AN ATP-DEPENDENT DEOXYRIBONUCLEASE FROM ESCHERICHIA COLI WITH A POSSIBLE ROLE IN GENETIC RECOMBINATION

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Abstract.—A DNase which requires ATP for its function was purified from *E. coli* and some of the characteristics of the purified enzyme were studied. This enzyme activity could not be detected in certain (*recB* and *recC*) recombination deficient mutant strains. The possible role of the enzyme in genetic recombination is discussed.

It has been widely accepted that the molecular process of genetic recombination consists of several sequential steps which may include nuclease and repair enzyme actions. A few classes of recombination deficient mutants have been isolated from *E. coli*,[1-3] and there is physiological and genetical evidence that the genes impaired in these mutants code for enzymes (proteins) involved in the process of genetic recombination. Similar mutations have also been reported in the bacteriophages T4[4] and λ.[5, 6] There has been essentially no report on the isolation or characterization of possible recombination enzymes in bacteria, although Buttin and Wright[7] reported that in an extract of a certain *E. coli* recombination deficient strain (*recB21*), there is less of a DNase activity which can be stimulated by a mixture of deoxyribonucleoside triphosphates, than in the wild-type strain. In bacteriophage λ, at least two proteins (λ-exonuclease[8, 9] and β-protein[10]) were purified and are believed to be involved in λ-specific genetic recombination.

This paper reports the purification and characterization of an ATP-dependent nuclease from *E. coli*. The *recB* and *recC* group of recombination deficient mutant strains of *E. coli* lack any detectable activity of this enzyme.

Materials and Methods.—Bacterial strains: The following *E. coli* K12 strains, provided by Dr. B. Low, were used: (*1*) F^- strains: AB1157 (*rec*$^+$), AB2463 (*recA13*), AB2470 (*recB21*), AB3109 (*recB23*), and NH4033 (*recC22*). All of these strains have the following genetic markers in addition to the recombination mutations: *thr*$^-$, *leu*$^-$, *his*$^-$, *arg*$^-$, *thi*$^-$, *pro*$^-$, and *Sm*^{R}. (NH4033 differs only by having *his*$^+$ and *T6*^{R} markers.) (*2*) Hfr strains: KL16 (*rec*$^+$), KL168, (*recB21*), JC5084 (*recB73*), KL169 (*recC22*), and KL168-UV^R-3.

Preparation of P^{32}-labeled DNA: P^{32}-*E. coli* DNA was purified from *E. coli* B grown in nutrient broth (Difco) with P^{32}-phosphate (1–5 μc/ml) according to Saito and Miura,[11] followed by isopropanol precipitation as described by Marmur.[12] P^{32}-T4 and T7 DNA were purified by the method of Grossman *et al.*[13]

Enzyme assays: The DNase was assayed by measuring the acid-soluble product released from P^{32}-labeled DNA. The incubation mixture (0.5 ml) contained 12.5 μmoles of Tris-maleate buffer, pH 7.5, 3.5 μmoles of $MgCl_2$, 3 μmoles of 2-mercaptoethanol, 250 mμmoles of ATP, 15 mμmoles of P^{32}-labeled DNA, and 0.5 to 5.0 units of enzyme. (In order to obtain reproducible results, P^{32}-DNA was sheared by a Sorvall mechanical mixer to a mean molecular weight of 8×10^6 prior to use.) The reaction mixture was incubated for 20 min at 37° unless otherwise specified and the reaction was terminated by adding

0.1 ml of a carrier solution (DNA, 5 mg/ml and bovine serum albumin, 10 mg/ml) and 0.5 ml of cold 3.5% perchloric acid. After 5 min at 0°C, the resulting precipitate was removed by centrifugation (2000 × *g* for 5 min), all the supernatant fluid was transferred to a planchet, and after addition of one drop of 5 *N* KOH, the solution was evaporated to dryness. The radioactivity was measured by a gas flow counter (Nuclear-Chicago, model 4342). One unit of enzyme is defined as the amount catalyzing the conversion of 1 mμmole of DNA nucleotide equivalent to an acid-soluble product in 20 min at 37°.

