The Heterogeneity of Cytoplasmic Deoxyribonucleic Acid Polymerase from *Physarum polycephalum*

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Cytoplasmic DNA polymerase (DNA deoxynucleotidyltransferase, EC 2.7.7.7) was partially purified from *Physarum polycephalum*. The first step of the purification procedure utilized the fact that the enzyme on gel filtration behaves in anomalous fashion. The second step was either ion-exchange chromatography or sucrose-density-gradient centrifugation. The partially purified DNA polymerase was heterogeneous and at least four species with different sedimentation coefficients (5.5S, 7.2S, 8.6S and 11.5S) were detected. Calculated molecular weights indicated a tendency for stoichiometric polypeptide aggregation, accompanied by an alteration of the three-dimensional structure from a compact spheroid to a more open elliptical form. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and computed molecular weights suggest an active protomer in the range of 113000 daltons; all data pertain to I 0.045, which was maintained during the whole procedure.

There have been many attempts to purify and characterize DNA polymerase in order to elucidate regulatory aspects of enzyme actions in the DNA-replication machinery. All cells so far studied have shown more than one enzyme with DNA polymerase properties (Holmes & Johnston, 1975; Craig & Keir, 1975). Further, it has been demonstrated that enzymic activities can be associated with subcellular structures such as nuclei (Bernard *et al.*, 1974), mitochondria (Meyer & Simpson, 1970) and cytoplasmic membrane fractions (Matsukage *et al.*, 1974). Moreover it has been established that in DNA-containing virions multiple activities of DNA-dependent DNA polymerase can be separated by ion-exchange chromatography (Lin *et al.*, 1973). In addition there was a report that a physically heterogeneous, high-molecular-weight (6S–12S) DNA polymerase activity, or family of activities, is recoverable from an organelle-free cytoplasmic fraction (Sedwick *et al.*, 1975). This observation prompted us to look in more detail at the molecular parameters of partially purified, but heterogeneous, cytoplasmic DNA polymerase from the lower eukaryote *Physarum polycephalum* (slime mould).

Experimental

Materials

Preparation of 105000g concentrated supernatant. Microplasmidia of *Physarum polycephalum* (subline

M3c) were cultured in semi-defined medium (Daniel & Baldwin, 1964), containing yeast extract and tryptone (Difco, Detroit, MI, U.S.A.); the cultures were maintained in 20ml of medium in 500ml Erlenmeyer flasks, which were shaken in the dark. Plasmodia were harvested in exponential phase at room temperature (20°C). Subsequent procedures were carried out at 0–5°C. Plasmodia were pelleted by low-speed centrifugation (3000g for 30min) and washed four times with buffer A [50mM-Tris/HCl (pH 7.5 at 20°C)/250mM-sucrose/3mM-2-mercaptoethanol]. The final pellet was suspended in 4vol. of buffer A and homogenized. The homogenate was centrifuged in a fixed-angle rotor at 105000g for 1h. The pellet was discarded and the supernatant concentrated up to 10-fold by pressure dialysis in an Amicon apparatus, equipped with a PM-10 membrane. Further purification procedures were carried out by using the concentrate, called the DNA polymerase extract. Unless stated otherwise, all columns were developed by buffer B (buffer A without 250mM-sucrose).

Methods

Column chromatography. Method (1): combined Sephadex G-200, Sepharose 6B and DEAE-cellulose column chromatography. DNA polymerase extract (7.2ml), containing 90mg of protein, was filtered through a combined series, one beside the other, of Sephadex G-200 (70cm × 2.0cm) and Sepharose 6B (70cm × 2.0cm) columns (Pharmacia, Uppsala, Sweden) at a flow rate of 8.5ml/h, regulated by a Vario-Perpex pump (LKB). The combined columns (V_0 137ml) were previously equilibrated and

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developed with buffer B. Fractions (4.0ml) were collected and 100 μ l samples tested for DNA polymerase activity. The total amount of DNA polymerase activity from both columns was allowed immediately to penetrate into a DEAE-cellulose (DE-52) column (8cm $\times$ 2cm; Whatman, Maidstone, Kent, U.K.). After an elution volume of 200ml had passed through the three columns, the DEAE-cellulose column was separated, prewashed with 180ml of buffer B and eluted with a 220ml linear gradient of 0–300mM-KCl in buffer B. Fractions (4.0ml) were collected and 100 μ l samples were used to determine DNA polymerase activity. Conductivity was measured in each tube and converted into KCl concentration.

