Volume 6 Number 4 April 1979 Nucleic Acids Research

The binding of tyrosinyl-5'-AMP to tyrosyl-tRNA synthetase (E.coli)

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Received 15 January 1979

Abstract

The binding between tyrosyl-tRNA synthetase (E.coli) and the alkylanalogue of the aminoacyladenylate, tyrosinyl-5'-AMP, has been investigated by fluorescence titrations and rapid mixing experiments. Tyrosyl-tRNA synthetase has two equivalent and independent binding sites for tyrosinyl-5'-AMP. The intrinsic binding constant is $4 \times 10^{7} M^{-1}$. The binding sites for $tRNA^{Tyr}$ and tyrosinyl-5'-AMP are independent of each other, the anticooperative mode of tRNA binding being preserved in the presence of tyrosinyl-5-AMP.

Introduction

The relationship between the subunit structure and number of active sites of the aminoacyl-tRNA synthetase has found considerable interest in the past years. One of the most thoroughly studied synthetases with this respect are the tyrosyl-tRNA synthetase from E.coli and B.stearothermophilus. On the basis of solution studies a half-site reactivity has been postulated for the tyrosyl-tRNA synthetase from B.stearothermophilus (1,2). Recent crystallographic investigations provide evidence that the enzyme contains two identical, symmetrically arranged subunits and two equivalent binding sites for tyrosine and tyrosinyl-5'-AMP(3). The tyrosyl-tRNA synthetase from E.coli resembles that from B. stearothermophilus very closely in having a similar architecture and exhibiting half-site reactivity and nonequivalent binding sites for the small substrates (2,4). With respect to the tRNA binding it could be demonstrated that tyrosyl-tRNA synthetase from E.coli binds two tRNA-molecules in an anticooperative manner (5).

Knowledge about the stoichiometry and interdependence of the

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binding sites for the various substrates is essential for an understanding of the mechanism of the highly specific aminoacylation reaction. To investigate the mutual influence of the binding sites in the tyrosine system from E.coli we used an alkylanalogue of the activated aminoacid, tyrosinyl-5'-AMP. In utilizing this analogue we intended to obtain information concerning the equivalence of the active sites of this enzyme. Furthermore we wanted to study the influence of the analogue on the tRNA binding process. In the phenylalanine-specific system from yeast it was recently shown that the presence of the aminoalkyladenylate on the enzyme influences the binding affinity and the mechanism of complex formation between tRNA and synthetase (6). In the present paper we report binding parameters for the binding of tyrosinyl-5'-AMP to tyrosyl-tRNA synthetase (E.coli). We also deal with the effect of this analogue on the binding between $tRNA^{Tyr}$ and the cognate synthetase.

Materials and Methods

Tyrosyl-tRNA synthetase and $tRNA^{Tyr}$ were purified and characterized as outlined earlier (7). All reagents were of the highest commercially available purity. Experiments were performed in 3C mM potassium phosphate buffer pH 7.2, 5 mM $MgCl_2$ and o.5 mM dithioerythritol if not otherwise stated. The aminoacylation assay was performed as described in (8).

Preparation of Tyrosinyl-5'-AMP

Tyrosinyl-5'-AMP was synthesized using a similar procedure as described for the synthesis of phenylalaninyl-5'-AMP (6). N^6, $O^{2'}$, $O^{3'}$, triacetyl-5'-AMP was condensated with O-benzyl-N-t-butyoxycarbonyl-L-tyrosinol in the presence of triisopropylbenzenesulfonylchloride. The O-and N-protection groups of the reaction product were removed by hydrogenolysis over palladiumoxide and subsequent treatment with 8o % aqueous trifluoroacetic acid at room temperature. The tyrosinyl-5'-AMP obtained was purified by ether precipitation from an acetone solution, followed by silica gel chromatography and G-10 desalting. The molar absorption coefficient of tyrosinyl-5'-AMP was determined to be

11900 cm^2/mM at 261 nm. The UV spectrum (λ_{max}, 261 nm; λ_{min}, 235,5 nm) was identical with that reported by Sandrin and Boissonas (9).

Fluorescence titrations were performed in a Schoeffel RRS 1000 spectrofluorimeter equipped with a 1000 W lamp. Titrations monitoring the tryptophane fluorescence of the protein were carried out similarly as described in (10) and (19). In order to correct for the inner filter effect due to the tRNA added two titrations were carried out simultaneously: one in the absence, the other in the presence of 1 M KCl. It is known from ultracentrifugation experiments (12) that $tRNA^{Tyr}$ does not bind to tyrosyl-tRNA synthetase in the presence of 1 M KCl. No correction for an inner filter effect was necessary in titrations with tyrosinyl-5'-AMP. At very low enzyme concentrations ($<$0.05 μM) cuvettes coated with dichlorodimethylsilane were used. Binding constants and stoichiometries were evaluated by using a non-linear computer fitting procedure (11). Rapid mixing experiments were carried out in a high sensitivity stopped-flow spectrofluorimeter as described (5). All binding experiments were performed at 20 ^{o}C.