Purification of the enzyme: All manipulations were carried out at 0 to 5°C, unless otherwise stated. (*1*) *Preparation of crude extract: E. coli* K12 strain AB1157 (*rec*$^+$), was grown in Difco antibiotic medium 3 at 37° and the cells were harvested in late log phase (8×10^8 cells/ml) after chilling the medium with ice. After washing the cells with TMS buffer (0.01 *M* Tris-HCl buffer, pH 7.8, 0.01 *M* $MgCl_2$, 0.002 *M* 2-mercaptoethanol, 0.0001 M EDTA), the cells (115 gm wet weight) were mixed with 10 ml of TMS buffer and 230 gm of acid-washed glass beads (Superbrite, type 100, 3M Co.) and were disrupted by shaking them in a cell mill (Edmund Bühler Co. Tübingen, Germany) with a cooling jacket for 15 min (5 min × 3 with 1-min intervals) at maximum speed. The disrupted cell-glass mixture was extracted repeatedly with TMS buffer. The combined cell extract (250 ml) was centrifuged at 27,000 × *g* for 10 min and the supernatant (Fraction I, 225 ml) was collected. (*2*) *Ultracentrifugation:* Fraction I was centrifuged at 82,000 × *g* (25,000 rpm) for 150 min using a Beckman SW 27.1 rotor and the supernatant (Fraction II, 206 ml) was collected. (*3*) *Streptomycin precipitation:* To 205 ml of Fraction II, 27.4 ml of streptomycin sulfate solution (5%) were added with constant stirring over a 10-min period. (The volume of streptomycin solution to be added was calculated as 30 μg of streptomycin sulfate to be added per OD unit at 260 mμ of the Fraction II.) After centrifugation at 12,000 × *g* for 10 min, the tightly packed precipitate was resuspended in 53 ml of TMS buffer which contained 0.6 *M* $(NH_4)_2SO_4$ and was stirred over 12 hr with a magnetic stirrer until the suspension became homogeneous. The resulting viscous suspension was then sonicated (Sonifier, Branson Co., 185-C) for 60 sec (output 90 watt). The solution was dialyzed against 20 volumes of TMS buffer containing 0.2 *M* $(NH_4)_2SO_4$ and twice against 20 volumes of TMS buffer (Fraction III, 60 ml). (*4*) *Autolysis:* To the 60 ml of Fraction III, Tris-HCl buffer (1 *M*, pH 7.8), and ATP (50 mM), were added to give a final concentration of 25 and 0.1 mM, respectively, and the solution was incubated at 30°C with gentle stirring. Every 2 hr, ATP was added in the same amounts as described above, and after 10 hr of incubation the solution was chilled. Centrifugation followed (27,000 × *g* for 10 min) and the supernatant was collected (Fraction IV, 58 ml). Incomplete autolysis causes poor separation of the enzyme at step (7). (*5*) *First* $(NH_4)_2SO_4$ *precipitation:* To Fraction IV, solid $(NH_4)_2SO_4$ (28 gm) was added to give 70% saturation, and the resulting precipitate was collected by centrifugation and resuspended in 13 ml of TMS buffer (Fraction V). (*6*) *Second* $(NH_4)_2SO_4$ *precipitation:* To Fraction V, solid $(NH_4)_2SO_4$ was added to give 50% saturation, and the precipitate was centrifuged and resuspended in 2.9 ml of TMS buffer and dialyzed against 500 volumes of TMS buffer (Fraction VI, 2.9 ml). (*7*) *Sucrose gradient centrifugation:* Fraction VI of 0.5 to 1.0 ml was layered on 36 ml of a linear (10–20%) sucrose gradient solution in TMS-II buffer (0.01 *M* Tris-HCl buffer, pH 7.8, 0.001 *M* $MgCl_2$, 0.0001 *M* EDTA, 0.002 *M* 2-mercaptoethanol, 0.05 *M* NaCl) in centrifuge tubes (2.54 × 8.89 cm) and centrifuged at 88,000 × *g* (26,000 rpm) for 28 hr using a Spinco SW 27.1 rotor. Two-milliliter fractions were collected from the bottom of the tubes, and the 5th to 8th fractions were pooled (Fraction VII). The purified enzyme loses 5–10% activity per day at 0°C.

