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Original Research Article

Chain Length Analysis of ADP-Ribose Polymers Generated by Poly(ADP-Ribose) Polymerase (PARP) as a Function of β-NAD$^+$ and Enzyme Concentrations

Hilda Mendoza-Alvarez, Susana Chavez-Bueno, and Rafael Alvarez-Gonzalez
Department of Molecular Biology and Immunology and Institute for Cancer Research, University of North Texas Health Science Center at Fort Worth, Fort Worth, Texas

Summary

Bireactant autopoly(ADP-ribosyl)ation of poly(ADP-ribose) polymerase (PARP) (EC 2.4.2.30) was carried out by using either increasing concentrations of β-NAD$^+$ (donor substrate) at a fixed protein concentration or increasing concentrations of PARP (acceptor substrate) at a fixed β-NAD$^+$ concentration. The [^{32}P]ADP-ribose polymers synthesized were chemically detached from PARP by alkaline hydrolysis of the monoester bond between the carboxylate moiety of Glu and the polymer. Nucleic acid–like polymers were then analyzed by high-resolution polyacrylamide gel electrophoresis and autoradiography. The ADP-ribose chain lengths observed displayed substrate concentration–dependent elongation from 0.2 θM to 2 mM β-NAD$^+$. Similar results were observed at fixed concentrations of 4.5, 9, 18, 27, and 36 nM PARP. Therefore, we conclude that the concentration of the ADP-ribose donor substrate determines the average chain length of the polymer synthesized. In contrast, the polymer size was unaltered when the concentration of PARP was varied from 4.5 to 18 nM at a fixed β-NAD$^+$ concentration. However, when PARP concentrations >18 nM were used, the total amount of monomeric ADP-ribose produced was noticeably less. Therefore, we conclude that high concentrations of PARP lead to acceptor substrate inhibition at the level of the ADP-ribose chain initiation reaction.

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Keywords Mechanism; poly(ADP-ribose) polymerase, polymer length; regulation; substrate availability.

INTRODUCTION

The poly(ADP-ribosyl)ation of DNA-binding proteins, including poly(ADP-ribose) polymerase (PARP), is a posttranslational covalent modification catalyzed by PARP itself (EC 2.4.2.30) (*1*). Other polypeptides with ADP-ribose polymerizing activity have recently been reported, for example, PARP-2 (*2*, *3*), tankyrase (*4*), and VAULT-PARP (*5*), but little is known about their biochemical properties.

In the process of poly(ADP-ribose) synthesis, PARP catalyzes three chemically distinct reactions (*6*, *7*). The first step, or "initiation" reaction, involves the specific attachment of one ADP-ribose unit to a free carboxylate moiety on an acceptor protein (*8*). For example, the intermolecular modification of PARP, the main protein target for poly(ADP-ribosyl)ation in DNA-damaged cells, occurs stoichiometrically and quantitatively at four separate Glu residues (*9*), the result of formation of a catalytic homodimer of PARP (*10*) on nicked or broken DNA (*11*). The second and most efficient reaction catalyzed by PARP is the protein-distal ADP-ribose polymerization (*12*, *13*) reaction or "chain elongation". In this reaction, the 2′-hydroxyl group of the adenine proximal ribose of the acceptor unit, for example, mono(ADP-ribosyl)ated-PARP (*12*, *13*), is used as the target for ADP-ribose chain elongation, and a 2′-1″ O-glycosidic linkage is formed (*12*). The elongation step of ADP-ribose polymer synthesis is highly processive (*14*) and occurs with an enzymatic efficiency (k_{cat}/k_{NAD+}) > 2×10^4 (*9*). Therefore, PARP may catalyze >200 rounds of elongation for every initiation step (*15*). Interestingly, Glu residue 988 of the carboxy–terminal catalytic domain of PARP has recently been proposed as the key residue in ADP-ribose chain elongation (*16*, *17*). Finally, the last step of poly(ADP-ribose) synthesis corresponds to the polymeric "branching" reaction (*17*–*19*). This step involves the enzymatic formation of a (2″–1‴) ribose–ribose glycosidic bond (*18*, *19*) and is believed to occur with a frequency of one branching point per 40 rounds of elongation.

