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INHIBITION OF E. COLI RNA POLYMERASE BY Fab FRAGMENTS FROM SUBUNIT SPECIFIC ANTIBODIES

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Summary: Fab fragments from antibodies directed against the subunits of E. coli RNA polymerase were prepared. They were used to study the inhibition of RNA synthesis and of DNA-binding. It was found that Fab fragments from antibodies against the β, β', and σ subunit, and against holo enzyme, inhibit RNA synthesis, but to different extents, while anti α Fab fragments do not inhibit enzyme acticity. DNA-binding is reduced only in the presence of anti σ and anti holo Fab fragments. The inhibition of RNA synthesis by anti σ Fab fragments can be neutralized if the enzyme is incubated with DNA before adding the Fab fragment. The anti β and anti β' Fab fragments still inhibit the enzyme under these conditions. It is concluded that these Fab fragments directly inhibit the catalytic steps in RNA synthesis, whereas the inhibition by anti σ and anti holo Fab fragments originates from a reduced accessibility to the enzyme of the template in the presence of these Fab fragments.

INTRODUCTION

RNA polymerase from E. coli consists of four different subunits ($\alpha_2\beta\beta'\sigma$). The specific contribution of each subunit to the intricate process of DNA-dependent RNA synthesis still cannot be described unambigously in all cases (1-4). Genetics has been a powerful tool, introducing mutations specifically into subunits and investigating the properties of the mutant enzyme. The properties of a subunit may also be changed specifically by antibodies directed against it. It has been found that antisera against the subunits α, β, and β' inhibit RNA synthesis to different degrees (5). More recently, monovalent Fab fragments have been employed in inhibition (6). However, the previous study focused mainly on immunological comparisons between the enzymes from two different bacteria, one of which was E. coli.

In my study, I used Fab fragments from antibodies directed against the isolated E. coli RNA polymerase subunits α, β, β',

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σ, and against the complete enzyme to investigate the inhibition of RNA synthesis and the influence on the binding of DNA to the enzyme. Furthermore, it is expected that the sequence of addition of Fab fragments or DNA might allow distinction between the contributions of the subunits to DNA-binding and involvement in catalysis.

MATERIALS AND METHODS

Chemicals. Poly[d(A-T)], ATP, and UTP were purchased from Boehringer(Mannheim). [^{3}H]ATP (22 Ci/mmol) was obtained from Amersham-Buchler(Braunschweig) and [^{14}C]poly[d(A-T)] from P·L Biochemicals(St. Goar).

Enzymes. E. coli RNA polymerase holo enzyme was prepared according to Burgess & Jendrisak (7) with a heparin-sepharose chromatography after the DEAE-step according to Sternbach et al.(8). The specific activity on calf thymus DNA was 7500 units/mg. One unit gives the rate of GMP incorporation of 1 nmol/h at 37°. Papain was purchased from Boehringer (Mannheim) and desoxyribonuclease I (RNase free) from Worthington.

Antibodies and Fab fragments. The antibodies against the subunits α, β, β', and σ were produced in rabbits and purified by protein A-sepharose chromatography as described previously (9,10). The papain cleavage was performed in 0.01 M sodium acetate buffer (pH 5.5) in the presence of 0.01 M cysteine and 0.002 M EDTA using 1 μg papain per 100 μg IgG-fraction (11). After exhaustive dialysis against 0.01 M phosphate buffered saline (pH 7.2) containing 0.15 M NaCl, under vigorous stirring, the Fab fragments were purified on protein A-sepharose. To this material only Fc fragments are bound. Fab fragments from the flow through during elution with 0.1 M glycine-HCl (pH 3.0) were dialyzed against phosphate buffer, precipitated by ammonium sulfate and dissolved and dialyzed again with phosphate buffer. The final preparation no longer precipitates RNA polymerase. The protein concentration was determined by ultraviolet absorption measurements using an $E_{280}^{0.1\%}$ of 1.4.

RNA synthesis. 50 μl of the assay mixture (in 40 mM Tris-HCl(pH 7.9), 4 mM $MgCl_2$, 1mM $MnCl_2$, 12 mM β-mercaptoethanol and 0.4 mM K-phosphate) contained 0.75 μg RNA polymerase holo enzyme. The concentration of poly[d(A-T)] was 0.38 mM (nucleotides) and of the substrates ATP and UTP 1 mM each. The assay contained about 500,000 cpm per 50 μl.