Results: When a sonic extract of *E. coli* K12 was incubated with P^{32}-DNA it was found that if ATP was present there was a considerable release of radioactivity into the acid-soluble fraction (Fig. 1*A*). This result suggested the existence of a DNase in the cell extract which required ATP for its function. In order to explore the biological significance of the enzyme, its activity was assayed in various *E. coli* strains with mutations affecting either DNA synthesis or recom-

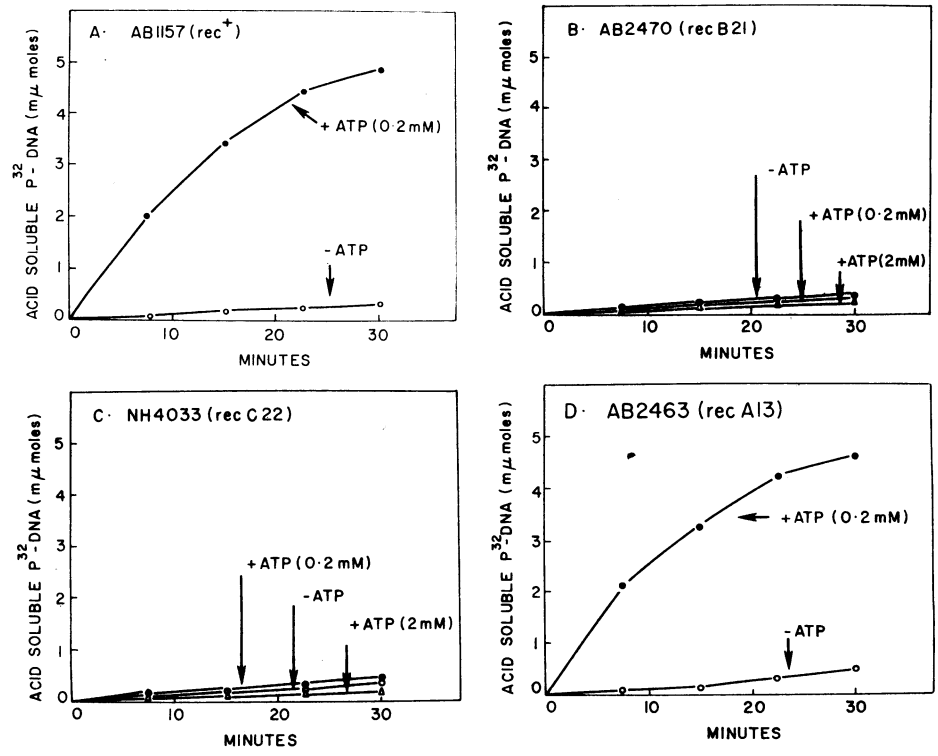

FIG. 1.—The degradation of P^{32}-DNA by cell-free extracts (with and without ATP) from various recombination deficient mutant strains. Cells grown in Difco antibiotic medium 3 (300 ml) were chilled and harvested by centrifugation at the middle of log growth phase (3–5 times 10^8 cells/ml). After washing once with TMS buffer, they were resuspended in the same buffer (3 ml), then were disrupted by sonication (Raytheon, model DF101, maximum power for 10 min). The supernatant fraction, after removing cellular debris (40,000 × *g*, 20 min), was dialyzed against 1000 volumes of TMS buffer overnight. The enzyme reaction, using this extract (1 mg protein/ml reaction mixture), and assay, are described in the *Materials and Methods* except that in this case incubation was carried out at 30°C. At various time intervals, 0.5-ml portions were withdrawn for assay.

