An Adenosine Triphosphate-Dependent Deoxyribonuclease from *Alcaligenes faecalis*

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A deoxyribonuclease was purified approx. 800-fold from crude extracts of the bacterium *Alcaligenes faecalis*. The enzyme requires ATP and Mn^{2+} ; ATP could be replaced by any other ribo- or deoxyribo-nucleoside triphosphate, and Mn^{2+} could be replaced by Mg^{2+} in 0.1 M-Tris/HCl, pH 8.0 at 37°C. The enzyme could degrade linear duplex or denatured DNA, but was inactive with closed-circular duplex DNA from bacteriophage PM-2. In the course of nucleolytic activity, ATP was hydrolysed. We have measured deoxyribonuclease and adenosine triphosphatase activity in the presence of various salts, and found that the amount of ATP hydrolysis associated with a given amount of deoxyribonuclease activity was decreased in the presence of tetraethylammonium ions. Since these ions decrease the stability of the DNA helix, we conclude that one function of the ATP hydrolysis is to unwind the DNA.

While studying a DNAase† activity of the bacterium *Alcaligenes faecalis* originally described by Catlin & Cunningham (1958), we identified at least three DNAases, one of which requires ATP and bivalent metal ions for activity. In the present paper we describe a purification and characterization of this ATP-dependent DNAase, together with results of studies on the role of ATP in the nucleolytic process. The results are discussed in relation to those obtained by others in studies of similar bacterial nucleases.

Materials

Bacteria and bacteriophage

Alcaligenes faecalis (A.T.C.C. 8750) was obtained from the N.C.I.B. Torry Research Station, Aberdeen, Scotland, U.K.; the bacteria were grown in the proteose/peptone medium described by Catlin & Cunningham (1958). *Escherichia coli* K12.1100 *thi* was the endonuclease I-deficient strain of Dürwald & Hoffmann-Berling (1968). Bacteriophage PM-2 and its host *Pseudomonas* sp. BAL-31 were obtained from Dr. M. J. Waring, Department of Pharmacology, University of Cambridge.

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† Abbreviations: DNAase, deoxyribonuclease; ATPase, adenosine 5'-triphosphatase; AMP-P(NH)P, adenylyl-5'-yl imidodiphosphate; SDS, sodium dodecyl sulphate; SSC, standard saline citrate solution; Et_4N^+ and Et_4NCl are tetraethylammonium ion and its chloride salt respectively.

Chemicals

Sigma (London) Chemical Co. (Kingston-upon-Thames, Surrey, U.K.) supplied ATP, CTP, UTP, GTP, ADP, CDP, dATP, dTTP (all as sodium salts), calf thymus DNA, salmine sulphate, Triton X-100 and ethidium bromide (3,8-diamino-5-ethyl-6-phenyl-phenanthridinium bromide). $(NH_4)_2SO_4$ (AristaR enzyme grade), Et_4NCl bisacrylamide, SDS and *E. coli* tRNA were from BDH Chemicals Ltd. (Poole, Dorset, U.K.). Acrylamide and 2-deoxyribose were from Koch-Light (Colnbrook, Bucks., U.K.). Boehringer Corp. (London) Ltd. (Lewes, East Sussex, U.K.) supplied AMP, UMP, dCTP, dGTP (disodium salts), UDP (dipotassium salt) and AMP-P(NH)P (tetralithium salt). Sephadex G-100 and AH (aminohexanyl)-Sephadex 4B were from Pharmacia (G.B.) Ltd., London W.5, U.K. DEAE- and CM-cellulose (as DE52 and CM52) were from Whatman Biochemicals Ltd., Maidstone, Kent, U.K. Bovine serum albumin came from Armour Laboratories, Eastbourne, Sussex, U.K. DARCO activated charcoal was a product of I.C.I. United States Inc. and was supplied by Honeywell-Atlas, Carlshilton, Surrey, U.K. Other chemicals were analytical grade from Fisons, Loughborough, Leics., U.K. Phenol was redistilled and stored under N_2 in the dark at 0°C. $[\gamma\text{-}^{32}P]\text{ATP}$ and $[6\text{-}^3H]\text{-thymidine}$ were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

Buffers

Buffer R contained 0.05M-Tris/HCl, pH 8.0, 0.01M-2-mercaptoethanol and 0.001M-EDTA. Buffer J was buffer R supplemented with 0.2M-NaCl. SSC medium contained 0.15M-NaCl, 0.015M-sodium citrate, pH 7.00.

