Multiple forms of DNA polymerase from the thermo-acidophilic eubacterium *Bacillus acidocaldarius*: purification, biochemical characterization and possible biological role

Mariarosaria DE FALCO*, Pasquale GRIPPO†, Mosè ROSSI*‡ and Pierangelo ORLANDO*[1]

*Istituto di Biochimica delle Proteine ed Enzimologia del Consiglio Nazionale delle Ricerche, Via Toiano 6, 80072 Arco Felice, Naples, Italy, †Istituto di Biochimica delle Macromolecole, II Università di Napoli, Via Costantinopoli 16, 80138 Naples, Italy, and ‡Dipartimento di Chimica Organica e Biologica, Università di Napoli 'Federico II', Via Mezzocannone 16, 80134 Naples, Italy

Two DNA polymerase isoenzymes, called DpA and DpB on the basis of their elution order from DEAE cellulose, were purified to homogeneity from the thermo-acidophilic eubacterium *Bacillus acidocaldarius*. The enzymes are weakly acidophilic proteins constituted by a single subunit of 117 and 103 kDa respectively. DpA and DpB differ in thermostability, in thermophilicity, in sensitivity to assay conditions and in resistance to sulphydryl-group blocking agents such as *N*-ethylmaleimide and *p*-hydroxymercuriobenzoate. They differ also in synthetic template–primer utilization, in the apparent K_m for dNTPs and in processivity. In particular, DpA utilizes more efficiently synthetic templates–primers such as poly(dA)·poly(dT), poly(dT)·$(rA)_{12-18}$ and poly(rA)·$(dT)_{12-18}$ and presents a greater tendency to accept dNTP analogues modified in the sugar or in the base ring, such as cytosine β-D-arabinofuranoside 5′-triphosphate, 2′,3′-dideoxyribonucleosides 5′-triphosphate, butylphenyl-dGTP and digoxigenin-conjugated dUTP. In addition, DpA presents an exonuclease activity that preferentially hydrolyses DNA in the 5′–3′ direction, whereas DpB lacks this activity. The possible biological role of the enzymes is discussed.

INTRODUCTION

DNA polymerases are enzymes involved in the mechanisms of DNA replication and repair that ensure faithful transmission and integrity of genetic information [1]. Since the discovery in 1950 of *Escherichia coli* DNA polymerase I by Kornberg and colleagues [1], several DNA polymerases from prokaryotic and eukaryotic organisms have been studied, providing fundamental knowledge of DNA metabolism. In particular, the study of the replicative *E. coli* DNA polymerase III holoenzyme, comprising at least 20 subunits, and the discovery of the eukaryotic α-like DNA polymerases δ and ϵ have clarified several aspects of nucleic acid–protein interactions and have provided a better understanding of the original replication model of Okazaki [1,2].

Despite the great differences in molecular mass, subunit composition and catalytic properties, studies of amino acid sequence alignment and X-ray crystallographic analysis indicate the presence of conserved sequences and of characteristic structural motives for all DNA and RNA polymerases, including RNA-dependent viral DNA polymerases [1,3]. These studies support the hypothesis of an ancestral precursor for the DNA–RNA polymerizing enzymes; accordingly, a classification based on the alignment of DNA polymerase sequences has been proposed [4].

Recently several DNA polymerases from archaebacteria [5–12] and thermophilic eubacteria [13–17] have been isolated and characterized. These enzymes have attracted the attention of investigators both for their intrinsic interest (correlated to questions arising from the study of DNA replication and repair mechanisms at high temperatures [18,19] and to evolutionary implications of amino acid sequence studies [20,21]) and because of their potential biotechnological applications. In fact, thermostable DNA polymerases have been usefully utilized for DNA and mRNA amplification by PCR [16], for sequencing by the ddNTPs method [22] and for DNA labelling by modified dNTPs (*Thermus thermophilus* DNA polymerase, according to Boehringer-Mannheim product information).

The present paper is one of the first reports on the isolation and characterization of multiple forms of DNA polymerase in a thermophilic micro-organism: the thermo-acidophilic eubacterium *Bacillus acidocaldarius* isolated in the thermal acid environment of Pisciarelli-Solfatara (Pozzuoli, Italy) by De Rosa and co-workers [23].

EXPERIMENTAL

Materials

PMSF, benzamidine, aphidicolin, *N*-ethylmaleimide (NEM), cytosine β-D-arabinofuranoside 5′-triphosphate, *p*-hydroxymercuriobenzoate (PHMB), Triton X-100, dNTPs, rNTPs, and salmon-sperm and calf-thymus DNAs were from Sigma (Milano, Italy); λ DNA and the sedimentation standards were from Boehringer-Mannheim (Mannheim, Germany).

[γ-^{32}P]ATP (3000 Ci/mmol), [8-^{3}H]dATP (10 Ci/mmol), [methyl-^{3}H]dTTP (30 Ci/mmol) and [8-^{3}H]dGTP (20 Ci/mmol) were from Amersham (Amersham, Bucks., U.K.). Acrylamide, Bio-lyte ampholytes, native isoelectric-focusing and SDS/PAGE standards were from Bio-Rad laboratories (Segrate, Milano, Italy). DEAE cellulose (DE 52), phosphocellulose (P11) and GF/C filters were from Whatman Biochemical (Springfield Mill, Kent, U.K.). Native-DNA–cellulose, M13mp18(+) single-stranded DNA, BSA (DNAse/RNAse free) and synthetic templates–primers were from Pharmacia Biotech (Milwaukee, WI, U.S.A.). M13mp18 24-mer sequencing primer (−47) number 1224 was custom synthesized by Primm (San Raffaele biomedical,

Abbreviations used: DpA and DpB, *Bacillus acidocaldarius* DNA polymerase (or deoxynucleoside-triphosphate:DNA deoxynucleotidyl transferase; EC 2.7.7.7) A and B respectively; NEM, *N*-ethylmaleimide; PHMB, *p*-hydroxymercuriobenzoate; Kelletinin A, ribityl pentakis(*p*-hydroxybenzoate); ^{w}dNTP, wrong dNTP.

[1] To whom correspondence should be addressed.

Milano, Italy) 'Digoxigenin–DNA labelling and detection kit' and nylon membranes were from Boehringer-Mannheim. Microconcentrators Centricon 30 were from Amicon (Grace Italiana S.p.A., Passirana di Rho, Italy).

Kelletinin A [ribityl pentakis(*p*-hydroxybenzoate)] was prepared as previously described [24]; butylphenyl-dGTP was kindly given by Dr. George E. Wright (Department of Pharmacology, University of Massachusetts, Worcester, MA, U.S.A.).

Enzymes

DNA polymerase I minimal nuclease (*E. coli* CM 5197), alkaline phosphatase, *E. coli* exonuclease III, T4 polynucleotide kinase, pancreatic and micrococcal DNAses were from Pharmacia.

Micro-organism and growth

The thermo-acidophilic eubacterium *B. acidocaldarius* originally isolated from the hot springs of Pisciarelli-Solfatara (Pozzuoli, Italy) [23] was grown as previously described [25]. Cells were harvested by centrifugation (at 20000 rev./min, 14 °C, using a Padberg model Z41 continuous-flow centrifuge) in the late logarithmic phase of growth, corresponding to the highest DNA polymerase specific activity of cell extracts. The biomass obtained after 20 h of growth was 1.5 g/l wet weight. Cell paste was stored at −80 °C until use.

Templates–primers preparation

Salmon-sperm and calf-thymus activated DNAs and exonuclease III-treated salmon-sperm DNA (gapped DNA) were prepared as previously described [26]. Micrococcal DNAse-treated salmon-sperm DNA was prepared as described for the activated DNA.

3′-^{3}H-labelled DNA was prepared by incubating activated DNA (1.4 mg) with 30 units of *E. coli* DNA polymerase I in the presence of 10 μM [8-^{3}H]dATP (specific activity 2 μCi/nmol) as labelled dNTP. After 1 h of incubation at 37 °C, the reaction was stopped by adding EDTA (15 mM final concentration), and the DNA was precipitated by adding 0.4 vol. of 5 M ammonium acetate and 1 vol. of isopropyl alcohol. The pellet was extensively washed with isopropyl alcohol, dried and dissolved in 10 mM Tris/HCl buffer (pH 8)/1 mM EDTA. The specific activity of 3′-^{3}H-labelled DNA was 6000 c.p.m./μg, and the resultant radioactivity was $> 99\,\%$ acid precipitable.