bination. Among the strains tested, recombination deficient strains with mutations of either the *recB*(*recB21*) or *recC* (*recC22*) genes lacked any detectable level of this enzyme activity, as shown in Figures 1*B*, 1*C*. Another recombination deficient strain, with a mutation of the *recA* gene, (*recA13*), had almost the same level of the enzyme activity as the rec^+ strain (Fig. 1*D*). The *recB* and *recC* mutant strains are classified as a cautious type rec^- strain which display only limited (less than normal) DNA breakdown following ultraviolet or X-ray irradiation.[14, 15] The *recB* and *recC* mutations, which map very close to each other [15,16] and result in essentially identical physiological phenotypes, have been distinguished only by complementation.[17]

In order to verify the absence of the enzyme activity in *recB* and *recC* type mutants, two more independently isolated mutants (*recB23* and *recB73*) were also tested for the ATP-dependent DNase activity, and neither of them showed any detectable enzyme activity. The lack of the enzyme activity in *recB*(*recB21*)

and *recC(recC22)* mutants was also demonstrated in the extracts from different stages of the cell growth. The enzyme activity was also not detected in the Hfr strains with *recB* or *recC* mutations (KL168, *recB21* and KL169, *recC22*). However, a reversion of *recB21*, which now showed resistance to ultraviolet light (KL168-UV^R-3, kindly provided by Dr. B. Low), restores the DNase activity to the same level as found in the wild type. The possibility that extracts of *recB* and *recC* mutants contain an inhibitor of the enzyme seems unlikely because adding these extracts to that of the wild-type strain did not produce any significant inhibitory effect. All of the above results indicate that there is a strong correlation between the state of the *recB* and *recC* genes and the ATP-dependent DNase activity which is observed in extracts of *rec*$^+$ *E. coli*.

Purification of the enzyme: The ATP-dependent DNase was purified 200-fold from the extracts of the *rec*$^+$ strain (AB1157) as described in *Materials and Methods* (Table 1). The enzyme attacks the DNA in an exonucleolytic fashion. However, since the purified enzyme preparation (Fraction VII) contains a small amount of an endonuclease activity, the possibility cannot be excluded of an association of the two types of nucleases in the overall DNA degradation process. The detailed study of the enzyme action on DNA will be reported elsewhere. During the process of purification, the ATP-dependent DNase activity was separated from exonuclease-I[18], exonuclease-II[19] (DNA polymerase), and exonuclease III[20] and a DNase which attacks oligodeoxyribonucleotides.[21]

Properties of the enzyme: (*1*) *Substrate specificity:* DNA (native and heat-denatured) from *E. coli* and from bacteriophages T4 and T7, and RNA (ribosomal RNA) were tested for their susceptibility to the enzyme. As is shown in Table 2, native double-stranded DNA from different sources including glucosylated T4 DNA, all serve as good substrates for this enzyme. In every case, the reaction is completely dependent upon the presence of ATP. On the other hand, single-stranded DNA (except for T4 DNA) can be degraded to a small extent without ATP although ATP does have a weak stimulatory effect on the degradation. RNA does not serve as a substrate for this enzyme.

(*2*) *Requirement for metal ions and pH optimum:* Besides ATP this enzyme has an absolute requirement for either magnesium or manganese ions. Under the conditions described in *Materials and Methods*, maximum activity was observed at Mg^{++} concentrations ranging from 5×10^{-3} *M* to 2×10^{-2} *M*. At 1×10^{-3} *M* and 2×10^{-3} *M*, 47 and 68 per cent respectively, of maximum activity was

TABLE 1. *Purification of the enzyme.*

Fraction		Protein		Total activity (units)	Specific activity (units/mg protein)
		Total (mg)	mg/ml		
I	Crude extract	5,740	25.5	13,460	2.4
II	82,000 × *g*	4,120	20.0	12,670	3.1
III	Streptomycin	318	5.3	13,210	41.5
IV	Autolysis	267	4.6	12,990	48.6
V	Ammonium sulfate (first)	185	14.0	9,110	49.2
VI	Ammonium sulfate (second)	94	32.3	8,110	86.3
VII	Sucrose gradient	7.2	0.29	3,470*	481.7

* When assayed, 0.5–1% of sucrose was present in the reaction mixture.

