Purification and properties of DNA polymerase from *Bacillus caldodenax*

Judith A. BURROWS and Christopher R. GOWARD

Division of Biotechnology, PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wilts. SP4 0JG, U.K.

A thermostable DNA polymerase was prepared from *Bacillus caldodenax* by using a four-step chromatography procedure. The protein exists as a monomer of M_r 94000, has a pI of 4.9 and has no associated 3′–5′ or 5′–3′-exonuclease activities or endonuclease activity. The temperature optimum of the enzyme was about 70 °C and the pH for maximum activity was about 7.5. The enzyme has an absolute requirement for a bivalent cation, and maximum activity was obtained at the unusually high concentration of 70 mM-MgCl₂. Mg²⁺ could be replaced by MnCl₂ or CoCl₂, with decreased activity, at the lower optimal concentrations of 1 mM and 2.5 mM respectively. Enzyme activity was inhibited in the presence of 2′,3′-dideoxy-TTP, arabinosyl-CTP and aphidicolin. Enzyme activity was stimulated with KCl concentrations of about 100 mM, and concentrations of univalent salts above about 150 mM inhibited activity. The enzyme could use activated calf thymus DNA, poly(dA)·p(dT)₁₀ or primed single-stranded phage M13 DNA as a template and maximum activity was obtained with poly(dA)·p(dT)₁₀. The enzyme was inactive on unprimed single-stranded DNA, double-stranded DNA and polyribonucleotide template/primer. The apparent K_m values for individual dNTPs, determined with the other dNTPs at saturating concentrations, were 5.7 μM (dCTP), 6.3 μM (dATP, dGTP) and 6.4 μM (dTTP). The K_m value for the overall incorporation of each dNTP from an equimolar mixture of all four dNTPs was 24.7 μM. The k_{cat} value was about 1.05 s⁻¹. The k_{cat}/K_m value was 0.16–0.18 M⁻¹·s⁻¹ for individual dNTPs and 0.04 for the incorporation of an equimolar mixture of all four dNTPs. Some of the properties of the enzyme show it may be classified as an α-Type DNA polymerase.

INTRODUCTION

DNA polymerases (deoxynucleoside triphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7) are required by organisms for replication and repair of DNA. They catalyse the addition of a deoxynucleotide to the 3′-hydroxy end of a primer DNA sequence to pair with the next base in line on the template. Since the discovery of DNA polymerase I from *Escherichia coli* [1], DNA polymerases have been purified and characterized from many prokaryotes, eukaryotes and archaeobacteria. The mechanism of action is similar, but properties such as M_r , subunit composition and sensitivity to inhibitors differ between organisms. For example, aphidicolin is an inhibitor of eukaryotic DNA polymerases α and δ and some animal virus, bacteriophage and archaeobacteria polymerases [2]. Some of these properties have been used to classify DNA polymerases into groups, either as similar to *E. coli* DNA polymerase I (pol I-Type) or to eukaryotic DNA polymerase α (α-Type) [3].

DNA polymerases and the replication mechanisms of mesophilic organisms have been widely reported, particularly DNA polymerase I from *E. coli*. DNA polymerases from thermophilic organisms have also been studied from a wide range of bacteria. These include *Bacillus stearothermophilus* [4,5], *Thermus aquaticus* [6,7], *Thermus flavus* [8], *Thermus ruber* [9] and *Thermus thermophilus* [10]. DNA polymerase from *T. aquaticus* (Taq polymerase) was cloned and expressed in *E. coli* [11]. A number of archaeobacterial DNA polymerases have also been isolated and purified, including those from *Sulpholobus acidocaldarius* [12,13], *Thermoplasma acidophilum* [14], *Methanobacterium thermotrophicum* [15], *Sulpholobus solphataricus* [16], *Pyrococcus furiosus* [17] and *Thermococcus litoralis* (Vent polymerase; New England Biolabs, Beverly, MA, U.S.A.). These organisms grow at temperatures between 70 °C and 105 °C [18], which implies that

they possess thermostable DNA polymerases, and are therefore of interest (although the yield of biomass is low) because of their potential application in recombinant-DNA technology work. DNA polymerase is a prerequisite for many techniques used in recombinant DNA technology such as PCR [19] and DNA sequencing [20].

We have an interest in the growth and DNA replication of *Bacillus caldodenax*. The organism is the most thermophilic of the *Bacillus* spp., capable of growth at 80 °C, and was first isolated from the same environment as for *T. aquaticus* [21]. The aim of the work described in this report was to purify a DNA polymerase from *B. caldodenax*, and to provide protein for characterization of the enzyme in terms of physical properties, inhibition, stability, kinetic constants and optimum conditions for assay.