In the first protocol, RNA polymerase was incubated for 10 minutes at 37° with the respective Fab fragment. After addition of template and substrates incubation was continued for 60 minutes. 5 μl of DNase I (100 μg/ml in 100 mM $MgCl_2$) was added and the solution incubated for 5 minutes at 37°. Finally, 5 μl of 0.1 M EDTA (pH 7.9) was added and 10 μl samples were spotted on 20 cm x 20 cm polyethyleneimine thin layer sheets (Schleicher & Schüll, Dassel) which had been prespotted on the origin with EDTA and sodium pyrophosphate (0.05 M each) and dried. Ascending chromatography was performed with 2 M LiCl, 0.01 M EDTA (pH 6.5)(12) until the front reached the end of the sheet. After drying, the origins were cut out, and radioactivity was measured in toluene-based scintillation fluid.

The second protocol differed from the first only in that the enzyme was first incubated with poly[d(A-T)]. There were no indications of abortive formation of short oligonucleotides which should have been detected by scanning the distribution of radioactivity on the chromatogram (12).

Nitrocellulose filter binding assay. The assay was performed according to Strauss at al.(13). 1.5 μg RNA polymerase holo enzyme were incubated with and without 35 μg Fab fragment for 10 minutes at 37°, and 0.053 pmol [^{14}C] poly[d(A-T)] were added. After an additional incubation for 20 minutes at

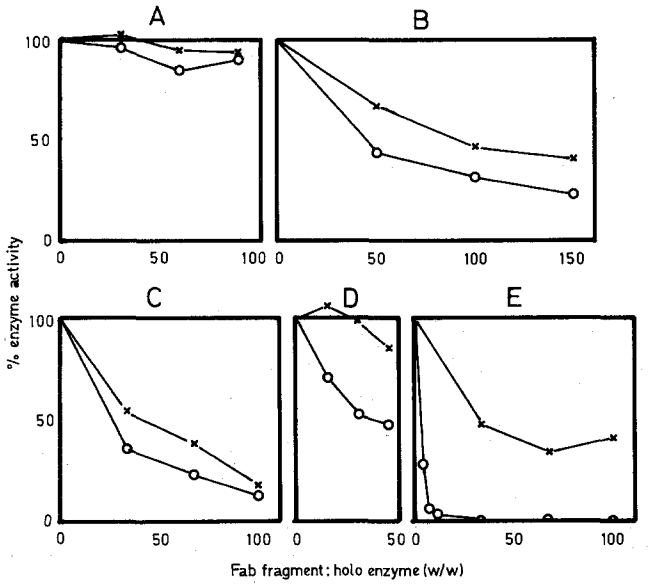

Figure 1: Inhibition of RNA synthesis by Fab fragments from A: anti α, B: anti β, C: anti β', D: anti σ, E: anti holo. The order of incubation was: enzyme - Fab fragment - DNA and substrates (o); enzyme - DNA - Fab fragment - substrates (x).

37°, the solution was diluted and filtered on Millipore filter type HA. The filter was dried and radioactivity was counted in a toluene-based scintillation fluid. Under these conditions about 50 % of the radioactivity applied was retained on the filter in the absence of Fab fragment.

RESULTS

Since I expected the inhibition of RNA polymerase by the Fab fragments to be dependent on the order of addition of DNA and Fab fragments, I used two different protocols for the inhibition of poly[d(A-T)]-directed RNA synthesis. In the first run of experiments RNA polymerase holo enzyme was incubated with increasing amounts of Fab fragments. Then template and substrates were added simultaneously and RNA synthesis measured. Fig. 1 shows the extent of inhibition by each Fab fragment. In the second run, holo enzyme was incubated with the template first, then with an Fab fragment, and finally RNA synthesis was started by adding substrates. The pattern of inhibition does not change very much for α, β, and β'. In the case of σ and holo, however, the inhibition is remarkably reduced.

In order to see whether the inhibition measured in the first protocol is due to a decrease in the DNA-binding capacity of

Table 1: Inhibition of DNA-binding activity of RNA polymerase holo enzyme in percent of the control without Fab fragment.

Fab fragment from	%
anti α	98.4
anti β	103.0
anti β'	114.0
anti σ	71.4
anti holo	81.3
control serum	96.0

the enzyme after it has bound with the respective Fab fragment, holo enzyme-Fab fragment complexes were incubated with radioactive poly[d(A-T)]. The results in table 1 fully correspond to the data from the inhibition experiments. DNA-binding is reduced in the presence of anti σ and anti holo Fab fragments. Fab fragments from a control IgG preparation do not inhibit RNA synthesis nor DNA-binding. Table 2 shows a compilation of all experiments where the complementarity of the data from inhibition and from DNA-binding can be recognized.