5′-^{32}P-labelled DNA was prepared by treating activated DNA with alkaline phosphatase, T4 polynucleotide kinase and [γ-^{32}P]ATP as described previously [27]; the specific activity of the resultant 5′-^{32}P-labelled DNA was 11600 c.p.m./μg, and the radioactivity was $> 96\,\%$ acid precipitable.

M13mp18 24-mer 1224 sequencing primer was 5′-^{32}P-end-labelled as described previously [27] and annealed to M13mp18(+) single-stranded DNA. The solution [10 mM Tris/HCl buffer (pH 8)/1 mM EDTA] containing 67 nM M13mp18 (+) single-stranded DNA and 32 nM 5′-^{32}P-labelled M13mp18 24-mer 1224 sequencing primer was heated at 80 °C for 5 min and annealed at 30 °C for 20 min. The resulting substrate was purified as described above and had a specific activity of 80000 c.p.m./μg of DNA and was over $90\,\%$ acid precipitable.

DNA polymerase activity assay

DNA polymerase activity was assayed essentially as previously described [26,28] in a reaction mixture containing, in 300 μl, 50 mM Tris/HCl buffer, pH 9, 3 mM β-mercaptoethanol, 10 mM $MgCl_2$, 70 mM KCl, 0.3 mg/ml DNase/RNase-free BSA, 160 μM each dGTP, dCTP and dTTP, 50 μM [8-^{3}H]dATP (0.1–1 μCi/nmol) and 0.5 mg/ml activated DNA. The reaction was started by addition of the enzyme to the mixture pre-warmed at 65 °C and was stopped by adding 1 ml of cold $10\,\%$ (w/v) trichloroacetic acid/2 ml of cold 0.1 M sodium pyrophosphate. The acid-precipitable product was collected on GF/C filters; the filters were washed with $1\,\%$ (w/v) cold trichloroacetic acid and dried, and the radioactivity was measured by scintillation counting. One unit of DNA polymerase was defined as the amount of enzyme catalysing in 20 min, under the standard assay conditions, the incorporation of 1 nmol of [8-^{3}H]dAMP in the trichloroacetic acid-precipitable product.

DNA polymerase activity in the presence of synthetic templates–primers [poly(dA)·poly(dT), poly(dA)·$(dT)_{12-18}$, poly(dT)·$(rA)_{12-18}$, poly(rA)·$(dT)_{12-18}$ and poly(dT)] was assayed in the standard reaction mixture (100 μl final volume) by replacing the activated DNA with 100 μg/ml each synthetic template–primer and utilizing the complementary dNTP (i.e. [8-^{3}H]dATP or [*methyl*-^{3}H]dTTP at a concentration of 50 μM and specific activity 0.5 μCi/nmol). After incubation, the reaction was stopped by adding 500 μl of a cold mixture containing 100 mM EDTA, pH 8, 2 mM dTTP or dATP, 1 mg/ml BSA and 1 mg/ml calf-thymus DNA. Acid-insoluble material was precipitated by adding 1 ml of cold $10\,\%$ (w/v) trichloroacetic acid/2 ml of cold 0.1 M sodium pyrophosphate and was processed as previously described.

Digoxigenin–dUTP labelling of DNA was performed by using the 'Digoxigenin–DNA labelling and detection kit' and *B. acidocaldarius* DpA. The enzyme (1 unit) was assayed in a modified standard reaction mixture containing, in 20 μl, linearized pBR328 DNA or denatured salmon-sperm DNA, both random-primed by a mixture of hexanucleotides, as templates–primers, 35 μM digoxigenin–dUTP and 65 μM dTTP, as the labelling mixture. After incubation for 30 min at 40 °C and 2.5 h at 55 °C, the reaction was stopped by adding 2 μl of 0.5 M EDTA, pH 8; the DNA was ethanol precipitated in presence of 0.5 M LiCl, dissolved in 50 mM Tris/HCl buffer (pH 8)/1 mM EDTA and blotted on nylon membrane. Digoxigenin–dUTP-labelled DNA was detected according to Boehringer-Mannheim product information.

Nuclease activity assay

3′–5′ exonuclease activity of DpA and DpB was tested by monitoring the release of acid-soluble radioactivity from 3′-^{3}H-labelled DNA. The assay was performed in a reaction mixture (50 μl) containing 50 mM Tris/HCl buffer, pH 9, 3 mM β-mercaptoethanol, 10 mM $MgCl_2$, 75 mM KCl, 200 μg/ml BSA (DNAse/RNase free), 20 μg of 3′-^{3}H-labelled DNA and 50–250 ng of DpA or DpB fraction VI (see the Enzymes purification procedures section of the Experimental section). The mixture was overlaid with paraffin oil and incubated, up to 1 h, in sealed Eppendorf tubes at 65 °C. The reaction was stopped by adding 100 μl of a mixture containing 100 mM EDTA (pH 8)/1 mg/ml BSA/1 mg/ml calf-thymus DNA/100 μl of $10\,\%$ (w/v) trichloroacetic acid. After 20 min of incubation in ice, the mixture was centrifuged for 20 min, at 2 °C and 38000 ***g***, and aliquots of supernatant were subjected to scintillation counting for radioactivity.

5′–3′ exonuclease activity of DpA and DpB was tested by monitoring the release of acid-soluble radioactivity from 5′-terminal-^{32}P-labelled DNAs by the same procedure described for 3′–5′ exonuclease activity.

Endonuclease activity was assayed by utilizing up to 500 ng of DpA or DpB fraction VI and the mixture described for 3′–5′

exonuclease activity, in which 2 μg of λ DNA replaced 3′-^{3}H-labelled DNA. After incubation for 1 h at 65 °C, aliquots of the reaction mixtures were electrophoresed in an agarose gel {1 % (w/v) agarose in 0.5 × 89 mM Tris/89 mM borate/2 mM EDTA buffer system [27]}, and DNA bands were revealed by UV light after ethidium bromide staining.

Enzyme purification procedures

All extraction and purification procedures were performed at 4 °C.

Extraction

The cells (100 g) were frozen and thawed three times, resuspended in 300 ml of extraction buffer [200 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.4, 6 mM β-mercaptoethanol, 0.5 mM EDTA, 5 % (v/v) glycerol, 0.2 mM each PMSF and benzamidine, and 0.2 % (v/v) Triton X-100] and homogenized by a tissue homogenizer (ULTRA-TURRAX T25; Janke and Kunkel, IKA Werke, Steussen, Germany). The homogenate was sonicated with ten 30 s bursts at 60 s intervals by Branson SONIFIER model J-17 A, using a scalpel tip at maximum power, and centrifuged at 38000 ***g***, 4 °C, for 20 min. The first supernatant was further centrifuged for 1 h, 4 °C, at 180000 ***g*** in a Beckman 60 Ti rotor. The supernatant obtained was dialysed against buffer A [50 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.4, 4 mM β-mercaptoethanol, 0.5 mM EDTA, 5 % (v/v) glycerol, and 0.2 mM each PMSF and benzamidine] and was assayed for DNA polymerase activity and protein concentration (fraction I).

DEAE cellulose chromatography

Fraction I was gently stirred in 300 ml of DEAE cellulose equilibrated in buffer A, and the suspension was centrifuged for 20 min, 4 °C, at 5000 ***g***. The resin was washed four times with 500 ml of buffer A and poured in a 5-cm-width column. After cellulose sedimentation, the column was eluted with buffer B (buffer A in which the K_2HPO_4/KH_2PO_4 buffer concentration was 200 mM), and 5 ml fractions were collected and assayed for DNA polymerase activity. The active fractions were pooled and dialysed overnight against 4 l (two changes) of buffer A (fraction II). After dialysis, the solution was layered on the top of a DEAE cellulose column (2.5 cm × 35 cm) equilibrated in buffer A; the column was washed with 500 ml of buffer A and was eluted with 450 ml of a linear gradient of buffer A and B. Two peaks of DNA polymerase activity were eluted at about 80 mM (DpA) and 120 mM (DpB) K_2HPO_4/KH_2PO_4 buffer respectively (see Figure 1). The active fractions were pooled, dialysed against buffer A and assayed for DNA polymerase activity and protein concentration (fraction III).

