ORDER OF SUBSTRATE BINDING TO TYROSYL-tRNA SYNTHETASE OF *ESCHERICHIA COLI* B

Daniel V. SANTI and Van A. PEÑA
Department of Chemistry, University of California, Santa Barbara, California 93106, USA

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1. Introduction

The formation of aminoacyl-tRNA is catalyzed by the aminoacyl-tRNA synthetases via activation of an amino acid with ATP to give an enzyme-bound aminoacyladenylate. These enzymes also catalyze an amino acid dependent incorporation of ^{32}P-pyrophosphate into ATP. We have initiated studies of certain mechanistic aspects of individual aminoacyl-tRNA synthetases, as well as a search for common features which may exist among this family of enzymes. One of the fundamental parameters of an enzymic reaction is the order in which substrates and products interact with their respective binding sites. It has recently been proposed that the binding of ATP to leucyl-tRNA synthetase from *E. coli* B [1] and threonyl-tRNA synthetase from rat liver [2] precedes the binding of the substrate amino acids. In contrast, the studies described herein demonstrate that the order of addition of substrates to tyrosyl-tRNA synthetase proceeds by a rapid equilibrium random mechanism.

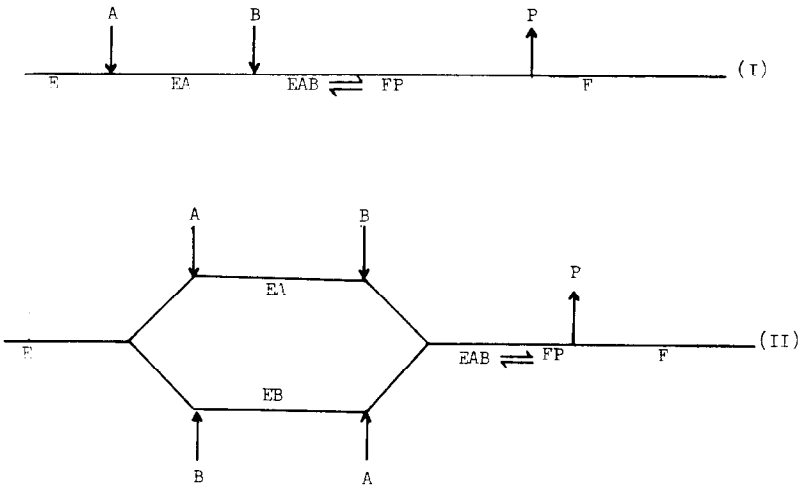
2. Materials and methods

A 550-fold purified preparation of tyrosyl-tRNA synthetase was isolated from *E. coli* B (General Biochemicals, Chagrin Falls, Ohio) by the method of Calendar and Berg [3] (with the exception that C-γ gel fractionation was omitted). Assays of ATP–^{32}PPi exchange activity were conducted according to the procedure of Calendar and Berg [3]; velocities are expressed as the μmoles of ^{32}P-ATP formed per min per 1.0 ml. Unless otherwise stated, the assay contained 2.0 mM ATP, 0.10 mM tyrosine and 2.0 mM PPi. The concentration of Mg^{2+} was kept 1.0 mM in excess of the sum of PPi and ATP (2′-dATP) concentrations; under these conditions the ligands are present almost exclusively as their mono-magnesium salts. Tyrosyl-AMP was prepared as described by Sandrin and Boissonnas [4]. All other materials were obtained commercially. The necessary kinetic expressions for ATP–^{32}PPi exchange are identical to those derived for asparagine synthetase [5], and the terminology used is that proposed by Cleland [6].

3. Results and discussion

In recent years a *modus operandi* has been developed to ascertain, in a relatively simple manner, the order of substrate binding to an enzyme and the sequence of product release [7, 8]. Through kinetic experiments it is first determined whether an enzymic reaction proceeds by a sequential or ping-pong pathway; after this segregation, mechanisms are further separated by initial velocity experiments, isotope exchange studies and/or competitive inhibitors of individual substrates.

Tyrosyl-tRNA synthetase-catalyzed ATP–PPi exchange does not occur in the absence of tyrosine, and it appears reasonable to surmise that the release of pyrophosphate from the enzyme occurs only after



Scheme 1: Mechanisms for ATP–PPi exchange.

the binding of both substrates. Further evidence for this has recently been obtained [9] by the isolation of a stable enzyme–tyrosyl-AMP complex which reacts with pyrophosphate to give ATP and tyrosine. Kinetic evidence [7] for a sequential pathway was obtained by variation of ATP and L-tyrosine at different fixed levels of the other substrate. Double-reciprocal plots of both experiments yielded lines which intersected at the left of the 1/velocity axis (fig. 1; L-tyrosine and ATP had limiting * Michaelis constants of 3.6×10^{-6} M and 2.9×10^{-4} M, respectively); since parallel lines would have been obtained if the reaction proceeded by a ping-pong mechanism, it can be concluded that both substrates must add to the enzyme before a product is released.

