

# Nucleoside triphosphatase activity associated with the N-terminal domain of mammalian tryptophanyl-tRNA synthetase

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Bovine tryptophanyl-tRNA synthetase (EC 6.1.1.2) deprived of  $Zn^{2+}$  by chelation with the phosphonate analog of  $Ap_4A$  hydrolyzed ATP(GTP) to ADP(GDP) although its ability to form tryptophanyl adenylate was impaired. This hydrolytic activity is stimulated by  $Mg^{2+}$  and  $Mn^{2+}$  ions and inhibited by  $Zn^{2+}$ . Monoclonal antibody Am1 against the N-terminal domain of the enzyme completely abolished ATP(GTP)ase activity. The core peptide generated after proteolytic splitting of the N-domain lacks this activity. We suggest that the nucleotide binding site(s) different from ATP sites involved in aminoacylation reaction reside(s) at the N-terminal domain(s) of the enzyme.

Mammalian aminoacyl-tRNA synthetase; ATP/GTP hydrolysis; Non-canonical enzymatic activity;  $Zn^{2+}$  chelation;  $Ap_4A$  phosphonate analog

## 1. INTRODUCTION

Aminoacyl-tRNA synthetases (E.C. 6.1.1) of mammalian cells exhibit a peculiar feature in their structure which does not occur in their prokaryotic counterparts, that is, they possess extensions at the N and/or C-termini of the polypeptide chains (for reviews see [1,2]). It was assumed that these extra domains typical for the enzymes of higher eukaryotes may be involved in the formation of multi-enzyme complexes [1–3] although it cannot be a general function since certain synthetases do not associate into multi-synthetase complex but nevertheless exhibit a large N-terminal domain (for example, bovine TrpRS). Therefore, it seems quite possible that the N-domains of synthetases from higher organisms may be involved in still unknown functions which could be called 'non-canonical' [4].

In this work, we attempted to approach this problem by investigation of the unusual enzymatic activity revealed in certain homogenous preparations of bovine TrpRS, mostly after prolonged (>2 months) storage at  $-70^{\circ}C$  in the presence of stabilizing reagents (see [5]): TrpRS became capable of catalyzing ADP formation

along with partial (activation of tryptophan) and complete (tRNA<sup>Trp</sup> charging) reactions. Furthermore, ADP formation was noticed in the reaction mixture containing E(Trp~AMP), tryptophan and ATP- $Mg^{2+}$  in experiments aimed to study the stability of the adenylate complexes and of dinucleoside tetraphosphate synthetase activity of TrpRS (preliminary results were reported by G. Kovaleva et al. [6]). The non-stoichiometric ATP consumption accompanied by ADP formation was reported earlier for ArgRS from *Bacillus stearothermophilus* [7]. This unusual enzymatic activity of ArgRS remained unexplained.

ADP may be considered neither as a final nor as an intermediate product of the reactions catalyzed by aminoacyl-tRNA synthetases (see [8]). However, during the last decade non-canonical functions were described for a number of aaRS (see [4] and references therein). For example, certain aaRS can catalyze in vitro phosphorolysis of the cognate aminoacyl adenylates and/or amino acid-dependent phosphorolysis and hydrolysis of  $Ap_4A$ . ADP is one of the products of these reactions. In fact, ADP formation in the presence of phenylalanine,  $Mg^{2+}$  and ATP or  $Ap_4A$  was observed for PheRS from *E. coli* and yeast [9,10]. For the *E. coli* GlyRS it was shown [11] that ADP is the product of phosphorolysis of the glycyl adenylate enzyme complex formed in the presence of glycine,  $Mg^{2+}$  and ATP or  $Ap_4A$ . A similar mechanism of ADP formation was shown for yeast PheRS [12]. In all these experiments [9–12]  $P_i$  was formed in the reaction mixture from  $PP_i$  hydrolysed by inorganic pyrophosphatase added to the reaction mixture or by other contaminating phosphatases. In these reactions ADP was formed from ATP indirectly as a secondary product. However, ATP hydrolysis leading

