Purification and Characterization of a DNA Polymerase From the Cyanobacterium *Anacystis nidulans* R2

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Purification and characterization of a DNA polymerase from the cyanobacterium *Anacystis nidulans* R2

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ABSTRACT

A DNA polymerase has been highly purified from *Anacystis nidulans* R2. Electrophoretic analysis in sodium dodecyl sulfate-polyacrylamide gels revealed that the final fraction contains three bands of M, 107,000, 93,000, and 51,000, respectively. Analysis of purified DNA polymerase activity *in situ* indicates that of the three polypeptides the M, 107,000 species has the catalytic activities. The native molecular weight of the enzyme was estimated by glycerol gradient sedimentation to be 100,000. The enzyme has an absolute requirement for a divalent cation. Mg\(^{2+}\) can be replaced with Mn\(^{2+}\), but the DNA polymerase is less active. Potassium chloride stimulates the enzyme, while potassium phosphate has no apparent effect. The enzyme is active over a pH range from 7.5 to 9.5 in 50mM Tris-HCl buffer. The ability of the cyanobacterial DNA polymerase to use activated DNA as a template, its associated 3'→5' and 5'→3' exonuclease activities, as well as its resistance to N-ethylmaleimide, dideoxynucleotides, adenosyl-CTP and aphidicolin suggest a similarity between this enzyme and *E. coli* DNA polymerase I. This is the first characterization of a DNA polymerase from a cyanobacterium.

INTRODUCTION

The characterization of DNA polymerase genes from a variety of organisms has led to predictions about their origin and evolution (1–5). Wang et al. (5) detected several regions of conserved amino acid sequence among eukaryotic and prokaryotic replicative DNA polymerases and predicted that these DNA polymerases evolved from a single primordial gene. Furthermore, Miller et al. (6) demonstrated the presence of consensus nucleic acid sequences in DNA polymerase genes from widely divergent vertebrates and invertebrates, but was unable to detect homologies in plant DNA. These findings suggest that the replicative DNA polymerases from plants may be derived from a different primordial gene.

The presumed evolutionary ancestors of modern photosynthetic organelles of higher plants and eukaryotic algae are unicellular members of a group of photosynthetic prokaryotes, the cyanobacteria (7–10). Although many genes, mostly those encoding proteins involved in photosynthesis, of these bacteria have been sequenced, to date nothing is known about proteins mediating DNA replication, in particular their DNA polymerases. We are currently characterizing a cyanobacterial DNA polymerase in order to compare this enzyme with known prokaryotic and eukaryotic DNA polymerases. We are particularly interested in establishing similarities or differences between the cyanobacterial enzyme and the DNA polymerases from higher plant chloroplasts. The latter enzymes resemble DNA polymerases of the γ class which are found in animal mitochondria, but they have some distinctive ‘plant-like’ features as well (11,12,13). We describe here the purification and characterization of a cyanobacterial DNA polymerase.

EXPERIMENTAL PROCEDURES

Material

*Anacystis nidulans* R2 was a kind gift from Dr. L. Sherman, University of Missouri. Poly(A)*^+^ RNA was a gift from Drs. Tornow and Santangelo, University of Southern Mississippi. Unlabeled nucleotides, calf thymus DNA, heparin-agarose, single stranded DNA-cellulose, protease inhibitors, aphidicolin, adenosyl-CTP, N-ethylmaleimide, β-amylase, alcohol dehydrogenase, and bovine serum albumin were purchased from Sigma Chemical Company. [\(^{3}H\)]dTTP and [\(^{32}P\)]dCTP were from DuPont/NEN. Synthetic homopolymers and primers were purchased from Pharmacia Fine Chemicals. DEAE-cellulose (Whatman), phosphocellulose (Whatman) and GF/C glass fiber filters (Whatman), as well as sodium dodecyl sulfate (molecular biology grade) and Scintiverse BD scintillation fluid were purchased from Fisher Scientific. *E. coli* DNA polymerase I and its Klenow fragment came from Promega.

