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Adenosine Triphosphate Consumption by Bacterial Arginyl-Transfer Ribonucleic Acid Synthetases

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ATP consumption by arginyl-tRNA synthetases from *Escherichia coli* and *Bacillus stearothermophilus* has been investigated by the firefly luciferin–luciferase assay. Arginyl-tRNA synthetase from *E. coli* utilizes ATP only for aminocylation of tRNA with a 1:1 stoicheiometry. In contrast, we have shown an adenosine triphosphatase activity of arginyl-tRNA synthetase from *B. stearothermophilus* in the absence of $tRNA^{Arg}$. Dowex chromatography revealed the formation of ADP by the thermophile enzyme; under aminoacylation conditions, AMP was also formed in amounts stoicheiometric with arginyl-tRNA formation.

The catalytic competence of the aminoacyl-tRNA synthetases may be measured either by tRNA charging or by reactions based on aminoacyl-adenylate formation. Arginyl-, glutamyl- and glutaminyl-tRNA synthetases from different organisms differ from other synthetases because no ATP–PP_i exchange is catalysed in the absence of tRNA. This fact leads to the question of whether the aminoacyladenylate–enzyme complex exists as an obligatory intermediate during aminoacylation.

Isolation of the enzyme–aminoacyladenylate complexes is done usually by gel filtration (Norris & Berg, 1964) or filtration on nitrocellulose discs (Yarus & Berg, 1970). These procedures require quite stable complexes, and, owing to the dynamic nature of the enzyme–aminoacyladenylate complex, the stoicheiometry could only be estimated. On the basis of the stoicheiometric liberation of 1 mol of PP_i and depletion of 1 mol of ATP by the formation of 1 mol of aminoacyladenylate, Fersht *et al.* (1975) detected the labile as well as stable aminoacyl-adenylate–enzyme complexes and quantified their catalytically active sites by measurements of residual [γ-^{32}P]ATP either by adsorption on charcoal or by t.l.c.

In the present study we propose a more economical procedure that determines the residual ATP by bioluminescence. The products formed during ATP consumption by the synthetases were analysed by Dowex chromatography using [^{14}C]ATP.

Materials and Methods

ATP (disodium salt), ADP, AMP, dithioerythritol, Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], phenylmethanesulphonyl fluoride, streptomycin sulphate and firefly-lantern extract as source of ATP luciferase were Sigma (St. Louis, MO, U.S.A.) products. [^{14}C]Arginine (sp. radioactivity 50 μCi/μmol) and [^{14}C]ATP (sp. radioactivity 318 μCi/μmol) were Schwarz/Mann (Orangeburg, N.Y. 10962, U.S.A.) products. The liquid-scintillation fluid used for aqueous fractions from the Dowex chromatography was Unisolve from Koch–Light Laboratories Ltd. (Colnbrook, Bucks., U.K.). Acrylamide/agarose (Ultrogel AcA 44) was from LKB (Industrie Biologique Française, Paris, France) and phosphocellulose (P11) was a Whatman (Maidstone, Kent, U.K.) product. The concentration of ATP was determined spectrophotometrically (ε_{259} 15.5 litre·mmol^{-1}·cm^{-1}). tRNA from *E. coli* K12 was obtained from Schwarz/Mann and purified by chromatography on DEAE-cellulose (Stephenson & Zamecnik, 1961). tRNA from *Bacillus stearothermophilus* was extracted by the phenol method (Brubaker & McCorquodale, 1963) and chromatographed on DEAE-cellulose. tRNA specific for arginine from *B. stearothermophilus* was purified by chromatography on benzoylated DEAE-cellulose as described by Gillam *et al.* (1968).

[^{14}C]Arginyl-tRNA formation was assayed as described previously (Charlier & Gerlo, 1976; Godeau, 1976).

Arginyl-tRNA synthetase was purified about 1000-fold from *E. coli* K12 cells after lysis with a Parr disruption bomb and precipitation of the nucleic acids with streptomycin sulphate, by chromatography on DEAE-cellulose, affinity elution on phosphocellulose (an adaptation of Von der Haar, 1973) gel filtration and a second affinity elution on

phosphocellulose. The sodium dodecyl sulphate/polyacrylamide-gel electrophoresis pattern shows one major band (greater than 95%) corresponding to a mol.wt. of 60000. The specific activity was determined to be 50000 units/mg, under the conditions defined previously (Charlier & Gerlo, 1976).

