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Physical and Biochemical Characterization of a Purified Arginyl-tRNA Synthetase–Lysyl-tRNA Synthetase Complex from Rat Liver†

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ABSTRACT: Arginyl- and lysyl-tRNA synthetases copurify throughout a six-step chromatographic procedure resulting in a purification of 605- and 559-fold, respectively. The purified enzymes were estimated to be 98% pure with a stoichiometry of 1:1 from acrylamide gel electrophoresis under denaturing conditions. On the basis of a native molecular weight of 285 000 calculated from $s_{20,w}$, R_s, and $\bar{v}$ and subunit molecular weights of 73 000 and 65 000 obtained by sodium dodecyl sulfate gel electrophoresis, the synthetases appear to exist as a tetramer. The tetrameric structure was also supported by cross-linking studies. These results are consistent with an $\alpha_2\beta_2$ structure, but an $\alpha\beta$ structure has not been ruled out.

In general, aminoacyl-tRNA synthetases from mammalian cells have been isolated as partially purified complexes (Bandyopadhyay & Deutscher, 1971; Vennegoor & Bloemendal, 1972; Som & Hardesty, 1975; Denny, 1977; Dang & Yang, 1979; Saxholm & Pitot, 1979) and as multiple forms (Dang & Yang, 1979; Ussery et al., 1977; Roberts & Olsen, 1976). At the present time it is not known if these complexes exist within the cell, nor is the structural basis for these complexes known. Thus, for these questions to be answered, the complexes must be purified to homogeneity so that physical and biochemical studies can be performed.

Accordingly, we have purified a complex which contains only arginyl- and lysyl-tRNA synthetase. Our results suggest that the synthetases exist as a tetramer with a molecular weight of 285 000. Also kinetic studies have been performed on the synthetases. Studies such as these on small complexes are necessary in order to understand larger complexes and the organization of aminoacyl-tRNA synthetases within the mammalian cell.

Experimental Procedures

Materials. Female Long Evans rats (120–150 g) were purchased from Charles River Breeding Laboratories, Inc. All radioisotopes were purchased from Schwarz/Mann. Dimethyl suberimidate dihydrochloride was purchased from Pierce Chemical Co. All other chemicals and supplies were of analytical grade and were obtained from standard chemical sources.

Aminoacyl-tRNA Synthetase Assays. Assays for both arginyl- and lysyl-tRNA synthetase have been optimized and are as follows. Arginyl-tRNA synthetase assays were performed in a volume of 100 μL containing 25 μmol of Hepes,[1] pH 7.0, 0.5 μmol of ATP, 3.5 μmol of $MgCl_2$, 10 μg of bovine serum albumin, >550 μg of unfractionated beef liver tRNA, and 0.025 μmol of [^{14}C]arginine (8–12 cpm/pmol). The reactions were initiated by the addition of the enzyme; after incubation at 37 °C, they were terminated with 3 mL of cold 10% trichloroacetic acid (Cl_3CCOOH) containing 0.02 M sodium pyrophosphate. After 10 min on ice the precipitate was collected on a Whatman R/A glass-fiber filter, washed 6 times with 3-mL portions of 2.5% Cl_3CCOOH containing 0.2 M sodium pyrophosphate and washed 1 time with 5 mL of ethanol–ether (1:1), dried, and counted in a Beckman liquid scintillation counter. A unit is defined as 1 nmol of arginine incorporated/min at 37 °C.

Lysyl-tRNA synthetase assays were performed in a volume of 100 μL containing 25 μmol of Hepes, pH 8.0, 0.5 μmol of ATP, 0.5 μmol of $MgCl_2$, 10 μg of bovine serum albumin, 650 μg of unfractionated beef liver tRNA, 0.02 μmol of EDTA, 0.5 μmol of KCl, and 0.025 μmol of [^{14}C]lysine (8–12

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[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate.

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cpm/pmol). The assay was performed as described above for arginyl-tRNA synthetase.

Preparation of Blue Sepharose. The Blue Sepharose chromatographic material was synthesized according to the method of Ryan & Vestlig (1974). CNBr-activated Sepharose 4B beads were swollen in 1 mM HCl and then collected on Whatman filter paper by aspiration. The beads were then slowly stirred at 4 °C for 18 h in 50 mL of a 400 mM sodium carbonate buffer (pH 10) containing 1 g of Blue Dextran. The Blue Sepharose beads were then collected by aspiration on Whatman filter paper and washed extensively with 1 M KCl. Blue dye was visibly removed only during the first use of the beads.

Isolation of Multiple Forms of Arginyl- and Lysyl-tRNA Synthetases. To isolate the multiple forms of aminoacyl-tRNA synthetase from fresh or frozen rat liver, we minced the liver in 5–10-g portions and homogenized it with 2 volumes of medium containing 0.02 M K_2HPO_4 (pH 7.0), 8 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.2 mM phenylmethanesulfonyl fluoride (PMSF), and 10% glycerol (final volume). Homogenization consisted of six up-to-down strokes on a motor-driven Teflon homogenizer. The homogenate was centrifuged at 12000*g* for 15 min to remove cellular debris.

Purification of Arginyl- and Lysyl-tRNA Synthetases. Unless otherwise stated all steps were performed at 4 °C. Rats were sacrificed by decapitation and the livers were removed, blotted, and weighed (80–100 g of liver). After weighing, the livers were minced in 2 volumes of buffer I, which contained 20 mM K_2HPO_4 (pH 7.0), 10 mM β-mercaptoethanol, 8 mM $MgCl_2$, 0.2 mM PMSF, and 10% glycerol (final volume), and homogenized by six strokes with a Teflon homogenizer. The homogenate was then centrifuged at 27000*g* for 30 min. This supernatant is referred to as the crude extract or fraction I in Table I.

ω-Aminohexylagarose resin was prepared as previously described (Hilderman, 1977). Crude extract was applied to the ω-aminohexylagarose column (5 × 23 cm). This column was run at room temperature with a flow rate of 75 mL/h. After the crude extract was loaded on the column the column was washed with buffer I until the A_{280} fell below 0.1, at which time the synthetases were eluted off the column by 0.5 M NaCl in buffer I. Fractions of 10 mL were collected and assayed as described with 5 μL of enzyme. Fractions containing arginyl- and lysyl-tRNA synthetase activities of at least 80 pmol were pooled (fraction II).

