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LIMITED DIGESTION OF RNA POLYMERASE FROM ESCHERICHIA COLI BY TRYPSIN (EFFECT OF RIFAMYCIN AND DNA ON THE INTEGRITY OF SIGMA SUBUNIT)

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A modified polyacrylamide gel electrophoresis technique is used to separate the polypeptides after digestion of E. coli RNA polymerase with various concentration of trypsin. The subunits β and β' and two large breakdown products of molecular weight of 147,000 and 141,000 are distinctly separated. At a very low level of trypsin σ and α are not cleaved while two major breakdown products of molecular weights of 110,000 and 43,000 appear from the larger subunits. At a still higher level of trypsin σ is converted to a polypeptide of molecular weight of 86,000 and other small fragments. DNA protects, to some extent, the σ and this polypeptide and also β and the two large breakdown products from trypsin digestion. It is also observed that rifamycin, an inhibitor of RNA synthesis, enhances the tryptic digestion of σ, only in the absence of $MgCl_2$.

INTRODUCTION

E. coli DNA-dependent RNA polymerase (ribonucleoside 5'-triphosphate:RNA nucleotidyl transferase, EC 2.7.7.6) is an oligomeric protein consisting of four core subunits $\alpha_2\beta\beta'$ (1). The subunit σ interacts catalytically with the core enzyme and confers specificity to the enzyme in recognizing the promoter site on the DNA template (2). The functions of the individual subunits of this enzyme have been studied extensively (3-6) yet their topographical arrangement is not well established.

Limited proteolysis is a simple technique which could be applied to study indirectly the association of subunits or their interaction with template or other components. The rate of hydrolysis of the subunits of RNA polymerase and its polymerizing activity during hydrolysis have been reported (7).Lill and

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Hartmann (8) proposed a tentative model for the protomer-protomer interaction based on the susceptibility of the subunits to proteolytic digestion. Recently Fisher and Blumenthal (9) applying this method have presented evidence in support of β and σ association in the holoenzyme.

Studies using proteolysis as a probe to investigate the quaternary structure of RNA polymerase have produced conflicting conclusions mainly because of the lack of resolution of β and β' subunits on sodium dodecyl sulphate polyacrylamide gel. In this investigation we have used a modified polyacrylamide gel system which allowed a distinct separation of β and β' subunits and the high molecular weight breakdown products. By using limited tryptic cleavage technique we have observed two high molecular weight polypeptide fragments not observed before. We also show that σ subunit is hydrolyzed to polypeptide of mol. wt of 86,000 and its breakdown is reduced by the presence of DNA. The effect of rifamycin - an inhibitor of RNA chain initiation - on the holoenzyme suggests that there is an enhanced breakdown of subunit in the absence of Mg^{2+}.

MATERIALS AND METHODS

RNA polymerase was isolated from E coli K12 cells (Grain Processing Corporation, Iowa) following the procedure of Burgess and Jendrisak (10). The enzyme was further purified on an Ultrogel AcA 34 (LKB) column (1.1 x 60 cm) equilibrated with TG'ED buffer (0.01 M Tris-HCl pH 7.9, 0.1 mM EDTA, 0.1 mM dithio - threitol , 20% glycerol). One ml of RNA polymerase in storage buffer (50% glycerol, 0.1 M NaCl, 0.01 M Tris-HCl pH 7.9, 0.1 mM EDTA , 0.1 mM dithiothreitol) containing 2.7 mg protein was applied on the column. The column was eluted with equilibrating buffer. The holoenzyme appeared in the flow - through fraction. The peak A_{280} absorbing material was pooled and dialyzed against storage buffer and kept at -20°C. This enzyme was more than 95% pure as judged by polyacrylamide gel electrophoresis. The enzyme fraction obtained from Biogel A-5m step (10) was used for the isolation of core enzyme according to an established procedure of Burgess and Jendrisak (10). The peak fractions of core and σ subunit were pooled, dialyzed against storage buffer and kept at -20°C.

