Arginyl-tRNA Synthetase from *Escherichia coli* K12. Purification, Properties, and Sequence of Substrate Addition

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ABSTRACT: Arginyl-tRNA synthetase from *Escherichia coli* K12 has been purified more than 1000-fold with a recovery of 17%. The enzyme consists of a single polypeptide chain of about 60 000 molecular weight and has only one cysteine residue which is essential for enzymatic activity. Transfer ribonucleic acid completely protects the enzyme against inactivation by *p*-hydroxymercuribenzoate. The enzyme catalyzes the esterification of 5000 nmol of arginine to transfer ribonucleic acid in 1 min/mg of protein at 37 °C and pH 7.4. One mole of ATP is consumed for each mole of arginyl-tRNA formed. The sequence of substrate binding has been investigated by using initial velocity experiments and dead-end and product inhibition studies. The kinetic patterns are consistent with a random addition of substrates with all steps in rapid equilibrium except for the interconversion of the central quaternary complexes. The dissociation constants of the different enzyme–substrate complexes and of the complexes with the dead-end inhibitors homoarginine and 8-azido-ATP have been calculated on this basis. Binding of ATP to the enzyme is influenced by tRNA and vice versa.

The proper attachment of amino acids to their cognate tRNAs catalyzed by the aminoacyl-tRNA synthetases is one of the crucial steps for a fidel translation of the genetic code. Arginyl-tRNA synthetases from *Escherichia coli* (Mitra & Mehler, 1966, 1967; Mehler & Mitra, 1967) and from yeast (Mitra & Smith, 1969) together with glutamyl- and glutaminyl-tRNA synthetases from *E. coli* (Ravel et al., 1965; Lapointe & Söll, 1972), yeast (Lee et al., 1967), and liver (Deutscher, 1967) were well known to differ from the other synthetases for not catalyzing an ATP–PP_i exchange in the absence of tRNA. This focused much research on the detailed mechanism ("sequential" or "concerted") of this class of enzymes, which is not yet completely elucidated. In particular, for the arginyl-tRNA ligase, a concerted mechanism has been suggested for the *Bacillus stearothermophilus* enzyme (Parfait & Grosjean, 1972), while formation of arginyladenylate as an intermediate of the reaction has been proposed for the enzyme from *Neurospora crassa* (Nazario & Evans, 1974) and yeast (Fersht et al., 1978). An ordered sequence of substrate addition for both was indicated by kinetic analysis. A random sequential mechanism was found for the corresponding enzyme from *E. coli* B (Papas & Peterkofsky, 1972). No conclusion of whether the reaction involves an arginyladenylate or proceeds by a concerted mechanism could be drawn. In previous studies, Mehler & Mitra (1967) suggested a model in which binding of tRNA to the enzyme was necessary for the initial activation step to form enzyme-bound arginyladenylate. Loftfield (1972), on the other hand, argues that the requirement of tRNA for the ATP–pyrophosphate exchange was an important argument in favor of a concerted mechanism.

This paper describes an improved method for the purification of *E. coli* K12 and some of the physical and chemical properties of the enzyme. A detailed kinetic analysis by the methods outlined by Cleland (1963b,c, 1970) was performed with the pure enzyme. The results are consistent with a random rapid equilibrium mechanism.

Experimental Procedure

Materials. [^{14}C]Arginine and other radioactive amino acids were Stan Star Schwarz/Mann products. *E. coli* K12 tRNA from Schwarz/Mann was purified as described by Zubay (1962). Crystallized bovine plasma albumin used as a marker in gel-filtration experiments was from Armour Pharmaceutical Co. (England). Firefly lantern extract was from Sigma. The *E. coli* K12 strain we used was an *argB argG* mutant from Hfr P4X (Crabeel et al., 1975). Cells were cultured at 37 °C in the Labline fermentor in rich medium containing bactotryptone (11 g/L), yeast extract (23 g/L), and glycerol (4 ml/L). The pH was adjusted to 7.3 with NaOH. The 0.066 M (final concentration) potassium phosphate buffer (pH 7.3) was sterilized separately. tRNA extracted (Brubaker & McCorquodale, 1963) from this strain and purified by DEAE-cellulose chromatography (Stephenson & Zamecnik, 1961) followed by stripping (Sarin & Zamecnik, 1964) was used for the kinetic measurements. The concentration of $tRNA^{Arg}$ was about 5%. Periodate oxidation of total tRNA was performed according to Norris-Baldwin & Berg (1966). 8-Azido-ATP (Freist et al., 1978) was a gift of Dr. W. Freist.

Enzymatic Measurements. Arginyl-tRNA synthetase activity was monitored as described previously (Charlier & Gerlo, 1976). One unit of enzyme catalyzes the aminoacylation of 1 nmol of tRNA in 1 min at 37 °C. The ATP consumption during the arginine charging of tRNA was followed by using the ATP-luciferase assay as described earlier for the isoleucine activating enzyme, but by omitting the heating step (Charlier & Grosjean, 1972; Godeau & Charlier, 1979).

Protein Determinations. Protein concentrations were determined both spectrophotometrically (Ehresmann et al., 1973) and by the method of Lowry et al. (1951) with bovine serum albumin as the standard. The two techniques were consistent within a 10% range for the different purification steps.

Polyacrylamide Gel Electrophoresis. Analytical polyacrylamide gel electrophoresis at pH 9.0 was performed according to Davis (1964). Sodium dodecyl sulfate gel electrophoresis after denaturation in the presence of dithioerythritol was performed as described by Godeau (1976).

