Purification and Properties of an (ADP-ribose)n Glycohydrolase from Guinea Pig Liver Nuclei*

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An (ADP-ribose)n glycohydrolase has been purified more than 3,000-fold from guinea pig liver nuclei with an 18% yield. The glycohydrolase activity present in the nuclei was solubilized only by sonication at high ionic strength and purified by sequential chromatographic steps on phosphocellulose, DEAE-cellulose, Blue Sepharose, and single-stranded DNA cellulose. The purified protein exhibited one predominant protein band on sodium dodecyl sulfate-polyacrylamide gels with an estimated molecular weight of 75,500. On Sephadex G-100 gel filtration, single coincident peaks of (ADP-ribose)n glycohydrolase activity and protein with a molecular weight value of 72,000 were observed. The $K_v$ value for (ADP-ribose)$n$, and the maximal velocity of the highly purified glycohydrolase were 2.3 $\mu$M and 36 $\mu$mol of ADP-ribose released from (ADP-ribose)$n$, $\cdot$ min$^{-1}$ $\cdot$ mg protein$^{-1}$, respectively. Hydrolysis of (ADP-ribose)$n$, by the enzyme was exoglycosidic in nature. The optimum pH for the enzyme activity was apparent at 6.8-7.0. Sulphydryl compounds and monovalent cations were required for the maximal activity. The enzyme was sensitive to Ca$^{2+}$ but not to Mg$^{2+}$. The enzyme activity was inhibited by ADP-ribose, cyclic AMP (adenosine 3':5'-monophosphate) and diadenosine 5',5'-diphosphate. Denatured DNA and histones were inhibitory, but native DNA and its histone complex were not inhibitory. Our data indicate that the glycohydrolase is present only as a minor protein in nuclei, being present in perhaps about 50,000 molecules/nucleus.

The chromatin functions in eukaryotic cells vary temporally and spatially in progression of the cell cycle and differentiation. These variations are thought to be regulated by conformational changes of chromatin structure (1). Posttranslational modification of chromosomal proteins have been implicated in these changes (2). Since chromosomal proteins are ADP-ribosylated (3-5) and the modification causes structural changes in chromatin (6, 7), it is assumed that the turnover of (ADP-ribose)n, on certain chromosomal proteins has some biological role which is essential to expression of a nuclear function. Although the physiological function of this modification has not yet been established, there are many indications that it may be involved in DNA repair and cell differentiation (3-5).

Our approach to better understanding of the biological function of ADP-ribosylation of chromosomal proteins has involved the extensive purification and characterization of the major enzymatic activities thought to be responsible for the degradation of (ADP-ribose)n, in vivo. The degradation of (ADP-ribose)n, has been thought to be catalyzed by two kinds of enzyme (3-5). One enzyme, (ADP-ribose) glycohydrolase, catalyzes hydrolysis of the glycosidic (1""2") linkages of (ADP-ribose)n (8-11). A second type is ADP-riboosyl-protein lyase, which has been purified from rat liver cytoplasm and is capable of splitting only mono-ADP-ribose-protein (glutamic acid residues) linkages (12, 13).

(ADP-ribose)n, glycohydrolases have been purified from several tissues to a varying degree of purity (8-11). However, none has achieved purification to homogeneity. Furthermore, postnuclear fractions were employed as the enzyme sources. As yet no detailed procedure has been reported for the purification of (ADP-ribose)n, glycohydrolase from nuclei. We present here a reproducible and efficient method for extensive purification of the major (ADP-ribose)n, glycohydrolase present in nuclei. The nuclear (ADP-ribose)n, glycohydrolase has now been purified to apparent homogeneity; some of its physical and catalytic properties differ from those of cytoplasmic enzymes.

EXPERIMENTAL PROCEDURES AND RESULTS*

Purification of Nuclear (ADP-ribose)n, Glycohydrolase

We developed procedures that essentially completely solubilize the nuclear (ADP-ribose)n, glycohydrolase activity and then purified the enzyme by chromatography. Details of the purification procedures are presented in the Miniprint. A summary of the purification is shown in Table I. The specific activity of the final preparation assayed at the substrate, (ADP-ribose)n, (average chain length of 15), concentration of 10 $\mu$M was 32.5 $\mu$mol$^{-1}$ $\cdot$ mg protein$^{-1}$.

