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Biochemistry

Novel enzyme from rat liver that cleaves an ADP-ribosyl histone linkage

[ADP-ribosylation/poly(ADP-ribose) glycohydrolase/histone H2B/NAD]

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ABSTRACT **A novel enzyme that splits a bond between ADP-ribose and histone was discovered and partially purified from rat liver cytosol. The 105,000 × *g* supernatant of rat liver homogenate was precipitated by 45% saturated ammonium sulfate and then chromatographed on a DEAE-cellulose column. The enzyme activity was eluted in a single peak at about 0.2 M NaCl and clearly separated from poly(ADP-ribose) glycohydrolase which came out at 0.13 M NaCl. In contrast to the latter enzyme, this new enzyme catalyzed the splitting of a linkage between ADP-ribose and a protein portion in mono ADP-ribosylated histone H2B but little, if any, of the glycosidic ribosyl(1″-2′)ribose bonds within poly(ADP-ribose). Analysis of the reaction product by paper chromatography and Dowex 1 column chromatography indicated that the split product contained the ADP-ribose moiety but was not exactly identical with ADP-ribose. Available evidence suggested that it was either an altered ADP-ribose molecule produced by a structural rearrangement or ADP-ribose itself linked to an unidentified compound. The enzyme had a pH optimum of about 6.0 and was inhibited by 80–90% in the presence of 5 mM ADP-ribose.**

Poly ADP-ribosylation is a unique covalent modification of nuclear proteins by poly(ADP-ribose), a linear homopolymer of ADP-ribose units synthesized from NAD (1, 2). Histones H1 and H2B are the major acceptors (3–8). In spite of extensive studies, no enzyme has so far been found that splits the linkage between the polymer and the histones.

Recently we succeeded in selectively isolating ADP-ribosylated histone H2B from rat liver nuclei by using covalent chromatography on dihydroxyboryl-polyacrylamide beads (8). Using this modified histone as a substrate, we have looked for the splitting enzyme and discovered in rat liver cytosol an enzyme that cleaves a bond between ADP-ribose and histone. The present paper describes preliminary results concerning its purification and some properties.

MATERIALS AND METHODS

[*Adenine-U-*^{14}C]NAD (237 Ci/mol) was purchased from the Radiochemical Centre, Amersham. [*Ribose(NMN)-U-*^{14}C]-NAD (100 Ci/mol) was prepared from [*U-*^{14}C]glucose (9). DEAE-cellulose (DE52) was obtained from Whatman; AG 1-X2 (200–400 mesh) was from Bio-Rad Laboratories.

[*Adenine-*^{14}C]ADP-ribosylated histone H2B and ADP-[^{14}C]ribosylated histone H2B were purified from rat liver nuclei preincubated with the respective radioactive NADs by covalent chromatography on a borate gel column and by CM-cellulose column chromatography (8). ADP-ribosylated H2B thus obtained was then precipitated with 10% CCl_3COOH to remove both free mono- and oligo(ADP-ribose) and some contaminating nonhistone proteins and was used as substrate for the assay of the splitting activity. More than 80% of ADP-ribose in this preparation was associated with H2B and the rest with nonhistone proteins. Approximately 60% of the H2B molecules was bound to ADP-ribose. The average chain length was 1.1 ADP-ribosyl units; more than 90% of the ADP-ribose chains were monomers. The linkage between ADP-ribose and H2B was susceptible to mild alkali and its half-life at pH 9.0 and 37° was approximately 20 min.

The enzyme activity of splitting the ADP-ribosyl histone linkage was assayed as follows. The reaction mixture contained 0.1 M potassium phosphate buffer (pH 6.0), 1 mM dithiothreitol, [*adenine-U-*^{14}C]ADP-ribosylated H2B (5 μM as ADP-ribose, 6.6 cpm/pmol), and enzyme in a total volume of 50 μl. The reaction was carried out at 37° for 5 min and was terminated by the addition of 40 μl of 45% CCl_3COOH. After the solution was left at 0° for 20 min, the precipitate formed was removed by centrifugation at 12,000 × *g* for 20 min. A 70-μl aliquot was removed from the supernatant and its radioactivity was quantified with a liquid scintillation spectrophotometer.

