Characterization of Polymers of Adenosine Diphosphate Ribose Generated in Vitro and in Vivo†

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Received June 27, 1986; Revised Manuscript Received December 22, 1986

ABSTRACT: Methods have been developed and applied to determine the size and branching frequency of polymers of ADP-ribose synthesized in nucleotide-permeable cultured mouse cells and in intact cultured cells. Polymers were purified by affinity chromatography with a boronate resin and were fractionated according to size by molecular sieve high-performance liquid chromatography. Fractions were enzymatically digested to nucleotides, which were separated by strong anion exchange high-performance liquid chromatography. From these data, average polymer size and branching frequency were calculated. A wide range of polymer sizes was observed. Polymers as large as 190 residues with at least five points of branching per molecule were generated in vitro. Polymers of up to 67 residues containing up to two points of branching per molecule were isolated from intact cells following treatment with the DNA alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Cells treated with hyperthermia prior to DNA damage contained polymers of an average maximum size of 244 residues containing up to six points of branching per molecule. The detection of large polymers of ADP-ribose in intact cells suggests that alterations in chromatin organization effected by poly(ADP-ribosylation) may extend beyond the covalently modified proteins and very likely involve noncovalent interactions of poly(ADP-ribose) with other components of chromatin.

Poly(ADP-ribose)[1] is synthesized in the nucleus of eukaryotic cells from NAD^+. A wide spectrum of DNA damage results in a rapid alteration of poly(ADP-ribose) metabolism due to the activation of poly(ADP-ribose) polymerase by DNA strand breaks (Benjamin & Gill, 1980; Jacobson et al., 1983). Following treatment of cells with DNA alkylating agents, inhibitors of poly(ADP-ribose) synthesis increase cytotoxicity (Durkacz et al., 1980; Jacobson et al., 1984a) and malignant transformation frequency (Lubet et al., 1984; Jacobson et al., 1985b), suggesting that this chromatin-associated process is necessary for cellular recovery from these agents. Inhibitors of ADP-ribosylation also affect cellular differentiation in a number of systems (Farzaneh et al., 1982; Althaus et al., 1982; Johnstone & Williams, 1982) and increase sister chromatid exchanges (Oikawa et al., 1980; Hori, 1981), suggesting that poly(ADP-ribose) metabolism is associated with chromatin changes required for many cellular processes. Conceptually, the function(s) of polymers of ADP-ribose in chromatin may be mediated by the covalent modification of chromatin proteins with the polymer and/or by noncovalent interactions of this negatively charged polymer with other chromatin components. A number of studies have begun to identify the covalent acceptors of poly(ADP-ribose) in intact cells (Wong et al., 1983; Tanuma & Johnson, 1983; Adamietz & Rudolph, 1984; Kreimeyer et al., 1984). The potential for stable noncovalent interactions should be highly dependent on the size of polymers generated, which is the subject of the studies described here. A preliminary report of this work has appeared (Alvarez-Gonzalez & Jacobson, 1985).

Experimental Procedures

Materials. [α-^{32}P]ATP (1000–2000 Ci/mmol) and [2,8-^{3}H]adenine (29 Ci/mmol) were obtained from International Chemical and Nuclear, Irvine, CA. Bio-Sil TSK-125 gel filtration columns (300 mm × 7.5 mm i.d.) and electrophoresis reagents were purchased from Bio-Rad, Richmond, CA. A Partisil-10 SAX column (250 mm × 4.5 mm i.d.) was obtained from Whatman Chemical Separation, Inc., Clifton, NJ). Oligoadenylic compounds were obtained from P-L Biochemicals, Milwaukee, WI. All other enzymes, chemicals, and equipment were obtained from sources described previously (Sims et al., 1980; Alvarez-Gonzalez et al., 1983).

Synthesis of Radiolabeled NAD$^+$. Radiolabeled NAD^+ was synthesized from [α-^{32}P]ATP with NMN^+-adenyl transferase under conditions described elsewhere (Alvarez-Gonzalez et al., 1983).

Preparation of Poly(ADP-ribose) from Permeabilized Cells. SV40 virus transformed Balb/c 3T3 fibroblasts (SVT2 cells) were grown as described previously (Jacobson & Jacobson, 1976). At a cell density of (20–25) × 10^6 cells/55.4-cm^2 dish, cells were washed with PBS, removed from dishes by treatment with trypsin, and collected by low-speed centrifugation. Cells were then made permeable for the in vitro synthesis of poly(ADP-ribose) as described previously (Alvarez-Gonzalez et al., 1983). The incubation mixture for polymer synthesis contained 2 × 10^7 cells/mL, 40 mM Tris-HCl buffer, pH 7.8, 0.6 mM EDTA, 1 mM 2-mercaptoethanol, 30 mM $MgCl_2$, 200 μg/mL DNase I, and 0.5 mM NAD^+ containing 100 μCi of [^{32}P]NAD^+ in a total volume of 2 mL. The suspension was incubated for 30 min at 30 °C, and the reaction was terminated by adjusting it to 20% TCA and chilling it on ice for 15 min. The TCA pellet obtained by centrifugation at 12000*g*