TABLE 2. *Substrate specificity of the enzyme.*

Substrate	Acid-soluble DNA or RNA (mμmoles*) −ATP	+ATP
Native *E. coli* DNA	0.12	2.63
Native T4 DNA	0.08	4.45
Native T7 DNA	0.05	3.28
Heat-denatured *E. coli* DNA	0.97	1.30
Heat-denatured T4 DNA	0.20	0.53
Heat-denatured T7 DNA	0.96	1.64
E. coli ribosomal RNA†	0.26	0.15

* Nucleotide equivalents.

† Mixture of 16S and 23S RNA.

Activity was measured using 2.5 units of the enzyme (Fraction VII) per reaction mixture (0.5 ml). All P^{32}-labeled DNA was sheared to a mean molecular weight of 8×10^6 daltons before use (see *Materials and Methods*). Denatured DNA was prepared by boiling the sheared DNA (300 mμmoles/ml of $^1/_{10}$ SSC) for 10 minutes and rapidly chilling in ice-water.

observed. The maximum activity with Mn^{++} was at 2×10^{-3} *M* and was 63 per cent of the maximum activity seen with Mg^{++}. This enzyme has broad pH optimum ranging from 7.5 to 9.5 for the DNase activity.

(3) Substitution for ATP by other compounds: As described above, this enzyme requires ATP for its function. Among various adenine derivatives tested in place of ATP, as seen in Table 3, only deoxyATP was found to be almost as active as ATP. As shown in Figure 2, the optimum concentration of ATP or deoxyATP under the standard assay conditions is approximately 2×10^{-4} *M*. The *Km* for ATP and deoxATP was calculated to be 4.3×10^{-5} *M* and 4.8×10^{-5} *M*, respectively. The effects of replacement of ATP with other ribo- and deoxyribonucleoside triphosphates and other possible substitutes were tested and are shown in Table 4 (column I). It was found that all of the ribo- and deoxyribonucleoside triphophates can substitute for ATP, although the effectiveness of the substitution is lower to varying degrees ranging from 16 (CTP) to 84 percent (deoxyGTP) and 87 percent (deoxyATP) of the value observed with ATP. Adding a mixture of the four ribonucleoside triphosphates or deoxyribonucleoside triphosphates did not show any significant effect beyond that obtained with ATP alone. The possibility remained that the effects of various nucleoside triphosphates might have been due to the formation of ATP from trace ADP, catalyzed by a nucleoside diphosphokinase[22, 23] present in the enzyme preparation. To check this possibility, the effect of nucleoside triphosphates was studied in the presence of hexokinase and glucose. It is known that hexokinase has a strict

TABLE 3. *Comparison of adenine nucleotides as substitutes for ATP.*

Compounds	Acid soluble DNA (mμmoles*)	Compounds	Acid soluble DNA (mμmoles*)
...	0.11	S-adenosylmethionine	0.19
Adenine	0.10	ADP	0.16
Adenosine	0.15	ATP	1.72
5′-AMP	0.13	deoxyATP	1.76
3′,5′-cyclic AMP	0.13		

* Nucleotide equivalents.

Enzyme activity was assayed with the enzyme (1.5 units of Fraction VII) and various adenine nucleotides and their derivatives at concentrations of 0.1 mM each in 0.5 ml of reaction mixture.

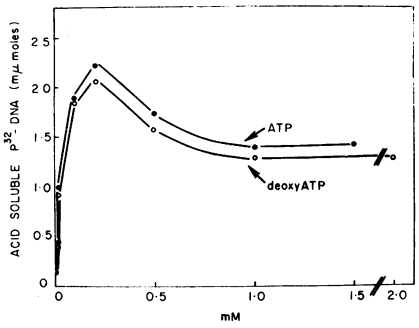

FIG. 2.—Effect of ATP and deoxyATP concentration on the DNase reaction. Each tube (0.5 ml) contained 2.0 units of the enzyme (Fraction VII) and ATP or deoxyATP at various concentrations in the standard reaction mixture as described in the *Materials and Methods*. Incubation was carried out at 37°C for 20 min.

