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The Enzymic Synthesis of Amino Acyl Derivatives of Ribonucleic Acid

II. THE PREPARATION OF LEUCYL-, VALYL-, ISOLEUCYL-, AND METHIONYL RIBONUCLEIC ACID SYNTHETASES FROM *ESCHERICHIA COLI**

FRED H. BERGMANN,† PAUL BERG,‡ AND M. DIECKMANN‡

From the Department of Microbiology, Washington University School of Medicine, St. Louis 10, Missouri

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The enzymic synthesis of amino acyl ribonucleic acid derivatives appears to take place in two steps, both of which are catalyzed by a single enzyme (1–4). The first step involves the reaction of adenosine triphosphate, an amino acid, and an enzyme specific for that amino acid, resulting in the formation of an enzyme-amino acyl adenylate complex and free inorganic pyrophosphate. In the subsequent reaction, the amino acyl moiety is transferred to the 2′- or 3′-hydroxyl group of a terminal adenylic acid in a specific ribonucleic acid chain. The present paper describes the isolation and characterization of the enzymes that catalyze the formation of leucyl-, valyl-, isoleucyl-, and methionyl ribonucleic acid.

EXPERIMENTAL PROCEDURE

Escherichia coli strain B or ML30 was grown in a medium containing 1.1% K_2HPO_4, 0.85% KH_2PO_4, 0.6% Difco yeast extract, and 1% glucose. The cells were grown with vigorous aeration and harvested near the end of the period of exponential growth. The cells were washed with a solution containing 0.5% NaCl and 0.5% KCl, recentrifuged, and stored at −15°.[1] Cells stored for several months in this way did not lose significant amounts of enzymatic activity.

Both the DL- and L-forms of the amino acids used were obtained from the California Foundation for Biochemical Research (CFP grade), or from Nutritional Biochemicals Corporation.

ATP was purchased as the crystalline sodium salt from the Sigma Chemical Company. DEAE-cellulose, type 40, was purchased from The Brown Company, and it was converted to the hydroxyl form before equilibration with the appropriate buffers. Superbrite glass beads, 200 μ average diameter, were obtained from the Minnesota Mining and Manufacturing Company, and they were washed, before use with 1 N HCl and then with water.

P^{32}-labeled sodium pyrophosphate was prepared by heating $NaHP^{32}O_4$ for 1 hour at 400° (5) and after separation from any remaining P^{32} inorganic phosphate by chromatography on a Dowex 1-Cl^- column (6), it was diluted with carrier sodium pyrophosphate to a final specific activity of 10^4 to 10^5 c.p.m. per μmole. Protein determinations were carried out by the method of Lowry *et al.* (7).

Paper chromatographic separation of isoleucine and valine was carried out with a mixture of pyridine, iso-amyl alcohol, and H_2O (35:35:30) (8).

The activity of each of the amino acyl RNA synthetases was assayed by the amino acid-dependent exchange of ATP and PP_i^{32} as described earlier (9). Potassium fluoride (0.01 M), which was not inhibitory to the ATP-PP_i^{32} reaction, was routinely added to the reaction mixtures to inhibit any inorganic pyrophosphatase. One unit of enzyme activity is equivalent to the incorporation of 1 μmole of PP_i^{32} into ATP in 15 minutes. As already pointed out (9), the initial rate of incorporation of PP_i^{32} into ATP was equivalent to the initial rate of amino acyl adenylate formation. Fig. 1 shows that for each assay the rate of the ATP-PP_i^{32} exchange was proportional to the amount of each amino acyl RNA synthetase preparation used. This proportionality was observed in the range of 0.02 to 0.3 μmole of ATP^{32} formation in 15 minutes (0.02 to 0.3 unit).

RESULTS

Crude extracts of *E. coli* catalyze the formation of the amino acyl adenylate derivatives of leucine, valine, isoleucine, and methionine, as well as the formation of the corresponding amino acyl RNA derivatives (10). The same initial fractionation procedures served for the purification of the valyl-, isoleucyl-, and leucyl RNA synthetases, and eventual separation of these activities was achieved by chromatography on a DEAE-cellulose column (11). The methionyl RNA synthetase was obtained by another procedure.

