other than the cycloheximide's effect might also prevent the function of factor A (5).

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Affinity Labeling of *Escherichia coli* DNA-Dependent RNA Polymerase with 5-Formyl-1-(α-D-ribofuranosyl)uracil 5′-Triphosphate†

Victor W. Armstrong, Hans Sternbach, and Fritz Eckstein*

ABSTRACT: 5-Formyl-1-(α-D-ribofuranosyl)uracil 5′-triphosphate has been used to affinity label *E. coli* DNA-dependent RNA polymerase. It is a noncompetitive inhibitor of the enzyme with K_i = 0.54 mM. A short preincubation of the enzyme and α-fo^{5}UTP is required to achieve maximum inhibition, and the extent of the inhibition is dependent upon the α-fo^{5}UTP concentration. When a preincubation mixture of α-fo^{5}UTP/enzyme is diluted, the enzyme regains activity with time showing that the inhibition is reversible, presumably occurring by Schiff base formation between an amino group on the enzyme and the formyl group. Upon sodium borohydride reduction of an enzyme/α-fo^{5}UTP preincubation mixture the enzyme is irreversibly inhibited. α-fo^{5}UTP is more effective in inhibiting the enzyme than α-fo^{5}U, and the inhibition is decreased by the presence of ATP, UTP, or GTP in the preincubation mixture, suggesting that inhibition is occurring at a triphosphate binding site. The stoichiometry of binding of α-fo^{5}UTP to the enzyme was determined using the γ-^{32}P-labeled derivative. After a 20-s preincubation of enzyme/α-fo^{5}UTP followed by $NaBH_4$ reduction the stoichiometry of binding was 1.1:1 (α-fo^{5}UTP bound:inactivated enzyme), and this rose to 2.42:1 after a 10-min preincubation. After a 20-s preincubation the [γ-^{32}P]-α-fo^{5}UTP was shown to be located on the β subunit of RNA polymerase by cellulose acetate electrophoresis in 6 M urea.

Much interest has been focussed upon the role of the different subunits of DNA-dependent RNA polymerase in the transcription process. In order to determine which subunits contain the catalytic site of the enzyme the technique of affinity labeling has been employed. Thus the substrate analogue 4-thiouridine 5′-triphosphate (Frischauf and Scheit, 1973) was shown to be covalently attached to the β and β′ subunits after photooxidation. The periodate oxidation product of 6-methylthiopurine ribonucleoside proved to be a noncompetitive inhibitor of RNA polymerase (Nixon et al., 1972), and it was suggested that this was due to inhibition of the initiation mechanism. After sodium borohydride reduction it was found to be attached to the β subunit. Using a fluorescent derivative, AMPR-OP, of this compound in which the methyl group was replaced by *N*-(acetylaminoethyl)-1-naphthylamino-5-sul-

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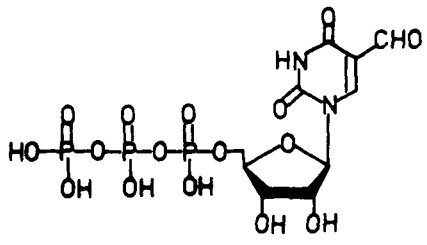
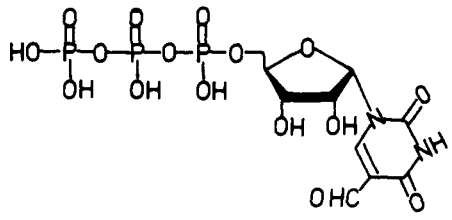
fonate it was found that the enzyme was still able to bind DNA and nucleoside triphosphates (Wu and Wu, 1974). Pyridoxal phosphate has also been shown to inhibit the enzyme by Schiff base formation with a crucial lysine residue on the enzyme (Bull et al., 1975).

We recently reported (Armstrong et al., 1974) an attempt to affinity label RNA polymerase using the substrate analogue 5-formyluridine 5′-triphosphate (**1**). Such a derivative would be able to function as an affinity label by reaction with an amino group at the active site of the enzyme, forming a Schiff base, and subsequent reduction with sodium borohydride, thus covalently linking the triphosphate to the enzyme.



