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SIZE AND SHAPE OF POLY(ADP-RIBOSE): EXAMINATION BY GEL FILTRATION, GEL ELECTROPHORESIS AND ELECTRON MICROSCOPY

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SUMMARY: Digestion of the poly(ADP-ribose) with snake venom phosphodiesterase yielded phosphoribosyl-AMP and AMP in a ratio of 30 to 1, indicating the "chain length" of 30 if the poly(ADP-ribose) is a linear molecule as previously supposed. Gel filtration and gel electrophoresis, however, revealed a very heterogeneous size distribution, the average size being, much longer than $(ADP\text{-ribose})_{30}$. Electron microscopy of poly(ADP-ribose) showed an irregularly branched figure. Molecules with more than ten branched portions were observed. These results are in agreement with the recent identification of the chemical structure of the branched portion in poly(ADP-ribose).

Poly(ADP-ribose) is a biopolymer, synthesized enzymatically from NAD^+(1-6). This polymer was found to be present in various tissues and in cultured cells. The biological functions of the polymer have been suggested to be involved in various chromatin functions including DNA repair, cell differentiation and cell transformation (7-11).

Poly(ADP-ribose) is resistant to alkaline treatment and to many types of DNase and RNase (7). However, it is sensitive to snake venom phosphodiesterase and poly(ADP-ribose) glycohydrolase (12), yielding phosphoribosyl-AMP and ADP-ribose, respectively, as major products. Because it has a ribose-phosphate-phosphate-ribose backbone and adenine base, this polymer is structurally related to, but distinct from, nucleic acids.

In many previous works, the size of the polymer was determined as the chain length, calculated from the molar ratio of total phosphoribosyl-AMP to terminal AMP residues, measured after complete digestion of the

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polymer with venom phosphodiesterase (13,14). This way of determining the chain length gives an average value, and the maximum value reported so far was about 30 (11). However, this chain length determination method is not suitable to estimate the size of a polymer with a branching structure, because we have recently identified and characterized the structure of branched portions of poly(ADP-Rib)(15,16).

In this work, we report that by electronmicroscopy poly(ADP-ribose), which has been proven very heterogenous in size by gel filtration and gel electrophoresis, showed a branching structure, with some molecules as large as 2×10^5 daltons or more.

MATERIALS AND METHODS

Preparation of Poly(ADP-ribose): Poly(ADP-ribose) was obtained as described previously (17). In brief, calf thymus nuclei were incubated with [adenine-U-^{14}C]NAD^+. They were then completely digested with DNase I, pancreatic RNase, nuclease P_1 and pronase E, and the digested material was extracted with phenol, precipitated with ethanol, and fractionated by hydroxylapatite column chromatography. High molecular weight poly-(ADP-ribose), which has a ratio of total adenine residues to terminal adenine residues of about 30, was eluted with 0.25 M to 0.5 M sodium phosphate buffer (pH 6.8). This fraction reproducibly yielded poly-(ADP-ribose) of at least 98% purity, as judged from its dry weight after desalting, absorbance at 260 nm, phosphorus content and radioactivity (17). About half the acid-insoluble radioactivity in the initial incubation mixture was recovered in this fraction.

Gel Filtration : This poly(ADP-ribose) was applied to a Bio-Gel A-50m column (1.6 x 100 cm) which had been equilibrated with 10 mM Tris-HCl (pH 8.5) in 1 mM Na_2EDTA and eluted with the same buffer at a flow rate of 5 ml/hr at 10°C. Fractions of 4 ml were collected and their A_{260} was measured.

Electrophoresis: Selected fractions of gel filtration were lyophilized, dissolved in water and precipitated with 2 volumes of ethanol. They were then dried in vacuo, dissolved in 10 % glycerol, 0.05 % bromophenol blue and 0.05 % xylene cyanol. These samples were then applied to 20 % polyacrylamide slab gel (0.15 x 20 x 40 cm) in a buffer of 7 M urea, 90 mM Tris-borate and 2 mM Na_2EDTA (pH 8.3) (18). Electrophoresis was performed at 800 volts for 6 hrs, then stained with Stains-All (Kodak) and photographed.

Electron microscopy : Fractions of gel filtration were also examined by electron microscopy by the method of Davis *et al.* (19). The spreading solution contained 90 % formamide and the hypophase was distilled water.

