TWO GROUPS OF DEOXYRIBONUCLEIC ACID POLYMERASES FROM *PHYSARUM POLYCEPHALUM* CLASSIFIED BY DIFFERENTIAL SENSITIVITY TO *N*-ETHYLMALEIMIDE, HEPARIN, CYTOSINE ARABINOSIDE TRIPHOSPHATE AND ETHIDIUM BROMIDE

Evidence for a β-like activity

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1. Introduction

Four forms of DNA-dependent DNA polymerase activity (A,B,C,D) have been found in preparations obtained from microplasmodia of the lower eukaryote *Physarum polycephalum* [1,2]. The preparation of DNA polymerase by the alternative procedure of polyethylene glycol precipitation of nucleic acids [3] reveals three forms of DNA polymerase. The properties of these three enzymes suggest a relationship between them which allows a classification of the DNA polymerases from *Physarum* into two groups. This classification is based on differential sensitivity to the agents *N*-ethylmaleimide, heparin, cytosine arabinoside triphosphate and ethidium bromide as well as on isoelectric points.

2. Materials and methods

2.1. *Reagents*

d[^{3}H]TTP (30 Ci/mMol) was purchased from Amersham-Buchler (Braunschweig). Polyethyleneglycol 6000 and ethidium bromide were obtained from Serva (Heidelberg). ara-CTP, heparin (sodium salt, grade 1, activity 170 USP/mg) and NEM were purchased from Sigma (Munich).

2.2. *Buffers*

The pH values of the following buffers refer to the final solution at 20°C. The composition of buffer A is: 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 10 mM 2-mercaptoethanol, 1.7 M NaCl. Buffer B is composed of 50 mM Tris–HCl (pH 7.5), 0.3 mM EDTA, 2 mM 2-mercaptoethanol, 20% (v/v) glycerol. Buffer C is identical to buffer B with glycerol omitted. Extraction buffer D is buffer B in 0.2 M KCl, 1 mg bovine albumin/ml.

Abbreviations: ara-CTP, cytosine-β-D-arabinofuranoside 5'-triphosphate; NEM, *N*-ethylmaleimide

2.3. *Cultures*

Microplasmodia of *Physarum polycephalum*, strain M_3c, were grown as in [2,4] in 20 l flasks containing 10 l medium. Innoculation of 100 g microplasmodia resulted in ~0.9 kg microplasmodia/flask within 3 days.

2.4. *Purification*

A scheme is given in table 1. All steps were performed at 4°C. Volumes given in the procedure are normalized to 1 kg washed microplasmodia.

Fraction 1: Microplasmodia were sedimented from 10–30 l medium, washed with deionized water and weighed. After suspension with 4 l buffer A the microplasmodia were homogenized in a Waring-type blender (MX 32, Braun, Frankfurt) for 30 s at 11 000 rev./min in the presence of 1 mM phenylmethylsulfonylfluoride.

Fraction 2: 2.5 l of 30% (w/v) polyethylene glycol in 2 M NaCl were added to the homogenate [3]. After stirring for 30 min the suspension was centrifuged for 30 min at 4000 × *g* (Stock, Marburg). The clear supernatant fluid was dialyzed against several changes

of buffer C. After 24 h dialysis NaCl was 40 mM. Material precipitated during dialysis was removed by centrifugation (30 min 4000 × *g*).

Fraction 3: Fraction 2 was mixed with 1 *l* DEAE–Sephacel slurry. After equilibration for 1 h the suspension was decanted through a polyamide tissue (mesh size 40 μm). The retained DEAE–Sephacel was washed with buffer C and the enzyme activity eluted with 4 l of 0.4 M KCl in buffer C. After dialysis of the clear filtrate against 40 l buffer B overnight, precipitated material was removed by centrifugation (10 000 × *g* for 25 min).

Fraction 4: Fraction 3 was pumped onto a DEAE–Sepharose column (3 × 28 cm) at 20 ml . h^{-1} . cm^{-2}. The column was washed with buffer B and the enzyme activity was eluted with 2 l of a linear 0–0.4 M KCl gradient in buffer B without change of the flowrate (fig.1). Eluate fractions (10 ml) corresponding to the peaks of enzyme activity were pooled and dialyzed against buffer B.

