NAD^+ analogs substituted in the purine base as substrates for poly(ADP-ribosyl) transferase

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Abstract **Poly(ADP-ribosyl) transferase (pADPRT) catalyzes the transfer of the ADP-ribose moiety from NAD^+ onto proteins as well as onto protein-bound ADP-ribose. As a result, protein-bound polymers of ADP-ribose are formed. pADPRT itself contains several acceptor sites for ADP-ribose polymers and may attach polymers to itself (automodification). In this study the influence of substitutions in the purine base of NAD^+ on the polymerization reaction was investigated. The adenine moiety of NAD^+ was replaced by either guanine, hypoxanthine or 1,N^6-ethenoadenine. These analogs served as substrates for polymer synthesis as judged from the extent of automodification of the enzyme and the sizes of the polymers formed. Time course experiments revealed that 1,N^6-etheno NAD^+ (ε-NAD^+) and nicotinamide hypoxanthine dinucleotide (NHD^+) were rather poor substrates as compared to NAD^+. Synthesis of GDP-ribose polymers from nicotinamide guanine dinucleotide (NGD^+) was more efficient, but still significantly slower than poly(ADP-ribosyl)ation of the enzyme using NAD^+. The size of the different polymers appeared to correlate with these observations. After 30 min of incubation in the presence of 1 mM substrate, polymers formed from ε-NAD^+ or NHD^+ contained up to 30 ε-ADP-ribose or IDP-ribose units, respectively. Using NGD^+ as substrate polymers consisted of more than 60 GDP-ribose units, an amount similar to that achieved by poly(ADP-ribosyl)ation in the presence of only 0.1 mM NAD^+ as substrate. These results suggest that the presence of an amino group in the purine base of NAD^+ may facilitate catalysis. Substitution of the nicotinamide moiety of NAD^+ with 3-acetylpyridine had no detectable effect on polymer formation. Oligomers of GDP-ribose and ε-ADP-ribose exhibited a slower mobility in polyacrylamide gels as compared to ADP-ribose or IDP-ribose oligomers.This feature of the two former analogs as well as their markedly attenuated polymerization by pADPRT provide valuable tools for the investigation of the enzymatic mechanism of this protein. Moreover, polymers of ε-ADP-ribose may be useful for studying enzymes degrading poly(ADP-ribose) owing to the fluorescence of this analog. Digestion of ε-ADPR polymers with snake venom phosphodiesterase was accompanied by a significant fluorescence enhancement.**

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Abbreviations: ε-NAD^+, 1,N^6-etheno NAD^+; 3-$APAD^+$, 3-acetylpyridine adenine dinucleotide; IDP, inosine diphosphoribose; NADase, NAD^+-glycohydrolase; NGD^+, nicotinamide guanine dinucleotide; NHD^+, nicotinamide hypoxanthine dinucleotide; NMN, nicotinamide mononucleotide; pADPRT, poly(ADP-ribosyl) transferase; PAG(E), polyacrylamide gel (electrophoresis); SDS, sodium dodecyl sulfate

Dedicated to Prof. Manfred Schweiger on the occasion of his 60th birthday.

Key words: ADP-ribosylation; NAD^+ glycohydrolase; 1,N^6-etheno NAD^+

1. Introduction

The enzyme poly(ADP-ribosyl) transferase (pADPRT) (EC. 2.4.2.30) catalyzes the transfer and the polymerization of ADP-ribose moieties from NAD^+ to protein acceptors (heteromodification), as well as to the enzyme itself (automodification). As opposed to monoADPRTs, this enzyme transfers ADP-ribose not only onto acceptor groups within the protein, but also onto protein-bound ADP-ribose, thus forming poly(ADP-ribose). Polymer sizes of up to 200–300 ADP-ribose units have been observed both in vitro and in vivo [1]. As yet, neither the actual biological role of this enzyme nor the physiological significance of the protein modification has been clearly established. Treatment of cells with DNA-damaging agents leads to depletion of the intracellular NAD^+ pool. This loss of NAD^+ can be attributed to the synthesis of poly(ADP-ribose) by pADPRT activated by DNA strand breaks. Such an enormous expense of energy would seem to indicate an important role of the enzyme in cellular defense mechanisms. Furthermore, its involvement in processes such as cell differentiation, proliferation or DNA repair is discussed (reviewed in [2,3]).

