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P^1,P^3-bis(5′-adenosyl)triphosphate (Ap_3A) as a substrate and a product of mammalian tryptophanyl-tRNA synthetase

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Abstract

Bovine tryptophanyl-tRNA synthetase (TrpRS, E.C. 6.1.1.2) is unable to catalyze in vitro formation of Ap_4A in contrast to some other aminoacyl-tRNA synthetases. However, in the presence of L-tryptophan, ATP-Mg^{2+} and ADP the enzyme catalyzes the Ap_3A synthesis via adenylate intermediate. Ap_3A (not Ap_4A) may serve as a substrate for TrpRS in the reaction of E·(Trp ~ AMP) formation and in the $tRNA^{Trp}$ charging. The K_m value for Ap_3A was higher than the K_m for ATP (approx. 1.00 vs. 0.22 mM) and V_{max} was 3 times lower than for ATP. The Zn^{2+}-deficient enzyme catalyzes Ap_3A synthesis in the absence of exogenous ADP due to ATPase activity of Zn^{2+}-deprived TrpRS. The inability of mammalian TrpRS to synthesize Ap_4A, might be considered as a molecular tool preventing the removal of Zn^{2+} due to chelation by Ap_4A and therefore preserving the enzyme activity.

Key words: Aminoacyl-tRNA synthetase; Tryptophanyl-tRNA synthetase; Ap_4A/Ap_3A synthesis

1. Introduction

Ap_3A and Ap_4A known since mid-1960s, are widespread in living cells (see [1,2] and references therein). These ubiquitous cell compounds belong to the family with a general formula Ap_4N or Ap_3N, where N is one of the purine or pyrimidine nucleosides. Ap_4A/Ap_3A seem to be implicated in cell metabolic pathways [3,4]. The ability to catalyze Ap_4A and Ap_3A synthesis was first reported for lysyl-tRNA synthetase from *E. coli* [5]. Later some other aaRS of different origin and amino acid specificity were shown to synthesize such dinucleoside oligophosphates in vitro (see [3]). It was suggested that these aaRS are also able to catalyze in vivo Ap_4A and Ap_3A synthesis. Ap_4A synthesis catalyzed by these aaRS proceeds through the stage of the aminoacyl adenylate·enzyme complex formation [6,7] as does the synthesis of aminoacyl-tRNA. At the same time, these enzymes are able to utilize at least in vitro [4] Ap_4A for the formation of aminoacyl adenylate. Besides Ap_4A synthesis, Ap_3A formation was reported for the same enzymes from the adenylate·enzyme complexes where ADP was generated in the process of Ap_4A hydrolysis [6,7]. No aaRS exhibited Ap_3A synthesis independent from Ap_4A formation.

AaRS of different amino acid specificities are subdivided into three groups with respect to their ability to form Ap_4A [6]. TrpRS is placed with the aaRS showing no significant rate of Ap_4A synthesis. Here we report that TrpRS is able to catalyze the in vitro synthesis of Ap_3A. Part of these results has been published elsewhere [8]. Furthermore, we found out that TrpRS is also able to use Ap_3A (not Ap_4A) instead of ATP in the reactions of E·(Trp ~ AMP) formation and $tRNA^{Trp}$ aminoacylation.

2. Experimental

TrpRS was purified from the bovine pancreas as described earlier [9,10]. Two types of enzyme preparations were used: (i) the preparations containing one Zn^{2+} atom per dimeric molecule isolated in the absence of EDTA [9] and (ii) Zn^{2+}-deprived preparations isolated either after addition of EDTA or after prolonged storage [10]. The latter samples possessed lower enzymatic activity and were able to hydrolyse ATP into ADP and P_i [11]. As tested by the denaturing polyacrylamide gel electrophoresis, both enzyme forms were homogeneous. Both preparations posssessed no Ap_4A-pyrophospho hydrolysing activity: labelled ADP, or ATP and AMP have been not detected after incubation of the enzyme with [^{3}H]Ap_4A.