Preparation of ATP-Sepharose

ATP was covalently coupled to Sepharose 4B by the method of Lamed *et al.* (1973), except that the Sepharose-aminohexane adduct was obtained from Pharmacia as AH-Sepharose 4B. ATP-Sepharose was stored as a suspension in buffer R at 4°C until required.

Polyacrylamide-gel disc electrophoresis

Samples of purified enzyme were subjected to polyacrylamide-gel disc electrophoresis as described by Ornstein (1964) and Davis (1964). Running gel (6cm×0.4cm diam.) contained 7.5% (w/v) acrylamide, 0.2% bisacrylamide in 0.375M-Tris/HCl, pH 8.5. Stacking gel (1cm×4cm diam.) contained 2.5% (w/v) acrylamide, 0.6% bisacrylamide in 0.06M-Tris/phosphate, pH 8.5, and was photopolymerized by adding 0.5mg of riboflavin/100ml of gel solution. Reservoir buffer contained 0.05M-Tris and 0.38M-glycine, pH 8.5. For electrophoresis, each sample of up to 0.2ml contained 0.1% Bromophenol Blue. Electrophoresis was performed on duplicate samples at 0°C and 1 mA per gel for 45 min to allow complete stacking, and then at 1.5 mA per gel for 2 h. By this time the Bromophenol Blue marker was usually within 1 cm of the bottom of the gel.

Treatment of polyacrylamide gels

After polyacrylamide-gel disc electrophoresis, duplicate gels were treated in one of two ways.

(1) *Staining.* The gels were fixed in 10% (w/v) trichloroacetic acid for 6 h, before staining in a solution of 0.0125% Coomassie Brilliant Blue in methanol/acetic acid/glycerol/water (9:2:1:1, by vol.). The gels were subsequently destained in 7.5% (v/v) acetic acid for 6–8 h and scanned at 620 nm in a Unicam SP. 500 spectrophotometer with a Gilford gel-scanner attachment.

(2) *Assay.* The duplicate gels were cut into 2.5 mm slices, which were macerated in 0.2 ml of buffer J containing bovine serum albumin (1 mg/ml), and left at 0°C for 6 h. ATPase and DNAase activities in the gel eluates were assayed as described below.

Preparation of ³H-labelled DNA

E. coli K12.1100 was grown in TPG medium (Godson & Vapnek, 1973) supplemented with 1 µg of

thiamin/ml, 1 µCi of [6-³H]thymidine/ml and 250 µg of 2'-deoxyadenosine/ml (Boyce & Setlow, 1962). ³H-labelled DNA was prepared by the method of Marmur (1961) except that: (i) after harvesting and washing, the bacteria were resuspended in a solution of 27% (w/v) sucrose in SSC medium; (ii) before lysis. Pronase (grade B; Calbiochem, Bishops Stortford, Herts., U.K.) was added to a final concentration of 2 mg/ml, and after lysis with SDS the mixture was incubated at 37°C for 5 h (Thomas *et al.*, 1966).

Preparation of bacteriophage PM-2 and its DNA

Bacteriophage PM-2 was prepared from lysates of *Pseudomonas* BAL-31 by the method of Richardson (1973), with the following modifications: (i) the bacteriophages were precipitated by centrifuging at 59000g for 80 min in the 40 rotor of a Spinco model L centrifuge; (ii) after resuspension, solid CsCl was added to give a solution that was 37.5% (w/v). The phages were banded by centrifuging for 24 h in the SW50.1 rotor of a Spinco model L centrifuge at 110000g.

DNA was prepared from purified bacteriophage PM-2 by the method of Espejo *et al.* (1969), and was finally dialysed into 0.02M-Tris/HCl (pH 8.0)/0.05M-NaCl. The amount of closed-circular DNA in the preparation was measured by the method of Paoletti & LePecq (1971).

Assay of ATPase activity

ATPase activity was assayed by measuring the amount of ³²P_i released from [γ -³²P]ATP, by using a method based on that of Goldmark & Linn (1972). The reaction mixture (0.1 ml) contained 10 µmol of Tris/HCl, pH 8.0, 1 µmol of MnCl₂, 20 nmol of DNA, 40 nmol of [γ -³²P]ATP (7000–20000 c.p.m./nmol) and enzyme. After incubation at 37°C for 15 min, the reaction was terminated by adding 0.1 ml of each of 1M-HCl, activated charcoal suspension (20% packed volume) and bovine serum albumin (1 mg/ml) in that order. After 5 min at 0°C, the charcoal was removed by centrifuging at 4000g for 30 min and the ³²P_i remaining in the supernatant was measured by liquid-scintillation counting of an aqueous emulsion (see below). ATPase activity was also measured in the absence of DNA (cf. above), and DNA-dependent ATPase activity is defined as the difference between the activity measured in the presence and absence of DNA.

