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Two pH Optima of Adenosine 5′-Triphosphate Dependent Deoxyribonuclease from *Bacillus laterosporus*†

Toshinobu Fujiyoshi,‡ Juichiro Nakayama,§ and Motoaki Anai*

ABSTRACT: The various catalytic activities of the ATP-dependent deoxyribonuclease (DNase) of *Bacillus laterosporus* have pH optima at 6.3 and 8.3. Although the pH profile of ATP-dependent DNase activity on duplex DNA is bell shaped with a maximum at about pH 8.3, ATP-dependent DNase activity on single-stranded DNA has optima at pH 6.3 and 8.3. ATPase activities dependent on double-stranded and single-stranded DNA have a high bell-shaped peak with a maximum at pH 6.3 with a low and broad shoulder at about pH 8.3. ATP-independent DNase activity also has optima at pH 6.3 and 8.3. The ratio of the amount of ATP hydrolyzed per number of cleaved phosphodiester bonds in DNA increases with decrease in the pH value of the reaction. The ratios obtained at pH 8.3 and 6.3 were respectively about 3 and 22 with duplex DNA as substrate and 5 and 17 with single-stranded DNA as substrate. Formation of a single-stranded region of 15 000–20 000 nucleotides, which is linked to duplex DNA and about half of which has 3′-hydroxyl termini, was observed at about pH 6.3, but not at above pH 7.5. Furthermore, the optimum concentrations of divalent cations for the activity producing the single-stranded region and the activity hydrolyzing ATP were identical (3 mM Mn^{2+} or 5 mM Mg^{2+}). Thus the two activities are closely related. These results indicate that the enzyme has two different modes of action on duplex DNA which are modulated by the pH.

The general properties of the ATP-dependent DNase[1] from *Bacillus laterosporus* have been described in previous papers (Anai et al., 1975a,b). Similar DNases are widespread among bacterial species, and the involvement of this class of enzymes in genetic recombination and DNA repair has been proved (Buttin & Wright, 1968; Oishi, 1969; Barbour & Clark, 1970; Goldmark & Linn, 1970; Vovis & Buttin, 1970). These enzymes have a variety of in vitro catalytic activities (Whitehead, 1979). Apart from its postulated role in recombination, the ATP-dependent DNase has aroused considerable interest

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[1] Abbreviations: DNase, deoxyribonuclease; Tris, tris(hydoxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl.

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because ATP is involved in its DNA-hydrolyzing activities.

We have improved the purification procedure for ATP-dependent DNase from *B. laterosporus* (Fujiyoshi & Anai, 1981), and using the resulting electrophoretically pure enzyme preparation, we have found that the enzyme has an essential lysine residue at a DNA-binding site and that there are two different active sites, a DNA-binding site and an ATP-binding site (Fujiyoshi et al., 1981). The enzyme hydrolyzes 2–3 mol of ATP to ADP and P_i per DNA phosphodiester bond cleaved during the exonuclease reaction on duplex DNA under standard reaction conditions (Anai et al., 1975a). Similar results were obtained on the enzymes from *Micrococcus luteus* (Anai et al., 1970b) and *Mycobacterium smegmatis* (Winder & Lavin, 1971). In contrast, the enzymes from *Escherichia coli*, *Hemophilus influenzae*, and *Bacillus subtilis* can hydrolyze up to 40 mol of ATP per DNA phosphodiester bond cleaved, although the exact ratio depends on the conditions (Whitehead, 1979). In connection with the role of ATP in DNA hydrolysis, we are interested in this remarkable difference in ATP consumption during DNA degradation by these enzymes.

To obtain more information about the role of ATP in the catalytic mechanism of the enzyme, we reinvestigated the effect of pH on the enzyme. In this paper, we describe the effect of pH on the in vitro catalytic activities of the ATP-dependent DNase from *B. laterosporus*. The enzyme has pH optima at pH 6.3 and 8.3, and the pH modulates the ratio of ATP hydrolyzed to DNA phosphodiester bonds cleaved as well as the nature of the products formed. Rosamond et al. (1979) recently reported modulation of the action of the *recBC* enzyme of *E. coli* by Ca^{2+}. The possible mechanistic significance of the observed effect of pH is discussed.

