DNA Strand Break-Mediated Partitioning of Poly(ADP–Ribose) Polymerase Function†

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ABSTRACT: The nuclear enzyme poly(ADP–ribose) polymerase participates in DNA excision repair. Following binding to DNA strand breaks through its amino-terminal Zn^{2+}-finger domain, the enzyme is activated to form polymerase-associated ADP–ribose polymers of various sizes. Focusing on this "automodification" reaction, we observed that optimal enzyme activity and maximal polymer formation were attained only at a strict stoichiometry of two polymerase molecules per DNA fragment. Using various linearized DNAs and nicked circular DNA, we show that this stoichiometric dependence is dictated by the number of enzyme activating sites, i.e., DNA strand breaks. Deviations from the optimal ratio inevitably resulted in decreased polymer formation, ruling out a strict automodification mechanism of poly(ADP–ribosyl)ation. Our results suggest that the mechanism of poly(ADP–ribose) formation on polymerase molecules entails DNA strand break-mediated partitioning of the polymerase into two functional populations: one bound to the DNA breaks and catalytically active, the other, catalytically inactive, functioning as polymer acceptors.

Poly(ADP–ribose) polymerase (EC 2.4.2.30) is an abundant DNA binding protein found in the nuclei of higher eukaryotes [for a review see Ueda (1987); Althaus & Richter, 1987; de Murcia *et al.*, 1991]. This enzyme and subsequent poly(ADP–ribosyl)ation plays a key role in DNA excision repair (Durkacz *et al.*, 1980; Satoh & Lindahl, 1992) and may also influence other DNA processing events entailing the formation of DNA strand breaks. With a molecular mass of 116 kDa, poly(ADP–ribose) polymerase contains three known functional domains. The NH_2-terminal DNA binding domain contains two Zn^{2+} fingers enabling the enzyme to selectively bind to DNA strand breaks (Gradwohl *et al.*, 1990; Ikejima *et al.*, 1990). The COOH-terminal domain contains the active site in which NAD^+ is bound and cleaved into nicotinamide and ADP–ribose. This region also catalyzes the initiation and polymerization of ADP–ribose molecules on acceptor proteins (Simonin *et al.*, 1993). The internal region of the molecule contains those sites that become modified with polymers of ADP–ribose and therefore is referred to as the automodification domain.

Poly(ADP–ribose) polymerase is dependent on DNA strand breaks for activation (Benjamin & Gill, 1980), and activation in turn leads to modification of polymerase molecules with long polymers of ADP–ribose. Through noncovalent interactions with histones (Wesierska-Gadek & Sauermann, 1988; Panzeter *et al.*, 1992a,b), these polymers disrupt DNA/histone complexes, making the DNA accessible to DNA processing enzymes (Realini & Althaus, 1992). Subsequent polymer degradation releases the histones for reassembly on the DNA. Since poly(ADP–ribose) polymerase is responsible for targeting polymer formation to DNA breaks and is itself the main target for poly(ADP–ribosyl)ation (Ogata *et al.*, 1980), it constitutes the key component of the histone shuttle mechanism in chromatin (Althaus, 1992). However, little is known concerning the macromolecular prerequisites for poly(ADP–ribose) synthesis.

In this report, we specifically address the question of whether the poly(ADP–ribose) polymerase molecules that become activated by DNA breaks are themselves modified, i.e., automodification, or whether activated poly(ADP–ribose) polymerases modify other non-self-polymerase molecules, i.e., homomodification, as well as catalyzing limited modification of other nuclear proteins [reviewed in Althaus and Richter (1987)], i.e., heteromodification. After having analyzed the dependence of poly(ADP–ribose) polymerase activity on defined DNA segments and strand breaks, and after carefully dissecting the characteristics and quantities of the polymeric products formed, we find that only a homomodification mechanism can account for the results obtained.

MATERIALS AND METHODS

Materials. Poly(ADP–ribose) polymerase was purified to homogeneity as previously described (Naegeli *et al.*, 1989) and stored at −80 °C. It is important to note that the use of 3-aminobenzamide–Affigel as the last purification step ensures the isolation of only active poly(ADP–ribose) polymerase. pUC19 DNA modified with CC-1065 was generously donated by Dr. Hanspeter Naegeli and Barbara Zweifel (this institute). Restriction endonucleases *Mbo*I and *Eco*RV, SV40 form I DNA, and pBR322 form I DNA were purchased from Gibco/BRL. [*adenine*-2,8-$^3H_2$]NAD^+ (31.3 Ci/mmol) and [*adenylate*-^{32}P]NAD^+ (800 Ci/mmol) were purchased from New England Nuclear. All other chemicals were of the highest quality commercially available.

