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^{31}P NMR STUDIES OF THE INTERACTION OF ATP WITH RNA POLYMERASE FROM *ESCHERICHIA COLI*

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1. Introduction

The transcription mechanism is based on the principle of complementary recognition of nitrogen bases. It is however obvious that this principle alone cannot guarantee a high fidelity of RNA synthesis catalyzed by RNA polymerase from *Escherichia coli* during copying of the natural and synthetic templates [1]. Since the RNA polymerase is a template-directed enzyme, most likely, the template must participate in forming the working conformation of the active enzyme center.

The application of the NMR method to investigations of the structure of the above system is based on a tight binding of a Mn^{2+} (which surves as a paramagnetic label) in the vicinity of the active center of RNA polymerase [2]. The changes in the relaxation times of various groups of substrates induced by this ion allow one to determine the distances between the Mn^{2+} and these groups of substrates of RNA polymerase [3–5]. In the present work the NMR method was used to study the effects of complementary and non-complementary templates on the structure of an enzyme–substrate complex.

2. Materials and methods

High-purified RNA polymerase from *E. coli* and polydeoxynucleotides (poly-dA and poly-dT) were purchased from SBDT BAC (Novosibirsk). The specific activity of the enzyme was about 1000 calf thymus DNA units per mg protein [6]. Enzyme concentrations were determined by ultraviolet absorption using $\epsilon_{280} = 0.65$ (mg/ml)$^{-1}$ assuming M_r 500 000 for the holoenzyme [7]. The dinucleotide pUpU was kindly supplied by Dr L. Boldyreva (Novosibirsk State University). ATP (Reanal, Hungary) was additionally purified by chromatography on a DEAE-Sephadex A-25 column. The concentrations of ATP, pUpU, poly-dA and poly-dT were determined by ultraviolet absorption at 259 nm and 267 nm (for poly-dT) using extinction coefficients of 15.4×10^3 M^{-1}, 20×10^3 M^{-1}, 10×10^3 M^{-1} and 7.6×10^3 M^{-1}, respectively.

All solutions used in NMR experiments were passed through Bio-Rad Chelex 100 before use to remove trace metal ion concentrations.

2.1. *Preparation of enzyme solutions for EPR and NMR measurements*

The enzyme (about 1 mg ml^{-1}) containing 50% (v/v) glycerol, 0.5 mM EDTA was diluted three-fold with cold 20 mM Tris–HCl (pH 7.8)–50 mM KCl buffer (buffer A), precipitated by adding 0.5 g ml^{-1} of solid $(NH_4)_2SO_4$ and centrifuged at 6000 rev./min for 1 h. The pellet was redissolved in buffer A and the $(NH_4)_2SO_4$ was removed by dialysis against the same buffer. The final concentration of the enzyme was about 5 mg ml^{-1}. No less enzyme activity was observed in the course of the magnetic resonance experiments.

For ^{31}P NMR experiments, 20% ^2H_2O was added to the enzyme solution for internal stabilization.

2.2.1. Magnetic resonance measurements

^{31}P magnetic resonance spectra were obtained at 81 MHz using a Varian XL-200 NMR spectrometer. The longitudinal relaxation rates ($1/T_1$) of the phosphorus of ATP and pUpU were determined by the pulsed Fourier transform 180°–τ–90° method of Carr and Purcell. The temperature for the relaxation measurements was 24 ± 1°C. To determine the

paramagnetic contribution to the relaxation rates of the phosphorus of ATP and pUpU, a $MnCl_2$ solution was added to the sample containing the enzyme and substrate after measuring the diamagnetic relaxation rates in the absence of the metal. These results were then subtracted from the relaxation rates observed with Mn^{2+} to obtain the paramagnetic contributions to $1/T_1$. The EPR spectra were recorded with an E-109 spectrometer (Varian) using a capillary at 23 ± 1°C.

3. Results and discussion

As mentioned in the introduction, the application of the NMR method to structure investigations of RNA polymerase is based on a strong binding of Mn^{2+} to the active center of the enzyme. Recently it has been shown by the ESR method [2] that RNA polymerase has only one binding of Mn^{2+} with K_D = 1.9×10^{-6} M.