Amino Acid Composition Analysis. The enzyme sample (90 μg) was dialyzed against 1 M acetic acid. After being freeze-dried, the protein was divided into three portions and subjected to hydrolysis in 6 N HCl containing phenol at 110 °C for 24, 48, and 72 h. The hydrolysates were analyzed with a D500 Durrum amino acid analyzer. Tryptophan was estimated spectrophotometrically (Beaven & Holiday, 1952). The sulfhydryl groups were determined spectrophotometrically

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0006-2960/79/0418-3171$01.00/0

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

enzyme fraction	total protein (mg)	total act. (units)	sp act. (units/mg)	yield (%)	purifcn (*x*-fold)
(1) crude extract	2800	12430	4.4	100	1
(2) streptomycin supernatant	2300	10780	4.7	87	1.1
(3) DEAE-cellulose	365	8620	23.6	69	5.4
(4) first phosphocellulose	5.0	5390	1078	43	245
(5) Ultrogel[b]	4.8	9670	2007	31[c]	450
(6) second phosphocellulose	1.1	5265	4984	17[c]	1120

[a] From 100 g of frozen cell paste. [b] Step 5 was performed on the combined extracts of two procedures (13.6 mg of protein, 13 280 units). [c] Calculated with the units loaded on the Ultrogel taken as 43%.

at 412 nm with Ellman's reagent (DTNB[1]) in 8 M urea at pH 8.0, after precipitation and washing of the enzyme with trichloroacetic acid. The SH content was calculated by using a molar extinction coëfficient of 13 600 (Ellman, 1959).

Inactivation of Arginyl-tRNA Synthetase by p-Hydroxymercuribenzoate. The inactivation of the arginyl-tRNA synthetase by pMB was studied by incubating the enzyme (90 ng/mL) in 0.01 M Hepes–NaOH, pH 7.4, in the presence of pMB (1 μM unless otherwise stated) at 25.6 °C. Aliquots of 10 μL were removed at different time intervals, and the residual arginine charging activity was measured immediately at 37 °C. The final volume of the assay mixture was 100 μL. It was verified that the observed inactivation was solely due to the reaction of pMB with the synthetase and not to any reaction with another component of the medium—for instance, tRNA (Stern et al., 1966)—by preincubating the reaction medium with 1 μM pMB for 15 min at 25.6 °C.

Kinetic Analysis. The kinetic nomenclature of Cleland (1963a) was used. In all initial velocity studies, conditions were selected that ensured linear reaction rates. The reaction mixtures contained, in a total volume of 0.2 mL, 0.1 M Hepes–NaOH buffer (pH 7.4), 10 μg of serum albumin, [^{14}C]arginine (different concentrations from 1 to 10 μM), ATP (0.49–4.9 mM), *E. coli* K12 tRNA (0.28–0.94 mg/mL), $MgCl_2$ (1.5 mM in excess over the ATP concentration), and 0.2 nM purified ligase (2.2 ng). For the experiments in the presence of inhibitors, the fixed substrate concentrations were 2.5 μM arginine, 1 mM ATP, 0.3 mg/mL tRNA. The following inhibitors were tested: homoarginine (1, 3, and 5 mM), 8-azido-ATP (1.4 and 2.8 mM), periodate-oxidized tRNA (0.28 and 0.84 mg/mL), AMP (0.9, 1.8, and 4.3 mM), and pyrophosphate (0.2, 0.4, and 0.6 mM). When 8-azido-ATP, AMP, or pyrophosphate was added, $MgCl_2$ was 1.5 mM in excess over ATP plus inhibitor. Incubations were at 37 °C for 2.5 min. The extent of reaction never exceeded 20% of the total $tRNA^{Arg}$ present and usually was considerably less. Reaction rates (v) are expressed in moles of arginyl-tRNA formed per mole of enzyme in 1 min (min^{-1}). Linear plots were calculated by using the least-squares method (Cleland, 1967a).

Results

Purification Procedure. All steps were carried out at 4 °C.

(1) Cell Disruption. Frozen *E. coli* K12 cells (100 g of cell paste) were resuspended in 100 mL of 0.01 M potassium phosphate, pH 7.5, containing 0.1 mM dithioerythritol and 2 mM phenylmethanesulfonyl fluoride to prevent proteolytic degradation. After addition of a few mg of DNase (RNase free), the cells were lysed by a two-step procedure of each 20 min under 1500 psi of N_2 pressure in a Parr lysis bomb, and the extract was centrifuged at 9000*g* for 45 min. When necessary, the supernatant (Table I, line 1) was diluted to 20 mg of protein per mL.

(2) Streptomycin Precipitation. One-fifth volume of 6% streptomycin sulfate solution was added dropwise to the crude extract under magnetic stirring. After 30 more min of stirring, the precipitate was removed by centrifugation for 30 min at 9000*g*.

(3) DEAE-cellulose Chromatography. The supernatant after streptomycin sulfate precipitation (Table I, line 2) was loaded onto a DEAE-cellulose column (2.5 × 50 cm), equilibrated with 0.01 M potassium phosphate, pH 7.5, with 0.1 mM dithioerythritol, at a flow rate of 90–100 mL/h. The column was washed with about 2 column volumes of the same buffer and eluted by using a potassium phosphate gradient with 500 mL of 0.05 M buffer, pH 7.5, in the mixing chamber and 500 mL of 0.3 M buffer, pH 6.3, in the reservoir. Arginyl-tRNA synthetase activity was eluted at about 0.15 M potassium phosphate. Approximately 80% of the peak fractions were pooled (Table I, line 3). The volume was reduced to less than 110 mL by ultrafiltration through a Diaflo PM 10 membrane, and the solution was passed through a Sephadex G-25 column (3.8 × 38 cm, equilibrated with 0.01 M potassium phosphate, pH 6.0, containing 0.1 mM dithioerythritol) at a flow rate of 120 mL/h, in order to change the buffer. Fractions containing synthetase activity (eluting in the void volume) were pooled. Low-molecular-weight, ultraviolet-absorbing material was removed at this stage.