† This work was supported by Grant CA43894 from the National Institutes of Health. R.A.-G. was supported by predoctoral fellowships from the Mexican National Council of Science and Technology (CONACYT) and The Samuel Roberts Noble Foundation.
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[1] Abbreviations: poly(ADP-ribose), poly(adenosine diphosphate ribose); PBS, phosphate-buffered saline; TCA, trichloroacetic acid; NH_4OAc, ammonium acetate; TEMED, *N,N,N',N'*-tetramethylethylenediamine; EDTA, ethylenediaminetetraacetic acid; XC, xylene cyanol; BPB, bromophenol blue; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; HPLC, high-performance liquid chromatography; PRAMP, 2'-*O*-α-D-ribosyladenosine 5',5''-bisphosphate; $(PR)_2AMP$, 2'-(2''-ribosylribosyl)adenosine 5',5'',5'''-trisphosphate; poly(A), poly(adenylic acid); $(Ap)_9A$, decamer of oligo(adenylic acid); $(Ap)_5A$, pentamer of oligo(adenylic acid); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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for 5 min was washed 5 times with 20% TCA and 3 times with diethyl ether and dissolved in 1 mL of 0.1 N NaOH containing 20 mM EDTA. After incubation at 60 °C for 30 min, the sample was diluted to 10 mL with 250 mM NH_4OAc, pH 9.0, containing 6 M guanidine hydrochloride and adjusted to pH 9.0 ± 0.2 by addition of concentrated HCl. It was applied to a 0.5-mL column of dihydroxyboryl-Bio-Rex previously equilibrated with the application buffer. Following sample application, the resin was washed with 10 mL of application buffer followed by 10 mL of 1 M NH_4HCO_3, pH 9.0. Bound material was eluted with 5 mL of H_2O at 37 °C and stored at −20 °C until used. The recovery of poly(ADP-ribose) was routinely about 90%.

HPLC Molecular Sieve Chromatography. A TSK-125 column (300 mm × 7.5 mm i.d.) was used to fractionate polymers of ADP-ribose. This column was preceded by a guard column (75 mm × 7.5 mm i.d.) containing the same material. For some experiments, two columns were connected in series. The running solvent used was 0.1 M sodium phosphate buffer, pH 6.8, at a flow rate of 1 mL/min. Samples were routinely injected in a volume of 1.0 mL, fractions of 0.5 mL were collected, and small aliquots were removed from each fraction for determination of radioactivity.

Polyacrylamide Gel Electrophoresis. Twenty percent polyacrylamide gels (20 × 20 × 0.15 cm) containing acrylamide and bis(acrylamide), in a ratio of 19:1, 100 mM Tris–borate buffer, pH 8.3, 2 mM EDTA, 4.4 mM ammonium persulfate, and 3.4 mM TEMED were used. Preelectrophoresis was carried out for 1–2 h at 400 V with 50 mM Tris–borate buffer, pH 8.3, containing 1 mM EDTA as the electrode buffer. Samples of 10–20 μL in 50% urea, 25 mM NaCl, 4 mM EDTA, pH 7.5, 0.02% XC, and 0.02% BPB were applied to the gels, and electrophoresis was carried out at 400 V until the BPB migrated 9 cm from the origin. At this point, electrophoresis was stopped, and the gel was exposed to Kodak BB5 X-ray film at −80 °C with the aid of an intensifying screen.

Analysis of the Structure of Poly(ADP-ribose) from Intact Cells. C3H10T1/2 cells were grown as described elsewhere (Jacobson et al., 1984a). Cells were grown to confluence, which resulted in a cell number of (2–3) × 10^6 cells/75-cm^2 flask. Cultures were labeled for 16 h with 20 μCi of [^{3}H]-adenine/mL of nicotinamide-free Dulbecco's modified Eagle's medium containing 10% dialyzed fetal calf serum. Although labeling was done in nicotinamide-free medium, the presence or absence of nicotinamide has no effect on the labeling of the pools under these conditions (M. K. Jacobson, unpublished results). Following radiolabeling, medium was removed from the flasks followed by two rinses with PBS, and 10 mL of Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 64 μM MNNG was added, and cells were incubated for 20 min at 37 °C. For hyperthermic treatment, 10 mL of Dulbecco's modified Eagle's medium containing 10% fetal calf serum preequilibrated to 45 °C was added, and cells were incubated at 45 °C for 30 min. Medium was replaced with medium at 37 °C, cells were then transferred to a 37 °C incubator, MNNG was added to a final concentration of 64 μM, and the mixture was incubated for 20 min. Medium was decanted, flasks were rinsed with cold PBS 3 times, and 20% TCA (w/v) was added. Acid-insoluble material was obtained by scraping the dishes followed by centrifugation at 1000*g*. ^{3}H-Labeled poly(ADP-ribose) was purified with dihydroxyboryl-Bio-Rex as described above except that release of polymers from protein was done by incubation for 2 h in 0.2 N NaOH and 20 mM EDTA at 37 °C to prevent loss of radiolabel by exchange reactions and the columns were washed with 20 mL of application buffer.

