AN EXTRACELLULAR NUCLEASE OF BACILLUS SUBTILIS : SOME NOVEL PROPERTIES AS A DNA EXONUCLEASE*

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An extracellular nuclease of the Marburg strain of Bacillus subtilis, capable of hydrolyzing both RNA and DNA, has been described by Nakai et al.(1963, 1965). The products of hydrolysis of RNA by this enzyme have been identified as 3'-ribonucleotides. A similar enzyme fraction prepared from a culture fluid of B. subtilis SB 19 grown in tryptose broth degraded both native and denatured E. coli DNA to yield 3'-deoxynucleotides in the presence of Ca^{++} (Sakabe and Okazaki, 1964). No oligonucleotides were found at various stages of degradation, suggesting an exonucleolytic action of the enzyme. Similar observations have been reported by Kerr, Pratt and Lehman (1965) on a nuclease purified from a culture fluid of B. subtilis SB 19 grown in a yeast-extract veal-infusion broth. They further presented evidence that their enzyme degrades denatured DNA in a stepwise manner from the 5'-terminus. This paper describes our recent observations on the exonucleolytic degradation of native, denatured, and various enzymatically modified DNA's with a nuclease preparation obtained by more extensive purification from the culture fluid of B. sub-

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tilis SB 19.

Materials: B. subtilis SB 19 is a wild type strain obtained from Dr. J. Lederberg. ^{14}C-labeled E. coli DNA was extracted by the method of Lehman (1960) from E. coli 15T$^-$ grown in an amino acid-glucose medium supplemented with ^{14}C-deoxythymidine. ^{14}C-labeled T7 DNA was isolated from ^{14}C-labeled T7 phage grown in the presence of ^{14}C-deoxythymidine and fluorodeoxyuridine. Purification of T7 phage and extraction of DNA were carried out according to Richardson, Inman and Kornberg (1964). A crystalline preparation of E. coli exonuclease I was kindly provided by Dr. I. R. Lehman. E. coli exonuclease III was a generous gift of Dr. C. C. Richardson. B. subtilis DNA polymerase used in the present study was Fraction VI-3 described by Okazaki and Kornberg (1964 b). ^{3}H-dTTP was prepared from ^{3}H-deoxythymidine according to Okazaki and Kornberg (1964a).

Purification of enzyme: The supernatant fluid of a culture of B. subtilis SB 19 fully grown in tryptose broth was used as the starting material. This culture fluid had an activity of 7.3 units/ml and its specific activity was 80 units/mg of protein*. DNase activity was first precipitated with ammonium sulfate from the culture fluid and then eluted in a stepwise manner from a DEAE-cellulose column with Tris-acetate, pH 6.8. This was followed by gradient elution from a second DEAE-cellulose column with NaCl at pH 6.7. In the second DEAE-cellulose chromatography there were two peaks showing DNase activity (Peaks I and II), which were eluted from the column at NaCl concentrations of 0.14 M and 0.21 M, respectively. No DNA endonuclease activity could be detected in Peak I with a very sensitive assay, which uses the inactivation of circular DNA of ØX174 phage as indicator. This was true even at an enzyme concentration 1000 times higher than that used in ordinary experiments. Material from Peak II, on the

* To assay DNase activity, a reaction mixture (100 µl) containing 5 µmoles of Tris, pH 8.8, 0.3 µmole of $CaCl_2$, 4 mµmoles of ^{14}C-labeled native E. coli DNA and 0.02 to 0.2 unit of enzyme was incubated at 37°C. A unit of enzyme is defined as the amount causing the formation of 10 mµmoles of acid-soluble nucleotide in 30 minutes under these conditions.

other hand, showed endonuclease activity that inactivates ØX174 DNA. Peak I was further purified by passing it through a column of Amberlite IRP-64 and then by gradient elution from a hydroxylapatite column, resulting in its separation into two chromatographic peaks (Peaks I-A and I-B). Both peaks are capable of hydrolyzing RNA as well as native and denatured DNA in the presence of Ca^{++}. Peak I-A, which had a specific activity of 1400 units/mg of protein, was used throughout the present investigation.