Thus far, a variety of NAD^+ analogs have been tested with respect to their ability to serve as inhibitors of pADPRT. $NADP^+$, 3-$APAD^+$ or NHD^+ did not exert a significant influence on pADPRT activity [4]. Bauer et al. [5] reported that 3-$APAD^+$ and several other NAD^+ analogs substituted in the pyridine base of NAD^+ served as forward activators in the initial (ADP-ribosyl)ation reaction, whereas the elongation of ADP-ribose chains seemed to be unaffected. Deoxy derivatives of NAD^+ have been employed to investigate the mechanism of chain elongation. 3′-deoxy-NAD^+ favored the synthesis of oligomers with only 4 or less ADP-ribose residues [6], whereas in the presence of 2′-deoxy-NAD^+ only mono-(dADP-ribosyl)ation occurred [7]. The latter compound was suggested to be a covalent inhibitor of pADPRT [8].

In this study the sensitivity of pADPRT to substitutions in the purine base of NAD^+ (Fig. 1) was examined. While the replacement of the nicotinamide moiety with 3-acetylpyridine had no detectable influence on the enzymatic reaction, the enzyme was sensitive to modifications of the purine base. The analogs tested (NGD^+, NHD^+ and ε-NAD^+) were utilized by the enzyme for the polymerization reaction, but less efficiently than NAD^+.

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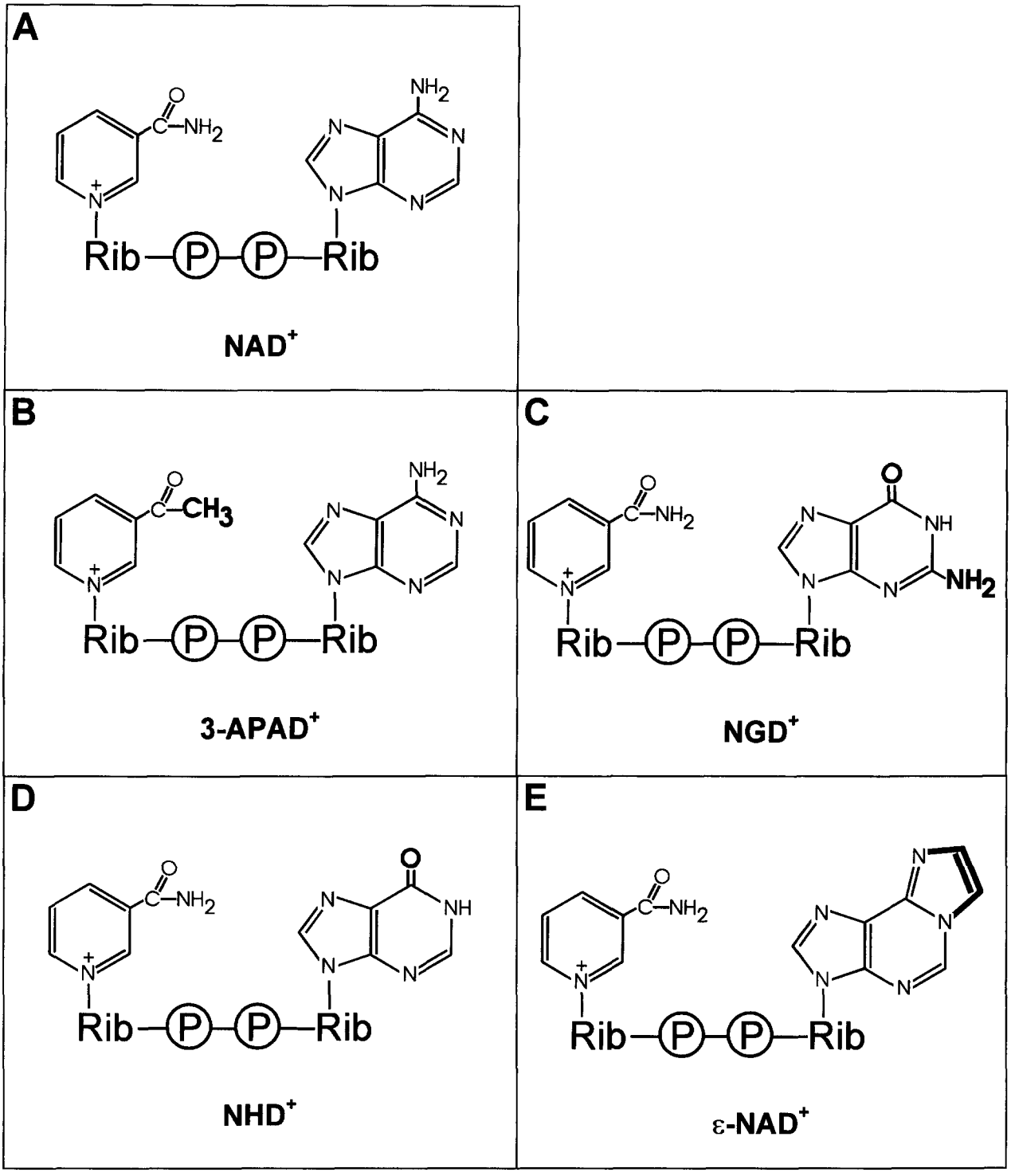