Clearly, one may anticipate that the hyperpoly(ADP-ribosyl)-ation of DNA binding proteins with these highly branched and complex nucleic acid–like molecules results in a dramatic

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Address correspondence to Dr. Rafael Alvarez-Gonzalez, Associate Director for Basic Research of the Institute for Cancer Research, University of North Texas Health Science Center at Fort Worth, 3500 Camp Bowie Boulevard, Fort Worth, TX 76107-2699. Fax: 817-735-2133. E-mail: ralvarez@hsc.unt.edu

electrostatic change of the acceptor protein surface. Not surprisingly, the covalent attachment of ADP-ribose (two negative charges per nucleotide) polymers to an acceptor molecule leads to the shuttling off, or release, of this protein from the helical phosphodiester backbone of DNA (*20*) (one negative charge per nucleotide). Further, as a result of this ionic repulsion between ADP-ribose polymers and DNA, chromatin structure and function may become uncoupled (*21*). This uncoupling of chromatin structure and function may directly alter the physiological course of DNA replication (*22*), DNA recombination (*23*), gene expression (*24–26*), DNA base excision repair (*27*, *28*), and cell survival or apoptosis (*29–31*).

In spite of the multiple roles that ADP-ribose polymers play in chromatin function, a termination of the ADP-ribose chain length(s) required to modulate or regulate a specific DNA function has not been documented. A major obstacle to identifying the metabolic signals that dictate the chain length and complexity of ADP-ribose polymers is our limited understanding of the role that the substrate concentrations (of β-NAD$^+$, DNA, and perhaps PARP itself) play in this process. Here, we report that the concentration of β-NAD$^+$ available to PARP determines the average chain length of the polymer synthesized and that an oversupply of PARP leads to substrate inhibition at the level of chain initiation.

EXPERIMENTAL PROCEDURES

Chemicals and Materials. Dithiothreitol, lithium dodecyl sulfate (LDS), and calf thymus DNA were obtained from Sigma; [α-^{32}P]β-NAD$^+$ was purchased from ICN. All other chemicals used were of the highest purity commercially available.

Enzyme Purification. PARP was purified to homogeneity from calf thymus by a previously published procedure (*32*).

Autopoly(ADP-Ribosyl)ation of PARP. Increasing concentrations of PARP from 4.5 to 36 nM were incubated with various concentrations of [^{32}P]β-NAD$^+$ in a 100-μl assay mixture containing 100 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 20 μg/ml calf thymus DNA. After incubation for 3 min at 37 °C, the reaction was stopped with ice-cold trichloroacetic acid (20%, w/v).

Size Distribution of Enzyme-Bound ADP-Ribose Chains. Acid-precipitable material was processed for qualitative analysis of the size distribution of the ADP-ribose polymers by a procedure published elsewhere (*15*).

RESULTS

The size distribution of ADP-ribose polymers synthesized in the autopoly(ADP-ribosyl)ation reaction catalyzed by PARP as a function of the concentration of either β-NAD$^+$ (ADP-ribose donor) or PARP (ADP-ribose acceptor) was analyzed by high-resolution polyacrylamide gel electrophoresis (*15*).