Results

Equilibria

1. Binding of Tyrosinyl-5'-AMP to Tyrosyl-tRNA synthetase

Tyrosinyl-5'-AMP is a strong inhibitor of the aminoacylation reaction: The K_I value, measured at constant and saturating ATP and $tRNA^{Tyr}$ concentration while varying the tyrosine concentration, was determined to be 2.8 x 10^{-7} M. The intrinsic fluorescence of the synthetase is strongly quenched upon binding of tyrosinyl-5'-AMP (fig 1). Under saturating conditions the quenching amounts to 32 %. A stoichiometric titration, i.e. a titration with C_{enz}, $C_{analogue} \gg 1/K_{Ass}$, yields a value of 1.7 ± 0.2 binding sites for tyrosinyl-5'-adenylate on the enzyme molecule (fig 1). This value is interpreted as a 2:1 stoichiome-

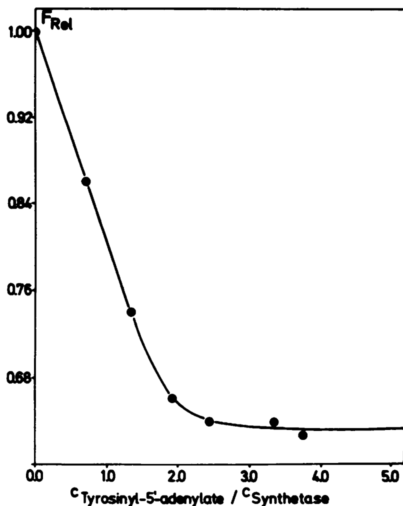

Fig 1: Titration of tyrosyl-tRNA synthetase with tyrosinyl-5'-adenylate monitored by the intrinsic fluorescence of the synthetase.

0.98 µM tyrosyl-tRNA synthetase were titrated with tyrosinyl-5'AMP in 0.03 M potassium phosphate pH 7.2, 5 mM $MgCl_2$, 0.5 mM dithioerythritol, at 20°C. The drawn line represents the computer fit to the titration data.

try of complex formation between tyrosyl-tRNA synthetase and tyrosinyl-5'-AMP. There was no experimental evidence that the two binding sites are nonequivalent. Titrations at concentrations of 0.01 up to 1µM synthetase and inhibitor in standard buffer yield a binding constant of 4.3 ($\pm$ 2.4) x 10^7 M^{-1}. The binding constant is not changed by lowering the pH 6 nor is it affected measurably by adding 3 M KCl.
There is also no change in the affinity of tyrosinyl-5'-AMP to the enzyme when the first tRNA binding site is occupied with $tRNA^{Tyr}$.

2. Binding of $tRNA^{Tyr}$ in the presence of tyrosinyl-5'-AMP.

In titration experiments on the binding of $tRNA^{Tyr}$ to the enzyme, the nonequivalence of the two tRNA binding sites, as reported earlier (5,12), is confirmed. The affinity of the two tRNA-molecules to the synthetase is not influenced upon addition of saturating amounts of tyrosinyl-5'-AMP (table 1). The fluorescence quenching produced by $tRNA^{Tyr}$ and the inhibitor is additive (fig 2). The final-level of fluorescence quenching in the ternary complex is independent upon whether $tRNA^{Tyr}$ or tyrosinyl-5'-AMP is added first. Mg^{2+} does not release the anticooperativity of tRNA binding in the presence of tyrosinyl-5'-

Table 1: Equilibrium parameters for the binding of tyrosinyl-5'-AMP and $tRNA^{Tyr}$ to tyrosyl-tRNA synthetase. Data from fluorescence titrations performed in 0.03 M potassium phosphate, 5 mM $MgCl_2$, 0.5 mM dithioerythritol, at 20°C. Other conditions as given below.

Titration	condition	Stoichiometry Ligand/enzyme	Binding constant M^{-1}
$tRNA^{Tyr}$ +enzyme	pH 7.2	2	1.6×10^7 3.2×10^5
tyrosinyl-5'-AMP + enzyme	pH 7.2 pH 6.0 pH 7.2 3M KCl	1.71 ± 0.17 1.7 1.7	$(4.3 \pm 2) \times 10^7$ 4×10^7 2×10^7
tyrosinyl-5'-AMP +(enzyme $2tRNA^{Tyr}$)	pH 7.2	1.7	3×10^7
$tRNA^{Tyr}$+ (enzyme 2 tyrosinyl-5'-AMP)	pH 7.2	2	2.3×10^7 3.2×10^5

adenylate: in standard buffer, the addition of $MgCl_2$ up to 25 mM to a solution containing 2 µM $tRNA^{Tyr}$, 1 µM enzyme and saturating amounts of the inhibitor results in a slight increase of the fluorescence. Under these conditions the first tRNA binding site is saturated to more than 90 %. A disappearance of the anticooperative tRNA binding in the presence of high concentrations of Mg^{2+}, as was detected in the methionine specific system from E. coli (13), should have shown up in a tighter binding of the second tRNA and thus in a further quenching of the fluorescence.

