# Mechanisms of poly(ADP-ribose) polymerase catalysis; mono-ADP-ribosylation of poly(ADP-ribose) polymerase at nanomolar concentrations of NAD

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Calf thymus and rat liver poly(ADP-ribose) polymerase enzymes, and the polymerase present in extracts of rat liver nuclei synthesize unstable mono-ADP-ribose protein adducts at 100 nM or lower NAD concentrations. The isolated enzyme–mono-ADP-ribose adduct hydrolyses to ADP-ribose and enzyme protein at pH values slightly above 7.0 indicating a continuous release of ADP-ribose from NAD through this enzyme-bound intermediate under physiological conditions. $NH_2OH$ at pH 7.0 hydrolyses the mono-ADP-ribose enzyme adduct. Desamino NAD and some other homologs at nanomolar concentrations act as 'forward' activators of the initiating mono-ADP-ribosylation reaction. These NAD analogs at micromolar concentrations do not affect polymer formation that takes place at micromolar NAD concentrations. Benzamides at nanomolar concentrations also activate mono-ADP-ribosylation of the enzyme, but at higher concentrations inhibit elongation at micromolar NAD as substrate. In nuclei, the enzyme molecule extensively auto-ADP-ribosylates itself, whereas histones are trans-ADP-ribosylated to a much lower extent. The unstable mono-ADP-ribose enzyme adduct represents an initiator intermediate in poly ADP-ribosylation.

*Poly(ADP-ribose) polymerase* *Mono-ADP-ribose transfer* *Initiation*

## 1. INTRODUCTION

It is generally assumed (cf. [1–4]) that the chromatin-bound enzyme poly(ADP-ribose) transferase or polymerase (EC 2.4.99) transfers ADP-ribose residues of NAD to acceptor proteins, and notably in the isolated form the enzyme protein itself can serve as an acceptor in the presence of coenzymic DNA [15,26], resulting in auto-poly-ADP-ribosylation. Since proteins other than the polymerase protein also contain labelled ADP-ribose [1,2] following incubation of nuclei with labelled NAD it must be assumed that a transfer mechanism is operative from NAD either directly ADP-ribosylating 'acceptor' proteins (e.g. histones), a reaction presumably catalyzed by the polymerase, or alternatively the auto-ADP-ribosylated enzyme could itself trans-ADP-ribosylate certain acceptor proteins. This question appears to be incompletely resolved. It is known from extensive studies from Hayaishi's laboratory [2,14] that once a polymer is formed on the enzyme it cannot be transferred to histones. Therefore, if a transfer occurs at all, it must happen at the mono-ADP-ribose level. It has also been demonstrated that histone-bound ADP-ribose can be elongated by purified poly(ADP-ribose) polymerase [5]. It seemed almost immaterial what reaction led to a histone–mono-ADP-ribose adduct, since even Schiff base adducts of histones [6] served as elongation templates (cf. [5]). It is now known that several, sometimes non-nuclear located, ADP-ribose arginyltransferases, distinct from poly-(ADP-ribose) polymerase, can transfer ADP-ribose to histones or casein and probably to other proteins with apparently no rigorous acceptor

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specificity (cf. [3]) and the histone adducts thus formed can be further elongated by poly(ADP-ribose) polymerase [7]. Mechanistic interpretation of the enzymology of poly-ADP-ribosylation and of important supramolecular events in chromatin that appear to coincide with shifts in protein patterns of ADP-ribosylation [8,9] depend on a more detailed understanding of the initial steps of ADP-ribosylation at the poly(ADP-ribose) polymerase level [10]. Pursuing this question, the existence of an early product [11] was indicated. This problem was further studied by incubation of the purified enzyme with 1 $\mu$M NAD for 20 s (at 37°C) followed by isolation of mono- and oligo-ADP-ribose that was bound to the enzyme protein, thus leading to the conclusion that the enzyme contained multiple 'initiator' sites [12]. However, a possibly unusual nature of the initiator site has not been considered. We have employed nanomolar concentrations of NAD as substrate for the poly(ADP-ribose) polymerase of various nuclei and found that almost exclusively mono-ADP-ribose adducts were synthesized [13], indicating that below micromolar concentration of NAD the polymerase appeared to catalyze mono-ADP-ribose transfer. Following this experimental approach we report here that purified poly(ADP-ribose) polymerase from 2 sources catalyzes the auto-mono-ADP-ribosylation of the enzyme protein resulting in highly unstable monomer adducts that especially under alkaline conditions, reported earlier to be optimal for the polymerase enzyme (cf. [1,2]), release ADP-ribose from NAD apparently accounting for the known NAD-glycohydrolase activity of this enzyme [14]. This paper deals with kinetic analyses and interpretation of these phenomena.