Method (2): Sephadex G-200 gel filtration and $(\text{NH}_4)_2\text{SO}_4$ precipitate layered on a Sephadex G-100 column. DNA polymerase extract (9.8ml), containing 30.4mg of protein, was loaded on a column (70cm $\times$ 2.0cm) of Sephadex G-200 (V_0 66.9ml), previously equilibrated with buffer B. Elution was carried out with the same buffer at a flow rate of 8.5ml/h. Fractions (4.1ml) were collected and 200 μ l of each fraction was assayed for DNA polymerase activity. The main activity of DNA polymerase appeared in tubes 17–25, which were pooled (Sephadex G-200 pool, 24.6ml). To a Sephadex G-100 column (65cm $\times$ 2.0cm, V_0 62.3ml), previously equilibrated with buffer B, 6.3ml of 90% satd. $(\text{NH}_4)_2\text{SO}_4$ solution was applied, followed by 5ml from the Sephadex G-200 pool (2.6mg of protein). The $(\text{NH}_4)_2\text{SO}_4$ concentration on top of the column was thus initially about 50% saturation; the column was run at a flow rate of 17ml/h with buffer B: 4.1ml fractions were collected and 200 μ l portions were assayed for DNA polymerase activity.

Velocity sedimentation. Velocity sedimentation of cytoplasmic DNA polymerase extract was performed in nitrocellulose tubes in a Heraeus Christ (Osterode, Germany) SW-40 rotor at 38000rev./min (143000g) for 15h at 4°C. Samples of the DNA polymerase extract from various purification steps were layered on top of a 12.2ml linear sucrose gradient (4–38%, w/v) in solution B. After the run, approx. 30 fractions (eight drops each) were collected from below. Human haemoglobin (Serva, Heidelberg, Germany) (4.4S), immunoglobulin G (Behring, Marburg/Lahn, Germany) (7S), catalase (Sigma, St. Louis, MO, U.S.A.) (11.3S), β -galactosidase (Boehringer, Mannheim, Germany) (15.9S) and immunoglobulin M (Behring) (19S) were centrifuged in companion tubes as markers. Enzymes were monitored by their catalytic properties, human globulins by specific antibodies (Behring) and haemoglobin was detected by measurement of A_{420} .

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. About 100 μ g of protein from the peak of material eluted by 100mM-KCl and approx. 140 μ g of

protein from that eluted by 150mM-KCl from the three combined columns (method 1) were loaded on top of two gels [see Figs. 5(a) and 5(b) respectively]. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed as described by Weber & Osborn (1969) with the following standards: β -galactosidase (polypeptide chain mol.wt. 130000), catalase (polypeptide chain mol.wt. 56000), bovine serum albumin (polypeptide chain mol.wt. 68000) (Behring), ovalbumin (polypeptide chain mol.wt. 43000) (Serva) and trypsin (polypeptide chain mol.wt. 23000) (Serva).

DNA polymerase assay. DNA polymerase activity was determined by the rate of conversion of ^3H -labelled dTTP into cold-0.5M- HClO_4 -insoluble material as a result of incubation with the enzyme. Reactants were incubated at 27°C for 30min in glass tubes and contained the following in a total volume of 300 μ l: 100 μ l of 300mM-morpholinepropane-sulphonate buffer (pH 7.5 with KOH), 50nmol each of dATP, dCTP, dGTP, 0.16nmol [*methyl*- ^3H]dTTP (30Ci/mmol, Amersham Buchler, Braunschweig, Germany), 10mM-magnesium acetate, 3mM-EGTA, bovine serum albumin (500 μ g/ml) and 40 μ g of activated calf thymus DNA (Loeb, 1969) as primer template. Radioactivity was measured as described by Schiebel & Schneck (1974). Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Results

