Volume 13 Number 14 1985 Nucleic Acids Research

Purification and characterization of DNA polymerase from the archaebacterium *Sulfolobus acidocaldarius*

Leszek J.Klimczak*, Friedrich Grummt+ and Klaus J.Burger

Institut für Genetik und Mikrobiologie and +Institut für Biochemie, Universität Würzburg, Röntgenring 11, D-8700 Würzburg, FRG

Received 16 May 1985; Accepted 20 June 1985

ABSTRACT

DNA polymerase has been purified about 25,000-fold from the thermoacidophilic archaebacterium Sulfolobus acidocaldarius. On SDS-PAGE the enzyme was observed to have a molecular weight of 100 kDa and to be about 90% pure. The native molecular weight was 108 kDa indicating that the enzyme is composed of a single polypeptide. Activity gel analysis showed an active polypeptide of about 100 kDa. Under conditions promoting proteolysis this polypeptide was degraded to a slightly smaller form of 98 kDa. The enzyme has been characterized in respect to optimal assay conditions, template specificity, sensitivity to inhibitors and associated nuclease activities. The high temperature optimum of 65°C should be emphasized. No substantial similarities have been found with other prokaryotic and eukaryotic DNA polymerases, although the enzyme bears certain resemblances to prokaryotic non-replicative polymerases.

INTRODUCTION

A substantial amount of information on DNA polymerases from different prokaryotic and eukaryotic organisms is known (1,2). Although several similarities among the different polymerases have been recognized and e.g. formed the basis of an extensive classification of eukaryotic enzymes, still not much is known about the evolution of DNA replication.

This is due to the fact that there are still significant groups of organisms in which DNA replication has not been substantially investigated. For example, only recently were DNA polymerases from plants purified and characterized (3,4). Nothing is known about the enzymology of DNA replication in archaebacteria which constitute a third urkingdom separate from eubacteria and eukaryotes (5), thus being a suitable object for investigation of evolutionary aspects of DNA replication.

© IRL Press Limited, Oxford, England.

To compare archaebacterial DNA polymerases with their eubacterial and eukaryotic counterparts we have initiated the purification and general characterization of these enzymes. In this report we describe a preparation of DNA polymerase from the thermoacidophilic archaebacterium Sulfolobus acidocaldarius.

MATERIALS AND METHODS

Materials.

Herring sperm DNA was activated as described by Aposhian and Kornberg (6). Deoxyribonucleotide triphosphates and polynucleotide templates were obtained from Boehringer, Mannheim, and radioactive substrates from Amersham. DEAE-cellulose (DE-52) and phosphocellulose (P-11) were purchased from Whatman; Blue Sepharose 6B-CL, Phenyl-Sepharose 4B-CL and Sephacryl S-200 from Pharmacia and hydroxylapatite (Ultrogel HA) from LKB. Specially pure SDS was from BDH and polyacrylamide from Bio-Rad.

Growth of cells.

Sulfolobus acidocaldarius cells (DSM 639) were obtained from Dr. D. Palm, University of Würzburg, Germany. They were grown at 77°C as described by Brock et al. (7).

Enzymatic assays.

DNA polymerase. The assay was performed in a 40 ul volume containing 40 mM Tris-HCl pH 8.0, 1 mM $MgCl_2$, 100 ug/ml bovine serum albumin, 10 uM each of dATP, dGTP and dCTP, 2.5 uM TTP, 0.2 uCi of (^3H)TTP (specific activity 30 Ci/mmole), 0.1 mg/ml DNaseI-activated herring sperm DNA to which 1 ul of a 1:10 dilution of the fraction to be assayed was added. After incubation at 56°C for 30 min, the reaction was terminated by addition of 10 ul 0.5 M EDTA containing 10 mg/ml tRNA and the acid-precipitable radioactivity was determined. One unit of DNA polymerizing activity is defined as the amount of enzyme catalyzing the incorporation of 10 nmole of TTP under these conditions.