Buffers

Buffer A is 30 mM Tris-HCl (pH 8.0 at 25°C), 20 mM NaCl, 10% glycerol, 2 mM dithiothreitol (DTT), 0.3 mM p-toluenesulfonyl fluoride (PMSF) and 0.3 mM phenylmethylsulfonyl fluoride (PMSF). Extraction Buffer is 0.7 M NaCl and 0.1% Triton X-100 in Buffer A. Buffer B is 20 mM potassium phosphate (pH 8.0 at 25°C), 0.5mM DTT, 0.1
mM PMSF, 0.1 mM PTSF and 10% glycerol. Buffer C is 0.1 M ammonium sulfate, 10% glycerol, 50 mM Tris-HCl (pH 8.0 at 25°C), 0.1 mM EDTA, 0.1% Triton X-100, 0.1 mM PTSF, 0.1 mM PMSF, and 10 mM β-mercaptoethanol. Buffer D is 20 mM KCl and 0.5 mM EDTA in buffer B.

Growth and Harvesting of Cells

*Anacystis nidulans* R2 cells were grown in liquid culture in BG-11 medium (14) under continuous illumination from two cold white fluorescent light banks (2,700 lux) and at ambient temperature. Starting material for enzyme preparations was obtained from cells at a density of 7×10⁷ cells/ml. Cells were harvested by centrifugation for 20 min at 10,000×g at 4°C, washed with cold buffer A, and stored at −70°C as a wet cell pellet until required. The yield was approximately 2.5 g (wet weight) of cell pellet per batch of 8 liters of medium.

Preparation of Cell Extracts

All steps of the preparation were carried out at 4°C. Ten grams of frozen cells were thawed and suspended in 25 ml of Buffer A and 25 ml of Extraction Buffer and sonicated in a steel beaker in a NaCl/ice/water bath with a Sonic Dismembrator Model 300 (Fisher Scientific). The total sonication time was 9 min, the cells being subjected to maximum power for 30 seconds at a time. The resulting suspension was then centrifuged for 20 min at 15,000×g and the supernatant was kept on ice, while the pellet was resuspended in 25 ml of Extraction Buffer and sonicated once more. A second 10 g batch of cells was treated identically, and the resulting supernatants were pooled.

DEAE-Cellulose Chromatography

The cleared cell extract was dialyzed against three two-liter changes of Buffer A and loaded onto a DEAE-cellulose column (4 mg of protein per ml of packed column material) which had previously been equilibrated with Buffer A. After a wash with two column volumes of the same buffer, a linear gradient of 20 to 400 mM sodium chloride in Buffer A was applied. DNA polymerase activity was observed at an elution concentration of 150–200 mM sodium chloride.

Phosphocellulose Chromatography

The peak fractions of DNA polymerase obtained from DEAE-cellulose chromatography were pooled, dialyzed against Buffer B and loaded onto a phosphocellulose column (4 mg of protein per ml of packed column material) previously equilibrated with Buffer B. The column was washed with two column volumes of Buffer B and developed with a linear gradient of 20 to 400 mM potassium phosphate in Buffer B. DNA polymerase activity was detected as a single peak at an elution concentration of 120–130 mM potassium phosphate.

Heparin Agarose Chromatography

The active DNA polymerase fractions eluted from phosphocellulose were pooled and dialyzed against Buffer C. The fraction was loaded onto a heparin agarose column (equilibrated with the same buffer) at 1.5 mg of protein per ml of packed column material. The column was washed with two column volumes of Buffer C, and a linear gradient of 100 to 600 mM ammonium sulfate in Buffer C was applied. DNA polymerase activity was noted at an elution concentration of 175–275 mM ammonium sulfate.

DNA-Cellulose Chromatography

The active DNA polymerase fractions from heparin agarose were pooled and dialyzed against Buffer D. The dialyzed solution was then loaded onto a single stranded DNA-cellulose column (0.4 mg of protein per ml of packed column material) equilibrated with the same buffer. After washing with two column volumes of 100 mM KCl in Buffer B, a linear gradient of 100 mM to 600 mM KCl was applied. The DNA polymerase activity was detected as a single peak at an elution concentration of approximately 0.35 M KCl. The final active fractions were pooled, dialedyzed against a buffer containing 20 mM potassium phosphate, pH 8 and 1 mM DTT, and stored at −70°C until required.

