An affinity matrix for the purification of poly(ADP-ribose) glycohydrolase

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ABSTRACT

The preparation of quantities of poly(ADP-ribose) glycohydrolase sufficient for detailed structural and enzymatic characterizations has been difficult due to the very low tissue content of the enzyme and its lability in late stages of purification. To date, the only purification of this enzyme to apparent homogeneity has involved a procedure requiring 6 column chromatographic steps. Described here is the preparation of an affinity matrix which consists of ADP-ribose polymers bound to dihydroxyboronyl sepharose. An application is described for the purification of poly(ADP-ribose) glycohydrolase from calf thymus in which a single rapid affinity step was used to replace 3 column chromatographic steps yielding enzyme of greater than 90% purity with a 3 fold increase in yield. This matrix should also prove useful for other studies of ADP-ribose polymer metabolism and related clinical conditions.

INTRODUCTION

Poly(ADP-ribose) is an homopolymer of repeating ADP-ribose units linked by (1″ 2′) ribosyl-ribose glycosidic bonds. In addition, branching residues linked by (1‴ 2″) ribosyl-ribose glycosidic bonds have been identified in large ADP-ribose polymers (1). Poly(ADP-ribose) polymerase, which is a chromatin-associated enzyme, uses NAD^+ as a substrate and catalyses the initiation and elongation of the poly(ADP-ribose) chains on various protein acceptors as well as the branching reaction. Although the function of poly(ADP-ribose) metabolism is poorly understood, poly(ADP-ribose) seems to be associated with chromatin changes required for many cellular processes, especially those involving nicking and resealing of DNA strands (see 2, 3). Poly(ADP-ribose) glycohydrolase, which cleaves ribosyl-ribose bonds and liberates ADP-ribose from both linear and branched portions of poly(ADP-ribose), is the primary enzyme involved in the catabolism of poly(ADP-ribose) *in vivo* (4–6). A second enzyme, ADP-ribosyl protein lyase, removes the proximal ADP-ribosyl moiety bound to the protein (7). The physiological importance of the glycohydrolase has been suggested by the observation that ADP-ribose polymers are rapidly turned over *in vivo* (8, 9). Further, studies have suggested that the enzyme is subject to regulation following hyperthermic treatment of cells (5).

Since the discovery of poly(ADP-ribose) glycohydrolase by Miwa and Sugimura in 1971 (10), several partial purifications of the enzyme have been reported (11–14). Only recently, purification to apparent homogeneity was achieved by Hatakeyama et al. from calf thymus (6). Difficulties in obtaining pure poly(ADP-ribose) glycohydrolase have been due to the very low cellular content of this enzyme and to its instability in late stages of purification (6). To date, no affinity matrix for the enzyme has been reported, thus purification of glycohydrolase has required numerous steps and pure enzyme was obtained only in small amounts (6). In this report, we describe the preparation and application of an affinity matrix which will facillitate detailed structure, function studies of poly(ADP-ribose) glycohydrolase.

EXPERIMENTAL PROCEDURES

Materials

[adenylate-^{32}P] NAD (10–50Ci/mmol) was obtained from New England Nuclear (Montreal, Canada). Dithiothreitol and β-NAD (grade 1) were from Boehringer Mannheim (Montreal, Canada). *Crotalus adamenteus* venom phosphodiesterase was obtained from Cooper Scientific (Montreal, Canada) and further purified by the method of Oka et al. (16). Histones (calf thymus), DNA (calf thymus, type 1) and ADP-ribose were from Sigma (St. Louis, MO). PEI-F cellulose was from British Drug Houses (Montreal, Canada). Blue, CM-, and Heparin-Sepharose CL-6B

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were obtained from Pharmacia (Montréal, Canada). Single-stranded DNA agarose (0.5 to 1.0 mg of DNA/ml bed volume of 4% agarose) was from BRL (Bethesda, Maryland). All of the material sources for the purification of poly(ADP-ribose) polymerase have been reported previously (17). DHB-Bio Rex was synthesized as described by Wielckens *et al.* (18) and DHB-Sepharose was synthesized as described by Jacobson *et al.* (19). Econo-columns, low and high molecular weight protein standards were from Bio-Rad (Toronto, Canada).

