MOLECULAR ASYMMETRY OF RAT LIVER CYTOPLASMIC DNA POLYMERASE

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Received 21 November 1972

1. Introduction

Recent work in several laboratories has shown that the high molecular weight DNA polymerase (polymerase S1 of [1]) found in high speed supernatants of mammalian cell or tissue homogenates is distinct and separable from the DNA polymerase activity of nuclei [1–5]. Circumstantial evidence implicates this enzyme in the process of replication; for instance its level of activity is very much higher in foetal [6] and regenerating rat [7] livers in both of which the number of cells involved in replication is about twenty times higher that in the adult. Further, in tumours of different growth rates levels of this polymerase, detected by its preference for denatured DNA, correlate well with rates of *in vivo* DNA synthesis determined using [^{3}H]thymidine [7, 8]. Although the cytoplasmic location of this polymerase is something of a paradox, it is possible either that it may have leaked from its nuclear environment during aqueous isolation procedures [9], or, as other evidence suggests, the enzyme is normally found in the cytosol *in vivo*, moving into the nucleus at the beginning of S-phase; this would appear to be the case in both the sea urchin [10] and in mammalian cells [11–13]. Although substantial purification of the corresponding enzyme from calf thymus [14] and human KB cells [15] has been reported, a more explicit view of the replication process requires that some idea of the molecular weight and sub-unit constitution of polymerase S1 be obtained. As a result of studies undertaken to purify the enzyme from rat liver we wish to report that it exhibits "non-standard" behaviour [16] on gel filtration. An estimate of molecular weight is obtained assuming it to be a pure protein and the implications of its deviation from standard behaviour are discussed.

2. Materials and methods

[^{3}H]TTP (26.4 Ci/mM), glucosamine [1-^{14}C] hydrochloride (55 mCi/mM), [1-^{3}H] galactose (5.7 Ci/mM), and choline chloride ([^{3}H] methyl) (15 Ci/mM) were obtained from the Radiochemical Centre, Amersham. Sepharose 6B was from Pharmacia. *E. coli* β galactosidase, beef liver catalase, and rabbit muscle lactate dehydrogenase were from Boehringer, horse spleen apoferritin from Calbiochem and hog thyroglobulin from Koch-Light Laboratories. Other materials were as described before [1].

2.1. Buffers

All buffers contained 20% w/v glycerol and 1.4 mM 2-mercaptoethanol. Buffer A is 0.1 M NaCl in 0.05 M Tris-HCl, pH 8.5 (23°). Buffer B is 0.5 M NaCl in 0.05 M Tris-HCl, pH 8.5. Buffer C is 0.5 M NaCl in 0.025 M potassium phosphate, pH 6.5.

2.2. DNA polymerase assay

25 μl aliquots of fractions were assayed as previously described [1] except that the calf thymus DNA was 15 to 20-fold activated before use and each assay contained 125 μg bovine serum albumin. 1 unit of activity is 1 nmole of [^{3}H]dTMP incorporated per hour.

2.3. DNA polymerase from rat liver

80 g Liver from 15–20 g albino rats or 50 g liver from 150 g rats 26 hr after two-thirds partial hepatectomy, were homogenised with 3 vol 0.25 M sucrose and the 105 000 *g* × 60 min supernatant (fraction I) used to prepare enzyme as follows. Supernatant was

made 0.05 M in potassium phosphate pH 7.0, and then adjusted to pH 5.0 with 1 N acetic acid; the resulting precipitate was dissolved in 0.05 M potassium phosphate, pH 7.0 (fraction II) and applied to a 12 × 2.4 cm phosphocellulose column. After washing with 0.1 M potassium phosphate, pH 7.0, the enzyme was eluted with 0.5 M potassium phosphate, pH 7.0 (fraction III). Following dialysis against several changes of 0.01 M potassium phosphate, pH 6.5, 0.001 M EDTA, the enzyme was loaded on to a 15 × 2 cm DNA–cellulose column [1] in the same buffer, washed with more of the same and finally eluted with 0.3 M NaCl in 0.05 M Tris-HCl, pH 8.5 (fraction IV, 100-fold purified). The enzyme was then concentrated against the appropriate buffer containing 30% w/v polyethylene glycol 6000 and chromatographed on a 61 × 1.8 cm Sepharose 6B column. 75 to 90% of the peak was taken and yielded fraction V (about 250–500-fold purified; overall yield 22%).