Fig. 1. Structures of NAD^+ analogs.

2. Materials and methods

Reagents were purchased from Sigma, unless otherwise noted. [α-^{32}P]NAD^+ was obtained from ICN. All chemicals were of analytical grade.

2.1. Overexpression and purification of human full-length pADPRT in E. coli

Standard recombinant techniques were used [9]. The cDNA encoding the wild-type human pADPRT [10] was cloned into the pQE31 vector system using the QIAexpress standard protocol (Quiagen). The resulting pQE31-pADPRT plasmid encoded the full-length pADPRT, at the N-terminal Met^1 tagged by 6 histidines and a further 16 amino acids [11]. *E. coli* M15 [pREP4+pQE31-pADPRT] cells were grown at 37°C to an $A_{600\ nm} = 0.8$ in 16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl with 50 mg/l ampicillin and 20 mg/l kanamycin. Overexpression was induced by adding 300 mg/l isopropyl β-thiogalactopyranoside (Appli Chem). The cells were grown for a further 5 h at 37°C. All subsequent steps were carried out at 4°C. The cells were harvested by centrifugation at 4000×*g* for 10 min, and the pellet was resuspended in extraction buffer (50 mM Tris-HCl pH 7.4, 1 M NaCl, 0.1 mM

phenylmethylsulfonyl fluoride, 1 mM β-mercaptoethanol). After French-press processing the lysate was cleared by centrifugation at 30000×*g* for 30 min. The crude extract was applied to a Ni^{2+}-nitrilotriacetic acid column (Quiagen) and was washed with extraction buffer. His-tagged protein was eluted with 100 mM EDTA, 20 mM Tris-HCl, pH 7.4, 20 mM NaCl and loaded onto a 3-aminobenzamide affinity column according to the method of Burtscher et al. [12]. The 116000 full-length pADPRT was eluted with 20 mM nicotinamide, 20 mM Tris-HCl, pH 7.4, 20 mM NaCl. Inhibitory nicotinamide was then removed using a hydroxyapatite column.

