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Heat-labile alkaline phosphatase from Antarctic bacteria: Rapid 5′ end-labeling of nucleic acids

(radioactive labeling/polynucleotide kinase)

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ABSTRACT **A heat-labile alkaline phosphatase has been purified to near homogeneity from HK47, a bacterial strain isolated from Antarctic seawater. The active form of the enzyme has a molecular weight of 68,000 and is uniquely monomeric. The optimal temperature for the enzymatic activity is 25°C. Complete and irreversible thermal inactivation of the enzyme occurs in 10 min at 55°C. By using this heat-labile enzyme for dephosphorylation followed by a 10-min heat treatment, rapid end-labeling of nucleic acids by T4 polynucleotide kinase has been achieved.**

The radioactive end-labeling technique of nucleic acids by T4 polynucleotide kinase is a useful tool in studies of the structure and function of nucleic acids (1–3). The technique involves key reactions: first, the removal of the existing phosphates at the 5′ termini of DNA, RNA, or oligonucleotides by alkaline phosphatase (APase), and second, the addition of the radioactive phosphates to the same 5′ termini by polynucleotide kinase in the presence of ATP (4). Following the first reaction, the APase activity must be eliminated before the second reaction in order to avoid both degradation of ATP and the loss of label from the substrates. However, elimination of the APase activity following dephosphorylation by either removal or inactivation has been difficult because of the great biochemical stability of the known APase enzymes. For example, *Escherichia coli* APase, which is the most widely used, can be heated for 8 min at 95°C with retention of 50% of the original activity (5), and it can also survive in acid conditions (6). Although APases from mammalian origins are more sensitive to physical and chemical treatment than bacterial APases, they are less widely used (ref. 7, p. 404).

In our laboratories, a total of 155 marine bacterial strains has been isolated from samples collected from Antarctic seawater. We studied the possibility of obtaining heat-labile APases from these Antarctic bacteria. This paper describes the purification and the general properties of one heat-labile APase we isolated from strain HK47 together with its use with end-labeling of nucleic acids.

MATERIALS AND METHODS

Materials. Plasmid pBR322 DNA and endonuclease *Hin*fI were obtained from P-L Biochemicals. T4 polynucleotide kinase was purified as described by Richardson (4). *E. coli* APase was purchased from Millipore. All additional chemicals were reagent grade or better.

Organisms and Culture Condition. Bacterial strains used in this study were isolated from samples taken from McMurdo Sound, Ross Sea, Antarctica. Field samples such as sea ice, seawater, sediments, and sea animals (isopod and pycnogonid) were obtained on two occasions: one from November through December 1980 and the other from October 1981 through January 1982, as described by Sullivan and Palmisano (8). Serially diluted samples were spread on 2216E plates that were incubated at 0°C for 2–12 weeks (9). Colonies were picked up from the plates and further purified. 2216E medium was also used for purification and maintenance of the bacteria. For APase purification, bacteria were grown at 4°C in TYS medium (10 g of Bactotryptone/2 g of Bacto yeast extract in 1 liter of 60% seawater adjusted to pH 7.6 with HCl). The detailed procedure for isolation and characterization of the Antarctic bacteria will be reported elsewhere.

Screening of Heat-Labile APase from the Antarctic Bacteria. A freshly prepared colony from each strain was suspended in 2 ml of mineral buffer containing 0.4 M NaCl, 10 mM KCl, 50 mM $MgCl_2$, 10 mM $CaCl_2$, and 0.1 M Tris·HCl (pH 7.6). The bacterial suspension was divided into two equal portions. One portion was preincubated at 65°C for 10 min and the other was kept on ice. One milliliter of 10 mM *p*-nitrophenyl phosphate was added to each of the bacterial suspensions. After 30 min of incubation at 25°C, production of *p*-nitrophenol was spectrophotometrically measured at 410 nm. Heat-labile APase producers were defined as those bacteria that liberated >20 nmol of *p*-nitrophenol in the unheated suspension but produced no detectable amount of *p*-nitrophenol in the suspension with heat treatment.