RESULTS AND DISCUSSION

Gel filtration of the poly(ADP-ribose)(Fig. 1) revealed a poly-dispersed pattern. More than 70 % of the polymer was eluted in fraction

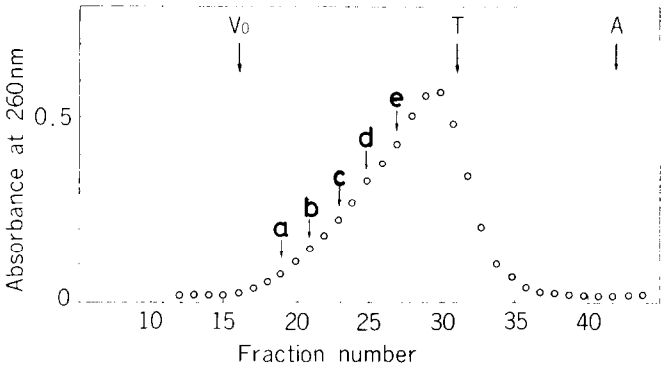

Figure 1. Gel filtration of poly(ADP-ribose). V_0 indicates the void volume, and arrows labeled T and A indicate the elution volumes of tRNA (a gift from Dr. Z. Ohashi of this institute) and ATP, respectively. Poly(ADP-ribose) fractions in a,b,c,d and e were subjected to electron microscopy.

No. 16 to 30, which had a smaller elution volume than tRNA. Thus, the elution pattern in Fig. 1 suggests that most of the poly(ADP-ribose) is much larger than tRNA and has a broad size distribution.

Polyacrylamide gel electrophoresis in the presence of 7 M urea (18) of selected fractions obtained by gel filtration confirmed these observations (Fig. 2). Using this electrophoretic method, molecules differing in size by one ADP-ribose residue can be resolved, thus giving multiple bands; bromophenol blue moves with $(ADP\text{-}ribose)_8$, while xylene cyanol moves with $(ADP\text{-}ribose)_{20}$ (20,21). As seen in Fig. 2, bands could be resolved up to $(ADP\text{-}ribose)_{70}$. Poly(ADP-ribose) in fractions 18 through 24 from the gel filtration did not enter the polyacrylamide gel. Poly(ADP-ribose) in fraction 26 created a smear from the origin to near $(ADP\text{-}ribose)_{50}$, while those in fractions 30 to 34 gave clear bands down to the position of xylene cyanol which is known to co-migrate with $(ADP\text{-}ribose)_{20}$, proving that the poly(ADP-ribose) has a very broad size distribution. It is also evident from Fig. 2 that the peak fraction in Fig. 1 (fraction 30), contained larger molecules than $(ADP\text{-}ribose)_{35}$, and that more than 70 % of the amount of poly(ADP-ribose) was recovered in fractions eluted before the peak fraction.

In order to visualize the size of poly(ADP-ribose) of higher molecular weight, the fractions indicated by arrows in Fig. 1 were examined by

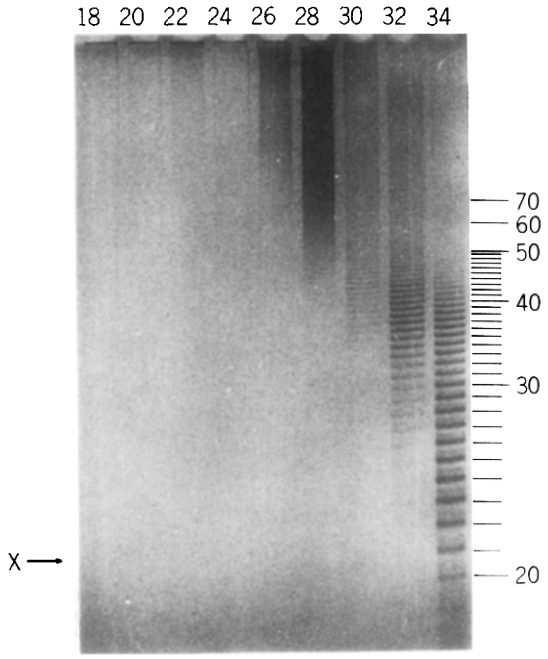

Figure 2. Electrophoresis of poly(ADP-ribose) fractions from gel filtration. Every other fraction from 18 to 34 in Fig. 1 was analyzed by 20 % polyacrylamide slab gel electrophoresis in a buffer of 7 M urea, 90 mM Tris-borate and 2 mM Na_2EDTA (18). This gel was stained with Stains-All and photographed. Numbers by slots correspond to fraction numbers in Fig. 1. The band number is indicated on the right. The arrow labeled X shows the position of the marker dye, xylene cyanol.

electron microscopy. Fig. 3 shows that poly(ADP-ribose) molecules all have an irregularly branched structure, large molecules having more than ten branches (Fig. 3a) and smaller ones having few (Fig. 3d to e). Essentially the same figures were obtained using different spreading solutions, such as 50 % formamide or formamide plus 0.15 M NaCl. Thus, this branched structure seems to be the true form of poly(ADP-ribose), not a collapsed state of linear molecules.