## 2. EXPERIMENTAL

Calf thymus poly(ADP-ribose) polymerase was purified to homogeneity by a published method and coenzymic DNA isolated simultaneously (cf. [15]). Partially purified rat liver poly(ADP-ribose) polymerase was isolated to stage IV as in [10]. ADP-ribose, NAD and poly(ADP-ribose) were identified by high-performance liquid chromatography (HPLC) [16,17]. Gel electrophoresis and enzymatic assays and the isolation of rat liver nuclei were carried out as in [18]. Rapid isolation of ADP-ribosylated proteins by Sephadex G-50 column centrifugation was performed as described [19].

HPLC identification of nucleotide products from neutral hydroxylaminolysis and base hydrolysis of mono-ADP-ribosylated nuclear proteins was carried out as follows. 60 $\mu$g nuclei were incubated with 100 nM [$^{32}$P]NAD (spec. act. 800 Ci/mmol, NEN) for 10 min. The reaction was stopped by addition of 1 ml of 20% trichloroacetic acid followed by centrifugation (2000 × $g$). The pellet was washed further with 3 additional portions of 20% trichloroacetic acid, until the supernatant did not contain any appreciable radioactivity, and then twice with 1.5-ml portions of diethyl ether to remove trichloroacetic acid and lipids. The pellet was then digested either with neutral hydroxylamine (1 M at 37°C for 1 h) or with aqueous potassium hydroxide (1 M at 56°C for 1 h). Samples were acidified to pH 5.0, centrifuged, and the clear supernatant injected into the HPLC system. The HPLC conditions were the same as in [16] except the flow rate was 1.50 ml/min.

Rat liver nuclei (protein content 10 mg/ml) were extracted with an equal volume of 0.5 M KCl, 50 mM Hepes, 5 mM DTT (pH 7.2) by incubation for 30 min at 4°C, then centrifuged at 105000 × $g$ for 30 min at 4°C. The supernatant (2.7 mg/ml protein content) was used for further experiments. To 200 $\mu$l nuclear extract, 60 $\mu$l of 750 mM Tris-HCl, pH 8.0, 50 mM $MgCl_2$, 10 mM DTT and [$^{32}$P]NAD were added (total volume 300 $\mu$l) and incubated for 10 min at 23°C. The final NAD concentration was 100 nM. Thereafter 250 $\mu$l of the sample was injected into a TSK W-2000 HPLC column and chromatographed with 25 mM Hepes, pH 7.4, 0.25 M KCl, 2.5 mM DTT elution buffer at 0.5 ml/min flow rate at 23°C. 0.5-ml aliquots were collected and 50 $\mu$l of each fraction was tested for radioactivity. Fractions 34–35 containing the adduct were collected and used for experiments shown in fig.2B. Alternatively, $5 \times 10^{-8}$ M purified calf thymus poly(ADP-ribose) polymerase was incubated with 100 nM [$^{32}$P]NAD for 20 min at 23°C under the conditions described in the legend to fig.1. The total volume was 100 $\mu$l. At the end of incubation, the macromolecules were separated from the unreacted [$^{32}$P]NAD by Sephadex G-50 column centrifugation [19] using 10.0 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, and

2.0 mM DTT as equilibration buffer. 90–95% ADP-ribose was bound to proteins. This was determined by hydrolysis of samples with 1 N NaOH for 1 h at 23°C, followed by neutralization and HPLC analyses [16] of products. In addition, products were also identified by PEI-cellulose TLC, developed with 0.9 M acetic acid, 0.3 M LiCl running buffer.

