TWO DISTINCT POLYNUCLEOTIDE LIGASES FROM RAT LIVER

Hirobumi TERAOKA, Mari SHIMOYACHI and Kinji TSUKADA*
Drug Research Institute, Toyama University, Gofuku, Toyama, Japan

Received 7 April 1975

1. Introduction

Polynucleotide ligase has been shown to be present in both soluble and nuclear fractions from rat liver [1,2], and the activities of these enzymes are increased in relation to the rise of DNA synthesis after partial hepatectomy [3].

With a view to studying the function of these ligases in regenerating rat liver, we have tried initially to purify and characterize them from both fractions of normal liver. During the course of these studies, Zimmerman and Levin [4] have described the properties of purified polynucleotide ligase from nuclei of rat liver.

The purpose of this report is to present evidence that the ligase from the soluble fraction is a distinguishable species from that of the nuclear fraction.

2. Materials and methods

[γ-^{32}P]ATP (sodium salt in 50% aqueous ethanol, 2.0 Ci/mmol) was obtained from the Radiochemical Centre. Phosphocellulose (P 11) was from Whatman. Sepharose 6B, Sephadex G-150 and Sephadex G-100 were Pharmacia products. Alkaline phosphatase from bovine intestine, β-galactosidase from *E. coli*, catalase from beef liver and lactate dehydrogenase from rabbit muscle were purchased from Boehringer Mannheim. Bovine serum albumin and cytochrome *c* from horse heart were from Sigma Chemical Company. 5′-[^{32}P] Phosphoryl-DNA was prepared as described previously [5]. Polynucleotide ligase activity was assayed by the method described previously [1].

Protein was determined by the method of Lowry et al. [6] and DNA by the procedure of Burton [7].

3. Results

Nuclear extracts and 105 000 *g* supernatant solutions from rat liver were prepared as described previously [5]. All purification operations were carried out at 0–4°C. Supernatant solutions (106 ml, 1590 mg of protein) were brought to pH 5.0 by the addition of 2 N acetic acid. The precipitate was dissolved in 50 ml of Buffer A (0.01 M potassium phosphate, pH 7.5, 0.5 mM dithiothreitol and 0.1 mM EDTA) containing 0.05 M KCl. The enzyme solutions were applied to a column of phosphocellulose (2 × 26 cm) equilibrated with Buffer A containing 0.05 M KCl, washed with the same buffer, and eluted with 51 ml of Buffer A containing 0.4 M KCl. The eluates were fractionated by $(NH_4)_2SO_4$ (25–55% saturation). The precipitates were dissolved in a minimal volume of Buffer A containing 0.2 M KCl and the solutions were applied to a column of Sepharose 6B (1.2 × 64 cm). Fractions of 3 ml were collected. Active fractions were pooled and diluted by the addition of an equal volume of Buffer A. The diluted solutions were applied to a column of phosphocellulose (2.1 × 3 cm) equilibrated with Buffer A containing 0.1 M KCl. After the column was washed with the same buffer, a linear gradient between 0.1 and 0.4 M KCl in Buffer A was applied. The ligase activities were eluted between 0.15 and 0.23 M KCl. Purification of nuclear ligase (nuclear extract, 100 ml,

* Present address; Department of Pathological Biochemistry, Medical Research Institute, Tokyo Medical and Dental University, Yushima, Bunkyo-ku, Tokyo, Japan. To whom correspondence should be addressed.

Table 1
Purification of soluble and nuclear polynucleotide ligases from rat liver

Step	Total activity (units)	Specific activity (units/mg)	Purification (-fold)
Soluble fractions			
I 10^5 *g* supernatant	795	0.5	1
II pH 5.0 precipitation	734	2.5	5
III 1st phosphocellulose	593	9.0	18
IV Ammonium sulfate (25–55% sat.)	438	27.3	55
V Sepharose 6B	300	39.5	79
VI 2nd phosphocellulose	147	119	238
Nuclear fractions			
I Nuclear extracts	6050	69	1
II pH 5.0 precipitation	3000	53	–
III Ammonium sulfate (25–75% sat.)	2083	70	1
IV Sepharose 6B	1434	338	5
V Phosphocellulose	816	2725	40

The reaction mixture (0.2 ml) contained 5 μg 5′-[^{32}P]-nicked DNA (8000–15 000 cpm), 3 μmol of $MgCl_2$, 0.04 μmol of ATP, 2 μmol of 2-mercaptoethanol, 50 μg of bovine serum albumin, 15 μmol of Tris–HCl buffer (pH 8.0), and 20 μmol of KCl (only in the assay of nuclear ligase), and was incubated at 37°C for 15 min. The reaction was stopped in ice. After boiling at 100°C for 10 min, 5 μg of alkaline phosphatase (1.5 units) were added, and the mixture was incubated for 20 min at 65°C, each mixture was cooled in ice, and 0.1 ml of 0.1 M sodium pyrophosphate was added, followed by 5% trichloroacetic acid. The precipitate was collected on a glass filter. After extensive washes with trichloroacetic acid, ethanol, and ether, the samples were counted in a Packard Tri-Carb liquid scintillation spectrometer. One unit of ligase activity was defined as that amount producing 1 pmol of alkaline phosphatase-resistant ^{32}P.