Assay of DNAase activity

(1) *Routine assay.* ATP-dependent DNAase activity was assayed as a routine by measuring the release of acid-soluble material from ³H-labelled DNA. The reaction mixture (0.1 ml) contained

10 μ mol of Tris/HCl, pH 8.0, 1 μ mol of MnCl_2 , 40 nmol of ATP, 20 nmol of ^3H -labelled DNA from *E. coli* K12.1100 (3000–5000 c.p.m./nmol) and enzyme. After incubation at 37°C for 15 min, the reaction was terminated by adding 0.2 ml of a cold solution of calf thymus DNA (2.5 mg/ml) and 0.1 ml of cold 20% (w/v) HClO_4 . This mixture was kept at 0°C for 10 min before the precipitate was removed by centrifuging at 2500g for 20 min; acid-soluble radioactivity in the supernatant fluid was measured by liquid-scintillation counting (see below). DNAase activity was also measured in reaction mixtures from which ATP had been omitted; ATP-dependent DNAase activity is the difference between the activity assayed with and without ATP.

A unit of enzyme is defined as the amount which produces 1 nmol of acid-soluble material in 15 min under these conditions, and activity was proportional to the amount of enzyme added, at least up to 1.4 units of enzyme.

(2) *By fluorimetry.* DNAase activity against the closed-circular duplex DNA from bacteriophage PM-2 was assayed by the fluorimetric method of Paoletti *et al.* (1971) and LePecq (1971), with a Perkin-Elmer MPF-2A fluorimeter at room temperature (19°C).

Other methods

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. DNA was measured by the method of Burton (1956) with deoxyribose as a standard, or spectrophotometrically, from the A_{260} , by assuming that $A_{260}^{1\%} = 200$ (Chargaff, 1955). DNA was denatured by heating at 100°C for 15 min, followed by rapid cooling in an ice/water bath for 10 min. All DNA concentrations are expressed as nucleotide equivalents.

Liquid-scintillation counting was performed by adding 1 vol. of aqueous sample to 10 vol. of scintillant [7.7 g of 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole, 1 litre of toluene and 500 ml of Triton X-100] and measuring radioactivity in either a Beckman or LKB Wallac 81000 liquid-scintillation counter. The specific radioactivity of labelled DNA and ATP solutions was determined under the same conditions as the respective assay samples. Quenching of radioactivity was monitored by external standardization.

Purification of an ATP-dependent DNAase

Growth of bacteria. *A. faecalis* was grown at 30°C with forced aeration in 18.5 litres of proteose/peptone medium to a density of 5×10^8 bacteria/ml, whereupon the culture was cooled rapidly to 0–4°C by adding ice. Bacteria were harvested in a Sharples continuous-flow centrifuge at a flow rate of 150–175 ml/min. The average yield of bacterial paste

was about 1.3 g wet wt./litre of culture; the paste was stored at –20°C until required.

All subsequent operations were carried out at 0–4°C, and all centrifuging was done in the SS-34 rotor of a Sorvall RC2-B centrifuge, unless noted otherwise. A summary of the purification procedure is given in Table 1.

Preparation of crude extract. Bacterial paste (200 g) was thawed and resuspended in 1 litre of buffer J. Bacteria were sonically disrupted in a Mullard L560 sonicator, in 50 ml portions, at 2.5 A output for three periods of 2 min each.

The resulting extract was centrifuged at 40000g for 40 min, and the supernatant fluid centrifuged again in the 30 rotor of a Spinco model L centrifuge at 73000g for 4 h. The resulting supernatant fluid was retained (fraction 1).

Salmine sulphate precipitation of nucleic acid. Salmine sulphate solution [17.2 ml of a 5% (w/v) solution in buffer J] was added slowly, with continuous stirring, to 880 ml of fraction 1. Gentle stirring was continued for 15 min after completion of the salmine sulphate addition; the mixture was allowed to settle for 10 min before the precipitate was removed by centrifuging at 9000g for 30 min in the GSA rotor of a Sorvall RC2-B centrifuge. To remove excess of salmine sulphate, the supernatant fluid was then passed through a column of CM-cellulose (1.6 cm² × 7.5 cm) that had previously been equilibrated with buffer J. The effluent was collected and the CM-cellulose column then washed with 25 ml of buffer J; the eluate was added to the effluent already collected (fraction 2).