Preparation of SV40 DNA Fragments. Eight micrograms of SV40 DNA were digested for 1.5 h by restriction endonuclease *Mbo*I. The resulting DNA fragments, having 4-base, 5′-overhanging ends, were separated in a 1% low melting point agarose gel. After visualization with ethidium bromide, the DNA bands were excised and the DNA extracted (Maniatis *et al.*, 1982). While the 59-bp, 236-bp, 609-bp,

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and 944-bp DNA fragments were cleanly separated, the 383-bp and 395-bp fragments, as well as the 1263-bp and 1346-bp fragments, respectively, could not be separated and were extracted as equimolar mixtures. In the text and figures, they are referred to by the appropriate average lengths, i.e., 389-bp and 1304-bp DNA fragments, respectively.

Poly(ADP–Ribose) Polymerase Activity Assays. Poly(ADP–ribose) polymerase activity was determined at 25 °C in the presence of DNA, NAD^+, 25 mM Tris (pH 8.0), 10 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.1 M NaCl. These conditions were used for all reactions involving poly(ADP–ribose) polymerase. DNA, NAD^+, and poly(ADP–ribose) polymerase concentrations as well as incubation times are specified in the figure legends. Unless otherwise noted, all assay components were added to a final reaction volume of 20 μL. Incorporation of radioactive ADP–ribose into acid-precipitable material was determined after the addition of trichloroacetic acid to a final concentration of 20% (w/v) and precipitation on ice for 20 min.

When [^{3}H]NAD^+ was used as the substrate, it was first dried to remove ethanol. The trichloroacetic acid-precipitated samples were applied to glass fiber filters; the filters were washed with 5% trichloroacetic acid and ethanol/ether (3:1) and dried. The dried filters were submerged in 7 mL of Beckman ReadySafe liquid scintillation cocktail and counted in a Beckman LS6000 counter.

When [^{32}P]NAD^+ was used as the substrate, trichloroacetic acid-precipitated samples were centrifuged at 14750*g*, 4 °C, for 30 min. The pellets were rinsed repeatedly with cold 5% trichloroacetic acid until no radioactivity could be detected in the supernatants. Each pellet was counted by the Cerenkov method.

High-Resolution Size Analysis of Poly(ADP–Ribose). Detachment of intact ^{32}P-labeled ADP–ribose polymers from protein, separation on 20% polyacrylamide gels, and autoradiography of the dried gels were performed as previously described (Panzeter & Althaus, 1990).

HPLC Analysis of Poly(ADP–Ribose). Detachment of ^{32}P-labeled poly(ADP–ribose) from protein, snake venom phosphodiesterase digestion of the polymers, and separation of digestion products on HPLC were carried out as previously described (Panzeter & Althaus, 1990; Alvarez-Gonzalez & Jacobson, 1987). Average polymer sizes were calculated using the published equation. The absolute number of polymers in a given sample was determined by subtracting the number of branch residues (diphosphoribosyl-AMPs) from the total number of terminal residues (AMPs).

RESULTS

To distinguish between poly(ADP–ribose) polymerase automodification and homomodification, we first analyzed the stoichiometric dependence of polymerase activity on DNA amount, length, and strand breaks. This was accomplished by altering the quantity of enzyme in the activity assay while the quantity of DNA remained constant. Second, the numbers and average sizes of ADP–ribose polymers formed were determined for a fixed amount of poly(ADP–ribose) polymerase with respect to the amount and length of cofactor DNA. These two approaches provided the information necessary to rule out an automodification mechanism.

Poly(ADP–Ribose) Polymerase Activity at Various Polymerase to DNA Ratios. DNA fragments of defined lengths were prepared and incubated with increasing amounts of poly(ADP–ribose) polymerase to give various polymerase to DNA molar ratios (Figure 1). The NAD^+ concentration was 2.8 μM to maximize polymer initiation while limiting polymer elongation (Ueda *et al.*, 1980). Assays with a 1304-bp DNA fragment revealed a peak in the polymerase activity at a polymerase to DNA molar ratio of 2:1. Identical results were obtained using 389-bp and 944-bp DNA fragments. Hence, optimal poly(ADP–ribose) polymerase activity is not dependent on DNA length *per se* nor the mass of DNA present, but responds to some feature on the DNA that stoichiometrically maximizes enzyme activity at a ratio of two polymerases for every DNA molecule. Since poly(ADP–ribose) polymerase is activated by DNA single- and double-strand breaks, the stoichiometric determinant of polymerase activity was thought to be DNA ends mimicking double-strand breaks.