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**Abbreviations:** aaRS, aminoacyl-tRNA synthetase;  $Ap_3A$ ,  $P^1, P^3$ -bis(5'-adenosyl)triphosphate;  $Ap_4A$ ,  $P^1, P^4$ -bis(5'-adenosyl)tetraphosphate; ATP(GTP)ase, adenosine (guanosine) triphosphatase; GlyRS, glycyl-tRNA synthetase; E(Trp~AMP), tryptophanyl adenylate enzyme complex; E(-Zn),  $Zn^{2+}$ -deprived enzyme; mAbs, monoclonal antibodies; PEI-cellulose, polyethylene iminocellulose; PheRS, phenylalanyl-tRNA synthetase; TrpRS, tryptophanyl-tRNA synthetase.

to immediate accumulation of ADP cannot be excluded a priori though it still is not confirmed for aaRS. The aim of this work was to analyze both the 'direct' and 'indirect' way of ADP formation catalyzed by bovine TrpRS.

## 2. EXPERIMENTAL

All solutions for experiments with the Zn-deprived form of the enzyme were purified as in [13]. The phosphonate analog of  $\text{Ap}_4\text{A}$  -  $\text{ApCH}_2\text{pppA}$  was synthesised and kindly provided by Dr. N. Tarusova. tRNA containing 2% tRNA<sup>Trp</sup> was prepared from total yeast RNA [14]. TrpRS was isolated from beef pancreas as described earlier [5,15]. The enzyme was homogeneous on SDS-PAGE [16]. 'Core' enzyme ( $M_r \sim 40 \text{ kDa} \times 2$ ) was isolated as described in [15]. The protein concentrations were determined according to Bradford [17] for E(-Zn) or by using the absorbion coefficient of  $0.90 \frac{\text{cm}^2}{\text{mg/ml}}$  for native TrpRS [5]. Isotopic ATP-PP<sub>i</sub> exchange and tRNA<sup>Trp</sup> aminoacylation activities were estimated as described [5].

Tryptophanyl-adenylate enzyme complexes were obtained as described earlier (see [5]). Stoichiometry of E(Trp~AMP) was determined by its retention on a nitrocellulose filter BA83 or by the 'burst' of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  hydrolysis [18]. In order to obtain the protein free from bound Zn ions, TrpRS (1.5–2 mg/ml) was incubated at 37°C with 6 mM  $\text{ApCH}_2\text{pppA}$  in 50 mM HEPES-NaOH, pH 7.5 containing 12 mM  $\text{MgCl}_2$  and 0.1 M KCl, then dialyzed against 50 mM Tris-HCl, pH 7.5, containing  $2 \cdot 10^{-4}$  M dithiothreite,  $10^{-4}$  M EDTA; the buffer solution was changed four times at 12 h intervals. The degree of enzyme deprivation from Zn ions and from excess dinucleotide was tested by CD spectra (absence of positive peak at 290–310 nm [19]) and by UV spectra ( $A_{280}/A_{260} \sim 1.8$ ).

The rate of ATP or GTP hydrolysis in the presence of E(-Zn) was estimated from  $[\text{C}^{14}]\text{ATP}$  or  $[\text{C}^{14}]\text{GTP}$  consumption or  $[\text{C}^{14}]\text{GDP}$  formation using TLC on PEI-cellulose F plates. A sample of 0.1 ml prepared for incubation contained: 0.8–2.5  $\mu\text{g}$  enzyme, 0.05–5 mM  $[\text{C}^{14}]\text{ATP}$  or  $[\text{C}^{14}]\text{GTP}$ , 0–10 mM  $\text{MgCl}_2$ , 80 mM KCl in 50 mM HEPES-NaOH buffer, pH 7.5. At the appropriate time intervals aliquots of the reaction mixture were applied together with the markers-carriers (ATP, ADP, AMP,  $\text{Ap}_4\text{A}$ , or GTP, GDP, GMP) onto PEI-cellulose TLC plates. The plates were washed with  $\text{H}_2\text{O}$  and than run in 0.75 LiCl. The resulting products were detected under UV-light and fluorographed. In order to estimate relative amounts of radiolabelled nucleotides corresponding pieces of PEI-cellulose were cut and counted in toluene scintillation liquid.