Gel filtration. To 860 ml of fraction 2 was added 445 g of $(\text{NH}_4)_2\text{SO}_4$ with continuous stirring. After addition of the $(\text{NH}_4)_2\text{SO}_4$ was complete, gentle stirring was continued for 30 min, before the precipitate was recovered by centrifuging at 10000g for 30 min (Sorvall centrifuge, GSA rotor). The precipitate was resuspended overnight in buffer J, to a final volume of 110 ml (fraction 2A).

A column of Sephadex G-100 (75 cm × 50 cm²) was equilibrated with buffer J. Fraction 2A (55 ml) was applied to the column, which was then washed with 4.5 litres of buffer J at a flow rate of 4 ml/min. Fractions (20 ml) were collected and subsequently assayed for DNAase activity with and without ATP. This procedure was repeated for the remaining 50 ml of fraction 2A.

In both cases, the ATP-dependent DNAase was eluted in a broad peak immediately after the void volume, and fractions containing this activity were pooled (fraction 3).

DEAE-cellulose chromatography. Before chromatography, fraction 3 was concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation and dialysed into a low salt buffer. $(\text{NH}_4)_2\text{SO}_4$ (815 g) was added in small portions to 1600 ml of fraction 3, to give eventually an 80%-

saturated solution. The mixture was stirred gently for 20 min, after which the precipitate was recovered by centrifuging at 10000g for 30 min, and resuspended overnight in buffer R to a final volume of 200 ml. This was dialysed against 20 vol. of buffer R for 24 h, with two changes of buffer (fraction 3A).

A column of DEAE-cellulose (12.5 cm² × 10 cm) was equilibrated with buffer R. Fraction 3A was applied to the column at a flow rate of 50 ml/h, followed by 150 ml of buffer R, to remove any unadsorbed protein. Bound protein was eluted from the column by a linear gradient of 0–0.4 M-NaCl in buffer R at a flow rate of 48 ml/h; the total volume of the gradient was 400 ml. Fractions (5 ml) were collected.

Protein was eluted from the column in two broad peaks, at 0.1–0.2 M-NaCl and 0.3–0.4 M-NaCl. Two DNAase activities were identified, only one of which was stimulated by ATP; this activity was eluted at 0.28–0.33 M-NaCl. Fractions which contained ATP-stimulated DNAase activity were pooled and dialysed overnight against 20 vol. of buffer R, with one change of buffer (fraction 4).

Over a number of enzyme preparations the ATP-stimulated activity was reproducibly eluted by NaCl in the concentration range indicated, and apparently independently of the amount of DNA remaining in the extract at the time. DNA was eluted from the column mainly after the enzyme and in the range 0.33–0.4 M-NaCl.

Affinity chromatography on ATP-Sepharose. A column of ATP-Sepharose (4.5 cm × 2 cm²) was prepared as described previously and equilibrated by washing with buffer R until all unbound ATP was removed. Fraction 4 (50 ml) was loaded on the ATP-Sepharose at a flow rate of 20 ml/h, and the column was washed with buffer R until no more protein was eluted. Material that did not bind to the column was collected in a single batch, and retained.

Bound material was eluted from the column with a solution of 2 M-NaCl in buffer R. Fractions (1 ml) were collected, and those containing the ATP-

dependent DNAase activity were pooled and dialysed overnight against 100 vol. of buffer R (fraction 5).

The column of ATP-Sepharose was re-equilibrated with buffer R, before fraction 5 was applied at a flow rate of 30 ml/h. The column was then washed with 50 ml of buffer R, 30 ml of 0.35 M-NaCl in buffer R and finally a linear gradient of 0.35–0.80 M-NaCl in buffer R (total gradient volume 200 ml). Fractions (7 ml) were collected; those containing more than 60 units/ml were pooled and dialysed against buffer J containing 10% (v/v) glycerol (fraction 6). This material was stored at –20°C with no detectable loss of enzyme activity after 2 months.

None of the ATP-independent DNAase activity bound to the ATP-Sepharose, and all of this activity was recovered in the initial wash. In contrast, almost all of the ATP-stimulated DNAase activity bound and was eluted at 0.45–0.55 M-NaCl. Fractions 5 and 6 are totally dependent on ATP for DNAase activity; fraction 6 was used in all subsequent experiments.

Results

General properties of the purified enzyme

Samples (0.1–0.3 ml) of fraction 6 were checked for their possible DNA content by adding 1 mg of bovine serum albumin as carrier, precipitating with cold 0.25 M-HClO₄ and testing the pellet for acid-hydrolysable deoxyribose (Burton, 1956). No DNA was found in assays which would have readily detected the equivalent of 0.1 mM acid-precipitable deoxyribose in fraction 6.

