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A NEW METHOD FOR OLIGO(ADP-RIBOSE) FRACTIONATION ACCORDING TO CHAIN LENGTH*

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SUMMARY A new method was developed to separate mono- and oligo-(ADP-ribose) with chain lengths below 11 ADP-ribose units by size difference of one ADP-ribose residue. The separation was performed on a DEAE-cellulose column by elution with a NaCl gradient (0-0.3 M) in the presence of 7 M urea at pH 7.6. Using this method, the chain length distribution of oligo(ADP-ribose) molecules attached to histones by incubation of isolated nuclei with radioactive NAD was determined. The average chain length estimated from this distribution coincided exactly with the value obtained by the phosphodiesterase digestion method, suggesting that the oligomers were synthesized directly on histones and not elongated from pre-existing ADP-ribose.

Poly(ADP-ribose) is a homopolymer synthesized from NAD by poly(ADP-ribose) synthetase in the nucleus of eukaryotic cells (1-4). This polymer is synthesized in a covalent association with various nuclear proteins (1-4). The size of polymer has been calculated from the ratio of isoADP-ribose to AMP after digestion with snake venom phosphodiesterase (5). This method, however, provides information only on the average size and not on the size distribution.

Resolution of poly(ADP-ribose) according to the chain length has been achieved using hydroxylapatite column chromatography (6) or polyacrylamide gel electrophoresis (7,8). The resolution by

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Abbreviation: isoADP-ribose, 2'-(5"-phosphoribosyl)-5'-AMP.

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the former method is not satisfactory especially for oligomers larger than a tetramer. The latter method effectively separates oligomers and polymers composed of up to 65 ADP-ribose units, but is not suitable for preparation of individual oligomers on a large scale.

DEAE-cellulose column chromatography in the presence of urea has been widely used to fractionate oligonucleotides according to their degree of polymerization (9). The fractionation is primarily based on the net negative charge. We applied this method to separation of oligo(ADP-ribose) fractions and obtained a better resolution than the previous methods mentioned above.

MATERIALS AND METHODS

[AMP-^{32}P]NAD was purchased from New England Nuclear; [Ade-U-^{14}C]NAD was from the Radiochemical Centre, Amersham; NAD, ADP-ribose, ATP, and adenosine 5'-tetraphosphate were from Sigma, DEAE-cellulose (DE 52) was from Whatman; DEAE-Sephadex A-25 was from Pharmacia. Crotalus adamanteus venom phosphodiesterase was obtained from Sigma and further purified by the method of Oka et al. (10). Oligo([^{32}P]ADP-ribosyl) histone H1 was prepared by incubating histone H1 and [^{32}P]NAD with purified poly(ADP-ribose) synthetase as described previously (11); the reaction mixture (2 ml) contained 100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μM [^{32}P]NAD (570 cpm/pmol), 50 μg/ml calf thymus DNA, 50 μg/ml calf thymus histone H1, and 3 μg of purified rat liver poly(ADP-ribose) synthetase. After incubation for 3 min at 37°C, the reaction was terminated by the addition of 0.75 N (final) $HClO_4$. The mixture was centrifuged, and histone H1 was precipitated from the supernatant with 20% Cl_3CCOOH (11). The precipitate was washed twice with 20% Cl_3CCOOH and once with ethyl ether. Oligo([^{14}C]ADP-ribosyl) histones H1 and H2B were prepared using isolated rat liver nuclei and purified with borate and CM-cellulose columns as described by Okayama et al. (12). Oligo(ADP-ribose) was released from histones by incubation in 0.2 ml of 1 N NH_4OH for 40 min at 25°C. The mixture was then lyophilized.

RESULTS AND DISCUSSION

Fractionation of oligo(ADP-ribose) Oligo(ADP-ribose) released from histone by an NH_4OH treatment was dissolved in 0.2 ml

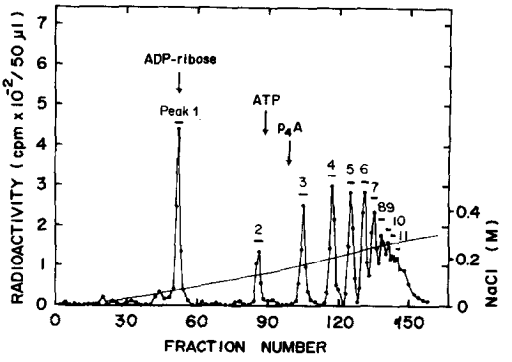

Fig. 1. DEAE-cellulose column chromatography of oligo(ADP-ribose) in the presence of urea. Oligo([^{32}P]ADP-ribose) (300,000 cpm, average chain length = 4.5) prepared with purified poly(ADP-ribose) synthetase was fractionated with a DEAE-cellulose column. Arrows indicate positions of markers: ADP-ribose, ATP, and adenosine 5'-tetraphosphate (p_4A).