(1)



(2)

However, we have since discovered that our original synthesis of fo^{5}UTP[1] led to a mixture of the α(**2**) and β(**1**) anomers of this triphosphate, the anomerization occurring during the synthesis of 5-formyluridine 5′-monophosphate (Armstrong and Eckstein, 1975) by the method of Tener (1961). In our preliminary communication (Armstrong et al., 1974) this mixture was used in the determination of the kinetic constants. We have now been able to separate the mixture of α- and β-fo^{5}UTP by DEAE-Sephadex chromatography. This present paper deals with the interaction of these two triphosphates with RNA polymerase.

Materials and Methods

Materials. ATP, UTP, and rifampicin were purchased from Boehringer and [^{14}C]ATP, [^{14}C]UTP, and [α-^{32}P]ATP from New England Nuclear. ^{32}P-labeled sodium pyrophosphate and $^{32}P_i$ were supplied by Amersham, and poly[d(A-T)] was a product of Miles Laboratories Inc. Nitrocellulose filters (0.45 μ, 2 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 was performed in 6 M urea as described (Rabussay and Zillig, 1969), and the cellulose acetate sheets were supplied by Chemetron. Glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle, 10 mg/ml, 80 units/mg) and phosphoglycerate kinase (yeast, 10 mg/ml, 450 units/mg) were supplied by Boehringer, Germany.

Enzyme Purification and Assay. E. coli RNA polymerase holo and core enzymes were purified according to a described procedure (Burgess, 1969) and were >95% pure according to sodium dodecyl sulfate gel electrophoresis. All experiments were performed with the holo enzyme unless otherwise stated. Enzyme activity was measured as the amount of [^{14}C]AMP or [^{14}C]UMP incorporated into acid-insoluble material (Bollum, 1966) after a 10-min incubation at 37 °C. The assay mixture contained in 0.1 ml: 40 mM Tris, pH 8.0, 8 mM $MgCl_2$, 5 mM DTE, 0.2 A_{260} unit of poly[d(A-T)], 0.05 M KCl, 1 mM ATP, and 1 mM [^{14}C]UTP. For the kinetic studies a fixed concentration of ATP (0.4 mM) was employed and the [^{14}C]UTP concentration was varied. Enzyme activity was then measured as the amount of [^{14}C]UMP incorporated into acid-insoluble material after 5 min.

Ternary Complex and Assay. The ternary complex consisting of RNA polymerase, poly[d(A-T)], and product poly[r(A-U)] was prepared as described by Rhodes and Chamberlin (1974). It was assayed in a reaction mixture (0.25 ml) containing 40 mM Tris-HCl, pH 8.0, 8.0 mM $MgCl_2$, 10 mM DTE, 0.05 M KCl, 2.5 μg of rifampicin, 0.2 mM [^{14}C]ATP (14 000 cpm/nmol), and 0.2 mM UTP. For the kinetic studies the UTP concentration was held constant at 0.1 mM and [α-^{32}P]ATP (3000 cpm/pmol) concentration was varied. The reaction was terminated after 5 min and the radioactivity determined as described.

Synthesis of α- and β-fo^{5}UTP. 10 500 A_{280} units of the anomeric mixture of fo^{5}UMP (Armstrong and Eckstein, 1975) were converted to their triphosphates by a standard procedure (Michelson, 1964). The crude reaction product was chromatographed over a DEAE-Sephadex column eluted with a linear gradient of 2.5 l. each of 0.05 M and 0.45 M triethylammonium bicarbonate solution. The α-fo^{5}UTP (856 A_{280} units) eluted between 0.39 and 0.42 M buffer and the β anomer (1190 A_{280} units) between 0.35 and 0.38 M buffer. These structures were assigned by comparison of their CD and NMR spectra to spectra of the nucleosides. For enzymatic studies they were further purified over Dowex ion-exchange resin as described for [γ-^{32}P]-α-fo^{5}UTP below.