Nuclease. A 20 ul assay mixture contained 40 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 100 ug/ml bovine serum albumin, 2 mM

2-mercaptoethanol, 0.02-0.1 units of DNA polymerase and the appropriate substrate. For measuring 3'->5' exonuclease, $2x10^4$ cpm of activated DNA labeled at the 3' end with (^{3}H)-TTP to a specific activity of $2x10^5$ cpm/ug was used, either native or heat-denaturated. For measuring 5'->3' exonuclease, $2x10^4$ cpm of (^{32}P)dA-poly(dA) (specific activity - 10^6 cpm/ug) was used, either alone or annealed to 5 times molar excess of poly(dT). Both assays were incubated for 30 min at 37°C as well as at 56°C and the acid-soluble radioactivity was determined. The endonuclease assay was performed with 0.2 ug of plasmid pBR322 DNA which was 70% supercoiled and 30% relaxed. After incubation for 30 min at 37°C or 56°C the DNA was analyzed on a 1% agarose gel.

Protein determination.

Protein was determined according to Sedmak and Grossberg (8) using bovine serum albumin as standard.

Purification of the DNA polymerase.

The preparation was performed at 4°C. No substantial loss of activity was observed when intermediate and final fractions were stored frozen at -20°C in the presence of 20% glycerol for several weeks.

1. Preparation of cell extract. 30 g cells were harvested by centrifugation at 5000 g for 15 min and were washed with 10 volumes of buffer A containing 50 mM Tris-HCl pH 8.0, 5 mM 2-mercaptoethanol, 20 mg/l benzamidine-HCl, 10 mg/l B-propionitrile fumarate, and 1 mg/l soybean trypsin inhibitor. The cell pellet was suspended in 3 volumes of fresh buffer A and the suspension was passed through a French press at 65 MPa. Immediately before loading the press, PMSF was added to the bacterial suspension to a final concentration of 50 ug/ml. The resulting viscous solution was centrifuged at 87000 g for 20 min to sediment all tiny, opalescent particles. The pellet was then extracted with 1 volume of buffer A containing freshly added PMSF (50 ug/ml) and the above centrifugation step was repeated. The two supernatants were pooled and saved as fraction I.

2. DEAE-cellulose chromatography. Fraction I was loaded on a DEAE-cellulose column (3x10 cm) equilibrated with buffer A and the flow-through, containing the polymerase activity, was collected as fraction II.

3. Phosphocellulose chromatography. Fraction II was applied to a phosphocellulose column (3x10 cm) equilibrated with buffer A. The column was washed with 5 volumes of the equilibration buffer and subsequently eluted with 8 volumes of a linear gradient of 0-1 M KCl in buffer A. A single peak of activity emerged around 300 mM KCl. Active fractions were pooled as fraction III.

4. Hydroxylapatite chromatography. Fraction III was loaded on a hydroxylapatite column (1.5x6 cm) equilibrated with 600 mM KCl in buffer A. The column was washed with the same buffer (5 volumes), then equilibrated with buffer B (10 mM potassium phosphate pH 7.0, 5 mM 2-mercaptoethanol, 20% glycerol and the protease inhibitors used in buffer A), and developed with 8 volumes of a linear gradient of 10-400 mM potassium phosphate pH 7.0 in buffer B. Fractions containing the activity eluted between 100-150 mM potassium phosphate. They were pooled and dialyzed against buffer D (50 mM Tris-HCl pH 7.0, 5 mM 2-mercaptoethanol, 20% glycerol and the protease inhibitors used in buffer A). The dialysate was designated fraction IV.

5. Blue Sepharose chromatography. To fraction IV, $MgCl_2$ was added to final concentration of 10 mM and the fraction was loaded on a Blue Sepharose column (1x12 cm) equilibrated with buffer D containing 10 mM $MgCl_2$. The column was washed with 3 volumes of equilibration buffer, 2 further volumes containing of 15 mM NAD, 1 volume containing 15 mM NAD and 15 mM NADP, and finally with 2 volumes of equilibration buffer alone. The column was then eluted with 12 volumes of a linear gradient of 0-400 mM KCl in the equilibration buffer. The activity emerged between 150-200 mM KCl and the active fractions were pooled as fraction V.