Ap_3A synthesis was examined in two ways. First, tryptophanyl[^{14}C]adenylate·TrpRS complex was obtained and isolated as described [9] and treated with the excess of ADP (see legend to Fig. 2). In the second assay, the sample initially contained ADP and all the components needed for the formation of the tryptophanyl[^{14}C]adenylate·enzyme complex in situ [9]. ATP/E and ATP/ADP ratios in the incubation mixtures varied from 100:1 to 1:1 mol/mol.

The reaction products were identified on the PEI-cellulose plates (Merck, Germany). Aliquots of the reaction mixture were applied together with the nucleotide markers. The plates were washed with H_2O and than dried and developed in 0.75 M LiCl, or in 0.75 M KH_2PO_4 (pH 3.5), or in 0.8 M LiCl + 7% H_3BO_3 (pH 4.6). The nucleotide derivatives were detected in UV light and fluorographed. In order to estimate the amounts of the labelled nucleotides, the corresponding areas of PEI-cellulose plates were removed and counted in the toluene scintillation liquid on SL-30 (Intertechnique, France) counter.

E·(Trp ~ AMP) formation from [^{14}C]tryptophan and Ap_3A was measured by the retention of ^{14}C-label of aminoacyl adenylate·enzyme complexes absorbed on BA83 nitrocellulose filters.

HPLC was performed on the 0.4 × 15 cm Shandon APC (UK) column. Nucleotides were eluted by the linear gradient of KH_2PO_4

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Abbreviations: aaRS, aminoacyl-tRNA synthetases (EC 6.1.1); Ap_3A, P^1,P^3-bis(5′-adenosyl)triphosphate; Ap_4A, P^1,P^4-bis(5′-adenosyl)tetraphosphate; ATPase, adenosine triphosphatase; E(Trp ~ AMP), tryptophanyl adenylate–enzyme complex; E(−Zn), Zn^{2+}-deprived enzyme; PEI-cellulose, polyethylene imino cellulose; TrpRS, tryptophanyl-tRNA synthetase (EC 6.1.1.2).

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(0.05–0.90 M) in 10% methanol. ^{31}P-NMR spectra free from the background proton noise and ^{1}H-NMR spectra were obtained on Varian XL-100-15 (USA). As the internal standards, trimethylphosphate and triethylbutanol, respectively, were applied as described [11].

3. Results

3.1. Ap_3A replaces ATP in the TrpRS-catalyzed E·(Trp ~ AMP) formation and $tRNA^{Trp}$ aminoacylation

If TrpRS is incubated with Ap_3A (1.4–10 mM) replacing ATP in the standard mixture for E·(Trp ~ [^{14}C]AMP) synthesis, the complex is formed with the molar ratio Trp ~ [^{14}C]AMP/TrpRS about 0.2 whereas in the presence of [^{14}C]ATP it was about 0.8. The E·(Trp ~ AMP) separated from low-molecular-weight substrates by gel filtration, rapidly and quantitatively reacts with PP_i forming ATP and with $tRNA^{Trp}$ forming Trp-$tRNA^{Trp}$. These observations imply that the complex formed is able to compete with common substrates of the reactions catalyzed by TrpRS.

The $tRNA^{Trp}$ aminoacylation at various Ap_3A concentrations in double reciprocal coordinates is presented in Fig. 1. The estimated K_m value for Ap_3A in this reaction is 1.0 ± 0.1 mM, whereas in the other experiments K_m for ATP was 0.22 ± 0.01 mM. V_{max} for Ap_3A is about 30% of that for ATP.

As shown by TLC and HPLC and by NMR spectroscopy Ap_3A preparation was not contaminated by ATP and the content of other nucleotides did not exceed 0.1%. Prior to experiments, the enzyme was purified additionally by gel-filtration followed by precipitation at pH 5.0. The preparations were free from ATP as demonstrated by CD and UV spectroscopy.