Synthesis of [γ-^{32}P]-α-fo^{5}UTP. This was prepared essentially according to the method of Glynn and Chappell (1964). 150 A_{280} units of α-fo^{5}UTP was incubated in a final volume of 1.2 ml containing 0.2 ml of 1 M Tris-HCl, pH 8.0, 12 μl of 1 M $MgCl_2$, 0.2 ml of 0.1 M NaOH, 3 μl of 0.1 M Na_2HPO_4, 20 μl of 3-phosphoglycerate (cyclohexylammonium salt, 200 mg/ml), 20 μl of glyceraldehyde-3-phosphate dehydrogenase, 12 μl of phosphoglycerate kinase, and 0.1 ml of $^{32}P_i$ (10 mCi/ml, carrier free). After 16 h at room temperature the mixture was diluted with 1 ml of H_2O and chromatographed over a Dowex 1-X4 (200–400 mesh, Cl^-) ion-exchange column (10 × 0.8 cm) with a linear gradient of 0.01 M HCl, 0.05 M LiCl and 0.01 M HCl, 0.45 M LiCl (180 ml of each). The fractions containing α-fo^{5}UTP were collected and neutralized with 1 M LiOH. After concentration in vacuo to ca. 5 ml the solution was transferred to a centrifuge tube and 5 drops of saturated $BaCl_2$ were added followed by 30 ml of EtOH to precipitate the barium salt of the triphosphate. After centrifugation, the supernatant was decanted and the residue was washed twice with 2 ml of water by centrifugation. Finally, the triphosphate was converted to its Na^+ salt with Merck-I (Na^+ form) ion-exchange resin and after evaporation in vacuo the α-fo^{5}UTP was dissolved in 1 ml of H_2O and stored as a frozen solution at −20 °C: yield = 92 A_{280} units; A_{280}/A_{260} = 1.98.

[1] Abbreviations used are: α-fo^{5}UTP, 5-formyl-1-(α-D-ribosyl)uracil 5′-triphosphate; α-fo^{5}U, 5-formyl-1-(α-D-ribosyl)uracil; β-fo^{5}UTP, 5-formyl-1-(β-D-ribosyl)uracil 5′-triphosphate; β-fo^{5}U, 5-formyl-1-(β-D-ribosyl)uracil.

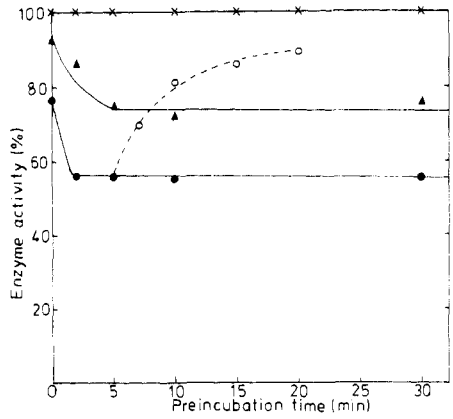


FIGURE 1: The inhibition of RNA synthesis by α-fo^{5}UTP. The enzyme (13 μg) was preincubated at 37 °C in a reaction mixture (0.1 ml) containing 40 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, 0.05 M KCl, and (a) H_2O (-x-x-); (b) 0.23 mM α-fo^{5}UTP (- ▲ - ▲ -); and (c) 0.46 mM α-fo^{5}UTP (- ● - ● -). After various times, 10-μl aliquots were removed and assayed as described in Materials and Methods. In the case of (b) and (c) the assay mixtures contained 0.23 and 0.46 mM α-fo^{5}UTP, respectively. In a further experiment (d) (- O - O), 10-μl aliquots were removed from (c) after 5-min preincubation and diluted with a solution (70 μl) containing 57.1 mM Tris-HCl, pH 8.0, 11.4 mM $MgCl_2$, 7.1 mM DTE, 0.07 1 M KCl, 1 mM ATP, and 1 mM [^{14}C]UTP. The incubation was continued at 37 °C and after various times the enzyme was assayed by the addition of a solution containing 0.2 A_{260} unit of poly[d(A-T)].

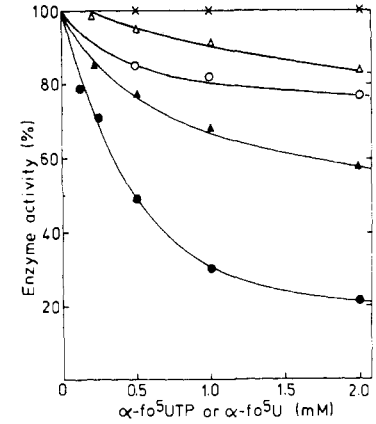


FIGURE 2: Inhibition of RNA synthesis after sodium borohydride reduction of an RNA polymerase/α-fo^{5}UTP (or α-fo^{5}U) incubation mixture. The reaction mixture (0.1 ml) was as described in Materials and Methods and contained 53 μg of enzyme and (a) 8 mM $MgCl_2$ and α-fo^{5}UTP (- ● -● -), (b) 8 mM $MgCl_2$ and α-fo^{5}U (- ▲ - ▲ -), (c) 2 mM EDTA and α-fo^{5}UTP (- O - O -), and (d) 2 mM EDTA and α-fo^{5}U (- Δ - Δ -). After a 5-min incubation at 37 °C the reaction mixtures were treated with 100 mM $NaBH_4$ solution as described in Materials and Methods.