Materials and Methods

Materials. *B. laterosporus* DNase was purified as described previously (Fujiyoshi & Anai, 1981); the specific activity of the enzyme preparation was 33000 units/mg. S1 nuclease was purchased from Seikagaku Kogyo Co., Tokyo. Snake venom phosphodiesterase was obtained from Sigma. *E. coli* alkaline phosphatase was purified from *E. coli* B by the method of Garen & Levinthal (1960). ^{3}H-Labeled T7 DNA was prepared by the method of Richardson et al. (1964); the specific activity was 1×10^3 cpm/nmol. Bacteriophage T7 was labeled with $^{32}PO_4$ by the procedure of Friedman & Smith (1972), and then the DNA was isolated by the method of Richardson (1966); its specific radioactivity was $(2–3) \times 10^4$ cpm/nmol. ATP, ADP, and AMP were products of Sigma. [γ-^{32}P]ATP was prepared by the method of Glynn & Chappell (1964). [8-^{3}H]ATP was obtained from Radiochemical Centre, Amersham. Bovine serum albumin was purchased from Armour. Poly(ethylenimine)–cellulose F thin-layer plate was a product of Merck. All other chemicals were of standard reagent grade.

Assay for B. laterosporus ATP-Dependent DNase. For DNase activity, the assay measures conversion of native T7 DNA to acid-soluble nucleotides. The reaction mixture (0.3 mL) contained 66.7 mM Tris-HCl buffer (pH 8.3), 66.7 mM Tris-HCl buffer (pH 7.3) or 66.7 mM cacodylate–HCl buffer (pH 6.3), 5 mM $MgCl_2$, 0.05 mM $MnCl_2$, 0.07 mg/mL bovine serum albumin, 0.33 mM ATP, 20 nmol of T7 [^{32}P]DNA, and enzyme preparation (diluted with 10 mM potassium phosphate buffer, pH 7.5, containing 10% glycerol). After incubation for 30 min at 37 °C, the mixture was cooled to 0 °C, and 0.2 mL of cold egg albumin (5 mg/mL) and then 0.5 mL of 0.5 N perchloric acid were added. The radioactivity of the supernatant was determined as described previously (Anai et al., 1975a). One unit of enzyme is defined as the amount catalyzing production of 10 nmol of acid-soluble nucleotides for 30 min under the above conditions. For ATPase activity, the assay measures the amount of [^{32}P]P_i not adsorbed to Norit by the procedure described previously (Anai et al., 1979).

Conditions for Other Nuclease Reactions. The reaction mixture for assay of S1 nuclease (0.3 mL) contained 0.05 M sodium acetate buffer (pH 4.6), 1 mM $ZnCl_2$, 0.1 M NaCl, 4.7 nmol of DNA, and 750 units of S1 nuclease. Incubations were carried out at 37 °C for 30 min, and then the radioactivity of the supernatant was determined as described for ATP-dependent DNase. Control reactions established that (i) under the conditions of the S1 nuclease reaction, the ATP-dependent DNase is inactive and (ii) the amount of S1 nuclease added to the reaction mixture is sufficient to degrade 20 nmol of denatured T7 DNA completely to an acid-soluble form within 30 min.

The mixture for treatment with snake venom phosphodiesterase (0.3 mL) consisted of 33 mM Tris–acetate buffer (pH 8.8), 30 mM $MgCl_2$, 7.5 nmol of DNA, and 10 units of venom enzyme. Incubation was carried out at 37 °C for 30 min and then the radioactivity of the supernatant was determined as described for assay of ATP-dependent DNase. Control reactions established that the amount of venom enzyme added to the reaction mixture was sufficient to degrade 20 nmol of denatured T7 DNA completely to an acid-soluble form in 30 min.