Isolation of Valyl-, Isoleucyl-, and Leucyl RNA Synthetases—Unless otherwise stated, all operations were carried out at 0–5°. A mixture of 100 g wet weight of *E. coli* cells, 100 ml of 0.025 M Tris buffer, pH 8.0, and 300 g of glass beads (200 μ) was stirred in a stainless steel Waring Blendor run at about 90 volts. If the temperature of the mixture approached 5°, stirring was stopped and the material was cooled in an ice bath to 0°. After a total of 15 minutes of stirring, 300 ml of Tris buffer were added, and the mixture was stirred at low speed for an additional 2 to 3 minutes. After the beads had settled, the supernatant fluid was

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† Postdoctoral Research Fellow of the National Institutes of Health, United States Public Health Service; present address, Department of Biochemistry, Brandeis University, Waltham, Massachusetts.

‡ Present address, Department of Biochemistry, Stanford University School of Medicine, Palo Alto, California.

[1] We are deeply grateful to Dr. A. Hanson of the Grain Processing Corporation for his help in obtaining *E. coli* cells grown in this way.

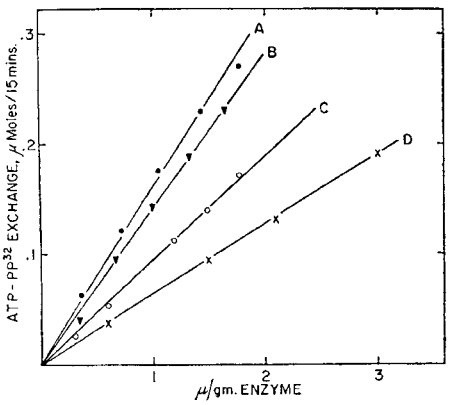

FIG. 1. The rate of ATP-PP_i^{32} exchange as a function of the amount of isoleucyl- (*A*), valyl- (*B*), leucyl- (*C*), and methionyl RNA synthetases (*D*) added. The reaction mixture contained, in a volume of 1.0 ml, 100 μmoles of Tris buffer, pH 8.0; 5 μmoles of $MgCl_2$; 5 μmoles of potassium fluoride; 2 μmoles of ATP; 2 μmoles of P^{32}-sodium pyrophosphate containing 10^4 to 10^5 c.p.m. per μmole; and 2 μmoles of the appropriate amino acid. The incubation was carried out at 37° for 15 minutes and the amount of radioactivity in the ATP was determined as previously described (9).

decanted, and the beads were washed twice with 150 ml aliquots of buffer. The first supernatant fluid and washings were combined and centrifuged at 20,000 × *g* in a Servall SS-1 centrifuge for 1 hour and the precipitate discarded. The total volume of extract was usually 600 to 700 ml.

For each 100 ml of cell extract, 5 ml of 1 M $MnCl_2$ were added with stirring, and after 20 minutes, the mixture was centrifuged for 15 to 20 minutes as described above. To every 100 ml of the supernatant fluid were added 31 g of ammonium sulfate, and after 20 minutes, the mixture was centrifuged at 30,000 × *g* for 1 hour and the precipitate discarded. To the supernatant fluid were added 12 g of ammonium sulfate, and after the mixture was stirred for 20 minutes, it was centrifuged at 30,000 × *g* for 15 minutes. The precipitate obtained from 100 g of cells was dissolved in 60 to 65 ml of 0.02 M phosphate buffer, pH 7.5, and dialyzed for 5 hours against the same buffer. The dialyzed solution was clarified by centrifugation (ammonium sulfate fraction 1).

To every 100 ml of ammonium sulfate Fraction 1 were added 16 ml of 0.5 M potassium phosphate buffer, pH 7.5. The solution was incubated at 30° for 1½ hours, chilled to 0°, and then centrifuged to remove any insoluble material. By this procedure most of the nucleic acid in ammonium sulfate Fraction 1 is converted to an acid-soluble form, facilitating the further purification of the enzymes. The breakdown of the nucleic acids results from the action of a ribonuclease present in the ribosomal particles (12). To each volume of the supernatant fluid were added 0.4 volume of 0.05 M ATP, pH 7.1, 0.2 volume of 0.1 M L-valine, 0.2 volume of 0.1 M $MgCl_2$, 0.14 volume of 0.05 M KF, and 0.06 volume of 0.5 M K_2HPO_4. The mixture was heated at 55° for 45 minutes, and after cooling to 0°, the insoluble material was removed by centrifugation. To each volume of the heated supernatant fluid, 1.6 volumes of ammonium sulfate solution, saturated at 4°, were added with stirring over a 10-minute period. The precipitate was removed by centrifugation at 30,000 × *g* for 10 minutes and dissolved in 20 ml of 0.02 M phosphate buffer, pH 7.5, containing 0.05 M 2-mercaptoethanol. This solution was dialyzed against the same buffer for 2 to 3 hours, centrifuged, and any insoluble material was discarded (ammonium sulfate Fraction 2).