DISCUSSION

The interaction between enzymes and their respective antibodies in general leads to a reduction in enzyme activity. The extent of inhibition may vary considerably from partial to complete, and in some cases there is no inhibition at all or even stimulation. The inhibition is very often the result of steric hindrance of substrate access. In such cases, the extent of inhibition is related to the size of the substrate. Another factor involves conformational changes imposed on the enzyme by its interaction with the antibody. Thus, antibodies combining outside the catalytic site can affect the integrity of the cataly-

Table 2: Summary of the inhibition data from figure 1 and table 1.

Fab fragment from	Inhibition of RNA synthesis protocol 1	protocol 2	Inhibition of DNA-binding
anti α	-	-	-
anti β	+	+	-
anti β'	+	+	-
anti σ	+	-	+
anti holo	++	+	+
control serum	-	-	-

tic center. On the other hand, there is no convincing data of antibody being elicited by the catalytic site of any enzyme (for a discussion of all aspects of enzyme inhibition by antibodies see (14) and (15)).

Both types of inhibition seem to be demonstrated in this study. First, the anti α Fab fragments seem to be an example of binding but not inhibiting antibodies (Fig. 1 A). Binding of anti α has been demonstrated unequivocally by complement fixation (9) and by electron microscopy (unpublished results). On the other hand, from the inhibition data alone one cannot deduce that α is without any importance for enzymatic activity. A possible explanation is that the functional site of the α subunit is not accessible to the corresponding antibodies. There are now three studies (including the one presented here) where it has been found that anti α either does not inhibit (the present one and (6))or inhibits only very slightly (5). The weak inhibition found in the last investigation may be the consequence of precipitation since complete antisera were used. It may be that α does not function directly in the catalysis of RNA synthesis or in DNA-binding (which is also not inhibited by the anti α Fab fragment, table 1) but serves to maintain an active conformation of the enzyme. This interpretation is supported by the existence of two α subunit mutants one of which is assembly defective, and in the other an altered structure of the assembled enzyme is suggested (16).

The Fab fragments of both anti β and anti β' inhibit RNA synthesis considerably and to a similar extent. This is found whether the enzyme combines with the Fab fragment before adding DNA or the reverse sequence of addition is performed, although in the latter case a small relief from inhibition is observed (Fig. 1 B,C). Furthermore, the binding of DNA to the enzyme is not inhibited (table 1). The Fab fragments may inhibit RNA synthesis directly by affecting the catalysis of RNA synthesis; β and β' have been identified as constituting the catalytic site of RNA polymerase.

Antibodies against the σ subunit have not been used previously in inhibition studies. RNA synthesis is inhibited if the enzyme combines with the Fab fragment before adding DNA, but this inhibition is largely relieved if the enzyme is bound to DNA

first. Furthermore, the anti σ Fab fragment is the only one (apart from the anti holo Fab fragments) that reduces the ability of the enzyme to bind DNA. Since this reduction amounts to only 30 % whereas the reduction in enzymatic activity is 50 %, one must assume that after adding Fab fragment some DNA can still bind but is probably not correctly aligned.

Inhibition of RNA synthesis and DNA-binding by anti holo Fab fragments appears to be a combination of the effects seen with the individual Fab fragments. Inhibition of holo enzyme incubated simultaneously with anti β and anti β' Fab fragments indicates a cumulative effect (data not shown). The relief of inhibition when incubation with DNA precedes the binding of Fab fragments is essentially a property of the anti σ Fab fragment.

One of the features characterizing the inhibition of most enzymes by their respective antibodies is the residual activity persisting in antibody excess (14). In this respect, the results of this study show that there are typical differences among the subunits. 100 % inhibition (i.e. zero residual activity) is obtained at very low concentrations of anti holo Fab fragments. For the anti β and anti β' Fab fragments there is a steady decrease in activity at increasing concentrations of Fab fragments and a low or even zero level of activity is reached, but at much higher concentrations of Fab fragments. The anti σ Fab fragments leave substantial residual activity (about 40 %), and the anti α Fab fragments do not inhibit at all.

The results presented here confirm the importance of the integrity of β and β' and their direct involvement in catalysis. If one accepts the inhibition by anti σ Fab fragments to be the result of reduced DNA-binding, the residual activity may be the consequence of a uniformly reduced DNA-binding capacity of the σ subunit, either by conformational change or steric hindrance. This interpretation does not consider the role of σ in promoter site selection although DNA-binding is an important aspect of this process. For such investigations the system described here could be applied to isolated promoters.

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