## 3. RESULTS

Automodification of purified poly(ADP-ribose) polymerase of calf thymus was determined at nanomolar concentrations of NAD. Initial velocities were plotted vs NAD concentrations as shown in fig.1. At nanomolar concentrations (lower curve) a concave curve was obtained, suggesting multiple binding sites for NAD. On the other hand, when $v_{init}$ is plotted vs micromolar concentrations of NAD, a practice generally followed, no anomalous relationship between $v_{init}$ and NAD concentrations could be detected (fig.1, upper).

The nature of the ADP-ribose enzyme adduct was studied with an extract of rat liver nuclei and

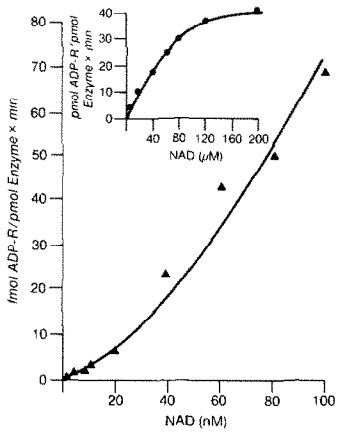


Fig.1. (Upper) $5.2 \times 10^{-9}$ M enzyme was incubated with [$^{14}$C]NAD (0–200 $\mu$M range) in the presence of 6 $\mu$g/ml coenzymic DNA for 1 min at 23°C and products assayed as in [18]. The volume of the incubation mixture was 100 $\mu$l. The specific activity of nicotinamide [U-$^{14}$C]adenine dinucleotide (Amersham) was 300 mCi/mmol. (Lower) $2 \times 10^{-9}$ M enzyme was incubated with [$^{32}$P]NAD (0–100 nM range) for 1 min at 23°C in the presence of 6 $\mu$g/ml coenzymic DNA, in a total volume of 30 $\mu$l.

with purified enzymes, keeping NAD concentrations at or below 100 nM. It is of interest that regardless of the purification stage of poly(ADP-ribose) polymerase or the time of incubation with 100 nM or lower concentration of NAD mono-ADP-ribose protein adducts represented 85–90% of protein products. With crude nuclear extracts, depending on the time of incubation, mono-ADP-ribosylated histones were also formed, apparently as a result of transesterification from enzyme–ADP-ribose (Bauer, P.I. and Kun, E., unpublished). Rat liver nuclei were extracted with 0.5 M KCl, the extract incubated with 100 nM NAD, and the ADP-ribosylated enzyme isolated on a TSK W-2000 HPLC column as shown in fig.2A. An identical peak was obtained when the homogeneous calf thymus enzyme was ADP-ribosylated. The same ADP-ribose–enzyme adduct was also isolated by Sephadex centrifugation at 4°C (cf. [19]) to maintain maximal enzymatic activity suitable for the identification of the pH stability of the ADP-ribose enzyme bond (fig.2B). It is evident that this bond was very sensitive to $OH^-$ and significant hydrolysis occurred above pH 7.0. The nature of the hydrolysis product was determined by HPLC (cf. [16,17]). Hydrolysis by hydroxylamine (cf. [21]) at pH 7.0 or hydrolysis at pH 8.0 yielded only ADP-ribose and at pH 14 ADP-ribose was cleaved to AMP + ribose phosphate as shown by the results from Hilz's laboratory (cf. [1]).

The kinetics of synthesis and hydrolysis of ADP-ribose-enzyme adducts are illustrated in fig.3A and B. Partially purified rat liver poly(ADP-ribose) polymerase (fig.3A) catalyzed the mono-ADP-

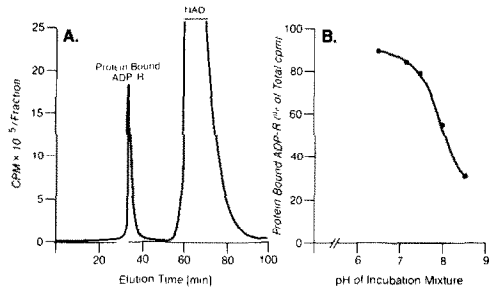


Fig.2. (A) Isolation of enzyme-bound ADP-ribose by HPLC. (B) The dependence of hydrolysis of ADP-ribose enzyme adduct on pH. Identical results were obtained with crude and purified enzymes.

