Studies on Protein Poly(ADP-ribosylation) Using High Resolution Gel Electrophoresis*

Teni Boulikas
From the Linus Pauling Institute of Science and Medicine, Palo Alto, California 94306

(Received for publication, February 2, 1990)

Analysis of poly(ADP-ribose) synthesized in cellular lysates or in isolated nuclei on 100-cm-long thin gels of 20% polyacrylamide, 2.5 M urea permits determination of the exact size of poly(ADP-ribose) molecules using labeled oligonucleotides as molecular weight markers. The size and concentration of poly(ADP-ribose) molecules increase at time intervals during its synthesis. Differences in the concentration of poly(ADP-ribose) size classes among cell lines are also shown. Inhibition of poly(ADP-ribose) degradation by ethacridine that directly interacts with the polymer and inhibits its hydrolysis by poly(ADP-ribose) glycohydrolase shows a dramatic increase in both polymer size and concentration. Use of alkaline conditions for the hydrolysis of poly(ADP-ribose)-protein linkages reveals a specific shortening of all size classes of poly(ADP-ribose) compared with its size in preparations obtained by extensive digestion of nuclei with nucleases, RNases, and proteases.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
They were labeled at their 5' ends using T4 polynucleotide kinase (Boehringer Mannheim). [γ-32P]ATP (Amersham Corp.), and the kinase exchange method (Perbal, 1988). An aliquot of labeled oligodeoxyribonucleotides was mixed with a large volume of sample buffer (10% glycerol, 0.05% bromphenol blue, 0.05% xylene cyanol in TBE buffer) and loaded directly on the gel.

RESULTS

Separation of Poly(ADP-ribose) and Oligothymidylic Acid on Long DNA Sequencing Gels—Nuclei from mouse or human cells in culture were isolated and incubated with [32P]NAD+ in order to label the poly(ADP-ribose) synthesized on numerous nuclear proteins, notably histones and poly(ADP-ribose) polymerase. The polymeric chains were then detached from the proteins by alkaline hydrolysis and analyzed for their size by electrophoresis on 100-cm-long 20% polyacrylamide, 0.67% bisacrylamide gels containing 2.5 M urea. Gels were dried and poly(ADP-ribose) molecules were viewed by autoradiography.

The electrophoretic mobility of poly(ADP-ribose) molecules is compared with the mobility of oligodeoxythymidylic acid in Fig. 1. The ladder of poly(ADP-ribose) is composed of doublets of bands (Fig. 1, slots 1–6) as a result of its alkaline-catalyzed detachment from proteins as will be shown below. The poly(ADP-ribose) bands are the upper species in each doublet (see Fig. 2).

As expected on the basis of its chemical composition, size classes of poly(ADP-ribose) give bands at approximately the same position as multimers of (dT)ₙ (Fig. 1). Di- to docosa-thymidylic acid (dT)ₙ₋₂₂ 5' end-labeled with ³²P was analyzed in the sequencing gel (Fig. 1, c and e). As can be seen in Fig. 1, the mobility of (dT)₁₀ is identical to the mobility of (ADP-ribose).⁰

NAD⁺ displays an unusually low mobility, migrating between dT and dT₀, or like (ADP-R), (Fig. 1a). This is presumably due to the positive charge on its nicotinamide ring. Alkaline hydrolysis of [α-³²P]NAD⁺ gives three ³²P-labeled products (φ, χ, ψ, Fig. 1b). Product φ, also present in the poly(ADP-ribose) preparations, is probably ADP-ribose arising by hydrolysis of the nicotinamide group from NAD⁺ (Fig. 1b) or from hydrolysis of mono(ADP-ribose) from nuclear proteins (Fig. 1, 1–6). Products χ and ψ (Fig. 1b) have not been identified; they are, in addition, present in the poly(ADP-ribose) preparations (Fig. 1, 1–6).

Synthesis of Poly(ADP-ribose) in the Presence of Glycohydrolase Inhibitors—Fig. 1 shows the poly(ADP-ribose) synthesized in cellular lysates from human or mouse cells during incubation at 20 °C for 1 h, followed by an incubation at 37 °C for 15 min in the absence or presence of poly(ADP-ribose) glycohydrolase inhibitors. In the absence of glycohydrolase inhibitors (Fig. 1, slots 1 and 5), the poly(ADP-ribose) is present in very low concentrations and is distributed in low size classes presumably due to its degradation by glycohydrolase. However, in the presence of 10 mM CAMP, considerably longer molecules of poly(ADP-ribose) are present (Fig. 1, slot 3), presumably due to inhibition of their degradation. CAMP is indeed known to inhibit poly(ADP-ribose) glycohydrolase (Miwa et al., 1974) and to cause a considerable increase in the length of poly(ADP-ribose) on histones when their labeling takes place in cellular lysates (Boulikas, 1989).