Purification of poly(ADP-ribose) polymerase

Poly(ADP-ribose) polymerase was purified from calf thymus according to Zahradka and Ebisuzaki (20) up to the DNA-Cellulose chromatography step. The active fraction was concentrated 6-fold on sucrose (17). The enzyme preparation had a specific activity of 480 U/mg.

Preparation of [^{32}P] poly(ADP-ribose)

[^{32}P] labeled poly(ADP-ribose) was synthesized according to Ménard and Poirier (17). The radiolabeled polymers were purified by affinity chromatography on a 0.5 ml column of DHB-BioRex as described by Aboul-Ela *et al.* (21). The eluted polymer was precipitated according to (17) and had a specific radioactivity between 20 and 25 mCi/mmol. An aliquot of the polymer was digested to nucleotides using snake venom phosphodiesterase and analyzed by strong anion exchange HPLC according to Alvarez-Gonzalez and Jacobson (22). The material had an average size of 90 ADP-ribose residues and an average number of branching points of 2.9 per molecule of polymer.

Assay of poly(ADP-ribose) glycohydrolase activity

Poly(ADP-ribose) glycohydrolase activity was determined essentially as described by Ménard and Poirier (17). The standard reaction mixture contained 50 mM potassium phosphate buffer (pH 7.5), 10 mM β-mercaptoethanol, 50 mM KCI, 100/μg/ml BSA, 0.1 mM PMSF, 10 μM [^{32}P] poly(ADP-ribose) and enzyme in a final volume of 60 μl. The reaction mixture minus enzyme was preincubated 5 min at 37°C, the reaction was carried out 5 min at 37°C and stopped by addition of 0.1% (final) SDS. An aliquot containing approximately 10 000 cpm was mixed with 40 nanomoles of ADP-ribose and applied to a PEI-F cellulose thin layer plate (10×20 cm). The plate was developed at room temperature in methanol and then transferred to 0.3 M LiCl-0.9 N acetic acid. The ADP-ribose spot and the origin were excised and counted. One unit of enzyme is defined as the amount which liberates 1 nmol of ADP-ribose from poly(ADP-ribose) per min at 37°C under the above conditions.

Preparation of poly(ADP-ribose) DHB-Sepharose

Poly(ADP-ribose) was synthesized by a modification of the procedure of Ménard and Poirier (17). The incubation mixture contained 100 mM Tris HCl (pH 8.0), 10 mM $MgCl_2$, 8 mM DTT, 10% glycerol, 225 μg of histones, 225 μg of activated DNA, 1 mM (^{32}P) NAD (0.3 μCi/μmol), 10% (V/V) ethanol and 180 units of poly(ADP-ribose) polymerase in a total volume of 9 ml. The reaction was carried out at 25°C for 30 min and terminated by the addition of TCA to a final concentration of 25% (W/V). The mixture was left on ice for 1 h, the TCA pellet was obtained by centrifugation, and dissolved in 0.4 ml ice cold 98% formic acid. The suspension was then diluted by the addition of 10 volumes of ice cold deonized H_2O and TCA was added to a final concentration of 20% (W/V) as described by Aboul-Ela *et al.* (21). The acid insoluble fraction was collected by centrifugation. The pellet was dissolved in 1 ml of 0.5 M. KOH containing 50 mM EDTA. After incubation at 37°C for 2 h, the preparation was diluted to a final concentration of 40 nmol of poly(ADP-ribose) per ml with 250 mM ammonium acetate, pH 9.0, containing 6 M guanidinium hydrochloride, 10 mM EDTA (buffer A). The pH was adjusted to 9.0 ± 0.2 by addition of concentrated HCl. Polymer preparation was stored at −20°C until used.

