INVOLVEMENT OF HIGHLY REACTIVE SULFHYDRYL GROUPS IN THE ACTION OF RNA POLYMERASE FROM *E. COLI*

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1. Introduction

Ishihama and Hurwitz [1] have shown that the DNA dependent RNA polymerase from *E. coli* is inhibited by sulfhydryl blocking reagents. Evidence was obtained that SH blocking reagents abolished the binding of the enzyme to DNA and also inhibited the initiation and elongation steps. In the present paper it is shown that the sulfhydryl groups of RNA polymerase from *E. coli* is effectively and reversibly inhibited by cystamine which reacts specifically with highly reactive sulfhydryl groups. The core enzyme is completely inhibited when 12 sulfhydryl groups are blocked. Association of the enzyme to the template, as well as the formation of the initiation complex, strongly reduces the reactivity of the sulfhydryl groups of the enzyme.

2. Materials and methods

2.1. *Enzyme preparation*

The enzyme was isolated from *E. coli* K12 growing in mineral glucose medium and harvested in the late log phase. The core enzyme was prepared and kept in storage buffer at -20° as described by Burgess [2]. Immediately before use the enzyme was passed through a short Sephadex G-25 column, equilibrated with Tris buffer containing 50 mM KCl, 5 mM $MgCl_2$ and 0.05 mM EDTA.

After polyacrylamide gel electrophoresis the characteristic subunit pattern of RNA polymerase was found, indicating that no contaminating protein was present. The specific activity was 450 units per mg protein when measured with calf thymus as template under the conditions given by Burgess [2]. Protein was determined according to Lowry et al. [3] as well as from the absorption at 280 nm, using $E^{1\%}_{1\,cm} = 6.5$ [4].

2.2. *Enzyme assay*

The assay mixture contained in a total volume of 0.2 ml: 40 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 100 mM KCl, 0.25 mM of GTP, CTP, and UTP, 0.25 mM ^{14}C-ATP (spec. act. 2 mCi/mmole) 100 μg calf thymus DNA and 10 μg RNA-polymerase. The mixture was incubated for 10 min at 37°, chilled in ice and precipitated with 5% TCA containing 10 mM Na-pyrophosphate. The precipitate was collected on Millipore filters previously soaked in 5% TCA and washed with 50 ml of 2% icecold TCA containing 10 mM Na-pyrophosphate. The filters were dried, placed in the scintillation solution (4.0 g 2.5 diphenyloxazole (PPO), 0.05 g 1.4-bis-(5-phenyloxazolyl-2) benzene (POPOP) in 1000 ml toluene) and counted in a Beckman scintillation system.

2.3. *Chemicals*

^{35}S-cystamine (RSSR), ^{14}C-ATP, and ^{3}H-UTP were obtained from The Radiochemical Centre, Amersham, England. Highly polymerized calf thymus DNA, unlabelled nucleoside triphosphates and unlabelled cystamine were obtained from Sigma Chemical Co., St. Louis, Mo, USA. Dithiothreitol (Cleland's reagent) was purchased from CalBioChem, Luzern, Switzerland.

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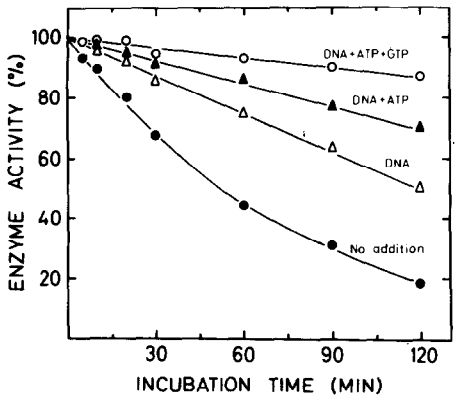

Fig. 1. Inhibition of RNA polymerase by cystamine. Native enzyme and enzyme pre-treated with DNA and purine nucleotides were incubated at 4° with cystamine and after different periods of time samples were taken for enzyme assay. The enzyme template complexes were formed by pre-incubating 100 μg of the enzyme for 2 min at 37° in a buffer containing 10 mM Tris-CHl, pH 7.9 (measured at 25°), 5 mM $MgCl_2$, 50 mM KCl, 0.05 mM EDTA, and DNA (500 μg) and nucleotides (0.4 mM) as indicated. The samples were then chilled, cystamine (5 mM) was added and the incubation was continued at 4° (final volume 2 ml).

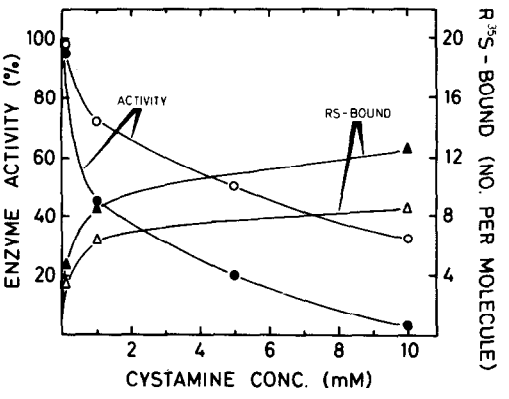

Fig. 2. Effect of cystamine concentration on the blocking of enzyme SH groups and on the enzyme activity. Native enzyme (250 μg) or enzyme pre-treated with DNA (250 μg enzyme plus 500 μg calf thymus DNA) were incubated in a total volume of 2 ml of buffer (same as in fig. 1) with increasing concentrations of ^{35}S-labelled cystamine at 4° for 120 min. The number of cysteamine residues bound was determined by measuring the radioactivity after dialysis of the samples (24 hr at 4° against 10 mM Tris-HCl buffer, pH 7.9 (measured at 25°) containing 50 mM $MgCl_2$, 50 mM KCl, and 0.05 M EDTA). 1 ml aliquots were added to 10 ml of Unisolve (Koch-Light Ltd.) and the radioactivity was measured. Conditions for assay of the enzyme were as in Materials and methods, except that ^{3}H-UTP was used as label. Closed symbols, native enzyme; open symbols, enzyme preincubated with DNA.