2.2. *ADP-ribosylation and (α-^{32}P) labeling of pADPRT by automodification*

In a standard reaction, 10 μM purified His-tagged pADPRT was preincubated in the presence of 1 μg sonicated salmon sperm DNA (Boehringer Mannheim) and 1 mM of the NAD^+ analog indicated for 30 min at 25°C in a total volume of 15 μl. The reaction mixture consisted of 10 mM Tris-HCl, pH 8.0, 7 mM $MgCl_2$, 50 μM $ZnCl_2$. After the addition of 0.1 μM [α-^{32}P]NAD^+ (0.3 μCi/nmol) the reaction was continued for a further 20 min at 25°C. For time course experiments the enzyme was first labeled in the presence of 0.1 μM [α-^{32}P]NAD^+. Incubation was then continued in the presence of the indicated substrate. The labeled protein was analyzed by SDS-polyacrylamide gel electrophoresis [13] and subsequent autoradiography. Size analysis of ADP-ribose polymers was performed as described by Panzeter and Althaus [14]. Briefly, after the automodification reaction the poly(ADP-ribosyl)ated protein was precipitated with trichloroacetic acid. ^{32}P-labeled polymers were detached from the protein by incubating the precipitate at 60°C for 3 h with 10 mM Tris, 1 mM EDTA, pH 12. After phenol/chloroform extraction the supernatants were lyophilized. Polymers were then resolved in 7 M urea, 25 mM NaCl, 4 mM EDTA (pH 8.0), 0.02% xylene cyanol, 0.02% bromphenol blue and loaded onto a 20% sequencing gel in 0.09 M Tris, 0.09 M boric acid, 2 mM EDTA (pH 8.3). After electrophoresis the gel was dried and subjected to autoradiography.

2.3. *Synthesis of ε-NAD^+ and poly(ε-ADP-ribose)*

^{32}P-labeled ε-NAD^+ was synthesized from ^{32}P-labeled NAD^+ according to a procedure described by Barrio et al. [15]. For the synthesis of poly(ε-ADP-ribose) 20 μg of purified His-tagged pADPRT, 5 μg DNA and 1 mM NAD^+ were incubated in a 50 μl reaction mixture (10 mM Tris-HCl, pH 8.0, 7 mM $MgCl_2$, 50 μM $ZnCl_2$) for 1 h at 25°C. After trichloroacetic acid precipitation modified proteins were treated with 100 μg/ml of proteinase K in the presence of 0.1% SDS at 37°C for 1 h, extracted with phenol/chloroform and precipitated with ethanol. Poly(ADP-ribose) was then incubated at ambient temperature for 16 h in 50 mM sodium acetate buffer, pH 5.0, 50 mM β-mercaptoethanol and 20% (v/v) freshly distilled chloroacetic aldehyde. Poly(ε-ADP-ribose) was then precipitated and washed with ethanol, and dried under vacuum.

2.4. *Fluorimetric activity assay*

Separation of the ε-adenine and the nicotinamide moieties of ε-NAD^+ results in a more than 10-fold increase in fluorescence [15]. Therefore, both NADase and phosphodiesterase activity can be measured fluorimetrically using this assay. Fluorescence was measured as described [16] at a wavelength of 410 nm, the excitation wavelength was set to 310 nm. The sample buffer contained 50 mM Tris-HCl, pH 7.5, and 0.05% lauryl dimethyl *N*-oxide. The fluorimetric assay of poly(ε-ADP-ribose) degradation was conducted under the same conditions. Fluorescence enhancement was interpreted as the release of ε-adenine-containing units from the packed structure of poly(ε-ADP-ribose).