DNAase activity in fraction 6 was totally dependent on the presence of both ATP and bivalent metal ions. Activity increased markedly as the concentration of ATP was raised within the range 1–20 μM, and reached a maximum between 0.4 and 2 mM (Fig. 1a). At higher ATP concentrations, DNAase activity was inhibited, and there was no detectable activity with 5 mM-ATP.

Table 1. *Summary of the properties of the fractions obtained during purification*

Fraction	Volume (ml)	Protein concn. (mg/ml)	ATP-dependent DNAase activity		Recovery (%)	Purification (fold)
			(total units)	(units/mg)		
1. Crude extract	900	25.8	24600	1.1	(100)	—
2. Salmine sulphate	950	16	22000	1.5	92	1.3
3. Sephadex G-100 eluate	1600	2.2	16000	4.6	67	4.2
4. DEAE-cellulose eluate	80	1.1	9000	102	38	95
5. ATP-Sepharose (high-salt eluate)	4	5.1	3000	150	26*	140
6. ATP-Sepharose (pooled gradient eluate)	28	1.10	2540	950	20*	820

* Corrected to allow for the further purification of only part of fraction 4.

Fig. 1. Variation of DNAase activity (a) with ATP concentration and (b) with temperature
DNAase activity was measured with 0.5 unit of enzyme and various concentrations of ATP in the reaction mixture in (a) and with incubation for 15 min at various temperatures between 8 and 45°C in (b). In this and all subsequent Figures the assay conditions were those described in the Methods section, but with exceptions noted in each case.

Fig. 2. Variation of DNAase activity with bivalent-metal-ion concentration
ATP-dependent DNAase activity was measured with 0.4 unit of enzyme; Mn²⁺ (●) was replaced by Mg²⁺ (▲) or Ca²⁺ (■) at various concentrations between 10 μM and 40 mM.

ATP-dependent DNAase activity was maximal between 30 and 40°C, and was assayed as a routine at 37°C (Fig. 1b). At this temperature, activity was proportional to the time of incubation for 15–20 min

Table 2. Nucleoside triphosphate requirements for DNAase activity

DNAase activity was assayed with 1 unit of enzyme, by using the reaction mixture described in the Methods section, except that ATP was replaced by 40 nmol of a variety of nucleoside derivatives. The activity with each nucleotide is expressed relative to the activity with 40 nmol of ATP.

Nucleotide	Activity (%)		No. of observations
	Mean	Range	
AMP	<1	<1	3
UMP	<1	<1	3
ADP	23	20–24	5
CDP	<1	<1	3
UDP	<1	<1	3
ATP	100	—	—
CTP	65	60–68	3
GTP	70	68–75	3
UTP	85	84–87	3
dATP	75	70–77	3
dCTP	75	75	2
dGTP	95	92–96	3
dTTP	50	40–55	2
AMP-P(NH)P	<1	<1	2
ATP + AMP-P(NH)P (40 nmol of each)	75	74–76	2

and was proportional to the amount of enzyme added, at least up to 1.4 units.

The requirement for bivalent metal ions could be satisfied by either Mg²⁺ or Mn²⁺. At concentrations below 1 mM there was no detectable activity with any of the metal ions used. Optimal activity was found at concentrations of 7–10 mM-Mn²⁺ and 15–30 mM-Mg²⁺. However, maximal activity with Mg²⁺ was only 55% of that with 10 mM-Mn²⁺ (Fig. 2). Ca²⁺ did not support detectable activity at any of the concentrations tested (0.01–40 mM).

Nucleoside triphosphate requirement

To determine whether the nucleotide requirement was specific for ATP, or whether any other nucleotide could activate the enzyme, the DNAase activity in fraction 6 was assayed with a variety of ribo- and deoxyribo-nucleoside derivatives. These were added to the reaction mixture to give a final concentration of 400 μM. This concentration was assumed to be near optimal, although this may not always be the case. The results are shown in Table 2.

Neither monophosphate tested was able to activate the enzyme; CDP and UDP were also ineffective. However, activity with ADP was about 25% of the activity with ATP, which is about the amount of activity that would be expected if the commercial preparation of ADP contained about 1% ATP.

Subsequent experiments suggested that this was indeed the case.

All ribo- and deoxyribo-nucleoside triphosphates activated the DNAase, with ATP the most effective and dTTP the poorest. AMP-P(NH)P, the non-hydrolysable analogue of ATP, was ineffective in stimulating the DNAase, and unlike ADP, AMP-P(NH)P decreased the DNAase activity when present with ATP (Table 2). This suggests that nuclease activity may require the hydrolysis of nucleoside triphosphate and that the nucleoside triphosphate is not acting simply as an allosteric effector.