DNA Polymerase Assays

Unless otherwise indicated, DNA polymerase activity was assayed in 0.05 ml of the following reaction mixture: 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 100 mM KCl, 140 mM BSA, 0.1 mM DTT, 125 µg/ml of calf thymus DNA, and 30 µM of each of the following: dATP, dCTP, dGTP and [3H]dTTP with a specific radioactivity of 78 cpm/pmol. Alternatively, using the primed homopolymer Poly(A)·dT₁₂₋₁₈ as a template, the assay contained 0.25 mM MnCl₂ instead of Mg²⁺, 64 µg/ml of Poly(A)·dT₁₂₋₁₈ instead of activated DNA, and 33 µM [3H]dTTP (specific radioactivity of 310 cpm/pmol) as the single nucleotide. The reaction was carried out at 37°C for 30 minutes. Aliquots of 40 µl were spotted onto glass fiber filters and nucleic acids were precipitated in 5% (w/v) trichloroacetic acid containing 1% (w/v) sodium pyrophosphate. The filters were washed twice with 5% trichloroacetic acid, once with ethanol, once with diethyl ether and dried under a heat lamp. Bound radioactivity on each filter was quantitated by liquid scintillation counting in 5ml of Scintiverse BD scintillation fluid. One unit of enzyme activity is defined as the incorporation of one nmole of deoxynucleotide into an acid-insoluble form per hour at 37°C.

Calf thymus DNA was activated by partial digestion with DNase I as described by Schlabauch et al. (15). Primed homopolymer templates were made by mixing solutions of template and primer in 10 mM Tris-HCl, pH 7, 10 mM NaCl, heating at 65°C for 10 min, and then slowly cooling to room temperature. The final concentration of Poly(A)·dT₁₂₋₁₈ was 0.58 mg/ml total nucleic acid with a molar nucleotide ratio of template to primer of four to one.

Protein Determination

Protein was determined by a modification of the dye binding method of Bradford (16), with bovine serum albumin as a standard.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis in the presence of SDS was performed according to Laemmli (17). The proteins were stained in the gel with silver according to the manufacturer’s (Bio-Rad) instructions.

Analysis of DNA Polymerase Activity in situ

The method for analysis of DNA polymerase activity *in situ* was essentially that of Karaywa and Wilson (18). The enzyme sample in 1.2% SDS, 12% glycerol, 0.7% β-mercaptoethanol and 5 µg of heterogeneous protein mixture (an extract from *A. nidulans*...
cells which was inactivated by incubation at 37°C for 16 hours and then at 95°C for 3 hours) was heated at 37°C for 3 min and electrophoresed in a 7.5% (w/v) polyacrylamide gel containing 0.1% SDS, 2 mM EDTA, and 125 μg/ml activated calf thymus DNA. Electrophoresis proceeded at 100 V for 40 min. SDS was removed by soaking the gel at room temperature in one liter of 50 mM Tris-HCl, pH 8.0, 2 mM EDTA for 90 min with two buffer changes, followed by gentle agitation in the same solution at 4°C for 24 hours with 4 changes of the liquid. To measure DNA polymerase activity, the gel was incubated for 17 hours at 37°C in 10 ml of 50 mM Tris-HCl, pH 8.0, 7 mM MgCl₂, 100 mM KCl, 0.1 mM DTT, and 12 μM of each of the following: dATP, dGTP, and TTP with 10 μCi of [³²P]dCTP (3,000 Ci/mmol). Unincorporated nucleotides were washed out by incubation in 0.5 liter of 5% (w/v) trichloroacetic acid containing 1% (w/v) sodium pyrophosphate at room temperature for one hour and for 40 hours at 4°C with four changes of the liquid. The gel was dried and autoradiographed at −70°C for 30 hours on Kodak X-Omat film using a Dupont Cronex Lightning Plus intensifying screen.