(4) Phosphocellulose Chromatography [Affinity Elution Adapted from Von der Haar (1973)]. Phosphocellulose was packed and equilibrated in a column (3.8 × 20 cm) with 0.01 M potassium phosphate, pH 6.0, containing 10% (v/v) glycerol and 0.1 mM dithioerythritol. The protein was applied, and the column was washed extensively with the initial buffer until no more ultraviolet-absorbing material was eluted. About 60% of the applied material is washed off. The arginyl-tRNA synthetase activity was eluted with a linear tRNA gradient (0–0.025 mg/mL crude tRNA) in the same buffer. The pH and ionic conditions for the adsorption of the synthetase are quite critical. Pooled active fractions were loaded on a small DEAE-cellulose column (1.3 × 10 cm, equilibrated with 0.01 M potassium phosphate, pH 7.5). Protein was eluted with the initial buffer containing 0.25 M NaCl, while tRNA remained adsorbed. At this stage (Table I, line 4), the enzyme preparation was stored at −20 °C in 50% (v/v) glycerol.

(5) Ultrogel AcA44 Gel Filtration. An Ultrogel AcA44 column (2.5 × 150 cm, equilibrated with 0.1 M potassium phosphate, pH 7.5, containing 0.1 mM dithioerythritol) was loaded at a flow rate of 8 mL/h with the combined and concentrated extracts from two procedures. The peak fractions containing arginyl-tRNA synthetase activity were pooled (Table I, line 5). At this stage, sodium dodecyl sulfate gel electrophoresis revealed two bands corresponding to molecular

[1] Abbreviations used: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); pMB, *p*-(hydroxymercuri)benzoate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

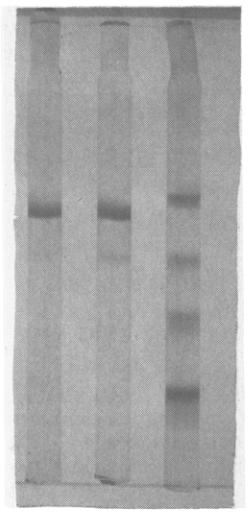

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of purified arginyl-tRNA synthetase (Table I, line 6). The left gel was loaded with 27 μg and the second with 45 μg of protein. The right gel shows the migration of marker proteins, serum albumin (67 000), ovalbumin (45 000), chymotrypsinogen (25 000), and cytochrome *c* (12 500).

weights of 60 000 and 40 000.

(*6*) *Second Phosphocellulose Chromatography*. In order to remove the contaminating 40 000-dalton protein, a second phosphocellulose affinity elution was set up. Buffer was changed by gel filtration through Sephadex G-25 (see above), and the enzyme extract was adsorbed on a P11 phosphocellulose column exactly as described in step 4. Most of the low molecular weight protein was washed out with the 0.01 M potassium phosphate buffer, pH 6.0, containing 10% (v/v) glycerol and 0.1 mM dithioerythritol, while arginyl-tRNA synthetase activity was eluted with the tRNA gradient. Active fractions were pooled and loaded on a small DEAE-cellulose column (1.3 × 5 cm). The enzyme was eluted with 0.25 M NaCl added to the initial 0.01 M potassium phosphate buffer, pH 7.5, while tRNA remained adsorbed. The fractions with arginyl-tRNA synthetase activity were pooled, concentrated by ultrafiltration by using a PM10 membrane, and dialyzed extensively against 0.01 M Hepes, pH 7.4, containing 0.1 mM dithioerythritol. The dialysate (Table I, line 6) was made 50% (v/v) in glycerol, aliquoted into 100-μL and 200-μL fractions, and stored at −20 °C.

The purification procedure is summarized in Table I. The enzyme has been purified 1000-fold from the crude extract with a recovery of 17%. In previously published procedures, the purifications of arginyl-tRNA synthetase from *E. coli* K12 (Hirshfield & Bloemers, 1969) or *E. coli* strain B (Mitra & Mehler, 1967; Marshall & Zamecnik, 1969; Craine & Peterkofsky, 1975) were 200- and 800-fold, respectively, with recovery of 3% and 12% of the activity. Our final preparation catalyzed the esterification of 5000 nmol of arginine to tRNA in 1 min/(mg of protein) (37 °C, pH 7.4), which is significantly more than the values previously reported: 1120 (37 °C, pH 7.0; Hirshfield & Bloemers, 1969), 1400 (37 °C, pH 7.8; Marshall & Zamecnik, 1969), 2000 (37 °C, pH 8; Mitra & Mehler, 1967), 235 (30 °C, pH 7.0; Craine & Peterkofsky, 1975). The preparation was about 95% homogeneous as judged by polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate: a single major band was observed and one minor band accounting for less than 5% of total protein (cf. Figure 1). The ratio of absorbance at 280 nm to the absorbance at 260 nm of the preparation was 1.38. The absorption spectrum was not altered by extensive dialysis, by gel filtration, by DEAE-cellulose chromatography, or by RNase treatment of the preparation. It could be an indication of the presence of a nonprotein substance tightly bound to the enzyme. The nature of this substance, however, could not be identified.

Activity with Other Amino Acids. When the preparation was tested for tRNA charging with 18 other amino acids (Asp, Asn, Ala, Cys, Gly, Glu, Gln, His, Ile, Leu, Lys, Phe, Pro, Ser, Tyr, Thr, Try, Val), less than 0.1% of the activity with arginine was observed.

Storage. The enzyme activity was affected by repeated freeze–thaw cycles when stored at −20 °C in the absence of glycerol or at −70 °C in the presence of 50% (v/v) glycerol. No appreciable loss of activity occurred for at least 6 months when the enzyme was stored at −20 °C in 50% (v/v) glycerol.

Molecular Weight Determination. The molecular weight of arginyl-tRNA synthetase from *E. coli* K12 was determined by different techniques. Gel-filtration experiments which use a Sephadex G-100 superfine column (2.5 × 95 cm) or an Ultrogel AcA44 column (2.5 × 146 cm), equilibrated with 0.1 M potassium phosphate buffer, pH 7.0, containing 10% (v/v) glycerol and 0.1 mM dithioerythritol gave identical results with different enzyme preparations. An average molecular weight of 63 000 was obtained from four different experiments with cytochrome *c*, ovalbumin, and bovine serum albumin as reference proteins. Disc gel electrophoresis in the presence of sodium dodecyl sulfate gave an average molecular weight of 60 000 ± 2000 with RNA polymerase, bovine serum albumin, and chymotrypsinogen as markers (average of nine determinations).