Purification of arginyl-tRNA synthetase from *B. stearothermophilus* NCA 1518 was as described previously (Godeau, 1976) with the following modifications: (1) a trypsin inhibitor (phenylmethanesulphonyl fluoride) was added for the lysis and the two following purification steps: (2) two additional steps were included, first a gel filtration on acrylamide/agarose and second an affinity elution chromatography on phosphocellulose and removal of the tRNA by a chromatography on DEAE-cellulose (Von der Haar, 1973). By polyacrylamide-gel electrophoresis under denaturing conditions (sodium dodecyl sulphate) one single band corresponding to a mol.wt. of 59000 was detected. This molecular weight is confirmed by Sephadex G-100 gel filtration and sucrose-gradient centrifugation by the method of Martin & Ames (1961). By this last technique a mol.wt. of 110000–130000 was revealed for the synthetase in the presence of its cognate tRNA (J.-M. Godeau, unpublished work).

Bioluminescence assay

The dried firefly-lantern extract was diluted with 5ml of water. After standing about 16h at 4°C, this extract was clarified by centrifugation or filtration and diluted 10-fold in 50mM-potassium arsenate buffer, pH 7.4, containing 50mM-$MgSO_4$. This enzyme was kept at room temperature during the measurements. To 100μl of luciferase–luciferin solution 10μl or 20μl ATP samples were added at zero time. The bioluminescence was measured in a Packard 2002 liquid-scintillation counter, with coincidence circuitry switched off. Samples were counted for luminescence for 0.1 min, and the bioluminescence decay was measured during 10min. This decay is about first-order, as can be seen in Fig. 1(*b*). The bioluminescence after 10min reaction time was recorded. The corresponding ATP concentration was extrapolated on a standard plot established before each set of measurements with known ATP concentrations (e.g. Fig. 1*a*). ADP gave some bioluminescence with the luciferase extract, due to the presence of myokinase; the method would be greatly improved and more specific for ATP if this contamination were eliminated from the luciferase preparation.

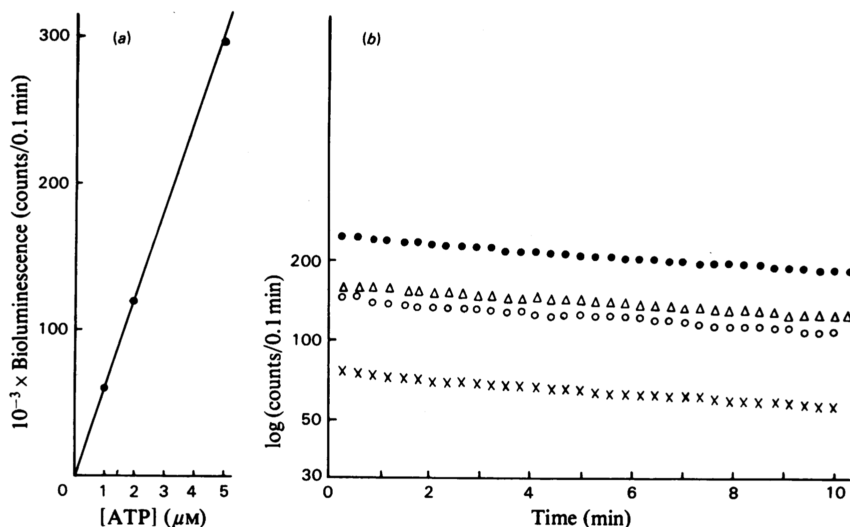

Fig. 1. *Bioluminescence assay of ATP*

(*a*) A standard curve for ATP was established with freshly made reference ATP solution in water before each set of measurements with synthetase. The luminescence counts per 0.1 min recorded after 10min reaction time at room temperature were plotted against the concentration of the reference ATP solution from which 10μl were added to 100μl of luciferase–luciferin extract. (*b*) Time course of bioluminescence. The bioluminescences (counts/0.1 min) were recorded by the repetitive mode of the Packard 2002 counter every 17s for 10μl of references 2μM-ATP (○) and 1μM-ATP (×) or for 10μl of ATP incubated with arginyl-tRNA synthetase from *E. coli* (0.225μg/ml) in presence of 0.1 M-Hepes, pH 8.35, 10μM-arginine and tRNA (3.5mg/ml) at zero time (●) or periodate-oxidized tRNA (3.5mg/ml) after 30min at 37°C (△), added to 100μl of luciferase–luciferin extract. The log (counts/0.1 min) was plotted against the time (in min).