TrpRS preparations also possessed no Ap_3A degrading activity: Ap_3A remained stable during the prolonged incubation with the enzyme in the absence of tryptophan (not shown). $tRNA^{Trp}$ was purified from the nucleotide contaminations as described [12].

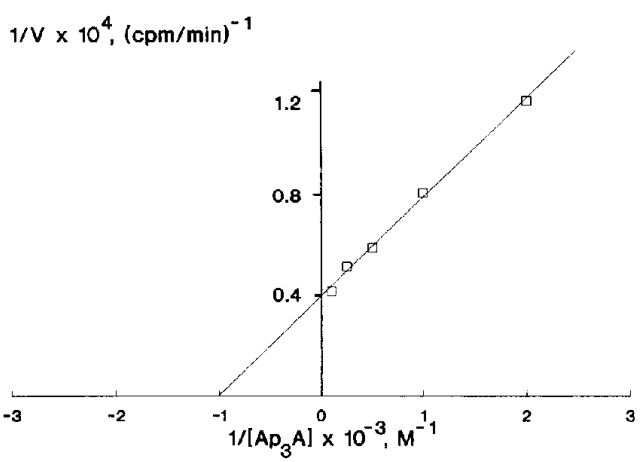

Fig. 1. The rate of $tRNA^{Trp}$ aminoacylation reaction versus Ap_3A concentration in double reciprocal coordinates.

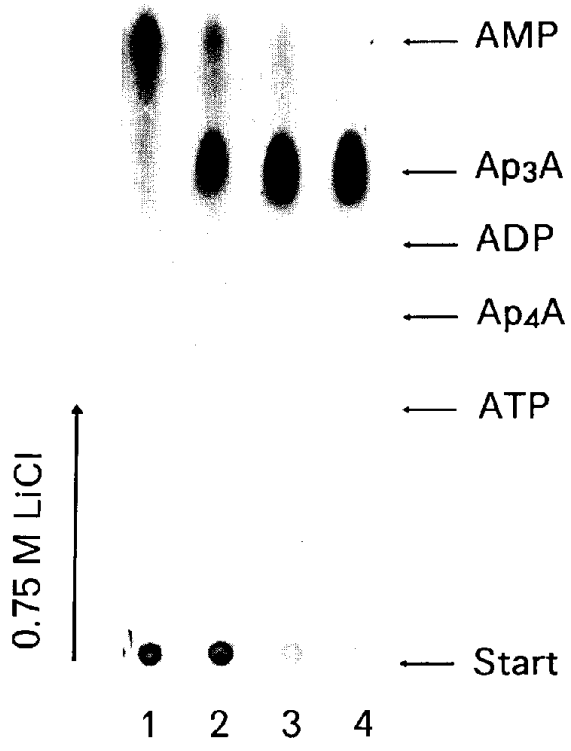

Fig. 2. Ap_3A synthesis from E·(Trp ~ [^{14}C]AMP) and ADP as seen on TLC fluorogram on 20 × 20 cm Merck PEI-cellulose plate: E·(Trp ~ [^{14}C]AMP), 2 μM (1); E·(Trp ~ [^{14}C]AMP) incubated 1 min (2) at 37°C and pH 7.5 with 2 mM ADP and 5 mM $MgCl_2$, 5 min (3), and 30 min (4).

Participation of Ap_3A as a substrate in the enzymatic reaction of Trp ~ AMP formation implies that in principle the TrpRS-catalyzed synthesis of Ap_3A from ADP and Trp ~ AMP might be also observed.