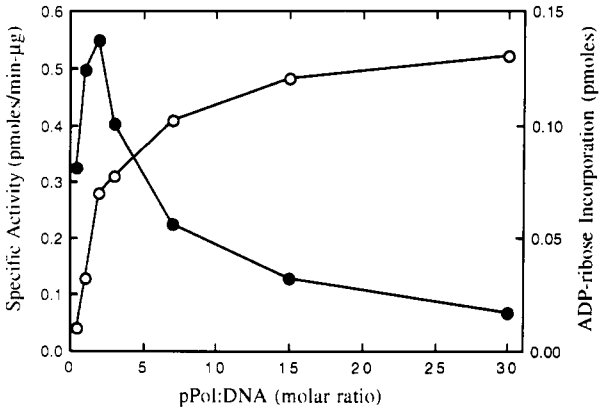

FIGURE 1: Optimal poly(ADP–ribose) polymerase activity at polymerase to DNA molar ratio of 2:1. Poly(ADP–ribose) polymerase activity assays were carried out in the presence of 50 ng of 1304-bp SV40 DNA fragments. The reactions contained 2.8 μM [^{3}H]NAD^+, and poly(ADP–ribose) polymerase (pPol) was added to the molar ratios indicated in the figure. After 10-min incubations, the reactions were stopped with trichloroacetic acid and processed as described under Materials and Methods. The specific activities of poly(ADP–ribose) polymerase (●) were calculated upon the determination of total ADP–ribose incorporation into acid-precipitable material (○). It should be noted that the use of poly(ADP–ribose) polymerase that was not completely active resulted in peak activities at ratios greater than 2:1. However, when the enzyme was fresh, activity inevitably peaked at a ratio of 2:1. Data from one experiment are shown (N = 4).

This hypothesis was verified by comparing enzyme activity in the presence of supercoiled or linearized pBR322 DNA (Figure 2). Some polymerase-mediated incorporation of ADP–ribose was observed using supercoiled DNA (Gradwohl *et al.*, 1987), but the enzyme specific activity remained constant, independent of the polymerase to DNA ratio tested. When the DNA was linearized, however, a sharp peak in poly(ADP–ribose) polymerase activity was detected at a polymerase to DNA molar ratio of 2:1, as seen in Figure 1, using a 3-fold shorter DNA fragment. Clearly, polymerase activity strictly depends on the ratio of polymerase molecules to the number of DNA ends such that any excess enzyme is not activated, thus reducing the apparent specific activity.

We tested whether the above stoichiometric dependence of polymerase activity on DNA ends was also characteristic for DNA single-strand breaks, the other known poly(ADP–ribose) polymerase activating site (Benjamin & Gill, 1980). First, plasmid DNA was prepared with a known amount of single-strand breaks per DNA molecule. CC-1065-modified DNA was chosen as an ideal substrate representative of damaged DNA *in vivo* (Swenson *et al.*, 1982). The nicked DNA was then incubated with increasing amounts of poly(ADP–ribose) polymerase at various polymerase to strand break molar ratios, and the enzyme activities were determined (Figure 3). Interestingly, peak enzyme activity occurred at a ratio of two