3. Results

Purified recombinant pADPRT was incubated in the presence of 1 mM of the various substrates shown in Fig. 1. The amount of poly(ADP-ribose) units covalently linked to pADPRT correlates with the retardation of automodified enzyme in SDS-PAGs. A high degree of modification resulted in a high molecular weight smear with no distinct protein band, which could only be detected by autoradiography. For example, in the presence of 1 mM NAD^+ pADPRT becomes highly modified and only a small amount of protein was able to enter the separating gel (Fig. 2A). In this time course experiment (Fig. 2) purified pADPRT was prelabeled in the presence of DNA with 0.1 μM [^{32}P]NAD^+ for 20 min at 30°C. 1 mM unlabeled NAD^+, NGD^+, NHD^+, or ε-NAD^+ was then added and incubation continued for the time intervals shown. Incubation of pADPRT with 0.1 μM [^{32}P]NAD^+ without subsequent addition of unlabeled substrate resulted in a single radiolabeled band with an apparent molecular weight of 116000 (Fig. 2A, first lane). This band remained unchanged over at least 2 h (Fig. 2A, last lane). In the presence of 1 mM NAD^+ long ADP-ribose polymers were formed within the first minute of reaction (Fig. 2A), whereas polymer synthesis was retarded using the NAD^+ analogs. NHD^+ and ε-NAD^+ were still poorer substrates than NGD^+ (compare Fig. 2C,D to B). When incubation was continued for 1 day, polymer lengths approached the extent of that seen with NAD^+ even with NHD^+ or ε-NAD^+ (data not shown). As expected, $NADP^+$ or NMN did not serve as substrates and 3-aminobenzamide, a known inhibitor of pADPRT, prevented the polymerization reaction (not shown). 3-$APAD^+$ was utilized as substrate as efficiently as NAD^+ (not shown).

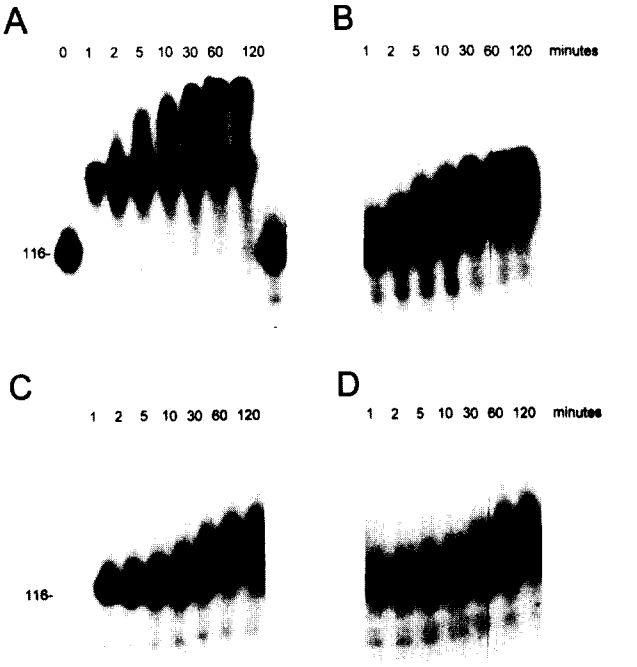

Fig. 2. Time courses of the automodification of pADPRT using different NAD^+ analogs as substrates. pADPRT (0.7 mg/ml) was first incubated in the presence of 1 mg/ml DNA and 0.1 μM [^{32}P]NAD^+ for 20 min at 30°C. Thereafter (time = 0), 1 mM of the analog indicated below was added and incubation continued for the time given at the top of each lane (in min). Samples were then subjected to SDS-PAGE (6%). Autoradiograms of the gels are shown. The rightmost lane of A contained a control sample that was incubated for 120 min without further addition of substrate. A, NAD^+; B, NGD^+; C, NHD^+; D, ε-NAD^+.

Polymer size analysis confirmed the results obtained by SDS-PAGE. 3-$APAD^+$ served as substrate for synthesis of long ADP-ribose chains with more than 60 residues, structurally equivalent to polymers obtained with NAD^+ (data not shown). The chain length of polymers formed in the presence of 1 mM NGD^+ were quite comparable to those obtained in