$Hg^{II}Cs_2SO_4$ Sedimentation Equilibrium Centrifugation. DNA samples were banded in Cs_2SO_4 by the Hg(II) banding technique of Nandi et al. (1965). The sample contained 17 nmol of T7 [^{32}P]DNA, which had been treated with ATP-dependent DNase, 76.8 nmol of native T7 [^{3}H]DNA (43000 cpm), 76.8 nmol of denatured T7 [^{3}H]DNA (43000 cpm), 0.4 mM Tris-HCl buffer (pH 8.2), 5 mM sodium borate buffer (pH 9.2), and 23 nmol of $HgCl_2$ (R_f 0.15). Saturated Cs_2SO_4 solution in 5 mM borate buffer was added to give a density of 1.500 g/cm^3, and the mixture was then adjusted to a final volume of 4 mL with Cs_2SO_4 solution of the same density in 5 mM borate buffer. The sample was overlaid with 1 mL of mineral oil and centrifuged at 25 °C at 25000 rpm for 48 h. Fractions were collected dropwise from the bottom of the tube on filter paper disks. The disks were dried, and the radioactivities of ^{32}P and ^{3}H on each disk were measured in a liquid scintillation counter.

Other Methods. Thin-layer chromatography was carried out as described by Goldmark & Linn (1972). Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Results

Effect of pH on the Various Activities of B. laterosporus ATP-Dependent DNase. First we investigated the effect of pH on the enzyme, and the results are shown in Figures 1 and 2. When double-stranded DNA was used as substrate, the pH profile of ATP-dependent DNase activity was bell shaped with a maximum at about pH 8.3 in Tris-HCl buffer (Figure 1A). In contrast, double-stranded DNA-dependent ATPase activity showed a low and broad shoulder at about pH 8.3 and a bell-shaped curve with maximum at pH 6.3 in cacodylate–HCl buffer, where slight DNase activity on duplex DNA is observed but no shoulder or small peak is detectable (Figure 1B). The ATPase activity at pH 6.3 was twice that at pH 8.3. No significant activity of DNA-independent ATPase was detectable at any pH.

When single-stranded DNA was used as substrate, the ATP-dependent and -independent DNase activities both

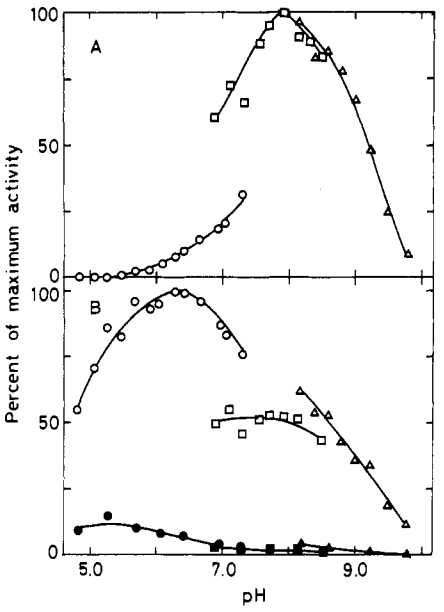

FIGURE 1: Effect of pH on enzyme activities with native DNA as substrate. (A) DNase activity. Assays were performed as described under Materials and Methods for 30 min, with native DNA as substrate and 0.28–23.6 units of enzyme, except that buffers and pH values were varied as follows: 66.7 mM cacodylate–HCl buffer (pH 4.9–7.3) (○); 66.7 mM Tris-HCl buffer (pH 6.9–8.3) (□); 66.7 mM glycine–NaOH buffer (pH 8.2–9.8) (△). (B) ATPase activity. Reaction conditions were as described above except that [γ-^{32}P]ATP and nonradioactive T7 DNA were used. Closed symbols indicate ATPase activity independent of DNA.

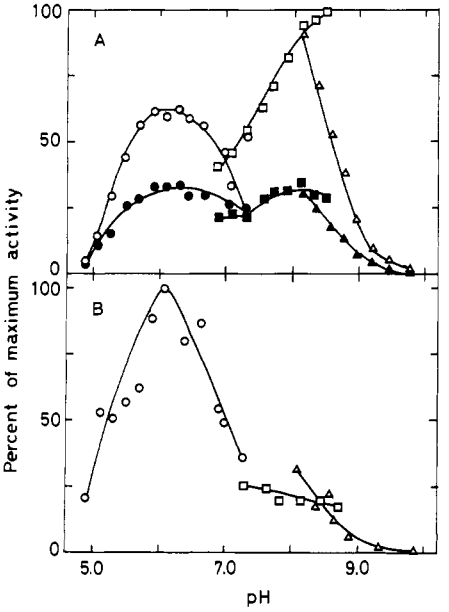

FIGURE 2: Effect of pH on enzyme activities with denatured DNA as substrate. Assays were as described for Figure 1 except that denatured DNA was used as substrate and 23.6 units of enzyme was used. (A) DNase activity in the presence (open symbols) or absence (closed symbols) of ATP. (B) ATPase activity.