The rate of ATP hydrolysis in presense of E(-Zn) was also estimated from  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  consumption as described by Fersht et al. [18]. The incubation sample (0.1 ml) contained: 0.1  $\mu\text{M}$  enzyme, 0.1 mM  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ , 0–10 mM  $\text{MgCl}_2$ , 80 mM KCl,  $10^{-4}$  M dithiothreite, 50 mM HEPES-NaOH (pH 7.5). The reaction was run at 37°C with or without inorganic pyrophosphatase (0.015 units/ml) added. At the appropriate time intervals the amount of radioactive ATP was measured in the aliquots as described for the reaction of isotopic ATP-PP<sub>i</sub> exchange [5].

The CD spectra were measured using the Jobin-Ivon Marck III dichrograph (France) at the conditions described in [19]. The  $K_m$ app. (ATP/GTP) were obtained by least square regression analysis of Lineweaver-Burk plots.

## 3. RESULTS AND DISCUSSION

### 3.1. TrpRS does not catalyze the phosphorolysis of E(Trp~AMP)

Two types of experiments were performed to test TrpRS phosphorolytic activity. First, E(Trp~ $[\text{C}^{14}]\text{AMP}$ ) was formed, isolated by gel filtration and incubated with  $\text{P}_i$ . As seen from Fig. 1 (lanes 2 and 3) no ADP

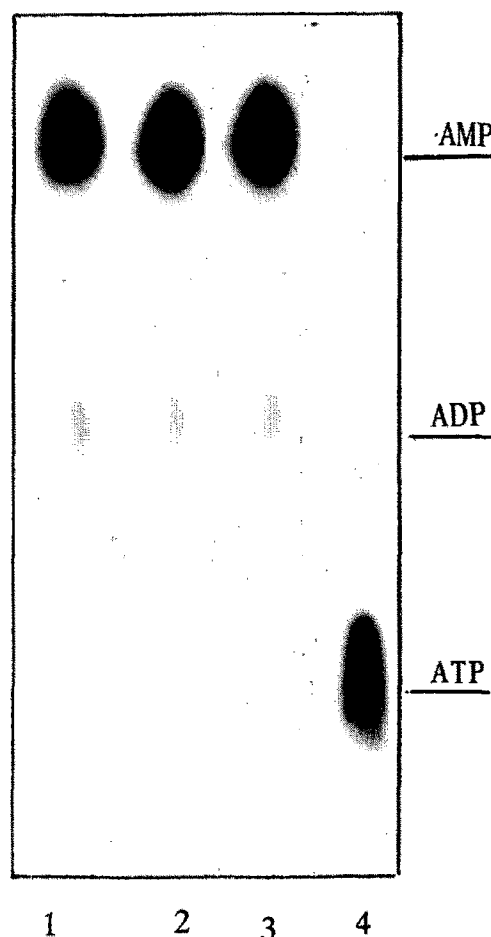


Fig. 1. The PEI-cellulose TLC fluorogram of ETrp~ $[\text{C}^{14}]\text{AMP}$  2  $\mu\text{M}$ , (it is quantitatively converted to AMP when fixed on PEI-cellulose (lane 1)); after treatment (pH 7.5, 10 h, 37°C) with 5 mM  $\text{P}_i$  and 10 mM  $\text{MgCl}_2$  (lane 2) or 5 mM  $\text{P}_i$  and 5 mM  $\text{MnCl}_2$  (lane 3); after treatment (pH 7.5, 10 min, 37°C) with 2 mM  $\text{PP}_i$  and 10 mM  $\text{MgCl}_2$  (lane 4).

formation was observed up to 10 h of incubation. However, when  $\text{PP}_i$  was added instead of  $\text{P}_i$  rapid and quantitative ATP formation was recorded (Fig. 1, lane 4) indicating the intactness of the complex and its location at the enzyme active site. Second, purified  $^{32}\text{P}_i$  was added to the reaction mixture containing TrpRS, tryptophan, ATP and  $\text{Mg}^{2+}$  to form in situ E(Trp~AMP). ATP was also replaced by  $\text{Ap}_3\text{A}$  or  $\text{Ap}_4\text{A}$ , because for TrpRS it is possible to use these dinucleotides for tryptophan activation [20]. Formation of  $^{32}\text{P}$ -labelled nucleotides was detected after filtration on charcoal discs (see [5]) and/or by TLC. No formation of radioactive ADP was recorded (not shown). In addition, TrpRS has no pyrophosphohydrolytic activity and incubation of the enzyme with  $[\text{H}^3]\text{Ap}_4\text{A}$  does not lead to formation of  $[\text{H}^3]\text{mono-}$  or dinucleotides.