The highly purified nuclear (ADP-ribose)n, glycohydrolase (Fraction VIII) was unstable on storage at 0 or -20 °C in buffer E without ethylene glycol and KCl. Repetition of freezing and thawing usually led to a considerable loss of the activity. The enzyme was routinely kept in 50 mM KCl-buffer E at -20 °C. Under the conditions, little decay of the catalytic activity was observed after storage of 6 months.

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* Portions of this paper (including "Experimental Procedures," part of "Results," Tables II and III, Figs. 2-6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-2184, cite the authors, and include a check or money order for $4.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
**Nuclear (ADP-ribose)$_n$ Glycohydrolase**

**TABLE I**

Purification of (ADP-ribose)$_n$ glycohydrolase from guinea pig liver

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein</th>
<th>Activity$^*$</th>
<th>Specific activity</th>
<th>Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>$\mu$mol $\cdot$ min$^{-1}$</td>
<td>$\mu$mol $\cdot$ min$^{-1} \cdot$ mg$^{-1}$</td>
<td>%</td>
<td>1-fo$^*$d</td>
</tr>
<tr>
<td>1. Cell homogenate</td>
<td>33,800</td>
<td>51.1</td>
<td>0.0015</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>2. Nuclear lysate</td>
<td>4,240</td>
<td>40.8</td>
<td>0.0096</td>
<td>79.8</td>
<td>(100)</td>
</tr>
<tr>
<td>3. Nuclear extract</td>
<td>1,290</td>
<td>35.5</td>
<td>0.0725</td>
<td>69.5</td>
<td>18.3</td>
</tr>
<tr>
<td>4. Phosphocellulose I</td>
<td>58.6</td>
<td>26.8</td>
<td>0.457</td>
<td>52.5</td>
<td>305</td>
</tr>
<tr>
<td>5. Phosphocellulose II</td>
<td>39.2</td>
<td>21.3</td>
<td>0.543</td>
<td>41.7</td>
<td>362</td>
</tr>
<tr>
<td>6. DEAE-cellulose</td>
<td>14.8</td>
<td>19.2</td>
<td>1.30</td>
<td>37.6</td>
<td>867</td>
</tr>
<tr>
<td>7. Blue Sepharose</td>
<td>2.73</td>
<td>13.1</td>
<td>4.80</td>
<td>25.6</td>
<td>3,200</td>
</tr>
<tr>
<td>8. Single-stranded DNA cellulose</td>
<td>0.23</td>
<td>7.47</td>
<td>32.5</td>
<td>14.6</td>
<td>(18.3)</td>
</tr>
</tbody>
</table>

$^*$The activity was measured according to assay 1.

**DISCUSSION**

An (ADP-ribose)$_n$ glycohydrolase has been extensively purified from nuclei of guinea pig liver. The procedure involved a cell fractionation step to yield pure nuclear fraction followed by solubilization of the glycohydrolase from nuclei and two ion-exchange and two affinity chromatography steps. The method described here has some important advantages over previously reported purification procedures for (ADP-ribose)$_n$ glycohydrolases from postnuclear fractions (8-11), for it is a reproducible high purity and yield method. The nuclear extract is already substantially purified. Our final purification
is more than 3,000-fold over the activity in the nuclear pellet in 18% yield. If based on the usual index of total cellular proteins, the degree of purification achieved for the glycohydrolase is actually at least 7-fold higher than the value, about 20,000-fold over the cell homogenate.

The purified enzyme exhibits one protein band on SDS-polyacrylamide gels and one major protein peak on Sephadex G-100 gel filtration which chromatographs with glycohydrolase activity. The molecular weight on the SDS gels based on the mobility of the protein relative to standards is 75,500. The specific activity for the glycohydrolase using free (ADP-ribose)₉, as the substrate is 32.5 μmol·min⁻¹·mg protein⁻¹. The specific activity and estimated protomeric molecular weight translate into a turnover number of about 4,000 mol of ADP-ribose released from (ADP-ribose)₉·min⁻¹·mol of enzyme⁻¹. However, since (ADP-ribose)₉, used here is free form, the maximal velocity for the glycohydrolase may be different with the natural intracellular substrates, (ADP-ribose)₉·protein adducts.