Analysis of Exonuclease Activities in situ
Activated DNA was 3’-end labeled as described in (19), while for the alkaline phosphatase digestion and 5’-end labeling procedures of Maniatis et al. (20) and Banks and Yarranton (21), respectively, were followed. The ³²P end labeled template DNA (spec. radioactivity 0.2 – 1.6 × 10⁸ cpm/μg) was polymerized in an SDS-polyacrylamide gel and 1.6 units of enzyme electrophoresed as described above. Renaturation was allowed to proceed for 90 min at room temperature followed by 5 hours at 4°C, with several buffer changes.

Exonuclease activity assays were performed as described for the in situ polymerase assay, but did not contain deoxynucleotides. Further processing of the gel was as described above.

Glycerol Gradient Sedimentation
A 150 μl aliquot of the pooled fractions from DNA cellulose was mixed with 50 μl of loading buffer containing 5% glycerol and 2 μl of BSA (10 mg/ml). The sample was layered onto a gradient consisting of 10–30% glycerol in 20 mM potassium phosphate buffer, pH 8.0, containing 0.5 mM DTT, 0.1 mM EDTA, 250 mM KCl, 1 mM PTF and 1 mM PMSF. The gradients were prepared in ultra-clear tubes which had previously been coated with BSA. Centrifugation was in a Beckman SW-40 rotor at 30,000 rpm for 62 hours at 4°C, after which ten-drop fractions were collected. Three gradients containing bovine serum albumin, β-amylase, and alcohol dehydrogenase, respectively, were run simultaneously as molecular weight standards. The molecular weight of the enzyme was estimated from the sedimentation data, assuming a globular structure of the protein (22).

RESULTS AND DISCUSSION
Purification of the Cyanobacterial DNA Polymerase
A DNA polymerase from A. nidulans R2 cells was highly purified by sequential column chromatography on DEAE-cellulose, phosphocellulose, heparin agarose and single stranded DNA-cellulose. The DNA polymerase eluted as a single peak from DEAE-cellulose at approximately 175 mM NaCl, from phosphocellulose at approximately 125 mM potassium phosphate, from heparin-agarose at approximately 250 mM ammonium sulfate and from single stranded DNA-cellulose at approximately 350 mM KCl. A summary of the purification scheme for the enzyme is shown in Table 1. DNA polymerase activity in cell extracts and in the fractions eluting from DEAE cellulose and phosphocellulose was routinely determined with activated DNA/Mg²⁺, as well as with Poly(A)·dT₁₂₋₁₈/Mn²⁺. Both assays were performed to screen the initial purification steps for further, possibly different DNA polymerase activities. We could detect only one peak of activity eluting from the two columns with both assay regimes.

Protease inhibitors such as PMSF and PTF were important for stabilization of the enzyme. When the final enzyme fraction (single stranded DNA cellulose eluate) was stored at −70°C without the protease inhibitors, two thirds of its initial activity was lost within one month, whereas the activity of an aliquot that had been kept in the presence of protease inhibitors was stable. After DEAE-cellulose chromatography, a remarkable increase in activity was observed (Table 1). This phenomenon

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<tbody>
<tr>
<td>Crude extract</td>
<td>920</td>
<td>774</td>
<td>0.8</td>
</tr>
<tr>
<td>DEAE cellulose</td>
<td>160</td>
<td>11004</td>
<td>69</td>
</tr>
<tr>
<td>Phosphocellulose</td>
<td>1.5</td>
<td>4252</td>
<td>2835</td>
</tr>
<tr>
<td>Heparin agarose</td>
<td>0.4</td>
<td>843</td>
<td>2108</td>
</tr>
<tr>
<td>ssDNA cellulose</td>
<td>0.035</td>
<td>681</td>
<td>19457</td>
</tr>
</tbody>
</table>

a. A unit of activity is defined as 1 nmole of deoxynucleotide incorporated into acid-insoluble material per hour. The assay was performed with activated DNA as a template and Mg²⁺ as the divalent cation.