The average length per branch was calculated for each fraction and found to have a constant value of 100 nm per branch in all fractions. Because the mass-length ratio of poly(ADP-ribose) is not known, the molecular weight of the polymer cannot be determined directly from the electron micrograph. We, therefore, tentatively assumed that the length occupied by one residue of poly(ADP-ribose) is twice that of single

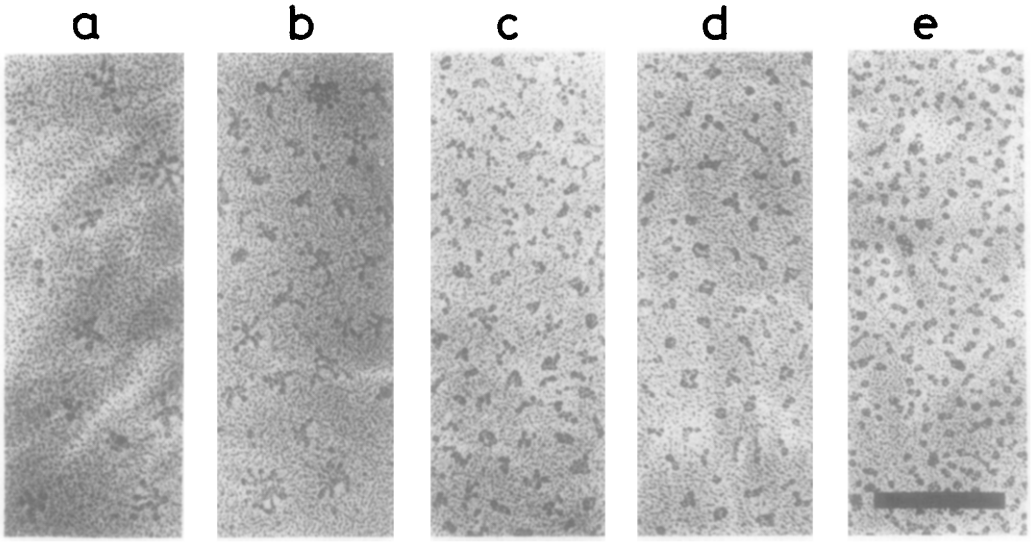

Figure 3. Electron micrograph of poly(ADP-ribose) fractions from gel filtration. The fractions shown in Fig. 1 were examined by electron microscopy by the method of Davis *et al.*(19). Photographs were taken at an instrument magnification of x 10,000. Bar indicates 1 μm; a, b, c, d and e correspond to fractions 19, 21, 23, 25 and 27, respectively, of Fig. 1.

stranded DNA, because the sugar-phosphate-phosphate-sugar backbone of poly(ADP-ribose) should be nearly twice as long as the sugar-phosphate backbone of single stranded nucleic acid. Making this assumption and measuring the length of the polymer relative to that of phage fd DNA (internal length reference in electron micrographs), we calculated the average length per molecule in the largest fraction (Fig. 3a) to be 350 residues, or 2 x 10^5 daltons. Similarly, the distance between two branched portions was calculated to be 35 residues per branch. This value is in close agreement with the ratio of total to terminal residues.

In agreement with these findings, we have isolated a product, 2'[1"-ribosyl-2"(1"'-ribosyl)]adenosine-5',5",5"' -tris(phosphate), among the venom phosphodiesterase hydrolysates of high molecular weight poly(ADP-ribose)(15,16). This product was most presumably derived from the branched portions of poly(ADP-ribose) and constituted about 2 % of the total hydrolysates (15).

This is the first report of the visualization of high molecular weight poly(ADP-ribose) and of its unusual branched shape. These findings

will contribute to better understanding of the structure of poly(ADP-ribose), which might be involved in the regulation of fundamental phenomena of gene expression (7-11).

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