Poly(ADP-ribose) glycohydrolase was assayed in a reaction mixture (100 μl) containing 0.1 M sodium phosphate (pH 7.0), 1 mM dithiothreitol, [*adenine-*^{14}C]poly(ADP-ribose) (5 μM as ADP-ribose, 5 cpm/pmol; average chain length, 24 ADP-ribosyl units) (10), and enzyme. The reaction was carried out at 37° for 10 min and was terminated by the addition of 70 μl of 50% CCl_3COOH. The precipitate formed was removed by centrifugation as mentioned above. The radioactivity in 150 μl of the supernatant was determined.

RESULTS

In the course of searching for an enzyme that splits the ADP-ribosyl histone linkage, we observed that, when ^{14}C-labeled ADP-ribosylated histone H2B was incubated with rat liver homogenate, considerable amounts of radioactivity were released into a 20% CCl_3COOH-soluble fraction. The major product released was then tentatively identified as ADP-ribose by paper chromatography, indicating that rat liver contains such an enzyme activity. When rat liver was fractionated into three major subcellular components—i.e., nuclei, mitochondria/microsomes, and cytosol (11)—20–30%, 20–30%, and 30–50% of the total activity were recovered in these fractions, respectively.

For the purpose of further characterization, this enzyme was partially purified from the cytosol by ammonium sulfate fractionation and DEAE-cellulose column chromatography. All operations were done at 0°–4°. Two livers obtained from Wistar rats weighing 300–350 g were homogenized with 3 volumes of 0.25 M sucrose containing 0.4 mM phenylmethylsulfonyl fluoride, an irreversible inhibitor of serine proteases. After centrifugation at 105,000 × *g* for 1 hr, a clear supernatant was obtained. The supernatant was then adjusted to 45% saturation by addition of solid $(NH_4)_2SO_4$. After being stirred for 30 min, the precipitate formed was collected by centrifugation at 12,000 × *g* for 20 min and dissolved in 25 ml of 50 mM potassium phosphate buffer (pH 6.0) containing 1 mM di-

Abbreviation: *iso*ADP-ribose, 2′-(5″-phosphoribosyl)-5′-AMP.

thiothreitol. More than 70% of the activity was recovered in this precipitate. After dialysis against 2 liters of 50 mM Tris·HCl buffer (pH 7.5) containing 1 mM dithiothreitol followed by centrifugation at 105,000 × *g* for 1 hr, the clear solution was applied to a DEAE-cellulose column (1.8 × 11 cm) preequilibrated with 50 mM Tris·HCl (pH 7.5) containing 1 mM dithiothreitol. The column was washed with the buffer and eluted with a linear gradient of 0–0.3 M NaCl contained in the equilibration buffer (total volume, 250 ml). The flow rate was maintained at 20 ml/hr. Fractions (3 ml) were collected. As shown in Fig. 1, the splitting activity was eluted in a single peak at a NaCl concentration of about 0.2 M whereas poly(ADP-ribose) glycohydrolase came out at 0.13 M NaCl. Both activities were thus completely separated from each other. The enzyme that splits the ADP-ribosyl histone linkage hardly degraded poly(ADP-ribose). Poly(ADP-ribose) glycohydrolase, by contrast, did not cleave the ADP-ribosyl histone linkage.

The time course of this splitting reaction is shown in Fig. 2. As mentioned previously, the linkage between ADP-ribose and histone was unstable even under the reaction conditions used. In fact, radioactivity was gradually released into a 20% CCl_3COOH-soluble fraction due to nonenzymatic cleavage of the bond. The addition of the enzyme accelerated the release at least 30-fold. Under these reaction conditions, the enzymatic reaction proceeded linearly for 5 min until approximately 20% of the total bonds was cleaved. Thereafter, the reaction slowed down and finally stopped when about 50% of the bonds was cleaved. Further addition of the enzyme at this time had no effect, indicating that this phenomenon was not due to inactivation of the enzyme during the reaction. The extent of the reaction varied depending on the preparation of the substrate and was independent of the preparation of the enzyme.

