KINETIC PARAMETERS OF TRYPTOPHAN : tRNA LIGASE CATALYZED ATP-[^{32}P] PYROPHOSPHATE EXCHANGE AS AN APPROACH TO ESTIMATION OF THE ORDER OF SUBSTRATE BINDING

O. O. FAVOROVA, L. L. KOCHKINA, J. A. MELDRAJS and L. L. KISSELEV
Institute of Molecular Biology, Moscow, 117312, USSR

V. V. ZINOVIEV, D. G. KNORRE, O. I. LAVRIK, E. G. MALYGIN and G. A. NEVINSKY
Institute of Organic Chemistry, Novosibirsk, 90, USSR

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1. Introduction

An effective method for elucidation of the order of substrate binding in enzymic multisubstrate reactions is based on the use of inhibitors competitive to one of the substrates [1,2]. This approach can be further augmented by statistical treatment of the results to give the relative probabilities for the possible reaction mechanisms [3,4].

The present paper describes an attempt to apply another approach to the same problem, namely a comparison of the kinetic parameters of the reaction as obtained for the substrate and substrate analog. This type of analysis has been discussed by Wong and Hanes [5]. We used the reaction of ATP-[^{32}P] pyrophosphate exchange catalyzed by tryptophan:tRNA-ligase from beef pancreas in the presence of tryptophan (Trp) and its 6-fluorinated analog (6-fluoro-Trp) demonstrated earlier to be a substrate of the enzyme [6,7].

It was shown in this work that changes in kinetic parameters observable with L-6-fluoro-Trp as compared with L-Trp were in agreement with ATP being the first substrate to be bound by the enzyme. Independently, by means of the inhibitory approach developed earlier [3,4] we showed that ATP is the first and 6-fluoro-Trp is the second substrate in the reaction mechanism.

Therefore we concluded that (a) the approach based on the comparison of kinetic parameters of the reaction in the presence of natural and non-natural substrate gave information on the order of substrate binding, and (b) non-natural substrate did not change the order of substrate binding with respect to its natural analog.

2. Materials and methods

Tryptophan:tRNA-ligase from beef pancreas was obtained as described elsewhere [8]. The enzyme form E_2 (mol. wt 120 000) homogeneous in polyacrylamide gel electrophoresis was used. D,L-6-Fluoro-Trp was synthesized according to [9]. The product was characterized by elementary analysis and IR and UV spectroscopy. L-6-Fluoro-Trp was isolated by means of D-amino acid oxidase [7]. All reagents were from the same sources as earlier [6].

Extent of the [^{32}P] pyrophosphate-ATP-isotope exchange reaction was measured at 25°C in a final vol. of 0.2 ml in the presence of 0.01 M $MgCl_2$, 0.05 M Tris–HCl (pH 7.6) and 0.16 mg/ml bovine serum albumin [10]. The concentrations of ATP, pyrophosphate, 6-fluoro-Trp and tryptamine are shown in table 1. The concentration of tryptophan:tRNA-ligase was 6 μg/ml. Under the conditions described the enzymic activity was proportional to the amount of the enzyme added. Reaction was stopped after 10 min (linear portion of the kinetic curve) by addition of 1 ml cold 0.2 M sodium pyrophosphate in 5% trichloroacetic acid and of 0.2 ml 5% Norit A. The suspension was filtered through a nitrocellulose disc. ^{32}P-radioactivity was counted in an 'Intertechnique' SL-40 scintillation spectrometer.

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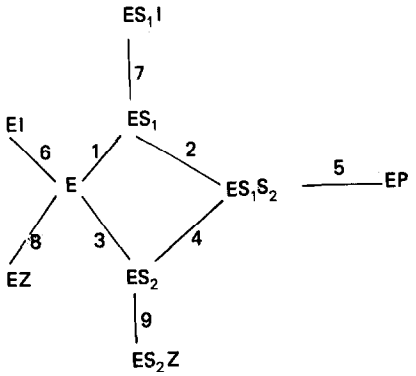
3. Results and discussion

The main idea of the approach proposed in the present paper is demonstrated using as an example ATP-[^{32}P] pyrophosphate exchange catalysed by amino acid:tRNA-ligase. The general scheme of the reaction including all possible orders of substrate binding as well as some additional non-reactive complexes postulated for some enzymes of this type can be represented as follows: where E stands for the enzyme, S_1 - ATP, S_2 - amino acid, Z - pyrophosphate, EP - complex of the enzyme with aminoacyladenylate, and I - inhibitor competitive to amino acid present in the reaction mixture in the case of the inhibitory approach.