Enzyme Treatment of TSK-125 Fractions. Fractions collected after molecular sieve chromatography (0.5 mL each) were diluted to 1.0 mL with water. Samples were adjusted to pH 8.0 and a final concentration of 10 mM $MgCl_2$ and subjected to treatment with 0.2 unit of snake venom phosphodiesterase for 2 h at 37 °C.

Nucleotide Analysis. The nucleotides obtained from poly(ADP-ribose) after phosphodiesterase digestion, $(PR)_2AMP$, PRAMP, and AMP, were separated on a Whatman Partisil-10 SAX column (250 mm × 4.6 mm i.d.) preceded by a guard column (50 mm × 1.5 mm i.d.) containing the same material. Samples were injected in a volume of 1.0 mL containing 50 mM KCl at a pH of 4.7. The column was eluted isocratically with 125 mM potassium phosphate, pH 4.7, containing 0.5 M KCl at room temperature at a flow rate of 1.0 mL/min. Radioactivity was monitored by collecting 1.0-mL fractions for scintillation counting, and the relative amount of radiolabel in each nucleotide was used for calculation of the average polymer size and average number of points of branching per molecule.

Calculations. The average size of poly(ADP-ribose) in a given fraction was calculated as the minimum average number of ADP-ribose residues per molecule from (Miwa & Sugimura, 1982)

$$\text{average polymer size} = \frac{[\text{AMP}] + [\text{PRAMP}] + [(\text{PR})_2\text{AMP}]}{[\text{AMP}] - [(\text{PR})_2\text{AMP}]}$$

The average number of branching points per molecule was calculated from (Miwa & Sugimura, 1982)

$$\text{average number of branching points per molecule} = \frac{[(\text{PR})_2\text{AMP}]}{[\text{AMP}] - [(\text{PR})_2\text{AMP}]}$$

RESULTS

In order to develop methods suitable for the characterization of polymer complexity, we utilized polymers of ADP-ribose generated in nucleotide-permeable cells incubated with [^{32}P]NAD^+. Labeled polymers were purified and then subjected to molecular sieve chromatography. Figure 1 shows the profiles obtained when absorbance at 254 nm and radioactivity were monitored. The distribution of radiolabel was identical with the absorbance profile, and it spanned the entire range from the void volume to the included volume. Fractions 13–25 were analyzed by polyacrylamide gel electrophoresis to examine the resolution obtained. Figure 2 shows that each fraction from 16 to 25 contained a narrow population of poly(ADP-ribose) molecules of a different average size as judged by relative migration. It was observed that the dyes BPB and XC comigrated with poly(ADP-ribose) molecules with a size of approximately 8 and 20 residues, respectively. This agreed well with previous studies (Tanaka et al., 1978). We also observed that fractions 13 to 15 contained primarily material that did not migrate into the gels. In fact, this material did not migrate in gels as low as 4% (data not shown). We characterized this material further to determine if it contained other chromatin macromolecules covalently bound to poly(ADP-ribose). It is known that most of the protein–poly(ADP-ribose) conjugates formed in vitro are linked via carboxylate ester linkages to either glutamic acid residues or carboxy-terminal lysine residues (Burzio, 1982). This bond is labile to mild alkaline conditions, and therefore, these linkages would have been released under the alkaline conditions utilized here for polymer preparation. However, the chemical

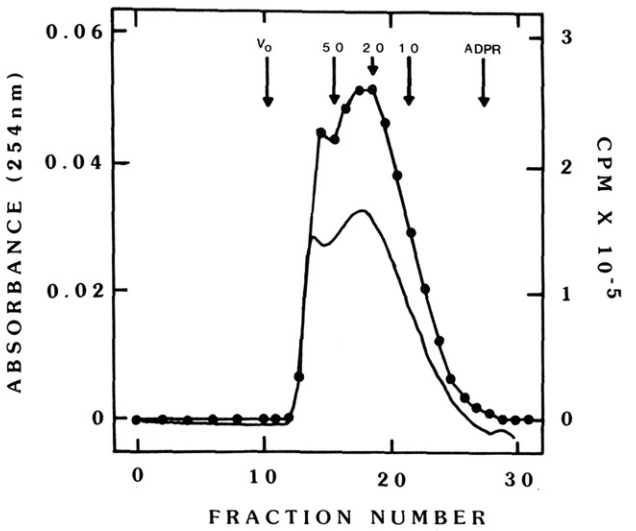

FIGURE 1: Molecular sieve chromatography of [^{32}P]poly(ADP-ribose) synthesized in nucleotide-permeable SVT2 cells. The arrows indicate the void volume, polymer size markers, and included volume, respectively. (●) Radioactivity; (—) absorbance.