Phosphocellulose chromatography

DpA activity from fraction III was loaded on to a phosphocellulose P-11 column (1.5 cm × 15 cm) equilibrated with buffer A. The column was washed with 100 ml of buffer A and eluted with 60 ml of a linear gradient of buffer A and C (buffer A in which the K_2HPO_4/KH_2PO_4 buffer concentration was 400 mM). The DNA polymerase activity was eluted as a single peak at about 250 mM K_2HPO_4/KH_2PO_4 buffer. The active fractions were pooled, dialysed against buffer A and assayed for activity and protein concentration (fraction IV).

DpB activity from fraction III was processed as described for DpA by utilizing a linear gradient of buffer A and B. The DNA polymerase activity was eluted as a single peak at about 130 mM K_2HPO_4/KH_2PO_4 buffer (fraction IV).

Preparative isoelectric focusing

Preparative isoelectric focusing was performed essentially as described by Haglund [29] by utilizing an LKB-Pharmacia isoelectric-focusing column (model 8101). DpA activity from Step IV was concentrated to 2 ml by Centricon 30. The solution was adjusted to 2.5 % (v/v) Bio-Lyte ampholytes (pH 3–10)/30 % (w/v) sucrose and layered at the right density in a 40–5 % (w/v) sucrose gradient (100 ml) containing 4 mM β-mercaptoethanol/2.5 % (v/v) Bio-Lyte ampholytes, pH 3–10. The run was carried out at constant power (2 W) for 19 h [cathodic solution: 0.250 M NaOH in 60 % (w/v) sucrose; anodic solution: 1 % (v/v) phosphoric acid]. Fractions of 2 ml each were collected from the bottom of the gradient. After evaluation of pH and DNA polymerase activity, the active fractions were pooled and dialysed against 50 mM Tris/HCl buffer (pH 9.0)/3 mM β-mercaptoethanol/1 mM EDTA/0.2 mM each PMSF and benzamidine/20 % (v/v) glycerol (buffer D) (Fraction IV bis).

Native-DNA–cellulose chromatography

DpA activity from fraction IV bis was adjusted to 5 mM $MgCl_2$ and loaded on to a native-DNA–cellulose column (1 cm × 7 cm) equilibrated with buffer D. The column was washed with 20 ml of buffer D/1 mM $MgCl_2$ and eluted by 30 ml of a linear gradient of 0–1 M KCl in buffer D. Active fractions eluted in the range of 0.2–0.3 M KCl were pooled and assayed for DNA polymerase activity and protein concentration (fraction V).

DpB activity from fraction IV was dialysed against buffer D, adjusted to 5 mM $MgCl_2$ and processed as described for DpA (fraction V).

Sucrose gradient

DpA (or DpB) activity from the DNA–cellulose column was equilibrated with 50 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.4, containing 3 mM β-mercaptoethanol, 1 mM EDTA, 0.5 M KCl, 0.02 % (v/v) Triton X-100, and 0.1 mM each PMSF and benzamidine and was concentrated to 0.25 ml by Centricon 30. The solution was layered on the top of a 12 ml linear gradient of 5–20 % (w/v) sucrose in equilibration buffer. The gradient was centrifuged for 40 h, 4 °C, at 181000 ***g*** in a SW 41 Ti (Beckman) rotor, and fractions of 0.25 ml were collected from the bottom. Active fractions were pooled and dialysed against two changes of buffer A containing 25 % (v/v) and 50 % (v/v) glycerol respectively (fraction VI).

The enzymes were stored at −20 °C for further analysis, and, under these conditions, they were stable for > 12 months.

SDS/PAGE electrophoresis

SDS/PAGE was performed on slab mini-gels (Miniprotean II; BioRad) by utilizing the discontinuous buffer system (separating gel: 7.5 % total and 2.67 % cross-linking monomer concentrations) described by Laemmli [30]; protein bands were revealed by R-250 Coomassie Brilliant Blue staining.

Determination of isoelectric point (pI) by isoelectric focusing

Native-disk-gel isoelectric focusing was performed in 10 % (w/v) acrylamide gels (0.5 cm × 11 cm) containing 40 % (v/v) glycerol and 1.5 % (v/v) Bio-Lyte ampholytes, pH 3–10. The gels were allowed to polymerize overnight in the presence of 0.02 % (w/v) riboflavin. The run was carried out at 4 °C for 15 h at 200 V and for 2 h at 400 V [cathodic solution: 0.1 M NaOH; anodic solution: 0.06 % (v/v) phosphoric acid]. Native isoelectric-focusing standards, including coloured proteins, were run in

parallel gels to monitor the focusing. The gels were stained according to Görg et al. [31], and the isoelectric points of DpA and DpB were evaluated by linear interpolation with the migration line of the standards. Alternatively, replicate gels were cut in 2.7-mm-wide slices; each slice was dialysed 30 min against 0.5 ml of distilled water, for pH evaluation, or 48 h at 0 °C against 0.5 ml of an elution buffer [100 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.5, 2 mM dithiothreitol, 1 mM EDTA, 1 mg/ml BSA and 4% (v/v) glycerol] for DNA polymerase activity determination.

Gradient sedimentation analysis

Sedimentation analysis of DpA and DpB activities was performed as previously described [28]. Samples of 50 μl each containing 2000 units of DpA or DpB, 5 μg of catalase (11 *s*, 250 kDa), 5 μg of yeast alcohol dehydrogenase (7.4 *s*, 148 kDa) and 50 μg of egg albumin (3.6 *s*, 43 kDa) as internal standards were layered on the top of a 5 ml pre-formed 10–30% (v/v) glycerol gradient in buffer A/1 M KCl. Centrifugation was carried out for 18 h at 170000 ***g*** (Beckman SW 65 K rotor, 4 °C). Fractions of 130 μl each were collected from the bottom of the tubes, utilizing a Buckler apparatus. The molecular masses were calculated according to the method of Martin and Ames [32].

Thermostability and thermophilicity

The thermal stabilities of DpA and DpB were evaluated by incubating each enzyme (50 units from fraction V) in 200 μl of 50 mM Tris/HCl buffer (pH 9)/75 mM KCl/3 mM β-mercaptoethanol/0.5 mg/ml BSA/25% (v/v) glycerol at temperatures ranging from 50 to 80 °C. The preincubation was performed in sealed Eppendorf tubes, and the mixture was overlaid with paraffin oil. At the indicated times, 20 μl aliquots of the pre-incubated mixtures were drawn off and assayed for DNA polymerase activity.

The temperature dependence of DpA and DpB activities was determined by assaying the enzymes at temperatures ranging between 40 and 80 °C, in the standard mixture where salmon-sperm activated DNA was replaced by calf-thymus activated DNA.

Determination of kinetic constants

Apparent K_m values were evaluated by Lineweaver–Burk plot analysis of the changes in the initial rate of polymerase reaction as a function of increasing concentrations of one of the two substrates (i.e. activated DNA or dNTPs) in the presence of saturating concentrations of the other.

Processivity was quantified according to the kinetic method of Bambara et al. [33] by comparing the rates of polymerization of DpA and DpB on gapped DNA in the presence of a limited (2 or 3 dNTPs) or a complete complement of dNTPs. Since a limited complement of nucleotides can also affect the cycling time of the enzyme, the reactions were performed in the presence or in the absence of micrococcal-DNase-treated DNA as reversible inhibitor (DNA hydrolysis by the micrococcal DNase releases 3′-phosphorus termini that are not utilizable by DNA polymerase), and the rate values obtained were utilized in equation 11 of [33]. The theoretical curves (equation 17 of [33]) were drawn taking into account the base composition of salmon-sperm DNA for the calculation of S_X and L parameters. The assays were performed in the standard conditions (final volume 100 μl) in presence of 350 μg/ml gapped DNA/2 units of enzyme and, for the inhibited reaction, 350 μg/ml of inhibitor DNA. The dNTPs omitted were dCTP or dCTP and dGTP.

Protein determination

Protein concentration was determined by the method of Bradford [34].