Purification of HK47 APase. All purification procedures were carried out at 0–4°C. Antarctic marine bacterium HK47 was grown at 0–4°C in 5.4 liters of TYS medium to late exponential phase. The cells were centrifuged at 10,000 × *g* for 15 min at 0°C and washed once with 5.4 liters of 1 M NaCl in 50 mM Tris·HCl buffer (pH 7.4). The pellet was suspended in 350 ml of hypertonic buffer containing 1.0 M NaCl, 1.0 M sucrose, and 50 mM Tris·HCl buffer (pH 7.4) and then stirred for 15 min at 0°C. The cell suspension was centrifuged and the pellet was rapidly suspended in 54 ml of cold shock buffer containing 50 mM NaCl, 10 mM $MgCl_2$, and 50 mM Tris·HCl (pH 8.0). After stirring for 15 min at 0°C, the mixture was centrifuged and the supernatant was collected. The pellet was further extracted with 27 ml of the cold shock buffer, and two supernatants were combined. The osmotic shock fluid was dialyzed against 50 mM Tris·HCl buffer (pH 8.4) containing 5 mM NaCl and 10% glycerol (T buffer) and centrifuged at 100,000 × *g* for 30 min. The supernatant was applied to an affinity chromatography column (0.8 cm^2 × 12 cm) equilibrated with 10 mM Tris·HCl buffer (pH 8.4). The affinity chromatography column was prepared by coupling the diazonium salt of 4-(*p*-aminophenylazo)phenyl arsonic acid to tyraminyl-Sepharose as described by Brenna *et al.* (10). The column was washed with 5 mM NaCl in 10 mM Tris·HCl (pH 8.4) and then with 5 mM NaCl in 100 mM Tris·HCl buffer (pH 8.4). The APase was eluted by a linear gradient from 20 to 100 mM sodium phosphate in 150 mM

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Abbreviation: APase, alkaline phosphatase.
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Tris·HCl buffer (pH 8) with 5 mM NaCl. The pooled APase fraction was dialyzed against T buffer and applied on a DEAE-Sephacel column ($0.5\ cm^2 \times 10$ cm) equilibrated with T buffer. The APase was then eluted by a linear gradient from 0 to 0.4 M NaCl in T buffer. The pooled fraction of the enzyme was dialyzed first against T buffer and then against 50% glycerol in 10 mM Tris·HCl (pH 8.4). The purified enzyme was stored at −20°C.

APase Assay. APase activity was routinely assayed throughout the purification procedure, at 25°C for 30 min in a 100-μl reaction mixture containing 2 mM *p*-nitrophenol phosphate, 5 mM $CaCl_2$, 0.1 M cyclohexylaminopropanesulfonic acid/NaOH buffer (pH 9.5). The reaction was stopped by adding 300 μl of 13% EDTA/1 M NaOH to the reaction mixture. Control mixtures were similarly prepared, except that 13% EDTA/1 M NaOH was added before the enzyme. APase activity was measured as the difference in absorbance at 410 nm between samples and control. One unit of APase activity is expressed as 1 μmol of *p*-nitrophenol liberated per min at 25°C under the assay condition. Protein concentration was determined by the Bio-Rad protein assay kit, using bovine serum albumin as a standard.

RESULTS

Isolation of Antarctic Bacteria and Screening of Heat-Labile APases. A total of 155 bacterial strains was isolated from various field samples from the Antarctic. Of these, 32 (23%) were psychrophiles, and 107 (77%) were psychrotrophic. [Psychrophilic bacteria are defined by their abilities to grow at 0°C but not above 18°C. Psychrotrophic bacteria are those able to grow at 0°C and 18°C or higher; see Morita (11).] One hundred thirty-nine strains were tested for the presence of APase and only 3 strains were found not to produce any detectable amount of APase. Heat-labile APases were found in 26 strains, including 3 psychrophiles. All of the heat-labile APases were inactivated at 60°C with 10 min of incubation, and no activity was restored after 3 days of incubation at 25°C. Among the 26 heat-labile APase producers, strain HK47 yielded the highest APase activity and was chosen for further studies.

HK47 is a motile, rod-shaped, Gram-negative bacterium having optimal, maximal, and minimal growth temperatures of 15°C, 22°C, and −2°C or lower, respectively. The maximal growth was seen in medium containing 60% seawater and the bacteria did not grow without seawater even after 3 days of incubation.