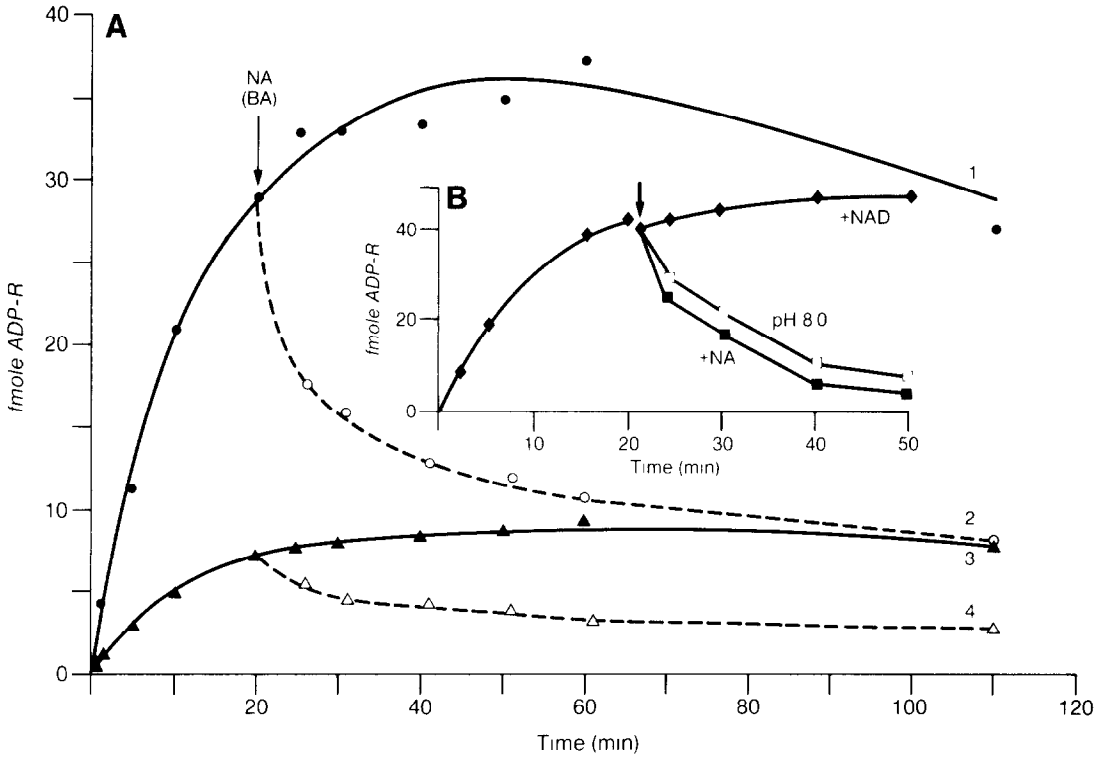


Fig.3. (A) Effect of nicotinamide and hydroxylamine on products synthesized by partially purified rat liver poly(ADP-ribose) polymerase (step 4 of [10]). 20 $\mu$g enzyme protein was incubated with 100 nM [$^{32}$P]NAD at 23°C, and aliquots were removed at specified times. ADP-ribose enzyme adducts were determined by 10% trichloroacetic acid precipitation. Curves: 1, without additions; 2, addition of 160 mM nicotinamide or 10 mM benzamide; 3, hydroxylaminolysis of experiment described in curve 1; 4, hydroxylaminolysis following addition of nicotinamide or benzamide. (B) (Inset) $4.5 \times 10^{-8}$ M purified calf thymus enzyme was incubated with 50 nM [$^{32}$P]NAD in a total volume of 150 $\mu$l under the conditions described in fig.1. 5-$\mu$l aliquots were withdrawn and the 10% trichloroacetic acid-precipitable radioactivity determined. Beginning at 20 min aliquots of ADP-ribosylated enzyme were withdrawn and $NH_2OH$ sensitivity of ADP-ribose protein bonds were assayed at pH 7.0 (Δ---Δ , O---O; see section 3).