Although the nonenzymatic cleavage was much accelerated under alkaline conditions, the enzymatic reaction had an optimal pH of about 6.0. Dithiothreitol activated the enzyme 1.5- to 2-fold at 1 mM concentration and also protected the enzyme from inactivation during chromatography. ADP-ribose was a potent inhibitor of the enzyme; the addition of 5 mM ADP-ribose inhibited the activity by 80–90%. In contrast to poly(ADP-ribose) glycohydrolase, this enzyme was hardly inhibited by cyclic AMP. Phenylmethylsulfonyl fluoride, which

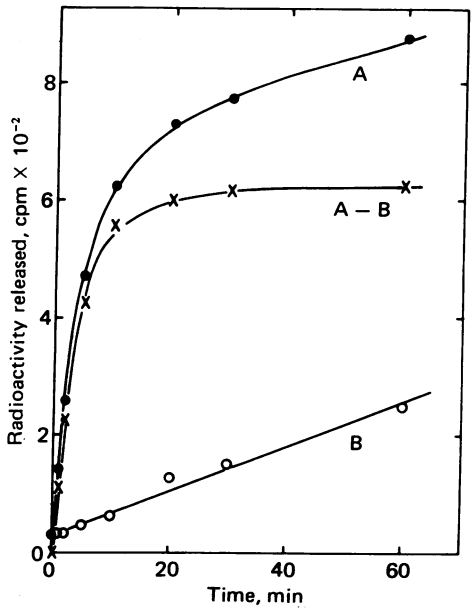

FIG. 2. Time courses of the splitting reactions. The reaction was carried out in the standard assay mixture in the presence (●—●) or absence (○—○) of the enzyme [5 μl of DEAE-cellulose fraction 74 (Fig. 1)]. ×—×, Net cleavage by the enzyme.

is used to prevent proteolytic degradation of histones (12), as well as unmodified histones from calf thymus did not inhibit the activity.

When ADP-[^{14}C]ribosylated H2B and [*adenine*-^{14}C]ADP-ribosylated H2B were used as substrates, almost equal amounts of radioactivity were released into a 20% CCl_3COOH-soluble fraction by the enzyme (Fig. 3). Because ADP-ribose is known to be bound to histones at its terminal ribose (1, 2), these results strongly indicate that the enzyme cleaved a linkage between ADP-ribose and a histone molecule and that the cleavage product probably contained at least the ADP-ribose moiety.

The split product was then analyzed by paper chromatography and Dowex 1 column chromatography. As shown in Fig. 4*A*, paper chromatography of the reaction product gave es-

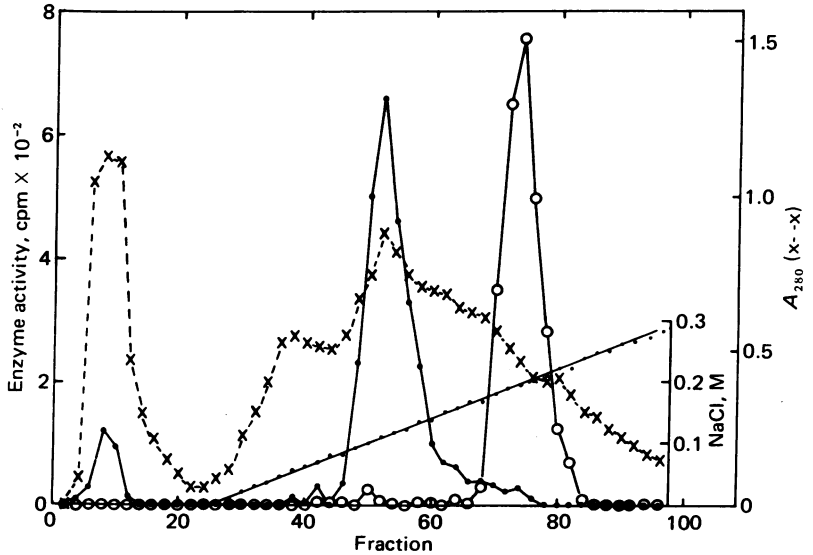

FIG. 1. DEAE-cellulose column chromatography of the 45% $(NH_4)_2SO_4$ precipitate of rat liver cytosol. Aliquots (10 μl) of alternate fractions were assayed for ADP-ribosyl histone splitting activity (○—○) and for poly(ADP-ribose) glycohydrolase activity (●—●). × - - - - - ×, Absorbance at 280 nm; —, NaCl concentration.