EXPERIMENTAL

Materials

Tryptone and yeast extract were obtained from Difco. Activated calf thymus DNA, dithiothreitol (DTT), phenylmethanesulphonyl fluoride (PMSF), Nonidet P40, Tween 20, trichloroacetic acid, DNA polymerase I, exonuclease III (EC 3.1.11.2), M13mp8 and pBR322 were from Sigma. Deoxynucleoside triphosphates were from Boehringer-Mannheim, and [³H]TTP was from Amersham. Optiscint Hisafe, Q-Sepharose FF, S-Sepharose FF, phenyl-Sepharose FF, poly(dA)·poly(dT), poly(dA)·p(dT)₁₀, polydeoxyadenylic acid, poly-(rA)·p(dT)₁₀ and M_r markers were from Pharmacia. Blue-Transacryl M was from Life Science Laboratories Ltd. (Luton, U.K.). 3MM chromatography paper was from Whatman. Acrylamide was from Fisons, bisacrylamide from Bio-Rad, and Ultra-Pure agarose from Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.). All other reagents were from BDH.

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; Tween 20, polyoxyethylenesorbitan; DTT, dithiothreitol; Capso, 3-(cyclohexylamino)-2-hydroxy-1-propanesulphonic acid; Caps, 3-(cyclohexylamino)-1-propanesulphonic acid; ddTTP, 2′,3′-dideoxy-TTP; arabinosyl-CTP, 1-(β-D-arabinofuranosyl)-CTP.

Measurement of DNA polymerase activity

A 50 μ l portion of assay mixture (50 mM-Tris/HCl buffer, pH 7.5, containing 70 mM-MgCl₂, 1 mM-DTT, 100 μ M-dATP, 100 μ M-dCTP, 100 μ M-dGTP, 25 μ M-dTTP, 6.25 μ g of activated calf thymus DNA and 0.5 μ Ci of [³H]dTTP, of specific radioactivity 43 Ci/mmol) was incubated with 12.5 μ l of enzyme solution (0.002–0.2 unit) at 70 °C for 30 min. The reaction was terminated by placing the reaction mixture on ice, and 50 μ l of the reaction mixture was transferred to a strip (3 cm $\times$ 1 cm) of 3MM chromatography paper. DNA was precipitated by washing in 10% (w/v) trichloroacetic acid for 1 h, followed by two further washes for 30 min each in 5% trichloroacetic acid. After a final wash in ethanol for 30 min, the chromatography paper strips were air-dried and counted for radioactivity in 4 ml of Optiscint Hi-Safe scintillant by using an LKB Wallac 1215 Rackbeta counter. One unit of DNA polymerase is defined as that amount of enzyme required to catalyse the incorporation of 10 nmol of dNTP into acid-insoluble radioactivity in 30 min at 70 °C.

Measurement of 3'-5'-exonuclease (EC 3.1.16.1) activity

Some 50 μ l of assay buffer (50 mM-Tris/HCl buffer, pH 7.5, containing 70 mM-MgCl₂, 1 mM-DTT and 1 μ g of activated calf thymus DNA labelled at the 3' end with [³H]TTP, specific radioactivity 2×10^4 c.p.m./ μ g) was incubated with 12.5 μ l of DNA polymerase (0.2–0.3 unit) at 70 °C for 2 h. Residual acid-insoluble radioactivity was determined as described above for the measurement of DNA polymerase activity. Exonuclease III (10 units) solubilized all radioactivity after 2 h incubation at 37 °C.

Measurement of 5'-3'-exonuclease (EC 3.1.4.1) activity

5'-3'-Exonuclease activity was measured using conditions similar to those for the determination of 3'-5'-exonuclease activity, except that the 3'-labelled calf thymus DNA was replaced with 1 μ g of ³H end-labelled thermostable primer DG48 [22], specific radioactivity 2×10^4 c.p.m./ μ g, annealed to a 3-fold excess of M13mp8 single-stranded DNA. *E. coli* DNA polymerase I holoenzyme (10 units) solubilized 92% of the radioactivity after 2 h incubation at 37 °C.

Measurement of endonuclease (EC 3.1.21.4) activity

DNA polymerase (0.2 unit) was incubated with 1 μ g of pBR322 in 10 mM-Tris/HCl buffer, pH 7.5, containing 50 mM-NaCl, 70 mM-MgCl₂ and 1 mM-DTT at a final volume of 20 μ l. After 16 h incubation at 70 °C, the reactions were terminated by addition of 0.4 μ l of 0.5 M-EDTA. Electrophoresis of the samples was carried out on 1% (w/v) agarose gels in 50 mM-Tris/borate buffer, pH 8.0, containing 1 mM-EDTA (running buffer) at 130 V and 30 mA for 16 h. The gel was stained for 15 min with ethidium bromide in the running buffer (1 mg/l) and observed under u.v. light.

