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Arginyl-tRNA Synthetase from *Escherichia coli*, Purification by Affinity Chromatography, Properties, and Steady-State Kinetics†

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ABSTRACT: A Blue Sephadex G-150 affinity column adsorbs the arginyl-tRNA synthetase of *Escherichia coli* K12 and purifies it with high efficiency. The relatively low enzyme content was conveniently purified by DEAE-cellulose chromatography, affinity chromatography, and fast protein liquid chromatography to a preparation with high activity capable of catalyzing the esterification of about 23 000 nmol of arginine to the cognate tRNA per milligram of enzyme within 1 min, at 37 °C, pH 7.4. The turnover number is about 27 s^{-1}. The purification was about 1200-fold, and the overall yield was more than 30%. The enzyme has a single polypeptide chain of about M_r 70 000 and binds arginine and tRNA with 1:1 stoichiometry. For the aminoacylation reaction, the K_m values at pH 7.4, 37 °C, for various substrates were determined: 12 μM, 0.9 mM, and 2.5 μM for arginine, ATP, and tRNA, respectively. The K_m value for cognate tRNA is higher than those of most of the aminoacyl-tRNA synthetase systems so far reported. The ATP–PP_i exchange reaction proceeds only in the presence of arginine-specific tRNA. The K_m values of the exchange at pH 7.2, 37 °C, are 0.11 mM, 2.9 mM, and 0.5 mM for arginine, ATP, and PP_i, respectively, with a turnover number of 40 s^{-1}. The pH dependence shows that the reaction is favored toward slightly acidic conditions where the aminoacylation is relatively depressed.

Since the 1960s, the mechanism of a group of three small aminoacyl-tRNA synthetases, arginyl-, glutamyl-, and glutaminyl-tRNA synthetases, has been widely studied and discussed. These synthetases require the cognate tRNA for the ATP–PP_i[1] exchange reaction (Zubay, 1962; Ravel et al., 1964, 1965; Mitra & Mehler, 1966, 1967; Parfait & Grosjean, 1972; Gangloff et al., 1976; Char & Gopuiathan, 1986) and are very different from the majority of aminoacyl-tRNA synthetases. Until now, the true mechanism has not yet been clearly elucidated. Generally, two different mechanisms have been proposed: (a) the aminoacyl adenylates being formed as in the other synthetases, e.g., the arginyl-tRNA synthetase from *Neurospora crassa* (Nazario & Evans, 1974) and baker's yeast (Fersht et al., 1978); (b) a concerted mechanism without formation of the aminoacyl adenylates, e.g., the arginyl-tRNA synthetase from *Bacillus stearothermophilus* (Parfait & Grosjean, 1972) and from brewer's yeast (Thiebe, 1983). We consider it very important to study the detailed properties, including the interactions between the enzyme and substrates,

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[1] Abbreviations: ArgRS, arginyl-tRNA synthetase; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; LeuRS, leucyl-tRNA synthetase; PMSF, phenylmethanesulfonyl fluoride; PP_i, inorganic pyrophosphate; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; $tRNA^{Arg}$, arginine-specific tRNA.

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as directly as possible. Such studies have not been done in many cases but would help to determine the true mechanism, and thereby avoid leading too fast to a conclusion, e.g., that the intermediates do not exist simply because they have not been found by our methods.

Escherichia coli K12 has a relatively low content of arginyl-tRNA synthetase. In the cell extract the total ArgRS activity approaches that of LeuRS, but the high specific activity of the former leads to its lower content in weight (only one-sixth of the latter). Thus, the efficiency of the purification is important for further investigations. Here, we present an improved, efficient preparation of this enzyme on a relatively larger scale than previously reported. The general properties and the steady-state kinetics are also studied. Results on the enzyme–substrate interactions will be reported in the following paper (Lin et al., 1988).

