Tryptophanyl-tRNA synthetase: pyrophosphorylation of the enzyme in the course of adenylate formation?

G.K. Kovaleva, E.G. Holmuratov and L.L. Kisselev

Institute of Molecular Biology, USSR Academy of Sciences, Moscow V-334, USSR

Received 5 November 1982

Aminoacyl-tRNA synthetase *Pyrophosphate* *Enzyme pyrophosphorylation*

1. INTRODUCTION

The intermediate role of aminoacyl adenylates in the course of enzymatic reactions catalysed by aminoacyl-tRNA synthetases has been proved (reviews [1,2]). However, the mechanisms of adenylate formation and that of transfer of amino acid to tRNA remain obscure. In particular, very little is known about the involvement of various functional groups of enzyme molecules in interconversions of substrates.

Bovine tryptophanyl-tRNA synthetase (EC 6.1.1.2), the best studied aminoacyl-tRNA synthetase of multicellular organisms [3], become pyrophosphorylated after incubation with PP_i [4,5]. Since PP_i is a normal substrate or the product of enzymatic reactions, the pyrophosphoryl enzyme, in principle, may also be formed in the course of catalysis. Here, we have discovered that the pyrophosphorylated derivative of enzyme is formed in the presence of ATP, tryptophan and Mg^{2+}; i.e., in condition of activation of substrate amino acid. It is assumed that the tryptophanyl adenylate synthesis may be accompanied by transient covalent attachment of the pyrophosphate moiety of ATP to the carboxylic group of enzyme via a phosphoanhydride bond.

Abbreviations: DTT, dithiothreitol; PP_i, inorganic pyrophosphate; SDS, sodium dodecylsulphate; TLC, thin-layer chromatography; E·(Trp~AMP), E·$(Trp\sim AMP)_2$, tryptophanyl adenylate·enzyme complexes

2. MATERIALS AND METHODS

The bovine tryptophanyl-tRNA synthetase (M_r 60000 × 2) was isolated and finally purified at pH 4.5 as in [3]. To obtain the enzyme·adenylate complex, 6.6 μM enzyme in 50 mM Tris–HCl (pH 7.5) buffer was incubated with 66 μM ^{14}C-labelled or cold tryptophan, different amounts of [^{14}C]- or [γ-^{32}P]ATP, 1 mM NaCl, 1 mM DTT and $MgCl_2$ as indicated below, in the total volume of 0.6 ml. After a 10 min incubation at 4°C the mixture was passed through the Sephadex G-50 (fine) column (30 × 0.8 cm) containing DTT and $MgCl_2$. Elution rate was 10 ml/h, fraction vol. 0.5 ml. ^{14}C-radioactivity was measured in aliquots in dioxane scintillator, ^{32}P-radioactivity – after precipitation of aliquots with 3% trichloroacetic acid as in [4] and in dioxane scintillator.

To prepare the enzyme free of bound tryptophanyl adenylate but containing the ^{32}P-label transferred from [γ-^{32}P]ATP, the synthetase·adenylate complex (1.5 μM) was treated with 1 mM PP_i in 50 mM Tris–HCl (pH 7.5) and 1 mM DTT followed by gel filtration as above. The ^{32}P-labeled protein isolated in such a way was further incubated with 0.4 mM AMP in 0.5 mM DTT and Tris–HCl (pH 7.5) at 37°C, the aliquots were applied to the PEI-cellulose plates (20 × 20 cm, Schleicher and Schüll); ascending TLC was run in 0.75 M NaH_2PO_4 (pH 3.5) followed by autoradiography.

Published by Elsevier Biomedical Press
00145793/83/0000—0000/$3.00

3. RESULTS AND DISCUSSION

The adenylate formation catalysed by tryptophanyl-tRNA synthetase was monitored by the binding of ^{14}C-labeled tryptophan and/or ATP to the protein-containing fractions after gel-filtration. The ^{32}P-binding to enzyme was measured by its incorporation into protein with [γ-^{32}P]ATP serving as label.

Depending on excess ATP over enzyme and tryptophan, either 1 or 2 mol adenylate are formed/native (dimeric) enzyme [6,7]. In both cases (10-fold and 100-fold molar excess ATP) along with adenylate formation a ^{32}P-binding was observed after gel-filtration of the protein containing fraction (fig.1). The ^{32}P-binding was completely tryptophan-dependent. [^{14}C]ATP and [^{14}C]Trp were bound in equimolar amounts to protein after gel-filtration. No additional ATP splitting over adenylate formation was observed by Fersht's procedure [8]. Taking into account all these observations we arrived at the conclusion that there was no intact ATP bound to enzyme after gel-filtration. If that was the case, the ^{32}P-binding from [γ-^{32}P]ATP might be derived from PP_i formed in the course of adenylate synthesis.

The value of the ^{32}P-binding was equal to 0.6 ± 0.2 equivalent for E·(Trp~AMP) and to 1.2 ± 0.2 equivalent for E·(Trp~AMP)$_2$/mol enzyme. This implies that the major part of the PP_i formed after ATP splitting was retained by the enzyme.