Although the heating procedure was designed to specifically protect the valyl RNA synthetase, about 50% of the activity with isoleucine and leucine remained after this treatment. Because there appeared to be considerable denaturation of protein and therefore some purification of each activity, the procedure was routinely used.

The dialyzed ammonium sulfate Fraction 2 was adsorbed to a DEAE-cellulose column (3.8 cm^2 × 16 cm) which had previously been equilibrated with 0.02 M phosphate buffer, pH 7.5, containing 0.05 M 2-mercaptoethanol. A linear gradient of decreasing pH and increasing phosphate concentration was used to elute the enzymes. The mixing chamber contained 550 ml of 0.02 M phosphate buffer, pH 7.5, whereas the reservoir contained 550 ml of 0.25 M phosphate buffer, pH 6.5; both solvents contained 0.05 M 2-mercaptoethanol. The flow rate was maintained at 50 to 60 ml per hour and fractions of approximately 25 ml were collected. The isoleucyl-, valyl-, and leucyl RNA synthetases were eluted after about 400, 600, and 800 ml of buffer, respectively, had passed through the column (Fig. 2). The major portion of each peak was contained in 75 to 100 ml. The fractions containing the major portion of each peak were pooled and the enzymes were concentrated by addition of solid ammonium sulfate to 0.85 saturation. After 15 minutes, the precipitate was centrifuged at 30,000 × *g* for 1 hour and then dissolved in 2 to 3 ml of 0.02 M phosphate buffer, pH 7.5, containing 10^{-3} M reduced glutathione. The individual enzymes were dialyzed overnight against about 100 volumes of the same buffer, and then against fresh buffer for another 3 to 4 hours. Any precipitate which formed during the dialysis was removed by centrifugation, and the enzymes were stored at −15°.

Isolation of Methionyl RNA Synthetase—Extract from 100 g of *E. coli* was prepared as described above, and then dialyzed overnight against 20 liters of 0.01 M Tris buffer, pH 8.0. For each volume of dialyzed extract, 0.9 volume of 0.05 M Tris buffer, pH 7.5, and 0.1 volume of 2 M potassium phosphate buffer, pH 7.2, were added. This mixture was incubated at 30° for 2.5 to 3 hours, and after it was chilled to 0°, the small amount of precipitate was removed by centrifugation. As previously pointed out, this procedure facilitated the subsequent fractionation steps by depolymerizing the nucleic acids present in the extract. To each 100 ml of this solution were added 21.1 g of ammonium sul-

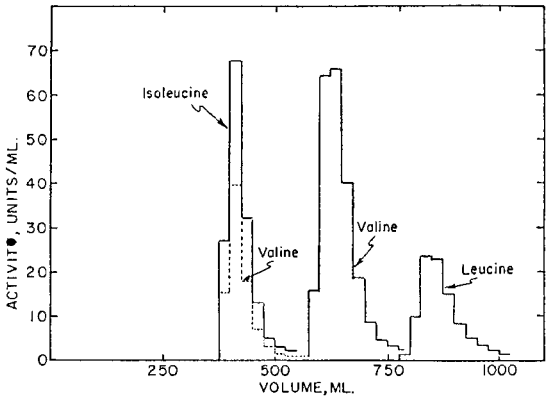

FIG. 2. Chromatographic separation of isoleucyl-, valyl- and leucyl RNA synthetases on a DEAE-cellulose column. See text for complete details.