Fraction II was dialyzed against 4 L overnight with two changes of buffer II, which contained 20 mM K_2HPO_4 (pH 6.5), 8 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.2 mM PMSF, 10% glycerol (final volume), and 0.1% Nonidet P-40 (v/v). Fraction II was applied to a DEAE-cellulose column (5 × 20 cm) equilibrated with buffer II and eluted with a 4-L linear gradient containing buffer II and buffer II at pH 7.0 containing 0.5 M NaCl. Fractions of 15 mL were collected. The column had a flow rate of 100 mL/h. Five microliters of enzyme was assayed as described. Fractions containing at least 80 pmol were pooled (fraction III).

Fraction III was dialyzed overnight against 4 L of buffer III, which contained 125 mM K_2HPO_4 (pH 7.5), 10 mM β-mercaptoethanol, 0.2 mM PMSF, 10% glycerol (final volume), and 0.1% Nonidet P-40 (v/v), with two changes. Fraction III was applied to a hydroxylapatite column (5 × 9 cm) equilibrated with buffer III, and the column was washed with buffer III until the A_{280} fell below 0.1. The enzymes were then eluted at a flow rate of 60 mL/h by a continuous gradient of 300 mL of buffer III and 300 mL of buffer III plus 400 mM K_2HPO_4. Fractions of 11 mL were collected, and 10 μL of enzyme was assayed as described. Fractions containing at least 80 pmol of activity were pooled (fraction IV).

Fraction IV was dialyzed against 2 L of buffer IV, which contained 20 mM K_2HPO_4 (pH 7.5), 10 mM β-mercaptoethanol, 0.2 mM PMSF, 10% glycerol (final volume), and 0.1% Nonidet P-40 (v/v), overnight with two changes.

Fraction IV was applied to a phosphocellulose column (2.5 × 30 cm) equilibrated with buffer IV, and the column was washed with buffer IV until the A_{280} fell below 0.1. The synthetases were eluted off the column with a linear gradient of 200 mL of buffer IV and 200 mL of buffer IV plus 400 mM K_2HPO_4 (pH 7.5) at a flow rate of 60 mL/h. Fractions of 6 mL were collected and 10 μL of enzyme was assayed for arginyl- and lysyl-tRNA synthetase activity. Fractions containing at least 60 pmol of activity were pooled (fraction V).

Fraction V was dialyzed overnight against 4 L of buffer V, which contained 10 mM Tris-HCl (pH 7.0), 10 mM β-mercaptoethanol, 12 mM $MgCl_2$, 0.2 mM PMSF, 10% glycerol (final volume), and 0.1% Nonidet P-40 (v/v), with two changes. Fraction V was applied to a Blue Sepharose column (0.9 × 10 cm) and washed with 4 bed volumes of buffer V. The synthetases were eluted off the column with buffer V containing 0.5 M NaCl at a flow rate of 40 mL/h. Fractions of 6 mL were collected and 5 μL of enzyme was assayed as described. Fractions containing greater than 80 pmol of synthetase activity were pooled and concentrated 3-fold by Amicon ultrafiltration with a YM-30 membrane under nitrogen (fraction VI). Greater than 90% of the enzyme activity was recovered after concentration.

Fraction VI was applied to a Sephadex G-200 column (1.5 × 83 cm) that had been equilibrated with buffer V. Fractions of 6 mL were collected at a flow rate of 3.5 mL/h, and 5 μL of enzyme was assayed as described. Only the three peak tubes of arginyl- and lysyl-tRNA synthetase activity were pooled and concentrated 6-fold by Amicon ultrafiltration with a YM-30 membrane under nitrogen (fraction VII). Greater than 90% of the enzyme activity was recovered after concentration.

Electrophoresis. Electrophoresis in the presence of sodium dodecyl sulfate ($NaDodSO_4$) was performed according to the method of Laemmli (1970). A slab of 0.75-mm thickness with a 9% polyacrylamide resolving gel was used in all cases. The samples (15–50 μg) in denaturing buffer were heated at 100 °C for 2 min before loading. A constant voltage of 90 V was applied until the sample entered the resolving gel, and the voltage was then increased and kept at 180 V for about 2 h. The gels were fixed in a methanol–acetic acid–H_2O (1.5:1:17.5 v/v) solution overnight. The protein bands were stained by Coomassie Brilliant Blue G-250 and scanned at 580 nm.

Isoelectric Focusing. Isoelectric focusing was performed in a 110-mL apparatus. The support matrix was a sucrose gradient from 5% to 50% and the ampholytes were from pH 5 to pH 8 at a concentration of 2.7%. Fraction VII (1.0 mg) was added in the middle of the gradient and the current run until the current fell below 2 mA. This took about 18 h. Fractions of 1 mL were collected and the tubes containing protein were dialyzed against buffer I overnight. Ten microliter samples were assayed for synthetase activity as described.

Glycerol Gradient Centrifugation. Samples from fraction VII were used in this experiment. The sedimentation coefficient was determined according to Martin & Ames (1961) in a Beckman SW 41 rotor at 35000 rpm for 20 h at 4 °C. β-Galactosidase (16 s) and catalase (11.3 s) were used as

standards. The gradient (10–30% glycerol) contained 10 mM Tris-HCl (pH 7.0), 10 mM β-mercaptoethanol, 12 mM $MgCl_2$, 0.2 mM PMSF, and 0.1% Nonidet P-40. Samples of 200 μL of enzyme were layered onto the gradients. Fractions of 0.4 mL were collected and 25 μL of enzyme was assayed as described. Greater than 90% of the activity applied to the gradient was recovered.