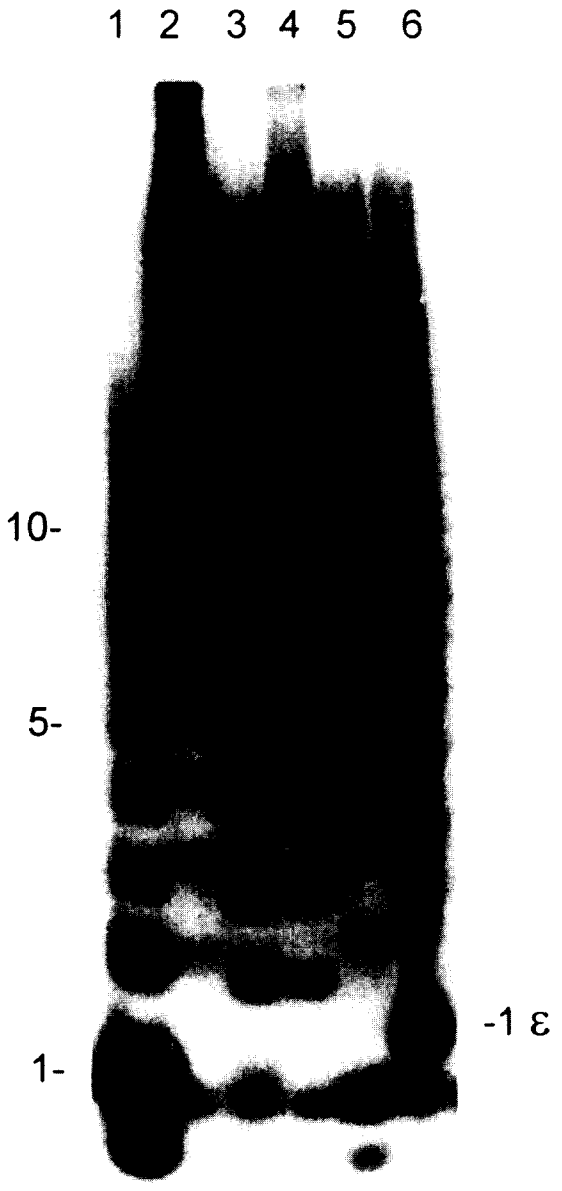

Fig. 3. Size analysis of polymers synthesized from NAD^+ analogs. pADPRT (0.7 mg/ml) was incubated under standard conditions (see Section 2) in the presence of the NAD^+ analog indicated below. After 30 min at 30°C, 0.1 μM [^{32}P]NAD^+ was added and incubation was continued for a further 20 min. This very low amount of [^{32}P]NAD^+ was used to attach a radioactive label to the polymers previously formed from the analogs. After the reaction had been stopped, the resulting polymers were detached from the protein by alkaline treatment as described in Section 2. The isolated polymers were then separated by 20% PAGE. The autoradiogram of the gel is shown. Lanes: 1, no preincubation; 2, 1 mM NGD^+; 3, 1 mM NHD^+; 4, 0.1 mM NAD^+; 5, 1 mM ε-NAD^+; 6, 0.1 mM [ε-^{32}P]NAD^+ only (no further addition of [^{32}P]NAD^+). The numbers on the left indicate the amount of ADP-ribose units in the ADP-ribose oligomers. 1ε designates the position of mono(ε-ADP-ribose).

the presence of 100 μM NAD^+ (Fig. 3, compare lane 2 to 4). However, these polymers showed a different electrophoretic mobility in 20% PAG as compared to poly(ADP-ribose) (Fig. 3, compare lane 2 to 1). The existence of shifted radiolabeled bands in the gel indicated that the polymers detected by autoradiography consisted of GDP-ribose oligomers labeled with one unit of [^{32}P]ADP-ribose. Polymer sizes obtained after preincubation with NHD^+ (Fig. 3, lane 3) ranged between 2 and 30 units. The electrophoretic mobility of these IDP-ribose oligomers radiolabeled with [^{32}P]ADP-ribose was found to be identical with ADP-ribose oligomers (Fig. 3, compare lane 3 to 4). The use of ε-NAD^+ (Fig. 3, lane 5) led to ε-ADP-ribose polymers with an average size of 25–30 units per polymer, and similarly to poly(GDP-ribose), retarded electrophoretic mobility (Fig. 3, compare lane 5 to 4). Using exclusively ^{32}P-labeled ε-NAD^+ with no further addition of ^{32}P-labeled NAD^+ yielded similar results (Fig. 3, lane 6). As can be seen, ε-ADP-ribose (denoted 1ε) exhibited a significantly lower mobility than ADP-ribose (marked 1) (Fig. 3, lanes 1,6). When the reaction was carried out using only 0.1 μM ^{32}P-labeled NAD^+, oligomers with chain lengths of less than 15 units of ADP-ribose were obtained (Fig. 3, lane 1). The presence of 1 mM 3-aminobenzamide in the reaction mixture resulted in mono ADP-ribosylated enzyme (data not shown), which is in accordance with results reported by Alvarez-Gonzalez [17].