These results suggest that the enzyme is not composed of subunits. Our value for the molecular weight of arginyl-tRNA synthetase is somewhat lower than the one reported by Hirshfield & Bloemers (1969) for the *E. coli* K12 enzyme (73 000) and is the same as the value found by Craine & Peterkofsky (1975) for the *E. coli* B enzyme. Marshall & Zamecnik (1969) reported a molecular weight of 40 000 for the *E. coli* B enzyme, as determined by gel filtration. By sedimentation equilibrium methods, their synthetase appeared to have a molecular weight of about 75 000. They suggested that this was caused by dimerization which was prevented by diffusion of the enzyme in the gel filtration. This interpretation is not in keeping with our observations and with those of Hirshfield & Bloemers (1969) and those of Craine & Peterkofsky (1975). The value of 60 000 is similar to the molecular weight of arginyl-tRNA synthetases from other organisms, for example, *Bacillus stearothermophilus* (59 000) (J. M. Godeau, manuscript in preparation), *Neurospora crassa* (85 000) (Nazario & Evans, 1974), and Baker's yeast (73 000) (Gangloff et al., 1976).

Amino Acid Composition. The number of amino acid residues, calculated for a molecular weight of 60 000, is shown in Table II. The amino acid analysis revealed one extra peak in a position not corresponding to one of the common amino acids. It might be possible that this position corresponds to a decomposition product of histidine, but this could not be proven. It is noted that Yue & Schimmel (1977) also found a "X residue" in the isoleucyl-tRNA ligase ATP binding site which does not correspond to any of the natural amino acids and has not yet been identified. The percentage composition (100 × number of residues of a particular amino acid/total number of residues) is very similar to that of the enzymes of Baker's yeast (Gangloff et al., 1976) and *Neurospora crassa* (Nazario & Evans, 1974). The values for the *E. coli* B enzyme (Marshall & Zamecnik, 1969), on the contrary, differ sig-

Table II: Amino Acid Composition of Arginyl-tRNA Synthetase

amino acid	no. of residues[a]	amino acid	no. of residues[a]
Asp + Asn	47	Ile[c]	21
Thr	24	Leu	47
Ser	24	Tyr	18
Glu + Gln	61	Phe	14
Pro	15	X[e]	46
Gly	50	His	13
Ala	57	Lys	28
Cys[b]	1	Arg	25
Val[c]	31	Trp[f]	8
Met[d]	18		

[a] Calculation of the number of residues was based on a molecular weight of 60 000. Results are the average of three determinations (24-, 48-, and 72-h hydrolysis). [b] Colorimetric determination with DTNB. [c] Value after 72-h hydrolysis. [d] Value obtained by extrapolation to zero-time hydrolysis. [e] Unknown. [f] Spectrophotometric determination.

Table III: Effect of Substrates on the Inactivation of Arginyl-tRNA Synthetase (90 ng/mL) by *p*-(Hydroxymercuri)benzoate (pMB) at 25.6 °C[a]

pMB (μM)	added substrate	% act.
0		86
1		22
1	tRNA	85
1	ATP + Mg^{2+}	4
1	Arg	37
1	ATP + Mg^{2+} + Arg	4
1	ATP + Mg^{2+} + tRNA	90
1	tRNA + Arg	87
1	dithioerythritol (1 mM)	93
10		0
10	tRNA	76

[a] Measurements were made at different time intervals as described under Experimental Procedure. The percentage activity remaining after 10-min incubation under different conditions is given. When added, substrate concentrations were 3.2 mg/mL tRNA, 5 mM $MgCl_2$, 5 mM ATP, 10 μM [^{14}C]Arg.

nificantly from our values for the *E. coli* K12 protein. As Marshall & Zamecnik (1969) found a molecular weight of only 40 000 in some experiments, it could be possible that their amino acid analysis was performed on a proteolyzed form of the native enzyme.

Effect of Buffer and pH on tRNA Esterification. The pH curve displays a broad pH optimum between pH 8.1 and 8.5 and is comparable with those previously published (Mitra & Mehler, 1967; Hirshfield & Bloemers, 1969). The activity at pH 8 is higher in Hepes than in Tris, glycine, or glycylglycine buffers (all 0.1 M).

Effect of Dithioerythritol and *p-(Hydroxymercuri)benzoate.* Titration of denaturated arginyl-tRNA synthetase with DTNB revealed one sulfhydryl group per enzyme molecule. Together with the identical results obtained by sodium dodecyl sulfate gel electrophoresis under reducing (presence of dithioerythritol) and nonreducing denaturation conditions, this indicates the probable absence of disulfide bridges in arginyl-tRNA synthetase from *E. coli* K12. The [^{14}C]arginyl-tRNA formation rate was identical whether the synthetase (stored in the presence of 0.1 mM dithioerythritol) was diluted in 0.01 M Hepes buffer with or without 0.1 mM dithioerythritol and was not dependent on the presence of dithioerythritol (1 mM) in the reaction mixture. This led us to investigate if the sulfhydryl group of the enzyme is essential for aminoacylation of tRNA by inhibition studies with pMB. pMB-inactivation kinetics were measured at 25.6 °C in the absence and in the presence of the different substrates (Table III).

There was no loss of catalytic activity during exposure to pMB (1 μM) in the presence of 1 mM dithioerythritol. The inactivation rate of arginyl-tRNA synthetase incubated with pMB was increased in the presence of ATP and of ATP plus arginine. A complete protection was afforded by tRNA, alone or in combination with ATP or arginine. This protection by tRNA was also effective when the pMB concentration was increased ten times. Further investigations are necessary in order to find out if the sulfhydryl group is involved in the binding of tRNA or in the formation of a thioester intermediate as suggested by Baltzinger & Remy (1977).