88 mg of protein) was carried out analogously except that in elution from the second phosphocellulose column between 0.1 and 0.5 M KCl in Buffer A was applied. The enzyme activities were eluted between 0.24 and 0.3 M KCL. A summary of the purifications is shown in table 1.

Gel filtration profiles of purified polynucleotide ligase activities from soluble and nuclear fractions are seen in fig.1. Purified ligase activity from the soluble fraction treated with 0.2 M KCl gave a wide peak (Ve/Vo, approx. 1.8) corresponding to an apparent mol. wt of more than 30×10^4 calculated using β-galactosidase (mol. wt, 520 000), catalase (mol. wt, 244 000) and lactate dehydrogenase (mol. wt, 140 000) as standards. The activity from the nuclear fraction emerged in a single peak (Ve/Vo, 2.1). In several different purifications, the position of each remained constant. The mol. wt of the nuclear ligase was estimated from Sephadex G-100 and G-150 column chromatography, a single peak of activity with a mol. wt corresponding to about 12×10^4 was obtained.

Active fractions from soluble and nuclear ligases that had been made in 0.2 M KCl were separately pooled, concentrated and the KCl removed by dialysis against buffer A. When those preparations were analyzed on Sepharose 6B that have been equilibrated only with Buffer A, the activity of soluble ligase again appeared as a peak in the same position as in fig.1 A, and the nuclear activity was eluted as a major peak near the void volume as shown in fig.2C. The dissociation of the nuclear ligase by high salt concentrations is reversible. These results suggested that nuclear ligase is a complex of high mol. wt which is reversibly associated and that the soluble ligase is a distinguishable species.

The ligase activities could also be distinguished by

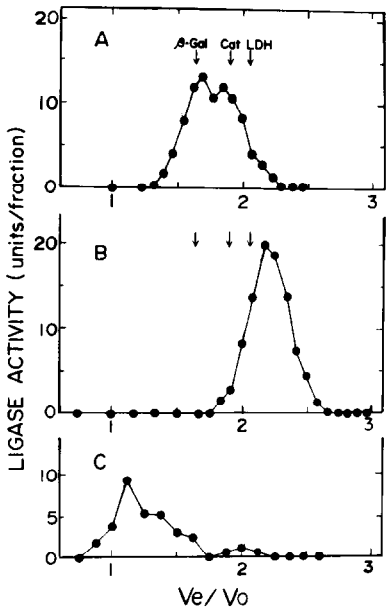

Fig.1. Sepharose-6B column chromatography of purified polynucleotide ligases from soluble and nuclear fractions. (A) Soluble ligase (150 units of step V enzyme in 2 ml) was applied to a column of Sepharose 6B (1.2 × 64 cm, Vo = 26 ml) equilibrated with Buffer A containing 0.2 M KCl, and eluted with the same buffer. Fractions of 2 ml were collected and assayed for enzyme activity according to the method described in Table 1. (B) Nuclear ligase (150 units of step V in 2 ml) was applied to a column of Sepharose 6B (1.2 × 60 cm, Vo = 24 ml). Elution and assay were as in (A). (C) Active fractions in (B) were pooled and concentrated in a collodion dialysis bag. The concentrated fraction (1 ml) was dialysed against Buffer A for 2 hr, applied to a column of Sepharose 6B (1.0 × 46 cm, Vo = 12 ml) equilibrated with Buffer A, and eluted with the same buffer. Fractions of 1.5 ml were collected. β-Galactosidase (β-Gal, 520 000), catalase (Cat, 244 000) and lactate dehydrogenase (LDH, 140 000) were used as protein standards. Vo, void volume; Ve, elution volume.

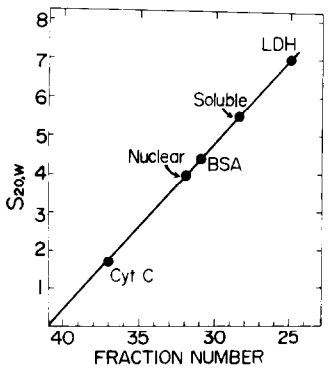

Fig.2. Sucrose density gradient ultracentrifugation of purified polynucleotide ligases from soluble and nuclear fractions. Sucrose density gradient centrifugation was carried out at 5°C and 38 000 rpm for 16 hr in the Beckman L-5 centrifuge in 13 ml of sucrose gradient (5 to 20%) containing Buffer A and 0.2 M KCl. Soluble enzyme (120 μg of step VI) or nuclear enzyme (10 μg of step V) was subjected to centrifugation with lactate dehydrogenase (LDH, 7 S), bovine serum albumin (BSA, 4.4 S), and cytochrome *c* (Cyt C, 1.7 S) as protein standards and 41 fractions (0.32 ml) were collected from the bottom of the tube.

their sedimentation characteristics in sucrose gradients. Sedimentation coefficients were determined by reference to cytochrome *c* ($S_{20,w}$ 1.7 S), bovine serum albumin ($S_{20,w}$ 4.4 S) and lactate dehydrogenase ($S_{20,w}$ 7 S). The nuclear ligase has an observed *S* value of about 4 S and the soluble enzyme about 5.5 S (fig.2).

