KINETIC MECHANISM OF THE [^{32}P]ATP–PP_i EXCHANGE REACTION CATALYSED BY YEAST PHENYLALANYL-tRNA SYNTHETASE

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1. Introduction

For various aminoacyl-tRNA synthetases (EC 6.1.1) an order of substrate binding has been studied in both [^{32}P]ATP–PP_i exchange and tRNA aminoacylation reactions (for reviews see [1,2]). Depending on enzyme specificity the order of substrate binding may be random or sequential. In the case of an ordered mechanism, ATP is the first bound substrate.

An approach has been previously developped [3–5] which is based on the statistical treatment of the kinetic data obtained in the presence of a competitive inhibitor, i.e. a substrate analog. In the present study, we have measured the kinetics of the [^{32}P]ATP–PP_i exchange reaction catalysed by yeast phenylalanyl-tRNA synthetase (PheRS) and the data obtained have been subjected to statistical treatment.

It is shown that PheRS binds low molecular weight substrates in the [^{32}P]ATP–PP_i exchange reaction mostly in a random order. However at the same time an ordered mechanism may be involved to a certain degree with ATP binding first followed by phenylalanine addition.

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2. Materials and methods

2.1. *Chemicals*

ATP (potassium salt) and adenosine were from Sigma. Phenylalaninol was from Schwarz/Mann. Sodium [^{32}P]pyrophosphate was purchased from Amersham.

2.2. *Enzyme*

Pure PheRS was prepared as previously reported [6]. Its activity was maximum and corresponded to 20 000 U/mg [7].

2.3. *Kinetic measurements*

The reaction media (0.1 ml) contained : 0.1 M Hepes buffer pH 7.0, 10 mM $MgCl_2$, 0.5 mM 2-mercaptoethanol as well as ATP, sodium pyrophosphate, phenylalanine at the concentrations indicated below and 5.6 nM enzyme. The ATP–PP_i exchange reaction was measured at 25°C for 10 min, with about 80 000 counts × min^{-1} of [^{32}P]pyrophosphate added.

3. Results and discussion

Kinetic measurements were performed in the following range of substrate and analog concentrations (in μM) : ATP 50–200, phenylalanine 20–80, pyrophosphate 40–120, phenylalaninol 200–800, adeno-

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sine 50–140. For further statistical treatment, 400 kinetic points were taken.

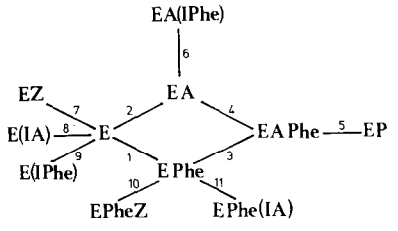
It was verified that: (1) the measured rates were initial steady-state rates; (2) the initial rates were not modified by prior incubation of the enzyme with the unlabeled species over a period ranging from 0 to 60 min; (3) under the conditions of incubation, we could not detect any phenylalanyladenylate by analysis of the reaction media on thin-layer chromatography.

Adenosine and phenylalaninol were found to be potent competitive inhibitors with respect to ATP and phenylalanine, their K_I corresponding to 7 μM and 170 μM respectively.

The common reaction scheme is presented below, where E stands for PheRS, A for ATP, Phe for phenylalanine, Z for pyrophosphate, P for aminoacyladenylate, (IA) for adenosine and (IPhe) for phenylalaninol. Combinations of these symbols with E stand for enzyme–ligand complexes.

Three main hypotheses on the reaction mechanism are considered. Hypotheses I and II involve stages 1, 2, 4, 5–9 and 1–3, 5, 7–11 respectively and correspond to ordered mechanisms of binding. In hypothesis I ATP binds first, whereas in hypothesis II phenylalanine is the first substrate to be bound. Hypothesis III corresponds to random binding of substrates and involves all stages, i.e. 1–11.

From table 1 one may see that the mean relative error Σ_i for hypotheses I and II with ordered mechanisms considerably exceeds the value of the experimental error found independently to be equal to 17%. This discrepancy allows to exclude both ordered mechanisms as the main route for the ATP–PP_i exchange reaction catalysed by yeast PheRS. In the case of the random mechanism (hypothesis III), the error is not far from the experimental one. Therefore in the case of yeast PheRS, one may establish to



Scheme 1

Table 1
The Σ_i and P_i values for various kinetic models

Hypothesis	Σ_i%	P_i
I	24.4	–
II	26.3	–
III	18.3	98.0
IV	18.2	2.0
V	18.6	0.0

The definition for mean relative error and all details concerning calculations of P_i are given earlier [3].

order of substrate binding by means of a simple statistical criterion, the mean relative error.

In contrast to PheRS it has been found in the case of tryptophanyl-tRNA synthetase [3] that all three hypotheses fit equally well with the experimental data : the mean relative error does not exceed the experimental one. Since the simple statistical treatment was not sensitive enough for the kind of rate equations describing the various mechanisms, the calculation of their relative probabilities P_i was done to discriminate between the hypotheses [3].

After the demonstration of the random order of substrate binding with PheRS, we decided to consider two additional hypotheses (IV and V) both of which are random too and contain the same set of enzyme–substrate complexes as hypothesis III. In addition to the latter, in hypothesis IV, the stage 3 is postulated to be very slow and hence the conversion of pyrophosphate into [^{32}P]ATP proceeds via stages 5–4–2. In hypothesis V the stage 4 is assumed to be very slow and the conversion of pyrophosphate into [^{32}P]-ATP takes place through the stages 5–3.