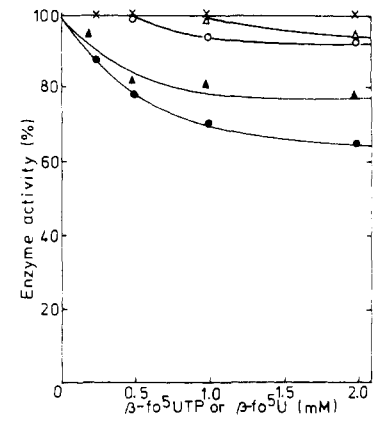


FIGURE 3: Inhibition of RNA synthesis after sodium borohydride reduction of an RNA polymerase/β-fo^{5}UTP (β-fo^{5}U) preincubation mixture. The conditions were as described in Figure 2 but using (a) 8 mM $MgCl_2$ and β-fo^{5}UTP (- ● - ● -), (b) 2 mM EDTA and β-fo^{5}UTP (- O - O -), (c) 8 mM $MgCl_2$ and β-fo^{5}U (- ▲ - ▲ -), and (d) 2 mM EDTA and β-fo^{5}U (- Δ - Δ -).

Specific activity = 2.34×10^8 cpm/μmol.

Sodium Borohydride Induced Inhibition. The enzyme was preincubated at 37 °C with the formyl derivative in a reaction mixture containing 40 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, 0.05 M KCl. The mixture was then treated with an equal volume of 0.1 M $NaBH_4$ solution and left at 0 °C for 15 min. Ten-microliter aliquots were then removed and assayed. For those experiments performed in the absence of Mg^{2+} the enzyme was dialysed against 0.15 M KCl, 0.05 M Tris, pH 8.0, 1 mM mercaptoethanol, 1 mM EDTA for 4 h at 4 °C with three changes of buffer. Mg^{2+} was omitted from the preincubation mixture to which 2 mM EDTA was added.

Results

Substrate and Inhibitory Properties of α- and β-fo^{5}UTP. α- and β-fo^{5}UTP were studied in their ability to replace UTP in the poly[d(A-T)] directed synthesis by RNA polymerase. The reaction was followed by the incorporation of [^{14}C]AMP into acid-insoluble material. At a concentration of 1 mM, β-fo^{5}UTP was able to replace UTP with an efficiency of about 55% that of UTP. Its K_m was determined to be 0.125 mM from a Lineweaver-Burk plot. On the other hand α-fo^{5}UTP showed less than 5% incorporation of [^{14}C]AMP. However, it inhibited the poly[d(A-T)]-directed synthesis in a noncompetitive manner with a K_i of 0.54 mM. This inhibition was most effective when the enzyme was preincubated with α-fo^{5}UTP for a short time prior to addition of the poly[d(A-T)], ATP, and [^{14}C]UTP, the inhibition rising from 24% with no preincubation to 45% after a 2 min or longer preincubation with 0.46 mM fo^{5}UTP (Figure 1). The enzyme was also preincubated with 0.46 mM α-fo^{5}UTP for 5 min and the solution was then diluted eightfold such that the final α-fo^{5}UTP concentration was 0.051 mM, and the preincubation was continued in the presence of ATP and UTP. The enzyme was then assayed by the addition of poly[d(A-T)]. In this case the enzyme regained activity such that the inhibition was only 11% after a further 15-min preincubation.

Inhibition of the poly[d(A-T)] Ternary Complex. At a concentration of 0.5 mM, α-fo^{5}UTP did not noticeably inhibit the ternary complex even after preincubation. However, at higher concentrations it was a competitive inhibitor of the incorporation of [^{32}P]AMP into poly[r(A-U)] with a K_i of 3.33 mM (K_m for ATP = 5.7 μM).

Inhibition after Sodium Borohydride Treatment. The effect of $NaBH_4$ on enzyme inactivation was studied with both α- and β-fo^{5}UTP and their corresponding nucleosides. After incubating the enzyme for 5 min at 37 °C with the formyl derivative, the incubation solution was then treated with 0.1 M $NaBH_4$ solution at 0 °C for 15 min and assayed. At a concentration of 2 mM, α-fo^{5}UTP inhibited the enzyme by 80%, α-fo^{5}U by 42% (Figure 2), β-fo^{5}UTP by 35%, and β-fo^{5}U by 24% (Figure 3). The inhibition in all cases was dependent upon the concentration of the formyl derivative. When Mg^{2+} was omitted from the preincubation mixture the extent of inactivation by all derivatives was markedly reduced. The presence of poly[d(A-T)] in the preincubation mixture did not affect the degree of inhibition by any of these derivatives.