Molecular Weight of Native Arginyl- and Lysyl-tRNA Synthetase. The molecular weight was obtained from the equation:

$$M_r = 6\pi\eta N R_s S/(1 - \bar{v}\rho)$$

where η, the viscosity, is 0.01 P, N, Avogadro's number, is 6.023×10^{23} mol^{-1}, R_s, Stokes radius, is 58 Å (determined by gel filtration with known standards: Blue Dextran, thyroglobulin, catalase, aldolase, ovalbumin, and ribonuclease A), S, the sedimentation coefficient, is 12.1 (determined by sedimentation experiments relative to catalase and β-galactosidase), $\bar{v}$, the partial specific volume, is 0.72 cm^3/g (calculated from amino acid and carbohydrate composition), and ρ, the density of water, is 1.00 g/cm^3. Values of f, f_0, and D were calculated from the relationships:

$$f = 6\pi\eta R_s$$

$$f_0 = 6\pi\eta(3M_r\bar{v}/4\pi N)^{1/3}$$

$$D = kT/f$$

where $k = 1.38 \times 10^{-16}$ erg/deg and $T = 277$ K.

Kinetic Studies. The apparent K_m values for the aminoacylation of arginyl- and lysyl-tRNA were determined from initial velocity measurements. These values were determined by varying the concentration of one substrate while the other two substrates were fixed at saturating amounts under optimal synthetase assay conditions. In determination of the kinetic parameters four to six time points per enzyme preparation were taken in the linear velocity range. The apparent K_m values reported in this paper are an average obtained from four different enzyme preparations by three different methods: a weighted linear least squares of the Lineweaver–Burk plot (Wilkinson, 1961), the method of Eisenthal & Cornish-Bowden (1974), and from $S_{1/2}$ values of Hill plots (Hill, 1925).

Since both arginyl- and lysyl-tRNA synthetases were very unstable after Blue Sepharose chromatography, all kinetic experiments were performed with enzymes isolated from the phosphocellulose chromatography step of the purification procedure. The enzyme preparations were stored in 0.1 M Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol, 0.2 mM PMSF, 0.1% Nonidet P-40, and 50% glycerol (final volume). The synthetases can be stored at −10 °C for at least 6 months without loss of activity. Since the enzyme preparation used in the kinetic studies was not homogeneous, it was necessary to demonstrate that there was no RNase activity, ATPase activity, pyrophosphate activity, or an enzymatic activity that degrades arginine or lysine. Also, assay of the enzyme preparation for other aminoacyl-tRNA synthetases using the assay procedures discussed above for arginyl- and lysyl-tRNA synthetases and the procedure described by Bandyopadhyay & Deutscher (1971) demonstrated it to be free (0.3% relative to arginine and lysine) of activity for Ala, Val, Leu, Ile, Ser, Thr, Phe, Tyr, Lys, Met, His, Asp, and Glu. Proteins were determined by the method of Lowry et al. (1951).

Results

Separation of Multiple Forms of Arginyl- and Lysyl-tRNA Synthetases. Multiple forms of arginyl- and lysyl-tRNA synthetases can be demonstrated by applying a crude extract of rat liver to a Sepharose 6B gel-filtration column as shown in Figure 1. Figure 1 demonstrated that both arginyl- and lysyl-tRNA synthetases exist in two forms: a large molecular weight form of 1.5×10^6 and a small molecular weight form in the range of 150 000. It is not unusual to isolate multiple molecular weight forms of the same synthetase from mammalian cells (Dang & Yang, 1979; Roberts & Olsen, 1976; Ussery et al., 1977). In this communication the small molecular weight form of the synthetases was purified.

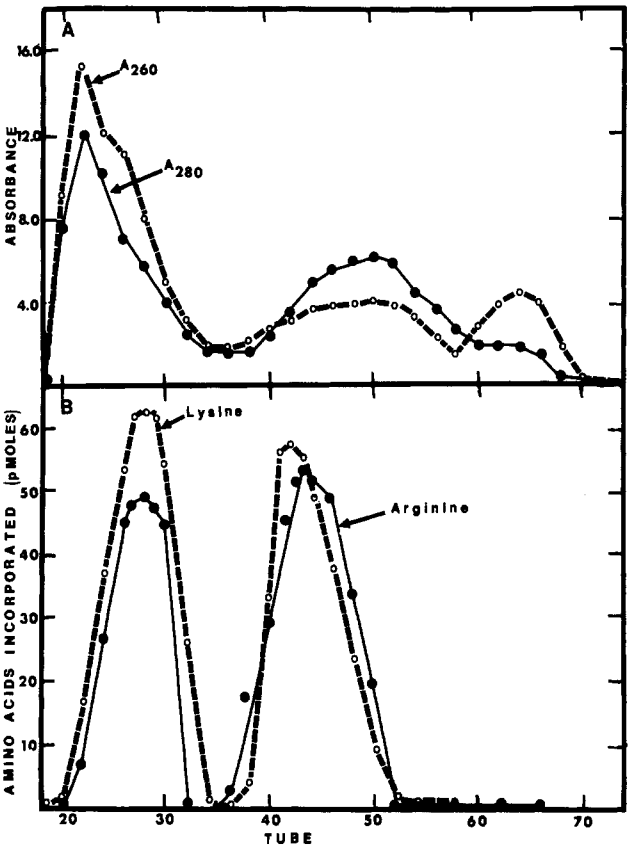

FIGURE 1: Sepharose 6B gel filtration of crude extracts. Crude extracts were prepared by Teflon homogenization as described in the text. One milliliter samples were applied to the column (0.9 × 51 cm). The column was eluted with buffer I. Fractions of 1 mL were collected. Aliquots of 10 μL were assayed for aminoacyl-tRNA synthetase activity as described in the text. (A) Absorbance profile at (●) A_{280} and (○) A_{260}. Standard proteins applied to the column were thyroglobin, aldolase, and hemoglobin. (B) (●) Arginyl-tRNA synthetase activity and (○) lysyl-tRNA synthetase activity. Greater than 90% of the activity applied to the column was recovered.