Purification and Characterization of HK47 APase. A summary of the purification scheme for HK47 APase is presented in Table 1. Because APase activity has been found to be localized in the periplasmic space outside the cytoplasmic membrane of cells in all Gram-negative bacteria reported so far, the first step in our procedure was to treat cells by osmotic shock (12). Sixty percent of the total APase activity was successfully released by the osmotic shock procedure, whereas only 6% of the total protein was solubilized from HK47 cells under the same condition. Therefore, we could accomplish a 10-fold purification of the first step. More than 90% of the total proteins in the osmotic shock extract passed through the affinity column, and the APase activity was eluted at ≈60 mM sodium phosphate (Fig. 1*A*). As shown in Fig. 1*B*, the pooled APase from the affinity column appeared as a

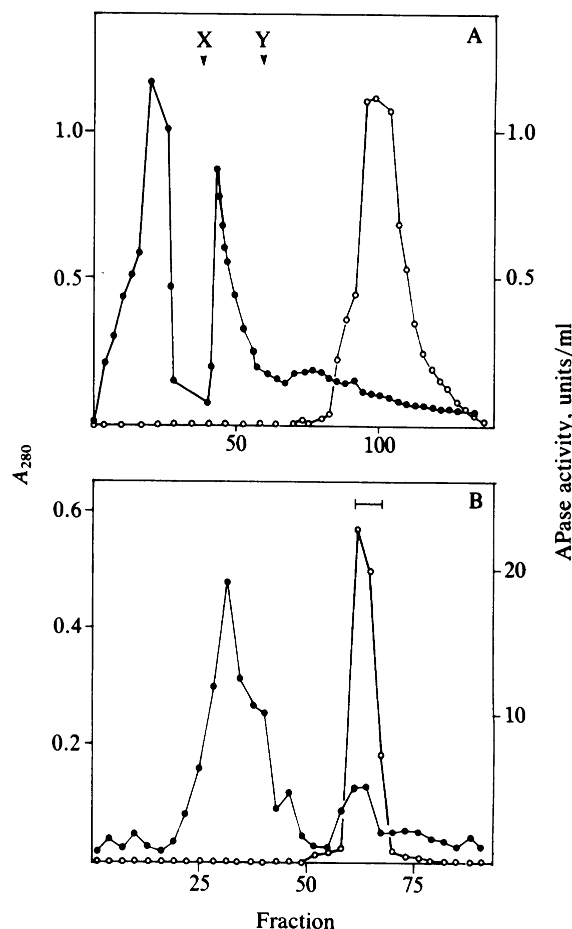

FIG. 1. (*A*) Elution profile of HK47 APase on 4-(*p*-aminophenylazo)phenyl arsonic acid/tyraminyl-Sepharose. The column was first washed with 5 mM NaCl/10 mM Tris·HCl, pH 8.4. At arrow X, 5 mM NaCl/100 mM Tris·HCl, pH 8.4, was applied, and at arrow Y, the enzyme was eluted by a linear gradient of 20–100 mM sodium phosphate in 5 mM NaCl/150 mM Tris·HCl, pH 8.4. (*B*) Elution profile of HK47 APase on DEAE-Sephacel. Fractions 60–66 (⊢⊣) were pooled and used for the studies. ○, APase activity; ●, A_{280}.

single peak at 190 mM NaCl of the DEAE-Sephacel column gradient. In separate experiments, when the osmotic shock extract was directly applied onto the DEAE-Sephacel column, APase activity was found in two peaks. It is not clear at present whether enzymes in the two peaks are isozymes, molecular aggregates, or modified enzymes. Further experiments are necessary to elucidate this point. Although the final specific activity varied from one experiment to another, we generally obtained 800–1600 units of APase activity per mg of protein. When the purified APase was reduced by 2-mercaptoethanol and analyzed by $NaDodSO_4$/10% polyacrylamide gel electrophoresis, a single band (M_r 68,000) was seen by Coomassie brilliant blue R250 staining, and one major band (M_r 68,000) and three faint bands were observed by silver staining. The apparent M_r of the native enzyme was 67,000 by P-200 gel filtration, suggesting that native HK47 APase is uniquely monomeric (Fig. 2).