When one of the substrates, e.g. S_2, is replaced with reactive analog S_2', only a part of the kinetic parameters chould change. For example if ATP is the first to bind to the enzyme the parameters of steps 1,6,7,8 remain unchanged, and the parameters connected with amino acid participation (steps 2,3,5) may be expected to alter with a suitably chosen amino acid analog. On the contrary, if the order of substrate binding is different, other kinetic parameters will change.

The approach was checked using the reaction catalysed by tryptophan:tRNA-ligase from beef pancreas and L-6-fluoro-Trp as an amino acid analog. To make possible a correct comparison of the kinetic parameters for the reaction with 6-fluoro-Trp with those obtained earlier with Trp [4], some kinetic measurements were performed in the presence of the competitive inhibitor tryptamine used in the previous paper. The experimental rates of the ATP-[^{32}P] pyrophosphate exchange at various concentrations of ATP, PP_i, 6-fluoro-Trp and tryptamine are presented in table 1.

To compare the data obtained with the natural substrate and the analog, the parameters of the reaction with 6-fluoro-Trp were calculated according to the scheme used before [4] including steps 1,2,3,5, 6,7 and 8. The kinetic and equilibrium parameters calculated according to [4] are presented in the table 2 as well as the corresponding parameters for Trp found in the previous paper. It may be seen that the parameters for ATP (k_1, k_{-1}, K_s), PP_i (K_z) and tryptamine (K_i^1, K_i^2) binding do not differ more than two times for sets with the substrate and analog which is within the error limits. At the same time parameters for the amino acid binding ($K_{S2}^{(1)}$ and $K_{S2}^{(2)}$) and equilibrium and kinetic parameters for the reaction within the enzyme –substrate complex differ significantly for Trp and its fluorinated analog. That means that ATP is bound to the complex independently of amino acid, and therefore may be regarded as the first bound substrate.

The $K_{S2}^{(1)}$ and $K_{S2}^{(2)}$ values (table 2) show that substitution of the 6-fluoro-Trp for Trp is not so strongly felt by the free enzyme as by the enzyme · ATP complex. This observation is in conformity with the results of other authors indicating the influence of ATP on the amino acid binding to the enzyme [11–13].

The method described earlier [3] permitted us to check independently whether the mechanism of the exchange established by comparison of kinetic parameters obtained with natural and non-natural substrates is indeed true. The data were used to calculate the relative probabilities of some mechanisms of the reaction. Four mechanisms: I,steps 1,2,3,5,6,7 and 8; II,steps 1,2,5,7 and 8; III,steps 3,4,5,6 and 9; IV – all steps, were considered.

The statistical treatment of the results was performed as before [3,4]. The values of the sum of the squares of the differences of the measured and calculated values (S), mean relative errors (ϵ%) and relative probabilities (P) of the hypotheses are represented in table 3.

The values S and ϵ% for three mechanisms are fairly consistent with the accuracy of the experimental data, while the mechanism with random order of substrate addition has the mean relative error 32%; this being much greater than experimental error. This apparently would exclude the random mechanism from the further analysis.

Table 1
Rates of [^{32}P]pyrophosphate-ATP exchange catalysed by beef pancreas tryptophan:tRNA-ligase in the presence of 6-fluoro-tryptophan

Substrate or analog	1	2	3
ATP	$0.5 \cdot 10^{-3}$	$1.0 \cdot 10^{-3}$	$1.5 \cdot 10^{-3}$
PP	$0.83 \cdot 10^{-4}$	$1.1 \cdot 10^{-4}$	$1.65 \cdot 10^{-4}$
6-Fluoro-Trp	$0.6 \cdot 10^{-6}$	$1.2 \cdot 10^{-6}$	$1.8 \cdot 10^{-6}$
Tryptamine	$0.14 \cdot 10^{-6}$	$0.28 \cdot 10^{-6}$	$0.56 \cdot 10^{-6}$

Substrates			Tryptamine			
ATP	PP	6-fluoro-Trp	0	1	2	3
		1	2.88*	2.59*	2.4	1.26
	1	2	3.55	3.12	2.76	2.54
		3	6.01	5.4	4.44	3.62
		1	2.54*	2.16	1.92*	1.51
1	2	2	4.70	4.2	3.61	2.76*
		3	5.88	5.4	4.38	3.12
		1	2.87	2.28	1.62	1.58
	3	2	3.95	4.44	3.44	3.44
		3	5.80	5.04	4.56*	3.84
		1	5.08	4.68	4.54*	3.56*
	1	2	5.66	5.52	4.62	5.28
		3	5.52	8.52	6.48	4.8
		1	5.72	4.68	3.80	3.6
2	2	2	8.65	7.44	5.71	4.03*
		3	10.56	9.6	8.40	6.12
		1	5.35	5.64*	4.08	2.16
	3	2	7.78	7.56	6.24	4.49*
		3	10.80	9.6	7.40	7.31
		1	5.15	4.56	4.49	2.82
	1	2	8.57	7.68	7.13	5.04
		3	11.64	9.72	9.79	7.25
		1	8.04	7.08	5.18	3.31
3	2	2	11.3	10.08	8.10	4.76
		3	12.66	11.16	9.6	6.35
		1	6.84	5.24	6.0	3.32
	3	2	9.25	6.24	8.4	5.84
		3	12.92	11.40	8.7	7.25

