α-AMINOPHOSPHONOUS ACIDS: THE SUBSTRATES OF ATP–PP_i EXCHANGE REACTION, CATALYSED BY AMINOACYL-tRNA SYNTHETASES

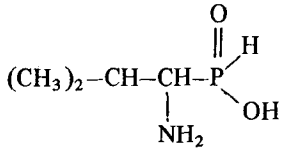
A. I. BIRYUKOV, T. I. OSIPOVA and R. M. KHOMUTOV*
Institute of Molecular Biology, USSR Academy of Sciences, Moscow 117312, USSR

Received 6 May 1978

1. Introduction

Studies on the specificity of aminoacyl-tRNA synthetases towards amino acid substrates have led to the conclusion that the α-carboxyl group of the substrate plays a secondary role in the binding with the enzymes [1]. Usually these studies involved such amino acid derivatives (e.g., esters, amides, hydrazides) [2] which could not participate in the activation reaction. However, substitution of the COOH group in the substrate amino acid by a phosphonyl group ($P(O)(OH)_2$) resulted in a sharp decrease (by 2–3 orders of magnitude) of the affinity for the enzyme [3], and these aminophosphonic acids were not activated by synthetases [4]. Therefore, the specificity of synthetases towards the COOH groups of substrates requires further investigations.

This work demonstrates that α-aminophosphonous acids (I) and (II) possess a high affinity for valyl- and methionyl-tRNA-synthetases, respectively, and are substrates in the ATP–PP_i exchange reaction.



I

α-aminoisobutylphosphonous acid

2. Materials and methods

2.1. *Chemical compounds*

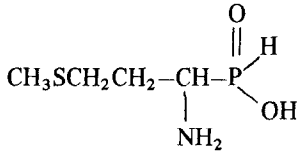
Standard ^{14}C-labelled amino acids were obtained from Chemapol (Czechoslovakia) and Amersham (England), and sodium [^{32}P]pyrophosphate, from Isotope (USSR). Phosphonous acids (I) and (II) were synthesized according to [5].

2.2. *Valyl- and methionyl-tRNA-synthetases*

These were isolated from *E. coli* B according to [6] and [7], respectively. Total *E. coli* tRNA was prepared as in [8]. *E. coli* B $tRNA^{Val}$ and $tRNA^{Met}$ were purified by the method in [9] with subsequent separation on a Sepharose 4B column [10].

2.3. *Determination of synthetase and acceptor activity*

(a) The activity of synthetases was determined using the ATP–PP_i exchange reaction according to [11]. Samples were analysed as in [7].



II

α-amino-γ-methylthiopropylphosphonous acid

* To whom reprint requests should be addressed

(b) The acceptor activity of $tRNA^{Val}$ and $tRNA^{Met}$ was assayed as in [11]. Incubation samples (0.5 ml) contained (μmol): Tris/HCl, pH 8.0, 50; $Mg(CH_3COO)_2$, 5; KCl, 5; KCl, 5; ATP, 1; β-mercaptoethanol, 2; ^{14}C-labelled amino acids, 0.001; tRNA, 0.01; and 0.005–0.01 mg corresponding enzyme. The mixture was incubated for 10 min at 37°C.

2.4. *The effect of I and II on the activity of synthetases*

Aqueous solutions of I and II were freshly prepared prior to use and the aliquots were added to the standard samples for ATP–PP_i exchange or tRNA aminoacylation, immediately before the addition of the enzyme. The enzyme activity was assayed according to (a) and (b) of 2.3, respectively.

2.5. *Determination of kinetic constants*

The K_m and V_{max} values for each amino acid substrate and α-aminophosphonous acids as well as the K_i value for each inhibitor, were determined from Lineweaver-Burk reciprocal plots of the data obtained in the standard ATP–$^{32}PP_i$ exchange and tRNA aminoacylation assays. No less than six points were used for each line in the plot and all the plots were done at least in duplicate. The values of V_{max} were determined in the presence of fixed concentrations of the enzyme, Mg^{2+} ions and ATP at constant pH. The apparent K_m and K_i values were compared with those of K_m for amino acids which were used to characterize the affinity of the substrate for the enzyme.