Materials and Methods

Materials

The strain we used for the arginyl-tRNA synthetase (EC 6.1.1.19) purification was *E. coli* K12. Total tRNA was extracted from the same strain according to the method of Holley et al. (1963). The charging capacity corresponded to a $tRNA^{Arg}$ content of about 5.2%. Pure *E. coli* $tRNA^{Arg}$ with a charging capacity of more than 1400 pmol/A_{260} unit was obtained from Subriden RNA Co., Rollingbay, WA. L-Arginine, ATP, and inorganic pyrophosphatase (EC 3.6.1.1, sp act. 500–600 units/mg) were from Sigma. Uniformly labeled L-[^{14}C]arginine (300–400 mCi/mmol), L-[*guanidino*-^{14}C]arginine (54.4 mCi/mmol), and [^{32}P]PP_i (1–100 mCi/mmol) were purchased from Amersham, England. DEAE-cellulose (DE-52) and phosphocellulose P11 were Whatman products. Blue Sephadex G-150 was prepared as described by Boehme and Bolton (1968), with Cibacron blue F3G-A, a reactive dye called K-GRS that was made in China. Protein markers for G-150 gel filtration were from Sigma and for the SDS electrophoresis from Pharmacia.

Methods

Determination of the Aminoacylation Activity. A reaction mixture of 90 μL contained 50 mM Tris-HCl, pH 7.4 (indicating the real pH at the reaction temperature, i.e., the temperature dependence of −0.028/°C, was considered), 80 mM KCl, 7–8 mM $MgCl_2$, 4 mM ATP, 0.1 mM EDTA, 0.5 mM DTT, 2.4 mg/mL total tRNA (corresponding to a $tRNA^{Arg}$ content of 5 μM), and 0.1 mM [^{14}C]arginine (25 μCi/μmol). The enzyme was first diluted in a solution containing bovine serum albumin, DTT (1 mM), and glycerol (10%), making final concentrations of about 0.06 μg/mL (0.8 nM) ArgRS and 0.2 mg/mL BSA in the reaction mixture. The reaction was initiated with the addition of ArgRS and conducted at 37 °C. At varying time intervals (usually 1–4 min) aliquots of 20 μL were applied to Whatman 3MM paper discs, followed by treatment according to the technique of Mans and Novelli (1961). The discs were then measured in PPO–POPOP/toluene (5 g of PPO and 0.3 g of POPOP per liter) by scintillation counting with a Beckman LS 5801. One unit was defined as the amount of enzyme which charges 1 nmol of $tRNA^{Arg}$ in 1 min under the above conditions.

Test of tRNA Charging. The procedure was similar to the determination of aminoacylation activity except an elevated enzyme concentration (50–100 nM) was used and the $tRNA^{Arg}$ or its equivalent was in the concentration range of 3–5 μM, giving a tRNA:enzyme molar ratio of approximately 50:1. Aliquots were taken after 10–20 min. After 15 min, no increase of tRNA charging could be found.

ATP–PP_i Exchange. The reaction procedure was similar to that of Fasiolo et al. (1981) as modified by Lin et al. (1983), except cognate tRNA was present. The incubation mixture contained 130 mM Tris-HCl, pH 7.2 (the actual pH at the reaction temperature), 6 mM $MgCl_2$, 2 mM ATP, 2 mM arginine, 2 mM [^{32}P]tetrasodium pyrophosphate (sp act. 1000–2000 cpm/nmol), and 10 μM purified $tRNA^{Arg}$. The reactions were initiated upon addition of arginyl-tRNA synthetase (ArgRS), diluted as described under Determination of the Aminoacylation Activity but to a final concentration of about 6 nM in the reaction mixture. After variable incubation times, 80-μL aliquots were pipetted out and treated as in Lin et al. (1983) and then counted by scintillation with 0.6% butyl-PDB [2-(4-*tert*-butylphenyl)-5-biphenyl-4-yl-1,3,4-oxadiazole] in toluene/ethylene glycol monomethyl ether (volume ratio 4:6).