The experimental values are expressed in sec^{-1} and multiplied by 100. Experiments used for calculation of the initial dispersion matrix are indicated by an asterisk. Substrate concentrations (in M) are shown in the upper part of the table.

Table 2
Kinetic and equilibrium parameters for [^{32}P]pyrophosphate-ATP exchange reaction in the presence of L-6-fluorotryptophan and L-tryptophan

Numbers in the scheme	Reaction	Parameter	Units	L-6-Fluoro-Trp	L-Trp
		k_{-1}	sec^{-1}	6.7 ± 4.4*	3.1 ± 1.5
1	$E + S_1 \rightleftharpoons ES_1$	k_1	$L \cdot M^{-1} \cdot sec^{-1}$	$8.7 \cdot 10^3$	$4.9 \cdot 10^3$
		K_{S1}	M	$(7.7 \pm 2.3) \cdot 10^{-4}$	$(6.3 \pm 3.1) \cdot 10^{-4}$
8	$E + Z \rightleftharpoons EZ$	K_Z	M	$(1.4 \pm 0.4) \cdot 10^{-4}$	$(1.6 \pm 0.5) \cdot 10^{-4}$
6	$E + I \rightleftharpoons EI$	$K_1^{(1)}$	M	$(1.1 \pm 0.5) \cdot 10^{-6}$	$(2.1 \pm 1.2) \cdot 10^{-6}$
7	$ES_1 + I \rightleftharpoons ES_1I$	$K_1^{(2)}$	M	$(1.7 \pm 0.4) \cdot 10^{-7}$	$(3.2 \pm 0.6) \cdot 10^{-7}$
2	$ES_1 + S_2 \rightleftharpoons ES_1S_2$	$K_{S2}^{(2)}$	M	$(1.4 \pm 0.6) \cdot 10^{-6}$	$(1.8 \pm 0.9) \cdot 10^{-7}$
3	$E + S_2 \rightleftharpoons ES_2$	$K_{S2}^{(1)}$	M	$(5 \pm 1) \cdot 10^{-7}$	$(1.2 \pm 0.5) \cdot 10^{-7}$
5	$ES_1S_2 \rightleftharpoons EP + Z$	K_p	M	$(1.4 \pm 0.6) \cdot 10^{-4}$	$(1.4 \pm 0.8) \cdot 10^{-5}$
		$\frac{k_2}{1 + \frac{k_{-2}}{k_3}}$	$L \cdot M^{-1} \cdot séc^{-1}$	$(1.4 \pm 0.3) \cdot 10^5$	$(5.1 \pm 1.5) \cdot 10^6$
2,5	$ES_1 + S_2 \underset{K_{-2}}{\overset{K_2}{\rightleftharpoons}} ES_1S_2$ $K_{-3} \Updownarrow K_3$ $EP + Z$	$\frac{k_{-3}}{1 + \frac{k_3}{k_{-2}}}$	$L \cdot M^{-1} \cdot sec^{-1}$	$1.4 \cdot 10^3$	$6.6 \cdot 10^4$

* Errors are indicated for parameters which are chosen to be independent in calculations [14].

The calculations of the relative probabilities for the three aforementioned hypotheses show that the probability of mechanism I approaches 100% while the probabilities of the other mechanisms drop to nearly zero.

Thus the structural alteration of the substrate does not change the kinetic mechanism of the reaction.

Table 3
The sum of squares of differences of the measured and calculated values (S), mean relative errors (ϵ%) and relative probabilities (P) for considering mechanisms

Mechanism	S	ϵ%	P
I	3.9	15.5	1
II	4.0	15.5	$0.16 \cdot 10^{-14}$
III	5.7	17.9	$0.84 \cdot 10^{-14}$
IV	17.1	32.0	Not calculated

It may be seen that the kinetic measurements with substrate analog and calculation of the kinetic and equilibrium parameters may be used to confirm additionally the order of the substrate binding obtained by the inhibitory approach [3,4]. It seems reasonable to assume that comparison of the kinetic parameters obtained with substrate and analog may serve as an independent method for the elucidation of the order of substrate binding, although the possibilities and restrictions of the approach should be studied using a variety of the multisubstrate reactions prior to final conclusions.

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