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On the functional role of the Tyr-639 residue of bacteriophage T7 RNA polymerase

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Substitution of Asp for a Tyr residue normally present at position 639 of the bacteriophage T7 RNA polymerase leads to a drastic drop in the enzymatic activity. This mutation does not affect the enzyme–promoter interaction but decreases the ability of the RNA polymerase to discriminate between GTP and ATP molecules, resulting in a decrease in the rate of the incorporation of the nucleotide into the RNA chain.

T7 RNA polymerase; Mutant form of enzyme; NTP binding

1. INTRODUCTION

Since bacteriophage T7 DNA-dependent RNA polymerase (T7RNAP) is one of the simplest RNA polymerases known, it is a prime candidate for the detailed study of the mechanism of transcription [1–3].

Earlier a two-plasmid system was constructed for the phenotypic selection of clones producing inactive T7RNAP [4]. One of the mutants isolated (Y639D) contains a Tyr-639→Asp substitution. The present paper reports a study of the influence of this substitution on the functioning of T7RNAP.

2. MATERIALS AND METHODS

Wild-type (wt) T7RNAP (200,000 U/mg) and the mutant, Y639D, were isolated from an *E. coli* strain containing the pACT7 plasmid according to our earlier paper [5]. The assay of T7RNAP activity and binding experiments were performed as in [5–9]. To avoid the synthesis of oligoG sequences in the GTP binding assay (the pGEM-2 plasmid has a transcribed region starting with GGG) a synthetic promoter (Pr) with a unique substitution ($\frac{G}{C} \rightarrow \frac{T}{A}$) in the +2 position of the transcribed region [6] was used. The kinetic and binding constants were calculated using the program, ENZFITTER (Elsevier Biosoft).

3. RESULTS AND DISCUSSION

The genetic system for the phenotypic selection of clones producing inactive T7RNAP [4] consists of two plasmids which belong to different incompatibility groups and confer resistance to different antibiotics. One of them (pGEM-4) contains the ColE1-type origin of replication and the T7RNAP promoter Φ10. The second plasmid (pACT7) is a derivative of pACYC184 which contains the gene for T7RNAP under the control of the inducible Pr promoter and the gene for the heat-sensitive λ repressor, cI857 [6]. It turned out that pGEM-4 was incompatible with pACT7 but could be stably maintained in the cells producing inactive T7RNAP. Using this property nine clones producing mutant T7RNAPs have been selected.

The sequencing of one of the mutant genes showed the transition, T→G, resulting in the substitution of Asp for the Tyr-639 residue of the enzyme. This residue belongs to a highly conservative region; it is invariant in the sequences of all the single-subunit RNA polymerases known (Fig. 1). Our recent data on affinity modification and oligonucleotide-directed mutagenesis of the Lys-631 residue suggest that this region participates in the formation of the NTP binding site of the T7RNAP [6].

Since a transcription includes the steps of the binding of RNA polymerase to promoter, initiation, elongation and termination of RNA chains it is of interest to determine which of these stages is affected by the mutation.

To evaluate the ability of the mutant to synthesize RNA the experiments on the incorporation of [^{32}P]GMP and [γ-^{32}P]GTP were carried out (Table 1). The incorporation of [^{32}P]GMP characterizing total RNA synthesis was about 0.5% with pGEM-2 and 5% with polydC. (The latter is known to be a good template for T7RNAP in promoter-independent poly G synthesis [1].) The incorporation of [γ-^{32}P]GTP characterizing the number synthesized de novo by both wt and mutant T7RNAP was about 70% for plasmid pGEM-2 and 100% for polydC templates. The data obtained suggest that the mutation does not affect the ability of the en-

Abbreviations: T7RNAP, bacteriophage T7 DNA-dependent RNA polymerase; Y639D, mutant T7RNAP containing Tyr-639→Asp substitution; Pr, synthetic promoter for T7RNAP.

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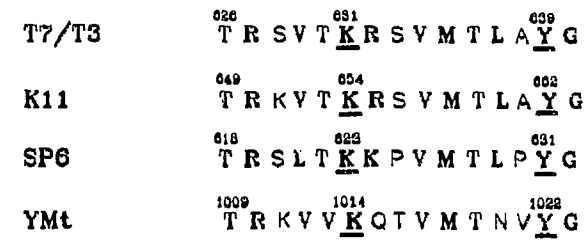

Fig. 1. Similarity of the region of the Tyr-639→Asp mutation in T7RNAP with the sequences of the RNA polymerases of phages T3 [10], K11 [11], SP6 [12] and the enzyme from *S. cerevisiae* mitochondria (YMt) [13]. Identical and homologous amino acid residues are in bold. Residues corresponding to Tyr-639 and Lys-631 (see text) are underlined.