ribosylation of the enzyme protein at pH 8.0 following a time course shown in curve 1, experimental points representing acid-precipitable ADP-ribose protein adducts. After about 50 min the rate declined, indicating an increased hydrolysis of enzyme ADP-ribose adducts. When the forward reaction was inhibited by nicotinamide (or benzamide) at 20 min, a rapid decay of adducts was evident (curve 2). Following acid precipitation, products were suspended in 1 M $NH_2OH$ (pH 7.0) and incubated at 37°C for 1 h. Controls, containing no $NH_2OH$, only buffer, were treated identically. About 80–90% of acid-precipitable materials was hydrolyzed by $NH_2OH$ at pH 7.0 in 1 h and curve 3 was obtained. Hydroxylaminolysis performed in the inhibited system (curve 2) resulted in curve 4. It was apparent that the $NH_2OH$-sensitive/resistant bond ratio was nearly the same in both control (curve 1) and inhibited (curve 2) systems. In a separate series of experiments, the ADP-ribose enzyme adducts were isolated (fig.3B) by Sephadex centrifugation [19], as indicated by a downward arrow. Without addition of NAD, the enzyme-ADP-ribose adducts hydrolyzed at pH 8.0 and this rate was not appreciably altered by nicotinamide (or benzamide). Added NAD maintained the steady-state level of ADP-ribose enzyme adducts. These experiments which are shown in fig.3B (inset) were done with the homogeneous calf thymus polymerase. The

sum of results (fig.3A and B) demonstrate that the first product formed at 100 nM NAD is an unstable monomeric adduct of ADP-ribose and the enzyme protein and this adduct spontaneously decomposes at pH 8.0 in the absence of NAD that supports the forward reaction. The same enzyme–mono-ADP-ribose adduct is formed in crude and purified enzyme preparations. It is noteworthy that even prolonged incubation with below 100 nM NAD only yields monomers with only traces of short oligomers, clearly discriminating this initiation step from elongation, that requires higher than 100 nM NAD as substrate. This was verified by previously developed techniques [16,17] tested within 10–100 min following enzymatic ADP-ribosylations (fig.3). The bond between ADP-ribose and protein was identified as an $NH_2OH$-sensitive ester (first-order rate constant of hydrolysis by $NH_2OH$ at pH 7.0 and 23°C is $K^1$ = 1.04 $h^{-1}$). Analyses of products formed at pH 8.0 or at pH 7.0 in the presence of $NH_2OH$ from enzyme-bound ADP-ribose yielded only ADP-ribose and no NAD was generated from added nicotinamide.

With the aid of NAD analogs and inhibitors, it was possible to discriminate kinetically between the initiation and elongation catalysis performed by the homogeneous calf thymus enzyme. Initiation was determined at nanomolar concentration and elongation at 9 $\mu$M NAD (table 1, expt 1). Formation of polymers at 9 $\mu$M NAD was ascertained by HPLC [16,17]. None of the NAD analogs had an appreciable effect on elongation, but at nanomolar concentrations the initiation reaction was notably activated, an effect that was especially pronounced at 12 nM with acetyl-pyridine-NAD (expt 3, 310%), and 12 nM nicotinic acid NAD (expt 5, 475%). Different effects on initiation and on elongation were obtained with nanomolar concentrations of benzamide and its amino analogs, which are known inhibitors of the enzyme [20]. As shown in expts 7–10, initiation determined at 25 nM NAD is activated by 263% by 30 nM benzamide and by 225% by 12 nM 3-aminobenzamide. On the other hand, elongation, that was determined at NAD concentrations varied between 0.01 and 1.0 mM and benzamide concentrations varied between 5 and 200 $\mu$M, exhibited competitive inhibition with benzamides with varying $K_i$ values. Our $K_i$ values differ from those originally reported with crude nuclear extracts as a source of polymerase (cf. [20]). This discrepancy is probably due to the experimental conditions since we applied homogeneous calf thymus enzyme and saturating concentrations of coenzymic DNA [15]. Due to the complication introduced by qualitatively different responses on initiation and elongation to different concentrations of an inhibitor (e.g. 3-aminobenzamide), the effects of these substances on the polymerase can be highly variable and activation or inhibition may be obtained as a function of NAD concentration.