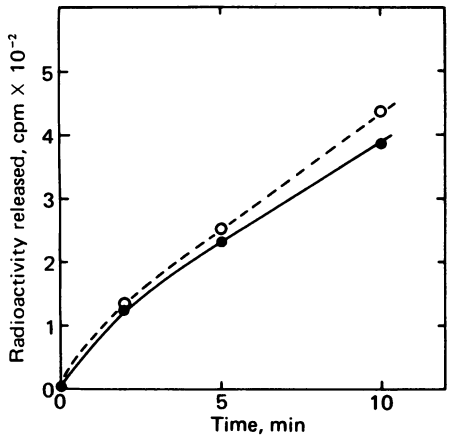

FIG. 3. Stoichiometric release of radioactivity from [*adenine*-^{14}C]ADP-ribosylated H2B and ADP-[^{14}C]ribosylated H2B. The reaction was carried out with the enzyme in the presence of [*adenine*-^{14}C]ADP-ribosylated H2B (10 μM as ADP-ribose, 6 cpm/pmol) (O - - - - - O) or ADP-[^{14}C]ribosylated H2B (10 μM as ADP-ribose, 6 cpm/pmol) (●—●). Other reaction conditions were the same as those of the standard assay. The data shown are the net values of radioactivity released by the enzyme.

sentially one radioactive peak that migrated in complete coincidence with authentic ADP-ribose. When the same product was analyzed with a Dowex 1 column, however, it did not cochromatograph with the marker ADP-ribose. It was eluted slightly later than ADP-ribose (Fig. 5). An identical elution pattern was obtained also with the split product from ADP-[^{14}C]ribosylated H2B. The product is clearly different from *iso*ADP-ribose because the latter compound is known to be eluted before ADP-ribose under the same conditions (13).

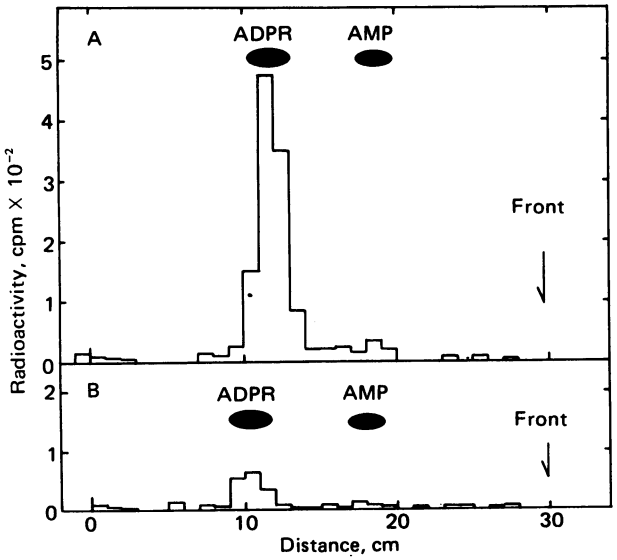

FIG. 4. Paper chromatography of the reaction products. The reaction was carried out at 37° for 20 min in the standard assay mixture containing [*adenine*-^{14}C]ADP-ribosylated H2B (15 μM as ADP-ribose, 6.6 cpm/pmol) in the presence (*A*) or absence (*B*) of the enzyme. The reaction was terminated by the addition of 40 μl of 45% CCl_3COOH. The supernatant obtained by centrifugation was washed several times with ethyl ether and applied to a Whatman 3 MM paper together with ADP-ribose and AMP as the markers. Chromatography was performed at room temperature in isobutyric acid/ammonia/H_2O/EDTA, 66:1:33:10^{-4} *M*, as the solvent system. After drying, the paper was cut into 1-cm strips and radioactivity was determined in a toluene-base scintillator.