The enzyme does not require the four major cations found

Table 1. Summary of the purification scheme for HK47 APase

Purification step	Protein, mg	Units	Specific activity, units/mg of protein	Yield, %
Osmotic shock fluid	119.0	668.5	5.6	100
Affinity chromatography	4.5	138.7	30.8	21
DEAE-Sephacel	0.08	72.5	906.3	11

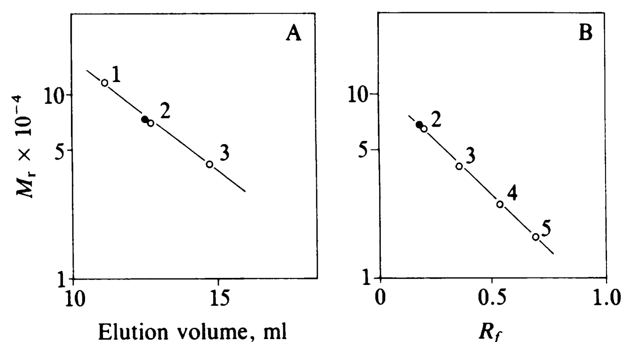

FIG. 2. Molecular weight determination of HK47 APase by Bio-Gel P-200 gel filtration (*A*) and $NaDodSO_4$/10% polyacrylamide gel electrophoresis (*B*). Molecular weight markers: D-glyceraldehyde-3-phosphate dehydrogenase (no. 1), bovine serum albumin (no. 2), ovalbumin (no. 3), chymotrypsinogen A (no. 4), and myoglobin (no. 5).

in seawater (Na^+, K^+, Ca^{2+}, and Mg^{2+}) for the manifestation of its activity. But the enzyme does require Ca^{2+} for its maximal activity. In the presence of 10 mM Ca^{2+}, the activity was 6-fold higher than the activity seen without addition of the cation to the assay mixture.

HK47 APase activity was inhibited 50% by as little as 0.1 mM EDTA and almost 100% by 1 mM EDTA. As 50% of the inactivation of *E. coli* APase occurs at 10 mM, HK47 APase is 100-fold more sensitive to EDTA than *E. coli* APase (13).

The enzyme was stable at a pH range of 7.0–9.5 at 0°C (Fig. 3*B*), and the activity was rapidly and irreversibly lost when kept at pH 4.5 or below (Fig. 3*B*). The enzyme was active within the narrow range of pH 9.0–10.0, and the optimal pH for the activity was 9.5 (Fig. 3*A*).

Many organic compounds of phosphorus are substrates for APase. By definition, APases are nonspecific to hydrolysis of substrates, regardless of the chemical nature of the leaving group. However, as shown in Table 2, as the molecules become bulkier, the rate of hydrolysis goes down. Poor hydrolysis was seen in nucleotide triphosphates. In the case

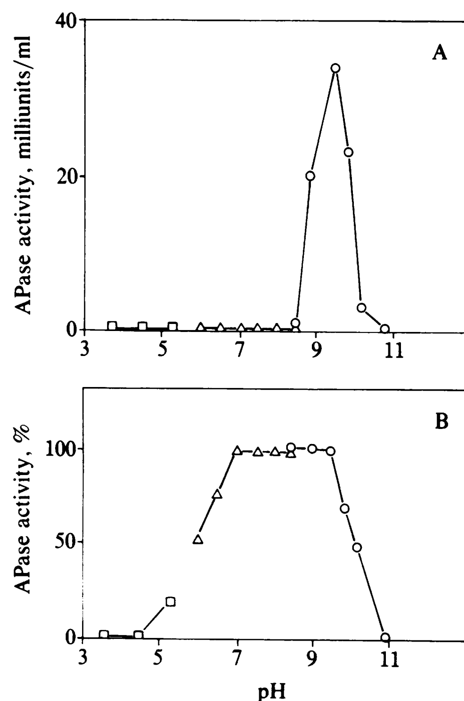

FIG. 3. (*A*) pH optimum of HK47 APase. (*B*) Stability of the APase at different pH values (relative to assay at pH 9.4). □, Sodium citrate buffer; △, Hepes buffer; ○, cyclohexylaminopropanesulfonic acid/NaOH buffer.

