

# Inhibition of the T7 DNA polymerase by insulin

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T7 DNA polymerase reduced insulin at the same  $K_m$  as thioredoxin, while the turnover number decreased. Recycling of the disulfide of thioredoxin subunit to its dithiol form was made by thioredoxin reductase. Incubation of T7 DNA polymerase with insulin decreases its ability to bind DNA and therefore inhibited polymerase and exonuclease activities. Thioredoxin reductase fully reversed this inhibition. Insulin did not induce dissociation of the T7 DNA polymerase subunits, which was tested by immunoadsorbent chromatography. No significant difference in single-stranded exonuclease compared to polymerase activity was seen in the flow through or the eluate, which had been expected if a dissociation of the subunits had occurred.

*T7 DNA polymerase*

*Thioredoxin*

*Thioredoxin reductase*

*Insulin*

## 1. INTRODUCTION

Upon bacteriophage T7 infection of *Escherichia coli* a novel DNA polymerase is induced. It consists of two subunits in a 1:1 ratio: the gene 5 protein ( $M_r$  79000) encoded for by the viral DNA and *E. coli* thioredoxin ( $M_r$  12000 [1,2]). The holoenzyme has besides its polymerase activity a 'proofreading' mechanism, a 3'-to-5' exonuclease activity on single- and double-stranded DNA [3,4]. We have reported the preparation of T7 DNA polymerase on immunoadsorbent chromatography as the main purification step [5,6].

The two subunits are firmly associated and separation demands denaturation of the enzyme in 6M guanidine hydrochloride and stepwise renaturation by dialysis of the separated subunits [4]. The gene 5 protein has in itself a 3'-to-5' exonuclease activity on single-stranded DNA [3]. Thioredoxin is a well-known protein, in several reactions demonstrated to participate as a reductant of protein disulfides in vitro [7]. Thioredoxin is specifically reduced by NADPH-thioredoxin reductase. The only metabolic role of thioredoxin that has been established by genetic studies is in T7 DNA replication.

So far, the nature of this host-virus relationship

has not been evaluated. The presence of thioredoxin with its known three-dimensional structure [8,9] as a component of the T7 DNA polymerase offers favorable possibilities for studying DNA synthesis and subunit interaction. This study has been directed towards finding out if the disulfide bridge in the thioredoxin subunit is within reach for reduction by NADPH-thioredoxin reductase and the extent to which a known substrate for free thioredoxin could interfere with the polymerase and exonuclease activities of the T7 DNA polymerase.

## 2. MATERIALS AND METHODS

Unlabeled T7 DNA and T7-[ $^3\text{H}$ ]DNA was prepared by infection of 1.2l of *E. coli* B/1 at  $A_{590} = 0.7$  nm with 3.5 T7 phages/*E. coli* cell. After 2 min, 2.5 mCi [methyl- $^3\text{H}$ ]thymidine was added. Growth was allowed to continue until full lysis of the cells had occurred. The phage solution was cooled on ice and solid NaCl was added to 2.5%. The phage particles were precipitated by 8% (w/v) polyethylene glycol 6000 and purified on a CsCl gradient [10]. The purified phages were dialyzed against 0.01 M Tris-HCl, (pH 7.5) 0.05 M NaCl/ 0.001 EDTA. Sodium dodecylsulfate (SDS)

was added to the sample to 0.5% (w/v) final conc. and the mixture was incubated for 10 min at 65°C. To remove the SDS-protein mixture from the DNA, 0.5 M KCl was added to the sample and it was cooled for 15 min on ice. The sample was centrifuged for 5 min at  $5000 \times g$  and the supernatant containing the purified DNA, was extensively dialyzed against 0.01 M Tris-HCl, (pH 7.5)/0.1 M NaCl/0.001 M EDTA. The DNA was free from contaminating protein according to spectrophotometric analysis [11]. Denatured DNA was made in a boiling water bath for 50 s, followed by rapid cooling on ice.

T7 DNA polymerase was purified to homogeneity as in [5,6]. Thioredoxin reductase from *E. coli* was prepared as in [12]. Bovine insulin (26.1 units/mg) was purchased from Vitrum (Stockholm). Solutions of insulin were prepared as in [13].