Measurement of protein concentration

Protein concentrations were determined by the Folin method of Lowry *et al.* [23] with BSA as the standard. The protein content of column eluates was also monitored by the A_{280} .

Bacterial cell culture

Bacillus caldotenax [21] was obtained from Dr. R. J. Sharp (Division of Biotechnology, PHLS Centre for Applied Microbiology and Research) and is a wild-type strain. Cells were grown in a 25-litre New Brunswick fermenter in 2 $\times$ Luria broth (30 g of tryptone/l, 10 g of yeast extract/l and 10 g of NaCl/l). The pH was maintained at 7.2 ± 0.1 with NaOH or HCl and the tem-

perature maintained at 60 °C. Samples (20 ml) were taken during the growth and the cells recovered by centrifugation, weighed and resuspended in 50 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-EDTA, 1 mM-DTT, 0.2 mM-PMSF, 10% (v/v) glycerol, 0.01% (v/v) Nonidet P40 and 0.01% (v/v) Tween 20 (buffer A) to a final concentration of 0.1 mg/ml. Enzyme was extracted from the cells by disruption with 3 $\times$ 30 s bursts at 18 MHz in an MSE Soniprep 150 sonicator. Cell debris was removed by centrifugation (11000 *g* for 45 min at 4 °C). The enzyme was partially purified on a Mono Q HR 5/10 column (f.p.l.c.), under the same conditions as described below for anion-exchange chromatography on Q-Sepharose FF, to remove a large proportion of the contaminating nucleolytic activities. The cells were harvested after 4 h, while still in the exponential phase of growth, quick-frozen and stored at -20 °C.

Preparation of DNA polymerase

A 125 g portion of cell paste was thawed at 4 °C in 455 ml of buffer A. The non-ionic detergents Tween 20 and Nonidet P40 included in buffer A appear to stabilize the enzyme; their absence from buffers used during the purification process resulted in a greatly decreased enzyme yield. The cells were disrupted by a single passage through a 15M-8BA Manton-Gaulin homogenizer at 55 MPa. Cell debris was removed by centrifugation for 45 min at 17500 *g* and at 4 °C. The cell-free extract was applied to a 500 ml Q-Sepharose FF column (15 cm $\times$ 6.5 cm internal diameter) equilibrated with buffer A, at a linear flow rate of 60 cm/h. The column was washed with buffer A and the protein eluted with a 10-litre linear gradient of 0–1 M-NaCl in buffer A. Active enzyme was eluted in about 230 mM-NaCl. Fractions of volume 200 ml were collected, active fractions were combined and (NH₄)₂SO₄ was added to a final concentration of 1 M. The solution was applied to a 150 ml phenyl-Sepharose FF column (9.5 cm $\times$ 4.5 cm internal diameter) equilibrated with buffer A containing 1 M-(NH₄)₂SO₄ at a linear flow rate of 25 cm/h. The column was washed with equilibration buffer and the protein was eluted with a 3-litre linear gradient of 1 to 0 M-(NH₄)₂SO₄ in buffer A, followed by 250 ml of buffer A; 50 ml fractions were collected. Active enzyme fractions, eluted at the end of the (NH₄)₂SO₄ gradient and during the wash with buffer A, were combined and dialysed exhaustively against Buffer A. The dialysed active fractions were applied to a 150 ml Blue-Trisacryl M column (9.5 cm $\times$ 4.5 cm internal diameter) equilibrated with buffer A, at a linear flow rate of 25 cm/h. The column was washed with buffer A and the protein was eluted with a 3-litre linear gradient of 0–1 M-NaCl in buffer A; 50 ml fractions were collected. Active enzyme fractions, eluted in about 580 mM-NaCl, were combined and dialysed exhaustively against buffer A. The dialysed active fractions were applied to a 150 ml S-Sepharose FF column (9.5 cm $\times$ 4.5 cm internal diameter) equilibrated with buffer A, at a linear flow rate of 25 cm/h. The column was washed with buffer A and protein was eluted with a 2-litre linear gradient of 0–1 M-NaCl in buffer A; 50 ml fractions were collected. Active enzyme was eluted in about 260 mM-NaCl. Fractions containing DNA polymerase were combined on the basis of activity and electrophoretic homogeneity, dialysed exhaustively against buffer A and stored in 50% (v/v) glycerol at -20 °C. All separations were conducted at ambient temperature (18–25 °C) unless otherwise stated.