DHB-Sepharose was synthesized as described by Jacobson et al. (19). Before use, 1 ml DHB-Sepharose columns were packed in econo-columns from Bio-Rad. Each column was prewashed with 5 ml H_2O, and 10 ml buffer A. After application of 400 nmol poly(ADP-ribose) in buffer A, each column was washed with 20 ml buffer A followed by 10 ml of 1 M ammonium acetate, 10 mM EDTA, pH 9.0 and 20 ml of 50 mM Tris-HCl, pH 9.0, 300 mM KCl, 5 mM 2-mercaptoethanol, 0.1% (w/v) triton X-100.

Purification of poly(ADP-ribose) glycohydrolase

Poly(ADP-ribose) glycohydrolase was purified from calf thymus up to the heparin-sepharose chromatography step according to Hatakeyama *et al.* (6) with slight modifications. Briefly, after ammonium sulfate fractionation and dialysis, gycohydrolase was applied onto a CM-Sepharose CL-6B column (1.6×80 cm) equilibrated with 20 mM potassium phosphate buffer (pH 8.0) containing 2 mM 2-mercaptoethanol (buffer 1). The column was washed with 400 ml of buffer 1 and eluted with a 1.2 l linear gradient (0−300 mM) of KCl in buffer 1 (flow rate 30 ml/h). After the polyethylene glycol # 6000 fractionation, enzyme was applied to an 8 ml DNA-agarose column (1.2×7 cm) equilibrated with 50 mM Tris/HCl, pH 9.0, 5 mM 2-mercaptoethanol and 10% glycerol (buffer 2) containing 50 mM KCl. The column was washed with 20 ml of buffer 2, 50 mM KCl, and eluted with a 100 ml linear gradient (50−250 mM) of KCl in buffer 2 (flow rate 7 ml/h). The active fraction from DNA-agarose was applied to a 2 ml column of Heparin-Sepharose Cl-6B equilibrated with 50 mM Tris-HCl, pH 9.0, 5 mM 2-mercaptoethanol and 10% glycerol (buffer 3) containing 150 mM KCl (flow rate 2 ml/h). The column was washed with 5 ml of the equilibration buffer (flow rate 5 ml/h) and eluted with a 14 ml linear gradient (150−500 mM) of KCl in buffer 3 (flow rate 5 ml/h). The enzyme activity was eluted at approximately 250 mM KCl. One ml poly(ADP-ribose) DHB-sepharose columns prepared as described above were equilibrated in 50 mM Tris-HCl pH 9.0, 5 mM 2-mercaptoethanol, 0.1% triton X-100 (buffer 4) containing 300 mM KCl. The active fractions from heparin-sepharose were adjusted to 0.1% (w/v) triton X-100 and applied (1500 units of glycohydrolase per column) to the poly(ADP-ribose) affinity columns. After loading, each column was washed with 3×1 ml of buffer 4 containing 400 mM KCl and eluted with 10×0.5 ml aliquotes of 1 MKCl (buffer 4) to maintain a steady flow rate. Flow rate was determined by gravity; 150 μl fractions were collected and the glycohydrolase activity eluted in the middle fractions. Glycerol was added to a final concentration of 10% to the active fractions. When necessary active fractions were pooled and concentrated on centriflo CF 25 cones as described by Hatakeyama *et al.* (6) without any loss of enzyme activity.

Protein Analysis

Electrophoresis was performed on slab gels (15 cm×15 cm×0.75 mm) containing 10% (W/V) polyacrylamide in the buffer system of Laemmli (23). Proteins were stained with silver (24) or with

Table 1. Purification of Poly(ADP-ribose) glycohydrolase from calf thymus

Step	Protein mg	Total activity Units	Specific activity Units/mg protein	Yield %	Purification -fold
1. Crude extract	33 600	36 500	1.09	100	1.0
2. Protamine sulfate	15 200	35 700	2.35	98	2.2
3. Ammonium sulfate	4 230	22 300	5.27	61	4.8
4. CM-Sepharose	166	12 300	74	34	68
5. Polyethylene glycol 6000	60.1	8 750	145	24	133
6. DNA-agarose	14.0	5 500	392	15	360
7. Heparin-Sepharose	4.92	5 290	1 080	14.5	990
8. Poly(ADP-ribose) DBH-Sepharose	0.067	3 330	50 000	9.1	45 900