2.4. Glycerol density gradient centrifugation

Following dialysis against either buffer B or buffer C (section 2.1) from which glycerol had been omitted, 0.4 mg (4 units) fraction IV enzyme in 0.5 ml was applied to a 12.5 ml 10–30% glycerol gradient made in the appropriate buffer and centrifuged at 4° in the SW 40 Ti rotor for 16 hr at 39000 rpm, in a Beckman L2-65B centrifuge. 0.5 ml fractions were collected from the top of the gradient by displacement from below with 60% w/v glycerol. Fractions were assayed as described [1]. The S-value of the enzyme was calculated

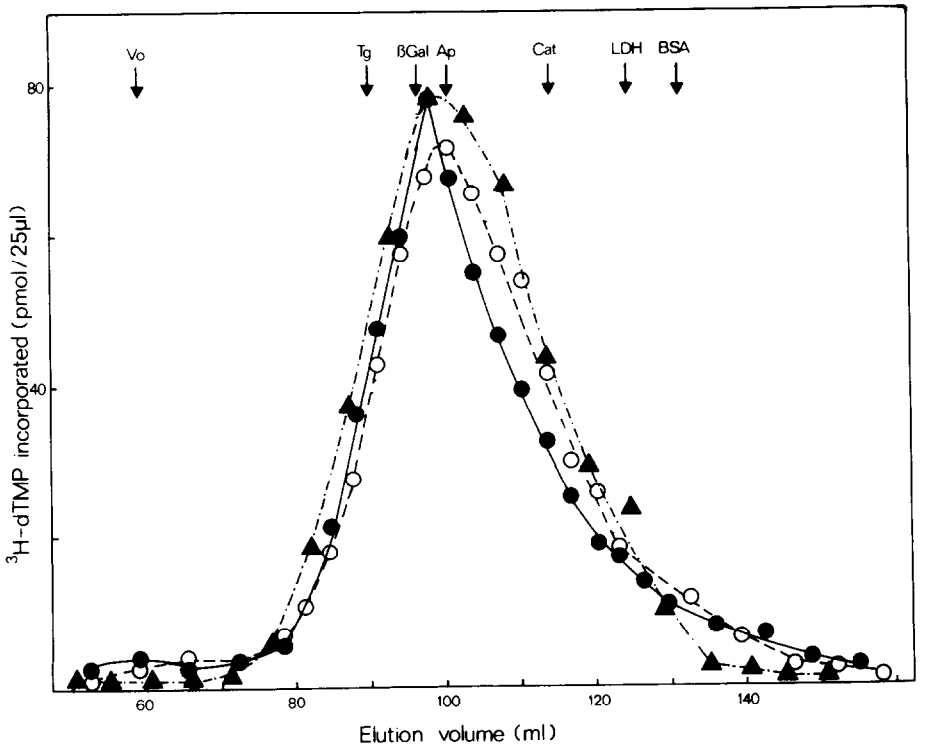

Fig. 1. Gel filtration chromatography of rat liver cytoplasmic DNA polymerase on Sepharose 6B. A Sepharose 6B column 61 × 1.8 cm was used. For runs shown in the figure enzyme was loaded, in 3% or less of the column volume, as follows; (▲—▲—▲) 4 mg fraction V enzyme (127 units), eluting with buffer A (see sect. 2.1); (●—●—●) 21 mg fraction IV enzyme (100 units), eluting with buffer B; (○—○—○) 18 mg fraction IV (118 units), eluting with buffer C. Markers used were, Tg, thyroglobulin; β-gal, β-galactosidase; Ap, apoferritin; Cat, catalse; LDH, lactate dehydrogenase; BSA, bovine serum albumin. They were detected by 280 nm absorption or by enzymatic activity. V_O, the void volume, was determined using blue dextran; V_e is elution volume. Recovery of enzyme activity from columns was 60–80% of that loaded.

using lactate dehydrogenase (7.3 S), catalase (11.3 S) and β-galactosidase (16 S) as standards [17]. Glycerol concentrations were obtained from refractive indices using an Abbé refractometer.

