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SYNTHESIS OF P^1,P^4-DI(ADENOSINE 5'-) TETRAPHOSPHATE BY LEUCYL-tRNA SYNTHETASE, COUPLED WITH ATP REGENERATION

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SUMMARY : A simple and practical procedure for the synthesis of P^1,P^4-di(adenosine 5'-) tetraphosphate from ATP by the catalysis of leucyl-tRNA synthetase from Bacillus stearothermophilus is described. Km for leucine was 6.7 μM and for ATP was 3.3 mM. The reaction yielded not only diadenosine tetraphosphate, but various byproducts such as P^1,P^3-(diadenosine 5'-) triphosphate, ADP and AMP. By coupling the reaction with an ATP regeneration system by acetate kinase and adenylate kinase with acetylphosphate as a phosphate donor, diadenosine tetraphosphate was prepared as a sole product at a high yield (96%).

P^1,P^4-di(adenosine 5'-) tetraphosphate (Ap_4A) was first discovered by Zamecnik et al in 1966 (1). Since then, a variety of biological activities have been reported, i.e., stimulation of DNA synthesis in G1-Arrested baby hamster kidney cell (2), function as a primer of DNA polymerase in HeLa cell (3), inhibition of protein kinase of Rous Sarcoma Virus (4), alarmone to the

ABBREVIATIONS : Ap_4A; P^1,P^4-di(adenosine 5'-) tetraphosphate, Ap_3A; P^1,P^3-di(adenosine 5'-) triphosphate, BICINE; N,N-bis(2-hydroxyethyl) glycine, HEPES; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, HPLC; High performance liquid chromatography LeuRS; leucyl-tRNA synthetase, PPi; inorganic pyrophosphate.

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oxidation and heat stress (5) of cell and inhibition of platelet aggregation (6).

Some reports on organic synthesis of Ap_4A were found in literature (7,8). AMP was first converted to an activated form like adenosine 5'-phosphomolibdate, which was allowed to react with ATP in anhydrous pyridine to give Ap_4A. Since the reactions were carried out in anhydrous pyridine, it was necessary to remove a trace of water by repeated azeotropic distillation of pyridine and to add hydrophobic amine such as tri(n-octyl)amine to increase the solubility of the substrates. Furthermore, the reaction was accompanied by a variety of by-products and the yield was poor (ca.25%).

The authors have been interested in enzymatic synthesis of Ap_4A which would be of practical value as a synthetic method. Ap_4A is synthesized, in vivo, by a reverse reaction from aminoacyl adenylatet-aminoacyl-tRNA synthetase complex with ATP (1). Therefore, the authors have examined the Ap_4A synthesis catalyzed by aminoacyl tRNA synthetase.

MATERIALS AND METHODS

Materials: Diadenosine tetraphosphate and acetylphosphate were obtained from Sigma. ATP was purchased from Oriental Yeast Co. Ltd., Tokyo. Acetate kinase and adenylate kinase from B. stearothermophilus were from Seikagaku kogyo Co. Ltd., Tokyo.

Enzyme preparation: Leucyl-tRNA synthetase was purified from B. stearothermophilus, NCA-1503, according to the method described previously (9) and gave single band on sodium dodecylsulfate-polyacrylamide gel electrophoresis. A unit of Leucyl-tRNA synthetase is defined as a amount of enzyme necessary to produce 1 nmol of leucine hydroxamate at 40°C in 10 min, and of all other enzymes are those to produce 1 umol of products at 30°C per min. .

Synthesis of diadenosine tetraphosphate by leucyl-tRNA synthetase: A standard reaction mixture (1 ml) contained 4000 unit/ml leucyl-tRNA synthetase, 10 mM leucine, 10 mM ATP, 10 mM $MgCl_2$, 50 mM BICINE (pH 8.5). The solution was incubated at 40°C for 5 hr. The production of diadenosine tetraphosphate was monitored on Waters HPLC system, employing a Novapak C_{18} column (8x100 mm). Ap_4A was eluted by a linear gradient from 10 % to 30 % acetonitrile in 5 mM tetrabutylammmonium bromide, 10 mM potassium phosphate (pH 8.0) solution and detected by absorbance at 260 nm.

Synthesis of diadenosine tetraphosphate coupled with ATP regeneration: A solution (80 ml) containing 3000 unit/ml leucyl-tRNA synthetase, 4 unit/ml acetate kinase, 2 unit/ml adenylate kinase, all from B. stearothermophilus, 1 unit/ml inorganic pyrophosphatase from Bakers yeast, 5 mM leucine, 30 mM ATP, 45 mM $MgCl_2$, 100 mM HEPES (pH 7.5) was incubated at 40°C for 8 hr. An acetyl phosphate solution in water (100 mg/ml) was added to the mixture, at the beginning of the reaction (1.5 ml) and at a interval of 2 hr (0.75 ml). The progress of the reaction was monitored by HPLC.

Isolation of diadenosine tetraphosphate: Prior to the purification of Ap_4A, leucyl-tRNA synthetase was recovered from the reaction mixture by ultrafiltration. The filtrate and the washings were combined and applied to a DEAE-Sepharose column (827 ml, 90x130 mm), which was previously equilibrated with 100 mM triethylammonium hydrogen carbonate solution. Ap_4A was eluted by a linear gradient from 150 mM to 400 mM triethylammonium hydrogen carbonate solution at a flow rate of 63 ml/min.