ATP Consumption. ATP used up by arginyl-tRNA synthetase during the aminoacylation reaction was measured by dosage of the residual ATP with firefly lantern luciferase. Since a net decrease of ATP concentration was to be observed, the substrate range which could be investigated was rather limited: in order to obtain an appreciable decay, the initial ATP concentration may not be too high. With an initial concentration of 10 μM, we were able to follow an ATP consumption by arginyl-tRNA synthetase of *E. coli* at different enzyme concentrations, over a range of 60 min. In these conditions, the substrate ATP was used up in an apparent first-order kinetic process, with a rate constant proportional to the enzyme concentration. The ATP decline was significant only when arginine and tRNA were present and aminoacylation occurred. Control experiments without enzyme, or in the presence of enzyme but by omitting arginine or tRNA, showed no ATP decay. When tRNA was replaced by a similar amount of periodate-oxidized tRNA, ATP was not consumed. The [^{14}C]arginine charging onto tRNA was measured in identical conditions. This allowed us to estimate the number of arginyl-tRNA molecules formed per ATP molecule consumed. Such an evaluation was based on the initial rate of [^{14}C]arginyl-tRNA formation (82 nM min^{-1} at a synthetase concentration of 0.45 μg/mL) and on the initial rate of ATP consumption ($v = k[ATP]_0$), derived from the apparent first-order rate constant for the ATP decay ($k = 0.008$ min^{-1} at the same synthetase concentration and an initial ATP concentration $[ATP]_0 = 10$ μM). The ratio of these values is equal to one (within experimental error, which was estimated to be less than 7%), indicating that one arginyl-tRNA is formed for every ATP consumed by arginyl-tRNA synthetase from *E. coli*. No "extra-degradation" of ATP (degradation of ATP which is not coupled to the formation of aminoacyl-tRNA) thus occurs under the given experimental conditions.

Kinetic Studies. Initial velocity studies were undertaken in order to determine the order of substrate addition. As nonenzymatic hydrolysis of arginyl-tRNA is appreciable at the optimum pH ($k = 0.13$ min^{-1} at pH 8.35), the kinetic experiments were performed in buffer of pH 7.4, where the rate of this hydrolysis is appreciably reduced ($k = 0.03$ min^{-1}). Figure 2 shows the dependence on $MgCl_2$ concentration of the rate of [^{14}C]arginyl-tRNA formation at different ATP concentrations at this pH. For each ATP concentration, the optimum $MgCl_2$ concentration which gave a maximum rate was about 1.5 mM in excess over ATP. Higher concentrations were inhibitory; however, the effect was very small at ATP concentrations lower than 1 mM. The optimum $MgCl_2$ concentration was identical at different tRNA concentrations up to 1 mg/mL. Based on these results, the $MgCl_2$ concentrations were adjusted to 1.5 mM in excess over the ATP concentrations which is consistent with the findings of others (Cleland, 1967b; Storer & Cornish-Bowden, 1976) that a constant excess of magnesium is to be preferred over a fixed

Table IV: Inhibition Characters of Homoarginine, 8-Azido-ATP, and Periodate-Oxidized tRNA

inhibitor	variable substrate	fixed substrates	pH	type of inhibn[a]	app K_i from slope	app K_i from intercept
Har	Arg	ATP (0.93 mM), tRNA (0.29 mg/mL)	7.4	C	2.4 mM	
	tRNA	ATP (0.93 mM), Arg (2.5 μM)	7.4	NC	3.3 mM	3.7 mM
	ATP	tRNA (0.29 mg/mL), Arg (2.5 μM)	7.4	NC	3.9 mM	4.9 mM
8-azido-ATP	Arg	ATP (1 mM), tRNA (0.55 mg/mL)	7.4	NC	1.0 mM	4.8 mM
	tRNA	ATP (1 mM), Arg (5 μM)	7.4	NC	0.9 mM	3.9 mM
	tRNA	ATP (1 mM), Arg (55 μM)	7.4	NC	1.6 mM	3.2 mM
	ATP	tRNA (1.4 mg/mL), Arg (50 μM)	7.4	C	1.8 mM	
$tRNA_{ox}$	Arg	ATP (0.95 mM), tRNA (0.30 mg/mL)	7.4	NC	0.49 mg/mL	1.7 mg/mL
	tRNA	ATP (0.95 mM), Arg (2.5 μM)	7.4	NC	0.19 mg/mL	2.4 mg/mL
	tRNA	ATP (0.6 mM), Arg (2.5 μM)	8.35	C	0.58 mg/mL	
	ATP	tRNA (0.30 mg/mL), Arg (2.5 μM)	7.4	NC	0.42 mg/mL	1.5 mg/mL

[a] C, competitive; NC, noncompetitive.

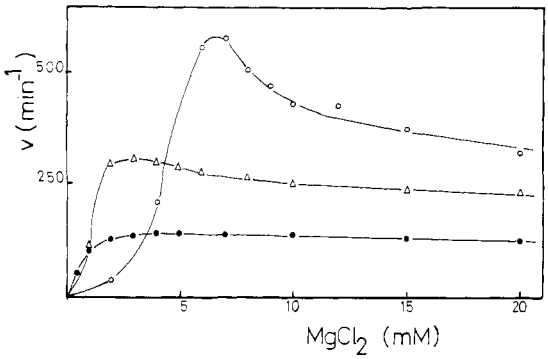

FIGURE 2: Dependence of initial aminoacylation rate of tRNA on $MgCl_2$ concentration. Reaction mixtures in 0.1 M Hepes–NaOH buffer (pH 7.4) contained serum albumin (50 μg/mL), dithioerythritol (5 μM), arginyl-tRNA synthetase (4.5 ng/mL), [^{14}C]arginine (25 μM), *E. coli* K12 tRNA (1 mg/mL), $MgCl_2$ as indicated and ATP [(●) 0.24 mM; (△) 1.2 mM; (○) 6 mM]. Incubation was for 2.5 min at 37 °C.