The activity of the purified enzyme was assayed in a 50 µl reaction mixture by measuring the incorporation of [8-^{14}C]ATP

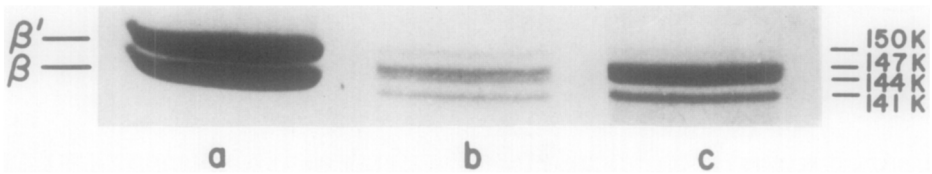

Fig. 1 Effect of trypsin on β and β' subunits. Fifty microliter sample containing 9 µg purified holoenzyme with or without 7.5 µg DNA was incubated in incubation buffer (0.05 M Tris-HCl pH 7.9, 0.1 mM EDTA, 0.01 M $MgCl_2$, 0.1 mM dithiothreitol, 15% glycerol (v/v), 0.05 M KCl) at 37°C for 30 min. with 2.7 µg of trypsin. The reaction was stopped by boiling for 2 min. with equal volume of 2X sample buffer and electrophoresed on 7.5% polyacrylamide gel as described under "Materials and Methods". In this figure only the ββ' region is taken from a regular gel and magnified. a) RNA polymerase, b) RNA polymerase cleaved with trypsin, c) RNA polymerase.DNA complex cleaved with trypsin. The molecular weights of the four polypeptides obtained from a standard plot are as follows: β' =150,000, β'a =147,000, β =144,000 and βa =141,000.

(New England Nuclear) into acid insoluble RNA using calf thymus DNA (Sigma Chemical Co) as template (11).

Sodium dodecyl sulphate discontinuous polyacrylamide gel was run as described by Sarma and Chatterjee (12) with some modifications. Several Bis (N,N'-Methylene-bis acrylamide) to acrylamide ratios were tested and a ratio of 1:86 was used. The gels were stained with 0.05% coomassie brilliant blue and destained overnight.

Proteolysis was carried out on 9-18 µg of purified holo- or core enzyme in an incubation mixture of 50 µl containing 0.05 M Tris-HCl pH 7.9, 0.05 M KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol and 15% glycerol with or without 7.5 µg calf thymus DNA. RNA polymerase in the above mixture was incubated at room temperature for 10 min and trypsin (Sigma Chemical Co) at a concentration of 0.01 to 0.37 parts by weight was added. The mixture was then incubated at 37°C for 30 min. In experiments where rifamycin was added, 5 µl of solution containing 22.5 µg Rifamycin SV in water was added and incubated at room temperature for 10 min prior to trypsin addition. The reaction was terminated by adding equal volume of 2X sample buffer (0.08 M Tris-HCl pH 6.8, 0.1 M dithiothreirol, 2% sodium dodecyl sulphate, 10% glycerol, 0.02% bromophenol blue) followed by boiling for 2 min and analyzed on 7.5% polyacrylamide gel.

RESULTS AND DISCUSSION

In our modified gel system there is adequate separation between β and β' subunits and their high molecular weight tryptic cleavage products (Fig. 1). We have investigated the rate of subunit breakdown in the presence and absence of DNA with a wide range of trypsin concentrations. At the lower limit of trypsin α