The three possible sequential pathways for ATP–PPi exchange are depicted in mechanisms I and II (scheme 1). For a two substrate sequential reaction the binding of substrates may proceed by ordered (mechanism I) or random (mechanism II) pathways, where P is magnesium pyrophosphate and F is the enzyme-bound aminoacyladenylate, or equivalent species; in mechanism I, A is the first substrate to add and B is the second.

Evidence supporting one of these may be obtained by analysis of the corresponding kinetic expressions [5] in terms of slope–intercept effects on double-reciprocal plots obtained when ATP is the variable substrate and tyrosine is the changing fixed substrate. High concentrations of tyrosine should result in an intercept effect for mechanism I where ATP is bound first, a slope effect for mechanism I where tyrosine must add initially, and a slope-intercept effect for the random mechanism II. The data presented in fig. 1, in

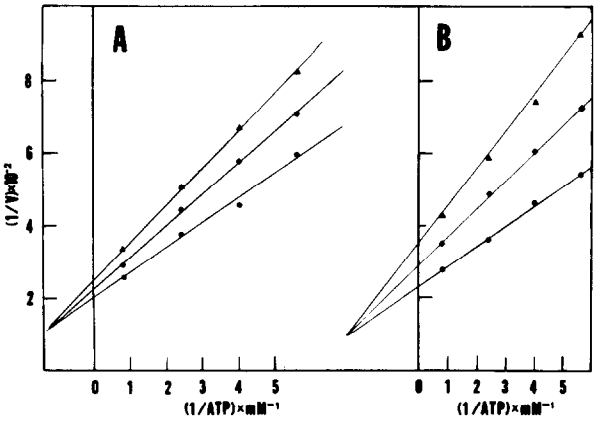


Fig. 1. Plot of reciprocal exchange velocity against reciprocal ATP concentration at constant PPi concentrations of 2.0 mM (A) and 0.50 mM (B). The concentrations of tyrosine were (mM): (A) ● 0.1, ◆ 0.05, ▲ 0.025. (B): ● 0.05, ◆ 0.13, ▲ 0.05.

* Obtained by extrapolation of changing fixed substrate to infinite concentration.

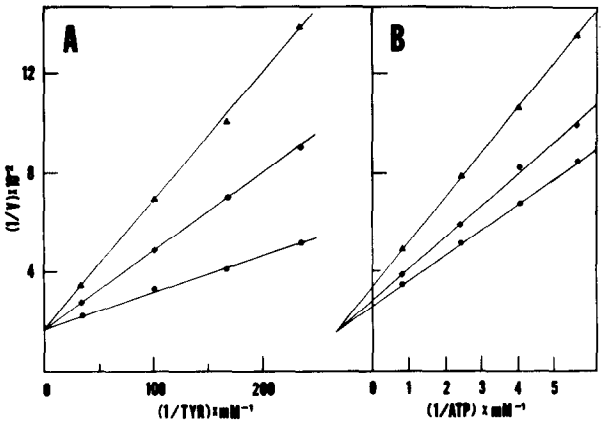


Fig. 2. Plot of reciprocal exchange velocity against reciprocal tyrosine (A) and ATP (B) concentrations at different concentrations of tyramine hydrochloride (M). (A): • no inhibitor, ♦ 5.0×10^{-6}, ▲ 1.0×10^{-5}. (B): • no inhibitor, ♦ 1.3×10^{-5}, ▲ 2.5×10^{-5}.

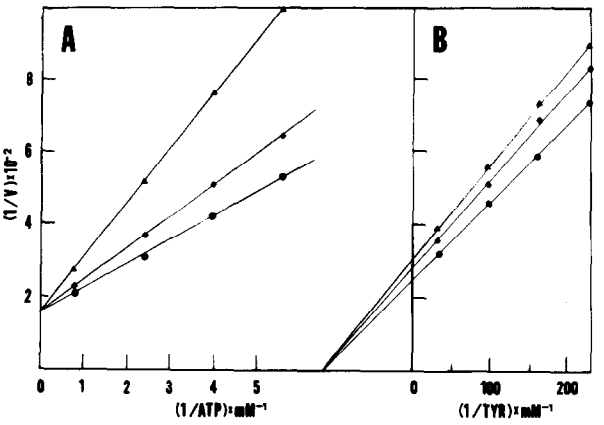


Fig. 3. Plot of reciprocal exchange velocity against reciprocal ATP (A) and tyrosine (B) concentrations at different concentration of 2′-dATP (mM). (A): • no inhibitor, ♦ 1.0, ▲ 2.9. (B): • no inhibitor, ♦ 2.0, ▲ 2.9.

which all lines intersect to the left of the V^{-1} axis, are in accord with a random order of addition for ATP and tyrosine.