Fraction 5: Pools from the DEAE–Sepharose column were loaded onto a DNA–cellulose column (2 × 25 cm) at 17 ml . h^{-1} . cm^{-2} [1]. The column was washed with buffer B and the enzyme activity was eluted with 600 ml of a linear 0–0.5 M KCl gradient in buffer B. Eluate fractions corresponding to the peaks of enzyme activity were pooled and dialyzed against buffer B. The dialyzed solution was referred to as fraction 5. In some cases the material was rechromatographed on a small DNA–cellulose column (8 ml) yielding fraction 6.

2.5. *Estimation of the Stokes radius*

Gel filtration was performed on an Ultrodex AcA 34 column (1.4 × 66 cm) equilibrated at 4°C with 0.4 M KCl in buffer B [1,5]. The column was calibrated using the following standard markers: ovalbumin (2.73); bovine albumin (3.55); alcohol dehydrogenase (4.5); pyruvate kinase (5.4); glutamic dehydrogenase (6.1); thyroglobulin (8.5). The values given in parentheses are Stokes radii in nm [6–8].

2.6. *Estimation of the sedimentation coefficient*

Sucrose gradient centrifugation was carried out using linear 5–20% (w/v) sucrose gradients in buffer B or in 0.4 M KCl in buffer B as in [1,9]. The material was centrifuged for 21 h at 280 000 × *g* at 4°C. Marker proteins were catalase (11.3 S), lactic dehydrogenase (6.93 S) and alcohol dehydrogenase (4.9 S).

2.7. *Isoelectric focusing*

Isoelectric points were estimated as in [4]. The method is based on standard procedures [10–12] and was modified as indicated. Gel rods (5 × 80 mm) consisted of polyacrylamide, $T = 5\%$, $C = 3\%$ [13], of 20% (w/v) glycerol, and the following amounts of ampholines/10 ml: 0.415 ml pH 3.5–10; 0.03 ml each of pH 4–6 and pH 5–7; 0.10 ml pH 9–11. The anodic buffer consisted of 10 mM H_3PO_4, and the cathodic buffer consisted of 0.1% (v/v) ethanolamine. The gels were run at 300 V for 3 h. For determination of DNA polymerase activity, the gels were cut into 2.1 mm slices. These were extracted with 200 μl each of extraction buffer D. For determination of pH, slices of a blank gel were extracted for 4 h with 1 ml each of degassed deionized water.

2.8. *Assay of DNA polymerase activity*

The assay was carried out with 15 μM d[^{3}H]TTP (2 Ci/mmol) as in [1]. One unit of activity was defined as the incorporation of 1 nmol dTMP into acid-insoluble material per 30 min at 37°C.

2.9. *Determination of protein*

Fractions 1 and 2 were precipitated by 0.5 M PCA. After digestion of the precipitate, ammonia was determined according to [14]. Estimation of protein in other fractions was performed according to [15] or the method in [16].

3. Results

3.1. *Preparation of three DNA polymerases*

Fractionation of homogenates with polyethylene glycol at 1.57 M NaCl [3] allowed removal of nucleic acids at low speed centrifugation. Up to 70% of the enzyme activity found in the homogenate were present in fraction 2 (table 1). Loading of this fraction onto a DEAE–Sepharose column clogged the column whereas previous application of a batch procedure (step 3) resulted in a reasonably high flowrate. The DNA polymerase activity was resolved into three species (DE-I, DE-II, DE-III) as seen in fig.1. In 6 different experiments the peaks were desorbed from the column at highly reproducible ionic strengths, KCl being at 99 ± 7 mM (DE-I), 129 ± 6 mM (DE-II) and 195 ± 6 mM (DE-III). From the DNA–cellulose column DE-I eluted above 0.175 KCl in buffer B, DE-II below this concentration. DE-III showed a broad