Thus, TrpRS is unable to catalyze ADP formation either via phosphorolysis of the tryptophanyl adenylate complex or via hydrolysis of  $\text{Ap}_4\text{A}$ .

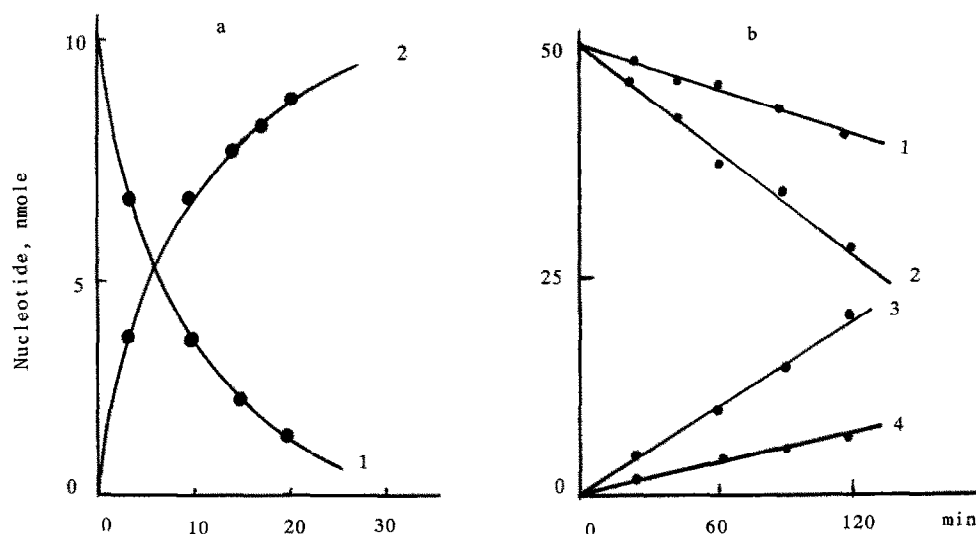


Fig. 2. Kinetics of ATP (a,b) and GTP (b) hydrolysis catalyzed by E(-Zn). (a) 1, [ $^{14}\text{C}$ ]ATP; 2, [ $^{14}\text{C}$ ]ADP; (b) 1, [ $^{14}\text{C}$ ]ATP; 2, [ $^{14}\text{C}$ ]GTP; 3, [ $^{14}\text{C}$ ]GDP; 4, [ $^{14}\text{C}$ ]ADP. Concentrations: [ $^{14}\text{C}$ ]ATP, 0.05 mM (a) or 1 mM (b); [ $^{14}\text{C}$ ]GTP, 1 mM; E(-Zn), 0.08  $\mu\text{M}$  (a) or 0.1  $\mu\text{M}$  (b).

### 3.2. ATP (GTP)ase activity of $\text{Zn}^{2+}$ deprived TrpRS

In the course of our work with TrpRS it was observed that the elevation of ATPase activity is associated with a loss of  $\text{Zn}^{2+}$  from the enzyme.  $\text{Zn}^{2+}$  binds to TrpRS rather loosely [19] and may be partially lost during enzyme isolation and/or its storage in the presence of EDTA, DTT and other  $\text{Me}^{2+}$ -chelating reagents. Enzyme preparations with low  $\text{Zn}^{2+}$  content (< 0.6 g-a per mol of dimeric TrpRS) hydrolyze ATP to various degrees (not shown). We suggested that ATPase activity of TrpRS is associated with  $\text{Zn}^{2+}$ -deprived enzyme molecules.

*o*-Phenanthroline and/or extensive dialysis were used earlier [19] to remove Zn ions from the protein. Here we used a more gentle and fast procedure for  $\text{Zn}^{2+}$  removal. TrpRS was treated with an  $\text{Ap}_4\text{A}$  phosphonate analog ( $\text{ApCH}_2\text{pppA}$ ) known to be a strong  $\text{Zn}^{2+}$ -chelating reagent [21]. During dialysis  $\text{Zn}^{2+}$  is completely removed as a  $\text{Zn}^{2+}$ - $\text{ApCH}_2\text{pppA}$  complex. The  $\text{Zn}^{2+}$ -deprived enzyme preparation, E(-Zn), exhibits the same dimeric composition as the native enzyme.