In order to purify the small molecular weight forms, it was necessary to separate the large molecular weight form from the small molecular weight form of arginyl- and lysyl-tRNA synthetases. To perform this separation, we used an ω-aminohexylagarose column. Arginyl- and lysyl-tRNA synthetase activity appeared in both the buffer- and salt-eluted fractions. For arginyl-tRNA synthetase 30–40% of the total synthetase activity was eluted with salt while 40–50% of the lysyl-tRNA synthetase activity was eluted with salt (data not shown). These proportions of enzyme activity were maintained over a 100-fold difference in protein concentrations. Thus the multiple forms were not due to simply overloading the column. If the fractions eluted from the affinity column by salt were pooled and concentrated by Amicon ultrafiltration with a PM-30 membrane under nitrogen and applied to a Sepharose 6B gel-filtration column, they eluted at essentially the same position as the small molecular weight forms of the enzyme. The fractions that eluted off the column with buffer alone were also pooled, concentrated by Amicon ultrafiltration with a

Table I: Purification of the Small Molecular Weight Form of Arginyl- and Lysyl-tRNA Synthetases[a]

step	tRNA synthetase	total units	total protein	sp act.	yield	purification
crude extract	Arg	4272 (1320)	9450	0.45 (0.14)	100	
(fraction I)	Lys	3888 (2008)		0.41 (0.21)	100	
ω-aminohexylagarose	Arg	1320 (1320)	506	3.34 (3.34)	30.9 (100)	7.4 (23.9)
(fraction II)	Lys	2008 (2008)		5.08 (5.08)	51.6 (100)	12.4 (24.2)
DEAE-cellulose	Arg	960 (960)	108	9.23 (9.23)	22.4 (72.7)	20.5 (65.9)
(fraction III)	Lys	1470 (1470)		14.13 (14.13)	37.8 (73.2)	34.5 (67.3)
hydroxylapatite	Arg	720 (720)	41	18.00 (18.00)	16.8 (54.5)	40.0 (128.6)
(fraction IV)	Lys	1156 (1156)		28.90 (28.90)	29.7 (57.6)	70.5 (137.6)
phosphocellulose	Arg	357 (357)	10.9	32.75 (32.75)	8.4 (27.0)	72.8 (233.9)
(fraction V)	Lys	660 (660)		60.55 (60.55)	17.0 (32.9)	147.5 (255.6)
Blue Sepharose	Arg	275 (275)	3.2	86.48 (86.48)	6.4 (20.8)	192.2 (617.7)
(fraction VI)	Lys	339 (339)		106.60 (106.60)	8.7 (16.9)	260.0 (507.6)
G-200 gel filtration	Arg	122 (122)	1.4	84.72 (84.72)	2.8 (9.2)	188.3 (605.1)
(fraction VII)	Lys	169 (169)		117.36 (117.36)	4.4 (8.4)	286.2 (558.9)

[a] The x-fold purification and yield of the enzyme have been presented in two different ways. The numbers outside the parentheses were arrived at by calculating the yield and purification considering the total enzyme activity to be composed of both the large and small forms. However, we feel that the numbers in parentheses are a more accurate indication of the yield and purification. They have been calculated relative to the amount of activity that exists in the small molecular weight forms, which is the only activity we wish to purify. The ω-aminohexylagarose column has been shown to be an effective technique for separating the large molecular weight synthetases from the small molecular weight enzymes. The small forms adhere to the column and the amount of activity recovered from the salt wash of the column is regarded 100% of the activity of the small molecular weight enzymes present in the crude extract.

PM-30 membrane under nitrogen, and applied to a Sepharose 6B gel-filtration column. These enzymes eluted at essentially the same place as the large molecular weight forms (data not shown). The hydrophobic column selectively binds the small molecular weight forms of the synthetases while allowing the large forms to pass through. Furthermore, the affinity column apprently does not alter the large molecular weight form of the enzymes since they elute identically from Sepharose 6B gel-filtration chromatography either before or after hydrophobic chromatography.

Purification of Small Molecular Weight Forms of Arginyl- and Lysyl-tRNA Synthetases. The purification procedure described here results in a 605-fold and 559-fold enrichment of arginyl- and lysyl-tRNA synthetase with a recovery of 9.2% and 8.4%, respectively (Table I). If the two synthetases exist as a complex, then the ratio of the specific activities should be relatively constant throughout the purification. The ratios of the specific activity calculated from Table I are as follows: 0.65 for fraction I, 0.66 for fraction II, 0.65 for fraction III, 0.62 for fraction IV, 0.54 for fraction V, 0.81 for fraction VI, and 0.72 for fraction VII. Except for two steps the ratios are within a 10% error of each other. It is not known at the present time why fractions V and VI vary; however, in a different enzyme preparation the ratio of these two fractions can fall within 10% of the other fraction (data not shown). It may therefore be due to the stability of the enzyme from preparation to preparation. Since ratios of the specific activities for five of the seven steps are constant, including the last step, this suggests that the two synthetases are copurifying as a complex. This does not necessarily suggest that they exist as a complex in vivo.

Criteria for Purity. Electrophoresis under denaturing conditions was performed on the enzymes after G-200 gel-filtration chromatography (fraction VII). The results of $NaDodSO_4$ gel electrophoresis in 9% polyacrylamide are shown in Figure 2. There are only two major bands seen. The protein concentration in fraction VII was varied from 15 to 50 μg, and still only two major bands were obtained. Fraction VII was estimated to be at least 98% pure on the basis of a densitometer tracing of a stained gel (Figure 2). Also, the scan indicated that the two bands were present quantitatively in a 1:1 ratio. Only the three peak tubes from the G-200 column were pooled (fraction VII) because $NaDodSO_4$ gel electrophoresis performed on tubes before or after these three tubes had minor bands present. $NaDodSO_4$ gel electrophoresis was performed on fraction VII in gels containing 12% and 15% polyacrylamide to give better resolution. These gels also showed the preparation to contain two bands and to be at least 98% pure (data not shown). Nondenaturing gel electrophoresis was attempted with 4.5% polyacrylamide, but the protein did