RESULTS

Comments on purification procedures

DpA and DpB were purified 4243 and 4980 fold, respectively, by the procedures summarized in Table 1. To reduce protein–nucleic acid interactions and to prevent proteolysis, the cell pellets were extracted in high-ionic-strength phosphate buffer containing

Table 1 Purification scheme of DpA and DpB

NA, not applicable; –, not done.

Fraction	Volume (ml)	Protein (mg)	Activity (10^{-3} units)	Yield (%)	Specific activity (units/mg)	Purification (-fold)
Crude extract (I)	400	6600	292.80	100	44.4	NA
I DEAE (II)	80	946.3	216.70	74.0	228.9	5
II DEAE (III)						
DpA	39	125.0	67.20	22.9	537	12
DpB	54	152.8	80.20	27.4	525	12
Phosphocellulose (IV)						
DpA	9.10	9.3	57.20	19.5	6150	139
DpB	13.00	6.4	60.90	20.8	9515	216
Isofocusing (IV bis)						
DpA	10.00	1.70	29.70	10.1	17470	393
DpB	–	–	–	–	–	–
DNA cellulose (V)						
DpA	0.25	0.11	15.70	5.4	142727	3214
DpB	0.27	0.14	29.30	10.0	209286	4714
Sucrose gradient (VI)						
DpA	0.23	0.05	9.42	3.2	188400	4243
DpB	0.25	0.09	19.90	6.8	221111	4980

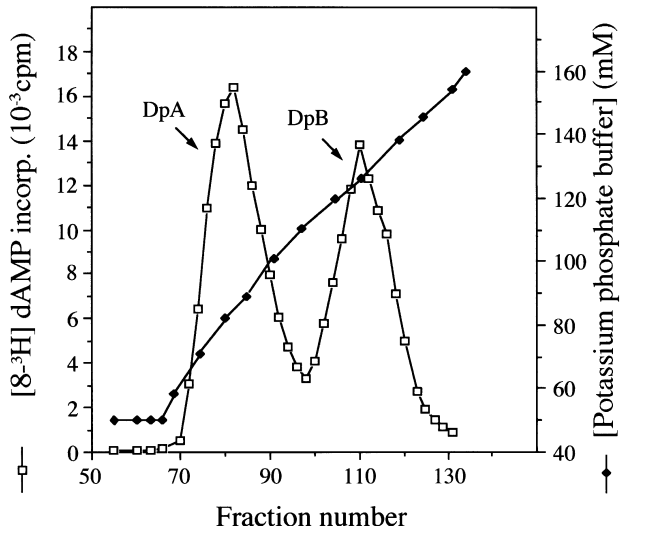

Figure 1 DEAE-cellulose chromatography of *B. acidocaldarius* DNA polymerases

DEAE-cellulose chromatography was performed as described in the Experimental section. Fractions of 2 ml each were collected, and 25 μl aliquots were assayed for DNA polymerase activity in the standard reaction conditions. incorp., incorporation.

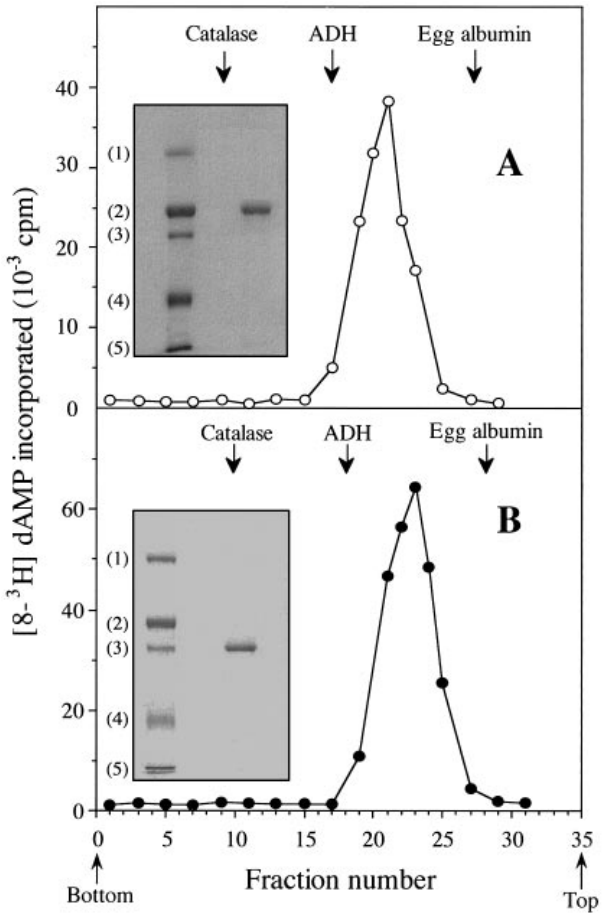

Figure 2 Glycerol-gradient sedimentation analysis and SDS/PAGE of DpA and DpB

Glycerol-gradient sedimentation analysis of DpA (**A**) and DpB (**B**) was performed as described in the Experimental section. Fractions of 130 μl each were collected from the bottom of the tubes, and 5 μl aliquots were assayed for DNA polymerase activity in the standard reaction conditions. The arrows indicate the positions of protein markers: catalase (11 *s*), yeast alcohol dehydrogenase (ADH; 7.4 *s*) and egg albumin (3.6 *s*). SDS/PAGE (insets) was performed as described in the Experimental section by using about 3 μg of DpA or DpB fraction VI. Protein standards were: (1) myosin (200 kDa), (2) β-galactosidase (116 kDa), (3) phosphorylase B (97 kDa), (4) BSA (66 kDa) and (5) egg albumin (45 kDa).

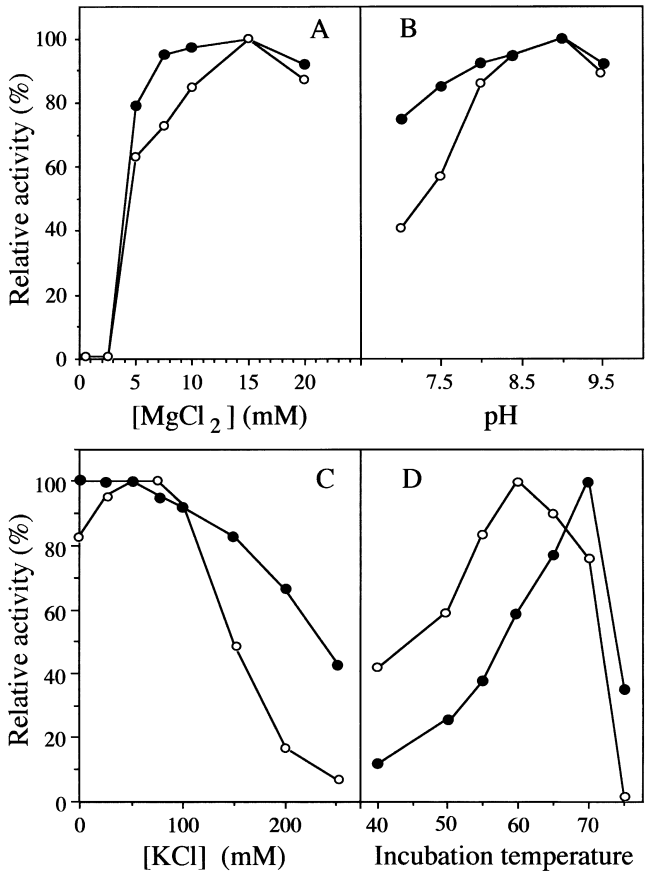

Figure 3 Reaction requirements of DpA and DpB

DpA (○) and DpB (●) were assayed in the standard conditions as described in the Experimental section, by varying the $MgCl_2$ concentration (**A**), the pH of the Tris/HCl buffer (**B**), the KCl concentration (**C**) or the incubation temperature (**D**). Relative activities were calculated by comparison of reaction velocities with the highest obtained, which was assigned the 100% value. In all the experiments the linear correlation coefficients were greater than 98%. The experiments were performed in triplicate, and the S.D. did not exceed 3% of the mean value.

EDTA and protease inhibitors. Two peaks of DNA polymerase activity with different sensitivity to salts and inhibitors and different thermophilicity were found in the absorbed fractions of DEAE cellulose column and were called DpA and DpB on the basis of elution order. Two steps of DEAE cellulose chromatography were performed: the first step was used in order to remove nucleic acids and DEAE-non-absorbed proteins from the extracts, and the second was column chromatography, eluted with a linear gradient, for analytical and quantitative separation (Figure 1).