Fig. 2 shows the kinetics of [ $^{14}\text{C}$ ]ATP hydrolysis and ADP formation in the presence of E(-Zn). ADP appears to be the immediate product of ATP hydrolysis.

The [ $\gamma$ - $^{32}\text{P}$ ]ATP assay indicated that the second product of ATP hydrolysis is  $\text{P}_i$  (not shown). ATPase activity was neither influenced by tryptophan nor by inorganic pyrophosphatase (not shown).

GTP also appears to be a substrate of E(-Zn). Under conditions described in the legend to Fig. 2, GTP hydrolysis (curve 2) is 2.5 times faster than ATP hydrolysis (curve 1). The  $K_m$  values were 0.17 mM for GTP and 0.9 mM for ATP. CTP was nonsignificantly hydrolyzed by E(-Zn) (not shown).

ATP(GTP)ase is rapidly and completely inhibited by  $10^{-4}$  M EDTA showing the importance of  $\text{Me}^{2+}$  for its

activity. The kinetics of GDP formation from GTP in the presence of  $\text{Mg}^{2+}$  and  $\text{Mn}^{2+}$  is presented on Fig. 3. The hydrolysis is significantly stimulated by both ions (curves 1 and 2). The background ADP formation recorded without external  $\text{Me}^{2+}$  added (curve 3) may be related to the traces of  $\text{Mg}^{2+}$  present even in the best purified nucleotide preparations [22]. The GTPase activity of E(-Zn) is inhibited by  $\text{Zn}^{2+}$  (Fig. 3, curve 4) and entirely disappears after short treatment of the reaction mixture with Chelex-100 (Fig. 3, curve 5), but may be almost completely recovered after addition of  $\text{Mg}^{2+}$  (not shown). As seen from the comparison of the linear parts of curves 1 and 5 (Fig. 3) the rate of GTP hydrolysis in the presence of 2 mM  $\text{Mg}^{2+}$  is about 3.0  $\mu\text{mol}/\text{mg}/\text{min}$ , more than 10 times higher than without  $\text{Me}^{2+}$ . The ATPase activity of E(-Zn) is slightly stimulated by 10 mM  $\text{Ca}^{2+}$  and 80 mM  $\text{K}^+$ , but not by  $\text{Na}^+$  ions (not shown).

While ATPase activity is inhibited by  $\text{Zn}^{2+}$  ions, the tRNA<sup>Trp</sup> charging is partially restored (not shown). The complete recovery of this activity was not achieved in these experiments due to protein precipitation at  $\sim 70 \mu\text{M}$   $\text{Zn}^{2+}$  concentrations. This observation corresponds with the data [23] on aggregation of TrpRS even at low  $\text{Zn}^{2+}$  concentrations.

### 3.3. ATP(GTP)ase activity is associated with the N-domain of TrpRS

Since E(-Zn) catalyzes neither tryptophan-dependent ATP- $\text{PP}_i$  exchange, nor tRNA charging and is unable to form a stable complex with  $\text{Ap}_4\text{A}$ , a compound closely resembling ATP [20,21], we proposed that the nucleoside triphosphates hydrolyzed by the enzyme bind to sites different from the substrate ATP binding site. Two types of nucleotide binding sites were described for bovine TrpRS [24]: the catalytic sites which