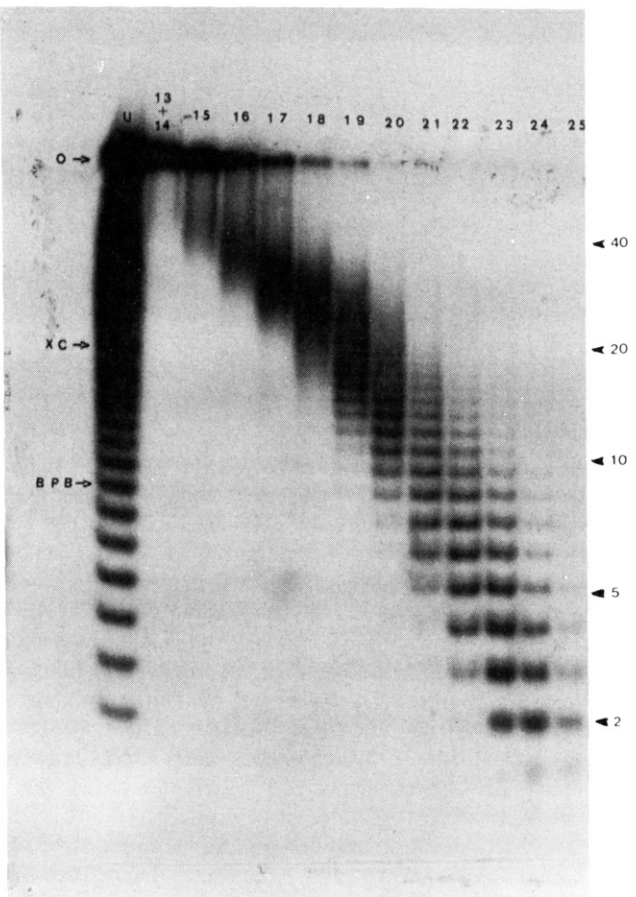

FIGURE 2: Polyacrylamide gel electrophoresis of [^{32}P]poly(ADP-ribose) fractionated by molecular sieve chromatography. (U) Unfractionated polymer. Numbers 13–25 represent fractions collected following chromatography as shown in Figure 1. (O) Origin. Polymer size markers are indicated.

stability of the linkages to protein has been shown to be heterogeneous (Hilz & Stone, 1976), and it was possible that the nonmigrating polymer was still attached to protein via an alkaline-stable linkage. The chromatographic and electrophoretic properties of the nonmigrating material were unaffected following treatment with either proteinase K or Pronase E. However, following treatment with snake venom phosphodiesterase, all of the radiolabel eluted from molecular sieve columns or migrated on gels at the positions of PRAMP and AMP (data not shown). To examine the possibility of covalent attachment to RNA, radiolabeled polymer was subjected to chemical hydrolysis prior to analysis by polyacrylamide gel electrophoresis. It has been shown that the phospho anhydride linkages in poly(ADP-ribose) are stable at alkaline pH in the absence of divalent cations but that the polymer is hydrolyzed under alkaline conditions in the presence of Mg^{2+} (Adamietz & Bredehorst, 1981). Following incubation with either 0.1 or 0.2 N NaOH for 3 h at 60 °C in the presence of 20 mm EDTA, we observed that the electrophoretic profile was unaffected. However, the nonmigrating material was completely digested to material that comigrated with PRAMP and AMP after 0.1 or 0.2 N NaOH treatments at 60 °C for 3 h in the presence of 10 mM $MgCl_2$ (data not shown). Because RNA is unstable under alkaline conditions even in the absence of Mg^{2+}, these data rule out the possibility that the poly(ADP-ribose) was covalently associated with significant amounts of RNA. In total, these experiments indicated that the nonmigrating material was not covalently bound to significant amounts of protein or RNA.

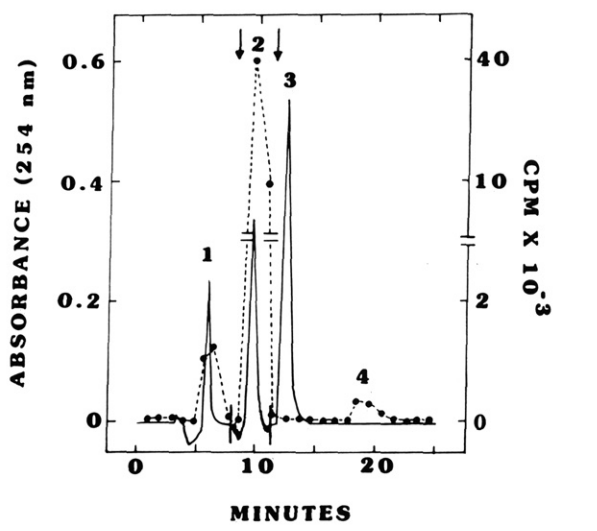

FIGURE 3: Analysis of nucleotides by strong anion exchange chromatography following treatment of [^{32}P]poly(ADP-ribose) with snake venom phosphodiesterase. The arrows indicate a 10-fold increase in absorbance sensitivity. Peaks 1–4 correspond to AMP, PRAMP, ADP, and $(PR)_2AMP$, respectively. (—) Absorbance; (●) radioactivity.