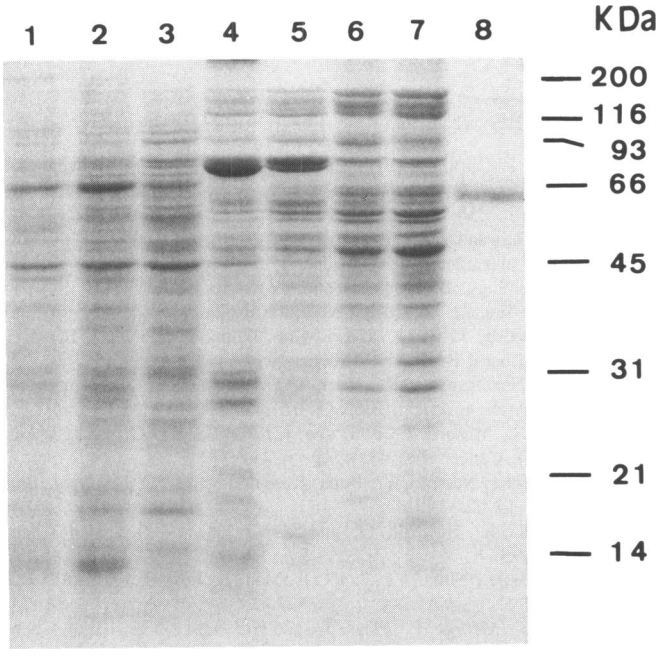

Figure 1. Analysis of Purification of Poly(ADP-ribose) glycohydrolase by SDS Polyacrylamide Gel Electrophoresis. Portions of the enzyme preparation from each step were subjected to electrophoresis on 10% gels, and the gel was stained with coomassie brillant blue, as described in Materials and Methods. Molecular weight markers are shown. Lane 1, crude extract; lane 2, protamine sulfate step; lane 3, ammonium sulfate step; lane 4, CM-Sepharose pool; lane 5, PEG step; lane 6, DNA agarose pool; lane 7, Heparin-Sepharose pool; lane 8, poly(ADP-ribose) affinity pool.

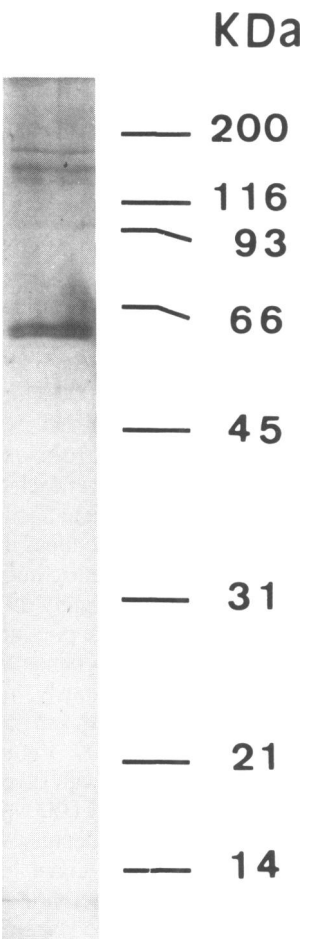

Figure 2. SDS-polyacrylamide gel electrophoresis of purified poly(ADP-ribose) glycohydrolase. The purified enzyme (200 ng of protein) was subjected to electrophoresis on a 10% gel and stained with silver as described in Materials and Methods. Molecular weight markers are shown.

coomassie blue R250. Prior to gel electrophoresis the purified enzyme was precipitated using TCA at a final concentration of 20% (w/v) with tRNA as a coprecipitant. Protein concentration was determined using bovine serum albumin as the standard (25).