3. Results

3.1. Agarose gel chromatography

During the course of purifying the enzyme using an Agarose gel filtration step it was noticed that the enzyme eluted well before catalase and close to β-galactosidase. Gel filtration runs were, therefore, carried out under a variety of conditions in an attempt to establish whether the enzyme was an easily reversible aggregate. By using standard proteins run under each set of conditions an estimate of apparent molecular weight was obtained. Some selected data are shown in fig. 1. The results of running partially purified enzyme (substantially freed of low molecular weight DNA polymerase by the DNA cellulose step [1]) at pH 8.5 in 0.1 M NaCl (buffer A) shows activity to peak at a V_e/V_0 of about 1.7, between β-galactosidase and apoferritin. This indicates an apparent molecular weight in the region of $460–520 \times 10^3$. Raising the NaCl concentration to 0.5 M at pH 8.5 (buffer B) was without effect on the elution position of the enzyme peak; lowering the pH to 6.5 while maintaining the NaCl at 0.5 M (buffer C) had only a slight effect on elution volume. At intermediate pH's or in 1 M NaCl the elution position was essentially similar. Since the ratio of 280 nm: 260 nm absorption was 1.6–1.7 across the peak, the elution position occupied by the enzyme was not due to the binding of nucleic acid. It would also seem unlikely that the enzyme is bound to lipid (cf. [5]) since, following the inclusion of 0.25% Brij-58 in the eluting buffer (buffer B) and in the loaded enzyme, there was again no alteration in V_e/V_0 although some loss in activity occurred. (0.5% Triton X-100 rapidly inactivated the 100-fold purified enzyme from fraction IV.) Variation of the amount of enzyme loaded onto the column by a factor of about ten (45 units loaded as opposed to 490 units) also had no effect on the elution position of the main peak. By omitting the steps we used in the enzyme preparation one or two at a time, we ascertained that this high apparent molecular weight did not arise as a result of any one of them. Similarly, the enzyme from each step of the preparation (that is, differing in degree of purity) when run or re-run on Sepharose 6B gave elution profiles and V_e/V_0 values essentially identical to those in fig. 1. Finally, since all the elution buffers contain 20% glycerol to stabilise the enzyme, this was omitted from buffer B and V_e/V_0 again determined in relation to standard proteins. The enzyme, in fact, ran closer to the position of β-galactosidase (V_e/V_0: 1.65). Therefore, as expected, its apparent molecular weight had not decreased in the absence of glycerol; it was nevertheless important to establish this point as regards the loading of enzyme, in 2–3% glycerol, on to glycerol density gradients.

It should be mentioned that on several occasions when smaller amounts of enzyme (45 units or less) have been run on Sepharose 6B, a shoulder of activity is observed at a V_e/V_0 of about 2.0. Although the significance of this minor activity remains to be assessed, its existence does not affect the conclusions drawn here regarding the properties of the main peak; it could, however, account for the width and asymmetry of the enzyme elution profile observed in both standard (fig. 1) and in glycerol-free buffers.