Polymer size was analyzed by subjecting the fractions obtained from molecular sieve chromatography to chemical analysis following digestion to nucleotides. Analysis of polymer size must take into account the fact that the polymer can have a branched structure. Digestion with snake venom phosphodiesterase results in the generation of the unique nucleotides PRAMP and $(PR)_2AMP$ from linear internal and branched residues, respectively, and AMP from terminal residues. Figure 3 shows an isocratic strong anion exchange HPLC system that was developed for the separation of AMP, PRAMP, and $(PR)_2AMP$. Table I shows data obtained from the analysis of fractions 13–23 of Figure 1. Using the formula described under Experimental Procedures, we calculated the average polymer size in each fraction. A wide range of sizes was observed from 6 to 190 residues. Note that fraction 19 contained a population of molecules that migrated near the dye XC on polyacrylamide gels. The calculated average size of this fraction was 20 residues, which agreed perfectly with the analysis on polyacrylamide gels (Figure 2). Further, a plot of the logarithm of molecular mass vs. elution volume was linear as would be predicted (not shown). It is also important

Table I: Analysis of Poly(ADP-ribose) Synthesized in Nucleotide-Permeable Cells[a]

fraction	cpm AMP	cpm PRAMP	cpm $(PR)_2AMP$	$(PR)_2AMP$ mol %	average polymer size	"average chain length"[b]	average number of points of branching per molecule
13	546	14 700	463	2.94	190	28	5.6
14	2 450	67 500	1980	2.75	154	28	4.2
15	7 760	203 000	5460	2.52	102	28	2.6
16	8 580	218 000	4330	1.87	54	26	1.0
17	11 500	257 000	4460	1.63	38	23	0.63
18	14 000	278 000	3470	1.17	28	21	0.32
19	17 400	273 000	2760	0.93	20	17	0.18
20	19 100	231 000	2090	0.82	15	13	0.12
21	21 000	206 000	1790	0.78	12	11	0.09
22	20 400	138 000	1160	0.73	8.5	7.7	0.06
23	18 600	87 000	667	0.62	5.9	5.7	0.04

[a] Results shown are from a single representative experiment. Samples containing radioactivity were counted to a preset error of 2%, and the values shown were corrected for background. [b] These values were calculated by a method that does not account for the presence of branching in poly(ADP-ribose) (Tanaka et al., 1977).

to note that when the average chain length was calculated by a method that does not correct for the fact that poly(ADP-ribose) is a branched molecule (Miwa et al., 1979), the estimated size of large polymers was as much as 6.8-fold smaller (Table I) with the maximum "average chain length" calculated to be approximately 28 residues. This is similar to what has previously been reported to be the maximum average chain length of polymers generated in vitro (Miwa et al., 1982; Tanaka et al., 1977; Adamietz et al., 1978). These data also permitted the calculation of the average number of branching points per molecule with the formula described under Experimental Procedures. The average number of points of branching per molecule increased with increasing polymer size with the majority of polymers below 30 residues being linear. Interestingly, fractions 13–15, which consisted of nonmigrating polymers, contained polymers that had more than one point of branching per molecule with fraction 13 containing polymers with an average of 5.6 points of branching per molecule.

In order to study polymers from intact cells, the approach described above was combined with radiolabeling of cultured cells with high specific activity [^{3}H]adenine. Cells in culture were radiolabeled with [^{3}H]adenine under conditions that allowed optimum labeling of the NAD pool (Jacobson et al., 1986). Since these experiments involved the radiolabeling of intact cells with [^{3}H]adenine, control experiments were done to determine if a significant amount of radiolabeled RNA contaminated the preparations. Such information was important since radiolabeled RNA would yield radiolabeled AMP upon phosphodiesterase treatment, which would cause an underestimation of polymer size. Preparations of labeled polymer were treated with alkali in the presence of EDTA or $MgCl_2$ as described above except that incubation was at 37 °C overnight instead of 60 °C to prevent exchange of radiolabel. Following this treatment, preparations were neutralized, treated with alkaline phosphatase, and subjected to reversed-phase HPLC as described previously (Jacobson et al., 1984b) to examine for the presence of radiolabeled adenosine. Radiolabeled adenosine was only detected in the alkali incubations containing $MgCl_2$, which would be expected for poly(ADP-ribose), while any radiolabel present in RNA would have also been present in the samples incubated with alkali and EDTA.