TABLE I

Purification of valyl-, isoleucyl-, and leucyl RNA synthetases from E. coli

Fractions	Volume	Protein concentration	Valyl RNA synthetase		Isoleucyl RNA synthetase		Leucyl RNA synthetase	
			Total units*	Specific activity	Total units*	Specific activity	Total units*	Specific activity
	ml	*mg/ml*		*units/mg protein*		*units/mg protein*		*units/mg protein*
Crude extract	635	13.4	11,400	1.3	16,600	2.0	22,500	2.7
Ammonium sulfate 1	105	16.4	6,850	3.4	11,550	6.7	12,000	7.0
Ammonium sulfate 2	22	17.1	6,060	16	5,320	14	5,230	14
DEAE-cellulose fractions†			3,420		3,770		2,965	
Valyl RNA synthetase	3.3	5.2	2,850	166				
Isoleucyl RNA synthetase	2.4	7.2			3,120	181		
Leucyl RNA synthetase	2.8	4.5					1,515	120

* Corrected for activity of isoleucyl RNA synthetase with valine. (The true valine-dependent ATP-PP_i^{32} exchange activity is equal to the valine-dependent ATP-PP_i^{32} exchange activity minus 0.55 of the isoleucine-dependent ATP-PP_i^{32} exchange activity.)

† The actual recovery of each enzyme from the column was: valyl RNA synthetase, 4860 units; isoleucyl RNA synthetase, 4680 units; leucyl RNA synthetase, 3875 units. The values shown in the table represent the amounts in the most active fractions which were pooled and used in the ammonium sulfate concentration step.

fate, and after stirring for 10 minutes the solution was centrifuged. To the supernatant fluid were added 6.3 g of ammonium sulfate, and after 10 minutes, the precipitate was removed by centrifugation and dissolved in about 20 ml of 0.02 M potassium phosphate buffer, pH 7.2 ammonium sulfate Fraction 1).

To each 100 ml of ammonium sulfate Fraction 1, 18.3 g of ammonium sulfate and then 6.3 ml of 1.8 M acetic acid were added. After about 5 minutes, the precipitate was removed by centrifugation, and 4.1 g of ammonium sulfate were added to the supernatant fluid; the mixture was again centrifuged, and the precipitate was dissolved in 40 ml of 0.02 M potassium phosphate buffer, pH 6.5 (ammonium sulfate Fraction 2).

To each 100 ml of ammonium sulfate Fraction 2 were added 250 ml of cold water, and then 25 ml of alumina Cγ gel (15 mg dry weight per ml). After 10 minutes, the mixture was centrifuged at 10,000 × *g*, the gel was washed with about 250 ml of cold water, and the washings were discarded. The enzyme was eluted from the gel with 150 ml of 0.1 M potassium phosphate buffer, pH 7.0, and this fraction was dialyzed overnight against 50 to 100 volumes of 0.02 M potassium phosphate buffer, pH 7.5 (Cγ eluate).

Of the dialyzed Cγ eluate fraction, 100 ml were adsorbed to a DEAE-cellulose column (3.2 cm^2 × 7 cm) which had previously been equilibrated with 0.02 M potassium phosphate buffer, pH 7.5. The enzyme was eluted with a linear gradient of increasing ionic strength produced with a mixing chamber with 300 ml of 0.07 M potassium phosphate buffer, pH 7.0, and a reservoir of 300 ml of 0.2 M potassium phosphate buffer, pH 7.0. The flow rate was maintained at 50 to 60 ml per hour, and 100 ml fractions were collected. The major portion of the enzyme was eluted in about 75 ml (Fractions 17 to 25). These fractions were pooled, and the enzyme was concentrated by precipitation with ammonium sulfate as described for the other enzymes. The pellet was dissolved in 5 ml of 0.02 M phosphate buffer, pH 7.5; any insoluble material was removed by centrifugation. The enzyme solution was stored at −15°.

The data relative to the purification of the valyl-, isoleucyl-, leucyl-, and methionyl RNA synthetases are summarized in Tables I and II.