Since ε-NAD^+ and its derivatives are fluorescent, they represent valuable tools for the determination of enzyme activities or mechanistic studies. It was, therefore, attempted to synthesize large polymers of ε-ADP-ribose. As the enzymatic synthesis from ε-NAD^+ was rather slow (see above), poly(ADP-ribose) was enzymatically synthesized and then chemically converted to poly(ε-ADP-ribose) by treatment with chloroacetic aldehyde (see Section 2). It is shown in Fig. 4B that degradation of these polymers by phosphodiesterase is accompanied by a substantial increase of fluorescence. While both snake venom phosphodiesterase and NADase from *Neurospora crassa* (Sigma) were able to hydrolyze ε-NAD^+ (Fig. 4A), the NADase did apparently not hydrolyze poly(ε-ADP-ribose).

4. Discussion

In this study analogs of NAD^+ were tested as substrates rather than as effectors (inhibitors) of poly(ADP-ribosyl)ation of pADPRT. It has been demonstrated that pADPRT may utilize NAD^+ analogs containing a modified purine base to form polymers similar to poly(ADP-ribose). However, the modifications analyzed here caused substantial retardation of the poly(ADP-ribosyl)ation reaction. It is striking that the deaminated form (NHD^+) was a rather poor substrate, whereas NGD^+ carrying an additional amino group at position 2 of the purine ring (cf. Fig. 1) appeared to be utilized more readily than NHD^+. These observations indicate that the presence of an amino group within the purine base may be important for efficient catalysis. This suggestion is further supported by the results obtained using ε-NAD^+. The free amino group of NAD^+ reacts to form the etheno derivative, ε-NAD^+ (cf. Fig. 1). As a consequence, this compound serves as a rather poor substrate, similar to NHD^+. The amino group within the purine base could be involved in the binding of NAD^+ to the enzyme.

The mobility of GDP-ribose and ε-ADP-ribose oligomers could be clearly distinguished from that of poly(ADP-ribose) in the gel system used. This observation provides direct evidence that indeed ε-NAD^+ and NGD^+ and not residual NAD^+ (ε-NAD^+ is synthesized from NAD^+) were used as substrate. Since, in addition, ε-NAD^+ and NGD^+ reacted only slowly, they may be valuable tools for kinetic and me-