The homogeneous nuclear (ADP-ribose)₉ glycohydrolase differs in several respects from the partially purified cytoplasmic glycohydrolase (8–11). The molecular weight of the nuclear glycohydrolase is higher than that of the cytoplasmic glycohydrolases. The K₅₀ for (ADP-ribose)₉ of the nuclear enzyme is estimated to be 2.3 μM. Apparent K₅₀ values previously reported for the cytoplasmic enzymes vary from about 0.5 to 5 μM (8–11). The optimal pH of the nuclear enzyme is slightly lower than that of the cytoplasmic ones. The nuclear enzyme activity is stimulated by monovalent salts and inhibited by CaCl₂ but not by MgCl₂. The other enzymological properties (the effects of nucleotides, sulfhydryl compounds, DNA, histones, and mode of hydrolysis) of the nuclear enzyme are similar to those of the cytoplasmic enzymes.

We find that the (ADP-ribose)₉ glycohydrolase is tightly bound to nuclei. Essentially complete solubilization of the nuclear glycohydrolase activity is obtained only when the step of high ionic strength and sonication is included. It is unclear what holds the glycohydrolase so tightly in nuclei. About 25% of the activity of glycohydrolase is extractable only by sonication without salt. Furthermore, the glycohydrolase is released from nuclei by a mild digestion of nuclei with micrococcal nuclease. So, we suspect that the nuclear glycohydrolase is mostly bound to the specific regions of nuclei which are readily destroyed by nuclease digestion and sonication.

By making the assumption that the Mₙ 75,500 band on SDS gels corresponds to the (ADP-ribose)₉ glycohydrolase protein and by estimating about 95% pure of the protein in the final preparation, the total mass of glycohydrolase in 400 g of guinea pig liver is able to be estimated using Table I. The result is (0.23 × 0.95 × (0.146⁻¹)) = 1.5 mg, which amounts to 0.004 and 0.035% total cellular and nuclear proteins, respectively. We can also make a rough estimate of the number of the nuclear (ADP-ribose)₉ glycohydrolase molecules present per nucleus. We estimate that 34 g of guinea pig liver cells contains 1 × 10¹⁶ molecules of 75,500 daltons each. Since 34 g of liver cells (or 4 g of nuclei) should contain 1.7 × 10¹⁰ cells (or nuclei) (based on a single cell mass of 2 × 10⁻¹⁰ g (16)), the number of glycohydrolase molecules/nucleus (or cell) is calculated to be 56,000, which is the equivalent of 1 molecule of every 600 nucleosomes.

There are many important problems left to be solved in the field of ADP-ribosylation of chromosomal proteins. The biological significance of the existence of nuclear and cytoplasmic (ADP-ribose)₉ glycohydrolase and the exact intracellular substrates for the nuclear glycohydrolase purified here are not known. Although the nuclear (ADP-ribose)₉ glycohydrolase is only a minor protein, the extensive purification of the enzyme reported here should make available enzyme in sufficient quantity and purity that one could raise monoclonal antibodies. By using these antibodies, some new approaches would be open for the understanding of the biological function of ADP-ribosylation of chromosomal proteins. Furthermore, such antibodies may permit genomic cloning of the (ADP-ribose)₉ glycohydrolase gene.

Acknowledgments.—We are grateful to Shuhei Nonaka for his conscientious assistance in the performance of these studies.

REFERENCES


3 S. Tanuma, K. Kawashima, and H. Endo, unpublished data.
Nuclear (ADP-ribose), Glycophospholipase

**EXPERIMENTAL PROCEDURES**

**Materials**
- (Tri-N,N,N-trimethyl-N,N-dipentaerythritol) 1,3-bis(2-aminooctanoyl) sorbitol (ADP-ribose) was purchased from New England Nuclear. Internal standards, solvents, buffers, enzymes, and other reagents were obtained from Sigma and other commercial sources.

**Preparation of Glycophospholipase**
- In a 50 ml Erlenmeyer flask, 2 ml of cell lysate was incubated with 200 ml of 0.02 M Tris Buffer (pH 8.5), 5 ml of 0.002 M Blue-Sepharose beads, and 5 ml of ADP-ribose. The reaction was allowed to proceed for 60 min at 37 °C. The reaction mixture was then poured through a column of Blue-Sepharose beads, and the eluate was collected.

