Resolution and reconstitution of the rec BC deoxyribonuclease of *Escherichia coli*

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Received 12 September 1983

The inactivation of rec BC nuclease activity and simultaneously the separation of 3 DNA-dependent ATPases and an ATP-independent DNases specific for single-stranded DNA have been observed after DEAE-cellulose chromatography of cell extracts from *Escherichia coli*. Two of the ATPases catalyze the strand separation of duplex DNA. Reconstitution of ATP-dependent DNase activity has been carried out by the combination of the separated enzymes.

Recombination *Unwinding* *ATPase* *DNase*

1. INTRODUCTION

Exonuclease V is the product of rec B and rec C genes [1] and plays an essential role as the 'recombination enzyme' in *Escherichia coli* [2]. Among the several catalytic properties of this ATP-dependent DNase are double-stranded and single-stranded exonucleolytic activities [3–5], single-stranded endonucleolytic activity [5] and DNA-dependent ATPase activities [3–6]. Moreover, the DNase reaction is accompanied by conformational changes of DNA [7].

The inactivation of ATP-dependent DNase activity during DEAE-cellulose chromatography [4,8–10] indicates the chromatographic dissociation of ATP-dependent DNase. Stoichiometry studies on ATP-dependent DNase reveal that ATPase and DNase reactions are not stringently coupled [5,11–13] suggesting either the existence of a protein with more than one active site [14] responsible for independent and separable functions or the presence of a multienzyme system that serves as a functional unit consisting of separable enzymes closely associated with DNA.

Our previous results with separated enzymes from *Bacillus cereus* indicate the ATP-dependent cooperation of separated DNA-stimulated ATPase and DNase [15]. We followed the inactivation of rec BC nuclease by simultaneously detecting both DNA-dependent ATPase and ATP-dependent DNase activities after DEAE-cellulose chromatography of cell extracts from *E. coli*. We here present evidence for the reconstitution of rec BC DNase activity by the combination of separated enzymes.

2. MATERIALS AND METHODS

2.1. *Buffers*

Buffer A contained 20 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 2 mM 2-mercaptoethanol and 30% (v/v) glycerol. Saline–sodium citrate buffer contained 150 mM NaCl, 15 mM sodium citrate (pH 7.0).

2.2. *Bacteria*

The *E. coli* strain described in [16] W 3110 thy^-, pol Al, which lacks polymerase was used. The other *E. coli* mutant MC 1–7 F^-, thr, arg^-m, Sm^R, lac^-, Bl^- was provided by J. Hofemeister Zen-

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tralinstitut für Genetik und Kulturpflanzenforschung, GDR. Cells were grown at 37°C in a Gallenkamp rotary incubator in 9 l of nutrient broth (Difco) supplemented with 10 μg/ml thymine. Bacteria were harvested in exponential phase at $3\text{-}5 \times 10^8$ cells/ml and stored at −20°C until further use.

2.3. *Materials and radioactive compounds*

Micrococcal nuclease was purchased from Boehringer, bovine serum albumin and Norit A from Serva, thymine from Merck and ATP from Calbiochem. [γ-^{32}P]ATP (200 Ci/mol) was prepared as in [17]. [^{3}H]DNA from *B. cereus 130 thy*$^-$ was isolated as in [18]. T7 DNA was prepared by the method in [19]. Heat denaturation of DNA was carried out at 100°C for 10 min in saline-sodium citrate buffer. Protein was determined as in [20], using bovine serum albumin as standard. Preparation of cell extracts and chromatography on DEAE-cellulose were the same as in [15]. DNA-dependent ATPase, ATP-dependent DNase and DNA unwinding activities were detected as in [15].

3. RESULTS

The properties of ATP-dependent DNase isolated from *E. coli* W 3110 were similar to those of rec BC enzymes described by others [3-6]. The enzyme had a pH optimum between 7-10 with a maximum at 9.0. Mg^{2+} was required, and maximal DNase activity was detected at 10 mM concentration of Mg^{2+}. The degradation of double-stranded DNA was stimulated 4-fold in the presence of ATP at concentrations between 0.2 and 4 mM (fig.1). The DNase activity on denatured substrate was higher than on duplex DNA. The degradation of denatured DNA was not influenced by the addition of ATP.