3.2. TrpRS catalyzes Ap_3A synthesis via formation of tryptophanyl adenylate intermediate

In order to reveal Ap_3A formation from aminoacyl adenylate and ADP, E·(Trp ~ [^{14}C]AMP) was isolated as described [9] and incubated with ADP. The reaction products were identified by TLC followed by fluorography. Addition of ADP to the E·(Trp ~ AMP) isolated by gel-filtration leads to its complete conversion into Ap_3A (Fig. 2). When ATP was added instead of ADP, no detectable amount of Ap_4A was formed at least during 5 h in agreement with the observation that TrpRS lacks Ap_4A-synthetase activity [6].

TrpRS-dependent Ap_3A synthesis was also observed when 10-fold molar excess of ADP over ATP was added to the reaction mixture to form E·(Trp ~ AMP) in situ (molar ratio ATP/E = 10, at 5–10 μM concentration of TrpRS) in the presence of 5–8 mM $MgCl_2$ and yeast

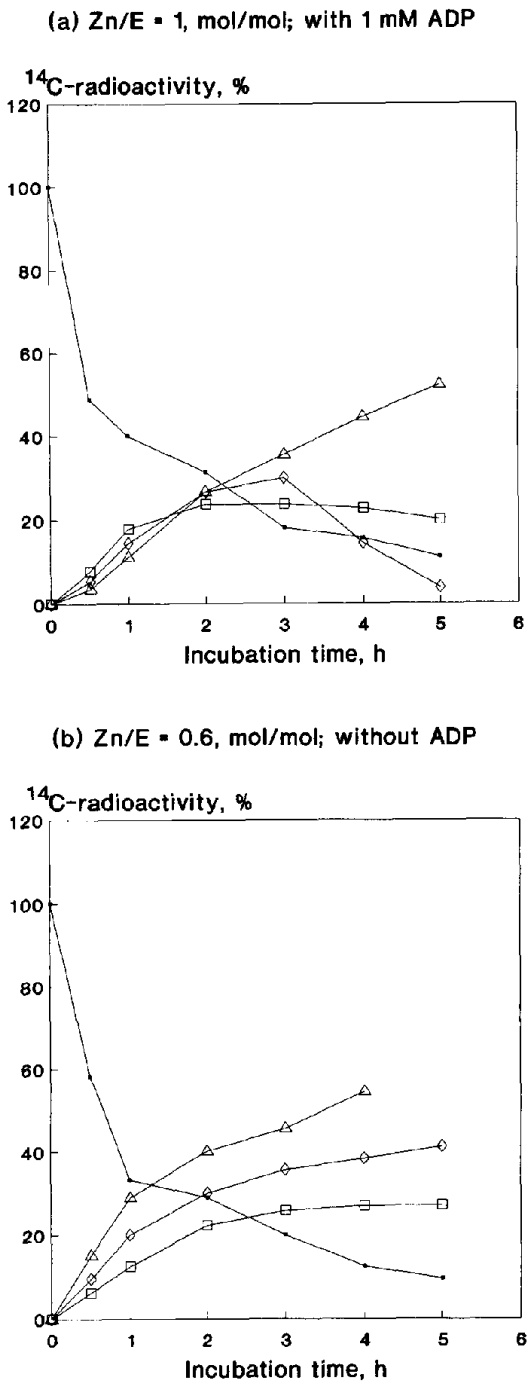

Fig. 3. Ap_3A synthesis at the conditions of E·(Trp ~ AMP) formation catalyzed by enzyme preparations with Zn/E ratio = 1 mol/mol of dimer in the presence of ADP (a), and with Zn/E = 0.6 mol/mol of dimer in the absence of ADP (b). (□) –ATP, (◇) –Ap_3A, (△) –AMP, (•) –ADP. Concentration of the reagents: [^{14}C]ATP, 0.1 mM; tryptophan, 5×10^{-5} M; $MgCl_2$, 8 mM; KCl, 150 mM; DTT, 0.1 mM; inorganic pyrophosphatase, 0.015 mg/ml; ADP, 1 mM. Incubation at 37°C, pH 7.5.

inorganic pyrophosphatase. As seen from Fig. 3A, the decrease of ATP concentration and increase of AMP concentration are accompanied by Ap_3A synthesis and de novo ADP formation. The curve of Ap_3A synthesis reaches a maximum after 3 h. The complex pattern of Ap_3A synthesis, as well as the elevated levels of AMP and ADP in the reaction mixture seem to indicate that after the decrease of ATP concentration Ap_3A takes part in the E·(Trp~AMP) synthesis as considered in the section 3.1.