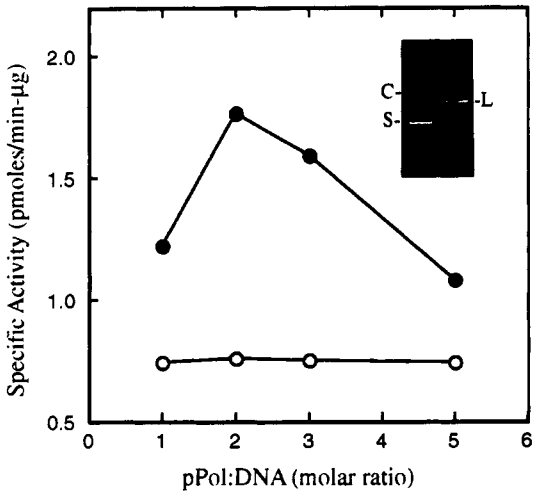

FIGURE 2: Dependence of poly(ADP–ribose) polymerase activity on the number of DNA ends. Poly(ADP–ribose) polymerase activity assays were performed in the presence of 100 ng of supercoiled pBR322 DNA (○) or *Eco*RV-linearized pBR322 DNA (●). Complete linearization of pBR322 was verified by 1% agarose gel electrophoresis (inset: S, supercoiled; C, relaxed circular; L, linear). The reactions contained 2.8 μM [^{3}H]NAD$^+$, and poly(ADP–ribose) polymerase (pPol) was added to the molar ratios indicated in the figure. After 10-min incubations, the reactions were stopped with trichloroacetic acid and processed as described under Materials and Methods. The specific activities of poly(ADP–ribose) polymerase were calculated upon the determination of total ADP–ribose incorporation into acid-precipitable material. Data from one experiment are shown ($N = 2$).

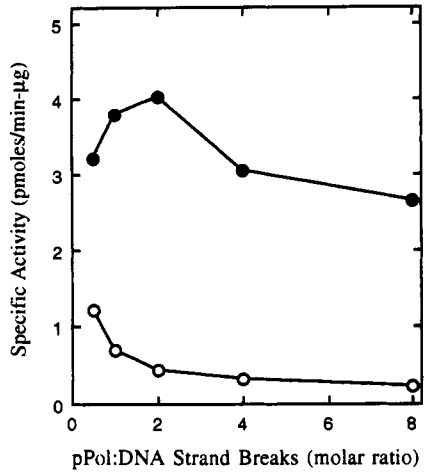

FIGURE 3: Optimal poly(ADP–ribose) polymerase activity at polymerase to DNA strand break ratio of 2:1. CC-1065 (8.6 μM) was coupled to pUC19 DNA (400 μg) at room temperature for 2 h to give an average of 100 adducts per plasmid (Swenson *et al.*, 1982). The subsequently purified DNA was heated at 70 °C for 50 min to induce the formation of single-strand breaks at modified sites. Poisson analysis of the DNA separated on a 1% agarose gel revealed an average of 0.2 strand breaks per plasmid. Poly(ADP–ribose) polymerase activity assays were carried out in the presence of 200 ng of the nicked DNA (●), 2.8 μM [^{3}H]NAD$^+$, and poly(ADP–ribose) polymerase (pPol) added to the molar ratios indicated in the figure. Control assays (○) contained unheated CC-1065-modified DNA in place of the nicked DNA. After 10-min incubations, the reactions were stopped with trichloroacetic acid and processed as described under Materials and Methods. The specific activities of poly(ADP–ribose) polymerase were calculated upon the determination of total ADP–ribose incorporated into acid-precipitable material.

polymerases per single-strand break, supporting a homo-modification mechanism of poly(ADP–ribosyl)ation. Agarose gel analysis showed only 2.8% of the control plasmid to be nicked (not shown), which accounts for the fact that peak polymerase activity using control DNA was not detected at the ratios tested (Figure 3).

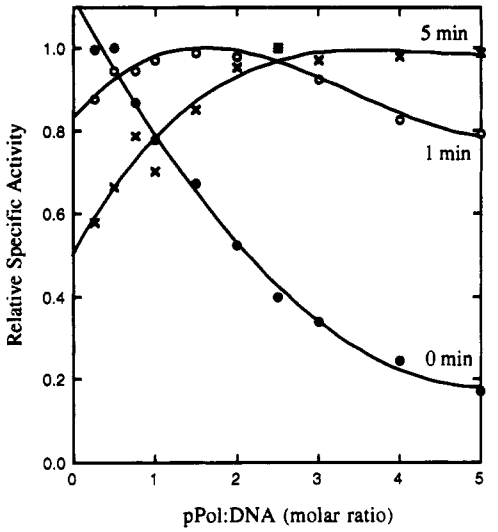

FIGURE 4: Dependence of poly(ADP–ribose) polymerase activity on the number of activating sites. Supercoiled pBR322 DNA was nicked with 4.25 units of DNase I/mg DNA at 25 °C for 0 (●), 1 (○), or 5 min (×). Nick formation was monitored by denaturing gel analysis (not shown). The reactions were stopped upon the addition of EDTA, DNase I was removed by extraction with phenol/$CHCl_3$/isoamyl alcohol, and the DNA was precipitated with ethanol. Poly(ADP–ribose) polymerase activity assays were carried out in the presence of 100 ng of each nicked DNA preparation, respectively. The reactions contained 2.8 μM [^{3}H]NAD$^+$, and poly(ADP–ribose) polymerase (pPol) was added to the molar ratios indicated in the figure. After 10-min incubations, the reactions were stopped with trichloroacetic acid and processed as described under Materials and Methods. The specific activities of poly(ADP–ribose) polymerase were calculated upon the determination of total ADP–ribose incorporation into acid-precipitable material. The specific activities determined using a given nicked DNA preparation were internally normalized by assigning a value of 1 to the peak specific activity value, thus allowing simple comparison between poly(ADP–ribose) polymerase activity curves from all three DNA preparations. Data from one experiment are shown.