Kinetics. When the K_m values for various substrates in the aminoacylation or ATP–PP_i exchange reactions were determined, the conditions were similar to those under Determination of the Aminoacylation Activity and under ATP–PP_i Exchange, respectively, except the concentrations of the corresponding substrate were varied and 10 mM ATP–Mg^{2+} was used in the aminoacylation kinetics. When the K_m values for ATP were measured, the concentration of Mg^{2+} varied with that of ATP, keeping an excess Mg^{2+} concentration of 3–4 mM. Pure $tRNA^{Arg}$ (*E. coli*) was used. Parallel experiments replacing $tRNA^{Arg}$ by total tRNA containing the same amount of $tRNA^{Arg}$ were taken as a control.

All experiments were carried out in Tris buffer (pH 7.4 and 7.2 for aminoacylation and exchange, respectively) except that, in the pH-dependence tests, Hepes buffer was employed to keep a unique buffer in the concerned pH region. The Hanes–Woolf plot was used for both reactions (Segel, 1975).

Protein Concentration Determination. The protein concentration of the enzyme was determined by a modified method of Lowry (Lowry et al., 1951) by Bensadoun and Weinstein (1976). During the purification procedure, the optical method of Warbourg and Christian (1942) ([protein] (mg/mL) = $1.55A_{280} - 0.76A_{260}$) was used.

SDS–PAGE. The polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out in a modified Laemmli discontinuous buffer system (Laemmli, 1970).

Purification Steps. The purification steps were carried out at about 4 °C. For all the chromatographies, except the Mono Q, a pH 6.8 potassium phosphate buffer (10 mM) containing the protecting reagents (0.1 mM EDTA, 5 mM mercaptoethanol, 0.1 mM PMSF, and 10% glycerol) was used. This buffer is referred to as Buffer A.

Results

Purification Procedure

Cell Disruption. Frozen *E. coli* K12 cells (500 g of cell paste) were thawed overnight in 500 mL of 10 mM potassium phosphate, pH 7.2, containing elevated protecting reagents (10 mM mercaptoethanol, 2 mM PMSF, 10% glycerol). The suspension was submitted to sonications (six times, 1.5 min each) with the MSE sonic oscillator (Soniprep 150) followed by centrifugation at 10000*g* for 45 min to separate the cell debris and then at 120000*g* for 90 min.

DEAE-cellulose Chromatography. This step was similar to that in Kern and Lapoine (1979). After the gradient chromatography, the fractions with ArgRS specific activity more than four to five times that of the applied cell extract

Table I: Purification of ArgRS with Blue Sephadex G-150[a]

	total protein (mg)	ratio to applied protein (%)	sp act. (units/mg)[b]	total act. ($\times 10^4$ units)
applied protein[c]	2641	100	119	31.5
nonretained protein	1432	54.2	0	0
2 mM ATP washing	332	12.6	0	0
60 mM KCl washing	218	8.3	0	0
150 mM KCl elution				
leading part	71	2.7	208	1.5
trailing part	148	5.6	1970	29.1
1 M KCl washing	373	14.1	0	0

[a] Column size 5.6 × 8 cm. [b] The same as footnote *b* in Table II. [c] Partially purified ArgRS after DEAE-cellulose chromatography.

were collected and precipitated by adding ammonium sulfate to 75% saturation.

Blue Sephadex G-150 Affinity Chromatography. (*A*) *Adsorption of ArgRS by the Column.* About 2600 mg of the partially purified DEAE fraction of ArgRS was desalted by Sephadex G-25 and then applied to the Blue Sephadex G-150 column (5.6 × 8 cm) previously equilibrated with buffer A at a flow rate of 80 mL/h. More than half of the protein passed through the column and had almost no ArgRS activity.

(*B*) *Washing with ATP and a Low Concentration of KCl.* The column was successively washed with 2 mM ATP in buffer A (~360 mL) and then buffer A until there was no absorbance at 260 nm. Then the column was washed with 60 mM KCl in the same buffer (~600 mL). The flow rate was kept at 60 mL/h. About 12.6% and 8.3% of the loaded protein came out with ATP and low KCl washings, respectively, in which no ArgRS activity was found (Table I).