showed optima at pH 6.3 and 8.3 (Figure 2A). In contrast, the pH profile of single-stranded DNA-dependent ATPase was similar to that of double-stranded DNA-dependent ATPase; that is, it showed a low, broad shoulder at about pH 8.3 and a bell-shaped curve with a maximum at pH 6.3 (Figure 2B). The activity at pH 6.3 was 4 times that at pH 8.3. When the buffers of low concentrations (6.67 mM) were used in this experiment, essentially identical pH–rate profiles were obtained (data not shown).

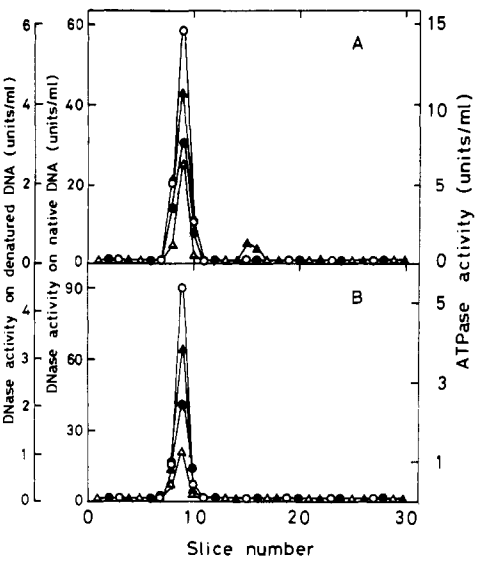

FIGURE 3: Polyacrylamide gel electrophoresis of the enzyme. The enzyme (37.5 μg) was layered onto the gel, and after electrophoresis, the gel was cut into 1.5-mm slices. Each slice was placed in 0.3 mL of 10 mM potassium phosphate buffer (pH 7.5) containing 10% glycerol for 2 days at 0 °C to elute the enzyme, and the supernatants were assayed for various enzyme activities under the respective standard conditions at pH 6.3 (A) and 8.3 (B). DNase on native DNA plus ATP (○); DNase on denatured DNA plus ATP (●); DNase on denatured DNA without ATP (△); ATPase dependent on native DNA (▲).

The enzyme preparation used in this experiment was nearly homogeneous as judged by polyacrylamide gel electrophoresis (Fujiyoshi & Anai, 1981). We further ruled out the possibility of the coexistence of two different enzymes with different pH optima as follows. We subjected the enzyme to electrophoresis on 7.5% polyacrylamide gel of pH 8.7 and, after electrophoresis, sliced the gel, eluted the enzyme from the segments with buffer, and assayed its activity at the respective pH values (Figure 3). Results showed that material with pH optima at 8.3 and 6.3 migrated to the same position on the gel. These results indicated that the activities at the two pH optima are embodied in the same molecular species.

Stoichiometry of ATP Utilization in the DNase Reaction. As shown in Figures 1 and 2, the ratios of DNase to ATPase activity at pH 8.3 and 6.3 are different. Therefore, we measured the amount of ATP hydrolyzed per number of cleaved phosphodiester bonds in the DNA substrate at each pH. The value obtained at pH 8.3 was about 3, whereas that obtained at pH 6.3 was about 22. ATP consumption per cleavage of phosphodiester bonds of DNA was about 10 times greater at pH 6.3 than at pH 8.3. When single-stranded DNA was used as substrate, the ratios were about 5 and 17 at pH 8.3 and 6.3, respectively. The results are summarized in Table I.