Stability of Purified Enzyme Fractions—Preparations of the most purified valyl- and isoleucyl RNA synthetases were reasonably stable when kept frozen at −15° in the presence of reduced glutathione (10^{-3} M) for periods as long as 3 to 4 months. The loss in activity ranged from 10 to 30%, and these preparations could not be reactivated by the addition of 2-mercaptoethanol, reduced glutathione, or cysteine. In the absence of reduced glutathione, approximately 95% of the initial activity was lost after 1 month, but such preparations could be reactivated to about 50% of the initial level by the addition of 10^{-3} M 2-mercaptoethanol to the assay mixture. The purified preparations of methionyl RNA synthetase showed little or no loss in activity on storage at −15°, and no stimulation of the activity with glutathione or 2-mercaptoethanol was ever observed.

In general, the purified leucyl RNA synthetase preparation was the most labile on storage. After several weeks at −15°, there was a marked dependence of the activity on 2-mercaptoethanol (or glutathione). With some aged and completely inactive preparations up to 90% of the activity could be restored by the addition of the 2-mercaptoethanol. This stimulation of the activity by 2-mercaptoethanol was found with Tris, glycylglycine, β,β-dimethylglutarate, and imidazole buffers, but there was marked inhibition by 2-mercaptoethanol when cacodylate buffer was used.

Requirements for ATP-PP_i^{32} Exchange Reaction—With each of the purified enzymes there was 1% or less of the activity of the complete system if either the amino acid, Mg^{++}, ATP, or the

TABLE II

Purification of methionyl RNA synthetase from E. coli

Fraction	Volume	Protein concentration	Total units	Specific activity
	ml	*mg/ml*		*units/mg protein*
Dialyzed crude extract	425	13.7	7245	1.2
Ammonium sulfate 1	172	6.2	3870	3.6
Ammonium sulfate 2	72	5.7	2304	5.6
Cγ gel eluate	100	1.4	1710	12.2
Methionyl RNA synthetase	5	3.0	1363	91

enzyme was omitted. The addition of 2-mercaptoethanol to the reaction mixture usually had a stimulatory effect. Leucyl RNA formation showed the greatest effect being activated 3 to 5 times in the presence of 2-mercaptoethanol. In the case of the valyl and isoleucyl RNA synthetases, the activation was between 1.5 and 3 times depending upon the age of the preparation.

Effect of pH and Type of Buffer on Rate of ATP-PP_i^{32} Exchange—Although each of the isolated enzymes displayed little variation in activity in the pH range of 7 to 9 there were marked differences with various buffers. For example, with the valyl RNA synthetase, maximal activity with Tris or glycine buffers was observed between pH 8.5 to 9.0, whereas with cacodylate or glycylglycine buffer, the maximal rate, which was about 10% higher than those found with Tris and glycine buffer, was found between pH 7.0 and 8.0.

In the case of the isoleucyl RNA synthetase, the activity was maximal at pH 8.5 (glycine or Tris buffers). There was approximately 50% of the activity at pH 9.5 (glycine buffer), whereas at pH 6.6 (cacodylate buffer) there was 55% of the maximal activity.

With the leucyl RNA synthetase there was little difference in the activity in the pH range of 6 to 9 with Tris, glycine, glycylglycine, or β,β-dimethylglutarate buffers.

The methionyl RNA synthetase had the same activity between pH 6.0 and 8.8 and was approximately 40 and 30% as active at pH 5.3 (cacodylate buffer) and pH 9.3 (glycine buffer), respectively.

Determination of K_m Values for ATP and Amino Acids—The K_m values for each of the amino acids were estimated by the Lineweaver-Burk (13) plot from data obtained in the standard ATP-PP_i^{32} exchange assay (Fig. 3). These are: L-isoleucine, 5×10^{-6} M; L-valine, 1×10^{-4} M; L-leucine, 5.6×10^{-5} M; and L-methionine, 2.4×10^{-5} M.

The K_m values for ATP in the formation of valyl-, leucyl-, and methionyl RNA under the conditions already described (2) are 2.3×10^{-5} M, 1.3×10^{-4} M, and 8.5×10^{-6} M, respectively.