ATPase activities in fraction 6

ATPase activity in fraction 6 was assayed in the presence and absence of DNA, as described in the Methods section. Without DNA, 0.5 μ g of fraction-6 protein released about 1 nmol of 32 P_i from [γ - 32 P]ATP after incubation at 37°C for 30 min. ATPase activity was stimulated approx. fourfold when DNA was added to the reaction mixture, and 32 P_i release was constant for about 20 min before decreasing in rate.

To determine the purity of the enzyme preparation, and whether both DNAase and ATPase activities were the properties of a single protein, samples of fraction-6 protein were subjected to polyacrylamide-

Fig. 3. Polyacrylamide-gel disc electrophoresis of fraction-6 protein

(a) Fraction-6 protein was electrophoresed on 7.5% (w/v) acrylamide gel, and material eluted from the macerated gel slices as described in the Methods section. The eluate from each slice was assayed for DNAase activity with ATP (●) and without ATP (○). A duplicate gel was stained in Coomassie Brilliant Blue and scanned at 620 nm (----). (b) As (a), except that the material eluted from the gel slices was assayed for ATPase activity with DNA (▲) and without DNA (△) as described in the Methods section.

gel disc electrophoresis as described in the Methods section.

After staining, two prominent bands and five very faint bands were seen; the major band corresponded to the ATP-dependent DNAase (Fig. 3a). On assay of eluates of gel slices, two ATPase activities were found, one of which was independent of the presence of DNA, and was the second major protein in fraction 6. The other ATPase activity was totally dependent on the presence of DNA, and coincided with the ATP-dependent DNAase previously identified (Fig. 3b).

Further evidence that a single protein has both ATPase and DNAase activities was obtained by determining the DNAase and ATPase activities at different pH values (Fig. 4). In the absence of DNA, the ATPase activity decreased as the pH was raised from 7.1. In contrast, both the ATP-dependent DNAase and the DNA-dependent ATPase showed a sharp peak of activity between pH 7.8 and 8.0, with the activities exhibiting a similar dependence on pH over the whole range tested.

If both DNAase and an ATPase activity reside in a single protein, and ATP hydrolysis is essential for nucleolytic activity, then very low concentrations of ATP ought to limit the DNAase activity to a value that is proportional to the initial ATP concentration. To examine this, DNA breakdown was measured after incubation at 37°C for various times with only 0.1–0.6 nmol of ATP in the usual reaction mixture. An experiment with initially 3 nmol of ATP/ml is

Fig. 4. Variation of ATPase and DNAase activity with pH. DNAase and ATPase activities were assayed with 1.2 units of enzyme, with 0.10M-Tris/HCl at various pH values from 7.1 to 8.9. ●, ATP-dependent DNAase; ▲, DNA-dependent ATPase; △, DNA-independent ATPase.

shown in Fig. 5. DNA breakdown reached a limit after 20–30 min. After 60 min the remaining reaction mixture was supplemented with extra buffer (0.25M-Tris/HCl, pH8), or extra DNA, or extra enzyme; none of these effected further DNAase activity. However, on addition of ATP (about 6 nmol/ml final concn.) there was an immediate increase in the production of acid-soluble material, the new initial rate being the same as that found in mixtures where the same concentration of ATP was present at zero time. DNA breakdown again ceased after about 30 min incubation. This shows that the limit in DNAase activity is not due to enzyme inactivation nor to

Fig. 5. Limitation of DNAase action by amount of ATP initially present

Reaction mixtures, in quadruplicate, contained (in 1 ml) 100 μmol of Tris/HCl, pH 8, 10 μmol of MnCl₂, 200 nmol of ³H-labelled DNA, 3 nmol of ATP (3 μM final concn.) and 6 units of enzyme. The mixtures were incubated at 37°C and 0.1 ml samples removed at various times for measurement of acid-soluble radioactivity. After 60 min the remaining reaction mixtures were supplemented with one of the following: (i) 3 units of enzyme, or (ii) 10 nmol of ³H-labelled DNA, or (iii) 0.03 ml of 0.25M-Tris/HCl, pH8, all represented by ● (iv) 3 nmol of ATP (giving about 6 μM final concn.), represented by ○. The Figure shows the amounts of acid-soluble radio activity present in 0.1 ml samples removed at intervals during a further 30 min incubation.

the removal of suitable DNA substrate, but rather is due to the removal of ATP, supporting the view that the hydrolysis of ATP is essential for nucleolytic activity.