Synthetase assays used for the bioluminescence measurement of ATP

The reaction medium for measuring ATP consumption by arginyl-tRNA synthetase from *E. coli* contained 0.1 M-Hepes/NaOH, pH 8.35, 4 mM-$MgCl_2$, 10 μM-ATP, 10 μM-[^{14}C]arginine and total tRNA from *E. coli* (3.5 mg/ml). The reaction was started by addition of synthetase, and, after incubation at 37°C, portions were taken at different times, diluted 2–5-fold in ice-cold water and measured for the ATP content by the bioluminescence assay. We have checked that a heating step (2 min at 65°C or 1 min at 100°C) after dilution and prior luciferase assay to inactivate the synthetase as described by Charlier & Grosjean (1972), is unnecessary (see Fig. 2). Control experiments without enzyme or in the absence of arginine or tRNA were also conducted. In some experiments, [^{14}C]arginyl-tRNA formation was measured simultaneously with the ATP decay on the same reaction mixture.

The reaction mixture utilized for *B. stearothermophilus* enzyme contained 0.05 M-buffer (Tris/HCl, pH 8.7), 5 mM-$MgCl_2$, 50 mM-NaCl, 1 mM-dithioerythritol, 2 μM-ATP and *B. stearothermophilus* tRNA and/or [^{14}C]arginine as stated in the legends to the Figures. During incubation at 37°C with the synthetase, portions were taken at different time intervals and treated as described for the *E. coli* enzyme.

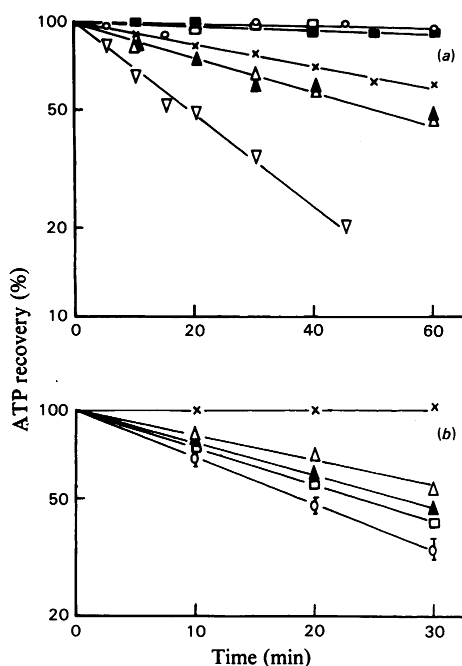

Fig. 2. *Kinetics of ATP consumption tested by the bioluminescence assay of residual ATP*

(*a*) ATP consumption by *E. coli* K12 arginyl-tRNA synthetase. ATP concentrations were measured by using the firefly luciferase–luciferin assay at different time intervals after dilution of the reaction medium in ice-cold water (△) and after an additional heating for 2 min at 65°C (▲). The results are shown as percentages of the initial concentrations and plotted logarithmically on the ordinate scale. The ATP decay was first-order over the time course considered. Control experiments without tRNA (■) or without arginine (□) gave the same results as ATP incubated in the absence of arginyl-tRNA synthetase (○). Three synthetase concentrations were measured: 0.45 (×), 0.90 (△, ▲) and 2.25 μg/ml (▽) in the reaction medium described in the Materials and Methods section. (*b*) Arginyl-tRNA synthetase from *B. stearothermophilus* (0.6 μg/ml) was incubated with 50 mM-Tris/HCl, pH 8.7, 50 mM-NaCl, 1 mM-dithioerythritol, 5 mM-$MgCl_2$ and 2 μM-ATP; the time-dependent ATP depletion at 37°C was analysed without any other substrate in the reaction medium (I) or with in addition: 1 mM-AMP (×), 4 μM-arginine (○), 167 μg of total tRNA (1 μM-$tRNA^{Arg}$) (□), 333 μg of total tRNA (2 μM-$tRNA^{Arg}$) (△), or 2 μM-$tRNA^{Arg}$ with 4 μM-arginine (▲). tRNA was from *B. stearothermophilus*. (Note: ○ and I coincide as ♀.)