Specificity of Isolated Amino Acyl RNA Synthetases in Formation of Amino Acyl Adenylates

(*A*) *Naturally Occurring Amino Acids*—Table III shows the results of experiments in which each of the isolated enzyme preparations was tested for its ability to catalyze the ATP-PP_i^{32} exchange reaction in the presence of the different naturally occurring amino acids. Aside from the reaction of L-valine with the isoleucyl RNA synthetase, and L-threonine with the valyl RNA synthetase, both of which will be discussed subsequently, each of the enzymes was found to show high specificity for a single amino acid. It should be pointed out that in certain instances anomalous results were obtained with certain commercially obtained samples of L-amino acids. For example, several samples of L-leucine gave significant amounts of exchange with the methionyl RNA synthetase ranging from 10 to 50% of that found with L-methionine, whereas other samples of the same amino acid were inert. Similar observations were made with samples of L-isoleucine and L-valine with the leucyl RNA synthetase. We believe that such anomalous results are due to contamination of these L-amino acid preparations by other L-amino acids. Since the K_m values for the amino acids by their respective enzymes (see above) were relatively low and because each amino acid preparation was routinely tested at a concentration of 2×10^{-3} M (with respect to the L-isomer), it is clear, for example, that an impurity of 1% of L-methionine in the L-leucine preparation or of L-leucine in the L-isoleucine and L-valine preparations would be sufficient to account for a significant exchange reaction. Moreover, since the isotope exchange is not accompanied by any net change in concentrations of the reactants, the trace impurity functions catalytically. Support for this conclusion comes from the fact that synthetically prepared DL-amino acids were found to be uniformly inactive. This was not due to inhibition by the D-isomer since mixing equal amounts of the D-amino acid with the active L-amino acid samples gave no decrease in the rate of reaction. These findings are being stressed inasmuch as several investigators have tested the spec-

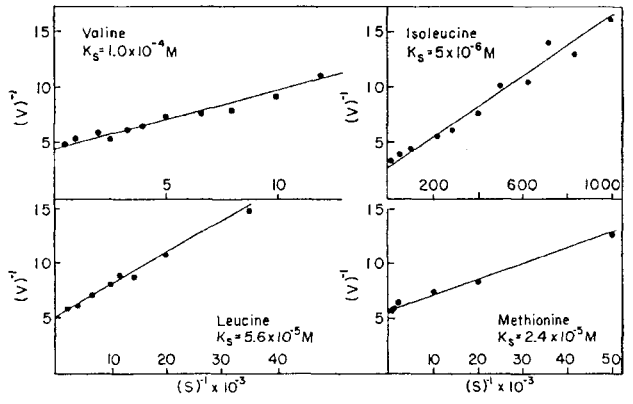

FIG. 3. Determination of Michaelis constants for valine, isoleucine, leucine and methionine in the formation of the corresponding amino acyl adenylate by the appropriate amino acyl RNA synthetase.

TABLE III

Specificity of valyl-, isoleucyl-, leucyl-, and methionyl RNA synthetases for amino acyl adenylate formation

The results have been expressed with the specifically activated amino acid as 100 and all others relative to that figure, so that results with different preparations carried out at different times could be tabulated. Each amino acid was tested under the conditions already described at a final concentration of L-amino acid of 2×10^{-3} M. The results with DL- or L-amino acids were the same except for certain instances noted in the text.

Amino acid tested*	Valyl RNA synthetase	Isoleucyl RNA synthetase	Leucyl RNA synthetase	Methionyl RNA synthetase
L-Valine	100	44–56	4	<1
D-Valine	<1	<1		
L-Isoleucine	1	100	<1	<1
D-Isoleucine		<1		
L-Leucine	<1	2–5	100	2
D-Leucine			<1	
L-Methionine	<1	5–10	2	100
L-Threonine	28	<1	<1	<1

* Experiments not included in the table with the L-isomers of phenylalanine, tyrosine, tryptophan, lysine, serine, alanine, arginine, histidine, cysteine, and glycine with each of the enzymes shown gave values less than 5% of that found with the appropriate amino acid. L-Proline, L-glutamic, and L-aspartic acids were tested only with the methionyl RNA synthetase and found to have less than 1% the activity of L-methionine.

ificity of these enzymes by the ATP-PP_i^{32} exchange reaction (14, 15) with commercially obtained L-amino acid preparations.