The distribution of DNA-dependent ATPase as well as the DNase activity was detected after elution of cell extract on DEAE-cellulose column (fig.2). Three DNA-dependent ATPases were eluted at 0.18, 0.24 and 0.37 M NaCl concentrations, denoted as ATPase I, II and III, respectively (fig.2A). ATPase I represents about 55%, ATPase II 40%, and ATPase III 5% of the total DNA-stimulated phosphohydrolytic activity. DNase activity eluted in a single peak at 0.31 M NaCl concentration (fig.2B). DNase activity detected in the

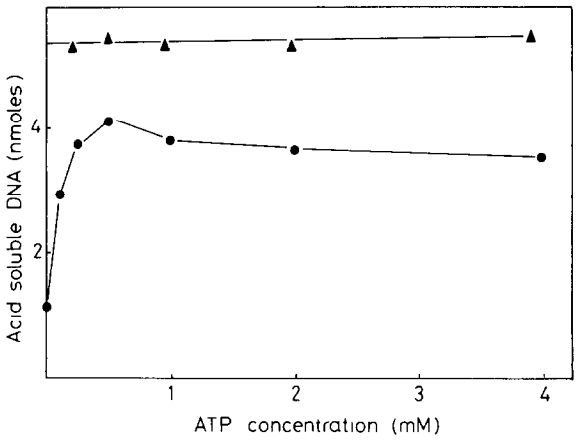

Fig.1. ATP-dependent DNase activity as a function of ATP concentration. The reaction mixture (0.15 ml) contained 10 nmol (in nucleotide equivalent) *B. cereus* [^{3}H]DNA, 1.5 nmol $MgCl_2$, 7.5 μmol Tris-HCl (pH 8.0) and 50% ammonium sulfate precipitate of ATP-dependent DNase. The frozen cells of *E. coli* were disrupted and the debris was removed by high speed centrifugation (110 000 × *g* for 2 h). The supernatant was first precipitated with streptomycin sulfate, then precipitated by 40% saturation with ammonium sulfate and, finally, the precipitate of the 50% ammonium sulfate saturation was dissolved in buffer A. ATP concentration changed as indicated. DNase activity in the presence of double-stranded DNA (●), and in the presence of denatured DNA (▲).

presence of either double-stranded or single-stranded substrate was not stimulated by ATP. Similar experiments were carried out with rec BC DNase from *E. coli* MC 1-7 strain and the chromatographic profiles that were obtained after DEAE-cellulose chromatography were the same as those shown in fig.2.

Active fractions of ATPase I (5-15, pool I) and ATPase II (21-32, pool II) were combined and dialyzed against buffer A. Peak fractions of DNase specific for single-stranded DNA (38-51) were also combined and dialyzed against buffer A (pool III).

Strand separation by ATPase I and by ATPase II was measured by following the generation, in the presence of ATP, of single-stranded DNA from duplex substrates in a micrococcal nuclease digestion assay. The basal activity of micrococcal nuclease on duplex DNA was stimulated about 5-fold in the presence of either ATPase I or