PAGE

Slab gels containing 12.5% (w/v) acrylamide were run in an LKB vertical electrophoresis unit under denaturing conditions [24]. The enzyme was electrophoresed under non-denaturing conditions using a Pharmacia-LKB PhastGel apparatus with PhastGel Gradient 8-25 gels and native buffer strips as described

by the manufacturer. The protein bands were stained with Coomassie Brilliant Blue R-350 and scanned with a Chromoscan-3 laser optical densitometer (Joyce-Loebl, Gateshead, Tyne and Wear, U.K.) to estimate the apparent M_r .

Determination of pI

A Pharmacia-LKB PhastGel apparatus was used to determine the pI by using broad-pH-range gels (pH 3.0–9.0) as described by the manufacturer. The pI was estimated from densitometer scans of the protein bands compared with appropriate Pharmacia-LKB calibration proteins. The theoretical pI values of various DNA polymerases were determined from amino acid sequences, retrieved from the NBRF Protein Identification Resources database as issued by the GCG Sequence Analysis Software Package, by using the program PEPTIDESORT [25]. Calculations were performed on a Digital Equipment Corporation Microvax II computer running the VMS v5.1 operating system.

Effect of pH

The activity of the enzyme (0.2 unit) was determined over a range of pH in appropriate assay buffers at a concentration of 50 mM: sodium citrate (pH 3.0–4.0), sodium acetate (pH 4.0–5.5), Mes (pH 5.5–6.5), potassium phosphate (pH 6.5–7.0), Tris/HCl (pH 7.0–9.0), Capso (pH 9.0–10.0) and Caps (pH 10.0–10.5). A similar profile was obtained with buffers of identical ionic strength, I , 0.1.

Determination of reaction kinetics

Kinetic data for the polymerization reaction were determined for each individual dNTP. The concentration of the dNTP of interest ranged from 0.8 to 240 μ M in the presence of the other dNTPs at the saturating concentration of 240 μ M. Kinetic data were also determined for the incorporation of each dNTP in the overall reaction from an equimolar mixture of all four dNTPs. The rate of incorporation was determined for an equimolar mixture of the four dNTPs over the concentration range 0.8–240 μ M. Assay conditions were otherwise as described above for the measurement of DNA polymerase activity. Kinetic parameters were evaluated using a non-linear regression-data-analysis program (Enzfitter, Elsevier-Biosoft, Cambridge, U.K.).

RESULTS AND DISCUSSION

Growth of *Bacillus caldodenax* and DNA polymerase production

A 25-litre bacterial cell culture produced 353 g of cell paste. DNA polymerase synthesis per cell was constant throughout the growth phase up to at least three-quarters exponential growth.

Purification of DNA polymerase

Chromatography elution profiles and a summary of the DNA polymerase purification are shown in Fig. 1 and Table 1 respectively. The apparent amount of enzyme increased to reach a maximum in the Blue-Trisacryl M eluate and this was due to the complete removal of nucleases after this step. Attempts to remove the contaminating nucleases before chromatography, by precipitation with polyethyleneimine, for example, were unsuccessful. Homogeneous enzyme was obtained after four chromatography steps, as shown by SDS/PAGE and isoelectric focusing (Fig. 2), with a final specific activity of 2083 units/mg (Table 1). The enzyme bound to anionic and cationic-exchange matrices in identical buffer composition at pH 7.5. As the enzyme has a pI of 4.9 (see below), it should only bind to the anion-exchanger Q-Sepharose FF, and thus binds to the cation-exchanger S-Sepharose FF by a mechanism other than cation exchange.

Native molecular mass and subunit composition

The native M_r determined by non-denaturing PAGE was 120000. A native M_r of 110000 was determined by f.p.l.c. gel-filtration chromatography on Superose 12 HR10/30, equilibrated with buffer A containing 100 mM-NaCl, at 8 cm/h, and calibrated with gel-filtration marker proteins from Pharmacia-LKB. The M_r of the enzyme determined by SDS/PAGE was 94000 (Fig. 2a). The discrepancy in M_r values suggests the native protein may be elongated or non-globular, or there may be disproportionate binding of SDS. The results indicate that the enzyme exists as a monomer, in common with other DNA polymerases, of M_r approx. 94000. This M_r value is similar to those reported for eukaryotic α -Type DNA polymerases [26] and the majority of bacterial DNA polymerases. For example, *E. coli* DNA polymerase I has an M_r of 109000 [27], the M_r of the DNA polymerase from *Sulpholobus acidocaldarius* is 109000 [13] and the DNA polymerase from *T. aquaticus* has an M_r of 97300 by SDS/PAGE, and a calculated M_r of 93910 from the DNA sequence [11].