The two DNA polymerases were further purified by phosphocellulose chromatography, performed at the same pH as DEAE-cellulose chromatography. DpA and DpB were eluted from phosphocellulose in opposite order with respect to DEAE-cellulose at about 250 and 130 mM K_2HPO_4/KH_2PO4 buffer respectively (results not shown). The enzymes were further purified by chromatography on DNA-cellulose and by sedimentation on a sucrose gradient. In order to achieve a complete purification of DpA, preparative isoelectric focusing was needed before the DNA-cellulose chromatography.

Native-DNA-cellulose chromatography was carried out at pH 9, since most of the contaminant acidophilic proteins did not bind to DNA-cellulose at this pH, thereby increasing by about 8-fold the DpA purification and about 22-fold the DpB purification with respect to the preceding step. Finally, 0.02% (v/v) Triton X-100 was included in the sucrose gradient buffer in order to improve the recovery of the enzymic activity.

Physical properties

Glycerol-gradient sedimentation analysis of DpA and DpB fractions VI, performed in high-ionic-strength buffer, gave sedimentation coefficient values of 6.0 and 5.6 *s* respectively (Figures 2A and 2B), from which molecular masses of 117 and 109 kDa were calculated. The same sedimentation coefficients were obtained by using low-ionic-strength buffer and/or partially purified preparations of the two enzymes (results not shown). These data suggest that DpA and DpB preserve their native structure during the purification procedures.

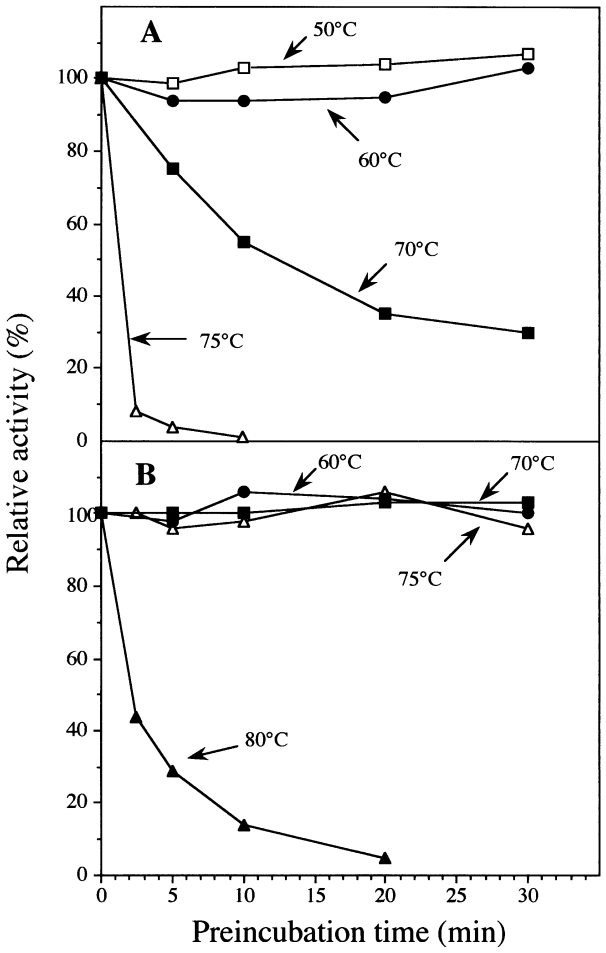

Figure 4 Thermal stability of DpA and DpB

DpA (**A**) and DpB (**B**) were preincubated at the indicated temperatures as described in the Experimental section. Aliquots of preincubated mixtures were drawn off at the indicated time and assayed for DNA polymerase activity. Relative activities were calculated by comparing [8-^{3}H]dAMP incorporation obtained after 10 min of incubation at 65 °C, 100% representing the activity of non-preincubated enzyme. This experiment is representative of three independent trials (S.D. $\leqslant$ 10%); each assay was performed in triplicate and the S.D. did not exceed 3% of the mean value.

SDS/PAGE analysis of DpA and DpB (Figures 2A and 2B, insets) showed single protein bands of 117 and 103 kDa, respectively, that co-migrated with the DNA polymerase activity (results not shown). These results indicate that the *B. acidocaldarius* DNA polymerases are each constituted by a single subunit of about 100 kDa, as described for other thermophilic DNA polymerases [5,9,10,17].

Both the enzymes are weakly acidophilic proteins, with an isoelectric point ranging from 5.9 to 6.1, which reflects the intracellular pH of *B. acidocaldarius* [23].

Reaction requirements, thermophilicity and thermostability

In the presence of activated DNA as template–primer, DpA and DpB required for maximum activity the four dNTPs, Tris/HCl buffer, pH 9, Mg^{2+} at a concentration of about 15 mM and KCl at a concentration of about 80 mM (Figure 3).

Both enzymes showed 80% of relative activity in presence of 5 mM Mg^{2+} and were inactive at a concentration of 2.5 mM Mg^{2+} (Figure 3A). Mn^{2+} ions were partially effective (about 20% of relative activity at the optimum concentration of 2 mM), whereas Ca^{2+} ions were completely ineffective.

The DNA polymerase activity of DpA was more strictly dependent on pH (Figure 3B) and KCl concentration (Figure 3C) than DpB activity was. In particular, only DpA was activated by KCl in the 100 mM range, and it was drastically inhibited by increasing the salt concentration; in contrast, DpB was more resistant to the KCl effect.

The dependence of DpA and DpB activities on the incubation temperature is shown in Figure 3D. First-order kinetics, with increasing velocity, were observed up to 60 and 70 °C for DpA and DpB respectively. At higher temperatures, a decrease in the initial velocity associated with premature arrest of the reaction, probably due to thermal inactivation, was observed.

The thermal stability of DpA and DpB at various temperatures is shown in Figure 4. DpA (Figure 4A) was less thermostable than DpB (Figure 4B). In fact DpB, up to 75 °C, was 100% resistant (for almost 30 min) to pre-incubation, and its half-life at 80 °C was about 3 min. In contrast, the half-life of DpA at 70 °C was about 10 min and the residual activity, after 2 min of incubation at 75 °C, was about 10%. One can observe that thermophilicity and thermostability of DpA and DpB appear to be strictly related to the growth-temperature range (58–80 °C) of *B. acidocaldarius* [23].

Table 2 Exonuclease activity of DpA and DpB on 3′-^{3}H- and 5′-^{32}P-terminal-labelled DNAs

DpA and DpB exonuclease activities were assayed on 3′-^{3}H- or 5′-^{32}P-terminal-labelled DNAs as described in the Experimental section. The incubation was performed for 20 min at [a]65 °C or [b]37 °C. [3′-^{3}H]DNA, 3′-^{3}H-terminal-labelled activated DNA; M13 [5′-^{32}P]DNA, M13mp18(+) single-stranded DNA annealed with [5′-^{32}P]24-mer 1240 sequencing primer; [5′-^{32}P]DNA, 5′-^{32}P-terminal-labelled activated DNA. The input acid-precipitable radioactivity was about 100 000 c.p.m. for [3′-^{3}H]DNA (specific activity 6000 c.p.m./μg), 170 000 c.p.m. for [5′-^{32}P]DNA (specific activity 11 600 c.p.m./μg) and 80 000 c.p.m. for M13 [5′-^{32}P]DNA (specific activity 80 000 c.p.m./mg). Two individual trials were performed, and each determination was performed in triplicate; the S.D. did not exceed 8% of the mean value. NA, not applicable; –, not done.

		Acid-soluble radioactivity released (% of input radioactivity)		
Enzyme	Units	[3′-^{3}H]DNA	M13 [5′-^{32}P]DNA	[5′-^{32}P]DNA
None[a]	NA	2.2	15	2.5
DpA[a]	20	5.0	49	19.8
DpB[a]	20	2.2	13	2.4
E. coli exonuclease III[b]	3	44.0	–	–

Nuclease activities

No degradation of circular λ DNA was observed in our reaction conditions when up to 500 ng of DpA or DpB fraction VI (corresponding to about 100 and 115 units, respectively, of DNA polymerase activity) were assayed for endonuclease activity.