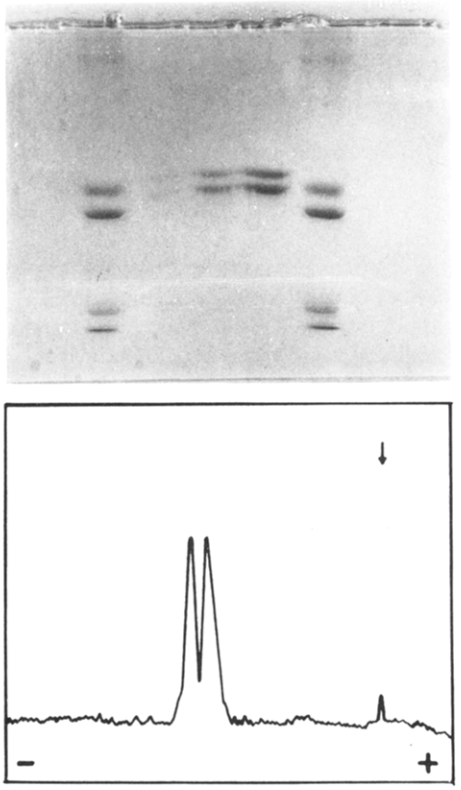

FIGURE 2: $NaDodSO_4$ gel electrophoresis in 9% polyacrylamide. The electrophoresis was performed as described under Experimental Procedures. Lanes 1 and 5 are protein standards. Lanes 2, 3, and 4 are samples from fraction VII, 15, 30, and 50 μg, respectively. The standards are (from top to bottom) thyroglobulin (330 000), ferritin (half-unit) (220 000), albumin (67 000), catalase (60 000), lactate dehydrogenase (36 000), and ferritin (18 500). The densitometer tracing is from the gel with 50 μg of protein. The arrow indicates the tracking dye.

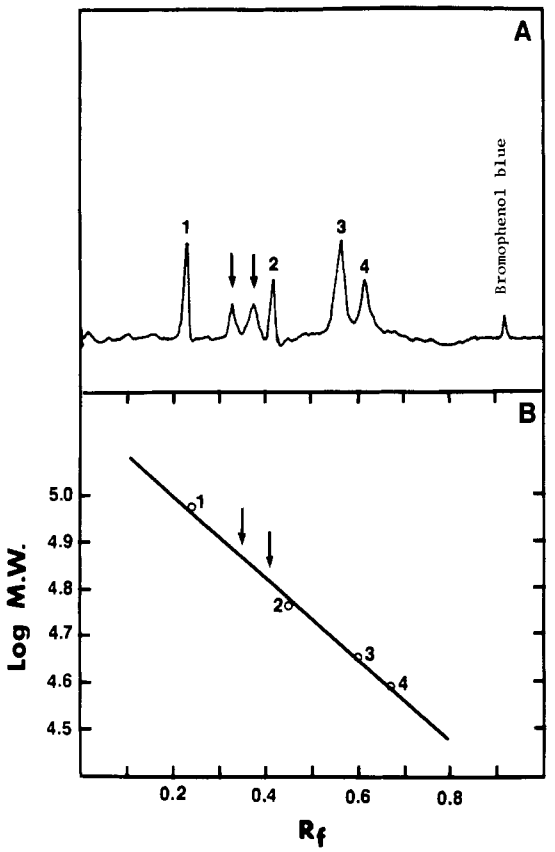

FIGURE 3: Molecular weight determination of purified arginyl- and lysyl-tRNA synthetase complex by acrylamide gel electrophoresis. The electrophoresis was performed as described under Experimental Procedures except a constant voltage of 70 V was applied until the sample entered the resolving gel, and the voltage was then increased and kept at 140 V. (A) A densitometric tracing of a stained gel, 15 μg of purified enzymes and 10–15 μg of each standard protein. (B) Plot of log of molecular weight vs. distance of migration relative to bromophenol blue (R_f). The standards used were (1) phosphorylase A (92 500), (2) pyruvate kinase (57 200), (3) ovalbumin (45 000), and (4) aldolase (39 000). The arrows indicate the positions of subunits of arginyl- and lysyl-tRNA synthetases.

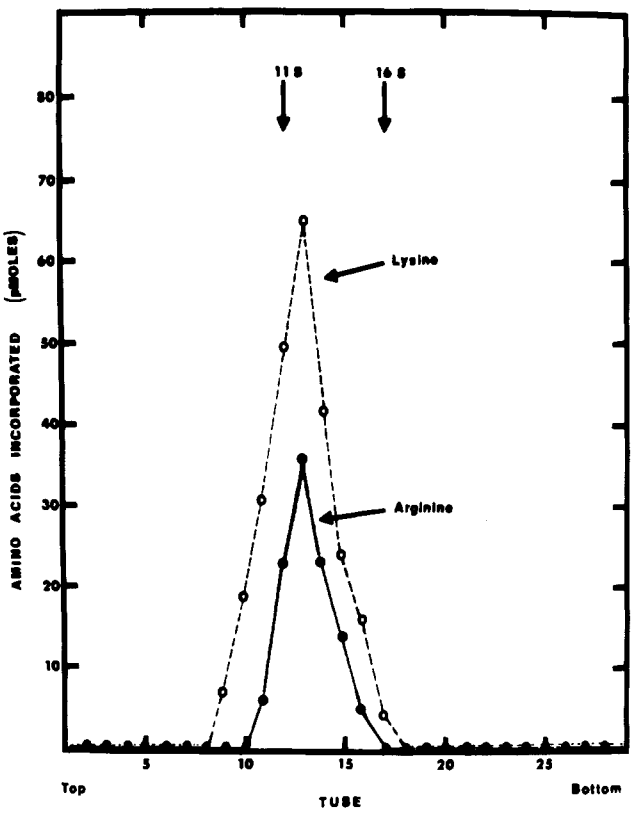

FIGURE 4: Glycerol gradient centrifugation. The experiment was performed as described under Experimental Procedures. (●) Arginyl-tRNA synthetase activity; (○) lysyl-tRNA synthetase activity. Recoveries for both enzymes were greater than 90%.

not penetrate the stacking gel, presumably due to aggregation of the enzymes. Reverse-polarity electrophoresis was also unsuccessful. Other workers have found that pure or partially pure mammalian synthetases would not enter low-percentage acrylamide native gels (Deutscher, 1967; Neale, 1970; Kane et al., 1978).

Assay of various fractions for other aminoacyl-tRNA synthetases demonstrated that no other synthetase activity could be detected on the phosphocellulose fraction, Blue Sepharose fraction, or the G-200 gel-filtration fraction.