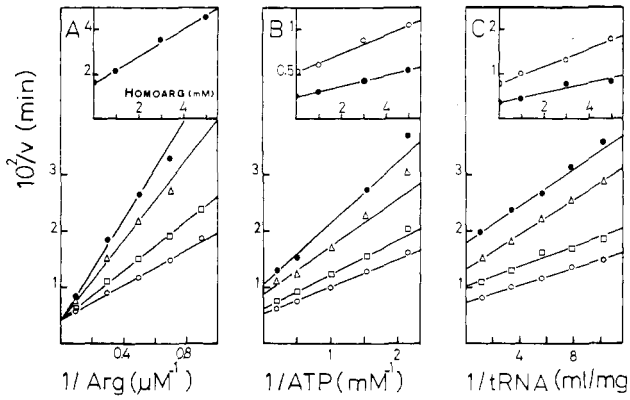

FIGURE 3: Effect of homoarginine [(○) 0; (□) 1 mM; (△) 3 mM; and (●) 5 mM] on the kinetics of aminoacylation. (A) Initial velocity pattern with arginine as the variable substrate and 0.3 mg/mL tRNA and 0.9 mM ATP. (B) Initial velocity pattern with ATP as the variable substrate and 0.3 mg/mL tRNA and 2.5 μM [^{14}C]arginine. (C) Initial velocity pattern with tRNA as the variable substrate and 0.9 mM ATP and 2.5 μM [^{14}C]arginine. Replots of slopes (●) and intercepts (○) vs. inhibitor concentration are shown in the inserts (ordinate in arbitrary units).

ATP/mg ratio. When experiments were made in the presence of 8-azido-ATP, AMP, or pyrophosphate, $MgCl_2$ concentrations 1.5 mM in excess over ATP plus the inhibitor were used.

Initial velocity studies were performed at pH 7.4 with various combinations of substrate concentrations. All double-reciprocal plots were linear. Clearly converging patterns were obtained when arginine was varied at different concentrations of ATP (tRNA fixed) and, at different concentrations of tRNA (ATP fixed), when tRNA was varied at different concentrations of arginine (ATP fixed), or when ATP was varied at different concentrations of arginine (tRNA fixed). These patterns indicate that arginine is bound to enzyme forms that are reversibly connected to the enzyme forms to which ATP and tRNA are bound, according to Cleland's analysis (Cleland 1963c, 1970). When tRNA was varied at different concentrations of ATP or when ATP was varied at different concentrations of tRNA (arginine fixed), Lineweaver–Burk plots gave lines with only slightly different slopes. However, the finding of two families of intersecting lines allows us to eliminate a ping-pong mechanism and seems to indicate that the aminoacylation reaction proceeds by a sequential mechanism, so that all three substrates must be added to the enzyme before any product is released. At pH 8.35 the same pattern was obtained. In order to determine whether the sequence of substrate addition is ordered or random, initial velocities were measured in the presence of dead-end inhibitors. As shown in the representative plot of Figure 3, homoarginine is a competitive inhibitor with respect to arginine and is noncompetitive with respect to ATP and tRNA. Similar results were obtained at pH 8.35. Replots of slopes and intercepts vs. inhibitor concentration were linear. The apparent inhibition constants derived from these replots are given in Table IV, together with those obtained for the other inhibitors used in this study. The inhibition pattern obtained establishes that arginine adds to the enzyme either first or randomly. Similarly, 8-azido-ATP was found to be a competitive inhibitor with respect to ATP and a noncompetitive inhibitor in relation to arginine and tRNA, indicating initial or random addition of ATP. Apparent inhibition constants are given in Table IV. In concert, the data obtained with homoarginine and 8-azido-ATP are only in accord with a random addition of both ATP and arginine. CTP, UTP, and TTP were also found to inhibit the reaction; GTP, on the other hand, was much less effective as inhibitor. As no uncompetitive inhibition with respect to tRNA was found in the above experiments, tRNA addition has to occur subsequent to the binding of both ATP and arginine, or randomly. Periodate-oxidized tRNA ($tRNA_{ox}$) demonstrated noncompetitive inhibition with respect to all three substrates. It was shown that $tRNA_{ox}$ can cause irreversible inactivation of arginyl-tRNA synthetase (Gerlo & Charlier, 1979). Under the conditions of the initial velocity experiments, however, this inactivation proceeds only to a negligible extent. The inhibition of $tRNA_{ox}$ in relation to tRNA, therefore, really is of the

Table V: Inhibition Characters of AMP and PP_i at pH 7.4

inhibitor	variable substrate	fixed substrates	type of inhibn[a]	app K_i (mM)	
				from slope	from intercept
AMP	Arg	ATP (1.5 mM), tRNA (0.3 mg/mL)	NC	1.5	1.7
	ATP	Arg (2.5 μM), tRNA (0.3 mg/mL)	NC	1.6	1.6
	tRNA	Arg (2.5 μM), ATP (1.5 mM)	NC	2.9	1.3
PP_i	Arg	ATP (1.5 mM), tRNA (0.3 mg/mL)	NC	0.05	0.16
	ATP	Arg (2.5 μM), tRNA (0.3 mg/mL)	NC	0.10	0.06
	tRNA	Arg (2.5 μM), ATP (1.5 mM)	NC	0.12	0.04

[a] NC, noncompetitive.

noncompetitive type and suggests that $tRNA_{ox}$ can bind to another site than the tRNA binding site, as saturation with tRNA cannot overcome this inhibition under the given reaction conditions. No information concerning the order of addition can be obtained from this inhibition pattern. However, contrary to the results obtained at pH 7.4, $tRNA_{ox}$ was found to be competitive with respect to tRNA when measurements were made at pH 8.35; in relation to ATP and arginine, the inhibition remained of the noncompetitive type, suggesting initial or random binding of tRNA in a sequential mechanism. Apparent K_i values at the two pHs are given in Table IV. Information on the sequence of product release may be obtained from product inhibition studies. Arginyl-tRNA was found to be competitive with tRNA in the aminoacylation reaction, indicating that arginyl-tRNA combines with the same enzyme form(s) as tRNA. Hence arginyl-tRNA may be the last product to be released from the enzyme. The inhibition by the other products of the reaction, AMP and pyrophosphate, was noncompetitive with respect to all three substrates. Replots of slopes and intercepts were linear. Apparent inhibition constants are given in Table V. These patterns do not provide a unique solution for the order of product release (see Discussion).

