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THE α-GLYCOSIDIC BONDS OF POLY(ADP-RIBOSE) ARE ACID-LABILE

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SUMMARY: The poly(ADP-ribosyl)ation system of higher eukaryotes produces multiple ADP-ribose polymers of distinct sizes which exhibit different binding affinities for histones. Although precipitation with trichloroacetic acid (TCA) is the standard procedure for isolation of poly(ADP-ribose) from biological material, we show here that poly(ADP-ribose) is not stable under acidic conditions. Storage of poly(ADP-ribose) as TCA pellets results in acid hydrolysis of polymers, the extent of which is dependent on storage time and temperature. The α-glycosidic, inter-residue bonds are the preferred sites of attack, thus reducing polymer sizes by integral numbers of ADP-ribose to yield artefactually more and smaller polymers than originally present. Therefore, poly(ADP-ribosyl)ation studies involving TCA precipitation, histone extraction with acids, or acidic incubations of ADP-ribose polymers must account for the impact of acids on resulting polymer populations.

Synthesis of ADP-ribose polymers in nuclei of higher eukaryotes is stimulated by DNA damage (1-3). These polymers vary considerably in size and can contain branches (4). We have recently shown that the numbers and sizes of polymers are strictly regulated by nuclear factors (5), and that they are major determinants for strong, non-covalent interaction with histones (6). Therefore, the precise analysis of polymer numbers and sizes in living cells are of great biological relevance in characterizing the functional consequences of poly(ADP-ribosyl)ation on chromatin.

The bonds involved in poly(ADP-ribose) attachment to proteins are alkali-labile but stable under acidic conditions (7). Poly(ADP-ribose) itself is stable under basic conditions (8). Although the maintenance of polymer integrity in the presence of acids has not been documented, methods recommended for isolating polymers of ADP-ribose from *in vitro* and *in vivo* systems include extraction, precipitation, and storage of polymers in the presence of acids (9, 10).

This report is the first to characterize the acid-lability of poly(ADP-ribose). We show that storage of acid precipitates and incubation in the presence of acid produced smaller, less-branched polymers than those originally isolated. Since hydrolysis occurred at the inter-

Abbreviations: TCA, trichloroacetic acid; EDTA, ethylenediamine tetraacetic acid; HPLC, high pressure liquid chromatography.

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monomer, α-glycosidic linkages, degraded polymers were indistinguishable from naturally-formed polymers, albeit smaller. A simple procedure to avoid polymer degradation by acids is presented which will enable us to study the significance of polymer sizes in DNA repair.

EXPERIMENTAL METHODS

Preparation of Protein-free Poly(ADP-ribose): Protein-attached polymers were synthesized in a 100 µl reaction mix containing 200 ng of calf thymus poly(ADP-ribose) polymerase (11), 2.78 µg of nicked calf thymus DNA (12), 100 µM [^{32}P]NAD$^+$ (45 Ci/mmole; New England Nuclear), 25 mM Tris (pH 8.0), 10 mM $MgCl_2$, 0.5 mM dithiothreitol, and 100 mM NaCl. After incubating at 25°C for 30 min, protein-attached polymers were precipitated with TCA (20% final concentration) for 20 min at 0°C. The samples were centrifuged at 10000 rpm, 4°C, for 30 min, and the precipitates were rinsed with ice-cold 5% TCA. Each pellet of protein-attached polymers was immediately resuspended in 150 µl of 10 mM Tris, 1 mM EDTA (pH 12) and incubated at 60°C for 3 h to detach poly(ADP-ribose) from protein. After extraction of protein with phenol:$CHCl_3$:isoamyl alcohol (49:49:2), the polymer-containing aqueous phase was dried in a Speed-Vac concentrator. The [^{32}P]polymers of ADP-ribose were dissolved in water and stored at 4°C. Polymers were stable for at least 1 month under these storage conditions.

Precipitation of Poly(ADP-ribose): An equal volume of 40% TCA was added to 10 µl of solution containing protein-free [^{32}P]poly(ADP-ribose) and 15 µg tRNA as carrier. Samples were precipitated on ice, centrifuged, and rinsed as described above. Recovery of radioactivity was always ≥95% using this precipitation procedure.