The two ligase activities showed identical pH dependence (7.5 to 8.5 in Tris–HCl buffer), and K_m for ATP (2×10^{-5} M), and both require Mg^{2+}. At 15 mM Mg^{2+}, the activity of the nuclear enzyme is stimulated by up to 100 mM Na^+, K^+, or NH_4^+, and the soluble enzyme is inhibited to about 50% at 100 mM of the cations.

4. Discussion

The characterization of polynucleotide ligase from various animal cells has been reported by [8–15]. Pedrali Noy et al. [16] isolated two molecular forms of ligase, having mol. wts of 190 000 and 95 000, from cultures of the human heteroploid line EUE, and two ligase activities were also found in extracts of calf thymus of mol. wt 175 000 and 85 000 [17]. In these cases, no interconversion between two molecules was observed. In our report, since gel filtration of the nuclear ligase provides evidence for the conversion from a high mol. wt to a low mol. wt molecule by salt treatment, the observed change may be explained by an association of one or more low mol. wt species or subunits. Soluble enzyme preparations showed no change in mol. wt when analyzed on Sepharose 6B in Buffer A or Buffer A containing

0.2 M KCl. The nuclear (4 S) and soluble (5.5 S) polynucleotide ligases from rat livers reported here are different from one another in mol. wt and charge properties. As these enzymes exibit anomalous gel filtration behaviour, it is suggested that this may be due to asymmetry of the ligase molecules. Similar results have been reported in several purified DNA polymerases from calf thymus, rat liver or rat spleen [18], T4 gene-32 protein [19] and DNA polymerase III star in *E. coli* [20].

During the course of these studies, a polynucleotide ligase has been purified from nuclei of rat livers by Zimmerman and Levin [4]. They found that at low Mg^{2+} concentrations the ligase activity showed a remarkable dependence upon the presence of monovalent cations. These results have been confirmed by our studies on purified nuclear ligase.

Acknowledgements

The authors are indebted to Miss Reiko Ishiwatari for the preparation of this manuscript. This work was supported in part by the Scientific Research Fund for Cancer from the Ministry of Education, Japan.

References

[1] Tsukada, K. and Ichimura, M. (1971) Biochem. Biophys. Res. Commun., 42, 1156–1161.
[2] Tsukada, K., Hokari, S., Hayasaki, N. and Ito, N. (1972) Cancer Res., 32, 886–888.
[3] Tsukada, K. (1974) Biochem. Biophys. Res. Commun. 57, 758–762.
[4] Zimmerman, S. B. and Levin, C. J. (1975) J. Biol. Chem. 250, 149–155.
[5] Ichimura, M. and Tsukada, K. (1971) J. Biochem., (Tokyo) 69, 823–828.
[6] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[7] Burton, K. (1955) Biochem. J. 61, 473–483.
[8] Lindahl, T. and Edelman, G. M. (1968) Proc. Natl. Acad. Sci. U.S. 61, 680–687.
[9] Sambrook, J. and Shatkin, A. J. (1969) J. Virol. 4, 719–729.
[10] Spadari, S., Ciarrochi, G. and Falaschi, A. (1971) Eur. J. Biochem. 22, 75–78.
[11] Beard, P. (1972) Biochim. Biophys. Acta. 269, 385–396.
[12] Tsukada, K. and Ito, N. (1972) J. Biochem. (Tokyo) 72, 1299–1305.
[13] Bertazzoni, U., Mathelet, M. and Campagnari, F. (1972) Biochim. Biophys. Acta. 287, 404–414.
[14] Pedrini, A. M., Nuzzo, F., Ciarrochi, G., Dalpra, L. and Falaschi, A. (1972) Biochem. Biophys. Res. Commun. 47, 1221–1227.
[15] Gazier, A. I. and Kuzin, A. M. (1973) Eur. J. Biochem. 37, 7–11.
[16] Pedrali Noy, G. C. F., Spadari, S., Giarrocchi, G., Pedrini, A. M. and Falaschi, A. (1973) Eur. J. Biochem. 39, 343–351.
[17] Söderhäll, S. and Lindahl, T. (1973) Biochem. Biophys. Res. Commun. 53, 910–916.
[18] Holmes, A. M., Hesslewood, I. P. and Johnston, I. R. (1974) Eur. J. Biochem. 43, 487–499.
[19] Delius, H., Mantell, N. J. and Alberts, B. (1972) J. Mol. Biol. 67, 341–350.
[20] Wickner, W., Schekman, R., Geider, K. and Kornberg, A. (1973) Proc. Natl. Acad. Sci. U.S. 70, 1764–1767.