The electrophoretic distribution of the ADP-ribose polymers synthesized with 4.5 nM PARP (a suboptimal amount of enzyme) at increasing concentrations of β-NAD$^+$ from 200 nM to 2 mM is shown in Fig. 1A. The short ADP-ribose oligomers of no more than 8 residues when synthesized with 200 nM β-NAD$^+$ (Fig. 1A, lane 1) increased to 18 ADP-ribose units

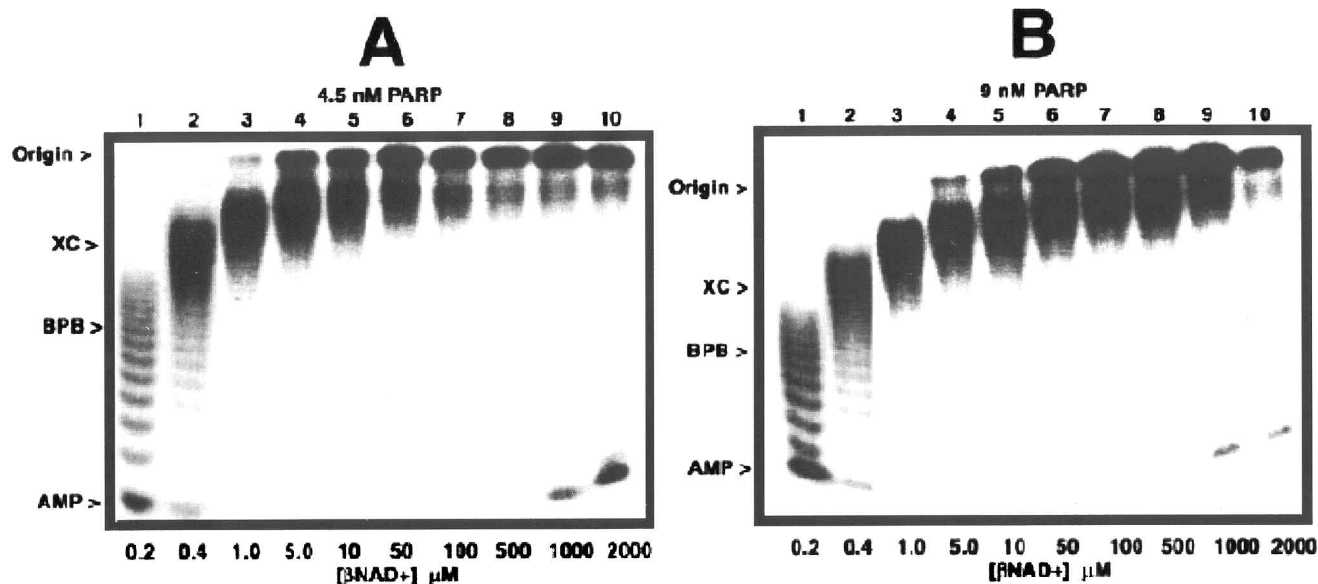

Figure 1. Effect of the β-NAD$^+$ concentration on the ADP-ribose polymer size distribution of the products generated by PARP at a fixed, suboptimal enzyme concentration of either 4.5 nM (A) or 9 nM (B), as shown by autoradiographic analysis of the protein-free polymers synthesized in the automodification reaction of PARP after high-resolution polyacrylamide gel electrophoresis. The relative electrophoretic migration of xylene cyanol (20-mer), bromophenol blue (8-mer), and AMP are indicated to the left of the autoradiograph. The concentration of β-NAD$^+$ is indicated at the bottom of each lane.

when β-NAD^+ was increased to 400 nM (Fig. 1A, lane 2). No sign of branched polymers at the origin of the gel (*15*) was detected in this population of polymers. In contrast, the presence of a radioactive signal at the origin of the gel (Fig. 1A, lane 3) indicated that branched polymers were synthesized with 1 μM β-NAD^+. As expected, greater micromolar concentrations of the ADP-ribose donor (1.0 μM to 2 mM) increased the length and complexity of ADP-ribose polymers generated, as shown by the band at the origin of the gel (Fig. 1A, lanes 3–10). Interestingly, the overall amount of [^{32}P]AMP observed in Fig. 1A, lanes 3–8, a hallmark of ADP-ribose chain initiation, gradually disappeared when β-NAD^+ concentrations were between 1 and 500 μM. That is, under those conditions, the elongation and branching reactions were favored. Surprisingly, however, the [^{32}P]AMP band reappeared at millimolar concentrations of β-NAD^+ (Fig. 1A, lanes 9 and 10). Thus, the efficiency of elongation and branching decreased as a result of substrate inhibition by millimolar concentrations of β-NAD^+.