Kinetics

The rate constant for the association of tyrosinyl-5'-AMP and the synthetase is determined from stopped-flow experiments to be $(3.8 \pm 1.0) \times 10^6\ M^{-1}\ sec^{-1}$. From this value and the binding constant derived from the fluorescence titrations, an overall rate constant of dissociation of 0.09 sec^{-1} is obtained.

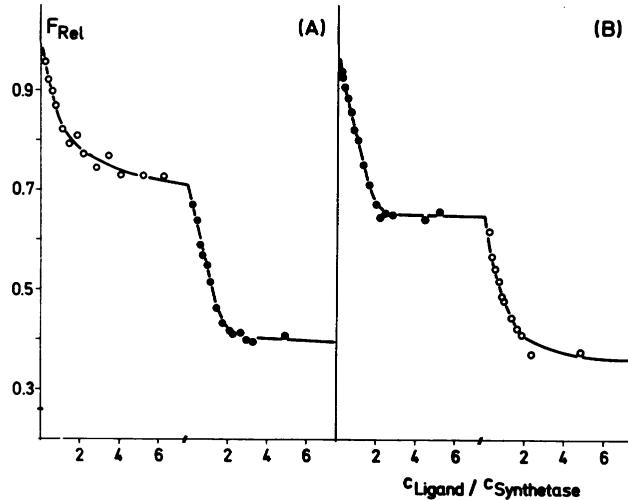

Fig 2: Titration of tyrosyl-tRNA synthetase with tyrosinyl-5'-AMP and $tRNA^{Tyr}$ monitoring the intrinsic fluorescence of the synthetase.

Buffer conditions as in fig 1. The drawn line represents the computer fit to the data.
(A): Addition of $tRNA^{Tyr}$ (o) followed by addition of tyrosinyl-5'-adenylate (•). (B): Addition of tyrosinyl-5'-adenylate (•) followed by addition of $tRNA^{Tyr}$ (o).

Discussion

The data presented in this paper show that tyrosyl-tRNA synthetase from E.coli contains two equivalent binding sites for tyrosinyl-5'-AMP. The affinity of tyrosinyl-5'-AMP for the two sites is comparable to that determined for aminoalkyladenylates in other synthetase systems (14). There is an apparent discrepancy between the binding constant and the K_I value for tyrosinyl-5'-AMP, the K_I value indicating a 10-fold lower affinity than the binding constant. However, the K_I value is a function of several reaction parameters. For instance, in the present case it may be increased due to a competition between ATP and the analogue. In the equilibrium studies we could not observe a mutual influence between the tRNA binding sites and the binding sites for the activated aminoacid. Evidently the tRNA binding sites and the catalytic sites are independent from each other.

The anticooperativity of tRNA binding is not a function of the occupancy of the active sites. Most probably this anticooperativity is retained also under catalytic conditions. The situation in the tyrosine-specific system from E.coli thus seems to be different from that in the phenylalanine-specific system from yeast, where the presence of the aminoalkyladenylate increases the affinity of the tRNA for the synthetase by a factor of 2-3 (6). The rate constants obtained from the stopped-flow experiments are similar to the values measured for the binding of aminoalkyladenylates and aminoacids in other synthetase systems (14,15,16,). The low value for k_R of 3.8×10^6 M^{-1} sec^{-1} is slower than the value one could estimate for a diffusion controlled binding between the two molecules (17). This indicates that the formation of the encountering complex is followed by a second step which could not be resolved separately in the stopped-flow experiments.

The present finding of two equivalent binding sites for tyrosinyl-5'-AMP seems to be in contrast to reports of Fersht et al. (15) and Mulvey and Fersht (2) regarding a half-site reactivity of this enzyme. These authors concluded from their experiments that tyrosyl-tRNA synthetase (a) binds only one tyrosine molecule and (b) forms two aminoacyladenylates, but at strongly different rates. Our results indicate that the two subunits of tyrosyl-tRNA synthetase are equivalent and symmetric with respect to the binding of tyrosinyl-5'-adenylate. In the case of tRNA binding it has been shown earlier (5) that the non-equivalence of the two a priori equivalent binding sites is introduced only after binding of the first $tRNA^{Tyr}$ molecule. The finding of only one binding site for tyrosine (18) may be explained in a similar manner. The non-equivalence of the two subunits during aminoacid activation may be due to conformational changes in one subunit that are induced by the formation of the tyrosyl-adenylate in the other subunit.

ACKNOWLEDGEMENTS

We thank Dr.A.Pingoud for the supply of tyrosyl-tRNA synthetase and for helpful discussions.
The expert technical assistance of Mrs.M.Möllering and Miss M.

Wehsling is gratefully acknowledged. This work was supported by grants from the Deutsche Forschungsgemeinschaft (Schwerpunkt-programm Struktur-Funktion von Biopolymeren) and the Fonds der Chemischen Industrie.

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