Inhibition of Pyrophosphate Exchange. After incubation

Table I: Inhibition of the Poly[d(A-T)]-directed Pyrophosphate Exchange by α-fo^{5}UTP/$NaBH_4$.[a]

α-fo^{5}UTP Concn (mM)	nmol of PP_i Exchanged	Enzyme Act. (%)
(a) —	2.26	100
(b) 0.5	1.16	49
(c) 2.0	0.38	17

[a] The enzyme (53 μg) was incubated with (a) H_2O, (b) 0.5 mM α-fo^{5}UTP, (c) 2.0 mM α-fo^{5}UTP at 37 °C for 5 min and then treated with $NaBH_4$ solution as described in Materials and Methods. A 40-μl aliquot was then removed and subjected to the poly[d(A-T)] pyrophosphate exchange according to the method of Krakow and Frank (1969).

Table II: Effect of Rifampicin on the Binding of [γ-^{32}P]-α-fo^{5}UTP to RNA Polymerase.[a]

	Preincubation Time (s)	pmol fo^{5}UTP bound	
		−Rifampicin	+Rifampicin
$+Mg^{2+}$	20	68	32
	40	74	30
	300	146	92
$-Mg^{2+}$	20	25	18
	40	42	45
	300	71	84

[a] The enzyme was preincubated with 0.5 mM [γ-^{32}P]-α-fo^{5}UTP in the presence and absence of rifampicin (4.3 μg) at 37 °C for various times and was then treated with sodium borohydride as described under Materials and Methods. The radioactivity bound to the enzyme was then determined as described in the legend to Figure 5.

with α-fo^{5}UTP and sodium borohydride reduction the enzyme was assayed for poly[d(A-T)]-directed pyrophosphate exchange (Krakow and Frank, 1969). The enzyme was inactivated by 51% at 0.5 mM and 83% at 2 mM α-fo^{5}UTP (Table I), values similar to those for the inactivation of [^{14}C]AMP incorporation (Figure 2).

Protection of α-fo^{5}UTP/$NaBH_4$ Inhibition by ATP and UTP. The results of the protection experiments are shown in Figure 4. The enzyme was preincubated with 0.5 mM α-fo^{5}UTP in the presence of varying concentrations of ATP or UTP prior to $NaBH_4$ reduction. The presence of increasing concentrations of ATP or UTP protected the enzyme against inactivation. Both triphosphates afforded similar protection at the same concentrations, and GTP was also equally effective (data not shown). The presence of poly[d(A-T)] had no effect on the ability of these nucleotides to protect the enzyme.

Stoichiometry of Labeling of RNA-Polymerase with α-fo^{5}UTP. The amount of [γ-^{32}P]-α-fo^{5}UTP bound to the enzyme after $NaBH_4$ reduction was found to be dependent upon the time of preincubation of the enzyme with α-fo^{5}UTP (Figure 5). After 20 s 82 pmol of α-fo^{5}UTP was bound to the enzyme (i.e., retained on nitrocellulose filters), but this rose to 240 pmol after 10 min. The inhibition of the enzyme on the other hand remained fairly constant over this time, only rising slightly between 20 and 40 s. The stoichiometry of binding after 20 s was 1.1:1 (α-fo^{5}UTP bound:inactivated enzyme) and 2.42:1 after 10 min. In the absence of Mg^{2+} in the preincubation solution only 20 pmol of α-fo^{5}UTP was bound after 20 s with a stoichiometry of 1:1 but this had risen to 150 pmol after 5 min with a stoichiometry of 5:1. The presence of poly[d(A-T)] in the preincubation mixture did not alter the binding results significantly.

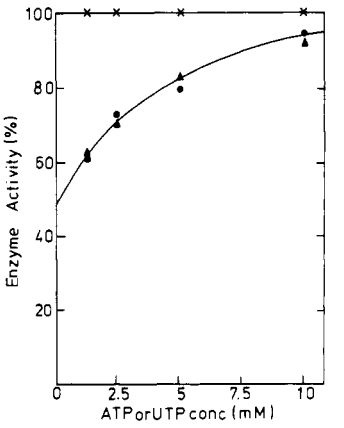


FIGURE 4: Protection of the α-fo^{5}UTP/$NaBH_4$ inhibition of RNA polymerase with ATP and UTP. The enzyme (53 μg) was preincubated in a reaction mixture as described in Materials and Methods containing (a) 0.5 mM α-fo^{5}UTP and varying concentrations of UTP (- ▲ - ▲) or ATP (- ● - ● -), (b) H_2O and varying concentrations of ATP or UTP (- x- x-). The mixture was incubated at 37 °C for 5 min prior to $NaBH_4$ treatment.