6. Phenyl-Sepharose chromatography. Ammonium sulphate was added to fraction V (20% saturation) and the solution was applied

to a Phenyl-Sepharose column (0.5x6 cm) equilibrated with 20% saturated ammonium sulphate in buffer D′ (without glycerol). The column was washed with 5 volumes of equilibration buffer and was developed with 10 volumes of a linear gradient of 20-0% ammonium sulfate and 0-50% ethylene glycol in buffer D′. The activity eluted as a single peak at 40-50% ethylene glycol. From the pooled active fractions ethylene glycol was removed using an Amicon microconcentrator and the solution was concentrated to 100 ul (fraction VI).

7. Glycerol gradient centrifugation. Fraction V was loaded on a linear gradient of 10-30% glycerol in buffer A containing 300 mM KCl, prepared in a siliconized tube. The gradient was centrifuged for 42 hours at 39000 rpm and 4°C in an SW40 Beckman rotor. 31 fractions were collected in siliconized tubes and the active fractions were pooled and concentrated to 100 ul.

RESULTS AND DISCUSSION

Purification of the S.acidocaldarius DNA polymerase.

The purification of the enzyme is summarized in Table I and the analysis of the polypeptide composition of the final fractions on a denaturating gel is shown in Fig.1. A 43 kDa protein copurified with the activity on several different columns (data not shown), and it was very diffucult to remove it. We

Table I.
Purification scheme of the S.acidocaldarius DNA polymerase.

Step	Fraction	Volume (ml)	Total activity (units)	Yield %	Total protein (mg)	Specific activity (units/mg)	Purification (-fold)
I.	Crude extract	150	2930	100	4069	0.7	1
II.	DEAE-cellulose	160	5083	173	1840	2.8	4
III.	Phosphocellulose	75	3280	112	32	102	146
IV.	Hydroxylapatite	20	4382	150	11	398	568
V.	Blue Sepharose	25	2608	89	0.66	3952	5646
VI.	Phenyl-Sepharose	3.1	1456	49	0.25	5824	8320
VII.	Glycerol gradient	0.1	858	29	0.048	17875	25536

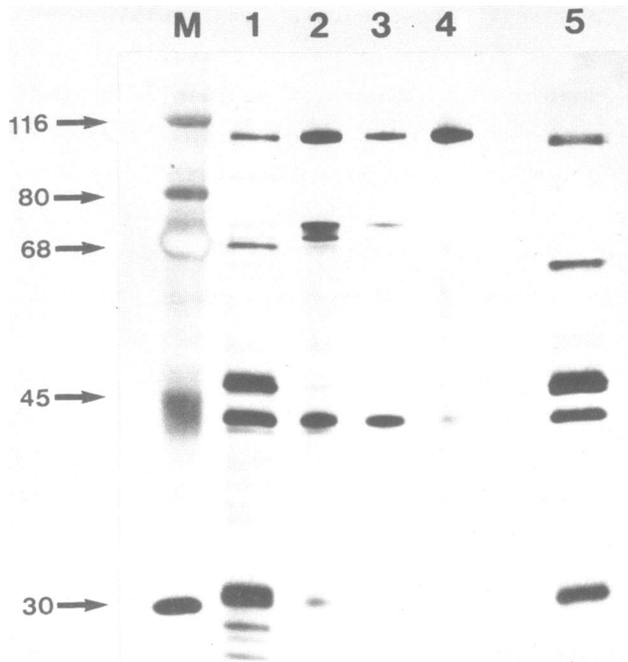

Figure 1. SDS-PAGE of final purification steps of the DNA polymerase. Electrophoresis under denaturating conditions was performed according to Laemmli (15) using slab gels containing 4% and 10% polyacrylamide in the stacking and separating gels, respectively. Proteins were visualized by a silver staining procedure described by Wray et al. (16). Lane labels: M - molecular weight standards given in kDa; 1 - hydroxylapatite pool (fraction IV); 2 - Blue Sepharose pool (fraction V); 3 - Phenyl-Sepharose pool (fraction VI); 4 - glycerol gradient pool (fraction VII); 5 - fraction IV from another preparation not sufficiently protected against proteolysis.

devised a procedure in which this contaminating protein was partially removed on the Blue Sepharose column during the wash with NAD and NADP and the remainder was almost entirely removed by glycerol gradient sedimentation. The resulting preparation was purified about 25,000-fold and a 100 kDa band comprised about 90% of it (Fig. 1).