Table 2. Substrate specificity of HK47 APase

Substrate	P_i formation, relative rate
p-Nitrophenyl phosphate	1.00
AMP	0.11
ADP	0.09
ATP, dATP, CTP	<0.01
GTP	0.01
UTP	0.04

Substrate specificity was analyzed at a concentration of 50 mM for each substrate. Orthophosphate released was determined at 0, 30, and 60 min by the method of Murphy and Riley (14). Rates of phosphorolysis were expressed relative to *p*-nitrophenyl phosphate (1.00).

of *E. coli* APase, all of the substrates listed in Table 2 are hydrolyzed at the same rate (15).

HK47 APase Is Heat-Labile. As shown in Fig. 4*A*, HK47 APase was assayed as described in *Materials and Methods* except that the reaction mixture was incubated at various temperatures from 0°C to 60°C. The optimal temperature for the activity was 25°C, and at 0°C only 17% of the maximal activity was seen. As the assay temperature was raised beyond 25°C, the activity was rapidly lost, and at 50°C virtually no activity was detected. Fig. 4*B* shows the temperature effect on the stability of the APase. The enzyme was preincubated for 10 min without the substrate at temperatures ranging from 0°C to 60°C and then assayed with the substrate at 25°C. Little loss of the activity was seen at 10°C incubation. However, at 15°C, 40% of the activity was lost during the incubation, and complete inactivation occurred at 55°C. In a separate experiment, the half-life of the enzyme was determined to be 2 min at 40°C. Unlike *E. coli* APase, the thermal inactivation of HK47 APase is irreversible. After 3 days, incubations at 0°C, 4°C, and 25°C showed no detectable activity restoration.

End-Labeling of Nucleic Acids After APase Treatment. Fig. 5 shows that simple heat treatment inactivates HK47 APase and subsequently allows phosphorylation of DNA fragments successively by polynucleotide kinase. Terminal phosphates of *Hin*fI-digested pBR322 DNA were removed by APases of HK47 or *E. coli*, and the reaction mixtures were then treated by heat at 60°C for 10 min as described in the legend to Fig. 5. After cooling at 0°C, end-labeling was carried out by adding [γ-^{32}P]ATP and polynucleotide kinase to the reaction mixtures. As a control, dephosphorylated DNA that was treated by phenol was used for the kinase reaction (lanes 1 and 6). Lane 2 shows successful end-labeling of the DNA after the heat treatment of HK47 APase. As seen in lane 4, the heat treatment was still necessary for the reaction. No

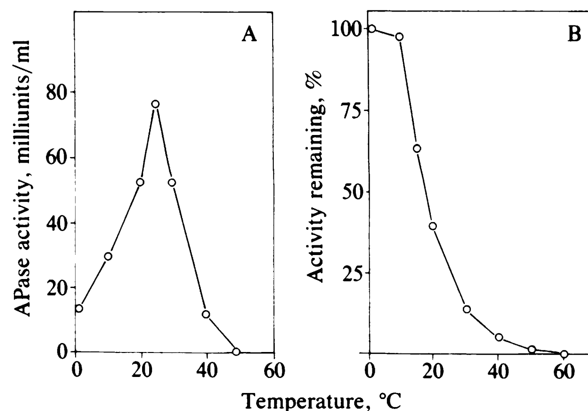

FIG. 4. Temperature optimum (*A*) and temperature sensitivity (*B*) of HK47 APase activity.