Discussion

The results of the kinetic studies of the aminoacylation reaction catalyzed by arginyl-tRNA synthetase from *E. coli* K12 in the absence and in the presence of inhibitors are consistent with a random order of substrate addition, with all steps in the sequence in rapid equilibrium relative to the interconversion of the quaternary complexes. The linear character of the double-reciprocal plots of initial velocity vs. substrate concentration allows us to exclude a steady-state random order mechanism to which corresponds a rate equation of higher complexity. The families of plots obtained when one substrate is varied at varying concentrations of the second substrate and a fixed concentration of the third substrate were converging for at least two of the three different combinations between variable and varying substrate, indicating a sequential mechanism. The results obtained with the substrate analogues homoarginine and 8-azido-ATP are only consistent with a random order of addition of these reactants. As parallel lines were not obtained when tRNA was the variable substrate in these inhibition experiments, it can be deduced that binding of tRNA is not a prerequisite for binding of either ATP or arginine. Addition of tRNA may occur randomly or subsequent to the addition of both ATP and arginine. This last possibility, however, can be eliminated as for a random addition of substrates A and B prior to the addition of substrate C Lineweaver–Burk plots with C as the variable substrate at different levels of A (or B) will converge at a common point on the $1/v$ axis (Fromm, 1967), which is not observed in our bisubstrate kinetics. Indications that tRNA may bind to the free enzyme come further from the protection afforded by tRNA against inactivation of the enzyme by heat (Gerlo & Charlier, 1979; Mitra et al., 1970), by pMB (Table III), and by periodate-oxidized tRNA (Gerlo & Charlier, 1979).

Table VI: Dissociation Constants for the Arginyl-tRNA Synthetase–Substrate Complexes at pH 7.4[a]

substrate	dissociation constants
Arg (A)	K_{ia} = [E][A]/[EA] = 5.5 ± 0.6 μM K_{ba} = [EB][A]/[EAB] = 2.9 ± 0.2 μM K_{ca} = [EC][A]/[EAC] = 3.7 ± 0.1 μM K_a = [EBC][A]/[EABC] = 3.4 ± 0.1 μM
ATP (B)	K_{ib} = [E][B]/[EB] = 0.58 ± 0.07 mM K_{ab} = [EA][B]/[EAB] = 0.30 ± 0.02 mM K_{cb} = [EC][B]/[EBC] = 1.47 ± 0.04 mM K_b = [EAC][B]/[EABC] = 1.35 ± 0.02 mM
tRNA (C)	K_{ic} = [E][C]/[EC] = 0.068 ± 0.007 mg/mL K_{ac} = [EA][C]/[EAC] = 0.046 ± 0.003 mg/mL K_{bc} = [EB][C]/[EBC] = 0.17 ± 0.02 mg/mL K_c = [EAB][C]/[EABC] = 0.20 ± 0.01 mg/mL

[a] By assumption of a random rapid equilibrium mechanism.

The observation that arginyl-tRNA is a competitive inhibitor with respect to tRNA suggests that arginyl-tRNA may be the last product released. The linear noncompetitive inhibition of AMP and pyrophosphate in relation to all three substrates of the synthetase is indicative of a random release of both these products prior to the release of arginyl-tRNA, if no dead-end complexes are formed. However, an ordered sequence of release with the second product involved in dead-end combinations would give a similar inhibition pattern. This possibility could not be excluded.

A random sequence of substrate addition has already been proposed for the phenylalanyl- (Santi et al., 1971) and arginyl-tRNA synthetase (Papas & Peterkofsky, 1972) from *E. coli* B. Simultaneous operation of a ping-pong mechanism was not excluded for the phenylalanyl-tRNA ligase (Santi et al., 1971). The data of Papas & Peterkofsky (1972) obtained from exchange studies at chemical equilibrium indicate that the substrates interact randomly with the arginyl-tRNA synthetase from *E. coli* B and that products are also released in a random order. A random order of product release for the enzyme of *E. coli* K12 can be excluded on the basis of the product inhibition patterns. AMP and pyrophosphate should give competitive inhibition with respect to all substrates if no dead-end complexes are formed, or else the replots should be parabolic. In the case of the *E. coli* B enzyme, the interconversion of the central complexes was not considered to be the only rate-determining step. For the *E. coli* K12 enzyme, the kinetic data are consistent with a rapid equilibrium random mechanism (k = 838 min^{-1}). The proposed sequential random addition of substrates differs more substantially from the ordered sequences postulated for the arginyl-tRNA synthetases

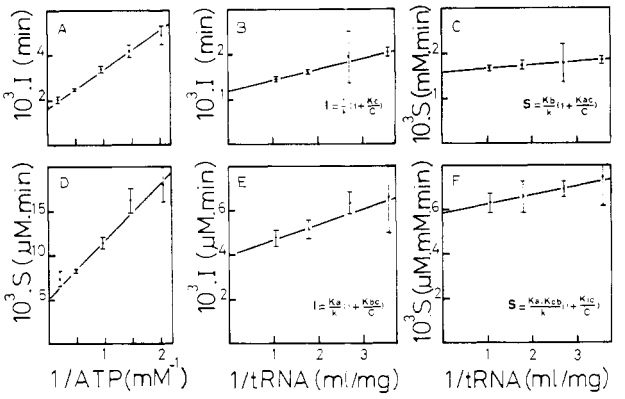

FIGURE 4: Secondary (A and D) and tertiary (B, C, E, and F) plots of slopes (S) and intercepts (I). Lines are calculated by using the weighted least-squares method (Cleland, 1967a). The length of the bars is equal to twice the standard error. (A) $1/v$ intercepts of Lineweaver–Burk plots with arginine as the variable substrate are replotted against the reciprocal of the ATP concentration (fixed tRNA concentration, 0.56 mg/mL). Similar experiments were performed at different tRNA concentrations (0.28, 0.37, 0.94 mg/mL). (B) Plot of the intercepts of the secondary intercept replots (Figure 4A) against the reciprocal of the tRNA concentration. (C) Plot of the slopes of the secondary intercept replots (Figure 4A) against the reciprocal of the tRNA concentration. (D) Slopes of Lineweaver–Burk plots with arginine as the variable substrate are replotted against the reciprocal of the ATP concentration (fixed tRNA concentration, 0.56 mg/mL). (E) Plot of the intercepts of the secondary slope replots (Figure 4D) against the reciprocal of the tRNA concentration. (F) Plot of the slopes of the secondary slope replots (Figure 4D) against the reciprocal of the tRNA concentration. The equations for the linear tertiary plots are derived from the rate equation for a random rapid equilibrium mechanism. The values of the different constants, calculated from ratio's of slopes and intercepts of the tertiary plots, are given in Table V.