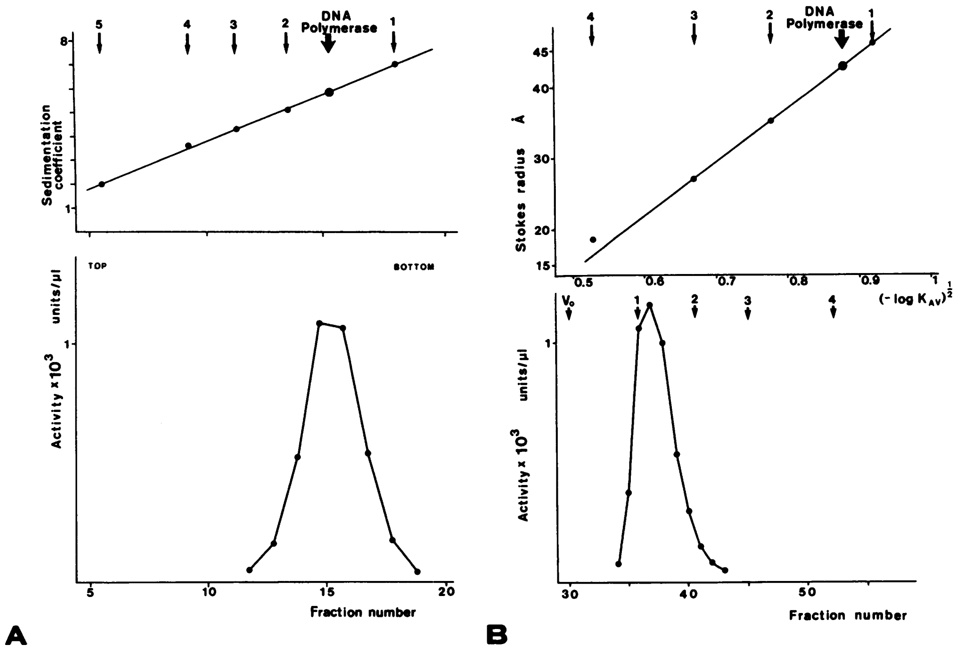

Figure 2. Physical properties of native DNA polymerase.
A. Glycerol gradient sedimentation. 2 units of fraction V were loaded on a 10-30% glycerol gradient in buffer A containing 300 mM KCl, preformed in a SW40 rotor tube, and were centrifuged at 4°C and 39000 rpm for 36 h. 31 fractions were collected from the bottom and the volume of the top fraction was taken into account. Sedimentation coefficient calibration was done with following standards: 1. human gamma-globulin ($s_{20,w}$=7.0), 2. transferrin (5.1), 3. bovine serum albumin (4.3), 4. ovalbumin (3.6) and 5. myoglobin (2.0).
B. Gel filtration. A Sephacryl S-200 column (0.7x50 cm) equilibrated with 300 mM KCl in buffer B was loaded with 2 units of fraction V (100 ul) and eluted overnight while 250 ul fractions were collected. Stokes radius standards were 1. yeast alcohol dehydrogenase (46 Å), 2. bovine serum albumin (35 Å), 3. ovalbumin (27 Å) and 4. myoglobin (18.7 Å).

Properties of the DNA polymerase.

Native molecular weight. - Centrifugation of the DNA polymerase through the glycerol gradient along with several marker proteins was used to determine its sedimentation coefficient (9). The activity sedimented as a single symmetrical peak exibiting an $s_{20,w}$ value of 5.8±0.2 (Fig.2A). From data obtained by gel filtration through a calibrated Sephacryl S-200