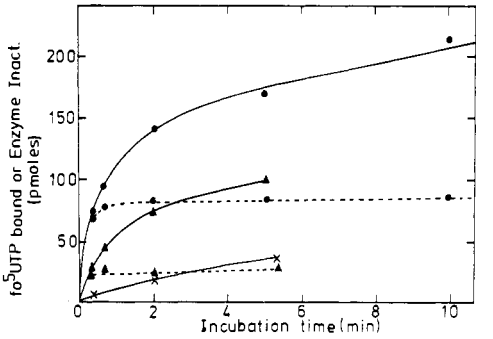


FIGURE 5. Stoichiometry of binding of [γ-^{32}P]-α-fo^{5}UTP to RNA polymerase after $NaBH_4$ reduction. The solid lines (—) represent the amount of α-fo^{5}UTP bound and the broken lines (- - -) the amount of enzyme inactivated. The enzyme (67.5 μg) was preincubated at 37 °C in a reaction mixture (0.1 ml) containing 40 mM Tris-HCl, pH 8.0, 0.05 M KCl and (a) (- ● - ● -) 8 mM $MgCl_2$, 0.5 mM α-fo^{5}UTP, (b) (- ▲ - ▲ -) 0.5 mM α-fo^{5}UTP, and (c) (- x - x -) 0.5 mM α-fo^{5}UTP, 10 mM UTP. After various times at 37 °C the mixtures were treated with 0.1 ml of 100 mM $NaBH_4$ solution and left at 0 °C for 15 min. Ten-microliter aliquots were then assayed as in Materials and Methods. In order to determine the amount of [γ-^{32}P]-α-fo^{5}UTP bound to the enzyme, 75-μl aliquots were removed and diluted with 1 ml of wash buffer (40 mM Tris, pH 8.0, 8.0 mM $MgCl_2$). After incubation at 37 °C for 10 min the mixture was filtered over a nitrocellulose disk, the disk washed with 30 ml of wash buffer and dried under an ir lamp, and the radioactivity determined in 10 ml of scintillation solution.

Effect of UTP on the Binding of [γ-^{32}P]-α-fo^{5}UTP. The effect of UTP on the binding of α-fo^{5}UTP is shown in Figure 5. The presence of 10 mM UTP in the α-fo^{5}UTP-enzyme incubation mixture caused a significant reduction in the binding of α-fo^{5}UTP. After a 20-s incubation period less than 5 pmol was bound, and after 2 and 5 min the values were 20 and 32 pmol, respectively.

Effect of Rifampicin on the Binding of [γ-^{32}P]-α-fo^{5}UTP. The introduction of rifampicin into the preincubation mixture of α-fo^{5}UTP and enzyme caused a reduction in the amount of α-fo^{5}UTP bound (Table II) in the presence of rifampicin. However, in the absence of Mg^{2+} the rifampicin had relatively little effect on the binding.

Location of the [γ-^{32}P]-α-fo^{5}UTP Binding Site. The en-

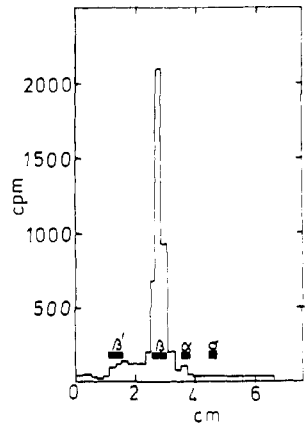


FIGURE 6: Cellulose acetate electrophoresis of RNA polymerase after labeling with [γ-^{32}P]-α-fo^{5}UTP. The reaction mixture contained, in 1.0 ml: 615 μg of enzyme, 0.5 mM [γ-^{32}P]-α-fo^{5}UTP, 40 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, 0.05 M KCl. After a 20-s incubation at 37 °C the mixture was treated with 0.5 ml of 100 mM $NaBH_4$ solution and left at 0 °C for 15 min. The mixture was then passed over a Sephadex G-100 column, equilibrated with 50 mM Tris, pH 8.0. The column was eluted with the same buffer and the enzyme containing fractions (detected by uv and radioactivity) which eluted with the exclusion volume were collected and concentrated in vacuo to approximately 100 μl. The enzyme was then subjected to electrophoresis in 6 M urea on cellulose acetate strips. After electrophoresis and destaining the sheets were cut into 2-mm strips and radioactivity was measured in Brays solution.