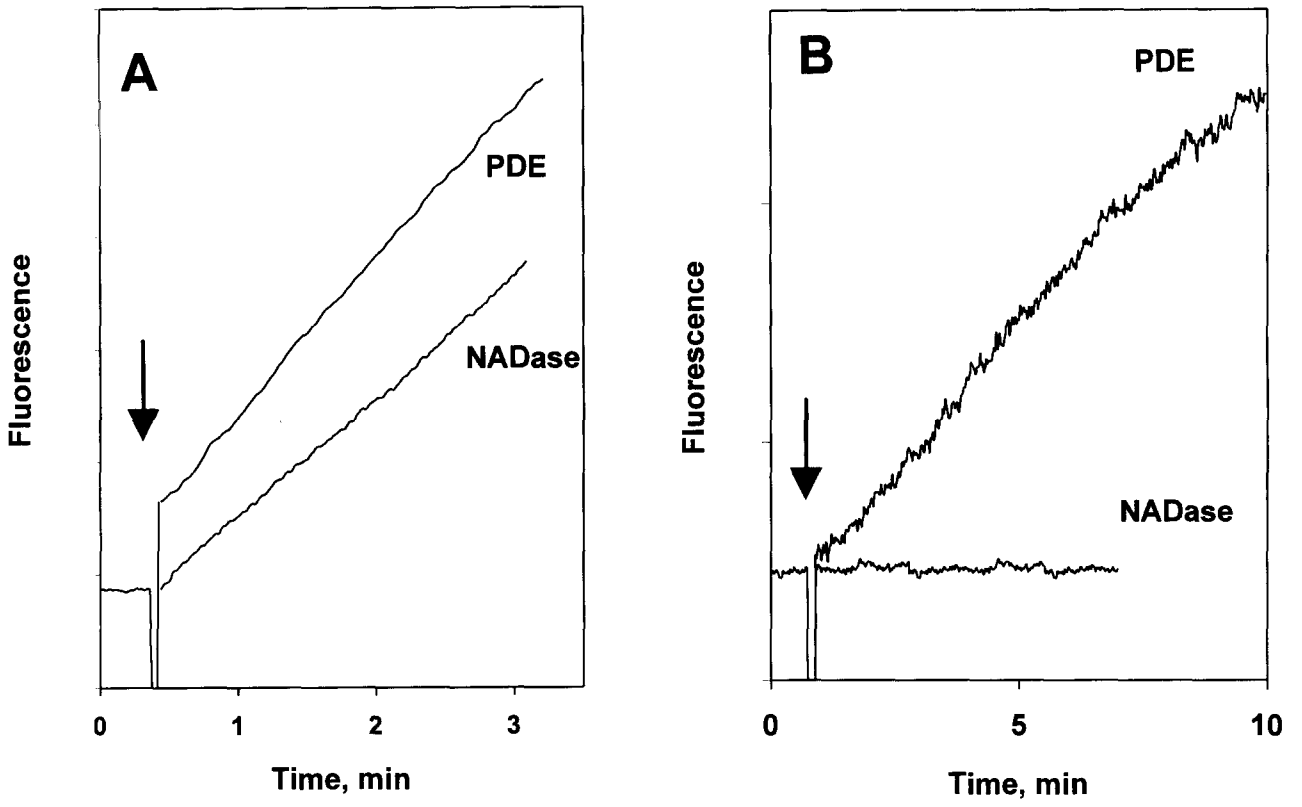

Fig. 4. Degradation of ε-NAD^+ and ε-ADP-ribose polymers by NADase from *Neurospora crassa* or snake venom phosphodiesterase (PDE). Fluorescence assays were carried out as described in Section 2 in the presence of 50 μM ε-NAD^+ (A) or poly(ε-ADP-ribose) (B). The concentration of poly(ε-ADP-ribose) corresponded to approx. 0.3 μM of ε-ADP-ribose units. The arrow indicates the addition of the respective enzyme.

chanistic studies, for example, of the mode of initiation, elongation, and branching [17]. To date, experiments that addressed these steps were conducted either by varying the concentration of NAD^+ [8] or using 3′-deoxy-NAD^+ [18]. However, 3′-deoxy-NAD^+ has the limitation that oligomers with only up to four ADP-ribose units may be synthesized by pADPRT.

This study extends the applications of $1,N^6$-etheno derivatives of adenine-containing nucleotides. When polymers of ε-ADP-ribose are hydrolyzed a significant fluorescence increase can be observed, presumably due to the separation of the packed fluorophores. As shown herein the combined use of ε-NAD^+ and poly(ε-ADP-ribose) chains permits one to distinguish between NADase and phosphodiesterase activities. While the NADase cleaved only ε-NAD^+, the phosphodiesterase from snake venom used both compounds as substrates. Poly(ADP-ribose) glycohydrolase from human placenta cleaved poly(ε-ADP-ribose) but not ε-NAD^+ (not shown). Therefore, this test should also provide a fast and non-radioactive alternative for the detection of poly(ADP-ribosyl) glycohydrolase activity.

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