Effect of pH on Formation of an S1 Nuclease Sensitive Fraction of Reaction Products. As described above, the ratio of ATP consumption per cleavage of phosphodiester bonds of DNA was about 10 times higher at pH 6.3 than at pH 8.3. This finding led us to examine the amounts of S1 nuclease sensitive products formed at the two pH values. Formation of products digestible with S1 nuclease was observed at about pH 6.3 but not at above pH 7.5 (Figure 4A). The acid-soluble products formed at pH 8.3 and 6.3 were analyzed by DEAE-Sephadex A-25 chromatography in the presence of 7 M urea, and essentially identical results with the previous results (Anai et al., 1975b) were obtained at both pHs. In further studies on formation of S1 nuclease sensitive products,

Table I: Stoichiometry of ATP Utilization in the DNase Reaction[a]

DNA present	pH	ATP consumed (nmol)	DNA termini produced (nmol)	ratio
native	6.3	20.3	0.92	22.1
	7.3	13.4	1.83	7.3
	8.3	1.7	0.71	2.4
denatured	6.3	8.9	0.51	17.5
	7.3	3.8	0.56	6.8
	8.3	1.5	0.30	5.0

[a] Reactions (0.3 mL) were carried out in standard assay mixtures with 4.05 units of enzyme, 100 nmol of [8-^{3}H]ATP, and 20 nmol of duplex or denatured T7 [^{32}P]DNA at pH 6.3, 7.3, and 8.3. The incubation times were 2.5 min at pH 8.3, 15 min at pH 7.3 and 6.3 for native DNA, and 30 min at each pH for denatured DNA. After incubation, the reaction mixtures were heated at 100 °C for 2 min. Then an aliquot (5 μL) of each reaction mixture was spotted onto a poly(ethylenimine)-cellulose thin-layer plate together with 20 nmol each of ATP, ADP, and AMP as markers. After development of the chromatogram, the spots were cut out, and the radioactivity of each spot was measured in a liquid scintillation counter. A second aliquot (80 μL) was used to determine the portion of ^{32}P susceptible to *E. coli* alkaline phosphatase, as described previously (Anai et al., 1970a).

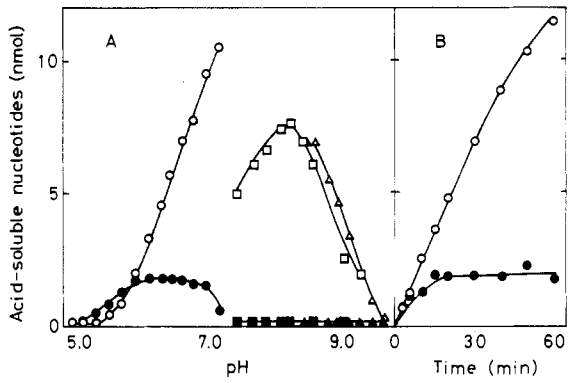

FIGURE 4: (A) Effect of pH on formation of S1 nuclease sensitive product. Reaction mixtures were as described under Materials and Methods except that the buffers and pH values were varied as indicated and 4.05 units of enzyme was added. Incubation was carried out for 5 min at pH 7.4–9.7 or for 15 min at pH 4.9–7.2. The mixtures were chilled to 0 °C. Then aliquots (70 μL) were removed from each tube for determination of acid-soluble (open symbols) and S1 nuclease sensitive (closed symbols) radioactivities, respectively, as described under Materials and Methods. (B) Time course of hydrolysis of DNA and formation of product susceptible to S1 nuclease. The reaction mixture (0.9 mL) contained 66.7 mM cacodylate–HCl buffer (pH 6.3), 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.07 mg/mL bovine serum albumin, 0.33 mM ATP, 60 nmol of native T7 [^{32}P]DNA, and 12.2 units of enzyme. Two aliquots (40 μL each) were removed at the indicated times for determination of acid-soluble (○) and S1 nuclease sensitive (●) nucleotides, respectively, as described under Materials and Methods.

we carried out experiments at pH 6.3, conditions where formation of these products and ATPase activity are nearly optimal.

Time Course of the Reaction Catalyzing Production of the Acid-Soluble Fraction and S1 Nuclease Sensitive Region. To examine the relation between production of the S1 nuclease sensitive region and the stage of the reaction, we estimated the production of S1 nuclease sensitive material during the course of the reactions at pH 6.3 and 8.3. At pH 8.3, no product susceptible to S1 nuclease was detected throughout the reaction, whereas at pH 6.3, production of an S1 nuclease sensitive portion was parallel to that of the acid-soluble fraction until about 10% of the input T7 DNA became acid soluble.