To test the presence of exonuclease activity and the directionality of hydrolysis, DNA substrates with 3′-^{3}H- or 5′-^{32}P-terminal labels were utilized in the optimum reaction conditions and in absence of the four dNTPs. An exonuclease activity that preferentially hydrolyses the 5′-^{32}P-terminal-labelled DNA was found for DpA fraction VI (Table 2). No exonuclease activity was revealed, in contrast, for DpB.

In this regard it is pertinent to mention that thermostable DNA polymerases have been described that: (i) do not present exonuclease activity [9,15,17,20]; (ii) present a 3′–5′ [10,12,20]

Table 3 Effect of inhibitors on the activity of DpA and DpB

DpA and DpB were assayed in the presence of inhibitors as described in the Experimental section. The standard reaction conditions were modified as indicated: [a]dCTP concentration was lowered to 25 μM; [b]the enzymes were preincubated with the inhibitors for 10 min at room temperature and then assayed. The 100% value represents the incorporation of 400 pmol of [8-^{3}H]dAMP in 20 min. Three individual trials were performed, and each determination was performed in triplicate; the S.D. did not exceed 8% of the mean value.

Inhibitor	Concentration	Residual activity (%) DpA	DpB
No addition		100	100
Aphidicolin[a]	50 μg/ml	100	100
NEM[b]	5 mM	71	100
	10 mM	44	100
PHMB[b]	2.5 mM	51	100
Kelletinin A	25 μM	88	85
	50 μM	42	64

exonuclease activity or a 5′–3′ exonuclease activity, strongly coupled to chain growth [1,12,35]; or (iii) present both the activities [8], such as *E. coli* DNA polymerase I [1].

Interestingly, DpB was more thermostable and thermophilic than DpA, suggesting that the lack of exonuclease activity could stabilize DpB against thermal inactivation.

Inhibitors

In Table 3 the effect of the tetracyclic diterpenoid aphidicolin, an inhibitor of α-like DNA polymerases [1], and of sulphydryl-groups-blocking reagents NEM and PHMB on DpA and DpB activities is shown. Both the enzymes were 100% resistant to 50 μg/ml of aphidicolin (a complete inhibition of *Xenopus laevis* DNA polymerase α [36] was achieved, with the same aphidicolin solution, at a concentration of 5 μg/ml).

DpA, but not DpB, was sensitive to preincubation with NEM and PHMB, thus suggesting the presence in DpA of sulphydryl groups involved in the catalytic activity.

In addition, the effect of Kelletinin A, an irreversible inhibitor of DNA–RNA polymerizing enzymes [24,37], was also tested. For both the enzymes the time course of reaction (results not shown) was linear during the first minutes and then declined with time, reaching a plateau. This mechanism is reminiscent of that observed for other DNA–RNA polymerizing enzymes [37].

Utilization of unusual or modified deoxyribonucleotides

A modified or 'unusual' deoxyribonucleotide ([w]dNTP, where w is wrong) can influence, or not, the rate of DNA synthesis. Generally speaking it can be recognized, or not, by DNA polymerase, it can be incorporated, or not, into DNA, and after incorporation it can influence, or not, chain elongation (for a review see [38]). The effect of [w]dNTPs on DpA and DpB activities was evaluated in the presence of saturating concentrations for all dNTPs and dATP or dTTP or dUTP or dGTP as labelled dNTPs (Table 4).

Both DpA and DpB presumably incorporate specifically dUTP, 7-deaza-dATP and dITP instead of dTTP, dATP and dGTP respectively. In fact the decrease in residual activity observed in the presence of these [w]dNTPs parallels the decrease in the specific activity of the corresponding labelled dNTP. For example, at equimolar ratio, 7-deaza-dATP decreased [8-^{3}H]dATP incorporation by about 40% but did not affect [*methyl*-^{3}H]dTTP and [8-^{3}H]dGTP incorporation even at a molar ratio of dATP/7-deaza-dATP = 5:1. Furthermore, 5-methyl-dCTP even at 1 mM concentration did not dilute labelled dTTP, but it was

Table 4 Effect of unusual or modified dNTPs on DpA and DpB activities

DpA and DpB were assayed in the standard reaction conditions, as described in the Experimental section (the assay volume was lowered to 100 μl), in the presence of activated DNA and [8-^{3}H]dATP, [*methyl*-^{3}H]dTTP, [5-^{3}H]dUTP or [8-^{3}H]dGTP as the labelled dNTP (20 μM and 0.2 μCi/nmol each). The concentration of the three complementary dNTPs was 100 μM or as otherwise indicated: [a]dCTP was replaced by 100 μM 5-methyl-dCTP; [b]the concentration of dCTP was lowered to 20 μM; [c]the concentration of dGTP was lowered to 20 μM. The 100% value represents the incorporation of 200 pmol of [8-^{3}H]dAMP, [*methyl*-^{3}H]dTMP, [5-^{3}H]dUTP or [8-^{3}H]dGMP in 10 min. Three individual trials were performed, and each determination was performed in triplicate; the S. D. did not exceed 7% of the mean value. Abbreviations: AraCTP, cytosine β-D-arabinofuranoside 5′-triphosphate; BuPdGTP, butylphenyl-dGTP.

Labelled dNTP	NTP added	Concentration (μM)	Residual activity (%) DpA	DpB
[*methyl*-^{3}H]dTTP	No addition	0	100	100
	dUTP	20	49	43
		100	30	25
	7-deaza-dATP	500	100	100
	dITP	500	100	100
	5-methyl-dCTP	1000	100	100
	5-methyl-dCTP[a]	100	100	100
[5-^{3}H]dUTP	No addition	0	100	100
	dTTP	20	50	55
[8-^{3}H]dATP	No addition	0	100	100
	7-deaza-dAT	20	68	57
		200	30	31
	dITP	500	100	100
[8-^{3}H]dGTP	No addition	0	100	100
	7-deaza-dATP	500	100	100
	dITP	100	75	75
		500	35	3
[8-^{3}H]dATP	AraCTP[b]	100	74	94
		200	60	87
	ddGTP[c]	50	64	88
		250	38	71
	BuPdGTP[c]	250	44	96

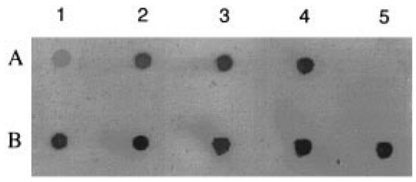

Figure 5 Incorporation of digoxigenin–dUTP into DNA by DpA

Dot-blot analysis of digoxigenin–dUTP incorporation into DNA by DpA and Klenow enzyme. Samples of 1 unit of DpA or 2 units of Klenow enzyme were used in all assays, and neo-synthesized DNA was processed as described in the Experimental section. Lane 1A, 3 μg of unlabelled DNA; lanes 2A, 3A and 4A, 3 μg of unlabelled DNA plus 5, 20 or 100 ng of digoxigenin–dUTP-labelled control DNA respectively. Lanes 1B and 2B, DpA and Klenow enzyme, respectively, assayed by using linearized pBR328 DNA primed by a mixture of random hexanucleotides; lanes 3B and 4B, DpA and Klenow enzyme assayed by using single-stranded salmon-sperm DNA primed by a mixture of random hexanucleotides; lane 5B, DpA assayed by using activated salmon-sperm DNA.

Table 5 Activity of DpA and DpB on synthetic templates–primers

DpA and DpB (1.25 units each) were assayed in the presence of the synthetic templates–primers as described in the Experimental section. The standard reaction conditions were modified as indicated: [a]the enzymes were assayed by replacing Mg^{2+} ions with 2 mM Mn^{2+} ions; [b]the assays were performed in the presence of 1 mM ATP. Each value represents the mean of three independent determinations (S.D. $\leqslant$ 7% of the mean value); in each determination the linear correlation coefficient was greater than 98%. Each value in parentheses is the percentage of efficiency of utilization of the template–primer.

Template–primer	Incubation temperature (°C)	Activity (pmol of dNTP incorporated/min)	
		DpA	DpB
Activated DNA	60	63.6 (100)	61.5 (100)
Poly(dA)·(dT)	60	56.2 (88)	16.3 (26)
Poly(dA)·$(dT)_{12–18}$	60	26.1 (41)	7.9 (13)
Poly(dT)·$(rA)_{12–18}$	60	> 1	14.3 (23)
	50	32.7 (51)	4.0 (6)
Poly(rA)·$(dT)_{12–18}$[a]	60	> 1	2.2 (3)
	50	11.0 (17)	> 1
Poly(dT)[b]	40 → 50 → 60	> 1	> 1

able to replace dCTP. rNTPs could not substitute for dNTPs even at high concentration (results not shown).