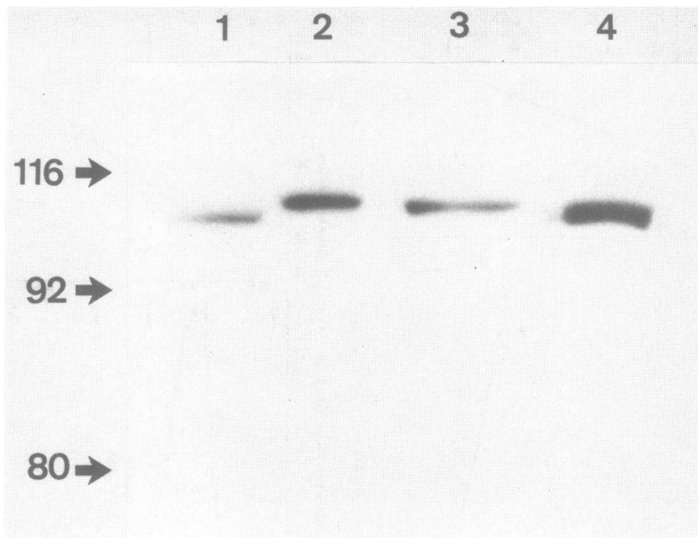

Figure 3. Detection of active subunits of the DNA polymerase in situ after SDS-PAGE. Activity gel technique was performed according to the protocol of Karawya et al. (17) with the in situ assay performed at 60°C. Protein molecular weight standards, given in kDa, were run in one lane, which was subsequently cut off and stained for 6 hours in 0.005% Coomassie Blue, 10% isopropanol, 10% acetic acid and destained overnight in 10% methanol, 5% acetic acid. Lane numbers: 1 - crude extract (fraction I); 2 - phosphocellulose pool (fraction III); 3 - Blue Sepharose pool (fraction V); 4 - fraction V from the proteolytically degraded preparation.

column a Stokes radius of 42.5±1.0 Å was calculated (Fig.2B). The native molecular weight of the enzyme was calculated to be 108±6 kDa using the two values in the equation of Siegel and Monty (10).

Subunit composition. - One protein band of molecular weight 100 kDa was highly enriched during consecutive purification steps and in the final fraction it was the major species (Fig.1, lanes 1 to 4). Based on the native molecular weight of the enzyme (108 kDa), this polypeptide was concluded to be the single subunit of the DNA polymerase.

Activity gels. - Direct detection of DNA polymerase catalytic subunits in situ after SDS-PAGE and protein renaturation revealed

in purified fractions one activity band of 100 kDa (Fig.3, lanes 2 and 3). This further confirmed the above identification of the polymerase polypeptide.

In crude extracts the 100 kDa activity band was not present, and an activity band of a slightly smaller molecular weight (98 kDa) was observed instead (Fig.3, lane 1). Later fractions containing the 100 kDa active polypeptide did not show any loss of DNA polymerase activity in relation to the crude extract. This excludes the possibility that a minor DNA polymerase species, not observed as an activity band in the crude extract, would have been enriched upon purification and would have substituted for the 98 kDa form. These observations suggest rather that the 98 kDa active polypeptide could be derived from the 100 kDa one by proteolytic cleavage. In the presence of protease inhibitors the 100 kDa polypeptide cannot be cleaved and persists in the preparation. However, the mild denaturation of the samples before loading on the activity gel strongly promotes proteolysis (for discussion see Pringle, 1975). During the preparation of the sample from the crude extract, degradation could occur, generating the 98 kDa fragment of the 100 kDa DNA polymerase. More purified fractions were not degraded, because the presumptive proteases had been removed during the purification.

In another independent DNA polymerase preparation, which was apparently not sufficiently protected against proteolysis, the 98 kDa activity band was observed even in fractions from the later purification steps in addition to the 100 kDa band (Fig.3, lane 4). In this preparation an additional 98 kDa protein was observed on the silver-stained polyacrylamide gel (Fig.1, lane 5). This protein must have accumulated in the preparation at earlier stages and therefore could be observed even after the removal of the proteases. These data demonstrate the exact correlation between the activity bands and the silver-stained bands.

The occurence of the smaller fragment is interesting because a DNA polymerase from another archaebacterium, *Methanobacterium thermoautotrophicum*, is also composed of a single polypeptide of

Table II.
Properties of the DNA polymerase from S.acidocaldarius.