Size Analysis of Poly(ADP-ribose): After treatment as indicated, samples were dissolved in 10 µl of loading dye and separated on high resolution polyacrylamide gels (13). Average polymer size, polymer number, and branches per polymer were determined using HPLC analysis (4).

RESULTS AND DISCUSSION

In order to characterize the influence of acid on the size distribution of ADP-ribose polymers, protein-free polymers were precipitated with TCA and stored under various conditions. Figure 1 shows that TCA degraded poly(ADP-ribose) in a time-dependent manner. While precipitation of polymers by TCA had no immediate effect on polymer sizes, there was a significant shift in the poly(ADP-ribose) size distribution toward the bottom of the gel, indicating shorter polymers, after only five days of storage. After two weeks storage at 4°C, the polymers, which had all originally migrated as molecules greater than 40 residues in length, were all shorter than 30 residues. Protein-associated polymers were just as susceptible to TCA degradation as protein-free poly(ADP-ribose). When TCA-precipitated polymers were rinsed with ether to remove residual TCA, significant polymer degradation was still observed. Also, degradation occurred to the same extent whether the TCA pellets were stored wet or were dried before storage. Apparently, even after drying, there was sufficient moisture present to facilitate acid hydrolysis.

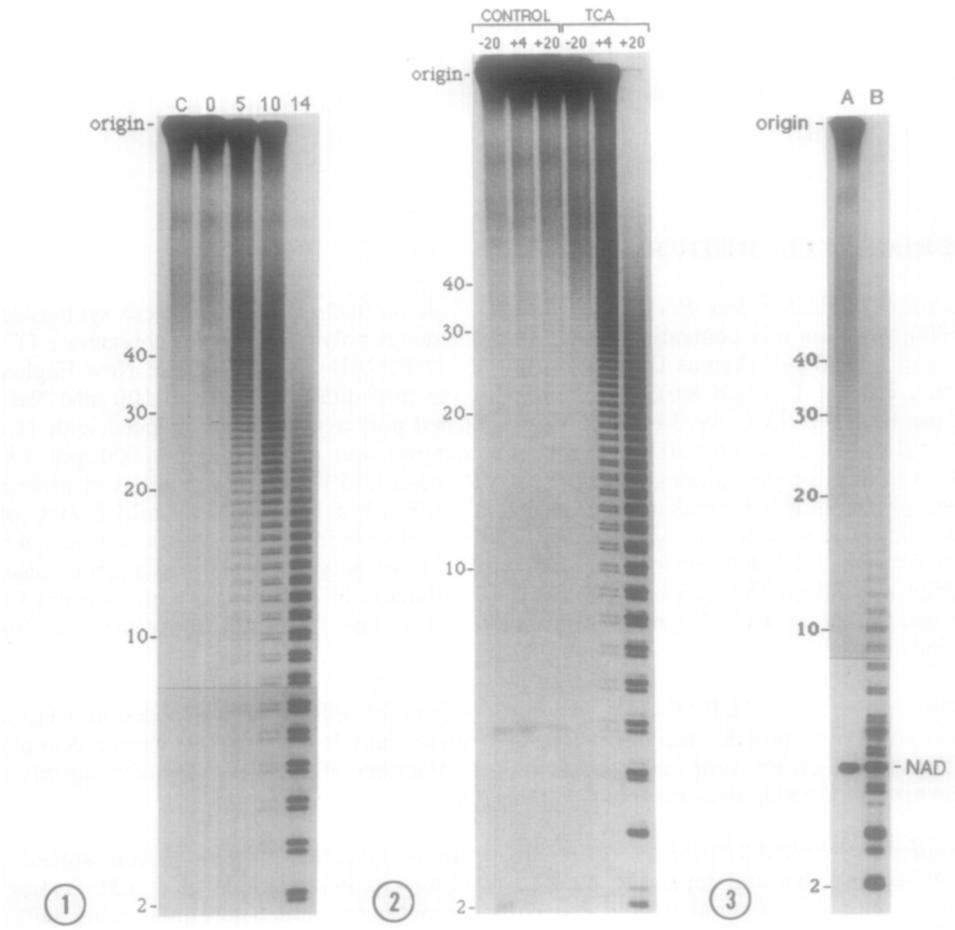

Figure 1. TCA Degradation of Poly(ADP-ribose) is Time-dependent. In each sample, 20000 cpm (16 pmoles ADP-ribose) of [^{32}P]poly(ADP-ribose) were precipitated by TCA (see EXPERIMENTAL METHODS) and stored at 4°C for the indicated number of days. The control sample, C, was not precipitated but was dried and analyzed immediately. Polymer lengths in terms of ADP-ribose residues are indicated on the left.

Figure 2. TCA Degradation of Poly(ADP-ribose) is Temperature-dependent. [^{32}P]ADP-ribose polymers (20000 cpm; 8 pmoles ADP-ribose) were dried, CONTROL, or precipitated with TCA (see EXPERIMENTAL METHODS). The polymers were stored for 7 days at the indicated temperatures. Polymer lengths in terms of ADP-ribose residues are indicated on the left.

Figure 3. Acid Hydrolyzes the α-Glycosidic Bonds of Poly(ADP-ribose). NAD^+ and [^{32}P]poly(ADP-ribose) (20000 cpm; 84 pmoles ADP-ribose) were incubated for 2 h at 40°C in water, **A**, or in 1 N HCl, **B**, and the products were analyzed on high resolution polyacrylamide gels (13). Scanning densitometry confirmed that >95% of the NAD^+ withstood the acid treatment. Polymer lengths in terms of ADP-ribose residues are indicated on the left.