DNAase and ATPase activities with single-stranded DNA

In the absence of ATP, no detectable acid-soluble material was produced from single-stranded DNA substrate; with ATP, denatured DNA was degraded at about 2.5 % of the rate with native DNA (Table 3). ATP was again hydrolysed in the course of the DNAase reaction, and about 1.5 nmol of ³²P_i was produced per nmol of ³H-labelled nucleotide made acid-soluble. Unlike the ATP-dependent DNAase from *E. coli* (Goldmark & Linn, 1972), the variation of DNAase activity with ATP concentration appears to be the same for both native and denatured DNA (Fig. 1a). This assay does not exclude the possibility that this ATP-dependent DNAase from *A. faecalis* can act endonucleolytically with single-stranded DNA.

DNAase activity against double-stranded circular DNA

DNAase activity was measured with bacteriophage PM-2 DNA by the fluorimetric method described by LePecq (1971). With pancreatic DNAase, there was an increase in the observed fluorescence intensity within 1 min, as the circular DNA was nicked. With fraction-6 enzyme in the absence of ATP, there was no observable change in the fluorescence intensity over the whole time-period studied (Fig. 6). With ATP in the reaction mixture, there was a small but measurable decrease in fluorescence intensity during 15 min incubation. This decrease could be due to a decrease in ethidium bromide binding as a result of exonuclease attack on linear molecules in the bacteriophage PM-2 DNA preparation. It was concluded that the enzyme cannot act endonucleolytically against circular, covalently closed duplex DNA of bacteriophage PM 2.

Table 3. Comparison of DNAase and ATPase activities with double-stranded and single-stranded DNA
DNAase and ATPase activities were measured by using either 1.0 or 1.25 units of enzyme with either 20 nmol of native *E. coli* K12.1100 DNA (ds), or 20 nmol of heat-denatured DNA (ss) in the standard reaction mixture.

Enzyme added (units)	DNA substrate	ATP	DNAase (mol of acid-soluble nucleotide)		DNA-dependent ATPase (nmol of ³² P _i released)		No. of expts.	
			Mean	Range	Mean	Range	DNAase	ATPase
1.0	ds	+	1.0	0.95–1.09	3.6	3.5–3.75	5	3
1.25	ds	+	1.25	1.18–1.35	4.4	4.2–4.65	2	3
1.25	ds	–	<0.01	<0.01	—	—	2	—
1.25	ss	+	0.035	0.03–0.04	0.04	0.035–0.05	2	2
1.25	ss	–	<0.01	<0.01	—	—	2	—

Fig. 6. DNAase activity with double-stranded circular DNA

The reaction mixture (1.0 ml) contained 7.5 μ g of bacteriophage PM-2 DNA, 8 μ mol of MnCl_2 , 400 nmol of ATP, 9.5 μ mol of Tris/HCl, pH 8.0, and 1 μ mol of EDTA. The reaction was initiated by adding either 0.01 ml of pancreatic DNAase (0.01 μ g/ml) or 0.5 unit of fraction-6 enzyme. Samples (0.1 ml) were taken at various times and the reaction was stopped by adding 0.9 ml of a solution of 1 μ g of ethidium bromide/ml in 0.01 M-Tris/HCl (pH 8.0)/0.02 M-NaCl/5 mM-EDTA. Fluorescence intensity was measured as described previously (see the Methods section). I_0 = intensity at zero time; I_t = intensity at time t . ●, pancreatic DNAase; ○, fraction 6+ATP; ■, fraction 6 without ATP.

DNAase and ATPase activities in the presence of tetraethylammonium chloride

To investigate whether a reagent believed to facilitate DNA unwinding could alter ATP utilization by the nuclease, DNAase and ATPase activities were measured in the presence of various concentrations of NaCl, NH_4Cl and tetraethylammonium chloride.

DNAase activity was inhibited as the concentration of NaCl or NH_4Cl was increased (Fig. 7a), with no apparent difference between the effect of NaCl and NH_4Cl . Some experiments suggested that DNAase activity was stimulated with 30–50 mM-NaCl, but this was not observed consistently. With Et_4NCl , DNAase activity was again inhibited, but the inhibition was less than with the same concentration of NaCl.

DNA-dependent ATPase activity was also inhibited by all three salts, although as with DNAase activity the inhibitory effect of Et_4NCl was less than that found with the same concentration of either NaCl or NH_4Cl . The effect of these latter two salts was again almost identical (Fig. 7b).