(B) Evidence for Formation of L-Valyl Adenylate by L-Isoleucyl RNA Synthetase—During the analysis of the enzyme fractions eluted from the DEAE-cellulose column, two separate peaks of valyl adenylate-forming activity were found (Fig. 2). One of these (*Peak 1*) was found to react with L-isoleucine as well as L-valine. Moreover, the ratio of activity with isoleucine and valine was constant (1.72 to 1.82) in all fractions of the peak (see Fig. 2). The material in this peak accounted for over 85% of the isoleucine-dependent ATP-PP_i^{32} exchange activity of ammonium sulfate Fraction 2 and about 30% of the valine-dependent activity. The finding of two separable activities for valyl adenylate formation and the constant ratio of activity with isoleucine and valine in the first peak suggested the existence of a single enzyme capable of forming both isoleucyl- and valyl adenylate. The following lines of evidence support this hypothesis.

1. The activity observed in the presence of equimolar but saturating amounts of isoleucine and valine are not additive but rather equal to the activity with isoleucine alone. This would be predicted on the basis of the 100-fold difference in K_m values for the two amino acids as mentioned below.

2. The ratio of activities with isoleucine and valine remains constant during inactivation and reactivation of the enzyme. In one preparation in which 2-mercaptoethanol was omitted from the eluting solutions, approximately 80% of the activity was lost whether tested with isoleucine or with valine as substrate. Moreover, incubation of the partially inactivated enzyme preparation with 2-mercaptoethanol resulted in equal restoration of the two activities.

3. The possibility of contamination of the valine samples with a trace of isoleucine as the basis for the observed activity with valine can be eliminated. With several different samples of L-valine or DL-valine, the ratio of the two activities (with isoleucine and valine) did not vary significantly. When a mixture of L-isoleucine and L-valine was chromatographed with a solvent which separates the two amino acids (8), the material recovered from successive 0.5-cm strips cut perpendicularly to the line of solvent flow gave two separate peaks of activity when tested in lieu of the amino acid in the standard assay system. The position of these two peaks corresponded to that for the isoleucine and valine.

4. As pointed out in the previous paper (2), although this enzyme produces both isoleucyl- and valyl adenylates, it catalyzes the formation of only isoleucyl RNA. Nevertheless, L-valine competitively inhibits the reaction linking L-isoleucine to RNA, and the K_i for this inhibition is 3.8×10^{-4} M, whereas the K_m for valyl adenylate formation under the same conditions is 3.9×10^{-4} M.

These experiments suggest that whereas the isoleucyl RNA synthetase can form both L-valyl- and L-isoleucyl adenylates, the enzyme differentiates between the two amino acyl adenylate derivatives in the subsequent transfer of the amino acid residue to RNA.

(C) Utilization of Analogues of Valine by Valyl RNA Synthetase—The purified valyl RNA synthetase preparation shows some activity in the ATP-PP_i^{32} exchange reaction with amino acid derivatives structurally related to valine (Table IV). DL-Threonine, which differs from DL-valine in the replacement of a methyl group by an hydroxyl group, has a 100-fold greater K_m (based on the L-isomer) than valine and the maximal rate is 30% of that found with DL-valine. DL-Allothreonine, in which the hydroxyl group is of opposite configuration to that of DL-threonine, is inert. DL-α-Amino-β-chlorobutyrate, in which the chlorine atom has the same configuration as the hydroxyl group of threonine, is at saturating concentrations as active as DL-valine, although the K_m is slightly higher. DL-Allo-α-amino-β-chlorobutyrate, which corresponds to DL-allothreonine, is only 15% as active as DL-threonine.

TABLE IV

Activity of analogues of DL-valine with valyl RNA synthetase

All derivatives were tested under the conditions described under "Experimental Procedure," except that the substrate concentrations were varied to determine the K_m values.

Substrate	K_m*	V_{max}
	M	%
DL-Valine	1×10^{-4}	100
DL-Threonine	1.2×10^{-2}	30
DL-Allothreonine		0
DL-α-Amino-β-chlorobutyrate	3.3×10^{-4}	100
DL-Allo-α-amino-β-chlorobutyrate	1×10^{-3}	15
DL-α-Aminobutyrate	3.7×10^{-3}	30
DL-α-Aminoisobutyrate		0
DL-β,β-Dimethylcysteine		0

* The K_m values are calculated in each case for the L-isomer.