Mono-ADP-ribosylation of proteins with 100 nM NAD as substrate can be readily demonstrated also with isolated rat liver nuclei (cf. [13]). The quantity of proteins isolated by gel electrophoresis [18] was determined by densitometry using the purified enzyme protein and histones as standard. Specific activity was determined for each histone species (cf. [18]). There was a marked difference in the specific activities (i.e. mol ADP-ribose/mol protein) of the enzyme (calculated from fig.1) and histones (0.05–0.1 mol ADP-ribose per mol enzyme and 0.002–0.004 mol ADP-ribose per mol histone $H_1$), clearly indicating the predominance of auto-mono-ADP-ribosylation of the enzyme protein even in nuclei.

## 4. DISCUSSION

Our experimental results are consistent with a mechanism that includes the formation of an unstable mono-ADP-ribose enzyme protein initiator adduct that is likely to be the first catalytic product of poly ADP-ribosylation. Recognition of this initiator adduct [11,12] has been made difficult in the past by the use of micromolar NAD concentrations, which support both initiation and elongation. Identification of the initiator mono-ADP-ribose adduct depends on nanomolar concentrations of NAD as substrate.

By accepted criteria of hydroxylaminolysis, developed in Hilz's laboratory (cf. [1]), the ADP-ribose protein bonds are identified as unstable ester bonds, in agreement with the bonds described for poly(ADP-ribose) protein adducts (cf. [1–4]) except we determined hydroxylaminolysis at pH 7.0 whereas others [21] did so at pH 7.5. We find that the mono-ADP-ribose enzyme adducts are in a dynamic state with respect to NAD even at

Table 1

Effects of NAD analogs on the initiation and elongation rates of the automodification of purified calf thymus poly(ADP-ribose) polymerase

| Expt no. | Analog | Substrate NAD | Initiation rate (fmol ADP-ribose/ pmol enzyme in 1 min) | % of no.1 | Elongation rate (pmol ADP ribose/pmol enzyme in 1 min) | % of no.1 |
|---|---|---|---|---|---|---|
| (1) | – | 9 μM | – | – | 6.0 | 100 |
| | – | 25 nM | 10.5 | 100 | – | – |
| (2) | Guanino-NAD | | | | | |
| | 12 nM | 25 nM | 13.0 | 125 | – | – |
| | 30 nM | 25 nM | 16.0 | 160 | – | – |
| | 1 mM | 9 μM | – | – | 5.6 | 93 |
| (3) | Acetylpyridine-NAD | | | | | |
| | 12 nM | 25 nM | 32.3 | 310 | – | – |
| | 30 nM | 25 nM | 19.3 | 190 | – | – |
| | 1 mM | 9 μM | – | – | 7.0 | 116 |
| (4) | Aldehyde pyridine-NAD | | | | | |
| | 12 nM | 25 nM | 17.8 | 171 | – | – |
| | 30 nM | 25 nM | 15.6 | 149 | – | – |
| | 1 mM | 9 μM | – | – | 6.5 | 110 |
| (5) | Nicotinic acid-NAD | | | | | |
| | 12 nM | 25 nM | 49.4 | 474 | – | – |
| | 30 nM | 25 nM | 28.3 | 270 | – | – |
| | 1 mM | 9 μM | – | – | 7.2 | 120 |
| (6) | Aminopyridine-NAD | | | | | |
| | 12 nM | 25 nM | 30.7 | 293 | – | – |
| | 30 nM | 25 nM | 28.2 | 269 | – | – |
| | 1 mM | 9 μM | – | – | 6.85 | 110 |
| (7) | Benzamide | | | | | |
| | 12 nM | 25 nM | 11.5 | 110 | | – |
| | 30 nM | 25 nM | 25.4 | 263 | | – |
| | 5–200 μM | 0.01–1 mM | – | – | $K_i$ = 39 μM | – |
| (8) | 2-Aminobenzamide | | | | | |
| | 12 nM | 25 nM | 8.6 | 82 | | – |
| | 30 nM | 25 nM | 10.6 | 100 | | – |
| | 5–200 μM | 0.01–1 mM | – | – | $K_i$ = 71 μM | – |
| (9) | 3-Aminobenzamide | | | | | |
| | 12 nM | 25 nM | 23.5 | 225 | | – |
| | 30 nM | 25 nM | 15.8 | 150 | | – |
| | 5–200 μM | 0.01–1 mM | – | – | $K_i$ = 12 μM | |
| (10) | 4-Aminobenzamide | | | | | |
| | 12 nM | 25 nM | 13.2 | 120 | | – |
| | 5–200 μM | 0.01–1 mM | – | – | $K_i$ = 65 μM | |