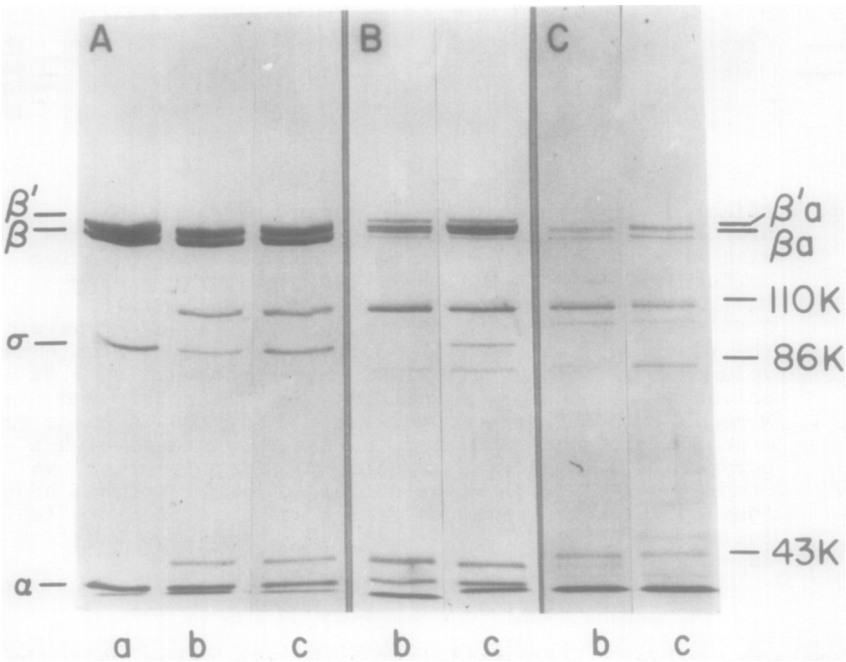

Fig. 2 Effect of various concentration of trypsin on RNA polymerase. Procedure for trypsin digestion is described under figure 1. Concentration of trypsin per 50 µl assay mixture was 0.1 µg for panel A, 1.0 µg for panel B and 3.0 µg for panel C. a) RNA polymerase, b) RNA polymerase cleaved with trypsin, c) RNA polymerase.DNA complex cleaved with trypsin.

and σ subunits are not digested (Fig. 2A) while they were completely cleaved at the higher level used (Fig. 2C). In all our experiments we do not observe any preferential digestion of β and β subunits in the absence of DNA as suggested by Lecocq (13) and Blumenthal (9). However, in the presence of DNA the rate of breakdown of β is slower than that of β' (Figs. 1 and 2).

As shown in figure 1, four high molecular weight polypeptides are separated at the top of the gel. The molecular weights of β'a and βa are derived from a standard plot of mobility vs. log of molecular weight of several marker proteins. The fragment designated β'a which is slightly larger than β appear rapidly in significant amount during proteolysis. A faint band of mol. wt of 147,000 however, is found in RNA polymerase stored at -20°C at a concentration lower than 1 mg/ml in storage buffer; the band of

mol. wt of 141,000 is not apparent in this sample. RNA polymerase solutions which are stored at a concentration of 2-4 mg/ml do not show any band of mol. wt of 147,000 even before the final purification step. The appearence of this faint band in dilute solution strongly suggests that it is generated from β' subunit. It is also known that one of the major breakdown products (polypeptide of mol. wt of 110,000 in our experiment) is sometimes present in very small quantity in the preparation of RNA polymerase. We have also used RNA polymerase obtained from Dr. S. A. Kumar (N. Y. State Division of Laboratories and Research) which is stored at a protein concentration higher than 2 mg/ml and does not show any band of mol. wt of 147,000 (not shown). This sample when treated with trypsin produces identical pattern of breakdown products as we observe. The polypeptide βa is a minor product of trypsin digestion. The molecular weight of this fragment is fairly high and it does not exhibit any significant change in its amount with increasing level of trypsin while β', β'a and β are progressively degraded. Further cleavage of βa, however, is reduced by DNA. DNA also protects β'a but β' is never protected. This indicates that a small segment (mol. wt of about 3000) at one end of β' is always exposed to the surface. The rate of breakdown of β subunit on the other hand is reduced when complexed with DNA as is evidenced by its accumulation. In addition to β'a and βa two other major polypeptides of mol. wt of 110,000 and 43,000 are generated from the high molecular weight subunits (Fig. 2). These two proteolytic products are probably the same as β_1 and β_2 of King et al (7) and the "indigestible core" of Lowe and Malcolm (14).

The subunit σ is known to be required for the recognition of promoter site on the DNA tamplate (2). When the holoenzyme is treated with trypsin σ is digested very rapidly (8). Isolated σ is shown to produce a series of major fragments with a relatively