of 20 m M Tris-HCl buffer (pH 7.6) containing 7 M urea. The mixture was applied to a DEAE-cellulose column (0.7 x 45 cm) equilibrated with the same buffer. The column was eluted with a linear gradient of 0 to 0.3 M NaCl in the above buffer (total 400 ml) at a flow rate of 6 ml/h, and fractions of 2.2 ml were collected. Fig. 1 shows the typical elution profile. Oligo(ADP-ribose) was resolved into 11 radioactive peaks, which were designated as peaks 1 to 11 according to the order of elution. Peak 1 co-eluted with authentic ADP-ribose. Peak 2 appeared just before ATP, and peak 3 after adenosine 5'-tetraphosphate, indicating that the oligomers of peak 2 and 3 had net charges of about -4 and -6, respectively. These 11 peak fractions were collected separately and deprived of salts and urea by the method of Rushizky and Sober (13); the sample was readsorbed onto a column of DEAE-cellulose (bicarbonate form) and then eluted with 2 M ammonium bicarbonate, and the effluent was dried in vacuo. Aliquots of the desalted samples were subjected to polyacrylamide gel electrophoresis. Upon autoradiography, each peak exhibited a single

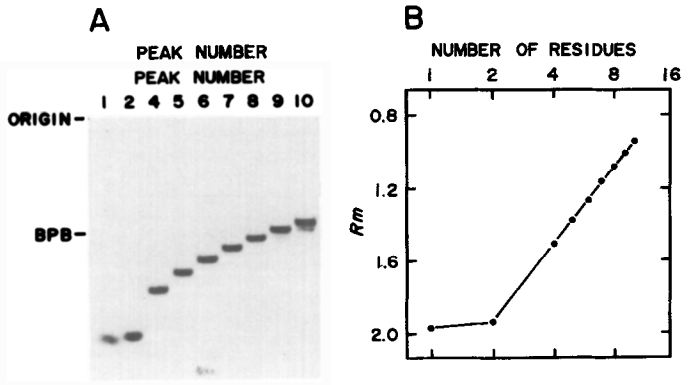

Fig. 2. Autoradiograms of peak 1 to 10 oligomers after gel electrophoresis (A) and the relation between the number of residues and the relative mobility (B). Aliquots (about 800 cpm each) of the desalted peak materials were dissolved in 10-µl solutions containing 0.5 mM EDTA/Na (pH 7.0), 25% glycerol, and 0.05% bromphenol blue. The mixtures were subjected to electrophoresis as described by Tanaka et al. (8) on a 20% polyacrylamide gel slab (12 X 14 X 0.15 cm) for 7 h at 120 V. After electrophoresis, the gel was dried and subjected to autoradiography. Relative mobility (Rm) was calculated with bromphenol blue (BPB) as a reference. The peak number was assumed to represent the number of residues.

radioactive band, except that peak 10 was contaminated by the peak 9 material (Fig. 2 A). Assuming that the peak number represented the number of residues, a linear correlation was obtained between the logarithm of the number of residues (2 to 10) and the electrophoretic mobility (Fig. 2 B). Other aliquots were digested with venom phosphodiesterase, and the chain lengths were calculated from the ratio of isoADP-ribose to AMP (5) (Table 1). A clear integer order was observed from peak 1 to 11 within a range of experimental error, indicating that each peak differed from its neighbor peaks by only one ADP-ribose unit. The fact that the estimated chain lengths of large oligomers were slightly less than the expected integers may suggest the presence of branched oligomers (14). Fractionation of oligomers was also achieved by DEAE-cellulose column chromatography in the buffer system of 10 mM sodium acetate (pH 5.4) in 7 M urea (9) (data not shown). Urea was added to eliminate secondary binding forces

Table 1. Chain lengths of oligo(ADP-ribose) fractions separated by DEAE-cellulose column chromatography. Aliquots of desalted peak materials were incubated for 6 h at 37°C in a solution (100 µl) containing 50 mM potassium phosphate (pH 7.5), 10 mM $MgCl_2$, 5mM AMP, and 0.3 unit of snake venom phosphodiesterase. The digests were analyzed by paper chromatography using the solvent system of isobutyric acid : conc. NH_4OH : H_2O : 0.1 M EDTA/Na_2 (66 : 1 : 32 : 1).