### 2.1. Enzyme assays

DNA polymerase activity was determined as in [14] with some modifications. The assay mixture contained 0.1 M Tris-HCl (pH 7.5) 0.3 mg/ml bovine serum albumin / 0.15 mM dATP, dCTP, dGTP and [ $^3\text{H}$ ]dTTP / (3 cpm/pmol) / 10 mM  $\text{MgCl}_2$  / 0.5 mM (calculated as phosphorus equivalents) of heat-denatured salmon sperm DNA. Enzyme was added to give 0.15 ml total vol. The incubation of the sample and termination of the reaction were as in [5]. Exonuclease activity was determined in an incubation mixture containing 0.1 M Tris-HCl, (pH 7.5)/ 10 mM  $\text{MgCl}_2$  / 29  $\mu\text{M}$  native or heat-denatured T7[ $^3\text{H}$ ]DNA (7 cpm/pmol,  $M_r$   $25 \times 10^6$ ). The enzyme was added to give 0.1 ml final vol. After incubation for 10 min at 37°C the reaction was terminated by addition of 0.1 ml of salmon sperm DNA (1 mg/ml) immediately followed by 0.3 ml of 10% trichloroacetic acid. The acid-soluble radioactivity was collected after centrifugation of the samples for 10 min at  $10000 \times g$ . Reduction of insulin by thioredoxin was measured spectrophotometrically at 412 nm with 5,5'-dithiobis-(2-nitrobenzoic acid) as in [13].

### 2.2. Nitrocellulose filter binding assay

Filter binding assays were used to detect complexes between T7 DNA and T7 DNA polymerase, the measurements were made by a modified

method [15,16]. Enzyme was added to a mixture consisting of 0.1 M Tris-HCl (pH 7.5) / bovine serum albumin 40  $\mu\text{g/ml}$  / 0.17 nM native T7[ $^3\text{H}$ ]DNA (38 cpm/pmol) to give 50  $\mu\text{l}$  final vol. The samples were incubated for 10 min at 37°C, 0.45 ml 10 mM Tris-HCl (pH 7.5) was added to the sample and a 0.45 ml aliquot was immediately taken after mixing and transferred to nitrocellulose filter. The aliquots were gently suctioned through the filters followed by 2 washes with 1 ml 10 mM Tris-HCl, (pH 7.5) / 0.1 mM EDTA. The filters were dried and immersed in a Permablend-toluene scintillation mixture.

### 2.3. Immunoabsorbent chromatography

The same procedure used for the purification of the enzyme [6], was also used for the chromatography of the T7 DNA polymerase. Anti-thioredoxin-Sepharose, 0.5 ml was equilibrated with 20 mM Tris-HCl (pH 7.5) / 10% glycerol / 0.15 M NaCl / 0.1 mM EDTA. After the sample was applied, the column was washed with 2 ml of the equilibrating buffer. Elution of the bound enzyme was made by 0.1 M glycine (pH 12.0) / 10% glycerol / 0.1 M NaCl/0.1 mM EDTA. Fractions of 0.5 ml were collected and immediately neutralized with 0.125 ml 1 M Tris-HCl (pH 7.0) / 0.02 M dithiothreitol.

### 2.4. Sources

dATP, dCTP, dGTP, dTTP and NADPH were purchased from Sigma. d[ $^3\text{H}$ ]TTP and [methyl- $^3\text{H}$ ]thymidine were from Amersham, (Bucks). Nitrocellulose filters 0.45  $\mu\text{m}$ , BA 85 were from Schleicher and Schull. Polyethylene glycol 6000 was from Kebo Grave (Stockholm).

