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SYNTHESIS AND PURIFICATION OF DEOXYRIBOSE ANALOGUES OF NAD^+ BY AFFINITY CHROMATOGRAPHY AND STRONG-ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Two different chemical analogues of NAD^+, containing either 2′-deoxyribose (2′-$dNAD^+$) or 3′-deoxyribose (3′-$dNAD^+$) were synthesized enzymatically with oxidized nicotinamide mononucleotide (NMN^+)–adenyl transferase (E.C. 2.7.7.18). These compounds were efficiently purified by affinity chromatography on a boronate gel, followed by strong-anion-exchange high-performance liquid chromatography under isocratic conditions. These chromatographic steps resulted in the elimination of unreacted deoxyadenosine triphosphates (dATP) and NMN^+, respectively. The purified $dNAD^+$ isomers were examined as possible substrates of homogeneous poly(ADP-ribose) polymerase, purified from calf thymus. 3′-$dNAD^+$ was effectively utilized as a substrate by the polymerase, which catalyzed the formation of protein-bound poly(3′-dADP-ribose) during automodification. However, 2′-$dNAD^+$ was not a substrate for the automodification reaction catalyzed by this DNA-dependent enzyme. Instead, 2′-$dNAD^+$ was a potent non-competitive inhibitor of NAD^+ in the elongation reaction.

INTRODUCTION

Several chromatin proteins of higher eukaryotes have been shown to be covalently modified with homopolymers of ADP-ribose[1]. This post-translational reaction is catalyzed by the DNA-dependent enzyme poly(ADP-ribose) polymerase (E.C. 2.4.2.30), which utilizes NAD^+ as the substrate[2,3]. Due to the molecular size of the modifying polymer, which can be as long as over 200 residues of ADP-ribose[4], it has been suggested that the poly(ADP-ribosylation) of proteins plays a major role in modulating changes in chromatin structure that take place during various cellular functions; *i.e.*, gene expression[5], cell differentiation[6,7], DNA replication[8], and DNA excision repair[9,10]. Cellular recovery from DNA damage seems to be more directly related to the poly(ADP-ribosylation) of chromatin proteins because of the sensitivity of poly(ADP-ribose) polymerase to stimulation by DNA strand breaks[11].

Poly(ADP-ribose) polymerase, a single polypeptide of 116 kilodalton, catalyzes the initial covalent attachment of an ADP-ribose residue to a protein acceptor, as

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well as the subsequent chain elongation[12]. In the latter reaction, the enzyme catalyzes the formation of a 2'-1" O-glycosidic linkage between two ADP-ribose residues[13]. Thus, the 2'-hydroxyl group of the adenine proximal ribose of one ADP-ribose residue is covalently bound to the 1"-hydroxyl group of the next residue. In order to characterize the elongation reaction catalyzed by this enzyme further, highly purified 2'-deoxyribose-NAD^+ (2'-$dNAD^+$) and 3'-$dNAD^+$ were examined as potential substrates for homogeneous poly(ADP-ribose) polymerase from calf thymus. The results obtained indicate that while 3'-$dNAD^+$ is a good substrate for the enzyme automodification reaction, 2'-$dNAD^+$ is not.

MATERIALS AND METHODS

Materials

Benzamide, oxidized nicotinamide mononucleotide (NMN^+), 2'-deoxy ATP (2'-dATP), 3'-dATP, inorganic pyrophosphatase, and bacterial alkaline phosphatase, Type II-S, were obtained from Sigma (St. Louis, MO, U.S.A.); Affi-gel 601 from Bio-Rad (Richmond, CA, U.S.A.); NMN^+–adenyltransferase from Boehringer Mannheim (Indianapolis, IN, U.S.A.); the Partisil 10-SAX column (250 × 4.6 mm I.D.) and packing material from Whatman (Clifton, NJ, U.S.A.); radiolabeled [α-^{32}P]2'-dATP (>3000 Ci/mmol) and [α-^{32}P]3'-dATP (>5000 Ci/mmol) from New England Nuclear (Boston, MS, U.S.A.). All other chemicals used were of reagent grade.