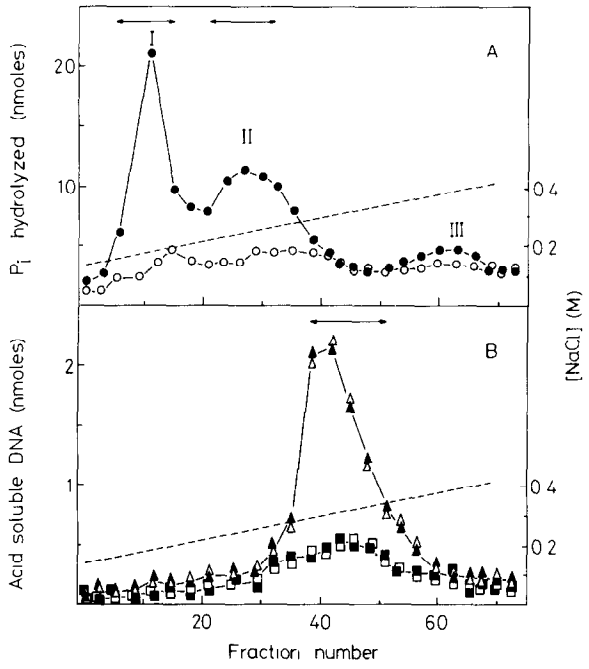

Fig.2. DEAE-cellulose chromatography of rec BC nuclease. The 50% ammonium sulfate precipitate containing 25 mg protein was loaded onto a DEAE-cellulose column (2.5 $cm^2 \times 2$ cm) equilibrated with buffer A. After washing with 30 ml of buffer A the proteins were eluted with a linear gradient of 0.15–0.40 M NaCl in buffer A (160 ml total volume). Two-ml fractions were collected. (A) ATPase activities were measured in the absence (○), and in the presence (●) of T7 DNA. (– – –) NaCl concentration gradient. (B) DNase activity on double-stranded substrate in the absence (□), and in the presence (■) of ATP. DNase activity on single-stranded DNA in the absence (△), and in the presence (▲) of ATP.

ATPase II (fig.3). Neither ATPase I nor ATPase II showed DNase activity by itself. The optimal concentration of ATP for the stimulation of DNA unwinding was 25 μM.

The reconstitution of ATP-dependent DNase activity was carried out by the combination of ATPase I (pool I) and of single-strand specific DNase (pool III) (fig.4). In the same way, ATP-dependent DNase activity was recovered by mixing ATPase II (pool II) and DNase (pool III) (fig.4). Single-strand specific DNase degraded double-stranded DNA slowly in an ATP-independent manner, while in the presence of either ATPase I or ATPase II an ATP-dependent hydrolysis of

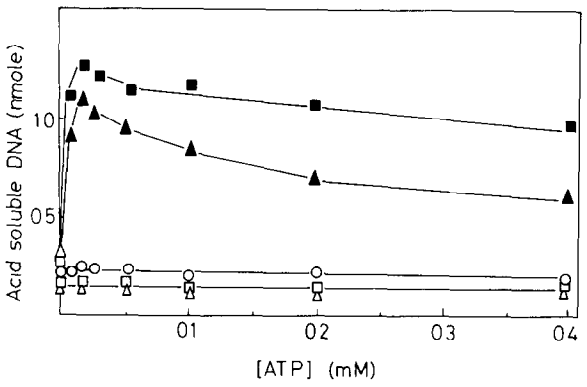

Fig.3. Effect of ATP concentration on micrococcal nuclease activity stimulated either with ATPase I or with ATPase II from *E. coli* W 3110. A standard micrococcal nuclease assay [15] was carried out by the addition of the indicated concentration of ATP in the absence of ATPases (○). As substrate 10 nmol double-stranded [^{3}H]DNA was used. DNase activity of ATPase I (pool I) without micrococcal nuclease (△), DNase activity of ATPase II (pool II) without micrococcal nuclease (□). DNase activity in the presence of 5 munits micrococcal nuclease and 30 units of ATPase I (pool I) (▲), DNase activity in the presence of 5 munits micrococcal nuclease and 30 units of ATPase II (pool II) (■). One unit represents the amount of DNA-dependent ATPase which degrades 1 nmol of ATP to ADP and P_i in 10 min, under defined conditions [15].

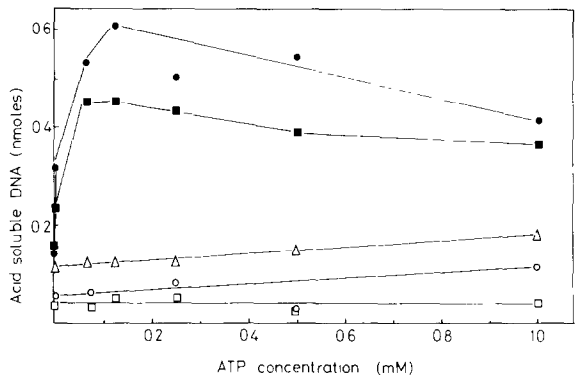

Fig.4. Reconstitution of ATP-dependent DNase activity. ATPase I (pool I), ATPase II (pool II) and single-strand-specific DNase (pool III) were dialyzed fractions of the DEAE-cellulose chromatography. Standard ATP-dependent DNase assay was carried out using *B. cereus* [^{3}H]DNA as double-stranded substrate. DNase activity of ATPase I (□), of ATPase II (○) and of single-strand-specific DNase (△). Stimulation of DNase specific for single-stranded DNA by ATPase I (■) and by ATPase II (●). ATP concentration changed as indicated.