Partial purification of cytoplasmic DNA polymerase

The enzyme activity of DNA polymerase extract, eluted from a Sephadex G-200 column at low ionic strength (buffer B) coincided almost with V_0 . A Sepharose 6B column was therefore used in series with the Sephadex G-200 column to remove contaminating globular proteins. The resultant bimodal activity profile covered a wide range of fractions (Fig. 1a), favouring the idea of heterogeneity of cytoplasmic DNA polymerase. At low ionic strength, the combination of gel filtration and ion-exchange chromatography allowed cytoplasmic DNA polymerase to be purified in a single elution step (method 1) to such a degree that only a limited number of protein bands can be detected when protein eluted by 100mM-KCl and 150mM-KCl is electrophoresed on a sodium dodecyl sulphate/polyacrylamide-gel [see Figs. 5(a) 5(b)]. No enzymic activity was detectable in the flow-through volume (200ml of buffer B) of the combined Sephadex G-200/Sepharose 6B/DEAE-cellulose column, but enzyme could be eluted by 100–150mM-KCl in buffer B (Fig. 1b).

Conversion of cytoplasmic DNA polymerase

DNA polymerase extract was loaded on a Sephadex G-200 column and eluted with buffer B. The major

Fig. 1. Combined column chromatography of DNA polymerase extract

First, DNA polymerase extract was run on a Sephadex G-200/Sepharose 6B column (a). Fractions 32–55 were allowed to penetrate immediately a DEAE-cellulose column, which was then eluted with a linear 0–300 mM-KCl gradient ($\square$). (b) Elution pattern of DNA polymerase extract ($\circ$) from this third column.

Fig. 2. Gel filtration and $(\text{NH}_4)_2\text{SO}_4$ precipitation on top of a Sephadex G-100 column

DNA polymerase activity emerges at the void volume of a Sephadex G-200 column (a). To samples of pooled DNA polymerase fractions (17–25) $(\text{NH}_4)_2\text{SO}_4$ was added to about 50% saturation and the mixture was applied to and eluted from a Sephadex G-100 column. The DNA polymerase activity penetrates the gel, indicating a shift of molecular weights to lower values (b).

activity was eluted mainly at the void volume. Another small and distinct peak of material suggested the possible presence of other DNA polymerase species (Fig. 2a). Fractions eluted at or close to V_0 of the Sephadex G-200 column were pooled and rechromatographed on a Sephadex G-100 column

with 50%-satd. $(\text{NH}_4)_2\text{SO}_4$ on top. DNA polymerase unaffected by $(\text{NH}_4)_2\text{SO}_4$ and/or pH-alterations should have been eluted with the void volume of the Sephadex G-100 column. The enzyme profile, however, was clearly separated from V_0 and revealed additional shoulders of activities (Fig. 2b). The

expected peak of activity chromatographing at V_0 had almost disappeared. To demonstrate that the elution pattern from the Sephadex G-100 column was not mainly due to immobilization or crystallization effects during $(\text{NH}_4)_2\text{SO}_4$ precipitation on top of the column, DNA polymerase extract was centrifuged in a sucrose density gradient before and after treatment with $(\text{NH}_4)_2\text{SO}_4$. Before this procedure, the DNA polymerase extract was filtered through a Sephadex G-25 column. Hereby, the 280 nm-absorbing material, which was eluted at the V_0 of the Sephadex G-25 column, was pooled, whereas a yellow pigment, which was clearly separated, was discarded. Portions (200 μl) of such a prepurified DNA polymerase extract were spun down on a sucrose gradient at low ionic strength (I 0.045) as detailed under 'Methods'. The result indicated a peak of activity sedimenting between human haemoglobin and immunoglobulin G. A sample of DNA polymerase extract in 50% satd. $(\text{NH}_4)_2\text{SO}_4$ was dialysed exhaustively overnight against buffer B. The sedimentation pattern shifted towards higher sedimentation coefficients and showed also a heterogeneous activity profile when analysed on a sucrose gradient (Fig. 3).