Peak number	Products of Phosphodiesterase digestion		Chain length	Nearest integer
	AMP (A)	IsoADP-ribose (B)	$(1+B/A)$	
	cpm	cpm		
1	8510	< 50	1.0	1
2	2876	2880	2.0	2
3	3097	6280	3.0	3
4	2731	8238	4.0	4
5	2124	7635	4.6	5
6	1236	5928	5.8	6
7	965	5641	6.9	7
8	696	4581	7.6	8
9	581	4339	8.5	9
10	ND	ND	ND	
11	317	3039	10.6	11

ND, not determined.

such as hydrophobic interactions and hydrogen bonds between the oligomer and the exchanger (9). In fact, the resolution of oligomers, especially larger ones, became poorer in the absence of urea. DEAE-Sephadex could not replace DEAE-cellulose, since higher NaCl concentrations were required to elute oligomers from DEAE-Sephadex than from DEAE-cellulose, and, at high salt concentrations, the Sephadex exchanger shrinked so much that the sharp resolution could not be achieved. Desalting of oligomers larger than a dimer could also be carried out using Bio-Gel P-2 as described by Uziel for oligonucleotides (15).

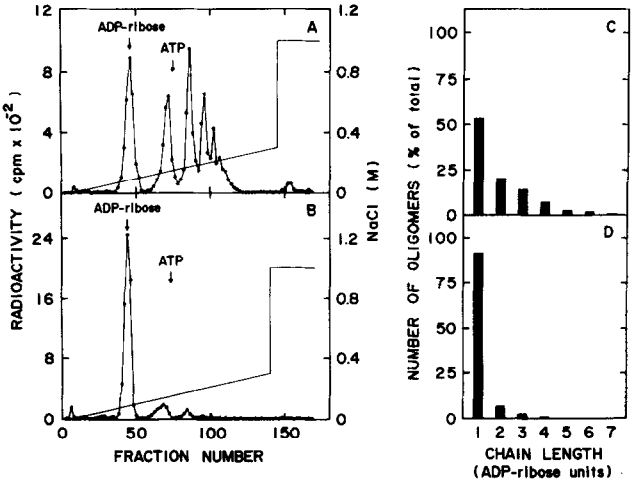

Fig. 3. Chain length distribution of oligo(ADP-ribose) bound to histones. Oligo([^{14}C]ADP-ribosyl) histones H1 and H2B were prepared using isolated rat liver nuclei as described under "Materials and Methods." The oligomers released from H1 (A) or H2B (B) were analyzed on a DEAE-cellulose column as described for Fig. 1, except that a shorter column (0.7 X 25 cm) and a smaller volume of the NaCl gradient (150 ml) were employed and that the column was finally washed with 1 M NaCl in the same buffer. The chain numbers of monomers and individual oligomers on H1 (C) or H2B (D) were determined from the peak areas devided by respective chain lengths, and presented as per cent of the total chain number.

Chain Length Distribution of Oligo(ADP-ribose) Attached to Histones in Isolated Nuclei ADP-ribose residues have been reported to be attached to histones in monomeric or short oligomeric forms (12,16,17). Using the present chromatographic method, we analyzed oligo(ADP-ribosyl) histones H1 and H2B produced in isolated rat liver nuclei (Figs. 3 A and B). The degree of ADP-ribose polymerization on histone H1 ranged from a monomer up to a heptamer, and that on H2B from a monomer to a tetramer. Relative chain numbers of monomers and individual oligomers could be estimated from the elution profiles and shown in Figs. 3 C and D. Based on these size distributions, the average chain lengths of oligomers attached to H1 and H2B were determined as 1.92 and 1.10 ADP-ribose units, respectively. These values coincided exactly with the values, 1.92 and 1.09, obtained by the

phosphodiesterase digestion method. The average chain length determined from the size distribution is based primarily upon the average negative charge of oligomers. On the other hand, the phosphodiesterase digestion method provides the average size of the radioactive portion of oligomers. The exact coincidence of the values obtained by these different methods, therefore, suggested that most, if not all, of the oligomers synthesized by the incubation of isolated nuclei with [^{14}C]NAD were initiated directly on histones and not produced by elongation from pre-existing, nonradioactive ADP-ribose chains. This result implies that histones in isolated nuclei were hardly modified with ADP-ribose and/or that ADP-ribose on histones turned over rapidly.

The present chromatographic method is suitable for separation of oligo(ADP-ribose) with chain lengths below 11, and makes possible a quantitative determination of the chain length distribution of oligo(ADP-ribose) on proteins. In view of the fact that ADP-ribose attached to histones and some nonhistone proteins *in vivo* is mostly monomeric and oligomeric (16-18), this method provides useful information about the relation between the degree of ADP-ribose polymerization and function of poly(ADP-ribosyl) proteins. Finally, this method can be easily scaled-up for preparation of individual oligomers in large amounts.

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