2.6. *Determination of radioactivity*

The radioactivity of samples was measured with an SL-30 liquid-scintillation counter combined with a Multi-20 computer in the Multimat system (Intertechnique, France).

3. Results and discussion

Compounds I and II are the first representatives of a new class of amino acid analogues, α-aminophosphonous acids, discovered in this laboratory. They are similar to aminocarboxylic acids in a number of properties. Therefore, enzymatic experiments followed the standard scheme [4].

Table 1
Comparison of kinetic parameters of valine and methionine with those of their phosphonous analogues

Compounds	K_m (M)	K_i (M)	V_{max}
tRNA aminoacylation reaction			
L-Valine	3×10^{-5}		
I		2×10^{-4}	
L-Methionine	3.4×10^{-5}		
II		6×10^{-4}	
ATP–PP_i exchange reaction			
L-valine	2.1×10^{-4}		1
I	8.3×10^{-4}		0.12
L-methionine	6.0×10^{-5}		1
II	1.0×10^{-3}		0.54

Compounds I and II were effective and specific inhibitors of the reaction of tRNA aminoacylation catalysed by valyl- and methionyl-tRNA-synthetases (table 1). The inhibition was reversible and competitive towards the substrates, L-valine and L-methionine (fig.1).

From the chemical point of view it seemed quite

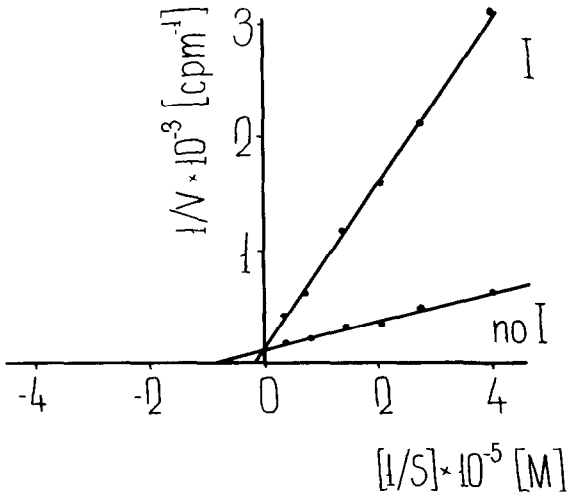


Fig.1. Lineweaver-Burk plot for the inhibition of aminoacylation of $tRNA^{Val}$ by I (5.5×10^{-4} M) measured by varying the [^{14}C]valine concentration in the presence of saturating amounts of ATP (2×10^{-3} M). The conditions for inhibition are described in section 2.

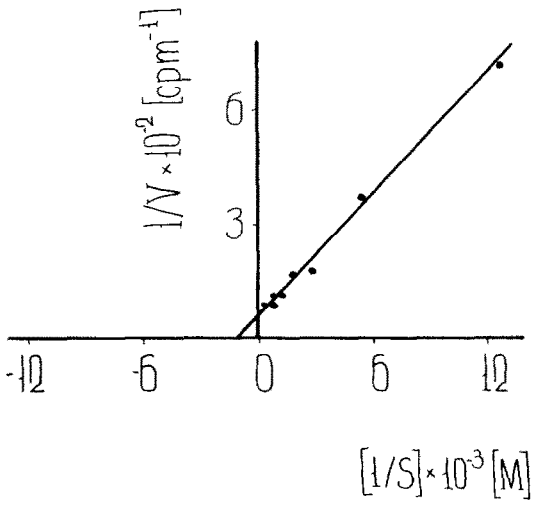


Fig.2. Determination of the Michaelis constant K_m for I in the reaction of ATP–PP_i exchange. Samples contained in 0.5 ml: Tris/HCl buffer, pH 8.0 (5×10^{-2} M), 0.01 mg enzyme, Mg^{2+} (2.3×10^{-3} M), NH_4^+ (3.5×10^{-2} M), ATP (9×10^{-4} M), $^{32}PP_i$ (2×10^{-3} M), β-mercaptoethanol (2.5×10^{-3} M) and I in the concentration range of 3×10^{-3} to 6×10^{-5} M. Incubation for 30 min at 37°C.