Physicochemical properties of cytoplasmic DNA polymerase (6S–12S enzyme)

Sedimentation rates of the pooled and pressure-concentrated fractions eluted by 100–150 mM-KCl from the combined columns were investigated. Different classes of enzyme activity were pooled from the sucrose gradient (Fig. 4), concentrated and filtered under constant buffer conditions through a calibrated Sepharose 6B column to estimate Stokes radii. Sedimentation and gel-filtration data were used to compute molecular-weight values and frictional coefficients (Table 1). The large values of the frictional ratio (f/f_0) could result from the presence of carbohydrates in the molecules. Analysis of a sample of the 6S–12S enzyme by affinity chromatography on a concanavalin A–Sepharose column (Pharmacia), equilibrated with buffer B, failed to reveal any carbohydrates in the cytoplasmic DNA polymerase molecules which were normally seen with concanavalin A under native conditions. A reaction for DNA (Burton, 1956) carried out from the Sepharose 6B gel-filtration samples was negative, excluding the possibility of errors from significant DNA–enzyme complex-formation.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Selected tubes or pools of activity after a series of purification steps were run on 10% sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The protein pattern for the enzyme eluted by 100 mM- (a)

Fig. 3. Sedimentation behaviour of DNA polymerase extract at low ionic strength (I 0.045)

A portion of the pooled DNA polymerase activity, shown in Fig. 2(a), was layered on top of a sucrose density gradient and spun down at low ionic strength. A dominant peak of activity co-sedimented with immunoglobulin G (7S), indicating an anomalous gel-filtration behaviour of these molecules when compared with the s value obtained. DNA polymerase extract treated with $(\text{NH}_4)_2\text{SO}_4$ revealed a sucrose-density-gradient pattern similar to that shown in Fig. 4. Δ , Reference proteins for s -values in companion tubes.

and 150 mM- (b) KCl from the Sephadex G-200/ Sepharose 6B/DEAE-cellulose columns is shown in Fig. 5. Noteworthy, several twin bands of protein can be seen in the separation gels of both KCl peaks. The difference in the molecular weights within the twin bands does not exceed 5000.

Fig. 4. Determination of physicochemical data for partially purified cytoplasmic DNA polymerases

Sedimentation rates of either the 100mM- or the 150mM-KCl-eluted enzyme were determined by sucrose-density centrifugation. Classes of different sedimentation coefficients were pooled as indicated in Table 1 and analysed for Stokes radii on a calibrated Sepharose 6B column. All experiments were carried out at low ionic strength (I0.045). The circled numbers label the three marker proteins trypsin (1), human haemoglobin (2) and catalase (3).

Discussion

The aim of this study was to throw some light on the heterodispersity of cytoplasmic DNA polymerase activity and to develop a simple and rapid purification procedure for further analysis of the 6S–12S enzyme.

Computed molecular weights agreed with the pronounced tendency of DNA polymerase to form oligomeric structures at low ionic strength. From a frictional-ratio consideration, it can be deduced that the aggregation of either isologous or heterologous polypeptide chains occurs along the longer axis to form high-molecular-weight DNA polymerases. The

Table 1. Physical characteristics of partially purified but heterogeneous cytoplasmic DNA polymerase (6S–12S) at low-salt conditions (50mM-Tris/HCl)

Sedimentation coefficients are the average of six independent measurements by sucrose-gradient centrifugation as described under 'Methods'. Stokes radii were obtained on a calibrated Sepharose 6B column (97cm × 1.5cm) with thyroglobulin (8.5 nm, Sigma), apoferritin (7.4 nm, Serva), alcohol dehydrogenase (4.5 nm, Boehringer) and bovine serum albumin (3.5 nm, Behring) as markers, traced by A_{280} measurement. Molecular-weight values and frictional ratios (f/f_0) were calculated assuming a partial specific volume ($\bar{v}$) of 0.725 cm³/g, neglecting the solvent factor δ .