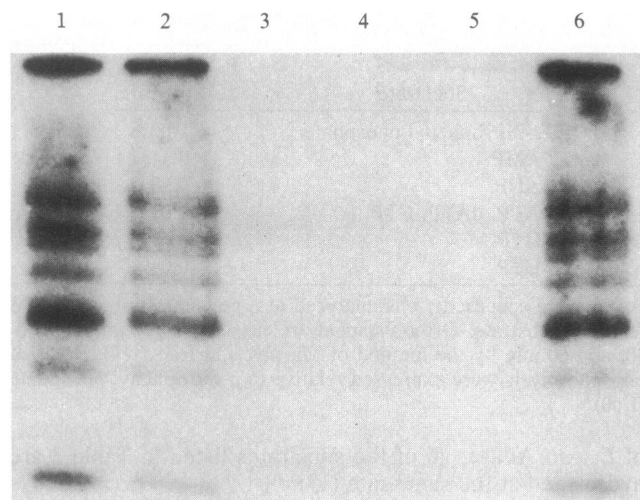

FIG. 5. Radioautograph of end-labeled *HinfI* DNA fragments with sequential treatments of HK47 APase and polynucleotide kinases. Five micrograms of pBR322 DNA was digested by 250 units of *HinfI* restriction enzyme in a 100-μl reaction mixture of 10 mM Tris·HCl, pH 7.5/50 mM NaCl/10 mM $MgCl_2$/1 mM 2-mercaptoethanol/15 μg of bovine serum albumin at 37°C for 16 hr. Terminal phosphates of *HinfI* fragments were removed by either HK47 or *E. coli* APase. In the case of HK47 APase, 0.2 μg of *HinfI* fragments was incubated in 10 μl of 100 mM cyclohexylaminopropanesulfonic acid/NaOH, pH 9.5/5 mM $CaCl_2$/0.04 units of HK47 APase at 25°C for 1 hr. *HinfI* fragments were also dephosphorylated in 10 μl of 100 mM Tris·HCl (pH 8.0) and 0.04 unit of *E. coli* APase at 37°C for 1 hr. Both reaction mixtures were incubated at 60°C for 10 min and then brought to 0°C. Phosphorylation was carried out by adding 10 μl of a mixture containing 25 mM Hepes/NaOH, pH 7.5/10 mM dithiothreitol/10 mM $MgCl_2$/0.36 μmol of [γ-^{32}P]ATP (3100 Ci/mmol; 1 Ci = 37 GBq)/1.6 units of polynucleotide kinase. Reaction mixtures were incubated at 37°C for 30 min and then chilled on ice. One microliter of the kinase reaction mixture was mixed with 4 μl of dye solution containing 0.05% xylene cyanol, 0.05% bromophenol blue, and 8.3 M urea in the TBE buffer (2.5 mM EDTA/89 mM boric acid/89 mM Trizma base). Samples were loaded onto a 10% polyacrylamide slab gel and electrophoresed in TBE buffer at 400 V until bromophenol blue reached 12 cm from the top of the gel. The ^{32}P-labeled DNA fragments were visualized by exposing the gel to Kodak XAR-5 film for 90 min at −70°C. Lanes 1 and 6, ^{32}P-labeled pBR322 *HinfI* fragments; lanes 2 and 3, *HinfI*-digested DNA, heat-treated after HK47 and *E. coli* APase, respectively; lanes 4 and 5, no heat treatment after HK47 and *E. coli* APase, respectively.

detectable phosphorylation occurred in the *E. coli* APase reaction mixture with or without heat treatment (lanes 3 and 5). A similar experiment was performed with oligoribonucleotide. Oligonucleotide ApApApCp was treated with HK47 APase and heated at 50°C for 10 min. The 5′ end of the molecule was phosphorylated by T4 kinase. As shown in Fig. 6, nearly 100% of the 3′-end phosphates were removed and 5′ termini were successfully phosphorylated.

DISCUSSION

HK47 APase was purified 160-fold from the osmotic shock fluid to near homogeneity with an 11% yield. Several characteristics of HK47 APase are unique among the known APases: the enzyme is monomeric; Ca^{2+} ions stimulate the activity; the enzyme is irreversibly denatured, even at pH 4.5; the optimal temperature for the activity is 25°C; and the enzyme is unusually heat-labile. The specific activity after the DEAE-Sephacel column is one of the highest among bacterial APases reported so far (ref. 7, p. 405). Perhaps an interesting aspect of the enzyme is its molecular structure. The active form of the enzyme is a single polypeptide with an M_r of 68,000. APase with a monomer as its active form has not been reported previously. One of the well-characterized *E.*