pI

A calibration curve was obtained from densitometric scans of an isoelectric focusing gel (Fig. 2b) and the pI was estimated to be 4.9. The pI value, calculated from amino acid sequences by using the program PEPTIDESORT [25], for the *T. aquaticus* enzyme [11] was 6.38, that for the *E. coli* DNA polymerase I [28] was 5.37, and that for *B. subtilis* DNA polymerase III [29] was 5.23. The DNA polymerase from *B. caldodenax* is therefore apparently more acidic than the other enzymes. Eukaryotic α -Type DNA polymerases are also acidic proteins [26].

Nuclease assays

3'-5'-Exonuclease activity was separated from the DNA polymerase activity during the anion-exchange chromatography step on Q-Sepharose FF, and 5'-3' exonuclease activity was removed during the triazine-dye affinity-chromatography step on Blue-Trisacryl M. The final preparation was free from contaminating endonuclease activity. The enzyme therefore does not have associated exonuclease or endonuclease activities, although these activities may be associated with the DNA polymerase *in vivo* as part of a complex and are subsequently separated during the purification procedure. Exonuclease activity is detected in the purified DNA polymerases of bacteria, bacteriophages and some animal viruses, but, with few exceptions, is not associated with α -Type enzymes [30].

Effect of pH

The enzyme showed highest activity between pH 7.0 and 8.0, in line with the optimum pH for growth of the organism. Maximum activity was obtained in Tris/HCl, pH 7.5; 75% and 25% of this activity was obtained at pH 8.5 (Tris/HCl) and pH 6.5 (Mes) respectively and 25% of the maximum activity was measured at pH 10 (Capso). The enzyme was inactive below pH 5, perhaps surprising in view of the fact that it derives from an acid-producing thermophile.

Thermal stability

The optimum temperature for activity of the enzyme in the assay system was determined by measuring the incorporation of dNTP at various temperatures between 0 °C and 100 °C, with the maximum activity observed at 70 °C. Some 65% of the maximum activity was measured at 55 °C, and 15% of the maximum activity was obtained at 37 °C. Activity was greatly decreased as the temperature was increased to 75 °C, with only 27% of maximum activity retained. The enzyme was also incubated for

Fig. 1. Chromatography elution profiles

The elution profiles of DNA polymerase (●) and the A_{280} (—) are shown for Q-Sepharose FF (a), phenyl-Sepharose FF (b), Blue-Trisacryl M (c) and S-Sepharose FF (d). NaCl and $(\text{NH}_4)_2\text{SO}_4$ gradients are indicated with a broken line.

Table 1. Summary of DNA polymerase purification

Step	Volume (ml)	Protein (mg)	Enzyme (units)	Specific activity (units/mg)
Cell-free extract	455	14333	532	0.04
Q-Sepharose	600	1065	2452	2.30
FF eluate				
Phenyl-Sepharose	300	420	63400	151
FF eluate				
Blue-Trisacryl	215	108	94700	877
M eluate				
S-Sepharose	100	40	83300	2083
FF eluate				

30 min at temperatures between 60 °C and 100 °C and then assayed at 70 °C. A similar temperature profile was obtained. This indicates that decreased enzyme activity at temperatures above 70 °C is due to its thermal instability at these temperatures

rather than denaturation of the DNA substrate. The thermal stability of the enzyme at 70 °C was investigated over 30 h in the presence and absence of 50 % (v/v) glycerol. The $t_{1/2}$ in the absence of glycerol was about 1.5 h, whereas in the presence of glycerol the $t_{1/2}$ was increased to about 20 h (Fig. 3).

Storage stability

The stability of the enzyme was investigated over a period of 50 weeks. The enzyme was stored at -70 °C, -20 °C, 4 °C and 20 °C with or without 50 % (v/v) glycerol. Data are presented for storage of the enzyme in the presence of glycerol (Fig. 4). The enzyme was less stable in the absence of glycerol.