![Figure 1](https://example.com/figure1.png)

Figure 1. SDS-polyacrylamide gel electrophoresis of the cyanobacterial DNA polymerase fractions. Lane A: DEAE cellulose; lane B: phosphocellulose; lane C: heparin agarose; lane D: ss-DNA cellulose, 5× concentrated. 5 μl of each fraction were loaded on a 7.5% polyacrylamide slab gel. Protein was detected by silver staining as described under 'Experimental Procedures.' Molecular weights of protein standards, indicated on the left (×10⁶), were phosphorylase B (97,400), bovine serum albumin (66,200), ovalbumin (42,699), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400).
has been observed for other DNA polymerases (13) and probably reflects the removal of inhibitory factors such as nucleases or endogenous nucleic acids in the crude extract by the anion exchanger.

SDS-polyacrylamide gel electrophoresis was performed to estimate the polypeptide composition of the various enzyme fractions (Figure 1). The final enzyme fraction contained three major bands upon silver staining, of Mr 107,000, 93,000, and 51,000, respectively. The appearance of the 107,000 dalton polypeptide could be followed starting with the phosphocellulose step (lane B), and was most likely hidden in the DEAE fraction due to the low proportional concentration of the enzyme. The polypeptide composition of the final fraction could be the result of proteolytic digestion of the enzyme, a common phenomenon among DNA polymerases (23–26). However, the 93,000 and 51,000 dalton polypeptides were visible in each enzyme fraction, suggesting that these two polypeptides might represent other proteins which were not removed by the final purification step. The native molecular weight of the enzyme was estimated by velocity sedimentation in glycerol gradients. Aliquots from the phosphocellulose, heparin agarose and from the final purified fraction were sedimented in parallel gradients. In each case, DNA polymerase activity was resolved into a single peak of an estimated molecular weight of 100,000 (data not shown).

Activity Gel Analysis
To determine which of the three polypeptides of the final purified fraction was responsible for the DNA polymerase activity, the pooled enzyme fractions from each purification step were subjected to an activity gel analysis. The procedure involves mild denaturation and electrophoresis in an SDS-polyacrylamide gel followed by renaturation and enzyme assay in situ (18). Figure 2 demonstrates that the DNA polymerase activity is associated with the largest of the three polypeptides (Mr 107,000) in the final enzyme fraction (lane D). No DNA polymerase activity was detected corresponding to the Mr 93,000 and 51,000 polypeptides. Furthermore, the molecular weight of the catalytic polypeptide was identical in each purification step (lanes A–D), suggesting that the 107,000 molecular weight species represents an unproteolyzed polypeptide of the DNA polymerase present in vivo. Besides the 107,000 dalton poly enzyme, an active species of Mr 74,000 was prominent in the heparin agarose fraction (lane C). Proteolysis of the large (Mr 107,000) polypeptide could account for this lower molecular weight species in this purification step. In fact, a faint band of the same molecular weight is visible on autoradiographs of the final enzyme fraction upon longer exposure (star in lane D). Furthermore, glycerol gradient sedimentation of an enzyme aliquot stored in the absence of protease inhibitors revealed a single peak of an estimated molecular weight of 76,000 (not shown), which again points to a possible proteolytic degradation of the 100,000–107,000 molecular weight enzyme to a smaller species of Mr 74,000–76,000. Taken together with the native molecular weight of approximately 100,000 estimated by sedimentation velocity analysis, our results suggest that the highly purified cyanobacterial DNA polymerase is a monomer of 100,000 to 107,000 daltons.

It is intriguing to speculate that the relationship between the smaller catalytically active polypeptide of Mr 74,000 and the 107,000 molecular weight species (Figure 3, lanes C and D) might be analogous to that of the Klenow fragment (Mr 76,000) and DNA polymerase I (Mr 109,000) from E. coli (27,28).