Table 1
Partial purification of DNA polymerase forms DE-I, DE-II and DE-III

Step	Fraction (Enzyme form)	Volume (ml)	Protein (mg)	Activity (units)	Spec. act. (units/mg)
1	Homogenate	5000	13 000	3100	0.24
2	Supernatant	11 400	8000	2200	0.28
3	DEAE–Sephacel	2800	4200	11 600	2.8
4	DEAE–Sepharose				
	DE-I	200	530	1500	2.8
	DE-II	176	270	1000	3.7
	DE-III	85	150	420	2.8
5	DNA Cellulose				
	DE-I	125	4	780	195
	DE-II	170	17	630	37
	DE-III	60	2	360	180

The values are normalized to 1 kg washed microplasmodia. An increase in enzyme activity in step-3 was also observed in [1] and may be due to the removal of an inhibitor. The activities of the pools DE-I, II, III obtained from the DEAE–Sepharose column were 2100, 1600 and 470 units, respectively, before dialysis

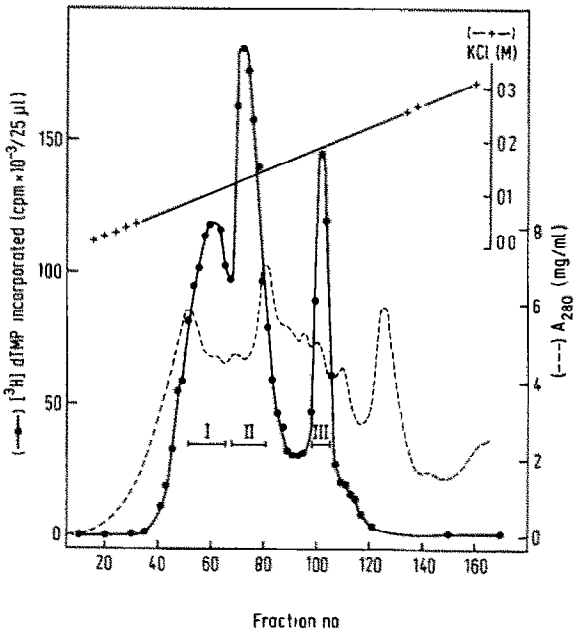

Fig.1. DEAE–Sepharose chromatography. Step-3 enzyme (4.1 l, 3800 units, 4.8 g protein) was loaded onto a column (3 × 45 cm): 1% of the activity was not adsorbed to DEAE–Sepharose; 69% were collected in the gradient. In other experiments the yield varied from 35–77%. (—•—) DNA polymerase activity; (—+—) KCl gradient; (----) A_{280}. The protein concentration was calculated with bovine albumin as standard. The fractions (13.5 ml each) were pooled as indicated by (——).

peak of activity. The purification of the three species at this step was 1200-, 300- and 5000-fold, respectively, assuming that the activities of the species in the homogenate were of the same magnitude as in the undialyzed fractions obtained in step 4.

3.2. *Hydrodynamic parameters*

Molecular weights and frictional ratios (table 2) were calculated using the Stokes radius, the sedimentation coefficient and a partial specific volume of 0.725 ml/g [5,9]. Gel filtration and gradient centrifugation revealed single uniform peaks of enzyme activity if thoroughly separated enzyme forms were used. No tendency of aggregation at low ionic strength was observed in the latter procedure using the enzyme forms of step 5 or 6.

3.3. *Isoelectric points*

The activity of form DE-I showed a homogeneous peak at pH ~8.1 (table 2). Form DE-II exhibited a heterogeneous pattern with peaks at pH 7.1, 7.7 and 8.0. The isoelectric point of form DE-III was 5.5. In some preparations an additional activity was found at pH 6.5.