Rate and Extent of Degradation: Table I summarizes the rate and extent of degradation of native and heat-denatured T7 DNA in the presence and absence of Ca^{++}. Denatured DNA was a better substrate than native DNA both in the presence and absence of Ca^{++}. The stimulation of nuclease activity by Ca^{++} was observed with denatured as well as native DNA substrate. In the absence of Ca^{++} native DNA was not degraded to any measurable extent even with a large amount of enzyme and prolonged incubation, indicating that the action of this enzyme is highly specific to denatured DNA under these conditions.

Partially single-stranded T7 DNA prepared by limited digestion with *E. coli* exonuclease III (Richardson, Lehman and Kornberg, 1964) was degraded by *B. subtilis* exonuclease to a limited

Table I. Rate and Extent of Degradation of T7 DNA with B. Subtilis Exonuclease

Substrate	Ca++	Relative Rate	Extent
Native T7 DNA	-	0	<0.01%
Native T7 DNA	+	16	100
Denatured T7 DNA	-	41	100
Denatured T7 DNA	+	100*	100

The reaction mixture (50 µl) contained: 2.5 µmoles of Tris, pH 8.8; 2 mµmoles of ^{14}C-labeled DNA; 0.075 unit of Fraction I-A; and 0.15 µmole of $CaCl_2$ when present. Incubation was carried out at 37°C.

* Actual rate was 24.3 µµmoles/minute.

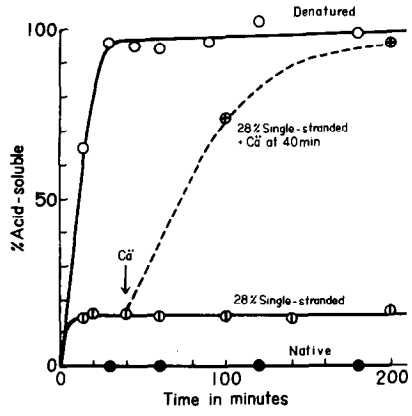

Fig. 1. Degradation of native, denatured, and partially single-stranded T7 DNA with B. subtilis exonuclease. The reaction mixture (50 µl) contained: 2.5 µmoles of Tris, pH 8.8; 2 mµmoles of ^{14}C-labeled T7 DNA and 0.19 unit of Fraction I-A. 0.15 µmole of $CaCl_2$ was added when indicated.

extent in the absence of Ca^{++}. Thus, T7 DNA, in which 28% of each strand was estimated to be single-stranded in the proximity of its 5'-terminus, was degraded to a limit of 15%, and no reaction beyond this point was observed unless Ca^{++} was added (Fig. 1).

Site of Initiation of Exonucleolytic Attack: Two kinds of experiments were performed to determine the terminus of the DNA at which the attack of B. subtilis exonuclease is initiated. In the first series of experiments, ^{14}C-T7 DNA, labeled at its 3'-terminus with ^{3}H-dTMP by "limited reaction"(Adler et al., 1958) with B. subtilis DNA polymerase, was subjected to exonucleolytic degradation and the time course of release of ^{3}H and ^{14}C-label was followed. The size of the ^{3}H-labeled region made by this "limited reaction" corresponded to 0.018% of the whole DNA. When this $^{3}H^{14}C$-labeled DNA was denatured by heating and then exposed to E. coli exonuclease I, which hydrolyzes denatured DNA in a stepwise manner from the 3'-terminus (Lehman and Nussbaum, 1964), the ^{3}H count was released rapidly, preceding the release of the bulk of ^{14}C.

By contrast, when the same denatured $^{3}H^{14}C$-labeled DNA was

treated with B. subtilis exonuclease either in the presence or absence of Ca^{++}, the release of ^{3}H showed a distinct lag in the early period of incubation, while ^{14}C was released linearly (Fig. 2a). Thus, when only 10% of ^{3}H had been released, 50% of ^{14}C was acid-soluble. On the other hand, when the native doubly-labeled DNA was degraded with the same enzyme, a rapid release of ^{3}H count preceded the release of the bulk of ^{14}C (Fig. 2b). For example, more than 90% of the ^{3}H-label was acid-soluble when 10% of the ^{14}C had been released.