Molecular Weight and Subunit Structure. The subunit molecular weights of the purified synthetases were determined by $NaDodSO_4$ gel electrophoresis. In order to obtain an accurate molecular weight, we applied fraction VII and the standards to the same well of an $NaDodSO_4$ slab gel. Figure 3A is the densitometer tracing of the stained gel, and as shown in Figure 3B the two polypeptides have molecular weights of 73 000 and 65 000. The molecular weight of the complex calculated from the sedimentation coefficient (Figure 4), the Stokes radius, and the partial specific volume was 285 000 (Table II). This molecular weight is in very good agreement with the native molecular weight calculated from the sum of the subunit molecular weight of 276 000. This suggests that the synthetases exist as a tetramer.

The tetrameric nature of the synthetase complex is further supported by cross-linking experiments (Davies & Stark, 1970). Exposure of arginyl- and lysyl-tRNA synthetases to dimethyl suberimidate, followed by $NaDodSO_4$ gel electrophoresis, as shown in Figure 5B, revealed a broad band that migrated between ferritin (220 000) and thyroglobulin (330 000) but closer to the latter, consistent with a tetramer. It is conceivable that the enzyme exists as a trimer; however,

Table II: Summary of Physical Parameters of Arginyl- and Lysyl-tRNA Synthetases

property	method	value
partial specific volume ($\bar{v}$)	amino acid and carbohydrate composition[a]	0.72 cm^3/g
Stokes radius (R_s)	gel filtration	58 Å
diffusion coeff ($D_{20,w}$)	calcd from R_s	3.50×10^{-7} cm^2/s
sedimentation coeff	sedimentation velocity	12.1 S
subunit mol wt	$NaDodSO_4$ gel electrophoresis	73 000 and 65 000
native mol wt (M_r)	calcd from $s_{20,w}$, R_s, and $\bar{v}$	285 000
frictional coeff ratio (f/f_0)	calcd from R_s	1.15
axial ratio (a/b)	calcd from f/f_0	3.5

[a] To determine the amino acid composition, we hydrolyzed the protein according to the method of Penke et al. (1974) with 3 N mercaptoethanesulfonic acid. The proteins were split into two equal parts, each dissolved in 2 mL of the acid. The solutions were then placed into tubes that were evacuated, and the samples were allowed to react for 24 and 72 h at 110 °C. Samples from both tubes were analyzed on a Phoenix Precision Instrument Co. amino acid analyzer, Model K-8000 VG. Values obtained from each hydrolysis time were used, and the numbers were extrapolated to zero on infinite time. Carbohydrate composition was determined by the method discussed by Glinski et al. (1979).

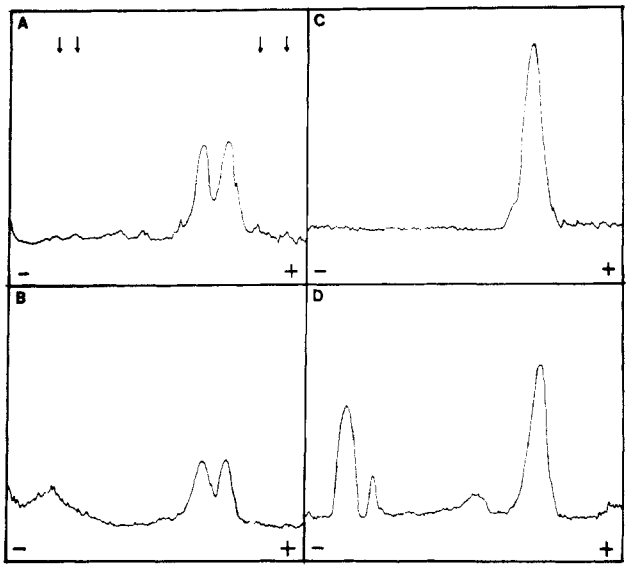

FIGURE 5: $NaDodSO_4$ gel electrophoresis of cross-linked arginyl- and lysyl-tRNA synthetases. (A) Densitometer tracing of 90 μg of fraction VII, not cross-linked. (B) Densitometer tracing of 90 μg of fraction VII, cross-linked at a protein to dimethyl suberimidate ratio of 1:1. (C) Densitometer tracing of 110 μg of rabbit muscle pyruvate kinase, not cross-linked. (D) Densitometer tracing of 110 μg of pyruvate kinase, cross-linked at a protein to dimethyl suberimidate ratio of 1:1. Samples from fraction VII were dialyzed overnight against 0.2 M triethanolamine hydrochloride, pH 8.5, 10 mM β-mercaptoethanol, 8 mM $MgCl_2$, 0.2 mM PMSF, 0.1% Nonidet P-40, and 10% glycerol (final volume). After incubation at 15 °C for 3 h, the samples were denatured and applied to a 9% $NaDodSO_4$–acrylamide gel as described under Experimental Procedures except after penetrating the stacking gels it was run for 4 h. Arrows in (A) indicate the standard proteins (from left to right) as follows: thyroglobulin (330 000), ferritin (220 000), albumin (67 000), and catalase (60 000).

this seems unlikely since the two synthetases are present in a 1:1 ratio of subunits based on a densitometer tracing of a stained gel before and after cross-linking with dimethyl suberimidate (Figure 5). Also, in support of this, a similar experiment was performed as described in Figure 5 except the protein to dimethyl suberimidate ratio was 2:1. The densitometer tracing showed a broad band in the tetrameric region plus the two monomers that were in a 1:1 ratio of subunits when gels were scanned at 580 nm (data not shown). As seen in Figure 5B, the dimeric form of arginyl- and lysyl-tRNA synthetase was not found, which is unusual and at the present time cannot be explained. However, the dimer was not found when the protein to dimethyl suberimidate ratio was varied from 1 to 5, nor was it found by varying the time of incubation from 0 to 180 min in the presence of the cross-linker (data not shown). As seen in Figure 5D, the monomeric, dimeric, trimeric, and tetrameric forms of pyruvate kinase were found. Since all four forms of the tetrameric pyruvate kinase can be seen, this demonstrates that the cross-linking experiments are working. It should also be noted that the dimeric form of pyruvate kinase is the minor species present; thus it seems that the cross-linking experiments described in Figure 5 favor the tetrameric form of the enzyme. Thus it is possible that the dimeric forms of arginyl- and lysyl-tRNA synthetases are present in very low amounts. Since there will be three different dimeric forms with different molecular weights (α_2, β_2, and $\alpha\beta$), the dimers may be too diffused to detect.