Effect of univalent and bivalent salts

Active enzyme has an absolute requirement for a bivalent metal cation. Maximum activity was observed with 70 mM- MgCl_2 . This concentration is extremely high compared with values reported for other thermostable DNA polymerases. High concentrations of Mg^{2+} may cause misincorporation of dNTP, and, although maximum activity is detected with 70 mM- MgCl_2 , the fidelity of the enzyme may be very low. *Taq* polymerase has

Fig. 2. SDS/PAGE and isoelectric focusing

(a) The Q-Sepharose FF (A), phenyl-Sepharose FF (B), Blue-Trisacryl M (C) and S-Sepharose FF (D) eluates were electrophoresed on a 12.5% (w/v) acrylamide gel in the presence of SDS with the following standards (1): phosphorylase *b* (M_r 94000), BSA (M_r 67000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000), soybean trypsin inhibitor (M_r 20100) and α -lactalbumin (M_r 14400). (b) A sample of S-Sepharose FF eluate (A) was focused on a PhastGel isoelectric-focusing gel (pH 3.0–9.0) with standards (B): amyloglucosidase (pI 3.50), soybean trypsin inhibitor (pI 4.55), β -lactoglobulin A (pI 5.20), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.55), horse myoglobin (pI 6.85 and 7.35), lentil lectin (pI 8.15, 8.45 and 8.65) and trypsinogen (pI 9.30).

Fig. 3. Stability of the DNA polymerase at 70 °C

The enzyme (0.2 unit) was assayed in the standard reaction mixture after incubation for various time periods at 70 °C in the presence (■) and absence (●) of 50% (v/v) glycerol. The incubation period includes the 30 min reaction time.

a reported optimum $MgCl_2$ requirement of 10 mM for the native enzyme [6,7] and of 2 mM for the recombinant protein [11]. *S. acidocaldarius* DNA polymerase has a bivalent-cation requirement of 4 mM- $MgCl_2$ for maximum activity [13]. DNA polymerase from *B. stearothermophilus* requires 20–30 mM- $MgCl_2$ for maximum activity [4,5] and the *T. flavus* enzyme requires 10–40 mM- $MgCl_2$ for DNA polymerase activity [8]. Mn^{2+} or Co^{2+} could replace the requirement of the *B. caldodenax* enzyme for Mg^{2+} , but lower activity was obtained and concentration optima were lower; 30% activity was obtained with $MnCl_2$ at the

Fig. 4. Storage stability

A number of enzyme samples of 50 μ l (1 unit) were stored at –70 °C (■), –20 °C (●), 4 °C (▲) and 20 °C (▼) in the presence of 50% (v/v) glycerol. Samples were removed for assay after various time intervals over a period of 12 months.

Fig. 5. Dependence of DNA polymerase activity on the concentration of KCl, NaCl or NH_4Cl

The activity of the enzyme (0.2 unit) was determined over a range of concentrations of KCl (●), NaCl (■) or NH_4Cl (▲) in the standard reaction mixture.

optimum concentration of 1 mM and 4% activity was obtained with $CoCl_2$ at the optimum concentration of 2.5 mM. Ca^{2+} , Ni^{2+} or Zn^{2+} could not replace Mg^{2+} . In the presence of Mg^{2+} , the *B. caldodenax* DNA polymerase was activated by the univalent salts NaCl, KCl and NH_4Cl at concentrations of 60–100 mM. The maximum activity was obtained with 100 mM-KCl and concentrations of the salts above about 150 mM inhibited the enzyme (Fig. 5).

Template specificity

The activity obtained with activated calf thymus DNA as substrate was used as a standard value of 100%. Maximum activity of 228% was recorded with the template/primer poly-(dA)·p(dT)₁₀. The enzyme was able to utilize M13mp8/DG48 template/primer as a substrate, with a relative activity of 36%. Higher activities for both poly(dA)·p(dT)₁₀ and M13mp8/DG48 were found when the enzyme was assayed at 50 °C, rather than 70 °C, because of the greater stability of the templates at the lower temperature. The enzyme was inactive on the unprimed single-stranded DNA polydeoxyadenylic acid and was also inactive on the polyribonucleotide template/primer poly-(rA)·p(dT)₁₀ when assayed at both 70 °C and 50 °C in the presence of either $MgCl_2$ (70 mM) or $MnCl_2$ (1 mM). The double-

Table 2. Kinetic data for DNA polymerase

Variation of reaction mixture	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1} \cdot \text{s}^{-1}$)
Equimolar mixture of all dNTPs: dATP, dCTP, dGTP and dTTP	24.7	1.02	0.041
dNTP varied (other dNTPs saturating):			
dATP	6.3	1.02	0.162
dCTP	5.7	1.04	0.182
dGTP	6.3	1.02	0.162
dTTP	6.4	1.08	0.168

stranded DNA substrate poly(dA)·poly(dT) was incubated with 10 units of DNA polymerase I at 37 °C to fill in protruding termini. The DNA was then used in the standard DNA polymerase assay system and the enzyme was found to be inactive.