zyme was incubated with α-fo^{5}UTP for 20 s and then reduced with $NaBH_4$. After separation of the labeled enzyme from nucleotide by Sephadex G-100 chromatography the enzyme was subjected to cellulose acetate electrophoresis (Rabussay and Zillig, 1969) in order to divide it into its various subunits. After destaining, the cellulose acetate sheets were cut into strips and the radioactivity associated with the various subunits was determined. As can be seen from Figure 6 the majority of the label was found to run with the β subunit.

Discussion

The evidence from the results strongly suggests that α-fo^{5}UTP is inhibiting RNA polymerase by reaction with a critical amino group on the enzyme forming a Schiff base. The noncompetitive inhibition produced by a short preincubation with α-fo^{5}UTP would be expected if α-fo^{5}UTP is inhibiting the initiation site of RNA polymerase. However, the enzyme has been preincubated with α-fo^{5}UTP with the subsequent formation of a Schiff base. Although this formation is reversible, the equilibrium may still be in favor of the Schiff base, and in this case noncompetitive inhibition might also be expected even if the α-fo^{5}UTP is binding to the elongation site.

In order to investigate this further, particularly with regard to the site of inactivation, we studied the effect of α-fo^{5}UTP on the poly[d(A-T)]-directed pyrophosphate exchange reaction (Krakow and Frank, 1969). The α-fo^{5}UTP was as effective in the sodium borohydride induced inhibition of pyrophosphate exchange as it was in the inhibition of [^{14}C]AMP incorporation into poly[r(A-U)] in the polymerization assay. Inhibition of the pyrophosphate exchange has been suggested as evidence that AMPR-OP primarily inhibits the initiation of RNA chains (Wu and Wu, 1974). However, this does not necessarily presuppose that the inhibition is caused by binding of the derivative to the initiation site on the enzyme since binding at the elongation (polymerization) site would equally suppress formation of the first phosphodiester bond.

It was therefore of interest to investigate the interaction of α-fo^{5}UTP with the poly[d(A-T)] ternary complex consisting of holo enzyme/poly[d(A-T)] template/poly[r(A-U)] product, where the elongation process can be studied independently of initiation (Rhodes and Chamberlin, 1974). No inhibition of the ternary complex occurred at an α-fo^{5}UTP concentration which produced 45% inhibition of [^{14}C]AMP incorporation in the normal polymerization assay. However, α-fo^{5}UTP proved to be a competitive inhibitor at higher concentrations, with a K_i = 3.33 mM. This is consistent with the results of Rhodes and Chamberlin (1974) who showed that the elongation site has an equal affinity for all noncomplementary nucleoside triphosphates. The failure to obtain inhibition of the ternary complex after preincubation with 0.46 mM α-fo^{5}UTP may be interpreted as evidence that α-fo^{5}UTP acts on the initiation site. However, it is still possible that this occurs at the elongation site, but that in the ternary complex the amino group is now no longer available for Schiff base formation with α-fo^{5}UTP.

The α-fo^{5}UTP is a more effective inhibitor than its nucleoside, suggesting that inhibition is occurring at a triphosphate binding site. The protection studies with ATP, UTP, and GTP, all of which are equally effective in protecting against inhibition, further support this suggestion. The concentrations required for protection are high, 50% protection occurring with 4 mM triphosphate. However, this was determined for enzyme which was not actively synthesizing RNA. Rhodes and Chamberlin (1974) have shown that the elongation site in the ternary complex has a general affinity for noncomplementary nucleoside triphosphates, the association constants being of the order of 2 mM.

The inhibition due to α-fo^{5}U alone is still appreciable. In this context it is worth noting that the oxidation products of 6-methylthiopurine ribonucleoside (Nixon et al., 1972) and the corresponding *N*-(acetylaminoethyl)-1-naphthylamine-5-sulfonate (Wu and Wu, 1974), both lacking phosphate groups, effectively inhibit RNA polymerase by reaction with an amino group. Furthermore, the latter compound which contains a large apolar group is a far more potent inhibitor with a K_i 100 times lower than the former. It has also been shown (Bull et al., 1975) that pyridoxal phosphate inhibits RNA polymerase by reaction with an amino group with a very low pK_a, and it was suggested that this amino group is located in an apolar environment or near another cationic group. It appears likely that all of these aldehyde derivatives are inactivating RNA polymerase at the same amino group.