Physical parameters	Classes of enzymes			
	5.0–6.0	7.0–7.4	8.4–8.8	11.3–11.7
$s_{20,w}^0$				
Stokes radius (nm)	5.0	6.5	8.3	9.8
Calculated mol.wts.	113 000	193 000	293 000	463 000
f/f_0	1.57	1.71	1.90	1.92

enzyme may undergo a conformational transition to a much more elliptical shape in discrete steps (Table 1). These findings are consistent with the growing acceptance of heterogeneity of DNA polymerase activity, isolated from cytoplasmic extracts (Holmes *et al.*, 1974; McLennan & Keir, 1975). It is still questionable whether the 125000-mol.wt. protein, which has been obtained by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, can be related to the smallest active protomeric form, calculated from physicochemical data. Moreover, it is uncertain whether this polypeptide band is identifiable with an active monomer. The elution pattern found from the (NH₄)₂SO₄ experiments on Sephadex G-100 suggests an active DNA polymerase molecule smaller than the calculated one, because it was eluted away from the void volume of the Sephadex G-100 column. The gel-filtration experiments, carried out on both Sephadex columns, clearly demonstrate that temporarily altered ionic strength causes a dissociation of high-molecular weight forms of cytoplasmic DNA polymerase activity into smaller pieces. The conversion of s values of cytoplasmic DNA polymerase activity, isolated from testis, under high- and low-salt conditions was first reported by Hecht (1973); the evaluation of gel-filtration characteristics of *Physarum* cytoplasmic DNA polymerase activity before and after treatment with (NH₄)₂SO₄ confirm this finding.

The purification procedure of cytoplasmic DNA polymerase activity indicates that the column chromatography, carried out by the three columns in series (method 1), is a powerful tool to obtain a rapid and acceptable yield of a protein preparation that contains a drastically decreased number of proteins

Fig. 5. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, derived from the enzyme eluted by 100 mM- and 150 mM-KCl from combined gel and ion-exchange chromatography

About 100 μ g of protein from the material eluted by 100 mM-KCl and approx. 140 μ g of that eluted by 150 mM-KCl (Fig. 1b) were loaded on top of gels (a) and (b) respectively. Molecular weights of the stained protein bands were extrapolated from a gel with marker proteins, run in a companion tube.

compared with the DNA polymerase extract as examined by sodium dodecyl sulphate/polyacrylamide gels. In some preparations the purification factor varied, which was a disadvantage. Whether this variation in specific activity of cytoplasmic DNA polymerase activity was due to limited proteolysis for which the symmetrical protein pattern on sodium dodecyl sulphate/polyacrylamide-gel might be an indication, could not be ascertained. Interestingly,

sucrose-gradient centrifugation after a Sephadex G-200/Sephadex 6B gel-filtration step shows a much lower increase in specific activity than ion-exchange chromatography. It cannot be ruled out that KCl stimulates DNA polymerase activity or, on the other hand, sucrose-density-gradient centrifugation separates a necessary factor with an accompanying change in the template specificity (Kiyokazu & Hiroshi, 1974); in practice this would mean decreased enzyme activity.

We emphasize that neither by gel filtration at initial NaCl concentrations up to 1.8 M, nor by sucrose-density-gradient sedimentation has an active enzyme species of 3S–3.5S been observed, but sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of preparations from the Sephadex/Sephadex/DEAE-cellulose columns in series reveal protein bands that might correspond to DNA polymerase activity found in nuclei (Chang & Bollum, 1971, 1972); in this case, however, the mechanism of such disaggregation, whether involving proteolysis or salt-conversion or both, remains obscure.

Our results support the view of the multiplicity of cytoplasmic polymerase species and the inter-conversion *in vitro* of *s* values of cytoplasmic DNA polymerase activity, isolated from the lower eukaryote *Physarum polycephalum*. There have been only a few investigations on the relationship of DNA polymerase species to the cell cycle (Bernard *et al.*, 1974; Craig *et al.*, 1975), but at least one DNA polymerase species seems to be an acute-phase protein, which shows maximum activity during DNA synthesis. Further work has to be done on the scheme of DNA polymerase regulation in order to understand the biological relevance of the heterogeneity of DNA-dependent DNA polymerase activity. It is difficult to assign a definite role to either enzyme on the basis of the results presented here. Although physicochemical data give ample evidence for heterogeneity of cytoplasmic DNA polymerase activity, the multiple species described have to be compared in the light of exhaustive examination of utilizing different primer-templates, requirement for bivalent cations, pH optimum and specific inhibitors. For this view it is an advantage to have a preparation procedure that allows rapid separation of different DNA polymerase species.

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