Treatment of cultured mouse cells with the carcinogenic alkylating agent MNNG results in a rapid alteration of poly(ADP-ribose) metabolism (Juarez-Salinas et al., 1979), which appears to be necessary for cellular recovery from the cytotoxic effects of this agent (Jacobson et al., 1984a). We have analyzed polymers following treatment of C3H10T1/2 cells with 64 μM MNNG. Confluent cultures were utilized under conditions where MNNG treatment is only potentially

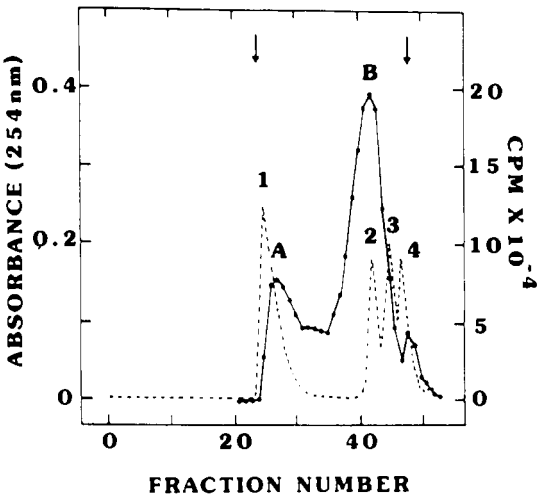

FIGURE 4: Molecular sieve chromatography of [^{3}H]poly(ADP-ribose) synthesized in C3H10T1/2 cells following DNA damage. Two TSK-125 columns in series were used. The arrows indicate the void volume and included volume, respectively. (●) Radioactivity; (--) absorbance. Peaks 1–4 represent poly(A), $(Ap)_9A$, $(Ap)_5A$, and AMP, respectively.

lethal as cell survival varies with the period of time the cells are held prior to plating for colony formation (Jacobson et al., 1985a). Under the conditions used, cells plated at 48 h posttreatment had a survival of approximately 50% relative to untreated controls. [^{3}H]Poly(ADP-ribose) preparations were fractionated according to size by molecular sieve HPLC (Figure 4). Two peaks, which we have labeled A and B, were observed. Also shown are the retention times for AMP, $(Ap)_5A$, $(Ap)_9A$, and poly(A), sizing standards routinely used to monitor resolution of the columns. Each fraction containing radiolabel was subjected to phosphodiesterase treatment and analysis by strong anion exchange HPLC. The data obtained and calculations are shown in Table II. A wide distribution of average polymer sizes was observed from 11 residues to 67 residues, which contained an average number of points of branching per molecule from less than 0.1 to 2.2. It is interesting to note that all the fractions contained in peak A of Figure 4 contained polymers with an average number of points of branching per molecule of one or more. Polymers of such size and complexity were somewhat unexpected since the rate of turnover of polymers under these conditions is 1 min or less (Jacobson et al., 1985c). The exposure of cells to hyperthermic conditions results in an alteration of poly(ADP-ribose) metabolism, which results in a decreased rate of turnover of poly(ADP-ribose) (Juarez-Salinas et al., 1984; Jacobson et al., 1985c). Polymers of ADP-ribose were isolated from cells

Table II: Analysis of Poly(ADP-ribose) Synthesized in Intact Cells following MNNG Treatment[a]

fraction	cpm AMP	cpm PRAMP	cpm $(PR)_2AMP$	$(PR)_2AMP$ mol %	average polymer size	average number of points of branching per molecule
24	345	6570	239	3.34	67	2.2
25	761	16200	500	2.86	61	1.9
26	833	16800	515	2.83	57	1.6
27	539	11100	327	2.73	56	1.5
28	776	14400	420	2.70	44	1.2
29	510	9510	275	2.67	44	1.2
30	392	7110	137	1.79	30	0.53
31	432	7970	141	1.64	29	0.48
32	397	7420	130	1.63	29	0.48
33	401	6290	110	1.61	23	0.38
34	414	6180	103	1.53	21	0.33
35	584	6690	105	1.42	15	0.22
36	830	9560	90	0.95	14	0.12
37	1718	12500	118	0.82	14	0.07
38	1293	11600	105	0.80	11	0.08

[a] Results shown are from a single representative experiment. Samples containing radioactivity were counted to a preset error of 2%, and the values shown were corrected for background.