Since the K_D of the RNA polymerase–Mn^{2+} complex depends on the enzyme preparation [2], we used the ESR method to study the binding between the Mn^{2+} and RNA polymerase, and the effect of poly-dA and poly-dT templates upon the binding parameters. The result of RNA polymerase titration by Mn^{2+} is shown in fig.1 in Scatchard's coordinates. Table 1 lists the K_D values and the number of Mn^{2+}-binding sites (n) calculated by these curves. Table 1 shows that the presence of a template in the system affects K_D and n negligibly. These data allow us to choose

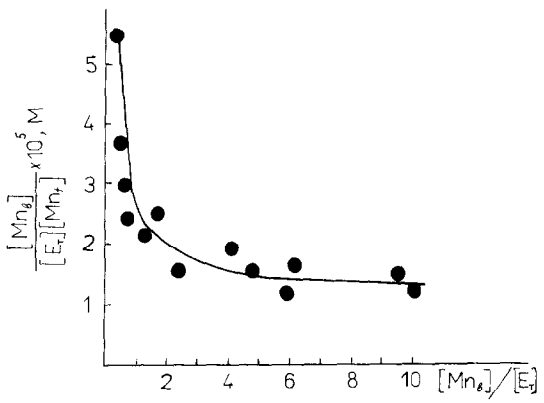

Fig.1. Scatchard plot of the binding of Mn^{2+} to RNA polymerase. Solution contained: RNA polymerase (10 μM), $MnCl_2$ (1–100 μM), 20 mM Tris–HCl buffer (pH 7.8), and 50 mM KCl. Mn_f indicates free Mn^{2+}, and Mn_b indicates the enzyme-bound Mn^{2+}.

Table 1
Dissociation constants and numbers of Mn^{2+}-binding sites of Mn^{2+}–RNA polymerase complexes

Complex	K_D (μM)	n
E–Mn^{2+}	1.8 ± 0.3	1 ± 0.2
E–poly-dA–Mn^{2+}	3.5 ± 0.7	1.2 ± 0.3
E–poly-dA–Mn^{2+}	2.0 ± 0.3	1.5 ± 0.3

Experimental conditions were as described in fig.1, poly-dA and poly-dT concentrations were 1 mM. E, enzyme

the ratio Mn^{2+}/RNA polymerase so that in the NMR experiments, more than 85% of Mn^{2+} are in strong complexes with the enzyme.

The relaxation time T_1 determined experimentally for α-, β-, and γ-phosphorus of ATP and internucleotides and the terminal phosphorus of pUpU in the complexes with RNA polymerase with complementary and non-complementary templates are given in table 2. The distances between the phosphorus and the coordinated Mn^{2+} were calculated using the Solomon-Blombergen equation [8,9].

$$\frac{1}{T_{1M}} = \frac{q\,A}{r^6}\,\tau_c \qquad (1)$$

Here $1/T_{1M}$ are the paramagnetic contributions to the longitudinal relaxation rates of a molecule bound near the paramagnetic ion; τ_c is the correlation time for the dipolar interaction, r is the metal–nucleus distance; q is the number of ligands bound at or near each metal ion; A is a constant.

For the Mn^{2+}–RNA polymerase system the shortest time averaging the dipolar interaction is that of the electron relaxation of Mn^{2+} [3]. Therefore, we calculated the distances using this value $\tau_c \approx \tau_e = 2.6\ 10^{-9}$ s and $q = 1$ [3]. Table 2 and 3 list T_{1M} calculated using these values, and r calculated by eqn 1.

It can be seen from tables 2 and 3 that the formation of enzyme–ATP–template complex is accompanied by a decrease in the T_1 of ATP phosphorus in the case of the complementary template (poly-dT). This is an evidence for the fact that the substrate (ATP) is less mobile in the complex with the complementary template as compared to that with the noncomplementary one. However, the nature of the template affects the Mn^{2+}–ATP distance negligibly (10% shortening in the presence of the complementary template).