DpA was more sensitive than DpB to the chain terminators cytosine β-D-arabinofuranoside 5′-triphosphate and ddGTP [1] and to dGTP-competitive inhibitor butylphenyl-dGTP [39], thus indicating a greater tendency to accept dNTP analogues that are modified in the sugar or that present large substituents in the base ring. In fact DpA was able to incorporate into newly synthesized DNA digoxigenin-conjugated dUTP by using as templates–primers linearized pBR328 DNA and denatured salmon-sperm DNA, both primed by a mixture of random hexanucleotides, or activated DNA (Figure 5).

Template–primer specificity

In Table 5 the specificity of DpA and DpB for some synthetic templates–primers with respect to the activated DNA is shown. DpA utilized the synthetic templates–primers tested more efficiently than DpB did. In particular, both the enzymes were able, at 60 °C, to utilize either poly(dA)·poly(dT) or poly(dA)·$(dT)_{12–18}$. Furthermore, DpA utilized efficiently at 50 °C (51% of efficiency) poly(dT)·$(rA)_{12–18}$; in contrast, at 60 °C, the polymerization activity of DpA on this template–primer was undetectable. Apparently this behaviour, also shown for poly(rA)·$(dT)_{12–18}$, is an intrinsic characteristic of the enzyme, and it is not correlated to a greater instability of these template–primers at 60 °C because (i) the rate of polymerization of DpB on these templates–primers was greater at 60 °C than at 50 °C; and (ii) DpA utilized efficiently at 60 °C the homologue substrate poly(dA)·$(dT)_{12–18}$. It is interesting to note that the DNA polymerases B and C from *T. thermophilus* [15] showed with synthetic polynucleotides optimal temperatures somewhat lower than with activated DNA, with the exception of poly(dA-dT).

No DNA primase activity was detected when the enzymes were assayed on unprimed poly(dT) in the presence of dATP and ATP [36]. To stabilize the putative DNA primase product by primer elongation, the reaction was performed by increasing progressively the incubation temperature from 40 to 60 °C (as shown, DpA retains 40% of the DNA polymerizing activity at 40 °C). Alternatively, aliquots of the incubation mixture were drawn off at different incubation temperatures and were incubated at 37 °C in the presence of 2 units of *E. coli* DNA polymerase I in order to amplify the DNA primase reaction products.

Both DpA and DpB were able, in the presence of Mn^{2+} ions, to use poly(rA)·$(dT)_{12–18}$ as template–primer in the reverse-transcription reaction (17% and 3.5% of relative efficiency respectively). Furthermore, DpA and DpB activity on this template–primer was strictly dependent on the temperature of incubation and the Mn^{2+} concentration.

Kinetic determinations and processivity

Table 6 shows DpA and DpB kinetic parameters compared with data available in the literature. Steady-state kinetic parameters were determined by using pseudo-first-order conditions, i.e. by varying the concentration of one substrate (gapped DNA or dNTP) in the presence of an excess of the second substrate. DpA and DpB gave comparable apparent K_m^{DNA} values and quite different apparent K_m^{dNTPs} values. By comparing the apparent K_m^{dNTPs} of DNA polymerases from *Bacillus* and *Thermus* species, it is evident that the affinity for dNTPs decreases with increasing thermostability.

Table 6 Comparison of kinetic parameters of thermostable DNA polymerases

DpA and DpB were assayed as described in the Experimental section. Apparent K_m values for dNTPs are related to the total concentration of the four dNTPs (present in an equimolar mixture). Data are from [a]the present paper and [b][17]. [c]Data from [12] were recalculated: the original apparent K_m values for dNTPs are expressed as mol of each dNTP in a equimolar mixture of the four dNTPs. Abbreviation: d.m., different (not comparable) methodology; –, not done. The number of independent trials is given in parentheses; K_m values are means ± S.D.; values separated by commas indicate individual determinations.

DNA polymerase (pol)	Apparent K_m^{DNA} (μg/ml)	Apparent K_m^{dNTPs} (μM)		Inactivation		Processivity (residues)
		Mean	Range	Temperature (°C)	Half-life	
DpA[a]	13 ± 2.2 (3)	19.5	22, 17 (2)	70	10 min	7.7 ± 2.1 (5)
DpB[a]	15 ± 1.7 (3)	57.0	61, 53 (2)	80	3 min	17.5 ± 3.3 (5)
B. stearothermophilus pol[b]	–	85 ± 10		80	–	–
Bacillus caldotenax pol[b]	–	85 ± 10		85	–	–
Bacillus caldovelox pol[b]	–	85 ± 10		85	–	–
T. thermophilus pol[b]	–	115 ± 12		95	–	–
Thermus aquaticus pol[c]	d.m.	60	56, 68	95	1.6 h	42 ± 30
Thermus litoralis pol[c]	d.m.	164 ± 108		95	8 h	7 ± 3

The processivity (the average number of nucleotides added by a polymerase in a single round of synthesis) was determined by the Bambara et al. [33] protocol that compares polymerization levels in the presence of a full or a partial complement of dNTPs. Because the binding of DNA polymerase to template–primer and the addition of the first nucleotide are generally slower steps than the addition of the subsequent nucleotides, the omission of one or more nucleotides slows the incorporation by a processive DNA polymerase, for which re-initiation events occur less frequently than for a distributive enzyme. Both the enzymes were processive on gapped DNA as template–primer, and DpB was more processive than DpA (Table 6).

DISCUSSION

The presence of multiple forms of DNA polymerase in thermophilic micro-organisms and their involvement in DNA replication and repair mechanisms is a question not yet explored enough. Three DNA polymerase isoenzymes that differ in sensitivity to NEM, in thermal stability and in the utilization of templates–primers have been characterized from the thermophilic eubacterium *T. thermophilus* [15]. Two DNA polymerases separable by DEAE cellulose have been described in the archaebacterium *Halobacterium halobium* [6,40]. One of the enzymes resembles the eukaryotic DNA polymerase α involved in DNA replication [1]: in fact it presents a DNA primase activity and it is inhibited by aphidicolin and NEM; the other enzyme resembles the eukaryotic DNA polymerase β, involved in DNA-repair mechanisms [1], because it is not inhibited by aphidicolin and NEM and it is sensitive to ddNTPs. On the other hand, several papers report the presence of a single DNA polymerase either in archaebacteria [5,7–10] or in thermophilic eubacteria [13,14], including *Bacillus* species and *T. thermophilus* [17].

Our data clearly indicate the presence in the thermophilic-acidophilic eubacterium *B. acidocaldarius* of two DNA polymerases distinguishable on the basis of their physical and catalytic properties.

Analysis of the physical properties of these enzymes shows that both DpA and DpB are weakly acidophilic proteins, each comprising a single subunit and differing in molecular mass, in behaviour on DEAE or phosphocellulose columns and in thermostability. The hypothesis that *B. acidocaldarius* DNA polymerases could be derived one from the other by non-physiological proteolysis, or that they could derive from a common precursor because of extraction and purification artefacts, can be excluded on the basis of the following considerations: (i) the use of different extraction buffers as well as different extraction procedures did not significantly influence the ratio of the two enzymes; (ii) no interconversion of DpA into DpB was observed during the purification procedures or during prolonged storage; (iii) DpA and DpB have similar molecular masses, and several native DNA polymerases each constituted by a single subunit of about 100 kDa have been isolated from thermophilic micro-organisms [5,9,10,17]. Furthermore, the presence of two bacterial strains in the culture can be excluded both by taxonomic considerations [23] and because a single β-galactosidase (F. La Cara, P. Orlando, F. Nazzaro, S. Stellato, R. Cozzolino, P. Grippo and M. Rossi, unpublished work) and a single alcohol dehydrogenase [25] were isolated from the same cellular preparations. In contrast with our data, despite the similarity of the first chromatographic steps, the presence of only one major DNA polymerase devoid of exonuclease activity has been reported in *Bacillus* species [17]. Differences in cell-growth techniques and/or extraction procedures or, rather, peculiarities of *B. acidocaldarius* DNA polymerases such as their acidophilic character could tentatively explain the discrepancy of the findings.