Hypothesis III

$$V = \frac{X_{11} + [\mathrm{Phe}]}{X_{12} + X_{13}\cdot[\mathrm{Phe}]} \Big/ \left(X_1 + \frac{1}{[Z]} + \frac{X_1\cdot X_2}{[\mathrm{Phe}]}\right)\left(1 + X_3\cdot[\mathrm{IPhe}] + \frac{X_4}{[A]}\left(1 + X_5\cdot[Z] + X_6\cdot[IA] + X_7\cdot[\mathrm{IPhe}] + X_8\cdot[\mathrm{Phe}]\cdot\left(1 + X_9\cdot[Z] + X_{10}\cdot[IA]\right)\right)\right) = \varphi_1/\varphi_2 \qquad (1)$$

where:
X_1 . . . . . X_{10} are association and dissociation constants for elementary stages,
and
X_{11}, X_{12} and X_{13} are the sum of kinetic parameters.

Hypothesis IV

$V = \varphi_1/\varphi_2$, where φ_2 is the same as in equation (1),

$$\varphi_1 = \frac{1}{X_{11} + X_{12}\cdot[\mathrm{Phe}]} \qquad (2)$$

X_{11} and X_{12} parameters do not coincide in hypotheses III and IV.

Hypothesis V

$V = \varphi_1/\varphi_2$, where φ_2 is the same as in equation (1) and φ_1 is constant. (3)

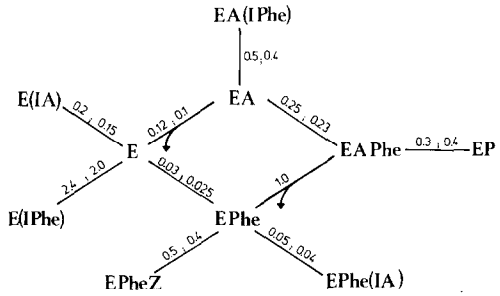
Practically the equations of hypotheses IV and V differ from that of hypothesis III by a 'kinetic' factor φ_1 due to the fact that, as mentioned above, all three random hypotheses contain the same set of enzyme forms. From equations (1), (2) and (3), one may notice that the dependence of the kinetic factor φ_1 versus aminoacid concentration varies according to the hypothesis under consideration.

Parameters for equations (1), (2) and (3) were found (see below) for all hypotheses fitting with the experimental data, with a mean relative error similar to that of hypothesis III (table 1).

In all cases the value for dissociation constant of EZ complex was high enough, around 10^{-1} M. Due to this observation all stages corresponding to this interaction may be omitted in equations (1), (2) and (3) without significant deterioration of the mean relative error. Therefore, a further analysis of hypotheses III to V has been done without the stage $E + Z \rightleftharpoons EZ$.

For selection of the most probable mechanism among the three above mentioned hypotheses, the Bayers approach was used which is based on the calculation of relative probabilities P_i for each hypothesis III to V. The results of sequential calculations of the probabilities of the reaction mechanisms are shown in table 1. Zero probability has been found for hypothesis V, where the route 5–3 dominates. The highest probability of 98% is given to hypothesis III according to which both routes function with comparable efficiencies. Hypothesis IV where conversion of pyrophosphate into [^{32}P]ATP proceeds via stages 5–4–2 has a low but non negligible probability of 2%.

The most probable kinetic scheme for the [^{32}P]-ATP–PP_i exchange catalyzed by yeast PheRS is shown below.



Scheme 2

The values above the connecting lines are dissociation constants expressed in mM and calculated as described earlier [4]. The first value corresponds to hypothesis III and the second to hypothesis IV. Arrows show stages where [^{32}P]ATP formed as a result of isotope exchange is released.

At first it seems that the probability of hypothesis IV is only 2%, hypothesis III with 98% probability is the only one to describe adequately the reaction mechanism. However from experience with similar calculations made earlier [3–5], we realized that a difference between two hypotheses in the range of one or two orders of magnitude is not sufficient for unequivocal rejection of the less probable one. Since the relative probabilities of the hypotheses are sensitive criteria, one should only take into account a big difference in the probability values, i.e. five or more orders.

Therefore, the small but meaningful probability found for hypothesis IV may indicate that part of the experimental data (for example at sharply increased ATP/Phe concentration ratio) is better described by a mechanism starting with the binding of ATP to PheRS. This assumption is in agreement with data [8] on isoleucyl-tRNA synthetase of *E. coli*, showing that ATP interacts with the enzyme much more rapidly than does the amino acid.

It was found earlier [9,10] that in the case of *E. coli* PheRS the order of substrate binding is random too, although the relative efficiencies of various possible routes for ATP–PP_i exchange were not compared.

Probably in the case of synthetases the order of substrate binding depends on the aminoacid specificity of the enzyme but not on the species from which it comes : for instance, PheRS from *E. coli* [9,10], yeast (this work) and rat liver [11] bind aminoacid and ATP randomly whereas tryptophanyl-tRNA synthetases from beef pancreas [3,5], *E. coli* and human placenta [12] bind substrates in a strictly ordered way, ATP first. These two groups of enzymes also differ in their subunit structures, belonging to $\alpha_2\beta_2$ and α_2 classes respectively.

These preliminary correlations need much more proofs since up to now the number of synthetases with correctly established order of substrate binding remains rather limited. For future work it will be very interesting to establish whether different mechanisms for substrate binding correspond to different catalytic mechanisms. Another important question is the structural basis for the differences in kinetic mechanisms for the enzyme carrying out the same function in protein biosynthesis with similar specificities.

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