specificity for ATP.[24] Thus, if the effect of various nucleoside triphosphates was due to the intermediary formation of ATP from contaminating traces of ADP, the presence of the hexokinase system would be expected to produce a competitive inhibition.[25] As shown in Table 4 (column II), when hexokinase was added at a concentration which inhibits over 98 per cent of the enzyme activity in the presence of ATP, the inhibition of the enzyme activity in the presence of the other nucleoside triphosphates ranged from only 8–34 per cent. These results suggest that all the other nucleoside triphosphates may be directly involved in the enzymatic reaction, although they are less effective than ATP.

(*4*) *Molecular size of the enzyme:* The molecular weight of the enzyme was tentatively calculated to be 350,000 based upon sedimentation rates of the enzyme. However, the ionic strength and the pH of the buffer affect the sedimentation pattern of the enzyme significantly, while causing changes in the enzymatic properties. These observations and the fact that the enzyme activity is affected by mutations which fall into two complementation groups (*recB* and

TABLE 4. *Comparison of nucleotide triphosphates and other compounds as substitutes for ATP and the effect of hexokinase.*

Compounds	Acid-soluble DNA (mμmoles*) (I) Control	(II) + Hexokinase (% inhibition†)
...	0.06	0.06(- - - -)
CTP	0.24	0.18(33.3)
UTP	0.81	0.56(33.3)
GTP	0.85	0.75(12.7)
ATP	1.16	0.08(98.2)
deoxyCTP	0.52	0.46(13.0)
TTP	0.51	0.45(13.3)
deoxyGTP	0.98	0.91(7.6)
deoxyATP	1.02	0.71(33.3)
DPN	0.04	
TPN	0.04	
Pyrophosphate	0.05	
Phosphoenolpyruvate	0.07	

* Nucleotide equivalents.

† Calculated after subtraction of the value obtained without nucleotide triphosphate.

Enzyme activity was assayed with the enzyme (1.5 units of Fraction VII) and various compounds tested at a concentration of 0.1 mM in 0.5 ml of reaction mixture. The hexokinase system was composed of yeast hexokinase (0.3 units, Sigma, Type C 300) and glucose (10 μmoles) in a volume of 0.05 ml.

recC)[17] strongly suggest that the active enzyme may be composed of at least two essential protein subunits. Studies on the separation of the possible subunits and reassembly of the enzyme are now being performed in this laboratory.

Discussion.—The correlation between the lack of the ATP-dependent DNase activity and the *recB* and *recC* type recombination deficient mutant strains reported here indicate that this enzyme may be responsible for one of several steps in the genetic recombination process. The fact that ultraviolet or X-ray irradiation of *recB* and *recC* mutants cause only 20 per cent of the amount of DNA degradation observed in the *rec*$^+$ strain[15] makes good sense if these genes code for a DNA degradation enzyme involved in that process. Of course, the experiments presented here cannot exclude the possibility that *recB* and *recC* genes control the DNase activity indirectly.

There are several previous reports on nucleases whose activities are affected by ATP or its derivatives. Among them, DNases purified from *Micrococcus lysodeikticus*[26, 27] and *Bacillus laterosporus*[28] and a DNase activity found in extracts of *E. coli*,[17] seem to be similar in several respects to the enzyme described here. However, a requirement for ADP as well as ATP or deoxyATP was reported for the *Micrococcus* enzyme[26] and deoxyribonucleoside triphosphates seem to be better activators than ATP for the *E. coli* extract activity.

The nature of the role of ATP in the functioning of the enzyme is not clear at the present time. The fact that this DNase attacks denatured DNA (except T4 DNA) at a detectable rate without ATP present may indicate that degradation of double-stranded DNA demands an energy-requiring configurational change of the substrate. In fact, DNA-dependent degradation of ATP into ADP and inorganic phosphate was observed during the enzyme reaction, but the elucidation of the stoichiometric relationship between ATP degradation and nucleotide release from DNA must await further investigation.

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