3.4. *Influence of inhibitors and univalent cations*

The DNA polymerase forms DE-I and DE-II were inhibited by NEM, heparin and ara-CTP whereas the form DE-III was only slightly influenced by the indicated concentrations of these agents (table 3). This

Table 2
Physical properties of DNA polymerases

Parameter	Form DE-I	Form DE-II	Form DE-III
Stokes radius, a (nm)	4.5 ± 0.17 (6)	5.6 ± 0.27 (5)	3.8 ± 0.13 (7)
Sedimentation coefficient, $s_{20,w}$ (S)	5.3 ± 0.13 (4)	5.9 ± 0.45 (10)	5.2 ± 0.31 (16)
Relative molecular mass, M_r	98 000	136 000	81 000
Frictional ratio, f/f_0	1.48	1.65	1.33
Isoelectric point (pH)	8.1 ± 0.2	7.1–8.1	5.5, 6.5

Stokes radii, sedimentation coefficients and isoelectric points were obtained as in section 2. The M_r values and frictional ratios were calculated according to eq. (1) and (2) in [5]. Data are presented as means ± SD with no. expt. in parentheses

difference between the two groups of enzymes was enhanced by the effect of ethidium bromide which strongly inhibited form DE-III but only weakly inhibited the other two forms.

All three enzyme forms were stimulated by 100 mM KCl. The forms DE-I and DE-II were also stimulated by 50 mM NaCl whereas the activity of DE-III was not increased and in some experiments even slightly reduced by 50 mM NaCl.

4. Discussion

The properties of the enzyme forms described suggest a relationship between them as well as some of those found in [2] and allow the classification of the DNA polymerase species from *Physarum polycephalum*. The forms DE-I and DE-II have the following features in common with form B: sensitivity to heparin; resistance against ethidium bromide; and isoelectric points at pH values above neutrality. Form DE-III shares with form D, a high sensitivity to ethidium bromide, the resistance against heparin and the isoelectric point of pH 5.5. The molecular weights of DE-III and D differ by <4%. Both species probably represent identical enzymes.

The two groups of DNA polymerases from

Table 3
Effect of various agents on DNA polymerases

Agent	Conc. (mM)	Activity (%) of		
		Form DE-I	Form DE-II	Form DE-III
NEM	10	20	7	105
Heparin	1 μg/ml	2	15	92
ara-CTP	0.3	16	18	122
Eth Br	0.05	71	90	13
NaCl	50	190	170	105
	200	20	20	40
KCl	100	290	330	160
	200	75	50	78

For assays with inhibitors the standard assay mixture contained 2.5 mM magnesium acetate and 50 mM KCl. Potassium morpholinopropanesulfonate was replaced by 50 mM Tris–HCl (pH 7.0). Otherwise the assay mixture was composed as in section 2. For assays with NEM the enzyme preparations were dialyzed against buffer B containing a total of 50% (w/w) glycerol. NEM was dissolved in 50 mM K-phosphate (pH 6.7). Of the enzyme preparation 15 μl were preincubated with 10 μl NEM solution for 30 min at 4°C. NEM was 10 mM during the preincubation and the pH of the mixture 7.0. In the tests with NaCl or KCl only the stated univalent cation was present

Physarum polycephalum resemble, in certain features, the polymerases α and β from mammalian cells. The group comprised of forms DE-I and DE-II is characterized by $M_r \geqslant 98\,000$ and relatively high sensitivity to NEM, heparin and ara-CTP as is polymerase α [17–20]. The form DE-III shows a M_r similar to that of the β-like polymerase B from *Dictyostelium discoideum* [21] and to polymerase II from *Tetrahymena pyriformis* [22] which is ~70 000. Only slight or no inhibition was observed with NEM, heparin and ara-CTP.

The differences between mammalian DNA polymerases α and β and *Physarum* polymerases are nevertheless profound. The isoelectric point for example, of DE-III is about the same as that of polymerase α [17,23], whereas DE-I and II focus like DNA polymerase β [17,24].

Using a similar method of preparation only one form of DNA polymerase from *Physarum polycephalum* was obtained in [25]. The enzyme resembled form B. Likewise, only a polymerase activity with sensitivity to 10 mM NEM was detected in supernatant fluids from extracts obtained with buffered 0.25 M sucrose [1] or 0.2 M potassium phosphate [26]. The finding of several species of DNA polymerase depends apparently on the state of growth as well as on the conditions of extraction.

The recent observation of a β-like DNA polymerase activity in *Dictyostelium* [21], *Tetrahymena* [22] and now in *Physarum* suggests a rather continuous line of evolution of the DNA polymerases throughout the eukaryotic organisms.

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