APPROACHES TO THE AFFINITY LABELLING OF *E. COLI* DNA-DEPENDENT RNA POLYMERASE

V. W. ARMSTRONG, H. STERNBACH and F. ECKSTEIN
Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, Göttingen, Germany

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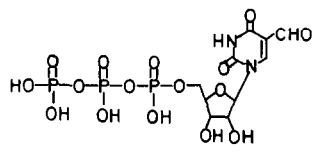
1. Introduction

For enzymes composed of non-identical subunits the elucidation of the function of the different subunits is an important problem. DNA-dependent RNA polymerase from *E. coli* is such an enzyme which has been shown to be composed of four major subunits in the stoichiometry $\alpha_2\beta\beta'\sigma$ [1]. The method of affinity labelling has been employed to identify the subunit(s) containing the active site of RNA polymerase. Scheit and Frischauf [2] using [^{35}S]4-thiouridine-5′-triphosphate found the label distributed between the β and β' subunits, the latter being preferentially labelled in the absence of DNA template and the former in the presence of template. Using the periodate oxidation product of [^{35}S]β-D-ribosyl-6-methylthiopurine Kimbal et al. [3] found 80% of the label to be located in the β subunit. Pyridoxal-5′-phosphate has also been employed [4] for the active site directed inhibition of RNA polymerase, but as yet no identification of the subunit involved has been reported.

We wish to report the application of 5-formyluridine-5′-triphosphate (fo^{5}UTP) to the affinity labelling of RNA polymerase.



5-formyluridine-5′-triphosphate (fo^{5}UTP)

The triphosphate bears a formyl group which can form a Schiff's base with a lysine residue at the active site of the enzyme, which on reduction with sodium borohydride leads to covalent attachment of the triphosphate to the enzyme. The binding of fo^{5}UTP to RNA polymerase, after sodium borohydride reduction has been studied, as well as the effects of ATP, rifampicin and streptolydigin on this binding. The subunits to which the fo^{5}UTP is attached have also been identified.

2. Materials and methods

E. coli RNA polymerase core enzyme was isolated according to Burgess [5]. Enzyme activity was assayed in a reaction mixture containing 0.15 ml 8 mM Tris–HCl, pH 8.0, 8 mM $MgCl_2$, 0.12 mM NH_4Cl, 6.8 mM DTE, 1 mM [^{14}C]ATP (2×10^3 cpm/nmole), 1 mM UTP, 1 A_{260} unit poly d(AT). Activity was measured as the amount of [^{14}C]AMP incorporated into acid-insoluble product after 9, 12 and 15 min incubation periods at 37°C. In a typical inhibition of the enzyme by fo^{5}UTP/$NaBH_4$ the reaction mixture contained in 0.1 ml 8 mM Tris–HCl, pH 8.0, 8 mM $MgCl_2$, 0.12 mM NH_4Cl, 74 μg enzyme and 0.5 mM fo^{5}UTP. After incubation for 5 min at 37°C the mixture was cooled to 0°C and treated with 0.1 ml 100 mM $NaBH_4$ solution for 10 min. A 10 μl aliquot was then removed and assayed as described above. For the labelling experiments fo^{5}UTP [7] was labelled enzymatically [8] by exchange of the γ-phosphate with $^{32}P_i$ Nitrocellulose membrane filters (pore size 0.45 μ, 25 mm diameter) were obtained from Schleicher and Schüll, and were soaked in wash buffer for at least 30 min at room temperature before use. Cellulose acetate electrophoresis in 6 M urea was performed according to Rabussay and Zillig [9].

3. Results

A Lineweaver–Burk plot showed fo^{5}UTP to be a competitive inhibitor (K_i = 6.9 × 10^{-5} M) of RNA polymerase as measured against the incorporation of [^{14}C]UTP into acid insoluble material. Reduction of the enzyme–fo^{5}UTP complex with $NaBH_4$ leads to inhibition of the enzyme (fig. 1) the extent of inhibition depending upon the concentration of fo^{5}UTP. This inhibition can be protected against by ATP (table 1) suggesting that the inhibition is occurring at the active site of the enzyme. Further evidence for the specificity of the inhibition is provided by a comparison of the inhibition produced by the nucleoside 5-formyluridine (11%) with that of the triphosphate fo^{5}UTP (69%) at the same concentration (1.5 mM) after $NaBH_4$ reduction.

The covalent attachment of [γ-^{32}P]fo^{5}UTP to RNA polymerase after $NaBH_4$ reduction was studied (table 1) using nitrocellulose filters [10]. Using 5 × 10^{-4} M fo^{5}UTP a stoichiometry of 2.5:1 for fo^{5}UTP:inactivated enzyme was found. The binding of fo^{5}UTP could be protected against by the presence of ATP in the incubation mixture. At a 20-fold excess of ATP over fo^{5}UTP, when no inhibition of the enzyme was recorded, the ratio of bound fo^{5}UTP: originally inactivated enzyme was 0.5:1 indicating some unspecific labelling. Rifampicin at a concentration of 10^{-5} M prevented the binding of 93 pmoles of fo^{5}UTP, a value similar to that recorded for the number of pmoles of enzyme (104) inactivated by fo^{5}UTP alone. Streptolydigin at a concentration of 10^{-4} M has relatively little effect on the binding of fo^{5}UTP.