It is interesting to note that the DL-α-amino-β-chlorobutyrate analogue of DL-threonine is a strong inhibitor of valine incorporation by animal cells *in vitro* whereas the DL-allo-α-amino-β-chlorobutyrate is only 20% as active.[2] Substitution of one of the methyl groups of DL-valine by hydrogen, as in DL-α-aminobutyrate, results in an increase of the K_m and a decrease in the V_{max}. DL-α-Aminoisobutyrate (norvaline) and DL-β,β-dimethylcysteine (penicillamine) are inert. These data show that the valyl RNA synthetase can differentiate between closely related structures in the formation of the enzyme-amino acyl adenylate complexes. Further studies are required to evaluate whether the analogues which are converted to the adenylate derivatives are transferred to the valine-specific acceptor RNA chains.

DISCUSSION

It is now generally accepted that the mechanism of the amino acid-dependent exchange of ATP and PP_i^{32} is explained by the reversible formation of an enzyme-bound amino acyl adenylate. To date, enzymes highly specific for the formation of L-methionyl- (9), L-tryptophanyl- (15), L-tyrosyl- (16), L-alanyl- (17), L-threonyl- (4), L-seryl- (18), D-alanyl- (19), L-valyl-, L-isoleucyl-, and L-leucyl adenylates have been isolated. A closer inspection of several of these enzymes revealed that they catalyze not only the formation of a specific amino acyl adenylate but also the synthesis of the corresponding amino acyl RNA derivative (2–4, 17, 19, 20), and therefore may be considered as amino acyl RNA synthetases. Although each enzyme acts stoichiometrically in the formation of the enzyme-amino acyl adenylate complex, it appears to be regenerated during the subsequent synthesis of the amino acyl RNA compound (2).

[2] Private communication from Dr. M. Rabinovitz of the National Institutes of Health. We are also grateful to him for the generous gift of the DL-amino-β-chlorobutyrate derivatives.

The present work has revealed a somewhat anomalous situation with respect to the isoleucyl- and valyl RNA synthetases. In the former case, L-valine is utilized at slightly less than one-half the rate of isoleucine, and in the latter, L-threonine reacts at about one-third the rate of L-valine. In both instances, however, the K_m of the less active amino acid is about 100 times higher than that of the "natural" substrate. It seems reasonable to suppose, therefore, that at conditions *in vivo* there is little competition between amino acids for the appropriate enzyme.

Of further interest is the finding that the isoleucyl RNA synthetase will not form valyl RNA even though it makes the enzyme-valyl adenylate (2). It is not yet clear, however, whether such discrimination in the second reaction occurs with other synthetases which show low level activity with "unnatural" amino acids and what the basis for this discrimination may be.

It might be pointed out that purified amino acyl RNA synthetases provide a relatively simple and specific tool for measuring the concentrations of a given L-amino acid in the presence of its D-analogue or in the presence of a mixture of amino acid. The relatively low K_m values for the amino acids in the ATP-PP_i^{32} exchange reaction (10^{-4} to 5×10^{-6} M) makes it possible to measure amino acid concentrations in this range. For example, Stevens *et al.* (21), using the methionyl RNA synthetase from yeast, have measured the biosynthesis of L-methionine by liver extracts.

SUMMARY

Amino acyl ribonucleic acid synthetases, relatively specific for either L-valine, L-leucine, L-isoleucine, or L-methionine, have been purified from extracts of *Escherichia coli*. The following statements summarize some of the pertinent findings made with the various synthetase preparations.

1. The purified valyl-, isoleucyl-, and leucyl RNA synthetases are relatively unstable in the absence of a sulfhydryl-containing compound, whereas the methionyl ribonucleic acid synthetase preparation shows no sulfhydryl requirement.

2. The K_m values of 1×10^{-4} M (L-valine), 5×10^{-6} M (isoleucine), 5.6×10^{-5} M (L-leucine), 2.4×10^{-5} M (L-methionine) were found with the respective enzymes in the formation of the amino acyl adenylates.

3. Specificity studies show that although the leucyl- and methionyl ribonucleic acid synthetases are highly specific for a single naturally occurring amino acid, isoleucyl ribonucleic acid synthetase forms L-valyl adenylate at about one half the rate of L-isoleucyl adenylate formation, and valyl ribonucleic acid synthetase utilizes threonine at about 30% the rate of valine. Nevertheless, valine is not converted to valyl ribonucleic acid by the isoleucyl ribonucleic acid synthetase.

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