Table II

Kinetic parameters for wt and mutant T7RNAPs

NTP	Template			
	pGEM-2		polydC	
	wt	Y639D	wt	Y639D
K_m (μM)				
G	160 ± 25	175 ± 25	300 ± 10	1,000 ± 25
A	40 ± 4	160 ± 10	–	–
C	77 ± 5	65 ± 10	–	–
U	40 ± 5	28 ± 5	–	–
V_{max} (relative units)	6.0 ± 1.0	0.2 ± 0.1	6.6 ± 0.6	0.6 ± 0.1

zyme to initiate RNA synthesis but influences mainly the elongation of the chain.

To characterize the mutant enzyme–promoter, binding experiments of two types were performed. First, the ability of the Y639D to compete with wt T7RNAP for the promoter was tested. Fig. 2 shows that the mutant competes with the wt enzyme with an 'inhibition constant' of 0.14 μM. Second, the dissociation constants (K_d) and rate constants of the dissociation (k_{-1}) of the enzyme–promoter complex were determined by the filter binding technique [9,14]. The values of these constants for wt T7RNAP and Y6539D were close (K_d 0.27 and 0.22 μM; k_{-1} $2.1 \cdot 10^{-4}$ s^{-1} and $2.3 \cdot 10^{-4}$ s^{-1}, respectively). The values obtained by both techniques are close and it appears that the decreased activity of Y639D does not result from the disturbance in the promoter binding.

To evaluate the T7RNAP–NTP interaction K_m^{NTP} and V_{max} values for wt and mutant T7RNAPs were compared (Table II). When plasmid pGEM-2 was used as a template K_m values were close, whereas V_{max} for Y639D was about 3% of that for wt T7RNAP. For polydC the V_{max} value for the mutant was 10% as compared to the wt enzyme.

Because of the complex kinetic mechanism of the reaction the K_m value could not serve as an accurate measure of substrate affinity. So we determined the K_d^{NTP}s of binary (E·NTP) and ternary (E·Pr·NTP) complexes using the nitrocellulose filter binding technique [8]. The dissociation constants for GTP and ATP binding to wt and mutant T7RNAPs are presented in Table III. K_d^{GTP}s for binary and ternary complexes for wt enzyme were 0.35 and 0.15 μM, respectively. These data are in keeping with that obtained earlier [14]. For the mutant the GTP binding did not depend on the presence of the promoter. The character of ATP binding differed from that of GTP. The K_d value of the binary complex with the wt enzyme was very high (>20 μM) indicating a lack of specificity of binding. In the presence of the promoter this value was about 3 μM. K_d^{ATP} values for both binary and ternary complexes for Y639D were close to K_d^{GTP} for wt T7RNAP (0.25 μM).

The data obtained enable us to conclude that the Tyr-639→Asp substitution decreases the ability of the enzyme to discriminate between GTP and ATP leading to the deceleration of nucleotide incorporation into the RNA chain. Taking into account a relatively high affinity of Y639D to ATP one could expect the ability of the mutant enzyme to carry on an efficient poly A synthesis on a polydT template (wt T7RNAP possesses such activity with an efficiency of about 4% as compared to that on pGEM-2 [15]). However, Y639D was completely unable to catalyze such a reaction.

We attempted to determine what part of the NTP molecule can interact with the amino acid residue at position 639 or T7RNAP. As GTP and ATP molecules differ in substituents at positions 2 and 6 of the purine base a number of NTP analogues were tested in compet-

Table I

Incorporation of [γ-^{32}P]GTP and [α-^{32}P]GMP into RNA

Template	[γ-^{32}P]GTP incorporated (pmol)		[α-^{32}P]GMP incorporated (nmol)		[α-^{32}P]GMP/[γ-^{32}P]GTP ratio	
	wt	Y639D	wt	Y639D	wt	Y639D
pGEM-2	2.2	1.9	3.36	0.016	1527	8.5
polydC	4.6	7.8	3.80	0.144	826	18.5

The incubation mixture (20 μl) contained 0.5 pmol of pGEM-2 or 0.6 μg of polydC and 0.7 pmol of wt or mutant T7RNAP. Incubation was at 37°C for 15 min.