Reaction kinetics

The K_m value for incorporation of a dNTP depends on the nucleotide composition of the DNA and the rate constants for the incorporation of all four dNTPs [31]. The kinetic data determined for incorporation of all four dNTPs at various concentrations and for incorporation of individual dNTPs with the other dNTPs at saturating concentrations are shown in Table 2. The activated calf thymus DNA template has a G + C content of 42 % and an A + T content of 58 %. The overall incorporation from an equimolar mixture of all four dNTPs, where all are in limited supply, is 24.7 μM per dNTP. The K_m values for incorporation of individual dNTPs, with the other dNTPs at saturating concentrations, may be predicted from the K_m value for the overall reaction based on the nucleotide content of the template. The predicted K_m value for dGTP and dCTP combined is 11.9 (about 5.9 μM per dNTP) and 12.8 μM for dATP and dTTP (about 6.4 μM per dNTP). These predicted results are in approximate agreement with those determined for incorporation of individual dNTPs (Table 2) and these data support the findings for DNA polymerase I of *E. coli* [31]. The K_m value for incorporation of an individual dNTP is lower than that for the overall reaction, because the other dNTPs in the reaction are at saturating concentrations and hence are freely available. The K_m value for *B. stearothermophilus* DNA polymerase, derived from a double-reciprocal plot for incorporation of all four dNTPs, was 7 μM [5].

Effect of inhibitors

The effect of several compounds known to be specific inhibitors of some DNA polymerases was determined. The enzyme activity (0.2 unit) was determined, in the presence of each concentration of inhibitor, as described for the standard assay method except that the dCTP concentration in the assay mixture was decreased to 2.5 μM in the presence of aphidicolin and arabinosyl-CTP. The DNA polymerase was inhibited by ddTTP (250 μM required for 50 % inhibition and equivalent to a dTTP/ddTTP ratio of 1:12), which terminates primer elongation and is used as the basis for DNA sequencing [20]. It is also inhibited by arabinosyl-CTP (100 μM required for 50 % inhibition), a strong competitive inhibitor, and by aphidicolin (50 μM required for 50 % inhibition), a tetracyclic diterpenoid which competes with each dNTP for binding to DNA polymerase.

Sensitivity to aphidicolin has been reported for the replicative α -Type DNA polymerases of eukaryotes, DNA polymerase δ (the α -like DNA polymerase of plant cells), yeast DNA poly-

merases I and II, viral-encoded DNA polymerases [32] and several archaeobacterial DNA polymerases [16,33,34]. Sensitivity to aphidicolin by a prokaryotic DNA polymerase has only been reported for *E. coli* DNA polymerase II, which has been classified as an α -Type DNA polymerase based on inhibition by aphidicolin and amino acid conservation [35,36]. As the *B. caldopenax* DNA polymerase is inhibited by aphidicolin, the enzyme may be involved in DNA replication, but *E. coli* DNA polymerase II is known to be involved in DNA repair and is not a replicative DNA polymerase [35]. All DNA polymerases are inhibited by ddTTP, and the degree of inhibition is used to distinguish between Pol I-Type and α -Type enzymes [30]. Pol I-Type enzymes are inhibited by a dTTP/ddTTP ratio in the range of 1:0.5 to 1:5, whereas α -Type enzymes require a higher ratio. The *B. caldopenax* enzyme requires a ratio of 1:12 for 50 % inhibition which is similar to the ratio required for α -Type enzymes [37].

The *B. caldopenax* enzyme is unusual in that it may be classified as an α -Type based on inhibition of activity by the inhibitors arabinosyl-CTP, aphidicolin and ddTTP (with a dTTP/ddTTP ratio of 1:12 for 50 % inhibition) in addition to the absence of associated nuclease activities. The classification of the enzyme as an α -Type may be confirmed once the gene has been cloned and the DNA sequenced, as shown for a number of DNA polymerases [3]. It is noteworthy that arabinosyl-CTP and aphidicolin may prove of use as structural probes for the study of substrate binding and catalysis in DNA synthesis.

We thank Professor T. Atkinson and Dr. M. D. Scawen for critical comments in the preparation of the manuscript, Mr. I. D. McEntee for culture of *B. caldopenax*, Miss H. C. Lovell for help in the determination of kinetic data, and DuPont for partial funding of the project.