Enzymatic activities were determined under the conditions described in the legend to fig.1. NAD analogs and their concentrations are given in column 1, NAD concentrations in column 2, $v_{init}$ of mono-ADP-ribosylation in column 3, $v_{init}$ of elongation in column 5. In expt 1, elongation rates and initiation rates are given (at 9 μM and 25 nM NAD, respectively) in the absence of NAD analogs, and column nos 4 and 6 express % activities of systems containing NAD analogs

physiological pH values and there is a constant flow of $OH^-$-catalyzed release of ADP-ribose from NAD at the ADP-ribose enzyme initiator template. This 'apparent NAD glycohydrolase' activity at the mono-ADP-ribose-binding site can provide ADP-ribose for Schiff base formation [6,22] with the enzyme protein or with proteins that are located near the initiation site. We have shown that the hydroxylamine-insensitive ADP-ribose-protein adducts, comprising 15–30% of total adducts, correspond to Schiff bases as identified by selective borotritiation [6] (Bauer, P.I. and Kun, E., presented elsewhere).

Rates of mono-ADP-ribosylation of the enzyme protein are greatly increased by NAD homologs which have no effect on polymerization (see table 1). Details of the abnormal kinetics at nanomolar NAD concentrations and the role of modifiers are subject to further studies. The large effect of desamino NAD as a 'forward' activator of initiation is probably physiologically significant, since this substance is a known metabolic precursor of NAD. This novel, probably allosteric effector phenomenon on poly-ADP-ribosylation initiation, which is predictably regulated by a probably localized decrease of NAD concentration at chromatin sites to nanomolar levels, may explain the 180-fold prevalence of monomer adducts over polymer-protein compounds, as found experimentally in AH7974 cells [21]. Another feature of the unstable mono-ADP-ribose enzyme initiation adducts is their propensity to serve as ester donors for ADP-ribose transesterification reactions to certain glutamate and lysine carboxyl end groups of histones as has been demonstrated [23,24]. In agreement with Kreimeyer et al. [21], on a molar basis ADP-ribosylation of histones appears to be minimal as compared to the enzyme protein. The large quantities of nuclear histones containing low molar specific activity of ADP-ribose (i.e. mol ADP-ribose/mol histone) will necessarily exhibit large spots of auto-radiograms. Similar transesterification mechanisms may account for ADP-ribosylation of topoisomerase [25]. It has been reported [26] that enzymatic cleavage of histone-ADP-ribose adducts can be catalyzed by a cytosolic enzyme, resulting in a rearranged product of ADP-ribose, ADP-3″-deoxypentose-2″-ulose, that can be chromatographically distinguished from ADP-ribose. However, hydroxylaminolysis at pH 7.0 of the same adduct yields only ADP-ribose [26]. Since no other substance except ADP-ribose was identified as a product of hydroxylaminolysis of the ADP-ribose enzyme initiator adduct, there seems to be no complicating interference by the reported ADP-ribosyl-protein lyase in our system. Actually this enzymatic reaction has been rigorously demonstrated to act on ADP-ribose adducts of histone $H_1$ and $H_2B$ (cf. [26]).

Demonstration of an unstable ADP-ribose-enzyme initiation adduct, detectable at nanomolar NAD concentrations and possessing special regulatory properties (e.g. forward activation by desamino NAD) provides a catalytically feasible model for initiation and limited trans-ADP-ribosylation and also explains the hitherto enigmatic 'extra'-NAD-glycohydrolase activity of poly(ADP-ribose) polymerase.

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