**Assays**
- The assay mixture was incubated with 200 ml of 0.02 M Tris Buffer (pH 8.5) and 5 ml of Blue-Sepharose beads. The reaction was allowed to proceed for 60 min at 37 °C. The reaction mixture was then poured through a column of Blue-Sepharose beads, and the eluate was collected.

**RESULTS**

**Purification of Nuclear (ADP-ribose), Glycophospholipase**

All the operations were performed at 5-6 °C unless otherwise indicated. To avoid violent reactions, glycophospholipase buffer used in the purification was supplemented with 10% ethylene glycol.

**Step 1: Cell Homogenate**
- Freshly minced guinea pig liver (about 500 g) was homogenized with a glass rod in a medium of 0.25 M sucrose, 0.025 M Tris-Cl (pH 8.0), 2.5 mM MgCl2, 1.25 mM EDTA, 12.5 mM dithiothreitol, 12.5 mM 2-mercaptoethanol, and 1.0 mM PMSF, containing 0.01 mg/ml of lipoamide dehydrogenase and 100 mg/ml of BSA, and passed through stainless steel (100 µM) yielding the cell homogenate (Fraction 1).

**Step 2: Nuclear Extracts**
- For the fractionation of nuclear extracts, the cell homogenate was fractionated into 2.0 M sucrose buffer A and 0.5 M NaCl buffer. The nuclear extract was assayed for its activity on the reaction mixture containing 0.002 M Blue-Sepharose beads, and 5 ml of ADP-ribose. The reaction was allowed to proceed for 60 min at 37 °C. The nuclear extract was labeled Fraction II.

**Step 3: Nuclear Extracts**
- The nuclear extract was further fractionated into 2.0 M sucrose buffer A and 0.5 M NaCl buffer. The nuclear extract was assayed for its activity on the reaction mixture containing 0.002 M Blue-Sepharose beads, and 5 ml of ADP-ribose. The reaction was allowed to proceed for 60 min at 37 °C. The nuclear extract was labeled Fraction III.
Nuclear (ADP-ribose)₉ Glycohydrolase

Fig. 2. Gel filtration of nuclear (ADP-ribose)₉ glycohydrolase on Sephadex G-100 and a calibration curve for molecular weight. Enzyme activity was measured according to Assay-1 (Δ). Protein was determined by absorbance at 280 nm (---). The molecular weight standards were: 1. thyroglobulin, 2. gamma globulin, 3. ovalbumin, 4. myoglobin, 5. vitamin B₁₂, and 6. blue dextran. Kᵥₚ is calculated according to the equation: Kᵥₚ = (Vᵥ/2/Vᵥ₁-Vᵥ₀), where Vᵥ₁ is the elution volume of the enzyme activity or the standards. Vᵥ₀ is the void volume and Vᵥ₁ is the total bed volume of the column. The data were fit to a line using least squares analysis (ΔΔΔΔ; the Kᵥₚ of nuclear (ADP-ribose)₉ glycohydrolase is ΔΔΔΔ).

Fig. 3. Effect of (ADP-ribose)₉ concentration on nuclear (ADP-ribose)₉ glycohydrolase activity. Glycohydrolase activity was assayed (Assay-1) in duplicate with 0.01 μg/ml of enzyme protein as described under "Experimental Procedures" except that (ADP-ribose)₉ concentration was varied as indicated.

Fig. 4. Time course and thin layer chromatograms of the reaction product of nuclear (ADP-ribose)₉ glycohydrolase. The reaction was carried out at 37°C for 0 (A), 20 (B), 30 (C), and 60 (D) min. An aliquot was applied to a thin layer plate. The plate was developed as described under "Experimental Procedures".

Fig. 5. Optimum pH of nuclear (ADP-ribose)₉ glycohydrolase. The reaction was carried out under the standard conditions except that pH value was varied with 50 mM 8-9-Na₄ (○) or 50 mM Tris-HCl (ΔΔΔΔ).

Fig. 6. Effect of sulfhydryl compounds on nuclear (ADP-ribose)₉ glycohydrolase. Fraction Y12 was dialyzed against 50 mM 8-9-Na₄ (pH 7.0), 100 mM sodium glycerol and 50 mM KCl. The reaction was carried out under the standard conditions (Assay-1) except that 2-mercaptoethanol (○) or dithiothreitol (ΔΔΔΔ) concentration was varied as indicated. The enzyme activity in 10 μl is taken as 100%.