In addition to their different thermophilicities and sensitivities to the assay conditions and sulphydryl-groups-blocking agents, DpA and DpB differ in synthetic template–primer and in modified dNTPs utilization, in the apparent K_m^{dNTPs} and in processivity.

DpA utilizes with good efficiency synthetic templates–primers such as the 'unnatural substrate' poly(rA)·dT_{12-18}. This ability, common to several DNA polymerases, including *E. coli* DNA polymerase I [41] and some thermophilic enzymes [42,43], is one of the arguments supporting the hypothesis of an 'ancestral precursor' for DNA and RNA polymerizing enzymes. It is interesting to note that Kelletinin A, a natural drug characterized in our laboratory, inhibits to a different extent DNA and RNA polymerizing enzymes [24], viral reverse transcriptases [37] and the *B. acidocaldarius* DNA polymerases. It has been suggested [37] that the drug could bind to the C-terminal active-site-associated structural motif, a cleft conserved among various DNA and RNA polymerases, to which the template–primer binds.

The results obtained do not permit the definition of the biological role of the enzymes. However, the presence in DpA of an exonuclease activity that preferentially hydrolyses the DNA in the 5′–3′ direction could suggest for this enzyme a potential role in DNA-repair mechanisms, such as removal of mismatched nucleotides or RNA primer from DNA [1]. In contrast, DpB could be involved in DNA replication, because of its greater thermostability, processivity and presumable fidelity (in fact evidence of a positive correlation between processivity and fidelity has been reported [44]). An alternative hypothesis is that DpB could derive from DpA by a post-translational modification, correlated with environmental conditions, in which DpA lacks part of the exonuclease domain. It is interesting to note that *B. acidocaldarius* is able to sporulate in adverse environmental conditions, and it is tempting to speculate that DpB could be stockpiled during sporulation.

Only genetic analysis and, possibly, the selection of lethal mutants may clarify the biological roles of DpA and DpB. It is our intention to carry on with an extensive study of this problem also, in view of the potential biotechnological applications in reverse amplification, in DNA sequencing (for reducing 'band compressions' [45]) and in non-radioactive DNA labelling.

We thank Dr. George E. Wright for kindly giving the butylphenyl-dGTP sample and Professor Paola Pierobon for helpful discussions and critical reading of the manuscript. We also thank Mrs. Rosamaria Parisi and Mr. Domenico Pollice for outstanding technical assistance.

REFERENCES

1 Kornberg, A. and Baker, T. A. (1992) DNA Replication, 2nd edn., W. H. Freeman and Company, New York
2 Bambara, A. R. and Jessee, C. B. (1991) Biochim. Biophys. Acta **1088**, 11–24
3 Joyce, C. M. and Steitz, T. A. (1994) Annu. Rev. Biochem. **63**, 777–822
4 Braithwaite, D. K. and Ito, J. (1993) Nucleic Acids Res. **21**, 787–802
5 Klimczak, L. J., Grummt, F. and Burger, K. J. (1985) Nucleic Acids Res. **13**, 5269–5282
6 Nakayama, M. and Kohiyama, M. (1985) Eur. J. Biochem. **152**, 293–297
7 Rossi, M., Rella, R., Pensa, M., Bartolucci, S., De Rosa, M., Gambacorta, A., Raia, C. A. and Dell'Aversano Orabona, N. (1986) Syst. Appl. Microbiol. **7**, 337–341
8 Klimczak, L. J., Grummt, F. and Burger, K. J. (1986) Biochemistry **25**, 4850–4855
9 Elie, C., De Recondo, A. M. and Forterre, P. (1989) Eur. J. Biochem. **176**, 619–626
10 Hamal, A., Forterre, P. and Elie, C. (1990) Eur. J. Biochem. **190**, 517–521
11 Lundberg, K. S., Shoemaker, D. D., Adams, M. W. W., Short, J. M., Sorge, J. A. and Mathur, E. J. (1991) Gene **108**, 1–6
12 Kong, H., Kucera, R. B. and Jack, W. E. (1993) J. Biol. Chem. **268**, 1965–1975
13 Chien, A., Edgar, D. B. and Trela, J. M. (1976) J. Bacteriol. **127**, 1550–1557

14 Kaledin, A. S., Slyusarenko, A. G. and Gorodetskii, S. I. (1981) Biokhimiia **46**, 1576–1584
15 Rüttimann, C., Cotorás, M., Zaldívar, J. and Vicuña, R. (1985) Eur. J. Biochem. **149**, 41–46
16 Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H. A. (1988) Science **239**, 487–491
17 Sellmann, E., Schröder, K. L., Knoblich, I. M. and Westermann, P. (1992) J. Bacteriol. **174**, 4350–4355
18 Elie, C., Salhi, S., Rossignol, J. M., Forterre, P. and De Recondo, A. M. (1988) Biochim. Biophys. Acta **951**, 261–267
19 Salhi, S., Elie, C., Forterre, P., De Recondo, A. M. and Rossignol, J. M. (1989) J. Mol. Biol. **209**, 635–644
20 Pisani, F. M., De Martino, C. and Rossi, M. (1993) Nucleic Acids Res. **20**, 2711–2716
21 Uemori, T., Ishino, Y., Toh, H., Asada, K. and Kato, I. (1993) Nucleic Acids Res. **21**, 259–265
22 Innis, M. A., Myambo, K. B., Gelfand, D. H. and Brow, M. A. D. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 9436–9440
23 Millonig, G., De Rosa, M., Gambacorta, A. and Bu'Lock, J. D. (1975) J. Gen. Microbiol. **86**, 165–173
24 Orlando, P., Carretta, F., Grippo, P., Cimino, G., De Stefano, S. and Strazzullo, G. (1991) Experientia **47**, 64–66
25 D'Auria, S., La Cara, F., Nazzaro, F., Vespa, N. and Rossi, M. (1996) J. Biochem. **120**, 498–504
26 Grippo, P., Locorotondo, G. and Caruso, A. (1975) FEBS Lett. **51**, 137–142
27 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor
28 Orlando, P., Geremia, R., Frusciante, C., Tedeschi, B. and Grippo, P. (1988) Cell Differ. **23**, 221–230
29 Haglund, H. (1967) Science Tools **14**, 17–23
30 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
31 Görg, A., Postel, W. and Westermeier, R. (1978) Anal. Biochem. **89**, 60–70
32 Martin, R. C. and Ames, B. N. (1961) J. Biol. Chem. **236**, 1372–1379
33 Bambara, R. A., Uyemura, D. and Choi, T. (1978) J. Biol. Chem. **253**, 413–423
34 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
35 Tindall, K. R. and Kunkel, T. A. (1988) Biochemistry **27**, 6008–6013
36 Orlando, P., Geremia, R., Frusciante, C. and Grippo, P. (1989) Cell Differ. Dev. **27**, 129–136
37 Orlando, P., Strazzullo, G., Carretta, F., De Falco, M. and Grippo, P. (1996) Experientia **52**, 812–817
38 Goodman, M. F., Creighton, S., Bloom, L. B. and Petruska, J. (1993) Crit Rev Biochem. Mol. Biol. **28**, 83–126
39 Khan, N. N., Wright, G. E., Dudycz, L. W. and Brown, N. C. (1984) Nucleic Acids Res. **12**, 3695–3706
40 Nakayama, M., Ben-Mahrez, K. and Kohiyama, M. (1988) Eur. J. Biochem. **175**, 265–270
41 Loeb, L. A., Tartof, K. D. and Travaglini, E. C. (1973) Nature New Biol. **242**, 66–69
42 Tse, W. T. and Forget, B. G. (1990) Gene **88**, 293–296
43 Myers, T. W. and Gelfand, D. H. (1991) Biochemistry **30**, 7666–7672
44 Eckert, K. A. and Kunkel, T. A. (1990) Nucleic Acids Res. **18**, 3739–3744
45 Mizusawa, S., Nishimura, S. and Seela, F. (1986) Nucleic Acids Res. **14**, 1319–1324

Received 15 May 1997/11 August 1997; accepted 19 August 1997