POLY ADP-RIBOSE GLYCOHYDROLASE FROM RAT LIVER NUCLEI, A NOVEL ENZYME DEGRADING THE POLYMER*

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SUMMARY: A novel enzyme, poly ADP-ribose glycohydrolase, has been discovered and partially purified from rat liver nuclei. The enzyme catalyzes hydrolytic cleavage of the ribosyl (1 → 2) ribose bond between neighboring ADP-ribose units, but does not split the linkage by which the polymer is bound to protein. The activity is specifically inhibited by adenosine 3',5'-cyclic monophosphate (cyclic AMP), the apparent Ki being around 1.5 mM. A kinetic study and product analyses have suggested that the glycohydrolase, rather than phosphodiesterase, plays a principal role in the degradation in situ of poly ADP-ribose.

Poly ADP-ribose is a nucleic acid-like macromolecule composed of repeating ADP-ribose units linked by a glycosidic bond of ribosyl (1 → 2) ribose (1 - 3). The polymer is synthesized by a successive transfer of ADP-ribose from NAD on to a protein receptor, presumably histone (4, 5), by chromatin-associated enzyme (6). As for degradation of this polymer, a phosphodiesterase activity which hydrolyzes the pyrophosphate linkage forming phosphoribosyl AMP was found in rat liver (7). The present study revealed that a hitherto unknown enzyme, poly ADP-ribose glycohydrolase, splits the ribose-

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ribose linkage existing in a nuclear soluble fraction and that is probably responsible for the in vivo degradation of poly ADP-ribose. Evidence is presented herein that the glycohydrolase is specifically inhibited by cyclic AMP.

MATERIALS AND METHODS

Rat liver nuclei and chromatin were prepared as described previously (8, 6).

^{14}C(adenosine)-NAD was prepared according to the method reported earlier (9). ^{14}C-labeled polymer and oligomer of ADP-ribose were prepared from ^{14}C-NAD using chromatin, followed by fractional extraction with dilute acid. The procedures were essentially identical with those employed previously (4) except that 7 mM cyclic AMP was added to the reaction mixture in order to inhibit the degradation of polymer once formed (see below). Both polymer and oligomer of ADP-ribose employed in this study were almost totally bound to protein. Their average chain lengths estimated from phosphodiesterase digests (4) were 8 and 1.2, respectively.

The activity of poly ADP-ribose degradation was assayed by measuring the decrease of acid-insoluble ^{14}C-ADP-ribose. The standard reaction mixture contained 50 µmoles of potassium phosphate buffer, pH 7.0, ^{14}C(adenosine)-poly ADP-ribose (1,500 cpm, 15 pmoles as ADP-ribose) and enzyme in a total volume of 0.5 ml. Incubation was carried out for 20 min at 37° and terminated by addition of CCl_3COOH (final 20%). The mixture was passed through a Millipore filter (pore size 0.45 µ) and the filter was washed with 5% CCl_3COOH. Radioactivity retained on the filter was determined using a Geiger-Müller gas flow counter.

Protein was determined after Lowry et al. (10) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Enzyme activities degrading poly ADP-ribose were found in various subcellular fractions. From a nuclear fraction two degrading enzymes, phosphodiesterase and glycohydrolase, were separated and the latter was partially purified. The purification procedures consisted of a disruption of nuclei in 0.01 M Tris-HCl buffer, pH 7.4, a high speed centrifugation (105,000 X g, 60 min, supernatant) and $(NH_4)_2SO_4$ fractionation (0.3 - 0.5 saturation). The final preparation was essentially free from ADP-ribose polymerase and phosphodiesterase activities.

When ^{14}C-poly ADP-ribose and the glycohydrolase were incubated, the acid-insoluble ADP-ribose was rapidly converted into acid-soluble material as shown in Fig. 1. The initial rate of this conversion was proportional to the amount of enzyme employed, and the