Further support for the order of substrate addition was obtained by analysis of the inhibition patterns of competitive inhibitors of each substrate. For a two substrate sequential reaction in which the binding of substrates is random, a competitive inhibitor for either substrate will demonstrate noncompetitive inhibition with respect to the other substrate. In the case of an ordered mechanism, a competitive inhibitor for the substrate which adds to the enzyme first will demonstrate noncompetitive inhibition relative to the second substrate; a competitive inhibitor for the second substrate will produce uncompetitive inhibition when the initially bound substrate is varied.

Tyramine and 2′-dATP were utilized as competitive inhibitors of tyrosine and ATP, respectively (figs. 2 and 3); K_i values of 5.6×10^{-6} M for tyramine and 3.0×10^{-3} M for dATP were obtained by the method of Lineweaver and Burk [10], and replots of 1/velocity versus inhibitor concentration were linear. Mitra and Mehler [11] have reported that 2′-dATP may serve as a substrate in the esterification of $tRNA^{Tyr}$ but not in the exchange reaction; similarly in the studies described here, we observed no incorporation of ^{32}PPi into dATP. As shown in fig. 2B, the inhibition by tyramine is clearly non-competitive with respect to ATP, and when tyrosine is varied in the presence of dATP, the inhibition obtained is also noncompetitive. These data are consistent with a random order of binding of tyrosine and ATP to tyrosyl-tRNA synthetase as depicted in mechanism II.

Cassio et al. [12] and Rouget and Chapeville [1] have observed that aminoalkyl adenylates are potent and specific inhibitors of the aminoacyl-tRNA synthetases, competitive with respect to the amino acid

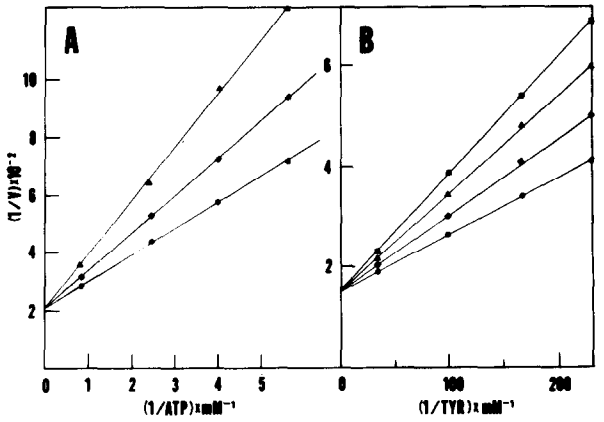


Fig. 4. Plot of reciprocal exchange velocity against reciprocal ATP (A) and tyrosine (B) concentrations at different concentrations of L-tyrosine-AMP (M). (A): • no inhibitor, ♦ 3.2×10^{-9}, ▲ 6.4×10^{-9}. (B): • no inhibitor, ♦ 6.4×10^{-9}, ▲ 1.6×10^{-8}, ■ 3.2×10^{-8}.

and, in cases examined, to ATP. Based largely upon the much lower K_i values of the animoalkyl adenylates than the corresponding aminoalcohols, and close structural resemblance to the aminoacyladenylate intermediates, they have implied that inhibitors of this type compete with aminoacyladenylate for its binding site of the enzyme. Fig. 4 shows the inhibition patterns obtained with L-tyrosyl-AMP on the ATP–PPi exchange. Since the inhibition is competitive with both L-tyrosine ($K_i = 2.3 \times 10^{-8}$ M) and ATP ($K_i = 2.2 \times 10^{-8}$ M) it must bind to the same enzyme form as do both substrates, a requirement fulfilled only by a random order of binding.

In fig. 1A, B are given double-reciprocal plots with varying ATP and tyrosine at two concentrations of PPi. The negative reciprocal of the abscissa values which define the points of convergence correspond to the apparent dissociation constants of ATP under these conditions, which vary from 0.8 mM at 2.0 mM PPi to 0.4 mM at 0.5 mM PPi. From these data it is clear that in addition to binding to the enzyme form F (mechanism II), PPi may act as a dead-end inhibitor by combining with the ATP binding site. Cole and Schimmel [13] have recently reported a similar inhibition of isoleucyl tRNA synthetase by $MgP_2O_7^{2-}$. Using the kinetic expression [5] for the random mechanism (mechanism II), values of 0.27 mM and 1.0 mM may be calculated for the true dissociation constant of ATP and the dead-end inhibition of PPi respectively. It is noted that the former value is virtually identical to the limiting K_m for ATP.

Acknowledgements

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