Analysis of products formed during ATP depletion

This was carried out on Dowex-1 resin (X8; 100–200 mesh) (De Muylder, 1975), a Fluka (CH-9470 Buchs, Switzerland) product that was washed and converted into the formate form as described by Bruening *et al.* (1970). The columns (4 cm × 1.5 cm) were packed with Dowex resin equilibrated in 1.2 M-formic acid (buffer A). To the [^{14}C]ATP samples were added known amounts of unlabelled AMP, ADP and ATP immediately before loading on to the column. These markers were detected spectrophotometrically at 260 nm. AMP was eluted with buffer A. Buffer B (4 M-formic acid/0.2 M-ammonium formate) was used to elute ADP, and buffer C (4 M-formic acid/0.8 M-ammonium formate) to elute ATP. Portions (500 μl) from each fraction were added to 5 ml of Unisolve and radioactivity was counted in a liquid-scintillation instrument.

Results and Discussion

Arginyl-tRNA synthetase catalyses arginine-dependent ATP–PP_i exchange in the presence of native tRNA only; periodate-oxidized tRNA does not induce the exchange. It is generally assumed that no arginyl-AMP forms in the absence of tRNA. To further investigate this process, we determined ATP consumption by using the bioluminescence method.

Initial ATP concentrations of 1–20 μM are suitable for following the kinetics of ATP decay over a time range of 30–60 min for different arginyl-tRNA synthetase concentrations (see Fig. 2). Fig. 2(*a*) shows

the ATP consumption by the arginyl-tRNA synthetase from *E. coli* K12. Clearly ATP only diminished significantly when arginine and tRNA were both present in the reaction mixture. Control experiments without arginine or tRNA showed no more ATP loss than in the control experiment without enzyme. Under aminoacylation conditions, ATP diminished by an apparent first-order kinetic process. The rate