resistant peptide of mol. wt of 40,000 at high level of trypsin (15). We also observe a high rate of breakdown of σ in the holoenzyme (Fig. 2). In our experiment a minor band of mol. wt of 86,000 is produced along with the proteolysis of σ. This poly - peptide, however, is not observed when isolated σ subunit is digested. Preincubation of the holoenzyme with DNA restores the σ subunit to some extent and also reduces further degradation of this minor polypeptide. It is observed that when core enzyme is treated with trypsin in presence or absence of DNA the polypeptede moving in the region of σ and the fragment of mol. wt of 86,000 are missing (not shown). This observation suggests that these two polypeptides are σ and its breakdown products and are not derived from β and β' subunits. To strengthen this observation holoenzyme is reconstituted from core and σ and subjected to trypsin digestion. Sigma subunit is again digested by trypsin and protected somewhat by DNA while the polypeptide of mol. wt of 86,000 reappears. Even though this is a very strong indirect evidence in favour of the origin of polypeptide of mol. wt of 86,000 from σ subunit one has to perform partial peptide mapping to obtain more definitive evidence. Simpson (16) has shown by photochemical cross-linking of RNA polymerase and promoter DNA that σ subunit is in close proximity of the template . Our observation that DNA reduces the breakdown of σ is in agreement with this observation.

Rifamycin - an inhibitor of RNA synthesis - has been used to study the association of RNA polymerase subunits during its reconstitution (17). Rifamycin forms a tight complex with RNA polymerase (18). Therefore, if this interaction produces a major conformational change in the molecule, peptide breakdown products could be altered. To test this possibility RNA polymerase is complexed with saturated concentration of rifamycin and subjected

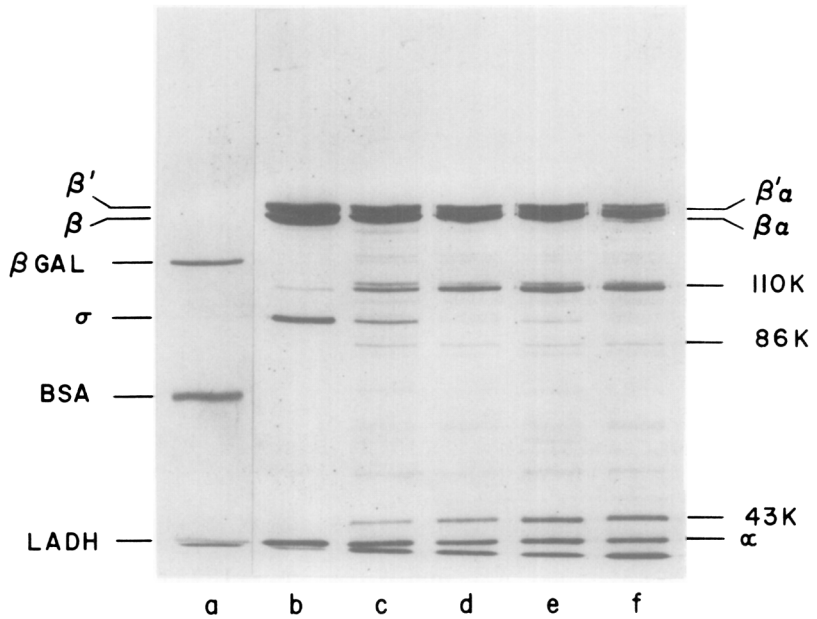

Fig. 3 Effect of Rifamycin SV on trypsin digestion of RNA polymerase. Nine microgram of purified RNA polymerase in 50 µl incubation buffer without $MgCl_2$ was digested with two concentrations of trypsin in presence or absence of 22.5 µg of Rifamycin SV as described under figure 1. a) Standard protein markers, 0.62 µg of LADH (Horse liver alcohol dehydrogenase) mol. wt=40,000, 0.62 µg of βGAL (E. coli βgalactosidase) mol. wt=116,000, 0.75 µg of BSA (Bovine serum albumin) mol. wt=67,000, b) RNA polymerase, c) RNA polymerase digested with 0.5 µg trypsin in absence of rifamycin, d) same as c) except 22.5 µg of Rifamycin SV was present, e) RNA polymeras digested with 0.75 µg trypsin in absence of rifamycin, f) same as e) except 22.5 µg of Rifamycin SV was present.

to trypsin digestion. As shown in figure 3. rifamycin does not produce any significant change in the fragments of high molecular weight. However, at low level of trypsin and only in the absence of magnesium ion breakdown of σ is enhanced by rifamycin. The effect of magnesium ion in this respect is not very clear. In our investigation absence of any distinctive difference in the proteolytic pattern with rifamycin does not indicate absence of conformational perturbation; it merely suggests that the effect is not extensive enough to produce any major spatial rearrangement. Further research is in progress to establish a definitive

precursor-product relationship between the subunits and the major breakdown products.

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