In an attempt to separate the two activities by charge, isoelectric focusing chromatography was attempted on fraction VII. The two synthetase activities copurify at a pI of 5.8 (Figure 6). Attempts were also made to separate two activities by using fraction VII in ultracentrifugation. Again, only one boundary was observed with an S value of 12.1 (Figure 4). It appears that the two synthetase activities cannot be separated on the basis of size, shape, or charge. This information along with the constant specific activity ratios throughout the purification and the 1:1 ratio of the two monomers on $NaDodSO_4$ gel electrophoresis suggests that the two activities are copurifying as a complex.

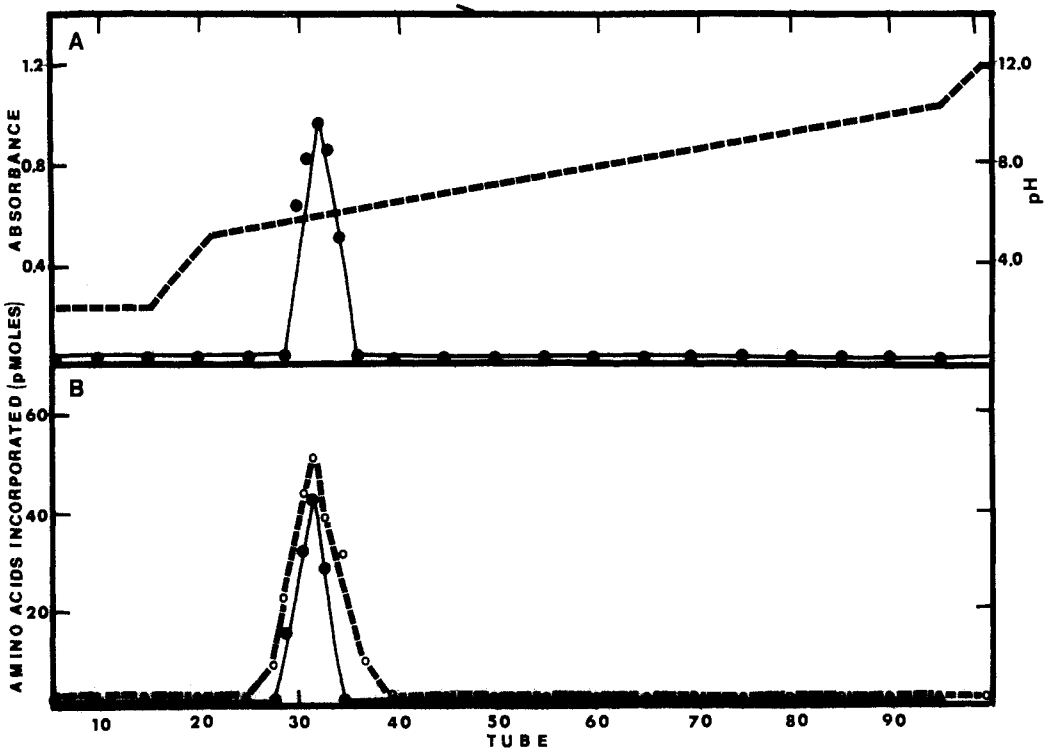

FIGURE 6: Isoelectric focusing. The experiments were performed as described under Experimental Procedures. (A) (●) A_{280} and (- - -) pH; (B) (●) arginyl-tRNA synthetase activity and (○) lysyl-tRNA synthetase activity. The recovery of arginyl-tRNA synthetase varied from 0% to 51% and that of lysyl-tRNA synthetase varied from 0% to 56% on different experiments.

Table III: Kinetic Affinity Constants of Arginyl- and Lysyl-tRNA Synthetases for tRNA, Amino Acid, and ATP

	arginyl-tRNA synthetase		lysyl-tRNA synthetase	
	K_m	$S_{1/2}$	K_m	$S_{1/2}$
tRNA	3.8 μM	4.0 μM	4.5 μM	5.0 μM
amino acid		8.2 μM		11.3 μM
ATP	1.4 mM	1.5 mM	6.0 mM	6.5 mM

Kinetic Parameters. The kinetic parameters for arginyl- and lysyl-tRNA syntehtase were determined from the initial velocity measurements for aminoacylation of tRNA by varying concentrations of one substrate and by using saturating amounts of the other two substrates. Table III summarizes the Michaelis–Menten constants of arginyl- and lysyl-tRNA synthetases with respect to tRNA, amino acid, and ATP. A K_m value was not reported for the amino acid because the Lineweaver–Burk plot was nonlinear and therefore would have no kinetic meaning. Therefore, only the $S_{1/2}$ value is reported for the amino acid (Table III). It is not apparent why the Lineweaver–Burk plots for both amino acids were nonlinear. The experiments were performed on four different enzyme preparations with freshly made tRNA, amino acid, and ATP solutions. The data have been analyzed by the Hill and Eadie–Hofstee plots. The information obtained from these plots suggests that negative cooperativity could exist for the synthetases in respect to arginine and lysine. As seen in Table III the K_m values for all three substrates for arginyl-tRNA synthetases are very similar to the K_m values obtained for the corresponding substrates of lysyl-tRNA synthetases. Also, these values are consistent with the range of reported values (Johnson et al., 1980; Choo & Logan, 1977; Chlumecka et al., 1969; Waldenstrom, 1968; Ikegami & Griffin, 1969; Allende & Allende, 1964; Papas & Peterkofsky, 1972).