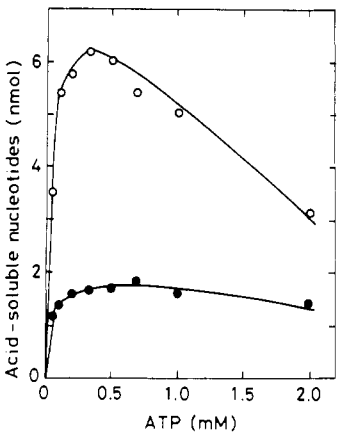

FIGURE 5: Effect of ATP concentration on activity at pH 6.3. Reaction mixtures were as described under Materials and Methods with cacodylate–HCl buffer (pH 6.3) except that the ATP concentration was varied as shown. After incubation for 30 min at 37 °C, two aliquots of 70 μL were removed for determination of acid-soluble (○) and S1 nuclease sensitive (●) radioactivities, respectively, as described under Materials and Methods. The amount of acid-soluble nucleotides formed by ATP-dependent DNase alone was subtracted from that formed by S1 nuclease treatment after incubation with ATP-dependent DNase.

During further hydrolysis, the amount of this S1 nuclease sensitive product remained constant (Figure 4B).

Effect of ATP Concentration on Product Formation at pH 6.3. DNA hydrolysis was maximal with 0.33 mM ATP, and formation of S1-sensitive products was also maximal at this ATP concentration. Because the rate of degradation of DNA to acid-soluble nucleotides was decreased, whereas the rate of formation of S1 nuclease sensitive nucleotides remained relatively constant on increasing the ATP concentration, the ratio of the portion susceptible to S1 nuclease to the amount of acid-soluble nucleotides increased at higher ATP concentration (Figure 5).

Effect of Concentration of Divalent Cations on Enzyme Activity at pH 6.3. The enzyme requires the presence of Mn^{2+} or Mg^{2+} for activity, and the maximum activity for formation of the acid-soluble fraction from duplex DNA was observed when Mn^{2+} (7.0×10^{-5} M) and Mg^{2+} (0.8×10^{-3} M) were present together. We examined the effect of divalent cations on the enzyme at pH 6.3. The maximum activity for formation of DNA acid-soluble products was obtained at 30 mM $MnCl_2$, but the maximum activity for producing S1-sensitive products was observed at 3 mM $MnCl_2$. ATP hydrolysis was also maximum at 3 mM $MnCl_2$. Similar results were also obtained with Mg^{2+}.

Characterization of Reaction Products Formed at pH 6.3. When T7 DNA which has been treated under the optimum conditions for production of the single-stranded region was sedimented through a neutral sucrose gradient, intermediate products were obtained between 23 S and 7 S as well as undegraded T7 DNA (Figure 6). The amounts of S1 nuclease sensitive and acid-soluble portions in each fraction were measured. S1 nuclease sensitivity was detected in fractions of intermediate size, but not in the fraction of undegraded T7 DNA. For determining the attachment of the S1 nuclease sensitive, single-stranded region to duplex DNA, two pools of fractions of intermediate size and one of undegraded DNA as a reference were banded in an $Hg^{II}Cs_2SO_4$ density gradient (data not shown). With decrease in the size of fragments, the bands exhibited increasing degrees of trailing toward the denser region. Although a little fully single-stranded material was observed, most of the single-stranded material was attached