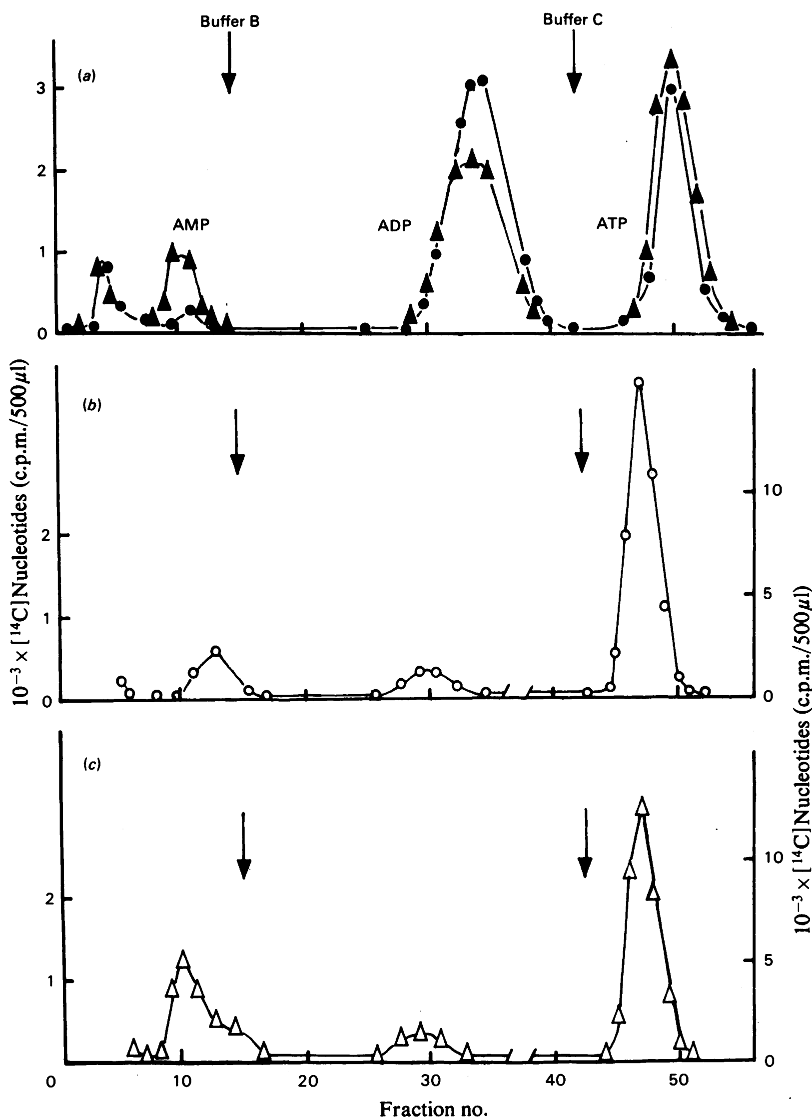

Fig. 3. *Dowex chromatography of* [^{14}C]*nucleoside phosphates*

(*a*) The samples submitted to ion-exchange chromatography were obtained from the incubation (20min at 37°C) of 120μl containing 3.3μM-[^{14}C]ATP and 0.98μg/ml of arginyl-tRNA synthetase from *B. stearothermophilus* mixed with 50mM-Hepes/NaOH, pH7.4, 5mM-$MgCl_2$ and 1mM-dithioerythritol (●, 1.56ml/fraction) or, in addition, 167μg of total tRNA from *B. stearothermophilus* (2μM-$tRNA^{Arg}$) and 20.8μM-arginine (▲, 1.62ml/fraction). (*b*) Dowex chromatography after incubation (20min at 37°C in 150μl) of arginyl-tRNA synthetase from *E. coli* (0.225μg/ml) with 6μM-[^{14}C]ATP in 0.1M-Hepes, pH8.35, containing 4mM-$MgCl_2$ and 10μM-arginine (1.62ml/fraction). (*c*) Same experiment as in (*b*), but in the presence of tRNA from *E. coli* (1 mg/ml) (1.56ml/fraction). The ordinate scales to the right of (*b*) and (*c*) refer to the curves to the right of the break.

Table 1. *[^{14}C]ATP consumption by arginyl-tRNA synthetase as determined by Dowex chromatography*

The reaction mixture (120 μl) for the synthetase from *B. stearothermophilus* is as follows: 50 mM-Hepes, pH 7.4, 1 mM-dithioerythritol and 5 mM-$MgCl_2$ and the additions listed. Arginyl-tRNA synthetase from *E. coli* was measured in the reaction mixture (150 μl) containing 0.1 M-Hepes, pH 8.35, 4 mM-$MgCl_2$ and the substrates listed. Incubation was for 20 (*) or 60 (†) min at 37°C. The net results (pmol) obtained in the eluted fractions were corrected for amounts (pmol) of [^{14}C]ATP destroyed or [^{14}C]AMP and [^{14}C]ADP contaminating the preparation; this was carried out by a blank chromatography without enzyme.

	Additions								[^{14}C]Nucleotide recovery from Dowex chromatography				
		tRNA homologue							Total		Net recovery (pmol)		
Source of arginyl-tRNA synthetase	[^{14}C]ATP (in pmol)	Total tRNA (mg/ml)	$tRNA^{Arg}$ (μg/ml)	Arginine (μM)	NaF (mM)	NaN_3 (mM)	AMP (mM)	ADP (mM)	(pmol)	(%)	ATP	ADP	AMP
B. stearothermophilus (0.98 μg/ml)	397*	—	—	—	—	—	—	—	372	94	126–134	209–222	—
	397*	—	—	20.8	4.2	—	—	—	385	97	124–128	221–228	—
	397*	—	—	—	—	12.5	—	—	357	90	115–128	206–229	—
	397*	1.39	48.6	20.8	—	—	—	—	371	93	165–177	146–156	23.1–24.7
	397*	0.38	13.5	—	—	—	—	—	359	90	106–117	219–242	—
	397*	0.37	150.0	—	—	—	—	—	356	90	210–223	125–133	—
	397†	—	—	—	—	—	—	—	353	89	35–39	287–314	—
	397†	—	—	—	—	—	—	1.7	358	90	238–261	88–97	—
	397†	—	—	—	—	—	2.15	—	358	90	116–123	224–236	—
E. coli (0.225 μg/ml)	896*	—	—	20.0	—	—	—	—	905	101	865	—	—
	896*	1.0	25.6	20.0	—	—	—	—	880	98	750	—	65