Synthesis and purification of $dNAD^+$ analogues

Radiolabeled [adenyl-^{32}P]2'-$dNAD^+$ and 3'-$dNAD^+$ were synthesized by incubating for 2 h at 37°C a total volume of 1.0 ml, containing 100 m*M* glycyl–glycine buffer (pH 7.4), 10 m*M* magnesiumchloride, 3.0 m*M* NMN^+, 0.5 m*M* [α-^{32}P]2'-ATP or 3'-dATP (3 Ci/mol), 3 units of inorganic pyrophosphatase, and 0.2 units of NAD^+ pyrophosphorylase. The reaction was stopped by cooling the mixture to 0°C. It was immediately loaded on a 1.0-ml column of Affi-gel 601 to erliminate unreacted dATP, as described previously[14]. The boronate purified-material was lyophilized and incubated with 3 units of bacterial alkaline phosphatase for 1 h at 37°C in 1.0 ml of 10 m*M* Tris–HCl buffer (pH 8.0). The incubation mixture was then analyzed by high-performance liquid chromatography (HPLC) on the Partisil 10-SAX column, preceded by a guard column (75 × 2.1 mm I.D.) containing the same material with 50 m*M* potassium dihydrogenphosphate (pH 4.7) as the buffer system at a flow-rate of 1.0 ml/min. The fractions containing the radiolabeled $dNAD^+$ analogue were collected, pooled and further concentrated on a 1.0-ml column of Affi-gel 601. The boronate-purified material was lyophilized, redissolved in a small volume of water and stored at −20°C until used. Typically, about 20–30% of the initial radiolabel was recovered as pure 2'-$dNAD^+$ or 3'-$dNAD^+$.

Purification and assay of poly(ADP-ribose) polymerase

The enzyme was purified from calf thymus by the procedures of both Niedergang *et al.*[15] and Zahradka and Ebisuzaki[16]. Similar results were obtained with both enzyme preparations when using either 2'-$dNAD^+$ or 3'-$dNAD^+$ as a substrate. Poly(ADP-ribose) polymerase activity was determined by measuring the total

amount of radiolabeled substrate incorporated into 20% (w/v) trichloroacetic acid (TCA)-isoluble material. Typically, a total volume of 0.1 ml, containing 100 m*M* Tris–HCl buffer, (pH 8.0), and 10 m*M* magnesium chloride, 1.0 m*M* dithiotreitol (DTT), 2.7 pmol of pure enzyme and either 10 μM of [^{14}C]NAD$^+$ (400 cpm/pmol) or 10 μM [^{32}P]2′-dNAD$^+$ or 3′-dNAD$^+$ (4000 cpm/pmol), was incubated at 25°C for 5 min. All incubations were minimally performed in duplicate. The reaction was stopped by the addition of 0.9 ml of 20% (w/v) TCA and 50 μl of 1% bovine serum albumin at 0°C. The suspension was then filtered through Whatman GF/C filter papers, 2.4 cm in diameter, the filters were rinsed three times with 20% TCA and twice with diethyl ether, and the radioactivity was determined by liquid scintillation counting. Alternatively, aliquots of the incubation were analyzed by electrophoresis on a 10% polyacrylamide gel.

Polyacrylamide gel electrophoresis

Pure poly(ADP-ribose) polymerase from calf thymus was electrophoresed on a 10% lithium dodecyl sulphate polyacrylamide gel for 6 h at 300 V (constant voltage), following incubation with either [adenyl-^{32}P]2′-dNAD$^+$ or 3′-dNAD$^+$ for 5 min at 25°C in the absence or presence of 1 m*M* benzamide. The gel was then stained with 0.1% Coomasie Blue, destained, dried on a filter paper, and exposed to X-ray film overnight, at room temperature.