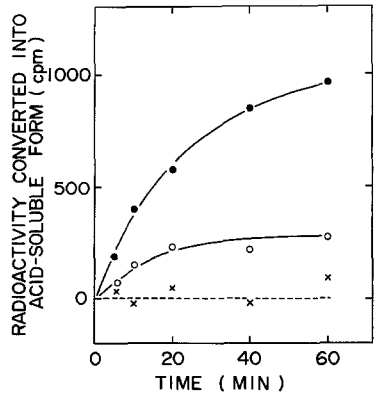

Fig. 1. Time course of enzymatic ADP-ribose degradation. The standard reaction mixture containing glycohydrolase (94 µg of protein) (●), glycohydrolase and 9 mM cyclic AMP (o), or no enzyme (×) was incubated for a specified period and assayed for acid-insoluble radioactivity.

boiled enzyme preparation was completely inactive. When histone-bound ^{14}C-oligo (or mostly mono) ADP-ribose was employed as substrate, little enzymatic hydrolysis was observed, suggesting that neither ADP-ribose unit nor the linkage between ADP-ribose and protein was split. The ribose-ribose linkage in phosphoribosyl AMP was resistant to this enzyme.

In Fig. 1, the effect of cyclic AMP on the degradation of poly ADP-ribose is also demonstrated. The addition of 9 mM cyclic AMP caused 70% inhibition of the enzymatic decrease of acid-insoluble ADP-ribose. The effects of varying concentrations of cyclic AMP, AMP and cyclic GMP are shown in Fig. 2. A complete inhibition

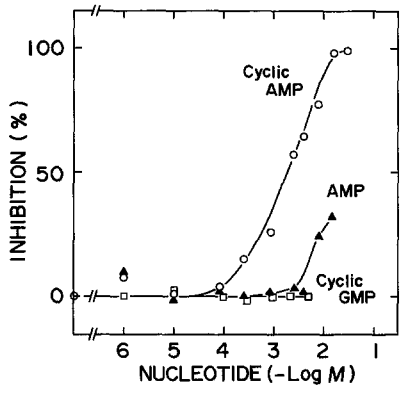

Fig. 2. Effects of nucleotides on poly ADP-ribose degradation. ^{14}C-poly ADP-ribose (1,500 cpm) was incubated with glycohydrolase (126 µg of protein) under the standard condition except that cyclic AMP (o), AMP (▲), or cyclic GMP (□) was added to a specified concentration. The inhibition was expressed as per cent decrease in the conversion of poly ADP-ribose into acid-soluble form. The conversion in the absence of nucleotide was 800 cpm.

was observed at approximately 20 mM of cyclic AMP and a half maximal effect at 1.5 mM. 5'-AMP was much less effective and cyclic GMP was completely inactive.

Fig. 3 is the Dowex 1 column chromatogram of glycohydrolase digests of poly ADP-ribose. The main product was eluted with ADP-ribose and identified as such by paper chromatography, high voltage paper electrophoresis, and treatment with venom phosphodiesterase

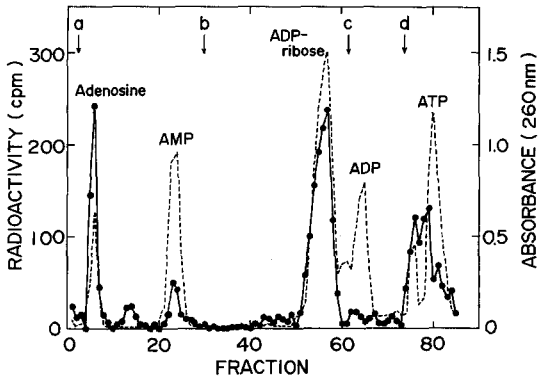

Fig. 3. Analysis of glycohydrolase products of poly ADP-ribose. The reaction mixture (0.8 ml) containing 100 μmoles of potassium phosphate buffer, pH 7.0, ^{14}C-poly ADP-ribose (15,000 cpm), 10 nmoles of ADP-ribose and glycohydrolase (1.3 mg of protein) was incubated for 20 min at 37°. The mixture was directly applied on to a Dowex 1 (X2, 200-400 mesh) formate column (0.8 X 35 cm) together with markers (adenosine, AMP, ADP-ribose, ADP and ATP). The column was eluted with linear gradients of HCOOH (a) 0 to 0.3 N (total volume 300 ml) and (b) 0.3 to 6 N (300 ml), followed by a stepwise elution with (c) 6 N HCOOH (100 ml) and (d) 6 N HCOOH-0.4 M $HCOONH_4$ (100 ml). Fractions (10 ml each) were collected and assayed for radioactivity (●———●) and absorbance at 260 nm (-------).

which produced AMP and ribose 5-phosphate. ADP-ribose oligomer was also detected as a peak of radioactivity appearing just before ATP. On further digestion of this fraction with glycohydrolase ADP-ribose monomer was produced. These facts present conclusive evidence that the ribose-ribose linkage in poly ADP-ribose was hydrolyzed by the nuclear enzyme. The name "poly ADP-ribose glycohydrolase" has been tentatively proposed for this enzyme. Other minor products were identified by paper chromatography as adenosine and AMP. The metabolic pathway to produce these compounds is under investigation.