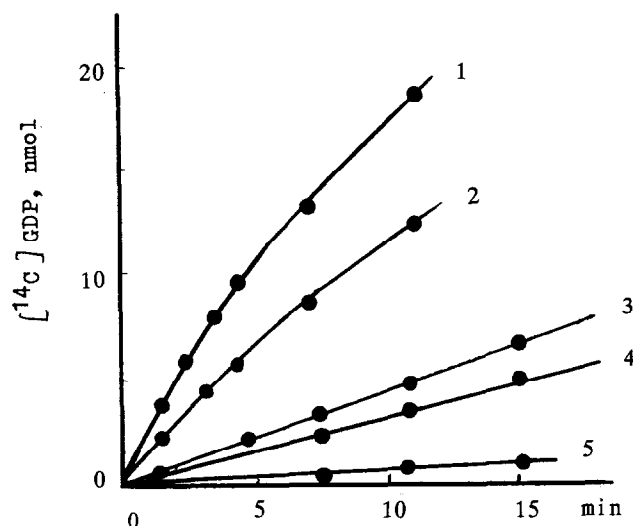


Fig. 3. Effect of some  $\text{Me}^{2+}$  ions on kinetics of  $[^{14}\text{C}]\text{GDP}$  formation from  $[^{14}\text{C}]\text{GTP}$  (1 mM) catalyzed by  $\text{E}(-\text{Zn})$  ( $0.07 \mu\text{M}$ ). 1,  $\text{Mg}^{2+}$  (2 mM); 2,  $\text{Mn}^{2+}$  (2 mM); 3, no  $\text{Me}^{2+}$  added; 4,  $\text{Zn}^{2+}$  ( $40 \mu\text{M}$ ); 5, endogenous  $\text{Me}^{2+}$  removed by Chelex-100.

are  $\text{Me}^{2+}$ -dependent and highly specific to adenosine (ATP and/or AMP moiety of  $\text{Trp}\sim\text{AMP}$ ) and the 'additional' nucleotide binding sites. The latter sites are less specific to nucleic base but possess higher affinity to various nucleotides.

Monoclonal antibodies against TrpRS characterized earlier [25] were applied to localize the binding sites of the nucleosides triphosphates hydrolyzed by  $\text{E}(-\text{Zn}^{2+})$ . It is known [25] that the enzyme activity is inhibited by mAbs Am2, but not by Am1 and Am3. It was supposed [25] that the antigenic determinant for Am2 is located at the catalytic C-domain (core) of the enzyme or nearby. Epitopes for Am1 and Am3 are located at the N-domain [25], which does not participate in tRNA aminoacylation [26,27].

The influence of each of the three mAbs on the ATP(GTP)ase activity of  $\text{E}(-\text{Zn})$  was tested (Table I). It is evident that the hydrolysis is significantly inhibited by Am1 and by Am3, whereas Am2 has no effect. Without  $\text{E}(-\text{Zn})$  almost no GTP hydrolysis occurred indicating the absence of GTPase active contamination in the mAbs preparations used.

The fact that GTP hydrolysis is inhibited by Am1, not by Am2 (Table I) indicates the participation of the N-domain in nucleotide binding because, as mentioned above, the epitopes for Am1 and Am3 are located at the N-domain of TrpRS, and for Am2 at the core domain [25]. Nucleotide binding site localization at the N-domain might be directly confirmed by detecting this activity in the isolated N-domain. However, we as well as earlier investigators (see [8]), failed to isolate it in the intact state. Polypeptide chain fragments of this domain seem to be localized at the surface of the compact protein globule (C-domain). They interact with 2/3 polyAbs

though their length is only about 1/3 of the total enzyme polypeptide [25]. They are easily cleaved by proteases of various amino acid specificity [25]. That is why even under conditions of limited proteolysis, the N-domain is separated from the molecule only as small fragments. The mixture of this fragments is neither able to bind the Abs [25], nor to hydrolyze ATP(GTP).

Thus, we tried to confirm our conclusion by independent evidence. Limited proteolysis was applied to obtain the core enzyme fragment with  $M_r$  about  $40 \text{ kDa} \times 2$  lacking the N-domain [26]. It was deprived of  $\text{Zn}^{2+}$  by prolonged dialysis against 5 mM EDTA and 5%  $\beta\text{-ME}$ . This preparation contained no  $\text{Zn}^{2+}$  as detected by CD spectroscopy and it neither catalyzed GTP nor ATP hydrolysis (not shown).

Therefore, ATP(GTP)ase activity of Zn-depleted preparations found in our experiments is associated with the N-domain of bovine TrpRS.