Table III: Analysis of Poly(ADP-ribose) Synthesized in Intact Cells following Heat Shock and MNNG Treatment[a]

fraction	cpm AMP	cpm PRAMP	cpm $(PR)_2AMP$	$(PR)_2AMP$ mol %	average polymer size	average number of points of branching per molecule
24	524	13900	463	3.10	244	6.6
25	3060	76400	2500	3.04	145	4.4
26	3450	81700	2690	3.06	115	3.5
27	3210	74700	2070	2.59	70	1.8
28	2690	56300	1520	2.51	52	1.3
29	2280	45500	1140	2.32	43	1.0
30	2420	45400	998	2.04	34	0.70
31	2430	42200	844	1.85	29	0.53
32	2650	44100	718	1.51	25	0.37
33	3580	49900	763	1.40	19	0.27
34	4680	57000	759	1.21	16	0.19
35	6050	67800	755	1.01	14	0.14
36	7200	76500	743	0.88	13	0.11
37	10230	84900	796	0.82	10	0.08
38	13380	90100	839	0.80	8.9	0.07

[a] Results shown are from a single representative experiment. Samples containing radioactivity were counted to a preset error of 2%, and the values shown were corrected for background.

subjected to a hyperthermic treatment and then probed with MNNG. Figure 5 shows the profile for molecular sieve chromatography and Table III the analysis of polymer complexity. Again, a wide range of polymer sizes was observed with average polymer sizes ranging from 9 to 244 residues. Polymers containing up to an average of over six points of branching per molecule were detected in these samples.

Discussion

The routine determination of the size of polymers of ADP-ribose in intact cells has not heretofore been possible for several reasons. One of these has been the lack of procedures for radiolabeling the NAD pool to a sufficiently high specific radioactivity. Tanuma and Johnson (1983) in a study designed to identify specific protein acceptors for poly(ADP-ribose) have previously described the labeling of poly(ADP-ribose) in vivo with [^{3}H]adenosine. In this study, we have utilized [^{3}H]-adenine, which labels the NAD pool of cultured mouse cells to a high specific radioactivity. A second problem is the need for isolation of radiolabeled poly(ADP-ribose) free of other interfering material such as RNA that would yield radiolabeled 5′-AMP upon phosphodiesterase treatment and thereby cause an underestimation of polymer size. We can conclude from this study that the boronate resin that we have utilized (Alvarez-Gonzalez et al., 1983) allows the retention of poly(ADP-ribose) without interference from other labeled polymers. In addition to the control experiments described under

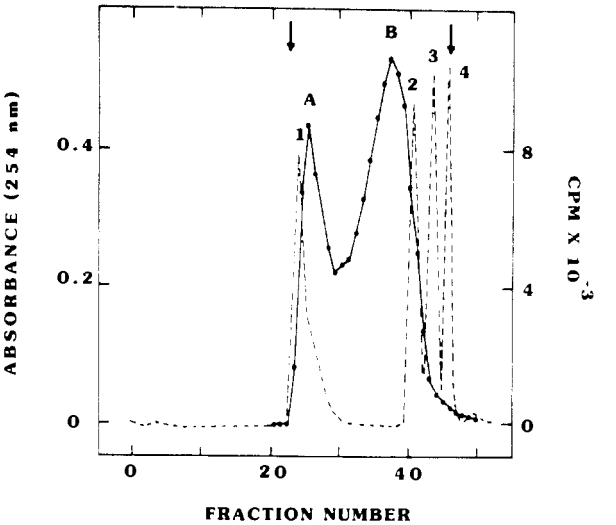

FIGURE 5: Molecular sieve chromatography of [^{3}H]poly(ADP-ribose) synthesized in C3H10T1/2 cells following hyperthermia and DNA damage. The arrows denote the void volume and included volume, respectively. (●) Radioactivity; (--) absorbance. Peaks 1–4 represent poly(A), $(Ap)_9A$, $(Ap)_5A$, and AMP, respectively.

Results, we observed excellent agreement of estimates of polymer size determined by electrophoretic migration and analysis by enzymatic digestion and HPLC (Figure 2, Table I). Thus, we can also rule out the possibility that the prepa-

rations of polymer from intact cells were significantly contaminated with other sources of radiolabeled 5′-AMP.

A third problem is the need for analytical methodology that can accurately determine branching frequency in polymers of ADP-ribose. Previously, estimates of the average chain length of polymers generated in vitro have been made with an analytical approach valid for linear polymers but not for branched polymers (Miwa et al., 1982). The precise quantification of branching frequency is difficult since points of branching occur at a maximum of approximately 1 in 40 residues (Miwa et al., 1982). Therefore, branched residues following phosphodiesterase digestion do not exceed 3 mol % and are generally less. The HPLC methodology described here allows the accurate measurement of branching frequency of poly(ADP-ribose) since $(PR)_2AMP$ is well separated from AMP and PRAMP.

As far as we are aware, this study has provided the first direct evidence for the existence of large, multibranched polymers of ADP-ribose in intact cells. It is important to note, however, that since we have determined only points of branching, we are unable to derive from these data information concerning the size of the branches in the polymer, i.e., a single residue or elongated branches with multiple residues. It is interesting to note that the branching frequency of poly(ADP-ribose) approaches one at an approximate polymer size of 40–50 residues, both in vitro and in vivo. This size corresponds to the TSK-125 fractions where large amounts of nonmigrating polymers are observed on polyacrylamide gels. Therefore, it is tempting to speculate that the branching is responsible for the anomolous electrophoretic behavior of these nonmigrating polymers. This anomalous behavior on polyacrylamide gels suggests that the branching points are indeed likely to be substantially longer than a single residue. Electron microscopic observations of multibranched polymers of ADP-ribose synthesized in vitro are also consistent with elongated branches although it was not clear if the complex structures observed represented single polymer chains (DeMurcia et al., 1983; Hayashi et al., 1983).