The effect of cyclic AMP on glycohydrolase provides a useful tool for investigation of the physiological role of this enzyme in nuclei. When ^{14}C-NAD was incubated with nuclei, the ADP-ribose portion was quickly incorporated into acid-insoluble material to form poly ADP-ribose (Fig. 4). This polymer was gradually degraded and rendered acid-soluble in the course of further incubation. Cyclic

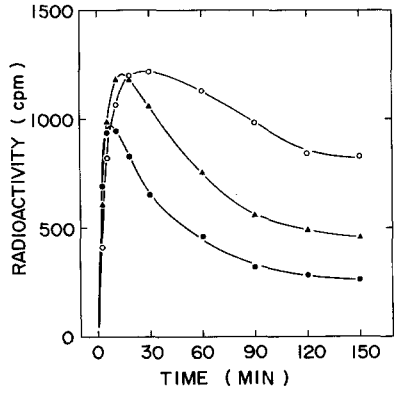

Fig. 4. Time course of poly ADP-ribose synthesis in the absence and presence of cyclic AMP or AMP. The reaction mixture (0.65 ml) containing 130 μmoles of potassium phosphate buffer, pH 7.0, ^{14}C-NAD (100,000 cpm, 10 nmoles) and nuclei (2.8 mg of protein) was incubated at 23° in the absence (●) or presence of either 6 m$\underline{M}$ cyclic AMP (o) or 6 m$\underline{M}$ AMP (▲). At intervals, aliquots (0.07 ml) were taken and subjected to assay of acid-insoluble radioactivity using a Millipore filter.

AMP effectively suppressed this conversion at a concentration of 6 m$\underline{M}$, but AMP, which was a potent inhibitor of phosphodiesterase (11), had less effect at the same concentration, although it enhanced the initial incorporation. Since cyclic AMP is almost inert to phosphodiesterase (12), this observation suggests that the disintegration of poly ADP-ribose $\underline{in\ situ}$ is carried out mainly by glycohydrolase, rather than by phosphodiesterase.

This interpretation was supported by analyses of acid-soluble and -insoluble products. The labeled acid-soluble products given by a prolonged incubation of nuclei and ^{14}C(adenosine)-NAD were mainly ADP-ribose, AMP and adenosine under a variety of reaction conditions. In some cases, ADP was also recovered in small quantity, but no appreciable amount of phosphoribosyl AMP nor its dephosphorylated metabolites were found, implying that phosphodiesterase hardly functioned in the polymer degradation. The average chain length of acid-insoluble products was remarkably increased by the presence of cyclic AMP at a concentration of 0.1 m$\underline{M}$ or higher.

The new enzyme, glycohydrolase, was clearly distinguished from phosphodiesterase by many enzymological properties. Besides the differences in mode of action and in nucleotide sensitivity as described above, subnuclear localization and pH optima were also different. The glycohydrolase was soluble in nuclei, but phosphodiesterase was bound to a nucleonemata structure. The optimal pH of the former was 6.5 to 7, whereas the latter was most active at approximately pH 10 (11). These differences could indicate that they are engaged in separate functions at different sites of nuclei.

The glycohydrolase reaction does not appear to be a partial or side reaction of the reversal of polymerization, since the glycohydrolase obtained is essentially free from ADP-ribose polymerase and no formation of NAD has thus far been detected in the presence of nicotinamide. The relations between the depolymerization and polymerization, e.g. a possible re-elongation of partially degraded polymer, are under investigation.

During the preparation of this manuscript, a similar observation was made by Miwa and Sugimura that calf thymus nuclei split the ribose-ribose bond in poly ADP-ribose (13).

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