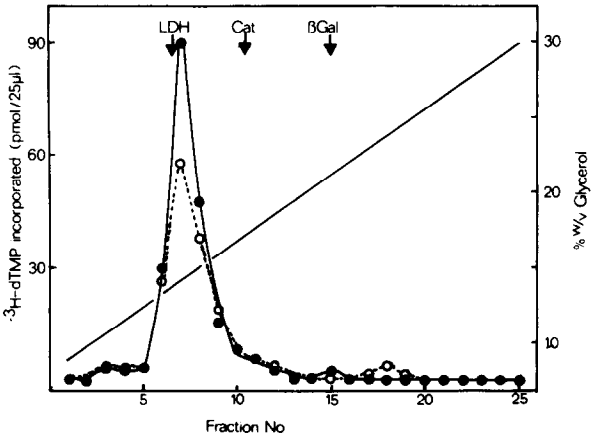

Fig. 2. Glycerol density gradient centrifugation. The enzyme was centrifuged as described in sect. 2.4. Sedimentation is from left to right. (●—●—●) 0.4 mg (4 units) enzyme from fraction IV run in buffer B (pH 8.5); (○—○—○) similar amount of fraction IV enzyme run in buffer C (pH 6.5). Recovery of enzyme activity gradients was 80–90% of that loaded.

3.2. Glycerol density gradients

Enzyme from the DNA cellulose step (fraction IV) or Sepharose 6B step (fraction V), was run as described in fig. 2 under the same conditions of pH (6.5 and 8.5) and salt concentration (0.5 M NaCl) used in some of the gel filtration studies. The sedimentation coefficient ($s_{20,w}$) of the peak enzyme activity was estimated as 7.6 ± 0.2 S (6 runs). Notably it ran just ahead of lactate dehydrogenase (7.3 S) but more slowly than catalase (11.3 S) whereas on Sepharose 6B it eluted from the column before catalase and close to β-galactosidase. The corresponding enzyme from rabbit bone marrow sediments at 6–8 S [18].

3.3. Molecular weight estimate

In order to gain some idea of the true molecular weight we obtained a value for the diffusion coefficient by plotting data from Sepharose 6B runs against the reciprocals of published $D_{20,w}$ values (fig. 3), as outlined by Andrews [19]. While such comparative estimates, and the Stokes radii derived from them, are subject to the errors of published values, they are probably in reasonable agreement with those obtained from more rigorous determinations [19, 20]. The $D_{20,w}$ value so obtained was $3.3 \pm .15 \times 10^{-7}$ cm²/sec.

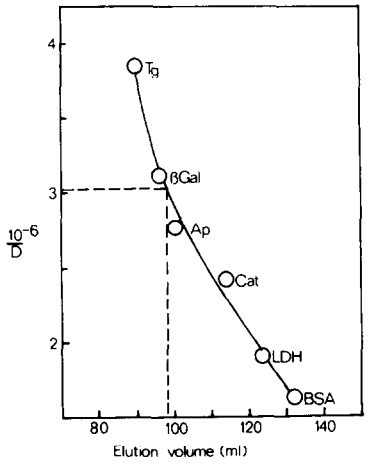

Fig. 3. Estimation of diffusion coefficient ($D_{20,w}$) for DNA polymerase. Elution volumes of standard proteins on Sepharose 6B column in fig. 1 were plotted against reciprocals of published $D_{20,w}$ values. Buffer A was used; exactly the same answers were obtained in buffer B or C. Abbreviations are as in fig. 1. Note that, unlike Andrews [16] we found thyroglobulin to exhibit normal behaviour on the column. D = diffusion coefficient ($D_{20,w}$ values were used [19, 21]).

The values for $s_{20,w}$ and $D_{20,w}$ can be substituted in the Svedberg equation

$$M = RTS/D(1 - \bar{\nu}\rho)$$

where M is molecular weight, R is the gas constant, T is absolute temperature, S is the sedimentation coefficient, D is the diffusion coefficient, ρ is the density of water at 20° and $\bar{\nu}$ is the partial specific volume, taken here as 0.725 ml/g [17]. The molecular weight is calculated to be 204,000. On the basis of this estimate, the deviation of the molecular weight determined by gel filtration is about 140–150%.