β-fo^{5}UTP and β-fo^{5}U are less effective in inhibiting RNA polymerase after $NaBH_4$ reduction. This may be due to the binding of these compounds to the enzyme which occurs in such a way that the NH_2 group is closer to the aldehyde group in the case of the α derivatives and thus can react more easily. The omission of Mg^{2+} from the incubation mixture causes a sharp reduction in the extent of inhibition by all four compounds. As this occurs with the nucleosides as well as the nucleotides it is unlikely that this is caused by the necessity of a Mg-formyl derivative complex for binding, but that the enzyme has undergone a conformational change preventing Schiff base formation.

The amount of α-fo^{5}UTP bound to RNA-polymerase is dependent upon the time of preincubation with the enzyme. After 20 s the stoichiometry is 1.1:1, which is what would be expected if α-fo^{5}UTP was binding to a unique site on RNA polymerase. With time the amount of bound α-fo^{5}UTP increases, although there is no corresponding increase in inactivation. This additional binding may represent the slow but effectively irreversible formation of Schiff base between α-fo^{5}UTP and amino groups not essential for enzyme activity.

It has been observed that 8 mol equiv of pyridoxal phosphate can bind to 1 mol equiv of enzyme (Venegas et al., 1973). In the presence of 10 mM UTP the binding of α-fo^{5}UTP during the first 20 s is virtually eliminated, providing further evidence that the inhibition is occurring at a triphosphate binding site. Even after 5 min the amount of α-fo^{5}UTP bound is much decreased. Similar results were obtained using 10 mM ATP (unpublished observations). Equilibrium dialysis and gel filtration experiments have indicated that there are several ATP binding sites on the enzyme (Ishikawa and Hurwitz, 1969). In the absence of Mg^{2+} in the incubation mixture the amount of α-fo^{5}UTP bound after 20 s is reduced by over two-thirds, but the stoichiometry of the inhibition remains 1:1.

The antibiotic rifampicin inhibits the initiation step in RNA polymerase although the mechanism of this inhibition is not yet fully understood. The presence of rifampicin caused a reduction in the amount of fo^{5}UTP bound in the presence of Mg^{2+}, whereas no effect was observed in the absence of Mg^{2+}. Thus, rifampicin has a direct effect on the binding of the α-fo^{5}UTP to the site at which inhibition occurs.

After a 20-s preincubation most of the α-fo^{5}UTP was located on the β subunit of the enzyme. This subunit has already been shown to be the site of rifampicin action (Zillig et al., 1970; Stender et al., 1975), and other affinity labeling studies (Frischauf and Scheit, 1973; Nixon et al., 1972; Wu and Wu, 1974) have implicated this subunit as containing at least part of the catalytic center of RNA polymerase.

The experiments described were all performed with holo enzyme. The inhibition of core enzyme by α-fo^{5}TP (data not shown) proved to be similar to that of holo enzyme, showing the same concentration dependence for inhibition and giving similar results for the stoichiometry of binding after various incubation times and subsequent sodium borohydride reduction. Thus σ factor has no influence on the binding of α-fo^{5}UTP to RNA polymerase.

The evidence presented does not allow a definite conclusion as to whether the inhibition is occurring at the initiation or elongation nucleoside triphosphate binding site. However, in view of the apparent purine specificity of the initiation site and the ability of UTP to protect the enzyme against α-fo^{5}UTP/$NaBH_4$ inhibition as effectively as ATP, it seems less likely that α-fo^{5}UTP is inhibiting at the initiation site.

In conclusion, the inhibition of RNA polymerase by the binding of α-fo^{5}UTP to the β subunit has been shown to satisfy several criteria for affinity labeling: (1) it forms a noncovalent complex prior to covalent attachment; (2) the inhibition and covalent attachment of α-fo^{5}UTP can be suppressed by the presence of nucleoside triphosphates; (3) the triphosphate α-fo^{5}UTP is a more potent inhibitor than the nucleoside α-fo^{5}U; and (4) the α-fo^{5}UTP stoichiometrically labels RNA-polymerase (after a 20 s preincubation).

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