We have previously shown that MNNG causes a rapid increase in total levels of polymers in cultured mouse cells (Juarez-Salinas et al., 1979) and that individual polymers are present only transiently with a half-life of 1 min or less (Jacobson et al., 1985). Since polymers are turning over very rapidly, it was not surprising that a wide range of polymer sizes are present in cells. The observation that a significant fraction of large multibranched polymers is present even under rapid turnover conditions suggests that large polymers are involved in poly(ADP-ribose) function. Since polymers of ADP-ribose carry two formal negative charges per residue, the results presented here argue that noncovalent interactions between the polymer and other components of chromatin must receive serious further consideration in future studies of poly(ADP-ribose) and regulation of chromatin functions. For example, it has been reported that two histone H1 molecules are apparent covalently cross-linked by poly(ADP-ribose) molecules of 15 or 16 residues in size, giving rise to a structure that has been referred to as a histone H1 dimer (Wong et al., 1983). Evidence that H1–poly(ADP-ribose) conjugates of higher complexity than the histone H1 dimer has also appeared (Smulson et al., 1983), and it has been suggested that these H1–poly(ADP-ribose) conjugates are involved in chromatin condensation. The nature of the cross-links in these conjugates has not been elucidated, and in view of the data presented here, it is possible that such complexes may contain highly branched polymers of ADP-ribose bound to histone H1 via noncovalent rather than covalent cross-links.

ACKNOWLEDGMENTS

We thank Dr. Elaine L. Jacobson for many helpful discussions and Dr. R. C. Benjamin for his advice concerning polyacrylamide gel electrophoresis and for his comments on the manuscript. We also thank Hilda Mendoza-Alvarez for technical assistance and Lynne Gracy and Kay Leck for assistance in the preparation of the manuscript.

Registry No. Poly(ADP-ribose), 26656-46-2.

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Electrostatic Control of the Rate-Determining Step of the Copper, Zinc Superoxide Dismutase Catalytic Reaction

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Received June 5, 1986; Revised Manuscript Received December 18, 1986

ABSTRACT: The dependence of the activity of bovine Cu,Zn superoxide dismutase on pH and ionic strength was extensively investigated in the ranges of pH 7.4–pH 12.3 and of ionic strength of 0.02–0.25 M. The results obtained indicate that two positively charged groups having pK values of approximately 10.1 and 10.8 are involved in the control of the activity. On the basis of previous work on the three-dimensional structure and on the chemically modified enzyme, these groups are likely to be lysine side chains, in particular Lys-120 and Lys-134. The oxidation state of the enzyme-bound copper ion at the steady state was found to be the same at either pH 7.4 or pH 11.5. The diffusion of superoxide ion into the active site, which is controlled by the positive charges around the active site itself, appears to be the rate-determining step of the dismutation reaction. NMR measurements of the relaxation rates of F^- showed that this control also applies to the access of F^- to the active site. Comparison of the nuclear relaxation rates of F^- with the enzyme activity indicates that F^- relaxation is controlled by the deprotonation of the group with p$K \sim 10.8$, which appears to be responsible for about 50% of the total activity measured at neutral pH.

The dismutation of the superoxide ion by Cu,Zn superoxide dismutase (SOD)[1] occurs according to (Fielden et al., 1974)

$$ECu^{2+} + O_2^- \xrightarrow{k_+} ECu^+ + O_2 \quad (1)$$

$$ECu^+ + O_2^- + 2H^+ \xrightarrow{k_-} ECu^{2+} + H_2O_2 \quad (2)$$

resulting in the overall process

$$2O_2^- + 2H^+ \xrightarrow[SOD]{k} H_2O_2 + O_2$$

The enzymatic reaction is very sensitive to ionic strength (Rigo et al., 1975; Cudd & Fridovich, 1982). This behavior, the decrease of the enzyme activity observed when positively charged lysines are modified to give neutral or negatively charged residues by carbamylation (Cocco et al., 1982) or succinylation (Marmocchi et al., 1982) and the lack or reversal of the ionic strength effect in enzyme samples with lysine charges neutralized or inverted, respectively (Cudd & Fridovich, 1982), indicate that the rate of the enzyme-catalyzed

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[1] Abbreviations: ECu^{2+}, oxidized form of the active site of Cu,Zn superoxide dismutase; ECu^+, reduced form of the active site of Cu,Zn superoxide dismutase; I, ionic strength; SOD, Cu,Zn superoxide dismutase.

0006-2960/87/0426-3224$01.50/0