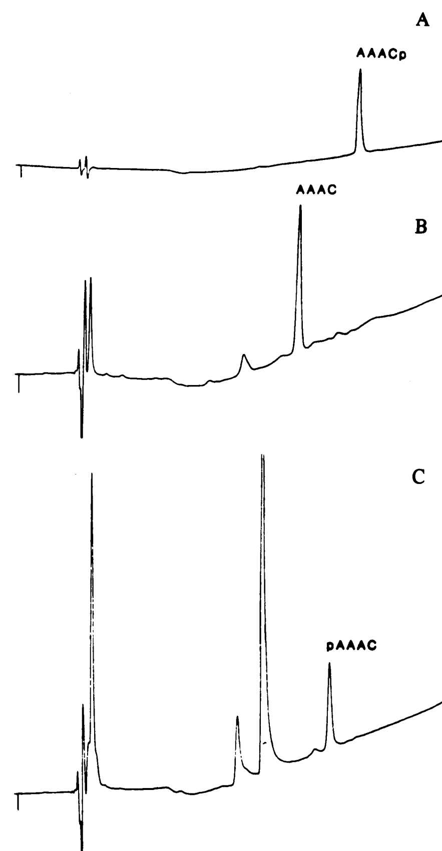

FIG. 6. Sequential treatment of an oligonucleotide with HK47 APase and T4 polynucleotide kinase. HPLC analysis was carried out on an IBM ternary gradient chromatograph with a Bio-Rad TSK 545 column. The linear gradient was 0.1–1.0 M ammonium acetate over 30 min. The phosphorylated oligonucleotide 5′-ApApApCp-3′ was prepared enzymatically by using RNA ligase. Reaction conditions were as described in the legend to Fig. 5, with the exception of the heat treatment temperature (lowered from 60°C to 50°C). (*A*) Control ApApApCp. (*B*) HK47 APase was used to dephosphorylate ApApApCp and heat treatment was done to inactivate the enzyme. (*C*) ApApApC from *B* was phosphorylated by using T4 polynucleotide kinase.

coli APase enzymes has two identical subunits with M_rs of 43,000 each. *E. coli* mutants that can form monomers, but are unable to associate the monomers spontaneously into dimers, do not produce active enzyme unless manipulations are performed to dimerize the subunits (16). This indicates that two subunits must associate before the molecule will exhibit enzymatic activity. The fact that HK47 APase does not require this dimerization for its activity suggests that the mode of action of the enzyme is different from that of *E. coli* APase.

Precisely how heat treatment inactivates the HK47 APase activity is not known. Thermal inactivation starts even at 15°C when the substrate is not in the assay mixture. The enzyme, in the presence of the substrate, has optimal activity at 25°C. However, even in the presence of the substrate, complete denaturation of the enzyme occurs in 10 min at 55°C. The half-life of the enzyme is 2 min at 40°C. Trout intestinal APase was previously the most heat-labile enzyme reported. It has a half-life of 10 min at 40°C (17).

The remarkable temperature sensitivity of HK47 appears to be a useful feature for 5′ end-labeling. The advantages of the use of HK47 APase are as follows: the enzyme is completely and irreversibly inactivated by relatively low temperature treatment; unlike calf intestinal APase, heat treatment does not require $NaDodSO_4$ for complete inactivation (18); the method is simple, safe, and quick and is therefore

appropriate for processing large numbers of labeled samples, and only a small amount of the nucleic acids is lost. This method is particularly useful for end-labeling of endonuclease fragments of DNA because endonucleolytic cleavage, dephosphorylation, and subsequent end-labeling can be performed sequentially in one reaction mixture.

A total of 155 marine bacterial strains was isolated from various samples obtained from Antarctic seawaters. Of these, 32 (23%) were psychrophiles and 107 (77%) were psychrotrophic. It seems paradoxical that in such a permanently cold environment a majority of the bacteria is not psychrophilic. Although taxonomical characteristics of the bacteria have not been studied yet, Gram-staining was negative for all 16 strains examined.

Of the total of 139 Antarctic bacterial strains tested, APase activity was found in 98% and only 3 strains did not produce detectable amounts of APases. Under the same conditions, we could not find a measurable level of *E. coli* APase whose production is normally inhibited by inorganic phosphate in the growth medium (19). Because the medium used in this experiment has enough inorganic phosphate for the enzyme production inhibition, it is suggested that APases from the Antarctic bacteria are synthesized constitutively in large quantities. Heat-labile APases were found in 26 strains of 139 Antarctic bacteria examined. In view of the rarity of heat-labile APase reported in the existing literature (ref. 7, p. 404), the Antarctic bacteria are valuable as a source for further isolation of heat-labile APases with useful characteristics.

In addition to the APase studied, bacteria from the Antarctic may provide sources for identifying and isolating new types of useful enzymes that are catalytically efficient at 0°C or that are able to be inactivated at moderate temperatures.

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