3.4. Basis of the deviation from "standard" behaviour

Using the $D_{20,w}$ value obtained from fig. 3 we derived the Stokes radius of the molecule using the equation $a = kT/6\pi\eta D$. Where a is Stokes radius, k is the Boltzmann constant, η is the coefficient of viscosity of water at 20° and the other symbols are as previously stated. The value for a is 65×10^{-8} cm. Substituting this value in the expression

$$f/f_0 = a/(3\bar{\nu}\,M/4\pi N)^{1/3}$$

where f/f_0 is the frictional ratio, N is the Avogadro number and other symbols have the meanings above, we obtain a value for f/f_0 of 1.66 which, allowing 0.2 g of water for hydration per gram of protein [21], indicates an axial ratio of about 10 to 1 [22] for a prolate ellipsoid molecule. In view of the uncertainties in the estimation of $s_{20,w}$, $D_{20,w}$ and therefore of molecular weight and Stokes radius, this ratio is at best an approximation; nevertheless, this degree of asymmetry would readily account for the anomalous gel filtration behaviour of the enzyme on Sepharose 6B.

However, deviation from standard behaviour on gel filtration can also arise because of the presence of carbohydrate in the molecule [16, 19]. We attempted to study this possibility by injecting, in separate experiments, a total of either 50 μCi [^{14}C]glucosamine or 1 mCi [^{3}H]galactose into rats (18 per experiment) 19 hr after two-thirds partial hepatectomy had been performed. The animals were killed 7 hr later. In both ex-

periments, acid-precipitable (phosphotungstic or trichloroacetic acids) radioactivity, present in the 105,000 *g* supernatant of the homogenised liver remnant, was eliminated completely when the enzyme was purified through to the Sepharose 6B step. However, this negative result could be inconclusive if the absolute amount of newly synthesized enzyme was too small to pick up labelling of it; a definitive answer would require milligram quantities of pure enzyme. Even so, it appears that the extent to which a molecule deviates from "standard" behaviour on gel filtration is strongly influenced by molecular asymmetry, while the influence of covalently-bound carbohydrate varies with individual glycoproteins [16]. Therefore, although the presence of carbohydrate (which would modify values of parameters used in the above calculations) is not ruled out, it seems necessary to envisage the cytoplasmic DNA polymerase as a relatively asymmetric molecule.

An experiment was also carried out identical to that described for labelled sugars, using 1 mCi [^{3}H]choline. Once again, however, the radioactivity of the high speed supernatant fractionated away from the enzyme during purification suggesting that choline-containing lipid is not part of the enzyme complex.

4. Discussion

In preliminary studies cytoplasmic DNA polymerase S1 appeared to have a molecular weight of the order of 400,000 or more as estimated by gel filtration of Sepharose 6B [1]. Others had also reported that cytoplasmic DNA polymerase from rat liver [4] and from HeLa cells [2] eluted close to the void volume of Sephadex G-200 columns; this is indicative of an apparent molecular weight in the region of 400,000–500,000 (compare for example, elution data in [16]). From the data presented in this paper, and bearing in mind the uncertainties involved, the true molecular weight of the enzyme is probably in the region 200–230,000. A more accurate estimate will have to await larger amounts of pure enzyme or a knowledge of subunit molecular weights. It should also be recognised that we have been unable to show whether or not the species described here is an associated form of the enzyme. Further, the polymerase moiety may be only a part of a complex of enzymes. Even allowing for this, the enzyme (or complex) probably exhibits considerable asymmetry. In this connection it is interesting to note that two other proteins involved in DNA synthesis, in T4 phage (the products of genes 44 and 62 [23]), bind tightly to form a complex which exhibits similar anomalous behaviour on gel filtration, probably as a result of the asymmetry of the complex. In addition T4 gene 32 protein, also involved in replication, exhibits some degree of asymmetry [24]. The possession of this property by all of these molecules may be entirely fortuitous.

Acknowledgements

We wish to thank the Medical Research Council for a research grant and for a studentship to A.M.H. We are grateful to Michael Rosemeyer for many valuable discussions.

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