double-stranded DNA occurred. The optimal concentration of DNA hydrolysis was 0.1 mM ATP in the reaction of the reconstituted ATP-dependent DNase.

4. DISCUSSION

Our studies with cell extracts from *E. coli* W 3110 and from *E. coli* MC 1-7 that were obtained by fractionation with ammonium sulfate precipitation confirm previous reports that ATP is required for the hydrolysis of linear duplex DNA [3-6, 12] in the ATP-dependent DNase reaction. DNase activity of the 50% ammonium sulfate precipitate was not inhibited at higher concentrations of ATP (1-4 mM). Chromatography of cell extracts of rec BC DNase on a DEAE-cellulose column using high chromatographic resolution resulted in the inactivation of ATP-dependent DNase activity. The unexplained inactivation of ATP-dependent DNase activity was also observed by others in this step of purification [4,8,10,21]. The resolution of DEAE-cellulose chromatography was characterized by the volume of elution buffer required to increase the NaCl concentration by 0.1 M on 1 ml gel. At high chromatographic resolution (10-12 ml caused 0.1 M increase in NaCl concentration on 1 ml gel) DNA-dependent ATPase activity eluted in two major and one minor peaks. DNase specific for single-stranded DNA, devoid of ATPase activity eluted between ATPase II and III and was not stimulated by ATP.

Three DNA-dependent ATPases catalyzing strand separation of duplex DNA have been described in *E. coli* [22-24]. Three DNA-dependent ATPases have also been found in *Ps. aeruginosa* [21], *B. subtilis* [25] and in *B. cereus* [26]. Two of the *B. cereus* ATPases were proved to be unwinding enzymes [15,27]. The unwinding by ATPase II from *B. cereus* was inhibited at higher ATP concentrations. Here the inhibition of ATPase I and II from *E. coli* was also detected at high concentration of ATP (fig.3). The analogy between unwinding and ATP-dependent DNase reactions is confirmed by the fact that the purified ATP-dependent DNase is also inhibited by a relatively high (500 μM) ATP concentration [3,5,6]. We found that ATP-dependent DNase in crude extracts was not sensitive to the increase of ATP concentration. However, the ATP-dependent DNase activity reconstituted by purified ATPase I and single-strand specific DNase or by mixing purified ATPase II and separated DNase was in fact hindered at high ATP concentration.

Based on inactivation and reconstitution data rec BC nuclease activity appears to be the cooperation of at least two dissociable subunits rather than the property of a single multifunctional enzyme. One of the cooperative enzymes is possibly a DNA-unwinding ATPase that separates strands as it moves along the helix. This is consistent with the fact that rec BC enzyme produces DNA duplexes with long single-stranded tails in the early stage of the ATP-dependent DNase reaction [9]. In this early stage the amounts of low M_r material and the acid-soluble DNA are low. Therefore it is reasonable to assume that an unwinding reaction is occurring. The unwinding activity of *E. coli* rec BC enzyme has been documented in [28] and [29]. A strand separation also would explain the accumulation of long, single-stranded fragments [9,30] and give a possible explanation for the origin of aggressive fragments in the early stage of recombination. The reaction that follows is probably the nucleolytic attack of the partially unwinded duplex by the DNase specific for denatured DNA. However, in this case it is possible that the rec B and rec C genes of *E. coli* are responsible for different dissociable subunits. This would be of importance in elucidating the roles of various activities of rec BC in recombination and cell viability.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Health (4.01 5). We thank Miss E. Krizsan and Mrs Z. Nigovicz-Pal for technical assistance.

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