of ATP decrease is expressed as the product of the kinetic first-order rate constant ($k = \ln 2/t_{\frac{1}{2}}$) by the ATP concentrations. When assaying [^{14}C]arginine charging on to tRNA and ATP consumption in the same reaction mixture, this allows an estimation of the amount of arginyl-tRNA formed per amount of ATP used. Such an evaluation based on the initial rate of [^{14}C]arginine charging gave a ratio of 1 mol of ATP consumed/mol of [^{14}C]arginyl-tRNA formed.

Similar experiments were performed with arginyl-tRNA synthetase from *B. stearothermophilus* (Fig. 2*b*), but with different results. (1) The synthetase from the thermophile causes an ATP depletion even in the absence of the other substrates; (2) this disappearance of ATP increases with synthetase concentrations, but the proportionality failed at high enzyme concentrations (9.7 μg/ml); (3) the ATP consumption is inhibited by tRNA and AMP. Calculation of the ratio (ATP used)/(arginyl-tRNA formed) is not meaningful under these conditions. To understand the nature of this ATP decay by pure arginyl-tRNA synthetase from *B. stearothermophilus* in the absence of the other substrates, we analysed the nature of the reaction product(s). Chromatography on Dowex exchange resin (Fig. 3*a* and Table 1) revealed an arginine-independent ATPase activity, since only ADP was produced under these conditions.

As Table 1 shows, in the presence of tRNA and arginine, less ADP was produced and AMP was detected. The decrease of ADP formation in the presence of total tRNA is due to the specific $tRNA^{Arg}$; for identical amounts of total tRNA, the fraction enriched in $tRNA^{Arg}$ (16 nmol/mg) gave 30% less ADP production than the preparation containing only 2.2 nmol/mg of $tRNA^{Arg}$. AMP, which is a product inhibitor of the aminoacylation reaction ($K_i = 0.15$ mM), also inhibited the ATPase activity ($K_i = 0.06$ mM), as well as ADP. These results support our contention that arginyl-tRNA synthetase participates in ATPase activity.

When the synthetase from *B. stearothermophilus* was incubated with [^{14}C]ATP, tRNA and arginine (Table 1), the amount of [^{14}C]AMP reached 23.6–24.7 pmol after 20 min; the same experiment performed with [^{14}C]arginine and unlabelled ATP gave a production of 23.6 pmol of [^{14}C]arginyl-tRNA in the same time interval. The AMP/arginyl-tRNA ratio is thus equal to 1. The same ratio was found for arginyl-tRNA synthetase from *E. coli*. No ADP was produced by this enzyme, either in the absence or in the presence of tRNA, as Figs. 3(*b*) and 3(*c*) show.

In conclusion, the ATP bioluminescence measurements enabled us to determine the 1:1 stoicheiometry of ATP utilization versus arginyl-tRNA formation for arginyl-tRNA synthetase from *E. coli*. For arginyl-tRNA synthetase from *B. stearothermophilus* we detected an ATPase activity in the absence of tRNA. A real advantage over the technique with [γ-^{32}P]ATP (Fersht *et al.*, 1975) is that the amounts of enzyme used were 400–1000-fold lower, but our assays did not allow us to estimate the stoicheiometry of the intermediate complex. As the initial ATP concentration is low, and the luciferin–luciferase method measures ATP decay, the latter is important, and has to be reliable (i.e. more than 10% ATP consumed in 30 min).

The combination of the luciferase assay with the chromatography on Dowex is noteworthy in that, once the reaction conditions are tested out with the bioluminescence assay, the products formed by ATP depletion could be analysed with [^{14}C]ATP, avoiding the disadvantage of the shorter half-life of [γ-^{32}P]ATP.

Small Dowex columns could be used, even in the presence of tRNA, which binds strongly on to the column, without retaining nucleotides, since the recovery from our columns was always at least 90% (Table 1).

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