As an independent approach to show that poly(ADP–ribose) polymerase activity is dependent on the number of activating sites, increasingly nicked pBR322 DNA was prepared using a low concentration of deoxyribonuclease to avoid the formation of double-strand breaks (confirmed by native *vs* denaturing gel analysis, not shown). Each of the nicked DNA preparations was incubated with increasing amounts of poly(ADP–ribose) polymerase to give various molar ratios, and the respective enzyme activities were determined (Figure 4). Sham processing of the undigested plasmid introduced some strand breaks. However, there were still relatively few nicks upon assay, and as in Figure 3, peak polymerase activity occurred below the range of ratios tested (<0.25 polymerases per DNA molecule). After 1 min of digestion with deoxyribonuclease, peak activity shifted to a molar ratio of approximately 1.5 polymerases per DNA molecule, and after 5 min of digestion it shifted to greater than 2.5 polymerases per DNA molecule. These data indicate that the specific activity of poly(ADP–ribose) polymerase is directly and stoichiometrically dependent on the number of single-strand breaks on the DNA.

From the results in Figures 1–4, we conclude that the specific activity of poly(ADP–ribose) polymerase strictly depends on the number of enzyme activating sites on the DNA and that once the maximum possible number of enzyme molecules has bound to these sites, no additional polymerases become activated. These results imply that once polymers are formed, other enzymes are not permitted to bind and become modified. Thus, poly(ADP–ribose) polymerase does not seem to cycle

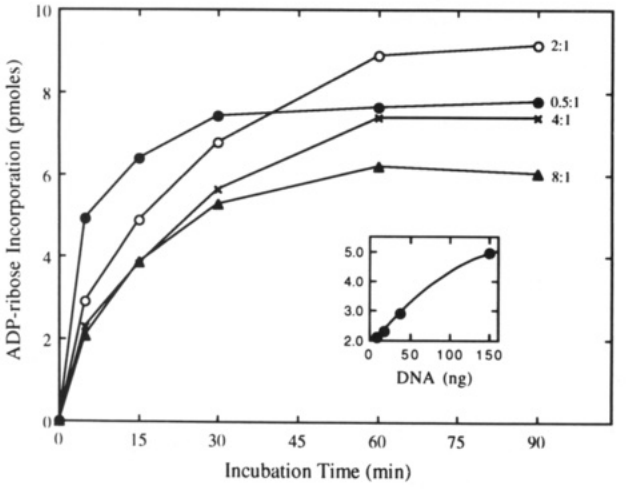

FIGURE 5: Maximal poly(ADP–ribose) formation at polymerase to DNA molar ratio of 2:1. The time-dependent incorporation of ADP–ribose into acid-precipitable material was monitored for poly(ADP–ribose) polymerase to DNA molar ratios of 0.5:1 (●), 2:1 (○), 4:1 (×), and 8:1 (▲). Each reaction contained 10 ng of poly(ADP–ribose) polymerase, 100 μM [^{3}H]NAD$^+$, and appropriate amounts of 1304-bp SV40 DNA fragments. The inset shows ADP–ribose incorporation after 5 min of incubation with respect to DNA content. Data from one experiment are shown (N = 3).

on and off the DNA. To explicitly address this question of polymer-mediated polymerase cycling, we turned from enzyme activity studies to product analysis.

Poly(ADP–Ribose) Formation at Various Polymerase to DNA Ratios. We gained insight into poly(ADP–ribose) polymerase cycling on a fixed number of activating sites by studying the quantities and characteristics of the enzyme product formed, namely, poly(ADP–ribose), at an NAD$^+$ concentration sufficient for polymer elongation.