From the data of Figs. 7(a) and 7(b), it is possible to determine the 'efficiency' of the DNAase reaction by calculating the amount of DNA-dependent hydrolysis of ATP relative to the amount of DNA made acid-soluble under the same conditions. This

Fig. 7. Variation of ATPase and DNAase activities with salt concentration

(a) ATP-dependent DNAase was measured with 1.4 units of enzyme by using the reaction mixture described in the Methods section, supplemented with various amounts of NaCl (●), NH_4Cl (○), or Et_4NCl (■). (b) The conditions were as in (a) except that ATPase activity was measured in the presence and absence of DNA for each salt at the concentrations shown. The difference between the amount of $^{32}\text{P}_i$ released from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence and absence of DNA is shown as DNA-dependent ATPase at each salt concentration. (c) From (a) and (b) the ratio of the amount of DNA-dependent $^{32}\text{P}_i$ release to the amount of ^3H -labelled acid-soluble material produced was calculated. This is represented by E ; variation of E with the concentration of NaCl (●), NH_4Cl (○) and Et_4NCl (■) is shown.

value is represented by E . However, since neither the reaction mechanism nor the size of the oligonucleotide products is known, it is not possible to relate the value of E more accurately to the number of ATP molecules hydrolysed per phosphodiester bond cleaved.

The variation in the value of E with salt concentration is shown in Fig. 7(c). In the absence of any salt, E is about 3.5 and remains approximately constant as the concentrations of NaCl and NH_4Cl are increased. As the concentration of Et_4NCl is increased, however, the value of E decreases from 3.5 to 1.2 with 600 mM Et_4NCl . Under these conditions, the amount of ATP hydrolysis needed to produce a given amount of DNAase activity is decreased by about 65%. We suggest that this is compatible with a model for DNAase activity in which ATP hydrolysis is coupled in some way to the unwinding of the DNA duplex.

Discussion

By using conventional procedures we have purified an ATP-dependent DNAase over 800-fold from crude extracts of *Alcaligenes faecalis*. Gel electrophoresis showed that the final preparation was not homogeneous with respect to this enzyme (Fig. 3), and although only a single DNAase activity was present, two ATPase activities were identified. One of these corresponded to the ATP-dependent DNAase, which suggests that a single protein has both the ATP-dependent DNAase and the DNA-dependent ATPase activities.

We found that this enzyme requires hydrolysis of ATP for nucleolytic activity to occur. This property is common to all other known ATP-dependent DNAases, and in all cases ATP is hydrolysed in excess of the number of phosphodiester bonds cleaved (Anai *et al.*, 1970; Vovis & Buttin, 1970; Winder & Lavin, 1971; Goldmark & Linn, 1972; Smith & Friedman, 1972; Ohi & Sueoka, 1973). Possible functions for such hydrolysis have been proposed for similar enzymes from other bacteria and include: (i) formation of an 'activated complex' by phosphorylation or adenylation (Anai & Takagi, 1971); (ii) unwinding of the DNA helix (Friedman & Smith, 1973; MacKay & Linn, 1974); and (iii) translocation of the enzyme along the DNA (Winder, 1972). Our experiments have been directed to examining the latter two possibilities.

Inclusion of Et_4N^+ in the reaction mixture for the ATP-dependent DNAase of *A. faecalis* led to a decrease in the amount of ATP hydrolysed relative to the amount of DNA made acid-soluble (Fig. 7c). It appears that Et_4N^+ is acting in some way to alleviate the need for ATP hydrolysis. Since there is evidence that Et_4N^+ facilitates unwinding of a DNA duplex (Melchior & von Hippel, 1973; Malcolm *et al.*, 1975), we suggest that the ATP hydrolysis which accom-

panies the nuclease action of the *A. faecalis* enzyme is used in some way to unwind the DNA strands, probably starting at the strand termini. MacKay & Linn (1974, 1976) have shown that this occurs with exonuclease V of *E. coli*, and that probably *in vivo* this unwinding is the principal function of this enzyme.

However, unwinding the DNA duplex seems unlikely to be the only function associated with hydrolysis of ATP, since the *A. faecalis* enzyme also requires ATP when it degrades linear single-stranded DNA, although less ATP is hydrolysed than with a duplex DNA substrate (Table 3). This implies that ATP may be used for a function other than unwinding. A possibility is enzyme translocation, as has been suggested by Winder (1972) in a general model of ATP-dependent exonuclease action.

In summary, our results for the ATP-dependent DNAase are consistent with ATP hydrolysis partly providing energy for DNA strand separation and partly for another process, which could be enzyme translocation along the DNA strands.

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