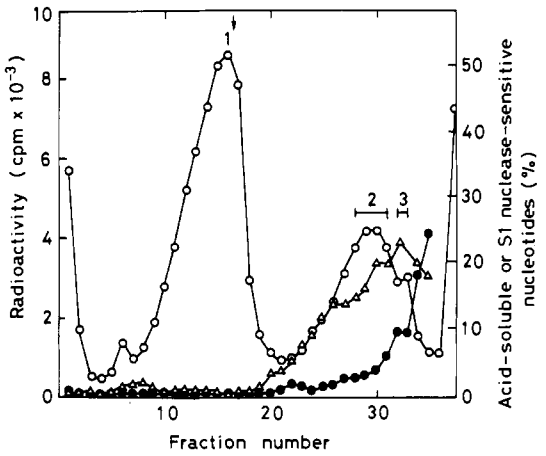

FIGURE 6: Sedimentation analysis of reaction products formed at pH 6.3. The reaction mixture (0.3 mL) was prepared as described under Materials and Methods with cacodylate–HCl buffer (pH 6.3), 60 nmol of native T7 [^{32}P]DNA, and 12.2 units of enzyme, except that bovine serum albumin was omitted. After incubation at 37 °C for 2.5 min, the reaction was stopped by cooling to 0 °C and adding EDTA to 20 mM, and then it was incubated at 55 °C for 10 min to inactivate the enzyme. Two aliquots (10 μL each) were taken for determination of acid-soluble and S1 nuclease sensitive radioactivity, respectively, as described under Materials and Methods. The acid-soluble and S1 nuclease sensitive radioactivities were 1.6% and 3.1%, respectively. Another aliquot (0.2 mL) was applied to a gradient of 5–20% sucrose (4.7 mL) in 0.25 M NaCl, 20 mM Tris-HCl buffer, pH 8.2, and 1 mM EDTA and centrifuged at 45 000 rpm for 2.5 h at 4 °C in a Hitachi RPS50 rotor. Fractions (6 drops each) were collected and the ^{32}P radioactivity of 10-μL aliquots (○) was determined. Three aliquots (20 μL each) were removed from each fraction for determination of acid-soluble (●), S1 nuclease sensitive (△) and venom phosphodiesterase sensitive (not shown) radioactivities, respectively, as described under Materials and Methods. The arrow indicates the position of untreated T7 DNA. Fractions were pooled as shown for use in the experiment of the $Hg^{II}Cs_2SO_4$ density gradient.

to double-stranded regions. As summarized in Table II, the single-stranded region had a relatively constant length in each fraction. About 50–60% of the region susceptible to S1 nuclease was sensitive to snake venom phosphodiesterase, indicating that half the single-stranded region had a 3′-OH terminus.

Although the ratio of enzyme to terminus of the DNA substrate was 1:1 in these experiments, neutral sucrose gradient sedimentation of the products did not indicate a continuous distribution of the products, but the formation of products of smaller size. Similar results were obtained with products treated with excess enzyme at high ionic strength (0.2 M NaCl) for a short period (data not shown).

Discussion

ATP-dependent deoxyribonucleases have diverse catalytic activities in vitro: (i) ATP-dependent exonuclease for duplex and single-stranded DNA, (ii) ATP-independent endonuclease for single-stranded DNA, (iii) DNA-dependent ATPase, and (iv) activity to unwind the DNA helix coupled to ATPase. In this work we examined the effect of pH on the various in vitro catalytic properties of the ATP-dependent DNase of *B. laterosporus* and found that the pH–rate profile of the activities of the enzyme showed pH optima at pH 8.3 and 6.3. We used electrophoretically pure enzyme for these experiments and confirmed that these properties resided in the same enzyme molecule. These interesting pH profiles have not yet been described for the other ATP-dependent DNases so far reported, although some differences in the effect of pH on various activities of the enzyme of *B. subtilis* have been observed (Shemyakin et al., 1979).

Table II: Molecular Size of Reaction Products

fraction no.[a]	M_r[b] (×10⁻⁶)	DNA susceptible to S1 nuclease[c] (%)	nucleotides in ss region[d]	DNA susceptible to SVP[e] (%)
23	17.0	8.1	4150	5.1
26	12.4	14.3	5370	6.6
29	8.9	20.0	4420	10.0
32	5.3	22.4	3600	15.9

[a] Fractions were obtained from the sucrose density gradient shown in Figure 6. [b] Molecular weight was estimated by the method of Friedman & Smith (1972). The molecular weight of intact T7 DNA was taken as 26.4 × 10⁶ (Studier, 1965). [c] The portion of DNA susceptible to S1 nuclease was determined as described under Materials and Methods. Values were as shown in Figure 6. [d] Numbers of nucleotides in the single-stranded (ss) region were determined as described by Friedman & Smith (1973). [e] Digestion with snake venom phosphodiesterase (SVP) was carried out as described under Materials and Methods.