REFERENCES

- Kornberg, A., Lehman, I. R., Bessman, M. J. & Simms, E. S. (1956) *Biochim. Biophys. Acta* **21**, 197–198
- Wright, G. E. & Brown, N. C. (1990) *Pharmacol. Ther.* **47**, 447–497
- Ito, J. & Braithwaite, D. K. (1991) *Nucleic Acids Res.* **19**, 4045–4057
- Stenesh, J. & Roe, B. A. (1972) *Biochim. Biophys. Acta* **272**, 156–166
- Kaboev, O. K., Luchkina, L. A., Akhmedov, A. T. & Bekker, M. L. (1981) *J. Bacteriol.* **145**, 21–26
- Chien, A., Edgar, D. B. & Trela, J. M. (1976) *J. Bacteriol.* **127**, 1550–1557
- Kaledin, A. S., Slyusarenko, A. G. & Gorodetskii, S. I. (1980) *Biokhimiya* **45**, 644–651
- Kaledin, A. S., Slyusarenko, A. G. & Gorodetskii, S. I. (1981) *Biokhimiya* **46**, 1576–1584
- Kaledin, A. S., Slyusarenko, A. G. & Gorodetskii, S. I. (1982) *Biokhimiya* **47**, 1785–1791
- Rüttiman, C., Cotorás, M., Zaldívar, J. & Vicuña, R. (1985) *Eur. J. Biochem.* **149**, 41–46
- Lawyer, F. C., Stoffel, S., Saiki, R. K., Myambo, K., Drummond, R. & Gelfand, D. H. (1989) *J. Biol. Chem.* **264**, 6427–6437
- Klimczak, L. J., Grummt, F. & Burger, K. J. (1985) *Nucleic Acids Res.* **13**, 5269–5282
- Elie, C., De Recondo, A. M. & Forterre, P. (1989) *Eur. J. Biochem.* **178**, 619–626
- Chinchaladze, D. Z., Prangishvili, D. A., Kachabaeva, L. A. & Zaalishvili, M. M. (1986) *Mol. Biol.* **19**, 1193–1201
- Klimczak, L. J., Grummt, F. & Burger, K. J. (1986) *Biochemistry* **25**, 4850–4855
- Rossi, M., Rella, R., Pensa, M., Bartolucci, S., De Rosa, M., Gambacorta, A., Raia, C. A. & Dell'Aversano Orabona, N. (1986) *Syst. Appl. Microbiol.* **7**, 337–341
- Lundberg, K. S., Shoemaker, D. D., Adams, M. W. W., Short, J. M., Sorge, J. A. & Mathur, E. J. (1991) *Gene* **108**, 1–6
- Brock, T. D. (1985) *Science* **230**, 132–138
- Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A. & Arnheim, N. (1985) *Science* **230**, 1350–1354
- Sanger, F., Coulson, A. R., Barrell, B. G., Smith, A. J. H. & Roe, B. A. (1980) *J. Mol. Biol.* **143**, 161–178

21. Heinen, W. (1971) Arch. Mikrobiol. **76**, 2–17
22. Innis, M. A., Myambo, K. B., Gelfand, D. H. & Brow, M. A. D. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 9436–9440
23. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
24. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
25. Devereux, J., Haerberli, P. & Smithies, O. (1984) Nucleic Acids Res. **12**, 387–395
26. Weissbach, A., Baltimore, D., Bollum, F., Gallo, R. & Korn, D. (1975) Science **190**, 401–402
27. Jovin, T. M., Englund, P. T. & Bertch, L. L. (1969) J. Biol. Chem. **244**, 2996–3008
28. Joyce, C. M., Kelley, W. S. & Grindley, N. D. F. (1982) J. Biol. Chem. **257**, 1958–1964
29. Hammond, R. A., Barnes, M. H., Mack, S. L., Mitchener, J. A. & Brown, N. C. (1991) Gene **98**, 29–36
30. Fry, M. & Loeb, L. A. (1986) Animal Cell DNA Polymerases, p. 26, CRC Press, Boca Raton, FL
31. Fersht, A. R., Shi, J.-P. & Tsui, W.-C. (1983) J. Mol. Biol. **165**, 655–667
32. Spadari, S., Sala, F. & Pedrali-Noy, G. (1982) Trends Biochem. Sci. **7**, 29–32
33. Nakayama, M. & Kohiyama, M. (1985) Eur. J. Biochem. **152**, 293–297
34. Zabel, H.-P., Holler, E. & Winter, J. (1987) Eur. J. Biochem. **165**, 171–175
35. Chen, H., Lawrence, C. B., Bryan, S. K. & Moses, R. E. (1991) Nucleic Acids Res. **18**, 7185
36. Iwasaki, H., Ishino, Y., Toh, H., Nakata, A. & Shinagawa, H. (1991) Mol. Gen. Genet. **226**, 24–33
37. Edenberg, H. J., Anderson, S. & DePamphilus, M. L. (1978) J. Biol. Chem. **253**, 3273–3280

Received 1 April 1992; accepted 8 May 1992