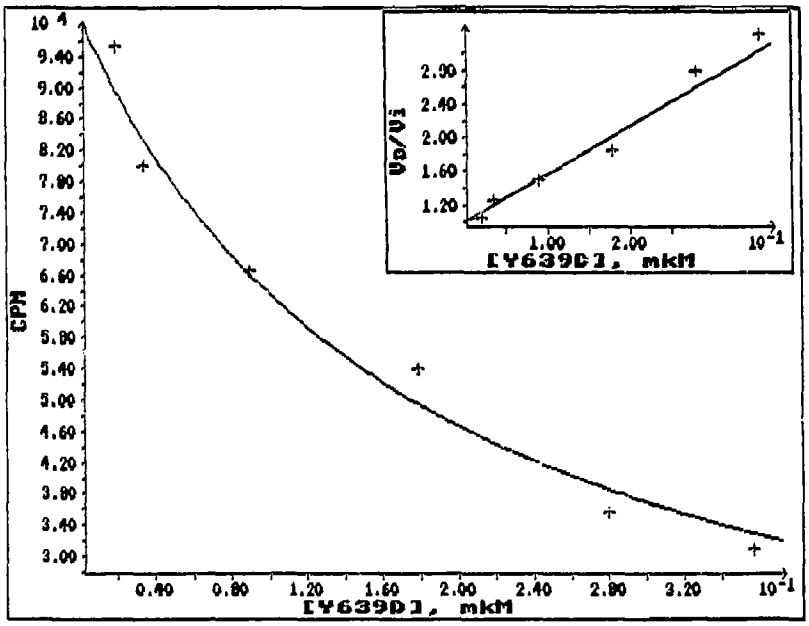

Fig. 2. Inhibition of [^{32}P]GMP incorporation by wt T7RNAP in the presence of Y639D (37°C, 15 min). The incubation mixture (20 μl) contained 0.5 pmol of pGEM-2 digested with *Hae*III and 0.05 μM of wt T7RNAP. The inset demonstrates the calculation of the 'inhibition constant' according to equation:

$$V_o/V_i = 1 + \frac{K_s\,[\text{Y639D}]}{K_i\,(K_s + [\text{wt T7RNAP}])}$$

(K_s = 0.27 μM). K_i calculated = 0.14 ± 0.02 μM.

itive binding experiments with GTP. It can be seen from the data presented in Table IV that nucleotides bearing an electron donor substituent at position 6 possess higher affinity to wt T7RNAP whereas those with electron acceptors are preferable for Y639D. The substitution of groups at position 2 affects nucleotide binding to a lesser extent. It seems that the Tyr-639 residue interacts with a substituent at position 6 of purine substrates.

One can speculate as to the nature of such an interaction. Since the stacking interaction between Tyr-639 and the purine base is hardly probable it seems that a hydrogen bond between the hydroxy group of Tyr-639 and the 6-oxo group of guanine is formed. This specific interaction can be the cause for the choice of a G residue to start most of T7 transcripts [1]. The substitution of Asp for Tyr abolishes the interaction but enables the formation of a hydrogen bond between the carboxy group of Asp and the 6-amino group of ATP. It should be noted that the inter-atomic distances between Cα and oxygens in Tyr and Asp residues are close (5.65 and 5.97 Å, respectively). However, the interaction between Asp

Table III

Dissociation constants (μM) for NTP of binary (E·NTP) and ternary (E·Pr·NTP) complexes of wt and mutant T7RNAP

NTP	wt		Y639D	
	E·NTP	E·Pr·NTP	E·NTP	E·Pr·NTP
GTP	0.35±0.07	0.15±0.04	0.10±0.02	0.10±0.03
ATP	>20	3.00±1.50	0.30±0.08	0.25±0.10

The synthetic promoter with a coding sequence starting with GUG (Pr) was used in these experiments.

Table IV

Competitive binding of purine NTP analogs by wt and mutant T7RNAP

NTP	K_i (μM)			
	wt		Y639D	
	E·NTP	E·Pr·NTP	E·NTP	E·Pr·NTP
ATP	11.0±2.0	7.0±2.0	0.10±0.04	0.16±0.05
εATP	1.3±0.2	0.9±0.2	0.40±0.15	1.00±0.20
ITP	1.1±0.3	1.6±0.1	2.50±0.40	4.00±0.55
XTP	0.4±0.1	0.4±0.1	0.20±0.07	0.22±0.09

Incubation mixtures contained 0.5 pmol of Pr, 0.7 pmol of wt or mutant T7RNAP, fixed (50, 125, 250 nM) concentrations of [α-^{32}P]- or [γ-^{32}P]GTP and variable (0.05–12.5 μM) concentrations of an analog. The inhibition constants were calculated from the plots of B_o/B_i (binding in the absence and in the presence of the NTP analog, respectively) vs. analog concentration. εATP denotes a fluorescent ATP analog, *etheno*ATP [16].

and ATP leads to the formation of an unproductive complex. Oligonucleotide-directed mutagenesis studies of the Tyr-639 residue are now in progress to verify this suggestion.

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