Fig. 3b shows the kinetics of the Ap_3A formation catalyzed by Zn^{2+}-depleted TrpRS in the absence of exogenous ADP. In this case, Ap_3A synthesis may be related to the ADP formation caused by ATP hydrolysis catalyzed by Zn^{2+}-deprived enzyme [10]. The curve (Fig. 3a,b) has an incline at half of the initial ATP concentration. Probably, when E·(Trp ~ AMP) complex is formed in situ, its interaction with ADP induces negative cooperativity for the two enzyme subunits as described earlier for the other low-molecular-weight ligands [9].

3.3. TrpRS is deficient in Ap_4A-synthetase activity

The lack of Ap_4A synthesis by TrpRS [6] might be in principle connected with the non-optimal experimental conditions chosen for its detection. Therefore, we decided to examine Ap_4A-synthetase activity of TrpRS at variable concentrations of TrpRS (1–10 μM), ATP (1–10 mM) and tryptophan (10^{-2}–1 mM). The effect of Mg^{2+} (1–10 mM), Mn^{2+} (0.1–1.0 mM) and inorganic pyrophosphatase (0.015 mg/ml) on Ap_4A synthesis was also studied. TLC showed no Ap_4A formation for up to 17 h incubation in all these assay conditions. Furthermore, no Ap_4A formation was noticed after incubation of E·(Trp ~ AMP) with ATP (data not shown).

In order to check the possibility of amino acid-independent but AMP-dependent Ap_4A synthesis described earlier for the lysyl-tRNA synthetase from rat liver [13] we studied the effect of AMP concentration (2–10 mM) on the reaction rate. Stimulation of Ap_4A synthesis was recorded neither in the presence, nor in the absence of tryptophan (data not shown).

Consequently, TrpRS in fact does not catalyze Ap_4A synthesis in accord with the earlier data [6].

4. Discussion

Contrary to the majority of the aaRS, mammalian TrpRS in a wide range of the reaction conditions is unable to catalyze Ap_4A synthesis. This property of TrpRS may be related to the fact that PP_i split off ATP in the course of Trp ~ AMP synthesis on the enzyme remains tightly bound to the TrpRS [14]. The bound PP_i is non-hydrolyzable by inorganic pyrophosphatase [15]. When PP_i and Trp ~ AMP are both bound to the same enzyme molecule, further binding of ATP molecule is precluded (see [15]); that accounts for the inability of TrpRS to promote Ap_4A formation. Furthermore, contrary to the majority of the aaRS, TrpRS is virtually unable to utilize Ap_4A instead of ATP in $tRNA^{Trp}$ aminoacylation reaction.

For Ap_4A-synthesyzing aaRS, Ap_3A synthesis is a secondary process: aminoacyl adenylate·enzyme complexes

react with ADP generated in prokaryotes from Ap_4A degradation to 2 ADP (see [2,6] and references therein). Therefore, as TrpRS does not promote Ap_4A synthesis it is unable to synthesize Ap_3A through Ap_4A decomposition.

Nevertheless, as shown here TrpRS is able to utilize Ap_3A instead of ATP as a substrate for the reactions of E·(Trp ~ AMP) formation and $tRNA^{Trp}$ aminoacylation, and to catalyze at certain conditions the Ap_3A synthesis as well. This Ap_3A-synthetase activity of the TrpRS is related neither to the presence of traces of ATP in the reaction mixture, nor to the contaminating pyrophospho hydrolase activity.