In the experiment shown in Figure 5, the quantity of poly(ADP–ribose) polymerase in each assay was kept constant, and varying amounts of 1304-bp DNA were added to give various polymerase to DNA molar ratios. ADP–ribose incorporation was measured at each molar ratio as a function of incubation time. If polymerase cycling were occurring, ADP–ribose incorporation plateaus of similar magnitude eventually should be attained, whereupon the majority of polymerases would be modified. However, after 60 min of incubation, polymer formation plateaued to reveal that ADP–ribose incorporation was maximal at a molar ratio of two polymerases per DNA molecule. Not only did the addition of DNA quantities in excess of that at the 2:1 molar ratio fail to result in further polymer production, it significantly limited polymer formation. Characteristically, poly(ADP–ribose) formation after 5 min of incubation (Figure 5, inset) directly correlated to the mass of DNA in the assay, as noted in earlier studies (Benjamin & Gill, 1980; Cohen *et al.*, 1982). Identical results were found using a DNA fragment of 389 bp, again emphasizing the dependence of poly(ADP–ribose) polymerase on DNA breaks, not length, for activity.

Since polymer formation on poly(ADP–ribose) polymerase is processive, *i.e.*, one polymerase molecule is completely modified with a conserved complement of polymers before modification of the next (Naegeli *et al.*, 1989), the results in Figure 5 may be due to (a) ratio-dependent variations in the number of polymerase molecules modified and, correspondingly, to the absolute number of polymers formed or (b) ratio-dependent variations in average polymer size. Polymers formed after 60 min at various polymerase to DNA ratios were visualized on high-resolution polyacrylamide gels and

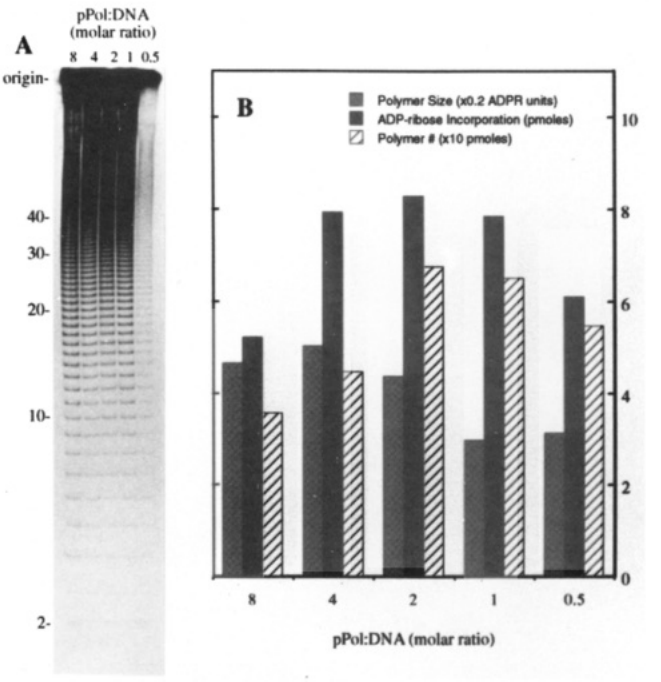

FIGURE 6: Maximal number of ADP–ribose polymers formed at polymerase to DNA molar ratio of 2:1. Ten nanograms of poly(ADP–ribose) polymerase (pPol) was incubated for 60 min with 1304-bp SV40 DNA fragments at the molar ratios given in the figure. Each reaction was carried out in the presence of 100 μM [^{32}P]NAD$^+$. The reactions were stopped with trichloroacetic acid, precipitated, and washed as described under Materials and Methods. After detachment from protein, the ADP–ribose polymers were visualized on a high-resolution gel (A) and analyzed by HPLC (see Methods) to determine the average polymer size and the total number of polymers formed (B). 2500 cpm was loaded in the last gel lane while 7500 cpm was loaded in all other gel lanes. Data from one experiment are shown (N = 2). The average error between experiments was 17.4%; in each experiment the trends were exactly as shown in the figure. ADPR = ADP–ribose.

analyzed using HPLC (see Materials and Methods) to determine the causative effect (Figure 6). Scanning densitometry of the gel autoradiogram (Figure 6A) revealed no significant differences in polymer size distributions with respect to the polymerase to DNA molar ratio. HPLC results (Figure 6B) indicated that the maximum in ADP–ribose incorporation observed at a molar ratio of 2:1 was due to a concomitant maximum in the total number of polymers formed. While there were observable changes in the average polymer size, these did not parallel changes in ADP–ribose incorporation. Conversely, when poly(ADP–ribose) polymerase was incubated with DNA of various lengths, but always at a molar ratio of 2:1, the total number of polymers formed remained constant (Figure 7B). In this case, variations in ADP–ribose incorporation could be attributed to differences in polymer size (Figure 7A,B).