In the second series of experiments, doubly-labeled DNA was prepared by the so called "repair reaction" (Richardson, Inman

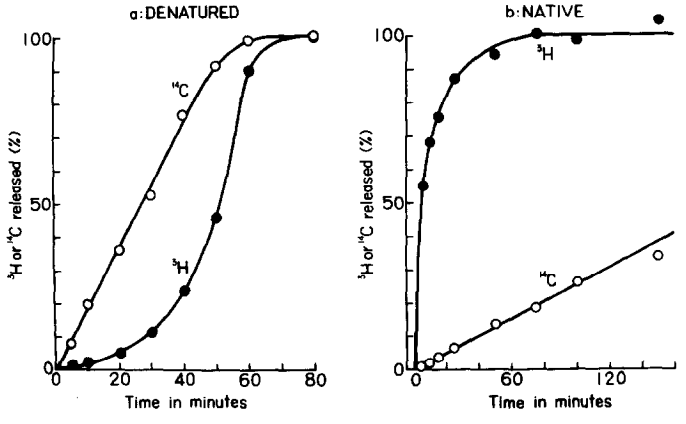

Fig. 2. Hydrolysis by B. subtilis exonuclease of $^{3}H^{14}C$-labeled T7 DNA prepared by "limited reaction". The 3'-terminal region of the $^{3}H^{14}C$-DNA corresponding to 0.018% of the whole molecule was labeled with ^{3}H and the remaining portion of the DNA chain was labeled with ^{14}C. The reaction mixture (50 µl) for denatured DNA contained: 2.5 µmoles of Tris, pH 8.8; 2 mµmoles of DNA; and 0.11 unit of Fraction I-A. The reaction mixture (50 µl) for native DNA contained: 2.5 µmoles of Tris, pH 8.8; 0.15 µmole of $CaCl_2$; 2 mµmoles of DNA; and 0.15 unit of Fraction I-A.

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and Kornberg, 1964) with DNA polymerase and the kinetics of its degradation with exonucleases was studied. ^{14}C-labeled T7 DNA was first partially hydrolyzed with E. coli exonuclease III to render a 28% portion of each strand single-stranded, and then used as a

primer of the "repair reaction" with B. subtilis DNA polymerase. The latter reaction was carried out at 20°C, using ^{3}H-dTTP as one of the substrates and was terminated when 0.8% synthesis was attained. When this "repaired" DNA was heat-denatured and treated with exonuclease I, ^{3}H was released rapidly preceding the release of the bulk of ^{14}C. This indicates that the terminal label with ^{3}H was made at the 3'-end of the DNA chain by the "repair reaction" as anticipated from the results of Richardson, Inman and Kornberg (1964) with E. coli DNA polymerase.

When the $^{3}H^{14}C$-labeled DNA, prepared by the "repair reaction",

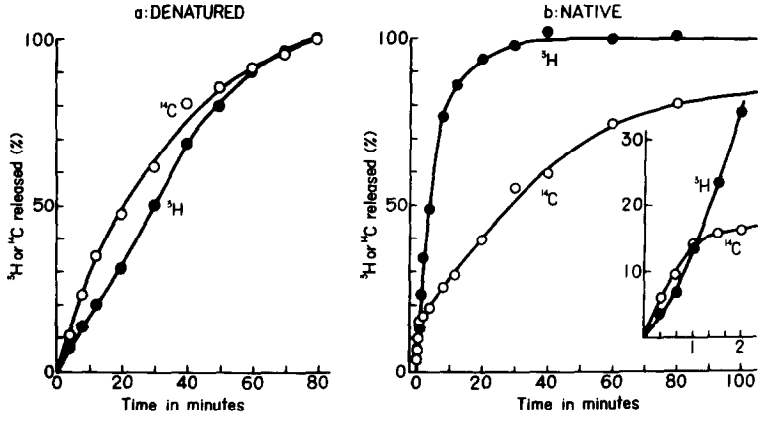

Fig. 3. Hydrolysis by B. subtilis exonuclease of $^{3}H^{14}C$-labeled T7 DNA prepared by "repair reaction". The 3'-terminal region of the $^{3}H^{14}C$-DNA corresponding to 0.8% of the whole molecule was labeled with ^{3}H and the remaining portion of the DNA chain was labeled with ^{14}C. In the "native" form the 5'-terminal region corresponding to 27% of the whole chain was estimated to be single-stranded. The incubation conditions were the same as those for Fig. 2 except that 0.15 and 0.38 unit of Fraction I-A were used for denatured and native DNA, respectively.

was degraded with B. subtilis exonuclease after heat denaturation, the proportion of ^{14}C found in acid-soluble form was higher than that of ^{3}H at many points during incubation (Fig. 3a). This result is similar to that obtained in the comparable experiment

with $^{3}H^{14}C$-labeled DNA prepared by the "limited reaction" (Fig. 2a), but the difference between ^{14}C and ^{3}H was smaller. This is presumably due to the fact that a greater proportion (0.8%) of the terminal region was labeled with ^{3}H in the "repaired" $^{3}H^{14}C$-DNA than in the doubly-labeled DNA prepared by the "limited reaction" (^{3}H-labeled region = 0.018%).