Lower stoichiometry for Trp ~ AMP/E observed for Ap_3A as compared with ATP could be caused by at least two factors: (i) E·(Trp ~ AMP) formed from Ap_3A appears to be less stable because it is known (see [14] and references therein) that aminoacyl adenylate·enzyme complexes are stabilized by the additional binding of PP_i from ATP; in case of Ap_3A the second product of the reaction is ADP, not PP_i; (ii) the second adenosine moiety present in Ap_3A molecule might generate stereochemical hindrance in the course of Ap_3A reaction with tryptophan.

In the $tRNA^{Trp}$ aminoacylation reaction, K_m for Ap_3A is almost the same as K_s for ATP, but is twice higher than K_m for ATP in the same reaction [9]. The maximal reaction rate (V_{max}) for Ap_3A is about 1/3 of V_{max} for ATP. This difference may be related to the difference of the reaction products (PP_i in the case of ATP and ADP in the case of Ap_3A): it is known that ADP is a TrpRS inhibitor non-competitive with ATP [16].

TrpRS catalyzes Ap_3A synthesis when ADP is added to the isolated E·(Trp ~ AMP) and also at the conditions of in situ formation of E·(Trp ~ AMP) in the presence of 10-fold molar excess of ADP over ATP. Consequently, Ap_3A synthesis proceeds via aminoacyl adenylate intermediate. This conclusion agrees with the dependence of this reaction on the presence of L-tryptophan, ATP-Mg^{2+} and inorganic pyrophosphatase.

The adenylate pathway of the Ap_3A synthesis might be expected a priori: from the fact that Ap_3A may serve as a substrate for the reaction of E·(Trp ~ AMP) formation it follows that the reversed reaction of E·(Trp–AMP) with ADP is eligible according to the principle of micro reversibility of chemical reactions. Therefore, Ap_3A may be regarded as both a substrate and a product of one of the non-canonical reactions catalyzed by TrpRS.

Ap_3A synthesis from E·(Trp ~ AMP) was observed both in the presence and in the absence of exogenous ADP for the Zn^{2+}-deprived TrpRS preparations (Zn^{2+}/E < 0.6 mol/mol per dimer). Recently, ATPase/GTPase activity of Zn^{2+}-depleted enzyme preparations was described [10]. These preparations were unable to form E·(Trp ~ AMP) [10]. The reaction of Ap_3A formation by the Zn^{2+}-deprived enzyme seems to proceed as follows. The preparations contain two types of molecules, and consequently the molecules with normal Zn^{2+} content (Zn/E about 1 mol/mol) are able to form E·(Trp ~ AMP), whereas the enzyme molecules lacking Zn^{2+} are able to catalyze ATP hydrolysis. The reaction of ADP formed from ATP with E·(Trp ~ AMP) leads to the accumulation of Ap_3A as a final product.

The ability of mammalian TrpRS to catalyze the synthesis of Ap_3A, not Ap_4A, might be considered as a molecular tool preventing the enzyme inactivation. Ap_4A, known to be a strong chelator for the Me^{2+} ions [17] may chelate Zn^{2+} ion essential for the enzymatic activity [11] and remove it from the enzyme [15].

If this is the case, the enzymes actively forming Ap_4A should not require Me^{2+} for their activity. In fact, aaRS of this type either contain no Zn^{2+}, or Zn^{2+} ion is not essential for their catalytic activity [18]. Contrary to Ap_4A, Ap_3A is not a strong chelator of Me^{2+}, and consequently does not affect the aminoacylation function of TrpRS. Ap_3A is often regarded as physiological antagonist of Ap_4A (see [3,19] and references therein). We assume that Ap_4A-synthesyzing (Lys, Pro, etc.) and Ap_3A-synthesyzing (Trp) enzymes might be implicated in regulation of Ap_3A/Ap_4A ratio in the cells.

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