## 3. RESULTS

### 3.1. T7 DNA polymerase catalyzes the reduction of insulin

The disulfide bridges in insulin are reduced by the thioredoxin system with a  $K_m$  of 11  $\mu\text{M}$  [13]. When associated with the gene 5 protein thioredoxin reduces insulin (fig. 1) with a  $K_m$  of 14  $\mu\text{M}$ . The turnover number for free and associated thioredoxin is 170 and 140  $\text{min}^{-1}$ , respectively. Recycling of the disulfide of thioredoxin to its dithiol form is necessary, as only catalytic amounts of T7 DNA polymerase are present (68 nM). The

disulfide bridge on thioredoxin must therefore be within reach both for the NADPH-thioredoxin reductase and the substrate, insulin. Without

cofactor and thioredoxin reductase present in the assay there is no measurable reduction of disulfides in the insulin molecule.

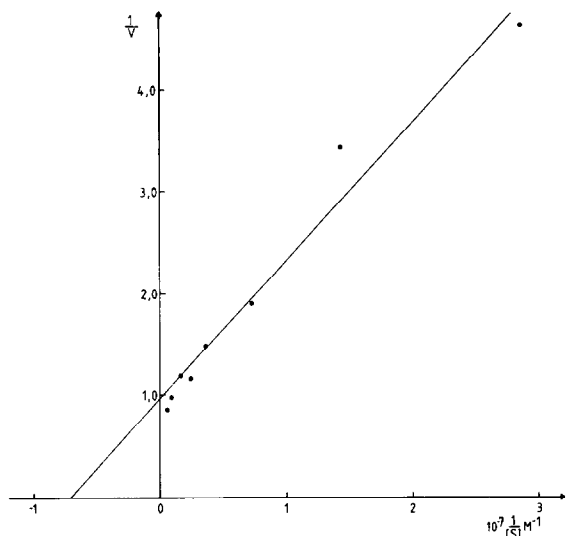


Fig. 1. Lineweaver-Burk plot of the reduction of insulin. Velocity is the increase in absorbance measured at 412 nm as in section 2. The points are the mean of 3 different experiments with duplicate samples in each. The line is derived by regression analysis. The correlation coefficient is 0.81,  $K_m$  for the reaction is  $13.9 \mu\text{M}$ .

### 3.2. Insulin acts as an inhibitor of T7 DNA polymerase

When T7 DNA polymerase is preincubated with low concentrations of insulin, its activities are inhibited (table 1). The binding of the enzyme to DNA is essential for polymerase and exonuclease activities. The decrease in the capacity for the T7 DNA polymerase to bind DNA is comparable to the decrease in activities. Thioredoxin reductase and NADPH can apparently reverse the inhibition of insulin. The enzyme even appears to activate T7 DNA polymerase to a certain extent. The increased binding of DNA in the filter binding assay may depend on the fact that thioredoxin reductase seems to have a certain ability to bind DNA in itself (not shown).

### 3.3. The inhibition is not due to dissociation of the subunits

The possibility that insulin inhibition of the enzyme depends on dissociation of the subunits was assayed by immunoadsorbent chromatography. T7 DNA polymerase ( $0.4 \mu\text{M}$ ) was incubated with  $0.2 \text{ mM}$  insulin in  $0.5 \text{ ml}$  for  $20 \text{ min}$  on ice. The enzyme was totally inactivated as measured by the

Table 1  
Inhibition of T7 DNA polymerase by insulin (activity as % of control)

	Control	Insulin	Insulin + NADPH-thioredoxin red.	NADPH-thioredoxin red.
DNA polymerase activity	100	48	105	96
Double-stranded DNA exonuclease activity	100	64	156	113
Single-stranded DNA exonuclease activity	100	49	185	117
DNA retained on nitro-cellulose filter	100	47	240	222

Insulin was  $0.2 \text{ mM}$ , thioredoxin reductase  $0.7 \mu\text{g/ml}$  and NADPH  $12 \text{ mM}$ . T7 DNA polymerase used here was freed from reductant by a buffer exchange to  $0.05 \text{ M}$  Tris-HCl (pH 7.5)/  $0.2 \text{ M}$  NaCl/  $0.001 \text{ M}$  EDTA saturated with argon on a Sepharose G-25 column. Prior to application to the column the sample was incubated for  $15 \text{ min}$  with  $5 \text{ mM}$  dithiothreitol to assure that the enzyme was fully reduced. The enzyme was inhibited by insulin in a  $0.02 \text{ ml}$  preincubation mixture in the above buffer. Immediately after the  $10 \text{ min}$  preincubation the appropriate assay mixture was added and the sample was incubated as in section 2.1.