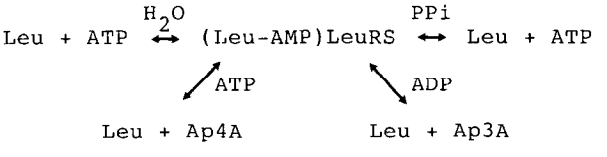
Ap_4A fractions were combined and evaporated to dryness by a rotary evaporator. The white solid was dissolved in methanol and treated with sodium iodide solution in acetone, according to the procedure of P. Feldhaus et al. (10), and converted to sodium salt of diadenosine tetraphosphate.

The salt gave a single peak on HPLC and had identical [^{1}H]NMR and [^{31}P]NMR spectra to those of the authentic sample.

RESULTS AND DISCUSSION

We have isolated several aminoacyl-tRNA synthetases from a thermophilic bacterium, Bacillus stearothermophilus (9). Since, in preliminary experiments, leucyl-tRNA synthetase showed the highest Ap_4A synthesis activity, we have employed the enzyme as the catalyst for the synthesis reaction of Ap_4A.

But, the synthesis of Ap_4A was accompanied with a variety of by-products. ATP was hydrolyzed to ADP or AMP at the reaction conditions. Furthermore, ADP thus produced interfered the synthesis of Ap_4A by producing P^1,P^3-di(adenosine 5'-) triphosphate (Ap_3A) (10), as shown in Scheme 1.



Scheme 1

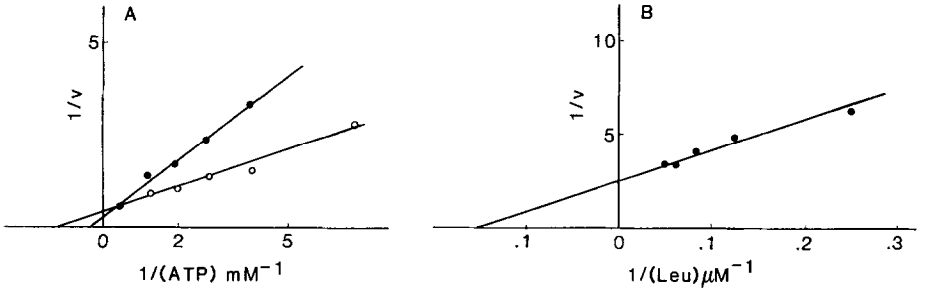


Figure 1, Lineweaver-Burk plots for ATP and leucine in the Ap_4A synthesis reactions by leucyl-tRNA synthetase from B. stearothermophilus. A; for ATP (● ; without correction for Ap3A synthesis, ○; with correction), B; for leucine.

Km value for leucine in the present conditions was 6.7 μM and for ATP was 3.3 mM (0.9 mM when corrected for Ap_3A production) as shown in Fig. 1.

Consequently, in order to produce Ap_4A selectively, it is necessary to remove ADP and AMP and, favorably, to regenerate ATP. Therefore, Ap_4A synthesis reaction was coupled with an ATP regeneration system employing thermostable acetate kinase and adenylate kinase from B. stearothermophilus as catalysts (11-13).

A typical reaction mixture (80 ml) contained leucyl-tRNA synthetase 3000 unit/ml, acetate kinase 4 unit/ml, adenylate kinase 2 unit/ml, leucine 5 mM, ATP 30 mM, $MgCl_2$ 45 mM, acetylphosphate 10 mM, HEPES buffer 100 mM and inorganic pyrophophatase 1 unit/ml. The mixture was incubated at 45°C for 8 hr.

In Fig.2, the result of HPLC analysis of the reaction mixture was shown. In contrast to the occurrence of many by-products without ATP regeneration, Ap_4A was produced as a sole product when the reaction was conducted with ATP regeneration system, (96% yield). Ap_4A was easily purified by the chromatography on DEAE-Cellulose with triethylammonium hydrogen carbonate as a eluent, and isolated as triethylammonium salt which was later converted to sodium salt by the addition of sodium iodide in acetone to a methanolic solution of the triethylammonium salt

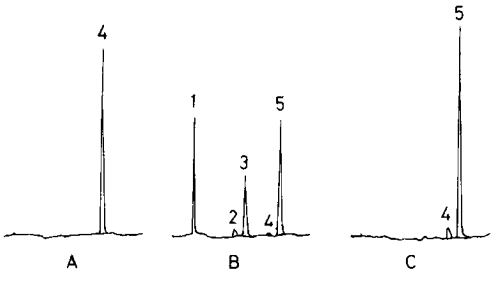


Figure 2, Results of HPLC analysis of the reaction mixture for Ap_4A synthesis by leucyl-tRNA synthetase, A; initial reaction mixture, B; after 5 hr without ATP regeneration system, C; after 8 hr with ATP regeneration system. 1; AMP, 2;ADP, 3; Ap_3A, 4; ATP, 5; Ap_4A

(10), (800 mg, 72% yield). At the end of the reaction, leucyl-tRNA synthetase retained most of the initial activity (>90%).

From our experimental data, following conclusion can be drawn. By coupling with ATP regeneration system catalyzed by thermostable acetate kinase and adenylate kinase, Ap_4A is prepared as a sole product from ATP in high yield by leucyl-tRNA synthetase. As all enzymes employed were derived from a thermophilisc bacterium, B. stearothermophilus, the whole system is quite stable. This method of Ap4A synthesis is straight forward compared with the conventional organic synthesis (7,8) and provide a practical tool for the synthesis of this biologically important compound, Ap_4A.

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