The ^{32}P-label bound to enzyme from [γ-^{32}P]ATP, could be removed from protein after denaturation of the enzyme in 2% SDS during 2 min. However, part of the label remained bound after precipitation with 3% trichloroacetic acid. Therefore, the PP_i binding might be connected, at least partially, with covalent attachment of PP_i to protein.

We assumed that the stability of this tentative pyrophosphorylated form of enzyme depended on [Mg^{2+}]. Indeed, the ^{32}P-incorporation into the trichloroacetic acid-precipitable fraction increased with [Mg^{2+}] and reached the same plateau at 2.0 mM for E·(Trp~AMP) (fig.2) and at 13 mM for E·(Trp~AMP)$_2$. At these [Mg^{2+}] the native enzyme retained its ability to form adenylates. The ^{32}P-binding measured after gel-filtration corresponded to 0.8 ± 0.1 equivalent for E·(Trp~AMP) at 2.0 mM Mg^{2+} and to 1.9 ± 0.1 equivalent for E·(Trp~AMP)$_2$ at 13 mM Mg^{2+}. Practically all the PP_i formed during adenylate formation was retained by the enzyme.

Most remarkably, in both cases one equivalent

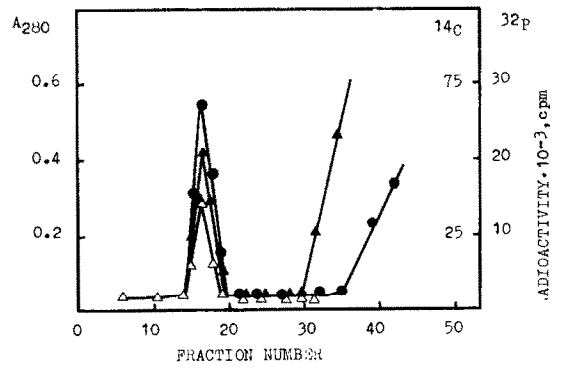

Fig.1. Chromatography of the tryptophanyl-tRNA synthetase on a Sephadex G-50 (fine) column after incubation of enzyme (6.6 μM) with [^{14}C]tryptophan (66 μM) and [γ-^{32}P]ATP (66 μM). Absorbance of eluate at 280 nm (△); ^{32}P-radioactivity (▲); ^{14}C-radioactivity (●). The column (30 × 0.8 cm) was equilibrated with the buffer containing 50 mM Tris–HCl (pH 7.5), 1 mM DTT. The elution rate 10 ml/h, the fraction volume 0.5 ml.

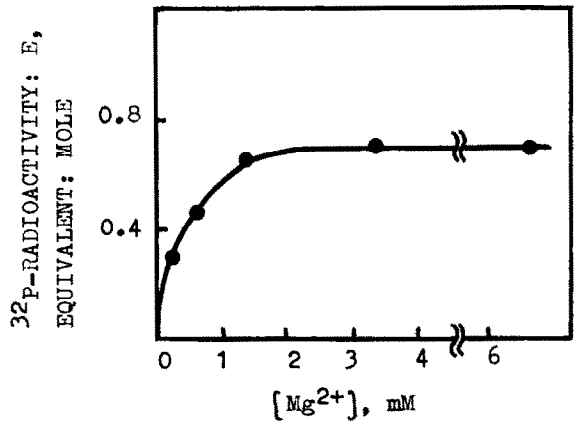

Fig.2. Mg^{2+} dependence of the degree of ^{32}P-incorporation from [γ-^{32}P]ATP into enzyme in the course of tryptophanyl adenylate formation. Incubation mixture contained 6.6 μM of enzyme, 66 μM tryptophan and 66 μM [γ-^{32}P]ATP. Protein-bound ^{32}P-radioactivity was measured in aliquots after precipitation of protein with 3% trichloroacetic acid.

of ^{32}P-radioactivity remained protein bound either after denaturation (2% SDS, 2 min, 90°C) or after acid precipitation with 3% trichloroacetic acid. We conclude that at stabilizing [Mg^{2+}] (2.0–13 mM) one molecule of PP_i released from ATP during adenylate synthesis was trapped by the enzyme molecule presumably via covalent bond formation.

To study the ^{32}P-labeled enzyme further, we treated the [^{32}P]E·(Trp~AMP)$_2$ complex with cold PP_i to pyrophosphorylate the tryptophanyl adenylate bound to the enzyme. After gel-filtration no [^{14}C]Trp or [^{14}C]ATP was revealed in protein-containing fractions whereas ^{32}P-label was retained almost quantitatively.

