher levels of lysine concentration[6]. The lysine induced dissociation of about half the enzyme bound ATP concomitantly with the synthesis of lysyl-AMP–enzyme from the other half of the ATP–enzyme, and the increase in the binding of lysine and ATP adenine to the enzyme on ageing suggests that two subunits might participate in the reaction mechanism in a manner unknown.

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