Discussion

In this communication arginyl- and lysyl-tRNA synthetases copurify as a complex. The native molecular weight is 285 000 (Table II) with subunit molecular weights of 73 000 and 65 000 in a 1:1 stoichiometry (Figures 2 and 3). From cross-linking studies it is apparent that the synthetases exist as a tetramer (Figure 5). At the present time we do not know in which tetrameric form they exits. It is likely that arginyl- and lysyl-tRNA exist as a tetrameric complex consisting of subunits of two different molecular weights since these two enzymes have been observed to copurify as partially purified in rat liver (Irvin & Hardesty, 1972; Goto & Schweiger, 1973; Dang & Yang, 1978), in sheep liver (Kellermann et al., 1979), in rabbit reticulocyte (Som & Hardesty, 1975), in rat mammary gland (Hele & Herbert, 1977), in Chinese hamster ovary cells (Ritter et al., 1976), in human placenta (Denney, 1977), and in bovine brain (Vadeboncoeur & Lapointe, 1980). However, conclusive results as to whether arginyl- and lysyl-tRNA synthetases exist as the α_4 and β_4 tetramers or as an $\alpha_2\beta_2$ complex require further studies. We are now in the process of determining this. It is important to point out that since in crude extracts we can isolate arginyl- and lysyl-tRNA synthetase in the range of 150 000 daltons no conclusion can be reached about the in vivo subunit organization of these two enzymes.

This is not the first report of the copurification of these two enzymes (Goto & Schweiger, 1973; Dang & Yang, 1979; Irvin & Hardesty, 1972). However, this is the first report of these two synthetases copurifying to homogeneity. Lysyl-tRNA synthetase has been purified to homogeneity from rat liver with a subunit molecular weight of 66 000 (Johnson et al., 1980). These authors report that arginyl-tRNA synthetase copurifies with lysyl-tRNA synthetase until the final step of their purification. Under Discussion of this paper the authors suggest that the subunit molecular weight of arginyl-tRNA synthetase is 48 000. From the data presented in this paper it seems unlikely that the 48 000-dalton polypeptide is arginyl-tRNA synthetase. This 48 000-dalton band may be due to the action of a nonserine protease, which is hard to control. Similar results have been obtained by various investigators working with alanyl- and methionyl-tRNA synthetase from *Escherichia coli*. Early reports suggested that methionyl-tRNA synthetase had a native molecular weight of 173 000 with a subunit molecular weight of 43 000 (Lemoine et al., 1968). More recently, it has been demonstrated that this enzyme had a molecular weight of 175 000 and a subunit size of 85 000 (Bruton et al., 1974). These investigators also demonstrated that this enzyme contains a significant amount of repeated sequence. In the case of alanyl-tRNA synthetase the subunit molecular weight was 95 000, but a 48 000-dalton fragment could also be isolated (Putney et al., 1981). This fragment had alanine-dependent pyrophosphate exchange activity but no aminoacylation. These investigators were able to perform limited proteolysis on the native enzyme and generate the 48 000-dalton fragment; this fragment had pyrophosphate exchange activity but no aminoacylation activity.

Recently the molecular weight of lysyl-tRNA synthetase has been reported as 69 000 (Johnson & Yang, 1981) and is in good agreement with the 73 000-dalton polypeptide isolated in this communication (Figure 3). Furthermore, their observation of a partially purified arginyl- and lysyl-tRNA synthetase complex with a sedimentation coefficient of 12 S (Dang & Yang, 1979) and the observation that partially purified arginyl- and lysyl-tRNA synthetase sediments at approximately 14 S in rabbit reticulocytes (Irvin & Hardesty, 1972) are in very good agreement with the 12.1S value we obtained with the pure arginyl- and lysyl-tRNA synthetase complex (Figure 6).

At the present time we do not know which band on the $NaDodSO_4$ gels is arginyl-tRNA synthetase and which band is lysyl-tRNA synthetase. Attempts to elute the enzyme of the $NaDodSO_4$ gels and assay them for aminoacylation have failed. However, Dr. David Yang furnished us with a sample of purified lysyl-tRNA synthetase isolated from rat liver (Johnson et al., 1980) and a sample of a purified 18S aminoacyl-tRNA synthetase complex containing lysyl-, arginyl-, leucyl-, isoleucyl-, and methionyl-tRNA synthetases (Johnson & Yang, 1981) so that we could identify our polypeptide. The lysyl-tRNA synthetase supplied by Dr. Yang from both the purified sample and the complex comigrated with the 73 000-dalton polypeptide that we have isolated (data not shown). Also our 65 000-dalton band comigrated with a minor species between bands VII and VIII of the 18S complex isolated by Dr. Yang (data not shown). Thus we tentatively identify the 73 000-dalton band as lysyl-tRNA synthetase and the 65 000-dalton band as arginyl-tRNA synthetase. We are in the process of raising antibodies from the two bands on the $NaDodSO_4$ gels to conclusively determine their identity.

The specific activity reported for lysyl-tRNA synthetase in this report of 117.4 (Table I) is lower than the 449 value reported for lysyl-tRNA synthetase in rat liver (Johnson et al., 1980). Since there are two synthetases present in stoichiometry of 1:1 (Figure 2), only half of the protein is lysyl-tRNA synthetase; thus the specific activity could be 234.8/mg of lysyl-tRNA synthetase or about half of that reported by Johnson et al. (1980). Why we obtain a lower

specific activity is not apparent at the present time. However, since we isolate arginyl- and lysyl-tRNA synthetase as a complex and the other investigators do not, it is conceivable that the synthetases are not as active in the complex. Therefore, it is important to be able to separate the two synthetases. Unfortunately, all attempts to separate them have failed. We have reported that there are carbohydrates associated with the complex (Glinski et al., 1979) and preliminary evidence suggests that lipid is also associated with the complex (C. V. Dang and R. H. Hilderman, unpublished observation). Since glycolipids (Saxholm & Pitot, 1979) and cholesterol ester have been found associated with aminoacyl-tRNA synthetase complexes (Bandyopadhyay & Deutscher, 1973) and free aminoacyl-tRNA synthetases (Hradec & Dusek, 1969) isolated from rat liver, we are attempting to determine if the carbohydrates and lipids are involved in holding the complex together. Even if the complex isolated in this communication was created during the purification, the interaction between the synthetases, carbohydrates, and lipids is of utmost importance since other investigators have found these molecules associated with partially purified aminoacyl-tRNA synthetase complexes.

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