We also determined the size distribution of ADP-ribose chains synthesized with 9 nM PARP (Fig. 1B), that is, at half of the optimal enzyme concentration (see below). Results of these experiments were similar to those using 4.5 nM PARP. Under closer analysis, however, an obvious difference between the patterns of ADP-ribose polymers synthesized at 4.5 (Fig. 1A) and 9 nM PARP (Fig. 1B) became evident when comparing lanes 9 and 10 of both panels. In Fig. 1B the intensity of the [^{32}P]AMP bands synthesized with 1 and 2 mM β-NAD^+ slightly decreased when the concentration of enzyme was increased to 9 nM PARP.

Fig. 2 (lanes 1–10) shows the results for polymer synthesis with increasing concentrations of β-NAD^+ at a fixed PARP concentration of 18 nM, the optimal enzyme concentration for PARP autopoly(ADP-ribosyl)-ation in vitro (*10*). These results confirm our preliminary observation (*10*) that the final chain length of the polymers synthesized is a βNAD^+ concentration–dependent phenomenon (lanes 1–10). Note that the intensity of the [^{32}P]AMP band observed with 1 and 2 mM β-NAD^+

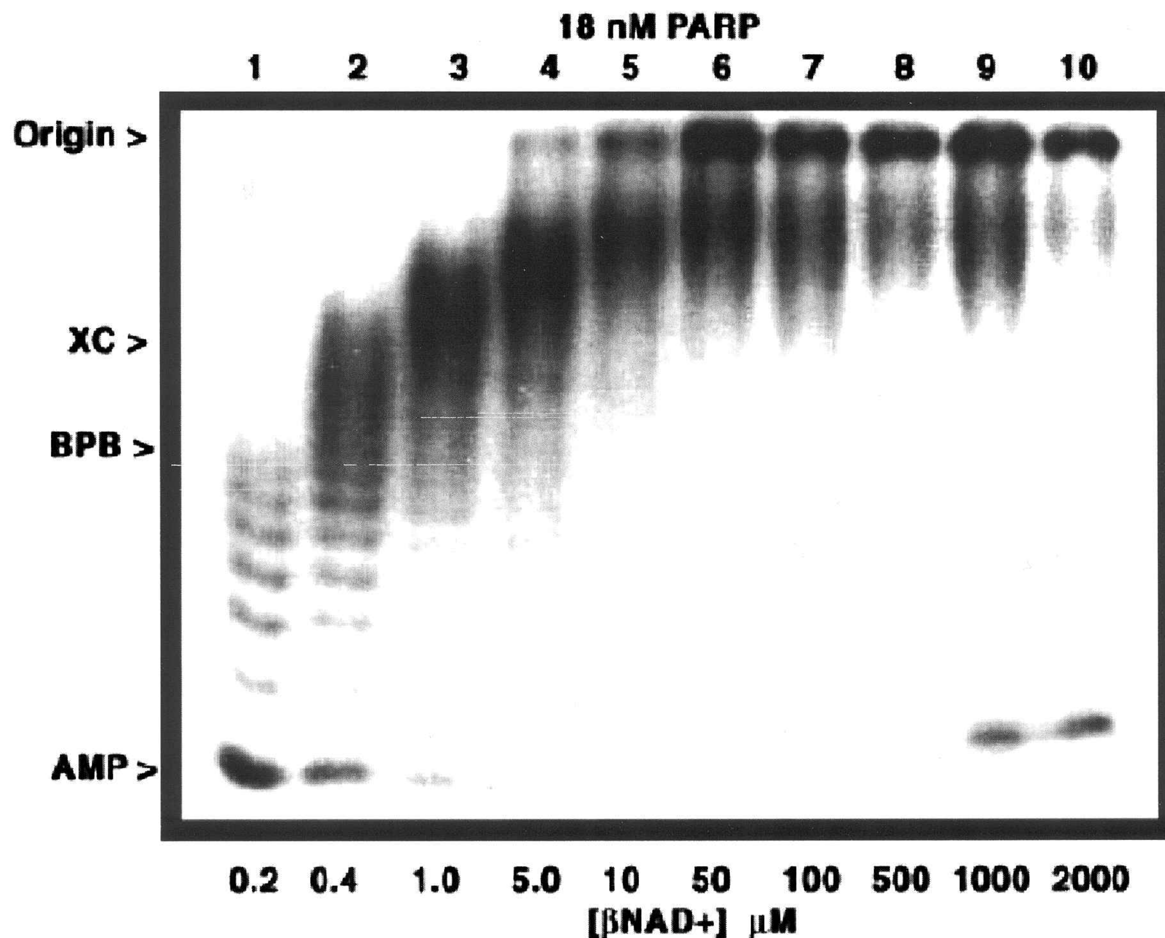

Figure 2. Effect of the β-NAD^+ concentration on the ADP-ribose polymer size distribution of the products generated by PARP at a fixed optimal enzyme concentration of 18 nM. The relative electrophoretic migration of xylene cyanol (20-mer), bromophenol blue (8-mer), and AMP are indicated to the left of the autoradiograph. The concentration of β-NAD^+ is indicated at the bottom of each lane.