It is noteworthy that the ATP(GTP)ase activity is not associated with traces of other proteins which might be present in the preparations since: (i) TrpRS preparations were homogeneous on SDS-PAGE and analytical gel-filtration; (ii) mAbs Am1 against TrpRS entirely inhibited the ATP(GTP)ase activity; (iii)  $\text{Zn}^{2+}$  ions while inhibiting ATP(GTP)ase activity, strongly stimulated the tRNA aminoacylation activity of TrpRS.

The appearance of ATP(GTP)ase activity in Zn-depleted TrpRS may be due to the involvement of  $\text{Zn}^{2+}$  amino acid ligands in the nucleoside triphosphate hydrolysis, or more likely, to the conformational changes in the protein molecule induced by Zn deprivation. The functional role of ATP(GTP)ase activity remains unclear.

However, several observations indicate that this non-canonical enzyme activity might take place in vivo: (i)

Table I  
GTPase activity of  $\text{E}(-\text{Zn})$  in the presence of mAbs against bovine TrpRS

n	$\text{E}(-\text{Zn})$ $0.15 \mu\text{M}$	Am 1 $5 \mu\text{M}$	Am 2 $4 \mu\text{M}$	Am 3 $5.5 \mu\text{M}$	GTP hydrolysis (%)	
1	+	—	—	—	33.0	0.8
2	+	+	—	—	0	
3	+	—	+	—	33.0	1.5
4	+	—	—	+	2.7	0.5
CONTROLS						
5	—	—	—	—	0.6	0.1
6	—	+	—	—	1.0	0.3
7	—	—	+	—	0.5	0.1
8	—	—	—	+	0.5	0.1

In addition to the components listed above the incubation mixture ( $50 \mu\text{l}$ ) contained:  $[^{14}\text{C}]\text{GTP}$ , 1 mM; KCl, 80 mM;  $\text{MgCl}_2$ , 10 mM; BSA, 0.5 mg/ml; HEPES-NaOH, pH 7.5, 50 mM. The mixture was incubated at  $35^\circ\text{C}$  for 12 min. The level of  $[^{14}\text{C}]\text{GTP}$  hydrolysis was estimated by TLC as described in section 2. Three separate experiments were averaged.

it is possible to isolate E(-Zn) as well as the common Zn-containing preparations even in the absence of Zn-chelating reagents; (ii) the enzyme rapidly becomes Zn-depleted even when stored at low temperature with L-tryptophane added for stabilization; (iii) not only phosphonate Ap<sub>4</sub>A analogs, but Ap<sub>4</sub>A itself may deprive the enzyme of Zn [28] and Ap<sub>4</sub>A levels in a cell may be rather high [29]. In addition, the specific activity of bovine TrpRS is low as compared to the activities of the other aaRS. At the same time the content of TrpRS polypeptides in the bovine pancreas as revealed by mAbs is much higher than the content of other aaRS from this organ and much higher than the TrpRS content in other bovine organs [30,31]. Thus, the additional function of TrpRS in bovine pancreas may be suggested besides the charging of tRNA<sup>Trp</sup>, the content of the latter in bovine pancreas being not increased [30]. The triggering of the canonical and non-canonical enzyme functions might be related to physiological regulation of Zn levels in the living cells.

We attempted to map the nucleotide binding site(s) along the known [32] polypeptide chain of bovine TrpRS. Many nucleotide binding motifs are suggested (see [33,34] and references therein), so we compared the TrpRS amino acid sequence with the proposed nucleotide binding motifs. We suggest that the glycine-rich sequence in positions 7 to 15 located near the N-terminus might be a putative additional nucleotide binding site.

TrpRS is assigned to class I aaRS [35], since it has two short sequence motifs 'HXGH' (X is V for TrpRS) and 'KMSXS' (X is K for TrpRS) [32] which indicate the presence of a structural domain (the Rossmann fold) that binds ATP, as is common for the structure of class I enzymes [35]. The two His residues of the HXGH tetrapeptide in class I synthetases are thought to interact with specific phosphates or bind substrate ATP [35]. This HVGH nucleotide binding motif was found [32] in the TrpRS core - C-domain (positions 75-79), where the active sites of TrpRS are presumably located [26, 27]. Thus, the deduced putative additional nucleotide binding motif GEQGCGSPL (positions 7-15) [32] located at the N-domain is different from the known structural motifs of class I aaRS.

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