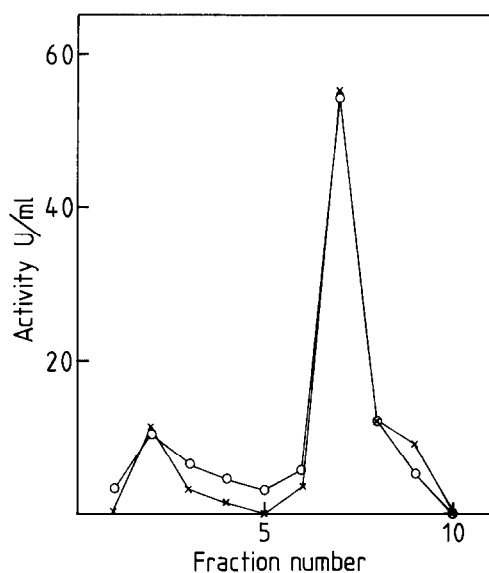


Fig. 2. Immunoabsorbent chromatography of T7 DNA polymerase inhibited by insulin. For details see section 2. (○—○) DNA polymerase activity; (×—×) single-stranded DNA exonuclease activity. Units as defined in [5].

DNA polymerase assay. The inactivated sample was applied to the immunoabsorbent column directly after incubation and treated as in section 2. The fractions were measured for polymerase and single-stranded DNA exonuclease activities, with 2 mM dithiothreitol included in the assay (fig. 2). No difference in the activities can be seen. The small leakage from the column (15% of total units) is seen also on a control column and is uniform for both activities. If insulin should induce a dissociation of the subunits, an increase in exonuclease activity compared to the polymerase activity would be expected in flow-through fraction.

#### 4. DISCUSSION

These results describe the capability of insulin to inhibit the T7 DNA polymerase activity and the reversibility of this inhibition by thioredoxin reductase and NADPH. The inhibition of the T7 DNA polymerase activities by insulin is not temperature-dependent over 0–37°C (unpublished). There is no difference in the reactivation of the polymerase if thioredoxin reductase and NADPH are added to the incubation at the same time as in-

sulin or after the preincubation period. Insulin could possibly react with the T7 DNA polymerase in two ways either with the thioredoxin subunit or with sulfhydryl groups on the gene 5 protein. Thioredoxin reductase specificity for thioredoxin [7,17] excludes the second possibility. The single-stranded DNA exonuclease activity is also inhibited by insulin. The filter binding assay shows that the decrease in the exonuclease activity correlates with the inability of the polymerase to bind DNA. The DNA binding site in the gene 5 protein that gives activity on single-stranded DNA is not independent of thioredoxin. The small difference in  $K_m$  between free and gene 5 protein-associated thioredoxin implies that the disulfide bridge in thioredoxin is within reach both by insulin and by the NADPH-thioredoxin reductase and that steric hindrance from the gene 5 protein must be very limited. Although the lowered turnover number shows that the reaction is not as fast as with free thioredoxin.

Oxidized thioredoxin does not compete with the reduced form for association with the gene 5 protein [18]. The immunoabsorbent chromatography experiment and inhibition of single-stranded DNA exonuclease activity is not contradictory to this, but shows that the enzyme does not dissociate into its subunits when thioredoxin is oxidized by insulin. This is further supported by the extreme conditions that have to be used to dissociate the subunits [4].

The conclusion of these experiments is that thioredoxin reduces insulin while associated to the gene 5 protein. In the absence of thioredoxin reductase and NADPH or any other reductant thioredoxin stays in the oxidized state and is thus not a functional subunit, although it is still bound to the gene 5 protein.

Stimulation of the T7 DNA polymerase activities by thioredoxin reductase and NADPH is probably due to the presence of a fractional amount of oxidized thioredoxin under the conditions we perform the experiments. Whether thioredoxin reductase does indeed take an active part in the DNA synthesis has to be further investigated.

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