As expected, TCA degradation of poly(ADP-ribose) was also dependent on storage temperature (Figure 2). Only slight degradation was observed after storage of TCA-precipitated polymers for one week at -20°C, whereas the same polymers stored at +20°C were dramatically altered. HPLC analysis determined that TCA-precipitated polymers initially averaging 45 ± 2.9 residues in size were reduced to an average 7.78 ± 0.3 residues

after only two days at room temperature. The average number of branches per polymer decreased from 1.03 ± 0.18 to 0.19 ± 0.02. In addition, the absolute number of polymers rose from 1.65 ± 0.17 pmoles to 11.6 ± 2.24 pmoles indicating internal cleavage of the polymers by TCA. The non-precipitated control samples were stable at all storage temperatures tested, as were polymers precipitated by ethanol (not shown).

O-glycosidic linkages of carbohydrates are known to be stable to base treatment but hydrolyzable by boiling with acids (14). Since each residue of an ADP-ribose polymer is joined by an α-(1"-2')-glycosidic bond to the next residue, it seemed likely that this bond was the site of attack. This hypothesis was tested by treating poly(ADP-ribose) and NAD^+ with acid (Figure 3). NAD^+ is the substrate from which ADP-ribose is enzymatically derived and possesses all bonds found in an ADP-ribose polymer except the inter-monomer, α-glycosidic linkages. While polymers were rapidly degraded by the acid treatment, >95% of the NAD^+ remained intact, indicating that the α-glycosidic linkages were indeed the sites for acid hydrolysis of poly(ADP-ribose).

Acid degradation of ADP-ribose polymers can be easily avoided by neutralizing samples before storage or by precipitating with ethanol instead of TCA. However, we find ethanol precipitation unsatisfactory for isolating newly-synthesized polymers since a significant amount of substrate (NAD^+) is co-precipitated. We now routinely precipitate protein-attached polymers with TCA and immediately thereafter adjust the pH to ≥ 7.0 with basic buffer.

In addition, we have used acid degradation to produce isolated polymer populations which are predictably enriched in shorter, less-branched polymers. Until now, researchers have been limited to working with polymer populations over which they had little control, i.e. polymer size distributions varied considerably between enzyme preparations, incubation conditions, and storage time. By avoiding artefactual polymer degradation by acid, and by using acid to manipulate polymer size distributions, we can now study the significance of polymer sizes in poly(ADP-ribose) turnover and DNA repair.

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