Ethacridine, a DNA intercalator resembling ethidium bromide, is highly efficient in protecting poly(ADP-ribose) toward enzymatic degradation by glycohydrolase or by snake venom phosphodiesterase through direct interactions with the polymer (Tavassoli et al., 1985). Fig. 1 (slots 2 and 6) shows that there is a tremendous increase in both polymer size and concentration in the presence of 2 mM ethacridine relative to that in its absence (compare slots 1 with 2 and 5 with 6 in Fig. 1). This

FIG. 1. Analysis of poly(ADP-ribose) molecules and oligothymidylic acid by high resolution polyacrylamide gel electrophoresis and effect of cAMP and ethacridine on polymer size and concentration. Human K562 or mouse P815 cells were continuously treated with 0.1 mM dimethyl sulfate for 1 h. Cellular lysates were incubated with [³²P]NAD⁺ in the presence of TLCK, Triton X-100, DTT, and in the absence or presence of cAMP and/or ethacridine at 20 °C for 1 h. Preparations were then directly digested at 37 °C for 15 min with micrococcal nuclease in order to reduce viscosity in subsequent steps. Poly(ADP-ribose) detached from total cellular proteins with 0.1 M NaOH at 60 °C for 30 min was analyzed on 20% acrylamide gels. Slot a contains NAD⁺ and slot b NAD⁺ partially hydrolyzed with NaOH. Slots c and d contain 5' end-labeled di- to docothymidylic acid molecules, (dT)ₙ₋₂₂, marked to the right-hand side of the figure. [γ-³²P]ATP was loaded on slot d. Poly(ADP-ribose) isolated from human (1–4) or mouse (5, 6) cellular lysates incubated with [³²P]NAD⁺ in the presence of ethacridine (2, 6) cAMP (3), ethacridine + cAMP (4), or in the absence of ethacridine and cAMP (1, 5) is shown and marked 1–27 to the far-right-hand side of the figure. BB, bromphenol blue; XC, xylene cyanol.
Astonishing difference indicates a very rapid turnover of poly(ADP-ribose) in cellular lysates from both human and mouse lymphoid cells at 37 °C in agreement with previous studies (Wielckens et al., 1982). Indeed, the data in Fig. 1 and Table I show that about 90% of the poly(ADP-ribose) that is synthesized is degraded over a 1-hour period at 37 °C. The 10% fraction of poly(ADP-ribose) that resists degradation probably represents the metabolically stable constitutive polymer fraction described by Alvarez-Gonzalez and Althaus (1989) with a half-life of 7.7 h.

Alkaline Hydrolysis Changes the Pattern of Poly(ADP-ribose)—Each band of poly(ADP-ribose) is present as a doublet (Fig. 1, slots 1–5). That the upper band in each doublet represents the intact polymer size classes and that the lower band is a product of poly(ADP-ribose) degradation caused by the alkaline hydrolysis is shown in Fig. 2. Poly(ADP-ribose) preparations obtained with the proteinase K method (Fig. 2, slots 2 and 3) show the presence of ladders of single bands up to penta(ADP-ribose). The single bands coincide in electrophoretic mobility with the upper bands in the doublets in poly(ADP-ribose) preparations liberated from proteins with 0.1 M NaOH at 60 °C for 20 min (Fig. 2, slots 4 and 5) or for 2 h (Fig. 2, slots 6 and 7).

Poly(ADP-ribose) detached from proteins with 0.1 M NaOH at 60 °C for 2 h shows virtually the presence of only the lower band in the doublets (Fig. 2, slots 6 and 7). However, when the time of alkaline hydrolysis is reduced to 20 min (Fig. 2, slots 4 and 5), the concentration of the upper band in each doublet is increased. It is therefore evident that alkaline treatment shortens by a specific size all chains of poly(ADP-ribose). Poly(ADP-ribose) prepared by the proteinase K method, however, shows the presence of double, triple, or even quadruple bands in molecules higher in size than hexa(ADP-ribose) (Fig. 2, slots 2 and 3). These bands may be due to single amino acids or short peptides that remain attached to the poly(ADP-ribose) because of incomplete digestion with proteinase K.