The ratio of the amount of ATP hydrolyzed per number of phosphodiester bonds cleaved in DNA increased when the pH of the reaction mixture was decreased. Thus the value obtained at pH 8.3 is about 3, which is similar to that we reported peviously for this enzyme (Anai et al., 1975a) and those of the enzymes of *M. luteus* (Anai et al., 1970a,b) and *M. smegmatis* (Winder & Lavin, 1971), whereas the value obtained at pH 6.3 is about 22, which is similar to that of the *recBC* enzyme of *E. coli* (Goldmark & Linn, 1972).

Different digestion products of duplex DNA were formed at pH 6.3 and 8.3, whereas similar acid-soluble products were formed at both pHs. The products formed at pH 6.3 had a long single-stranded region, but those formed at pH 8.3 did not. Half the terminus of the single-stranded region was 3′-OH, the remainder presumably being 5′-OH. These results are identical with those obtained with the *E. coli* enzyme. Furthermore, even at pH 6.3, where formation of an acid-soluble fraction from duplex DNA is very low, the activity hydrolyzing duplex DNA and the activity of DNA unwinding have different optimum concentrations of Mn^{2+} and Mg^{2+}, the optimum concentration for the latter activity being the same as that for the ATP hydrolyzing activity. These results indicate that the activity of ATP hydrolysis is closely related to that for unwinding of the DNA helix, but not to the DNA hydrolyzing activity. Similar relatedness of the former two activities depending on the $MgCl_2$ concentration was observed with *H. influenzae* ATP-dependent DNase by Wilcox & Smith (1976). Thus, at high concentrations of Mg^{2+} (5–20 mM), DNA degradation to an acid-soluble form is rapid and ATP hydrolysis is slightly depressed, whereas at low concentrations of Mg^{2+} (0.1–0.5 mM), the enzyme rapidly hydrolyzes ATP and converts linear DNA to single-stranded material. The *recBC* enzyme of *E. coli* forms intermediates which have duplex structures with long single-stranded tails in the presence of high concentrations of ATP (5 mM) and NaCl (100 mM), and under these conditions the release of acid-soluble material from duplex DNA is more strongly inhibited than under standard reactions. These observations suggest that the energy for the unwinding process is supplied by the hydrolysis of ATP, but this hypothesis is not consistent with our finding that the hydrolysis of denatured DNA involves as much consumption of ATP as that of native DNA at pH 8.3 and 6.3 and that the pH profiles of ATP hydrolyzing activities dependent on duplex DNA and single-stranded DNA are similar.

The activity for hydrolyzing single-stranded DNA in the presence of ATP has a distinct peak at pH 6.3. In addition,

it is interesting that ATP-independent DNase activity on single-stranded DNA also showed the same two pH optima with similar levels of activity in the two. Therefore, the possibility of the participation of these activities in DNA unwinding also cannot be dismissed at present.

Now we can separate, at least partially, the complex catalytic activities of the enzyme into two groups according to their optimum pH, and we may utilize these properties advantageously to extend our investigations on the reaction mechanism of these complicated activities of the enzyme.

We have recently reported that the enzyme has two binding sites, a DNA binding site and an ATP binding site, and the same DNA binding site of the enzyme participates in all DNase activities including ATP-independent, single-stranded DNA specific DNase activity (Fujiyoshi et al., 1981). From the results described in this paper, it is possible to speculate that the DNA binding or catalysis can be further divided into two modes or subsites: One mode or site, which has an optimum at about pH 8.3, catalyzes the hydrolysis of single- and double-stranded DNA with a small amount of ATP hydrolysis. The other mode or site, which has an optimum at pH 6.3, generates intermediates of duplex structure with a long single-stranded region from duplex DNA and hydrolyzes single-stranded DNA, with consumption of a large amount of ATP. The two catalytic activities which are modulated by pH may involve different enzyme conformations or different ionization of particular amino acid residues at or near the active site. In addition, the electrostatic effect of DNA and ATP, which exert their effect on the interaction between the enzyme and the substrates, must also be considered in interpreting these effects of pH on the catalytic activities of the enzyme. Although we have no evidence regarding the working hypothesis mentioned above, further work along this line is now in progress.

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