As shown in Fig. 3b, when the native "repaired" $^{3}H^{14}C$-DNA was treated with B. subtilis exonuclease, the release of ^{14}C proceeded at a rapid rate until about 14% of it had been made acid-soluble and then continued at a reduced rate. The release of ^{3}H count, on the other hand, proceeded in a more usual fashion and was completed when about half the ^{14}C count was still acid-insoluble.

All these results are in agreement with the hypothesis that B. subtilis exonuclease degrades denatured DNA in a stepwise manner starting at the 5'-terminus, while on native DNA it initiates exonucleolytic attack at the 3'-terminus. This does not imply that this enzyme does not carry out exonucleolytic attack from the 5'-terminus when native DNA is the substrate. It appears more likely that a single-stranded region is produced at the 5'-terminus of each strand of native DNA as the result of exonucleolytic cleavage initiated at the 3'-terminus of the opposite strand and that this single-stranded region is subject to exonucleolytic attack from the 5'-terminus. The initial rapid release of ^{14}C observed when the native "repaired" $^{3}H^{14}C$-labeled DNA was exposed to B. subtilis exonuclease (Fig. 3b) is very probably due to the degradation initiated at the 5'-terminus of the single-stranded region of this DNA.

Products and Mode of Hydrolysis: When native and denatured ^{14}C-labeled T7 DNA was degraded with B. subtilis exonuclease, more than 99% of the acid-soluble count was recovered as 3'-dTMP,

which was identified by paper chromatography in isopropanol-ammonia-water (7:1:2) and by the resistance to bull semen 5'-nucleotidase. Moreover, degradation of native T7 DNA, labeled with ^{3}H-dTMP at its 3'-terminus by the "limited reaction" resulted in the formation of radioactive deoxythymidine and 3'-dTMP.

These observations suggest that B. subtilis exonuclease is capable of carrying out two types of reactions: (a) Stepwise degradation beginning at the 5'-terminus of a single-stranded DNA chain producing 3'-deoxynucleotides; (b) Stepwise degradation initiated at the 3'-hydroxyl terminus of a double-stranded DNA, resulting in the liberation of terminal deoxynucleoside and 3'-deoxynucleotides. The latter reaction is different from the reaction of the known exonucleases which cleave 5'-nucleotides in a stepwise manner from the 3'-terminus. The decision as to whether or not the same protein carries out these two types of reactions requires further experiments. However, the observation that the ratio of activities for these two reactions* is constant throughout Peak I-A favors the possibility that both reactions are catalyzed by the same enzyme.

Summary: An exonuclease was extensively purified from a culture fluid of B. subtilis SB 19. The purified enzyme did not contain endonuclease activity detectable by inactivation of circular DNA of ØX174 phage. It degraded both native and denatured T7 DNA to completion in the presence of Ca^{++}. The reaction, however, was highly specific to denatured DNA in the absence of Ca^{++}. Partially single-stranded DNA, prepared by limited diges-

* Reaction (a) was assayed by the formation of acid-soluble products from ^{14}C-labeled T7 DNA. Reaction (b) was measured by the release of ^{3}H from the 3'-terminal region of ^{3}H^{14}C-labeled T7 DNA prepared by the "repair reaction".

tion with E. coli exonuclease III, was degraded to a limited extent under these conditions. The kinetics of the degradation of $^{3}H^{14}C$-labeled T7 DNA, which was prepared by labeling the 3'-terminus of ^{14}C-DNA with ^{3}H-dTMP using DNA polymerase reactions, suggested that this enzyme degrades a single-stranded chain of DNA in a stepwise manner starting from the 5'-terminus, whereas it degrades a double-stranded DNA chain from the 3'-terminus. The products of the degradation indicate the possibility that the linkage between the 3'-nucleotide and the adjacent nucleotide (or nucleoside) is hydrolyzed in both types of reactions.

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