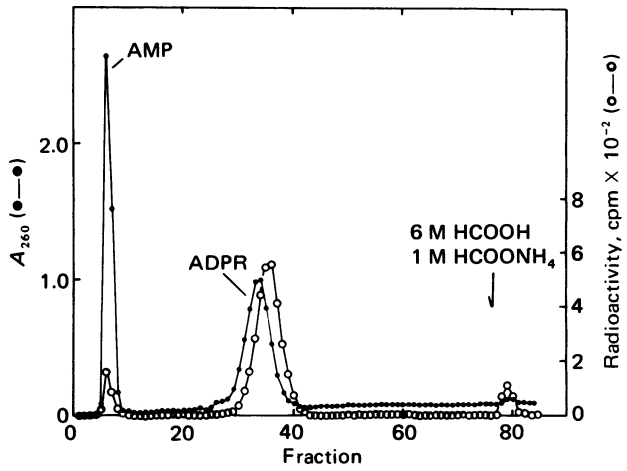

FIG. 5. Dowex 1 column chromatography of the reaction product. The reaction was carried out in 0.1 ml of the assay mixture under the same conditions as those in Fig. 4*A*. After washing with ethyl ether, the 20% CCl_3COOH supernatant was applied to a Dowex 1 (formate) column (0.6 × 14 cm) together with unlabeled ADP-ribose and AMP. The column was eluted with a linear gradient of 0–6 M HCOOH (total volume, 100 ml) and finally stepwise with 6 M HCOOH containing 1 M $HCOONH_4$. Fractions (1.3 ml) were collected, and absorbance at 260 nm and radioactivity in 1 ml of each fraction were determined. ADPR, ADP-ribose.

Under the same chromatographic conditions, radioactive ADP-ribose enzymatically prepared from the same [^{14}C]NAD exactly cochromatographed with unlabeled ADP-ribose used as the marker. These results taken together indicate that the split product contained the ADP-ribose moiety but was not exactly identical with ADP-ribose. The product could not be ADP-ribose bound to a peptide fragment because essentially no amino acid was detected in this product by dansyl method (14).

DISCUSSION

In the present study, we have found a new enzyme that splits a bond between ADP-ribose and histone. Several properties mentioned here clearly distinguish this enzyme from the already known poly(ADP-ribose)-degrading enzymes—i.e., phosphodiesterase (15, 16) and poly(ADP-ribose) glycohydrolase (17–23). The bonds split by these three enzymes are illustrated in Fig. 6. The enzyme reported in this paper splits a linkage between ADP-ribose and a protein portion in mono ADP-ribosylated histone H2B and releases the ADP-ribose moiety. Analysis of the product, however, has indicated that the split product is not exactly ADP-ribose itself. Judging from the chromatographic patterns, it appears to be either an ADP-ribose molecule modified by a structural rearrangement

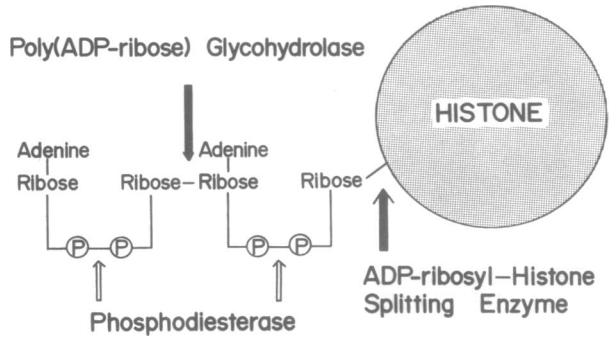

FIG. 6. Enzymes of poly ADP-ribosylated histone degradation.

or ADP-ribose itself linked to a small and almost neutral compound. This compound, if present, could not be a peptide fragment from H2B because essentially no amino acid was detected in the split product. The fact that the enzyme is strongly inhibited by ADP-ribose but not by phenylmethylsulfonyl fluoride or unmodified histones also indicates that proteolysis does not participate in this splitting reaction. ADP-ribose as well as poly(ADP-ribose) has been currently considered to be linked to histones, probably through an ester bond between its terminal ribose and a glutamic acid residue (5–7). The above results, however, suggest the possibility that ADP-ribose might not be directly attached to the amino acid but to some compound that could be present on the amino acid at the acceptor site.

Further studies on this enzyme will contribute to a better understanding of the metabolism of poly ADP-ribosylation and also the elucidation of the structure of ADP-ribosylated histones.

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