(*C*) *Elution of ArgRS.* The enzyme was eluted by 150 mM KCl in buffer A at a flow rate of about 100 mL/h. Nearly 90% of the applied ArgRS activity was found in the trailing portion of the eluted protein peak (Table I). This Blue Sephadex fraction had a specific activity of nearly 2000 units/mg of protein; i.e., the enzyme was purified at least 16–18-fold from the partially purified DEAE fraction with one affinity chromatography step. This main fraction was precipitated by adding ammonium sulfate to 75% saturation. Finally, the column was washed with 1 M KCl (Table I).

Sephadex G-150 Gel Filtration. The Blue Sephadex fraction in ammonium sulfate was desalted by Sephadex G-25 and then passed through a phosphocellulose column (3.5 × 20 cm), making use of the low-salt condition. About 60% of the loaded protein was adsorbed, and nearly all the ArgRS activity passed through the column; thus, a 2.3-fold purification was gained.

Part of the protein which passed through the P11 column was submitted to Sephadex G-150 gel filtration (column size 1.1 × 110 cm for 10 mg of applied protein) with a flow rate of 7.5 mL/h (the rest of the P11 fraction was submitted to FPLC directly in several batches) (Table II). The main active protein peak eluted at $K_{av} = 0.31$ (corresponding to a molecular mass of 7.05×10^4 Da). The K_{av} is the fraction of the stationary gel volume which is available for diffusion of a given solute species [see Pharmacia Fine Chemicals (1982)]. The Sephadex G-150 fraction thus obtained was nearly 90% homogeneous as judged by SDS–PAGE.

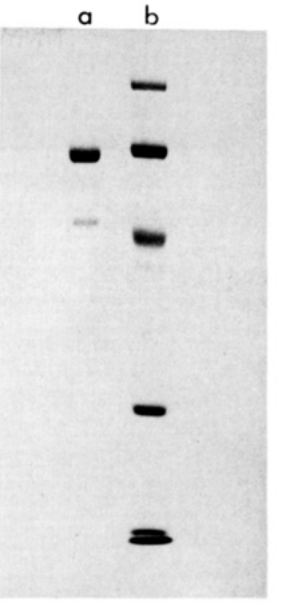

FIGURE 1: Sodium dodecylsulfate–polyacrylamide gel electrophoresis of purified ArgRS. (a) A major band of ArgRS and a minor band which accounted for about 3% of the protein content are shown. (b) The migration of marker proteins: phosphorylase *b* (94 000), serum albumin (67 000), ovalbumin (43 000), trypsin inhibitor (20 000) and 2-lactalbumin (14 000).

Chromatography with Mono Q. The trace impurity of the G-150 fraction could be easily separated by the Mono Q (HR 5/5) column (FPLC system). About 2–3 mg of the Sephadex G-150 fraction was applied each time with an equilibrium buffer consisting of 50 mM pH 7.8 Tris-HCl, 0.2 mM DTT, and a gradient of 0–0.8 M NaCl in the same buffer. The ArgRS activity was eluted in a symmetric peak yielding a preparation of more than 95% homogeneity (Figures 1 and 2a) which charged 22 000–24 000 nmol of tRNA in 1 min per milligram of protein at pH 7.4, 37 °C (the maximum velocity).

The FPLC system was also used as an alternative for the Sephadex G-150 gel filtration (Figure 2b). The P11 fraction (6–10 mg each time) could be applied to Mono Q directly, resulting in good separation of the M_r 70 000 band from all the contaminant proteins, except for a minor band. The yield in this step was about 60%, and the overall yield was higher (>40%) than those taking the G-150 gel filtration route. (The FPLC alternative method, however, resulted in a slightly greater content of the minor band.)