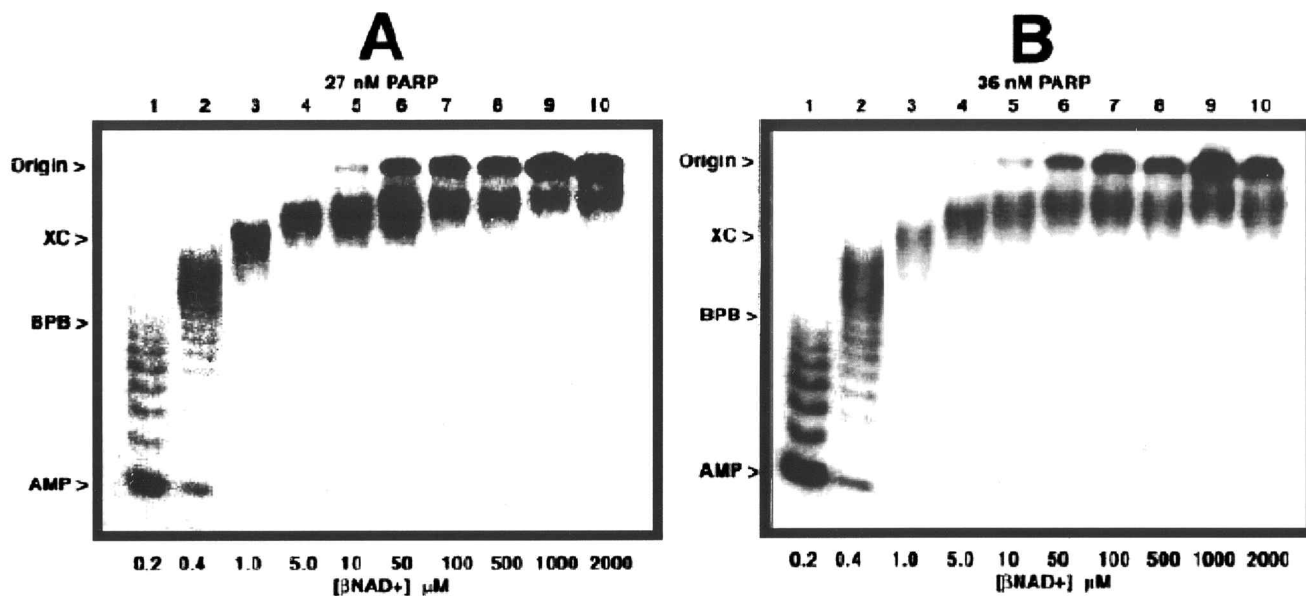

Figure 3. Effect of the β-NAD$^+$ concentration on the ADP-ribose polymer size distribution of the products generated by PARP at a fixed above-optimal enzyme concentration of either 27 nM (A) or 36 nM (B), as shown by autoradiographic analysis of the protein-free polymers synthesized in the automodification reaction of PARP after high-resolution polyacrylamide gel electrophoresis. The relative electrophoretic migration of xylene cyanol (20-mer), bromophenol blue (8-mer), and AMP are indicated to the left of the autoradiograph. The concentration of β-NAD$^+$ is indicated at the bottom of each lane.

(Fig. 2, lanes 9 and 10) is unchanged from that in Figs. 1A and 1B.

Finally, to fully evaluate the effects of the enzyme concentration on the structural complexity of the products formed by PARP, we also carried out enzymatic autopoly(ADP-ribosyl)-ation at 27 and 36 nM PARP. Indeed, we had previously reported a decline in overall enzymatic activity under these high nanomolar concentrations of PARP (*10*) apparently because of acceptor substrate inhibition. The results observed under higher nanomolar concentrations of PARP are shown in Figs. 3A and 3B. Again, even at high nanomolar PARP concentrations, our results indicate that the concentration of the ADP-ribose donor, β-NAD$^+$, determines the final size and complexity of the polymers synthesized (compare the overall polymer size distribution with that in Figs. 1 and 2). In addition, Figs. 3A and 3B show that, although the size distribution of poly(ADP-ribose) did not change substantially, the [^{32}P]AMP band observed at 1 and 2 mM β-NAD$^+$ decreased until undetectable. Thus, our results also indicate that the inhibition of the autopoly(ADP-ribosyl)ation reaction at high nanomolar concentrations of PARP (acceptor substrate) and millimolar concentrations of β-NAD$^+$ (donor substrate) is the result of inhibition by acceptor substrate of the initiation step of polymer synthesis.