Table 2
^{31}P Relaxation times of ATP and pUpU in RNA polymerase complexes

Complex	T_1 (s × 10^6)			T_{1M} (s × 10^6)				
	P_α	P_β	P_γ	P_α	P_β	P_γ	$P_{5'\to3'}$	$P_{5'}$
E–ATP	2.0 ± 0.08	1.85 ± 0.07	2.14 ± 0.05					
E–ATP–pdA	2.5 ± 0.37	1.85 ± 0.25	2.7 ± 0.25					
E–ATP–pdT	1.45 ± 0.27	0.78 ± 0.14	0.96 ± 0.21					
E–ATP–Mn				12.5 ± 2.4	7.8 ± 1.4	9.6 ± 1.1		
E–ATP–Mn–pdA				12.3 ± 3.9	8.8 ± 2.8	8.6 ± 2.0		
E–ATP–Mn–pdT				6.1 ± 2.6	4.4 ± 1.8	4.4 ± 2.1		
E–ATP–Mn–pdA–pUpU				6.8 ± 2.2	4.6 ± 1.4	5.0 ± 1.4	350 ± 150	620 ± 180
E–ATP–Mn–pdT–pUpU				4.6 ± 1.4	4.5 ± 1.4	3.7 ± 0.8		

Solutions contained: 13 ÷ 19 μM RNA polymerase, 30 mM ATP, 3 μM $MnCl_2$, 1 mM poly-dA or poly-dT, 5 mM pUpU, 20 mM Tris–HCl buffer (pH 7.8) and 50 mM KCl. pdA and pdT indicate poly-dA and poly-dT. E, enzyme

Table 3
Mn^{2+}–ATP and Mn^{2+}–pUpU distances in RNA polymerase complexes

Complex	r (Mn^{2+}–P(Å))				
	P_α	P_β	P_γ	$P_{5'\to3'}$	$P_{5'}$
E–ATP–Mn	4.0 ± 0.1	3.7 ± 0.1	3.8 ± 0.1		
E–ATP–Mn–pdA	4.0 ± 0.2	3.8 ± 0.2	3.8 ± 0.1		
E–ATP–Mn–pdT	3.6 ± 0.2	3.4 ± 0.2	3.4 ± 0.3		
E–ATP–Mn–pdA–pUpU	3.7 ± 0.2	3.4 ± 0.2	3.5 ± 0.2	7.0 ± 0.7	7.8 ± 0.6
E–ATP–Mn–pdT–pUpU	3.4 ± 0.2	3.4 ± 0.2	3.3 ± 0.2		

Concentrations of the components in the complexes are given in table 2. E, enzyme

It is very important to study the interaction of the substrates with RNA polymerase in the presence of a template and the product synthesized, i.e. under the conditions of transcription. However, the transcription requires a much higher (some 10^{-2} M) concentration of divalent cations (Mn^{2+} or Mg^{2+}). At this Mn^{2+} concentration, the basic contribution to ATP relaxation is made by the Mn^{2+} not bound to the enzyme. Diamagnetic Mg^{2+} cannot be used either, since, as shown by reference ESR experiments, at this concentration ratio of Mn^{2+} (3×10^{-6} M) and Mg^{2+} (some 10^{-2} M), a great fraction of Mn^{2+} is not bound with the enzyme. To simulate the transcription conditions, we measured the ATP relaxation times in the system enzyme–ATP–Mn^{2+}–template in the presence of dinucleotide pUpU which served as an initiator in the case of the poly-dA template [10]. We employed the method of equilibrium dialysis to show that pUpU substitutes half the ATP bound in the enzyme–Mn–ATP–poly-dA complex. According to the model of RNA polymerase with two substrate-binding sites (one being catalytic) [11], this result demonstrates that in the enzyme–Mn^{2+}–ATP–template complex (even with a non-complementary template) the ATP is bound in both RNA polymerase sites. Thus, the presence of a template alone does not suffice to form the working conformation of the active center of the enzyme, specific with respect to the true substrate.

In an enzyme–Mn–ATP–poly-dA–pUpU complex the dinucleotide substitutes the ATP in the catalytic site. Hence, the Mn^{2+}–ATP distances measured in the complex (3.4–3.7 Å) refer to the ATP which is in the non-catalytic substrate-binding site [11]. The catalytic site is about 7 Å distant from that of Mn^{2+} binding. This value is obtained from the paramagnetic relaxation time (T_{1M}) of the internucleotide phosphorus of dinucleotide pUpU.

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