3. Results and discussion

In the present study the weakly electrophilic agent cystamine was used to characterize the sulfhydryl groups of RNA polymerase. Since this disulfide has a low reactivity it can be used to detect small differences in the reactivity of sulfhydryl groups [5].

The data in fig. 1 show the inhibition of the RNA polymerase by cystamine under different conditions, as a function of the incubation time. It is seen that when the core enzyme was incubated with cystamine alone the activity initially decreased nearly linearly with time. The rate subsequently declined and the activity reached about 20% after 120 min. Since similar results were obtained with the holo-enzyme the subsequent experiments were carried out with the core enzyme which is obtained in a more pure state. When the enzyme was preincubated with calf thymus DNA for 2 min at 37° prior to addition of cystamine to permit the enzyme template complex to be formed, the initial rate of inactivation was reduced by a factor of almost 3.

It is known that purine nucleoside triphosphates, due to the formation of the first phosphodiester bonds, will stabilize the DNA-enzyme complex against dissociation at high ionic strength [6]. It is seen from fig. 1 that such stabilization results in further protection of the enzyme against inhibition by cystamine. On preincubation of the enzyme with DNA, ATP and GTP the initial rate of inactivation was reduced by a factor of 11, compared to that of the control. When GTP was omitted from the preincubation mixture an intermediate degree of protection was found. In all instances addition of cystamine or Cleland's reagent completely restored the activity (data not shown).

To study the role of the different sulfhydryl groups the RNA polymerase was incubated with increasing concentrations of labelled cystamine and the enzyme activity and the concomitant binding of cysteamine residues were measured (fig. 2). In the absence of DNA (closed symbols) 4 sulfhydryl groups were blocked at

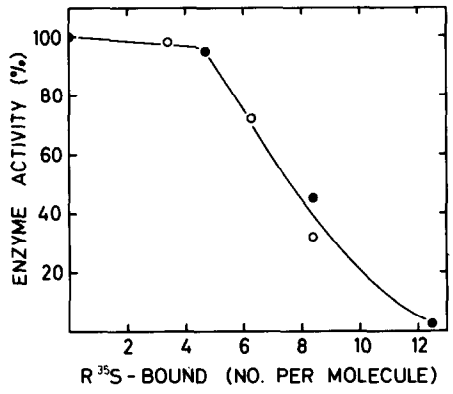

Fig. 3. The relationship between the number of sulfhydryl groups blocked and the enzyme activity. Data taken from fig. 2.

low (0.1 mM) cystamine concentration. At higher concentrations four additional sulfhydryl groups reacted with a concomitant rapid decline in the enzyme activity. When the enzyme was incubated with 10 mM cystamine, altogether 12 SH groups were blocked with complete loss of the enzyme activity.

When the enzyme was preincubated with DNA before the addition of cystamine (open symbols), similar curves were obtained. However, for each concentration of cystamine the number of sulfhydryl groups blocked and the concurrent enzyme inhibition was smaller than in the control.

The relationship between blocking of sulfhydryl groups and enzyme inactivation is more readily apparent from fig. 3. The curve clearly reveals that blocking of four sulfhydryl groups had little or no effect on the enzyme activity, and that on further blocking the enzyme activity decreased nearly linearly until no activity remained when altogether 12 sulfhydryl groups had reacted. The results indicate that the four most reactive sulfhydryl groups (fig. 2) are not essential for the catalytic activity of the enzyme.

The results in fig. 3 also demonstrate that the relationship between the enzyme activity and the number of sulfhydryl groups blocked is the same for the free enzyme and enzyme pre-incubated with DNA. Hence, the smaller inhibition observed by a certain cystamine concentration in the presence of DNA must be due to a lower extent of sulfhydryl blocking. This could mean that some of the sulfhydryl groups were unavailable after binding of the enzyme to DNA, or alternatively, that the binding of DNA reduced the reactivity of all the enzyme SH groups. The data in fig. 2 suggest that the latter is the case, since with increasing cystamine concentration the relative reduction in the binding of cysteamine residues in the presence of DNA appears to be the same.

For steric reasons it appears unlikely that all the enzyme sulfhydryl groups were covered by the template. The data therefore suggest that somehow the binding to DNA induces conformational changes in the enzyme, rendering its SH groups less reactive. The possibility should be considered that the sulfhydryl groups are involved in maintaining the quaternary structure of the enzyme and that treatment with sulfhydryl blocking agents will loosen the binding of the subunits. Conceivably, the binding of DNA may aid in maintaining the native structure of the enzyme.

Acknowledgement

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