RESULTS AND DISCUSSION

An affinity matrix which consisted of ADP-ribose polymers bound to dihydroxyboronyl-Sepharose (poly ADPR-DHB-S) was prepared for the application described here by loading approximately 400 nmol of polymer (calculated as ADPR residues) per ml of resin. The preparation of this matrix requires the synthesis of relatively large amounts of ADP-ribose polymers. However, this is not difficult since only partially purified poly(ADP-ribose) polymerase is required and the method of Zahradka and Ebisuzaki (20) allows the preparation of relatively large quantities of enzyme suitable for this purpose. In addition, we have utilized conditions previously determined to allow efficient conversion of NAD to large polymers of ADP-ribose (17). The preparation of poly ADPR-DHB-S is further quite simple since the polymers do not need to be extensively purified prior to loading onto the boronyl sepharose. As described here, crude mixtures containing polymers can be loaded onto DHB-

Sepharose in high concentrations of guanidinium chloride followed by washing with guanidinium chloride to remove proteins, DNA and other potentially interferring material. We have compared the affinity matrix prepared as described here with one prepared by first purifying the polymers and have obtained equivalent results. We have obtained the best results by preparing the columns the same day as used.

Previous studies have shown that the retention of ADP-ribose polymers in the presence of high concentrations of guanidinium chloride requires the formation of a borate complex as the polymers are only retained at pH values above 8.0 which are required for the formation of this complex (26). However, following removal of guanidinium chloride, the polymers are efficiently retained even at low pH values as the polymers are bound to the resin by other interactions. Thus, another advantage of the matrix described here is that, once prepared, it can be used under a wide variety of experimental conditions.

The utility of poly ADPR-DHB-S for purification of poly(ADP-ribose) glycohydrolase was tested using a partially purified fraction from calf thymus which was purified through the initial steps of the procedure of Hatakeyama et al. (6). The entire procedure of these workers involves 6 column chromatographic steps utilizing CM-Sepharose, DNA-agarose, Heparin-Sepharose, Sephacryl S-200, Blue Sepharose and Red Sepharose. In the application shown here an affinity step was done using the active fraction pool from the Heparin-Sepharose step. This fraction was subjected to affinity chromatography as described in Experimental Procedures. Data concerning the purification including the affinity step are shown in Table 1. The affinity step allowed an increase in fold purification from 990 to 45 900, which represented an approximately 50 fold purification for that step with over 60% recovery of enzymatic activity. Figure 1 shows analysis by SDS-PAGE of the glycohydrolase fractions at each of the steps of the purification described in Table 1. Lane 7 shows the material applied to the affinity matrix and lane 8 shows the material eluted. The affinity step resulted in a large enrichment of two closely migrating bands with M_r of approximately 59 000 and 60 000. The enzymatic activity eluting from the polymer affinity column corresponded exactly to the two bands which coeluted. Hatakeyama *et al.* (6) have previously reported that the preparation obtained by their entire purification procedure resulted in a broad band of M_r 59 000. Figure 2 shows SDS-PAGE of the affinity purified material with silver staining. Again, two major bands of M_r 59 000 and 60 000 were observed with minor bands detectable at higher molecular weight. We have consistently observed approximately equal intensities of both bands to co-migrate with enzymatic activities, making it likely that both bands have enzymatic activity. The enzymatic properties of the affinity purified material have also been characterized and found to be indistinguishable from the enzyme purified by Hatakeyama et al. (6) (unpublished results).

Taken together, the results of Table 1 and Figures 1 and 2 demonstrate that poly ADPR-DHB-S offers advantages for the preparation of poly(ADP-ribose) glycohydrolase of high purity. In the example shown here, we have been able to modify the procedure of Hatekayama *et al.* (6) to replace 3 time consuming conventional chromatographic steps with a single rapid affinity step while increasing the yield 3 fold. In another application, we have been able to purify the enzyme in even larger amounts for structural studies by combining an affinity step using the polyethylene glycol fraction (Table 1) with preparative gel electrophoresis. We would anticipate that this matrix should also prove useful for the isolation of the glycohydrolase from other sources. It also seems likely that this matrix should be useful for other studies related to ADP-ribose polymer metabolism. Further, the amino acid analyses of each band were very similar and following digestion of each band with endoproteinase lys C, very similar patterns of peptides were observed (unpublished results). Assuming that the two main bands present in our preparation are two forms of the same enzyme, the purity of the glycohydrolase was estimated to be 94% after silver staining of the gel, as judged by densitometry.

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