Conditions	Percent activity 100% - standard conditions (0.01 units per assay)
1. Salt optimum	
a. 0 mM KCl	100
b. 100 mM KCl	22
c. 200 mM KCl	6
2. Temperature optimum	
a. 37°C	7
b. 56°C	100
c. 65°C	140
d. 70°C	130
e. 80°C	12
3. Mg^{+2} dependence	
a. 0 mM $MgCl_2$	21
b. 0.1 mM $MgCl_2$	90
c. 1 mM $MgCl_2$	100
d. 5 mM $MgCl_2$	33
e. 0 mM $MgCl_2$+ 0.1 mM EDTA	1
f. 2 mM $MgCl_2$+ 1 mM EDTA	69
g. 0 mM $MgCl_2$+ 1 mM EGTA	6
4. Template specificity	
a. no DNA	1
b. activated DNA	100
c. poly(dA) and 1 mM UTP§	1
d. poly(dT) and 1 mM ATP§	1
e. poly(dA):oligo(dT)	30
f. poly(rA):oligo(dT)	2
5. Inhibitors	
a. 1 mM arabinosine-CTP	54
b. 1 mM N-ethylmaleimide#	56
c. 25 uM ddTTP$	70
d. 20 ug/ml aphidicolin	102

§ (^{3}H)-dATP incorporated
# 2-mercaptoethanol omitted
$ ddTTP:dTTP = 10:1

about 100 kDa, and has two forms differing by about 2 kDa, as well (Klimczak et al., in preparation). It remains to be established whether this similarity is a reflection of the relatedness of these polymerases.

pH, salt and temperature optima (Table II). - The enzyme was active in a broad range of pH values between 5.5 and 9.5 and the

optimum was dependent on buffer composition. For example: in 50 mM potassium phosphate the maximum activity was at pH 6.0, 70% of the maximal value was observed at pH 5.5 and 20% at pH 8.0. By contrast, in 50 mM Tris-HCl the optimal pH was 8.0 and 70% of the maximal activity was observed at 7.0 and 9.5.

The DNA polymerase required an extremely low concentration of Mg^{+2} cations for activity. A broad plateau was observed between 0.1 and 2 mM $MgCl_2$. Concentrations above 2 mM were already inhibitory. Interestingly, 20% of the activity of the plateau value was still observed even if no $MgCl_2$ was added to the assay (the activated DNA had been dialyzed to remove the $MgCl_2$ contained). No activity was, however, detected when 0.1 mM EDTA was additionally present. The inhibitory action of 1 mM EDTA could be reversed by an excess of $MgCl_2$ (2 mM). On the other hand, EGTA, which does not chelate Mg^{+2} ions strongly, did not inhibit the activity completely even at 1 mM concentration in the absence of added $MgCl_2$.

The enzyme was inhibited when KCl was added to the assay (80% inhibition by 100 mM), although it was not irreversibly inactivated by high salt treatment during elution from the columns.

The activity of the enzyme had its optimum at 65°C, where it was twenty times higher than at 37°C. A rapid tenfold decrease in activity occured between 70-80°C.

Template specificity (Table II). - The activated herring sperm DNA, used in the standard assay, was an optimal template and gave 3 times higher activity than that obtained with poly(dA):oligo(dT). The DNA polymerase could not use templates without primer (poly(dA) and poly(dT)), even when appropriate priming ribonucleotides (UTP or ATP, respectively) were supplied. It did not accept a polyribonucleotide template (poly(rA):oligo(dT)), either.

Inhibitors (Table II). - Aphidicolin did not inhibit the activity even at 20ug/ml (20 times higher than concentrations acting on eukaryotic polymerase alpha) either in crude extract

or in purified fractions. The enzyme was inhibited by dideoxy- and arabinosine-analogs and SH-blocking agents.

Nuclease activity. - DEAE-cellulose fraction contained all three nuclease activities tested. An endonuclease activity which generated the linear form of plasmid DNA was removed on phosphocellulose, and the remaining nicking activity was removed on Blue Sepharose. A 5'->3' exonuclease was removed by the phosphocellulose step and afterwards this activity was not detected either with single or double-stranded substrate, whereas the corresponding amount of *E.coli* polymerase I released about 25% of the acid-insoluble radioactivity from the double-stranded substrate.

A 3'->5' exonuclease activity persisted in all subsequent fractions and all fractions after the hydroxylapatite step showed a constant nuclease:DNA polymerase ratio at 56°C. This exonuclease solubilized about 40% of the input radioactivity per 0.1 units of DNA polymerase. Similar to the DNA polymerase, this activity was strongly inhibited by salt (90% inhibition by 100 mM KCl). At 56°C the nuclease activity was about 5 times higher than at 37°C both for native and denaturated substrate. At both temperatures the nuclease activity with the denaturated substrate was 5 times higher than with the native one. As a consequence, the same amount of activity was observed at 37°C on denaturated DNA as at 56°C on native DNA. These data indicate a preference for single stranded substrate and suggest the involvement of this activity in proofreading function.