Exonuclease activities of the cyanobacterial DNA polymerase

![Figure 2](image-url)

**Figure 2.** DNA polymerization by the cyanobacterial DNA polymerase in situ. Lane A: DEAE cellulose; lane B: phosphocellulose; lane C: heparin-agarose; lane D: ss-DNA cellulose. Fractions were denatured, electrophoresed in a 7.5% SDS-polyacrylamide gel, renatured and assayed for DNA synthesis in situ as described under 'Experimental Procedures'. *E. coli* DNA polymerase I (Mr, 109,000) and its Klenow fragment (Mr, 76,000) were used as DNA polymerization standards and internal molecular weight markers. Other protein markers were as indicated in the legend to Figure 1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>A. nidulans DNA Polymerase</th>
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<tbody>
<tr>
<td>pH optimum</td>
<td>7.5–9.5</td>
</tr>
<tr>
<td>Mg$^{++}$ optimum</td>
<td>5 mM</td>
</tr>
<tr>
<td>Mn$^{++}$ optimum</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>KCl optimum</td>
<td>150 mM</td>
</tr>
<tr>
<td>KPO$_4$ (0–100 mM)</td>
<td>no effect</td>
</tr>
<tr>
<td>Temperature optimum</td>
<td>42°C</td>
</tr>
<tr>
<td>N-ethylmaleimide (2 mM)</td>
<td>15% inhibition</td>
</tr>
<tr>
<td>Aphidicolin (20 µg/mL)</td>
<td>no inhibition</td>
</tr>
<tr>
<td>Ara-CTP (100 µM)</td>
<td>no inhibition</td>
</tr>
<tr>
<td>Ara-CTP (200 µM)</td>
<td>14% inhibition</td>
</tr>
<tr>
<td>ddTTP/TTP 1:1</td>
<td>no inhibition</td>
</tr>
<tr>
<td>ddTTP/TTP 10:1</td>
<td>10% inhibition</td>
</tr>
</tbody>
</table>

All assays were performed with activated DNA as a template.

<table>
<thead>
<tr>
<th>Template</th>
<th>Cation a)</th>
<th>% Activity b)</th>
</tr>
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<tbody>
<tr>
<td>Activated DNA</td>
<td>Mg$^{++}$</td>
<td>100</td>
</tr>
<tr>
<td>Poly(dA)&lt;sub&gt;12&lt;/sub&gt;-dT$_{12}$</td>
<td>Mg$^{++}$</td>
<td>48</td>
</tr>
<tr>
<td>Poly(dA)&lt;sub&gt;12&lt;/sub&gt;-dT$_{12}$</td>
<td>Mn$^{++}$</td>
<td>211</td>
</tr>
<tr>
<td>Poly(dA)&lt;sub&gt;12&lt;/sub&gt;-dT$_{12}$</td>
<td>Mn$^{++}$</td>
<td>1239</td>
</tr>
<tr>
<td>Poly(dC)&lt;sub&gt;12&lt;/sub&gt;-dT$_{12}$</td>
<td>Mg$^{++}$</td>
<td>-</td>
</tr>
<tr>
<td>Poly(dC)&lt;sub&gt;12&lt;/sub&gt;-dT$_{12}$</td>
<td>Mn$^{++}$</td>
<td>5</td>
</tr>
<tr>
<td>Poly(dC)&lt;sub&gt;12&lt;/sub&gt;-dT$_{12}$</td>
<td>Mn$^{++}$</td>
<td>169</td>
</tr>
<tr>
<td>M13mp2 ss-circular</td>
<td>Mg$^{++}$</td>
<td>4</td>
</tr>
<tr>
<td>M13mp2 ss-circular</td>
<td>Mn$^{++}$</td>
<td>11</td>
</tr>
<tr>
<td>Unprimed Poly(A)</td>
<td>Mg$^{++}$</td>
<td>-</td>
</tr>
<tr>
<td>Unprimed Poly(A)</td>
<td>Mn$^{++}$</td>
<td>-</td>
</tr>
</tbody>
</table>

a. The divalent cation concentrations were 5 mM for Mg$^{2+}$ and 0.25 mM for Mn$^{2+}$.
b. 100% activity corresponds to 0.024 units.
c. ss = single stranded DNA, ds = double stranded DNA.
were assayed in activity gels as well. Radioactivity was released from activated template DNA, either 3'– or 5'-end labeled, by the 107,000 molecular weight polypeptide species (not shown), suggesting that the cyanobacterial DNA polymerase possesses both 3’–5’ and 5’–3’ exonuclease activities. Our enzyme preparation did not contain an endonuclease activity, as evidenced by the stability of the covalently closed circular form of the plasmid pBR322 when incubated with the DNA polymerase (not shown).