Table II: Purification of *E. coli* K12 Arginyl-tRNA Synthetase[a]

	total protein (mg)	total act. (units)	sp act. (units/mg)[b]	yield (%)	purification (*x*-fold)
(1) crude extract	36 800	397 000	10.8	100	1
(2) DEAE-cellulose	2641	314 000	119	79	11
(3) Blue Sephadex G-150	148	290 000	1970	73	182
(4) phosphocellulose	63.2	290 000	4603	73	417
(5) Sephadex G-150	*c*	*c*	10 600	37	981
(6) Mono Q	*c*	*c*	12 500	30	1157

[a] From 500 g of frozen cell paste. [b] These comparative values of specific activity could be corrected by the saturation coefficients of both tRNA and ATP to give the V_{max} values; see text. [c] The preparation which passed through the phosphocellulose column was separated for Sephadex G-150 chromatography or used directly in Mono Q (in the alternative procedure) chromatography, so the total amounts are not indicated. The yield of each step and the details can be referred to in the text.

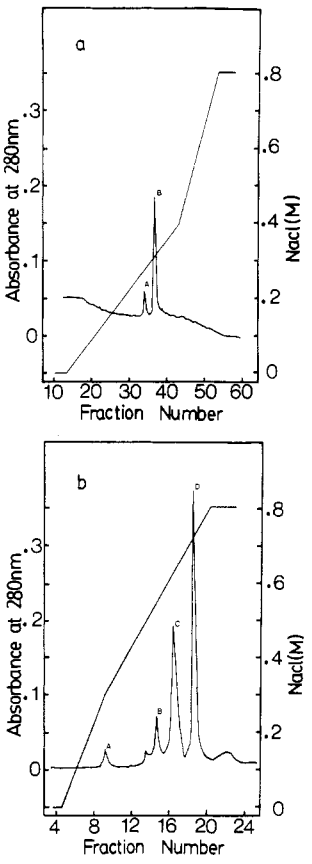

FIGURE 2: Purification of the ArgRS by an FPLC system with a Mono Q HR 5/5 column. The samples were applied to the column equilibrated with starting buffer (50 mM pH 7.8 Tris-HCl, 0.2 mM DTT) and then eluted with the indicated concentration gradient of sodium chloride (0–0.8 M) in the same buffer. (a) G-150 fractions used as the Mono Q sample are shown; peak B corresponded to the ArgRS activity. (b) Alternatively, P11 fractions were used directly as the Mono Q sample (see text); peak C corresponded to the ArgRS activity.

Our preparation had a single major band (molecular weight of about 70000 as judged by G-150 chromatography and SDS–PAGE) and a minor band which accounted for about 3% of the protein content, similar to the result of Charlier and Gerlo (1979). Since this minor band could not be separated by DEAE-cellulose, hydroxyapatite chromatography, or Mono Q in which our preparation showed a very symmetric peak, a substance may be tightly bound to the enzyme and will be of interest for further study.

Concentration and Storage

The enzyme preparation was concentrated by ultrafiltration and then dialyzed extensively against 20 mM potassium phosphate buffer (pH 7.2), containing 0.5 mM DTT, 0.1 mM EDTA, and 60% glycerol. With a change from 10 to 60% glycerol by dialysis, a 3-fold increase in concentration was gained. This preparation (about 2–3 mg/mL) was stored at −20 °C, and its activity was stable for 1–2 years.

Molecular Weight Determination

The molecular weight of the ArgRS was determined under nondenaturing conditions by Sephadex G-150 chromatography and denaturing conditions by SDS–PAGE as described under Materials and Methods. Sephadex G-150 (fine) chromatography (the column was 1.0 × 125 cm and equilibrated with 10 mM potassium phosphate buffer, pH 7.0) gave an average molecular weight value of 71 000 while the SDS–PAGE resulted in an average value of 67 000. The coincidence of these two values showed that arginyl-tRNA synthetase consists of a single polypeptide chain.

Absorption Coefficient Determination

We used an enzyme solution of known absorbance at 280 nm and measured its concentration by the method of Lowry modified by Bensadoun and Weinstein (1976) using BSA as the standard. The average value of A_{280} (1 mg/mL) was about 1.29.