Size of Poly(ADP-ribose) in Nuclei Isolated from Various Cell Types—Fig. 3A shows poly(ADP-ribose) molecules synthesized in isolated nuclei from various human and mouse lymphoid cell lines. A similar electrophoretic pattern is seen in spite of differences in poly(ADP-ribose) concentration among cell lines. In this experiment the same number of cells was used from each cell line for nuclei isolation, and the same conditions were followed for poly(ADP-ribose) labeling and sample preparation. The BB88 mouse cell line shows the highest concentration of poly(ADP-ribose) among all cell lines tested (slot 6).

Bands of poly(ADP-ribose) up to 50 residues long can be easily resolved. However, polymer bands seem to continue to the top of the gel in some samples especially in slot 7 (Fig. 3) indicating sizes of 200 residues or more. This finding is in

Table I

<table>
<thead>
<tr>
<th>Treatment</th>
<th>K562</th>
<th>P815</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>10 mM cAMP, 1 h</td>
<td>6.1</td>
<td>5.2</td>
</tr>
<tr>
<td>1 mM ethacridine</td>
<td>9.3</td>
<td>12.4</td>
</tr>
<tr>
<td>cAMP + ethacridine, 1 h</td>
<td>10.2</td>
<td>11.8</td>
</tr>
</tbody>
</table>

Fig. 2. Double bands of polymer appear on gels during alkaline hydrolysis of protein-poly(ADP-ribose) linkages. Slots 1–5, cultured P815 mouse cells were treated with 1 mM dimethyl sulfate at 37 °C for 1 h. Isolated nuclei digested with DNase I to an average of 20-kilobase DNA fragments were incubated at 20 °C for 40 min with 2 mM TLCK, 1% Triton, 10 mM DTT in nuclei buffer containing 1 mM Ca<sup>2+</sup>, 4 mM Mg<sup>2+</sup>. Nuclei were then extensively digested with RNase and micrococcal nuclease at 37 °C for 10 min in the presence of nicotinamide in order to inhibit poly(ADP-ribose) polymerase and AMP in order to inhibit poly(ADP-ribose) glycohydrolase. One aliquot was treated with 0.5 mg/ml proteinase K in 1% SDS at 37 °C for 4 h (2, 3). Another aliquot was treated with 0.1 M NaOH at 60 °C for 20 min (4, 5). Two independent experiments (2, 4 and 3, 5) are shown. Slots 6 and 7, same experiment as in slots 1–5 (f) except that nuclei were incubated with [32P]NAD<sup>+</sup> in the presence of 2 mM ethacridine instead of 10 mM cAMP and that poly(ADP-ribose)-protein linkages were disrupted in 0.1 M NaOH at 60 °C for 2 h. Separation was on 20% polyacrylamide, 2.5 M urea, 100-cm-long gels. Slot 1 contains NAD<sup>+</sup> partially hydrolyzed with NaOH.
Poly(ADP-ribose) was synthesized in nuclei isolated from the following cell lines in the presence of 0.2 mM cold NAD\(^+\), 0.2 mM ethacridine, 10 mM DTT, 1% Triton X-100, 2 mM TLCK in 1 mM Ca\(^{2+}\), 4 mM Mg\(^{2+}\), 10 mM Tris, pH 6.8. Incubation with \[^{32}P\]NAD\(^+\) was at 20 °C for 30 min. Nuclei were then digested with micrococcal nuclease to an average of tetranucleosomes, pelleted, and labeled with \[^{32}P\]NAD\(^+\) in the presence of 2 mM ethacridine. Aliquots from the reaction mixture were removed in SDS and NaOH for an immediate arrest of all enzymatic processes.

Poly(ADP-ribose) synthesized over a period of 0.5 min in isolated nuclei extends up to 30 mers of ADP-R (Fig. 3B, slot 1). A 6.3-fold higher amount of incorporation of \[^{32}P\] into trichloroacetic acid-precipitable material is found at 8 min of incubation compared with 0.5 min, and polymers having a chain length of 50 or more are found (Fig. 3B, slot 2). Polymer concentration continues to build up with time reaching an 8.9-fold increase at 15 min and a 15.7-fold increase at 40 min compared with 0.5 min of incubation. Poly(ADP-ribose) size also increases at 15 (slot 3) and 40 min (slot 4) of nuclei incubation with \[^{32}P\]NAD\(^+\) in the presence of ethacridine.