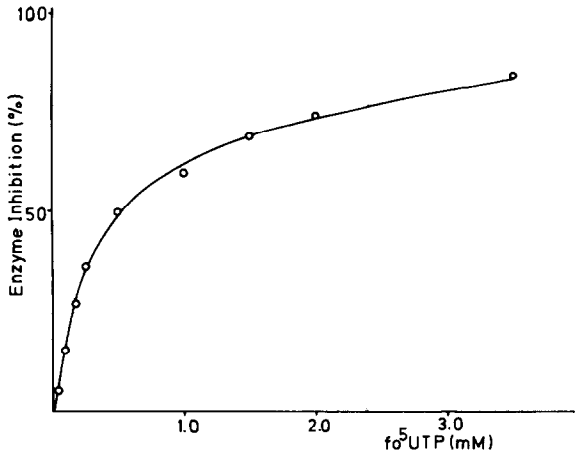


Fig. 1. Inhibition of RNA polymerase at different concentrations of fo^{5}UTP after $NaBH_4$ reduction. For procedure see Materials and methods.

Table 1
Determination of [γ-^{32}P]fo^{5}UTP covalently bound to RNA polymerase by retention on nitrocellulose filters

ATP (mM)	Inhibition (%)	Inactivated enzyme (pmoles)	fo^{5}UTP bond (pmoles)
0	56	104	254
1.25	47	87	179
2.5	29	54	116
5.0	13	24	96
10.0	0	0	55
Rifampicin (mM)			
10^{-2}	95	–	161
Streptolydigin (mM)			
0.12	95	–	240

Enzyme inhibition was performed as described in Materials and methods. The incubation mixture (0.1 ml) contained 0.5 mM [γ-^{32}P]fo^{5}UTP, 74 μg enzyme, and either ATP, rifampicin or streptolydigin at the above concentrations. After inhibition 0.03 μl aliquots were removed, diluted with 2 ml of wash buffer (8 mM Tris–HCl, pH 8.0, 8 mM $MgCl_2$, 2 mM EDTA) and incubated at 37°C for 10 min prior to filtration. The filters were washed with 50 ml of wash buffer after adsorption of the protein.

The distribution of [γ-^{32}P]fo^{5}UTP on the various subunits as determined by cellulose acetate electrophoresis is shown in fig. 2. When RNA polymerase was labelled with [γ-^{32}P]fo^{5}UTP alone the label was distributed between the β and β' subunits at a ratio of approximately 2.6:1. However, when the labelling procedure was repeated in the presence of rifampicin (10^{-5} M), the amount of label in the β subunit relative to the β' was significantly reduced such that the final ratio was 1.5:1 (β:β'). The rifampicin is, therefore, preventing the binding of fo^{5}UTP to the β subunit.

4. Discussion

The results indicate that fo^{5}UTP inactivates RNA polymerase on sodium borohydride reduction by

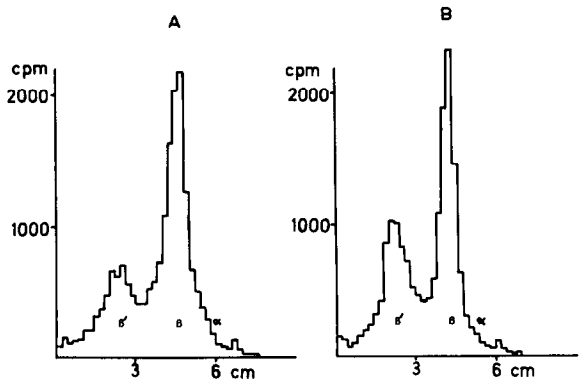


Fig. 2. Cellulose acetate electrophoresis of RNA polymerase after labelling with [γ-^{32}P]fo^{5}UTP (1×10^5 cpm/nmol). The reaction mixture contained in 0.5 ml, 370 μg enzyme and (A) 0.5 mM fo^{5}UTP or (B) 0.5 mM fo^{5}UTP and 10^{-5} M rifampicin. After reduction with 0.5 ml 100 mM $NaBH_4$ solution the samples were dialysed overnight against 5 mM Tris–HCl, pH 8.0, 1 mM DTE. After electrophoresis and destaining the sheets were cut into 2 mm strips and radioactivity measured in Bray's solution.

reaction at the substrate binding site. However, the stoichiometry of the inhibition shows that 2.5 moles of fo^{5}UTP are bound per mole of inactivated enzyme. Although this binding may be to a certain extent unspecific due to reaction with lysine residues not at the active site, 2 moles of fo^{5}UTP can be protected against by ATP. This represents at least two nucleotide triphosphate binding sites one of which (or possibly both) must be responsible for enzyme activity. Two substrate binding sites have been postulated for RNA polymerase [11], and several ATP binding sites have been measured by gel filtration, and equilibrium dialysis techniques [12]. Rifampicin inhibits the initiation step of RNA polymerase [13], and studies [9,14] with mutants have shown that the β subunit is involved in rifampicin inhibition. Streptolydigin on the other hand inhibits the polymerisation step [15, 16] of RNA polymerase. It was therefore of interest to study the effect of these antibiotics on the binding of fo^{5}UTP. As can be seen from table 1 streptolydigin had relatively little effect on the number of moles of fo^{5}UTP bound, but rifampicin prevented the binding of 0.9 moles fo^{5}UTP per mole of inactivated enzyme. Furthermore this rifampicin-protected site is located on the β subunit. Investigations are in progress to elucidate further the roles of these fo^{5}UTP-binding sites in RNA polymerase, and also the effect of DNA template on this binding.

Acknowledgements

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