Active Enzyme Site

The active-site titration (Fersht, 1977) could not be used with the arginyl-tRNA synthetase, since no aminoacyl adenylate was detected in the absence of tRNA. The active enzyme concentration was determined with equilibrium dialysis from the number of arginine binding sites as shown in the following paper. That method had been found to agree well with active site titration (Mulvey & Fersht, 1977). The enzyme binds 1 mol of arginine per polypeptide chain of ArgRS both in the absence and in the presence of tRNA. The stoichiometry of $tRNA^{Arg}$ binding was monitored by intrinsic fluorescence titration of the enzyme and found to be 1:1 (for detailed results, refer to the following paper).

Steady-State Kinetics

Aminoacylation. The reaction rate was optimal at pH 8 when 100 mM Hepes buffer was employed, and the optimal excess magnesium ion concentration was 3–4 mM, as deduced from Mg^{2+} titration curves (data not shown) in the presence of several ATP concentrations (2, 4, or 8 mM) in pH 7.4 Tris buffer. A higher Mg^{2+} concentration showed a slight inhibition for the aminoacylation reaction.

The determination of K_m and V_{max} values for the aminoacylation was carried out as described under Materials and Methods. When the K_m for ATP was measured, the ATP–Mg^{2+} complex was considered as the substrate varied, the concentration of which is close to the input ATP concentration under our conditions (Storer & Cornish-bowden, 1976). In the determination of K_m for tRNA in the aminoacylation, a higher specific activity (54 μCi/μmol) for radioactive arginine was used than for the other K_m determinations, and aliquots of larger volume (60 μL) were applied to Whatman 3 MM paper discs to raise the signal for the low tRNA concentration region. The initial velocity condition for the experimental work was always maintained (final aminoacylated tRNA < 15–20% input tRNA). This method was effective.

The K_m values at pH 7.4 for arginine, $tRNA^{Arg}$, and ATP were found to be 12 μM, 2.5 μM, and 0.9 mM, respectively. The K_m for tRNA is considerably higher than those of other aminoacyl-tRNA synthetase systems or even those of the arginyl-tRNA synthetases from other sources, e.g., 0.2 μM for the yeast phenylalanyl-tRNA synthetase (Fasiolo et al., 1970; Lefevre et al., 1980), 0.05 μM for the yeast valyl-tRNA synthetase (Kern et al., 1975), and 0.31, 0.7, 0.28 μM for the ArgRS of *B. stearothermophilus* (Parfait & Grosjean, 1972), *N. crassa* (Nazario & Evans, 1974), and *Saccharomyces cerevisiae* (Gangloff et al., 1986), respectively. The maximum velocity reached about 22 000–24 000 units/mg of protein or 25–28 s^{-1} for the turnover of moles of arginyl-tRNA per mole of enzyme at pH 7.4, 37 °C.

ATP–PP_i Exchange Reaction. This reaction proceeded with an optimal pH of 6.8 in 130 mM Hepes buffer (only in the pH optimum test was Hepes buffer employed). A significant displacement of the pH dependence of aminoacylation and exchange reaction could be seen in Figure 3. At pH 7.2 (Tris buffer), K_m values for the exchange reaction of 0.11 mM, 2.9

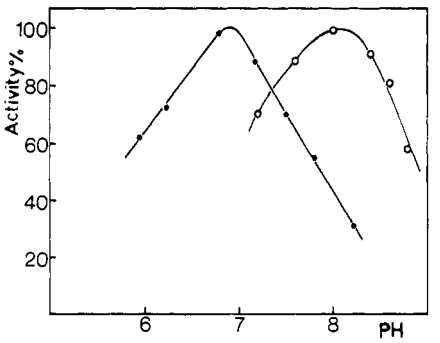

FIGURE 3: pH dependence of aminoacylation and ATP–PP_i exchange reaction with ArgRS at 37 °C. (○) Aminoacylation. The reaction mixture contained 100 mM Hepes buffer, 30 mM KCl, 7–8 mM $MgCl_2$, 4 mM ATP, 0.1 mM EDTA, 0.5 mM DTT, 5 μM $tRNA^{Arg}$, 0.2 mg/mL BSA, and 0.8 nM ArgRS. (●) ATP–PP_i exchange. The reaction mixture contained 130 mM Hepes buffer, 6 mM $MgCl_2$, 2 mM ATP, 2 mM arginine, 2 mM [^{32}P]tetrasodium pyrophosphate, 10 μM $tRNA^{Arg}$, 0.2 mg/mL BSA, and 6 nM ArgRS.

mM, and 0.5 mM were obtained for arginine, ATP, and PP_i, respectively. No difference was found for the exchange reaction in the presence of 10 μM and 20 μM $tRNA^{Arg}$. The V_{max} is about 34 000 units/mg of protein or 40 s^{-1} for the turnover number of [^{32}P]ATP production.