We therefore conclude that not only is maximum activation of poly(ADP–ribose) polymerase attained at a polymerase to DNA molar ratio of 2:1 but also the maximum possible number of polymerases is modified at this ratio. In addition, when there is less DNA present, fewer polymerases are modified, indicating that poly(ADP–ribose) polymerase does not cycle on and off of activating sites.

DISCUSSION

Poly(ADP–ribose) polymerase requires DNA breaks for activation, after which it catalyzes the formation of ADP–ribose polymers covalently linked to itself. In order to

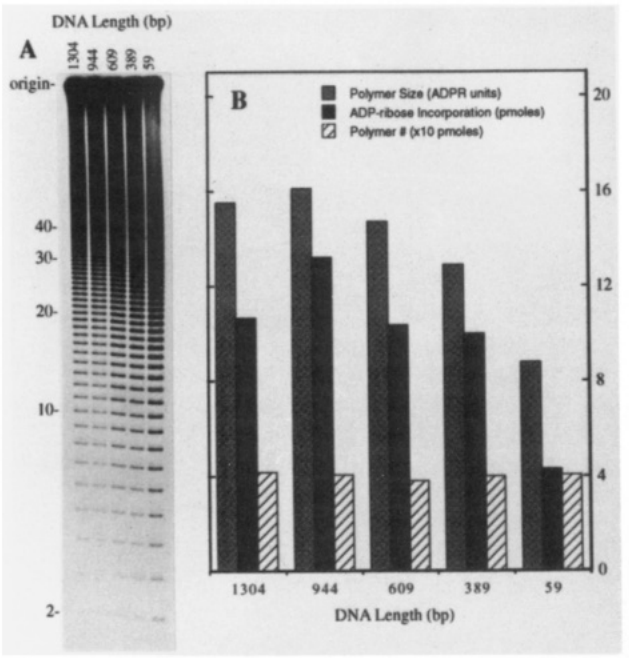

FIGURE 7: Number of ADP–ribose polymers formed independent of DNA length. Ten nanograms of poly(ADP–ribose) polymerase was incubated for 30 min with various SV40 DNA fragments, the lengths of which are indicated in the figure. In each reaction, the poly(ADP–ribose) polymerase to DNA molar ratio was 2:1, and [^{32}P]NAD$^+$ concentration was 100 μM. The reactions were stopped with trichloroacetic acid, precipitated, and washed as described under Materials and Methods. After detachment from protein, the ADP–ribose polymers were visualized on a high-resolution gel (A) and analyzed by HPLC (see Materials and Methods) to determine the average polymer size and the total number of polymers formed (B). 6500 cpm was loaded per gel lane. Data from one experiment are shown (N = 3). The average error between experiments was 13.0%; in each experiment the trends were exactly as shown in the figure. ADPR = ADP–ribose.

determine whether poly(ADP–ribose) formation occurs via an automodification mechanism (modification of the activated polymerase) or a homomodification mechanism (modification of another polymerase by the activated polymerase), we have performed a rigorous analysis of poly(ADP–ribose) polymerase activity and subsequent polymer formation with respect to the cofactor DNA.

Certain lengths of DNA (Berger & Petzold, 1985; Hengartner *et al.*, 1991) and certain DNA sequences (Hakam *et al.*, 1987; Sastry *et al.*, 1989) have been posited as optimal for poly(ADP–ribose) polymerase activity, but with no regard for polymerase to DNA molar quantities. Our results using linear DNA show that optimal activity of poly(ADP–ribose) polymerase always occurs at a polymerase to DNA molar ratio of 2:1, regardless of DNA length and sequence (Figures 1 and 2). As expected, activity is stoichiometrically dependent on the number of polymerase activating sites on the DNA, *i.e.*, DNA breaks (Figures 2–4).

The fact that optimal activity invariably occurs at a polymerase to DNA ratio of 2:1 implies that the activating sites (DNA breaks) become saturated by the enzyme, such that no additional enzyme molecules can be activated. Analysis of polymer formation at different polymerase to DNA molar ratios confirms this hypothesis. Not only is the specific activity of poly(ADP–ribose) polymerase optimal when two polymerases are present for every DNA molecule but also the number of polymers formed is maximal at this ratio (Figures 5 and 6). If less DNA is present, fewer polymers are formed (Figure 6). However, reducing the DNA length without varying the polymerase to DNA molar ratio has no effect on the number of polymers formed (Figure 7). Both of these observations indicate that poly(ADP–ribose) polymerase molecules saturate the activating sites on the DNA and do not dissociate to allow the activation of unactivated polymerases, *i.e.*, there is no poly(ADP–ribose) polymerase cycling on and off activating sites in this system.