The [^{32}P]E thus obtained was treated with various ligands (fig.3): $tRNA^{Trp}$, Trp and ATP did not cause a release of the ^{32}P-radioactivity. Inorganic pyrophosphatase in conditions of hydrolysis of phosphoanhydride bonds in organic compounds [9] split about half of the protein-bound radioactivity. The nucleophylic reagents, such as ADP or NH_2OH, possess a varying dephosphorylation effect. AMP, a normal product of the reaction and a nucleophile, caused an ~80%

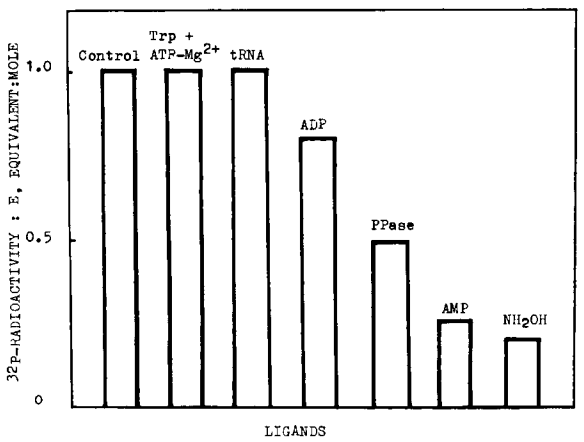

Fig.3. Action of various ligands on the ^{32}P-labeled tryptophanyl-tRNA synthetase (2 μM) after pyrophosphorolysis of adenylate. Concentrations (mM) of ligands were: tryptophan, 0.2; ATP, 0.6; tRNA, 0.2; ADP, 0.8; AMP, 0.4; NH_2OH, 600; inorganic pyrophosphatase, 0.01 mg/ml. The ^{32}P-labeled enzyme after pyrophosphorolysis was incubated with ligands during 30 min in the solution containing 50 mM Tris–HCl (pH 7.5) and 0.5 mM DTT. Control, incubation of the enzyme without ligands.

release of the ^{32}P-radioactivity after 30 min incubation. The radioactive products released by AMP were analysed by TLC and proved to be PP_i and ATP (not shown). Therefore, the ^{32}P-label was protein bound in a form of PP_i, rather than P_i. Furthermore, the formation of ATP from AMP and the [^{32}P]E strongly favoured the covalent nature of the bond between PP_i and the synthetase. The insignificant dephosphorylation of [^{32}P]E with ADP might be caused by partial splitting of the labile pyrophosphate bond leading to conversion of E–PP_i into E–P_i.

We observed that at stabilizing Mg^{2+} concentrations in the presence of ATP and L-tryptophan:

(i) PP_i formed from ATP in the course of tryptophanyl adenylate synthesis remains bound to enzyme;

(ii) The PP_i in tryptophanyl-tRNA synthetase exists in the two states – tightly bound (stable against denaturation, acid precipitation and action of the substrates) and non-stable which presumably corresponds to non-covalent enzyme·product complex;

(iii) The tightly bound PP_i does not exceed 1 mol/mol native dimeric enzyme and may be converted into ATP in the presence of AMP.

Based on these data, we conclude that in the given conditions tryptophanyl-tRNA synthetase is able to covalently fix the PP_i moiety in the course of enzymatic tryptophanyl adenylate formation.

What is the chemical nature of the bond linking PP_i and the protein? We observed that 0.6 M NH_2OH (pH 7.0) dephosphorylated the [^{32}P]E during 5 min, which is typical for the splitting of phosphoanhydride bonds in protein [10]. CH_3ONH_2 splits [^{32}P]E at 1.2 M of the reagent sufficient for complete removal of the ^{32}P-label. When $^{14}CH_3ONH_2$ was used for this treatment, nearly 1 mol reagent was incorporated into protein along with the release of the equivalent amount of PP_i (not shown). Since bovine tryptophanyl-tRNA synthetase has no Asn–Glu sequences sensitive to hydroxylaminolysis [11] the result obtained proves that the PP_i moiety is bound via a phosphoanhydride bond to the carboxylic group of the enzyme molecule.

When tryptophanyl-tRNA synthetase was treated with PP_i, the covalent derivative was also formed between the carboxylic group of the protein and the PP_i residue [4,5]. Moreover, some car-

boxylic groups of this enzyme are functionally important [12]. We suppose that carboxylic group(s) of the dicarboxyl amino acids are essential for catalytic functions of aminoacyl-tRNA synthetases. In the primary structures of aminoacyl-tRNA synthetases sequenced recently, di- and tripeptides are discovered composed of dicarboxyl amino acid residues (see [13]).

The formation of the covalent derivative of the enzyme and the substrate is not unusual for enzymes in general and, particularly, for aminoacyl-tRNA synthetases. Bovine tryptophanyl-tRNA synthetase forms a tryptophanyl-enzyme [11,14] and *E. coli* isoleucyl-tRNA synthetase makes a transient covalent complex with tRNA via U8 [15].

ACKNOWLEDGEMENTS

The authors are thankful to Dr S. Degtyarev and Mr M. Nurbekov who took part in some experiments at the initial stage of this work and to Dr A. Krayevsky for critical reading of the manuscript.

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