Possible relations of the DNA polymerase. - The finding that the archaebacterium *Halobacterium halobium* is sensitive to the drug aphidicolin, which inhibits its DNA synthesis (12,13), raises a question whether archaebacteria could possess a DNA polymerase similar to the eukaryotic polymerase alpha (14). The enzyme described in the present paper, however, is insensitive to aphidicolin and is composed of a single polypeptide rather than being a multisubunit complex. Therefore it does not resemble the alpha polymerase. Sensitivity to aphidicolin could not even be

detected in a fraction of total activity in crude extract. However, these results do not exclude the possibility that S.acidocaldarius does have an aphidicolin-sensitive DNA polymerase. If that putative enzyme would fulfill a replicative function, it could be present in low quantity like e.g. E.coli polymerase III being outweighed by an enzyme involved in DNA repair synthesis.

In fact, the described enzyme shows certain similarities to non-replicative prokaryotic polymerases. It is a single polypeptide of about 100 kDa and has an associated 3'->5' exonuclease activity. When these properties were compared to those of several prokaryotic DNA polymerases reviewed by Kornberg (1), a certain similarity was found with E.coli polymerase II, including also the sensitivity to inhibitors. However, no extensive conclusions can be based on this apparent analogy - the relations within that group of enzymes are not well established and are not defined by simple, unequivocal biochemical properties. More work is required to investigate this aspect, especially in connection with isolation of further archaebacterial DNA polymerases.

ACKNOWLEDGEMENTS

This work was supported by a grant from Deutsche Forschungsgemeinschaft to K.J.B. (SFB 105/A1-1). The authors thank Dr. D. Palm for providing S.acidocaldarius cells, Dr. W. Goebel for facilities and support and Dr. S. Lohmann for correcting the English.

*To whom correspondence should be addressed

REFERENCES

1. Kornberg, A. (1980) DNA Replication. Freeman. San Francisco
2. Kornberg, A. (1982) Supplement to DNA Replication. Freeman. San Francisco
3. Misumi, M. & Weissbach, A. (1982) J. Biol. Chem. 257, 2323-2329

4. McKown, R.L. & Tewari, K.K. (1984) Proc. Natl. Acad. Sci. U.S.A. 81,2354-2358
5. Fox, G.E., Stackebrandt, E., Hespell, R.B., Gibson, J., Maniloff, J., Dyer, T.A., Wolfe, R.S., Balch, W.E., Tanner, R.S., Magrum, L.J., Zablen, L.B., Blakemore, R., Gupta, R., Bonen, L., Lewis, B.J., Stahl, D.A., Luehrsen, K.R., Chen, K.N. & Woese, C.R. (1980) Science(Wash.) 209,457-463
6. Aposhian, H.V. & Kornberg, A. (1962) J. Biol. Chem. 237,519-525
7. Brock, T.D., Brock, K.M., Belley R.T. & Weiss R.L. (1972) Arch. Mikrobiol. 84,54-68
8. Sedmak, J.J. & Grossberg, S.E. (1977) Anal. Biochem. 79,544-552
9. Martin, R.G. & Ames, B.N. (1961) J. Biol. Chem. 236,1372-1379
10. Siegel, L.M. & Monty, K.J. (1966) Biochim. Biophys. Acta 112,346-362
11. Pringle, J.P. (1975) Methods Cell Biol. 12,149-184
12. Forterre, P., Elie, C. & Kohiyama, M. (1984) J.Bacteriol. 159,800-802
13. Schinzel, R. & Burger K.J. (1984) FEMS Microbiol. Letters 25,187-190
14. Huberman, J.A. (1981) Cell(Cambridge, Mass.) 23,647-648
15. Laemmli, U.K. (1970) Nature(Lond.) 227,680-685
16. Wray, W., Boulikas, T., Wray, V.P. & Hancock, R. (1981) Anal. Biochem. 118,197-203
17. Karawya, E., Swack, J.A. & Wilson, S.H. (1983) Anal. Biochem. 135,318-325