In accordance with the above results, three mechanistic scenarios for poly(ADP–ribose) polymerase binding to DNA and subsequent modification may be considered. (1) A single poly(ADP–ribose) polymerase molecule may bind to an activating site and, upon activation, form polymers on itself, *i.e.*, automodification. The now-modified polymerase remains bound to the DNA break, hindering the modification of subsequent polymerases. In this case, one polymerase binds per DNA end for an optimal ratio of two polymerases per DNA molecule. However, this scenario does not explain the results in Figure 3, where peak enzyme activity is observed when two polymerases are present for every single-strand break. (2) A poly(ADP–ribose) polymerase dimer may bind to an activating site, and activation of one polymerase molecule causes it to modify the other, *i.e.*, homomodification. Neither the activated polymerase bound to the DNA break nor the polymer-accepting polymerase dissociates to allow the modification of subsequent polymerases. In our system, one polymerase dimer would bind per linear DNA molecule for an optimal ratio of 2:1. Whether binding of one dimer to a DNA end precludes binding of another dimer to the other end or whether binding of one dimer involves both DNA ends simultaneously cannot be deduced from our experiments. (3) A single polymerase molecule may bind to a strand break, recruit an available acceptor such as an unactivated polymerase molecule, and thereby form polymers via the homomodification mechanism presented in the second scenario.

One line of evidence from our experiments rules out the first and second scenarios: additional molecules of DNA beyond the optimal polymerase to DNA ratio of 2:1 cause a decrease in the number of polymers formed (Figures 5 and 6). We would not expect such a dilution effect if poly(ADP–ribose) polymerase is automodified or if functional poly(ADP–ribose) polymerase dimers are formed before binding to strand breaks. Since incubation with longer molecules of DNA at a ratio of 2:1 did not cause a reduction in the number of polymers formed (Figure 7), it is unlikely that polymerase molecules bind to the increasing regions of double-stranded DNA and are not activated.

Therefore, we propose a homomodification mechanism involving DNA strand break-mediated formation of poly(ADP–ribose) polymerase dimers. In solution, all polymerase molecules are candidates for activation. However, once a polymerase binds to a DNA break and becomes activated, it requires the presence of another, unactivated polymerase to act as the polymer acceptor. Alteration of the ratio of activating sites to poly(ADP–ribose) polymerase simply alters the ratio of active *vs* acceptor molecules. When few DNA breaks are present, only few polymerases are activated and, subsequently, few are homomodified (see the evidence above for no polymerase cycling). When DNA breaks are present in excess, the majority of polymerase molecules are activated, leaving few unactivated enzymes to act as polymer acceptors.

Physical evidence supporting the intermolecular modification of poly(ADP–ribose) polymerase has been presented by Kawaichi *et al.* (1981) and Taniguchi (1987). In addition to previous data showing the formation of poly(ADP–ribose) polymerase dimers in solution (Bauer *et al.*, 1990), we propose that the dimer is a functional intermediate between the active

polymerase and its acceptor molecule. Dimer formation may be mediated through the recently discovered leucine zipper motif within the poly(ADP-ribose) polymerase automodification domain (Uchida *et al.*, 1993). Homomodification would disrupt the protein–protein association upon which a new protein–polymer association between the modifier and acceptor would be formed. Future studies involving site-directed mutagenesis of modification and acceptor sites on poly(ADP-ribose) polymerase may provide the direct physical evidence necessary to conclusively substantiate a homomodification mechanism.

The observations in this report have a significant bearing on the extrapolation from *in vitro* experiments to *in vivo* situations. The homomodification mechanism *in vivo* would be naturally adaptable for the rapid and efficient response of poly(ADP-ribose) polymerase to DNA damage. Calculations from experimental procedures presented in the literature, however, reveal that few laboratories work at optimal polymerase to DNA molar ratios precisely because this sensitive relationship has not been considered and because the percentage of activatable polymerase *vs* nonactivatable polymerase usually is not known. Our results show that if all polymerase molecules are activatable, optimal activity occurs at a polymerase to linear DNA ratio of 2:1. As the percentage of nonactivatable molecules increases, so too does the ratio necessary to attain optimal activity. Therefore, a standard activity assay performed at various polymerase to DNA molar ratios would not only optimize polymerase activity but also serve to quantify the functional potential of an enzyme population.

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