No exchange could be found in the absence of tRNA. For both aminoacylation and exchange reactions, parallel experiments with pure $tRNA^{Arg}$ or total tRNA containing the same amount of $tRNA^{Arg}$ produced the same results.

Discussion

With the procedure reported in this paper, the relatively low content of ArgRS of *E. coli* could be conveniently purified to more than 95% homogeneity with three gradient chromatographic steps yielding an ArgRS preparation with high specific activity. Our preparation charges about 22 000–24 000 nmol of $tRNA^{Arg}$/mg of protein in 1 min at pH 7.4, 37 °C, which is significantly more than the previously reported values: 2000 (pH 8, 37 °C; Mitra & Mehler, 1967), 1400 (pH 7.8 37 °C; Marshall & Zamecnik, 1969), 1120 (pH 7.0, 30 °C; Hirshfield & Bloemers, 1969), 2350 (pH 7.0, 30 °C; Craine & Peterkofsky, 1975), and 5000 (pH 7.4, 37 °C; Charlier & Gerlo, 1979).

The recovery for ArgRS of 30–40% (with a high purification ratio of about 1200-fold) was much better than those of previously reported procedures: 3% (200-fold) for *E. coli* K12 (Hirshfield & Bloemers, 1969), 2% (525-fold) (Mitra & Mehler, 1967) and 10% (780-fold) (Craine & Peterkofsky, 1975) for *E. coli* B, and even higher than the 17% yield (1000-fold) reported by Charlier and Gerlo (1979) for *E. coli* K12. This purification procedure with the affinity chromatography laid down a foundation for further research of this aminoacyl-tRNA synthetase (refer to the following paper).

The molecular weight of our preparation, about 70 000, is very close to the 73 000 value for the *E. coli* K12 ArgRS reported by Hirshfield and Bloemer (1969) and somewhat higher than the 60 000 value reported by Charlier and Gerlo (1979) for the *E. coli* K12 enzyme or the 63 000 value reported by Craine and Peterkofsky (1975) for the *E. coli* B enzyme. Our value is similar to the molecular weight obtained for ArgRS from other sources, e.g., 73 000 for baker's yeast by Gongloff et al. (1976), 72 000 for brewer's yeast by Thiebe (1983), and 78 000 for *B. stearothermophilus* by Parfait and Grosjean (1972).

Our preparation resulted in an ArgRS consisting of a single polypeptide chain, which agrees with most of the published results [e.g., Hirshfield and Bloemers (1969), Craine and Peterkofsky (1975), and Charlier and Gerlo (1979)].

Very different specificities even for the same aminoacyl-tRNA synthetase from the same origin can often be found in the literature (e.g., for arginyl-tRNA synthetase discussed above). This difference is likely related to the different purification procedures, and perhaps to proteolysis in some cases. We suggest that it is also partly induced by the diversity of tRNA concentrations used in different studies which might affect both the initial rate condition and the saturation coefficient (this coefficient lowers the observed v from V_{max}, but only the latter is significant for comparisons between different studies). In our study, we used a relatively high concentration of tRNA corresponding to 5 μM in $tRNA^{Arg}$, and the extent of the reaction never exceeded 15–20% of its charge, thus meeting the initial velocity condition for the experimental work. The influence of tRNA concentration becomes important with systems of high K_m for tRNA. For example, in our enzyme activity assay, in fact, if we use 5 μM $tRNA^{Arg}$, the saturation coefficient $S = 0.67$ lowered the observed velocity 33% below V_{max} (the comparative value in the purification procedure of our work could be corrected by this coefficient to give the true V_{max}).