of *Neurospora crassa* (Nazario & Evans, 1974) and *Bacillus stearothermophilus* (Parfait & Grosjean, 1972).

The rate equation in the absence of products for a rapid equilibrium random Ter mechanism may be written in the equilibrium constant form, with one term for each enzyme complex present during reaction:

$$\frac{1}{v} = \frac{1}{V}\left[1 + \frac{K_a}{[A]} + \frac{K_b}{[B]} + \frac{K_c}{[C]} + \frac{K_aK_{bc}}{[A][C]} + \frac{K_aK_{cb}}{[A][B]} + \frac{K_{ab}K_c}{[B][C]} + \frac{K_{ia}K_{ab}K_c}{[A][B][C]}\right]$$

The constants are defined in Table VI, which also gives their respective values as derived from the tertiary replots (Figure 4; Dalziel, 1969) of the bisubstrate kinetics at pH 7.4 and from the different relations that can be shown to hold between the dissociation constants:

$$K_{ia}K_{ab}K_c = K_{ia}K_bK_{ac}$$
$$= K_aK_{ib}K_{bc} = K_{ba}K_{ib}K_c$$
$$= K_{ca}K_bK_{ic} = K_aK_{cb}K_{ic}$$

These values may be related to interactions between the different binding sites: binding of ATP to the enzyme is made more difficult when tRNA is already bound, and binding of tRNA is hindered by ATP. Binding of arginine has virtually no effect on the binding of the other substrates and is not greatly influenced by the binding of other substrates. In the presence of a dead-end inhibitor I (e.g., homoarginine) that is competitive with substrate A (e.g., arginine) for the same site on the enzyme, the terms of the rate equation written in reciprocal form that contain [A] (these are the terms corresponding to the enzyme forms reacted with) are multiplied by $(1 + [I]/K_i)$, K_i being the dissociation constant of the given dead-end complex. The rate equation thus becomes

$$\frac{1}{v} = \frac{1}{V}\left[1 + \frac{K_b}{[B]} + \frac{K_c}{[C]} + \frac{K_{ab}K_c}{[B][C]}\right] + \frac{K_a}{[A]V}\left[\left(1 + \frac{[I]}{K_{i1}}\right) + \frac{K_{bc}}{[C]}\left(1 + \frac{[I]}{K_{i2}}\right) + \frac{K_{cb}}{[B]}\left(1 + \frac{[I]}{K_{i3}}\right) + \frac{K_{ib}K_{bc}}{[B][C]}\left(1 + \frac{[I]}{K_{i4}}\right)\right]$$

showing the competitive nature of the inhibition. The apparent inhibition constant obtained from the replot of the slope vs. the inhibitor concentration can thus be related to the different dissociation constants of enzyme–substrate and enzyme–inhibitor complexes and the fixed substrate concentrations. As five replots can be obtained from a study of the inhibition with respect to the three substrates (three slope replots and two intercept replots), a system of five equations with four unknowns can be generated (it is also possible to obtain different equations from inhibition studies with respect to only one substrate, at different fixed concentrations of the other substrates). The least-squares solution of such an over-determined system was computed for the data obtained from the inhibition studies with homoarginine and 8-azido-ATP, by using the values of the dissociation constants of the enzyme–substrate complexes given in Table VI. The results of these calculations are given in Table VII together with the definitions of the constants. Binding of homoarginine is not influenced by the binding of ATP or tRNA. The dissociation constants are 400–900 times larger than the values for the corresponding enzyme–arginine complexes. 8-Azido-ATP is inactive as a substrate. The introduction of an $-N_3$ moiety in position 8 of the base of ATP, however, does not significantly influence the values of the dissociation constants. This ATP analogue is a competitive inhibitor for the arginyl-tRNA synthetase from Baker's yeast also, but with a K_i value ten times higher than the K_m for ATP (Freist et al., 1978). The effect of tRNA binding on the ATP binding is maintained for the analogue (Table VII). The interactions between the different binding sites, deduced from the results of the bisubstrate kinetics, are thus fully confirmed by the results of the inhibition studies.

Table VII: Dissociation Constants for Arginyl-tRNA Synthetase–Inhibitor Complexes at pH 7.4

inhibitor	dissociation constants[a] (mM)
Har (I)	K_{i4} = [E][I]/[EI] = 2.2
	K_{i2} = [EB][I]/[EIB] = 2.5
	K_{i3} = [EC][I]/[EIC] = 3.3
	K_{i1} = [EBC][I]/[EIBC] = 1.6
8-azido-ATP (I)	K_{i4} = [E][I]/[EI] = 0.18
	K_{i2} = [EA][I]/[EAI] = 0.48
	K_{i3} = [EC][I]/[EIC] = 3.30
	K_{i1} = [EAC][I]/[EAIC] = 1.84

[a] A, Arg; B, ATP; C, tRNA.

Acknowledgments

The amino acid analyses were performed in the laboratory of Professor L. Kanarek at the Vrije Universiteit te Brussel. We also thank J. M. Godeau for introducing us to his NaDodSO$_4$ gel electrophoresis technique and Dr. V. Stalon

for discussions on enzyme kinetics. We are grateful to Dr. W. Freist for the supply of 8-azido-ATP.

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