Fig. 3B shows that treatment with NaOH at 50 °C results in a considerable decrease in the concentration of the lower bands in the doublets of poly(ADP-ribose) compared with Fig. 3A where NaOH treatment was performed at 60 °C. This is in agreement with the data shown in Figs. 1 and 2. Thus, the temperature and time of alkaline hydrolysis determine the relative concentration of the two bands in the poly(ADP-ribose) ladders.

**Discussion**

**Origin of the Doublets in Poly(ADP-ribose) Bands—Protein-poly(ADP-ribose) linkages in nuclei that have been determined seem to be carboxylate ester linkages. Such linkages are labile to mild alkaline conditions causing the release of poly(ADP-ribose) from proteins (Hilz and Stone, 1976; Riquelme et al., 1979).**

Doublets in the poly(ADP-ribose) bands are seen, and the relative concentration of the upper and lower bands in each doublet strongly depends upon the temperature and time of incubation with NaOH (Figs. 1–3). Alkaline treatment of phosphoribose from the head portion, an AMP moiety from the tail portion, both of these, or one adenine residue. Hydrolysis of adenine-ribose linkages is reminiscent of partial depurination of DNA promoted by methylation at the N-3 position of adenines and taking place by heating at neutral pH (Maxam and Gilbert, 1977). Alkaline treatment of poly(ADP-ribose) promotes degradation of the pyrophosphate linkages especially in the presence of Mg\(^{2+}\) (Adamietz and Brederhorst, 1981; Alvarez-Gonzalez and Jacobson, 1987). We have observed the presence of doublets of bands in the ladders of the poly(ADP-ribose) molecules even in the presence of EDTA (Fig. 3).

**Electrophoretic Pattern of Poly(ADP-ribose)—Analysis of free poly(ADP-ribose) by gel electrophoresis has been attempted in several laboratories (e.g. Tanaka et al., 1978; Butt and Smulson, 1980; Hayashi et al., 1983; Alvarez-Gonzalez and Jacobson, 1987; Huletsky et al., 1989). However, the fine analysis of poly(ADP-ribose) on 100-cm-long polyacrylamide gels conveniently employed for DNA sequencing (e.g. Mandel et al., 1982) that has been used in this work has revealed agreement with data from other laboratories showing the synthesis of large molecules of poly(ADP-ribose) (e.g. Alvarez-Gonzalez and Jacobson, 1987).**
several interesting characteristics of its electrophoretic mobility.

First, it has allowed us to analyze the small molecular weight products such as NAD$^+$ and its hydrolysis products, ATP, and (dT), as well as mono- and oligo(ADP-ribose) molecules (Fig. 1), that cannot be resolved by convenient polyacrylamide gel electrophoresis techniques.

Second, published studies use bromphenol blue and xylene cyanol in order to size poly(ADP-ribose) (e.g. Tanaka et al., 1978; Hayashi et al., 1983; Alvarez-Gonzalez and Jacobson, 1987; Huletsky et al., 1988). We find that the migration of these dyes with respect to poly(ADP-ribose) is altered according to the percentage of polyacrylamide in the gel. On 20% polyacrylamide gels bromphenol blue migrates between (ADP-R)$_5$ and (ADP-R)$_8$ whereas xylene cyanol migrates close to (ADP-R)$_17$. However, on 15% polyacrylamide gels of the same length and buffer components bromphenol blue migrates between (ADP-R)$_{12}$ and (ADP-R)$_{17}$ whereas xylene cyanol migrates close to (ADP-R)$_{21}$.

Third, our gel system has revealed doublets of poly(ADP-ribose) bands. The set of the lower bands in the doublets represents degradation products of poly(ADP-ribose) due to alkaline treatment used for its detachment from proteins (Fig. 2), and their concentration increases with increasing time or temperature of incubation in alkali. Alkaline treatments were used for poly(ADP-ribose) isolation in several other studies (e.g. Alvarez-Gonzalez and Jacobson, 1987; Huletsky et al., 1989).

Acknowledgments—I thank Felix Clairvoyant and Mike Sweeney for expert technical assistance and Jody Cox and Diane Read for processing the manuscript.

REFERENCES