Activity gel analysis of the active DEAE-cellulose fractions revealed the presence of two additional polypeptides with DNA polymerase activity (lane A). Their estimated molecular weights are 138,000 and 124,000, respectively. It is possible that these two larger polypeptides may represent other DNA polymerases in A. nidulans, which we failed to detect under the assay conditions used to scan the column fractions. Alternatively, these two activities may be very unstable and consequently were lost during the subsequent purification steps.

**Characterization of DNA Polymerase**

All characterization studies were performed with the final purified enzyme fraction. Table 2 summarizes the assay requirements for the cyanobacterial DNA polymerase. The enzyme was active over a broad pH range between 7.5 and 9.5 in 50 mM Tris-HCl. At pH 7 and pH 10, 70% of the activity still remained. The enzyme showed a complete dependence on a divalent cation and was inactive in the presence of 2 mM EDTA. The preferred metal ion in the standard assay was Mg2+ at an optimum concentration of 5mM, but Mn2+ (optimum concentration 0.1 mM) could partially fulfil the requirement for a divalent cation. In the absence of KCl, the enzyme activity was 35% of that observed at the optimum KCl concentration of 150 mM. Changes in phosphate concentration over a range of 0 to 100 mM had no apparent effect on enzyme activity. The enzyme showed highest activity at a temperature of 42°C, which is 190% of that seen at 37°C. At 55°C, between 10 and 25% of the activity still remained, indicating that this cyanobacterial DNA polymerase is remarkably heat stable. The sulfhydryl blocking agent N-ethylmaleimide had no appreciable effect on the activity of the enzyme, suggesting that this DNA polymerase does not depend on reduced sulfhydryl groups for its activity.

Aphidicolin, an inhibitor of eukaryotic DNA polymerase α, did not affect the cyanobacterial enzyme at 20 µg/ml. The enzyme was also resistant to another inhibitor of the nuclear DNA polymerase, arabinosyl-CTP (araCTP) at 200 µM. Likewise, the activity of the cyanobacterial DNA polymerase was unaffected by the DNA polymerase γ inhibitor deoxythymidine triphosphate (ddTTP), since the enzyme retained 90% of its activity at a ddTTP to TTP ratio as high as 10:1.

Table 3 summarizes the relative activity of the DNA polymerase from A. nidulans with different templates, arbitrarily setting the enzyme activity with activated calf thymus DNA as 100%. Assays were performed in the presence of either Mg2+ or Mn2+ because it has been demonstrated that the cation is able to influence template usage (29). The enzyme preferred activated DNA and Poly(dA)·dT12–18 over Poly(A)·dT12–18. An unprimed single stranded template, bacteriophage M13mp2 circular DNA or poly(A), as well as double stranded covalently closed circular DNA (not shown) could not be used. The enzyme did not show reverse transcriptase activity when dT12–18 primed Poly(A)·RNA was used as a template. As found with most DNA polymerases, Poly(dC)·dG12–18 was a better template than activated DNA (169% relative activity).

The DNA polymerase isolated from the cyanobacterium *Anacystis nidulans* is similar to DNA polymerase I from *E. coli* with regard to its ability to use activated calf thymus DNA, its resistance to aphidicolin, ddTTP, ara-CTP and N-ethylmaleimide, its stimulation by KCl, as well as its 3’–5’ and 5’–3’ exonuclease activities (30). Work is currently underway in our laboratory to determine the primary structure of the cyanobacterial DNA polymerase.

**REFERENCES**