DISCUSSION

To elucidate the biological role of ADP-ribose polymers in eukaryotic cells, we must uncover the regulatory mechanisms that modulate the activity(ies) of PARP—particularly, those that shift this versatile DNA-dependent enzyme from mono(ADP-ribosyl)ating activity (*9*) to ADP-ribose elongation and branching functions (*12*, *13*, *17*). A clear understanding of the signals that regulate PARP enzymatic behavior is crucial, given that the biological function of ADP-ribose polymers in transcription (*23–26*) and DNA base excision repair (*27*, *28*) may be chain-length dependent.

Here, we conclusively demonstrate that the size and complexity of poly(ADP-ribose) is specifically determined by the β-NAD$^+$ concentration and not by the concentration of PARP (Figs. 1–3). Indeed, the size distribution pattern of ADP-ribose polymers as a function of β-NAD$^+$ concentration from 200 nM to 2 mM, at five distinct fixed nM concentrations of PARP, remains unchanged. Therefore, the length and complexity of ADP-ribose polymers synthesized by PARP are determined by the ADP-ribose donor (β-NAD$^+$) concentration. Our results are also consistent with the notion that PARP is a highly processive enzyme (*14*) at high micromolar concentrations of β-NAD$^+$. Furthermore, our data suggest that when the ADP-ribose donor substrate concentration is limiting, PARP does not display much processivity. This is particularly relevant to the putative roles of PARP in DNA base excision repair and apoptosis after DNA damage, conditions in which the intracellular pools of β-NAD$^+$ are usually depleted. Finally, our data (Figs. 1 and 2, lanes 9 and 10) are also consistent with the conclusion that high nanomolar concentrations of PARP result in acceptor substrate inhibition at the level of ADP-ribose chain initiation.

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