plausible that phosphonous acids would react with ATP yielding corresponding adenylates. Compounds I and II were found to be capable of substituting for L-valine and L-methionine in the ATP–PP_i exchange reactions catalysed by the corresponding synthetases, their affinities for the enzymes and the V_{max} of the reactions being only several times lower than those of the substrates (table 1, fig.2). It is relevant to point out that I and II are racemates and their effectiveness therefore is in fact still higher.

The possibility that the ATP–PP_i exchange reaction for I and II might depend either on the nucleophilicity of the $-\overset{\overset{O}{\|}}{\underset{OH}{P}}-H$ group or on its steric parameters. Aminophosphonic acids cannot be involved in the reaction of ATP–PP_i exchange [4], however, their anhydrides with AMP, aminophosphonyl adenylates, prepared recently are effective inhibitors of synthetases [13]. Therefore, the similarity between the steric parameters of the $-\overset{\overset{O}{\|}}{\underset{OH}{P}}-H$ and $-\overset{\overset{O}{\|}}{\underset{OH}{C}}$ groups seems to be preferable. Phosphonous acids can exist in tautomeric forms:

However, under normal conditions, the equilibrium is almost completely shifted to the four-coordinated structure and the forms shown for I and II seem to be those participating in the enzymatic reaction.

The enzymatic activation of I and II suggests that these could be involved in the complete reaction. This possibility and the role of enzymatic deacylation in the process are being studied in our laboratory using new inhibitors of deacylation [14].

Therefore, compounds I and II are the first illustration of the participation of functional groups other than the carboxyl one in the activation reaction.

References

[1] Favorova, O. O., Parin, A. V. and Lavrik, O. I. in: Advances in Science and Technology (USSR), (1972) Ser. Biophys. 2, 6–102, Moscow.
[2] Baturina, I. D., Gnutchev, N. V., Khomutov, R. M. and Kisselev, L. L. (1972) FEBS Lett. 22, 235–237.
[3] Anderson, J. W. and Fowden, L. (1970) Chem.-Biol. Interact. 2, 53–55.
[4] Owens, S. L. and Bell, F. E. (1970) J. Biol. Chem. 245, 5515–5523.
[5] Khomutov, R. M. and Osipova, T. I. (1978) Izv. Akad. Nauk. SSSR Ser. Khim. in press.
[6] Yaniv, M. and Gros, F. (1969) J. Mol. Biol. 44, 1–15.
[7] Lemoine, P., Wallter, J.-P. and Van Rapenbusch, R. (1968) Eur. J. Biochem. 4, 213–221.
[8] Gutcho, S. (1968) Biochim. Biophys. Acta 157, 76–82.
[9] Gillam, J. C. and Tener, G. M. (1971) in: Methods Enzymol. 20, 55–57.
[10] Holmes, W. M., Hurd, R. E., Reid, B. R., Rimerman, R. A. and Hatfield, G. W. (1975) Proc. Natl. Acad. Sci. USA 72, 1068–1071.
[11] Bergmann, F. H., Berg, P. and Dieckmann, M. (1961) J. Biol. Chem. 236, 1735–1740.
[12] Kelmers, A. D., Novelli, G. D. and Stulberg, M. P. (1965) J. Biol. Chem. 240, 3979–3983.
[13] Khomutov, R. M., Osipova, T. I., Biryukov, A. I. and Ishmuratov, B.Kh. (1978) Bioorg, Khim. in press.
[14] Osipova, T. I., Biryukov, A. I., Gandurina, I. A., Tarusova, N. B. and Khomutov, R. M. (1978) Bioorg. Khim. in press.