In most aminoacyl-tRNA synthetase systems reported, the K_m for tRNA was on the order of 10^{-8}–10^{-7} M. The relative elevated K_m value is characteristic of this system. This value gave rise to some discussions in the following paper.

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Interactions between *Escherichia coli* Arginyl-tRNA Synthetase and Its Substrates†

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ABSTRACT: Interactions between *Escherichia coli* arginyl-tRNA synthetase and its substrates were extensively studied and distinctly demonstrated. Various approaches such as equilibrium dialysis, fluorescence titration, and substrate protection against heat inactivation of the enzyme were used for these studies. In the absence of other substrates, the equilibrium dissociation constants for arginine, ATP, and the cognate tRNA were about 70 μM, 0.85 mM, and 0.45 μM, respectively, at pH 7.5, in Tris buffer. The binding of arginine to the enzyme was affected neither by the presence of tRNA nor by the presence of ATP but was considerably enhanced when ATP and tRNA were both present at saturating concentrations. The dissociation constant in this case (about 16 μM) was very close to the K_m (12 μM) for arginine during aminoacylation. The binding of ATP (the equilibrium dissociation constant $K_D \approx 0.85$ mM) was not affected by the presence of arginine but was depressed in the presence of tRNA (K_D became 3 mM). Arginyl-tRNA showed a dissociation constant of (4–5) $\times 10^{-7}$ M which was not affected by the presence of a single other substrate. Possible explanations for the high K_m for tRNA in the aminoacylation are discussed. Our results indicated pronounced interactions between substrates mediated by the enzyme under catalytic conditions. Periodate oxidation did not alter the tRNA binding to the enzyme. The oxidized tRNA still afforded protection against heat inactivation of the enzyme.

The mechanism of a small group of aminoacyl-tRNA synthetases represented by arginyl-tRNA synthetase (and glutamyl- and glutaminyl-tRNA synthetases) has long been discussed [e.g., Mitra and Mehler (1967), Craine and Peterkofsky, (1975), Fersht et al. (1978), Charlier and Gerlo (1979), Thiebe (1983), and Char and Gopinathan (1986)]. Numerous studies have been devoted to the interactions between the synthetases and their substrates.

For example, for the arginyl-tRNA synthetase, Mitra et al. (1970) reported that arginine and tRNA protected the *Escherichia coli* B enzyme against heat inactivation but ATP did not (pH 5.5, Mes buffer). Arginine and tRNA showed a "synergistic protection" (pH 8, Tris, or pH 5.5, Mes).[1] Differently, Charlier and Gerlo (1979) reported that for the *E. coli* K12 enzyme no influence was observed between the binding of arginine and the binding of other substrates whereas the presence of ATP decreased the binding of tRNA and vice versa through steady-state kinetics with initial velocity and inhibition studies (pH 7.4, Hepes).

Parfait and Grosjean (1972) reported for the enzyme from *Bacillus stearothermophilus* that arginine was bound only after the binding of tRNA and ATP; the addition of substrates is ordered sequential (pH 7.4, Hepes). Nazario and Evens (1974) also reported the ordered sequential mechanism in *Neurospora crassa*, but more reports with *E. coli* B (Papas

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[1] Abbreviations: Arg, arginine; ArgRS, arginyl-tRNA synthetase; $tRNA^{Arg}$, arginine-specific tRNA; DTT, dithiothreitol; $K_{S_1}^{S_2}$, equilibrium dissociation constant of substrate S_1 in the presence of S_2; $K_{S_1}^{S_2S_3}$, dissociation constant of substrate S_1 in the copresence of S_2 and S_3; Mes, 2-(*N*-morpholino)ethanesulfonic acid; S_1–S_3, any of the three substrates—arginine, ATP, and $tRNA^{Arg}$; Tris, tris(hydroxymethyl)aminomethane; $tRNA_{ox}^{Arg}$, $tRNA^{Arg}$ of which the 3′-terminal adenosine is oxidized by periodate.

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