RESULTS

Two deoxyribose analogues of NAD$^+$, 2′-dNAD$^+$ and 3′-dNAD$^+$, were enzymatically synthesized from either 2′-dATP or 3′-dATP and NMN$^+$ with NMN$^+$–adenyltransferase (NAD$^+$ pyrophosphorylase), as specified in Materials and methods. As shown in Table I, the elimination of unreacted dATP was achieved by affinity chromatography on Affi-gel 601[14], which allows quantitative binding of ribonucleotides while deoxyribonucleotides, which lack a vicinal set of hydroxyl groups do not bind to the column. Thus, 2′-dNAD$^+$ and 3′-dNAD$^+$ effectively bind to the boronate gel via the nicotinamide ribose moiety. The large amount of NMN$^+$ contaminating dNAD$^+$ following boronate chromatography, was removed by strong-anion-exchange HPLC under isocratic conditions, following enzyme digestion of the boronate-purified material with bacterial alkaline phosphatase as described in Materials and methods. This enzymatic treatment resulted in the conversion of NMN$^+$

TABLE I

BINDING OF ADENINE-CONTAINING DEOXYNUCLEOTIDES TO AFFI-GEL 601

Adenine-containing nucleotide	*Loaded on column (cpm)*	*Bound to column (cpm)*	*Percentage of total bound*
2′-dATP	246 000	280	0.11
3′-dATP	223 000	187	0.08
2′-dNAD$^+$	249 000	219 840	88.28
3′-dNAD$^+$	248 000	221 430	89.29

TABLE II

HPLC RETENTION TIMES OF PYRIDINE NUCLEOTIDES ON A PARTISIL-10 SAX COLUMN

Nucleotide	*Retention time (min)*
Nicotinamide-ribose	3.45
NMN^+	6.85
NAD^+	7.60
2′-$dNAD^+$	9.22
3′-$dNAD^+$	9.75
NADH	18.25

to nicotinamide ribose, which was not retained by the Partisil 10-SAX column and thus eluted ahead of the phosphatase insensitive ^{32}P-radiolabeled $dNAD^+$, as indicated in Table II. Thus, the fractions containing the radiolabeled $dNAD^+$ analogue were collected, desalted, and stored as indicated above.

Pure 2′-$dNAD^+$ and 3′-$dNAD^+$ were separately incubated with homogeneous poly(ADP-ribose) polymerase from calf thymus, in a concentration of 10 μM for 5 min at 25°C in the absence or presence of 1.0 mM benzamide, a potent competitive inhibitor of poly(ADP-ribose) biosynthesis. Table III shows that while 3′-$dNAD^+$ was effectively used as a substrate by the polymerase in the automodification reaction, 2′-$dNAD^+$ was not incorporated into acid-insoluble material, indicating that it is not a substrate for the pure enzyme. It is important to note that, the total specific activity of the enzyme with 3′-$dNAD^+$ as a substrate is about 20 times lower than the specific activity obtained with the natural substrate, NAD^+, under identical conditions. This may be due to differences in the rates of elongation. Nevertheless, the inhibition of the incorporation of [adenylate-^{32}P]3′-$dNAD^+$ into acid-insoluble material by benzamide, demonstrates the enzymatic nature of the reaction. In order to substantiate that 3′-$dNAD^+$ is a substrate for the automodification of poly(ADP-ribose) polymerase and that 2′-$dNAD^+$ is not, aliquots of the enzyme incubation reactions were

TABLE III

UTILIZATION OF NAD^+, 2′-$dNAD^+$ AND 3′-$dNAD^+$ AS SUBSTRATES OF HIGHLY PURIFIED POLY(ADP-RIBOSE) POLYMERASE FROM CALF THYMUS AT A CONCENTRATION OF 10 μM AT 25°C IN THE PRESENCE AND ABSENCE OF 1 mM BENZAMIDE

Substrate	*Benzamide (1 mM)*	*Enzyme activity (pmol inc/min/μg protein)*
NAD^+	+	0.2 ± 0.1 (n = 5)
NAD^+	–	65.2 ± 1.8 (n = 5)
3′-$dNAD^+$	+	0.12 ± 0.01 (n = 5)
3′-$dNAD^+$	–	3.52 ± 0.4 (n = 5)
2′-$dNAD^+$	+	N.D.*
2′-$dNAD^+$	–	N.D.*

* No incorporation of high-specific-radioactivity [adenyl-^{32}P]2′-deoxy NAD^+ into 20% trichloroacetic acid-insoluble material.

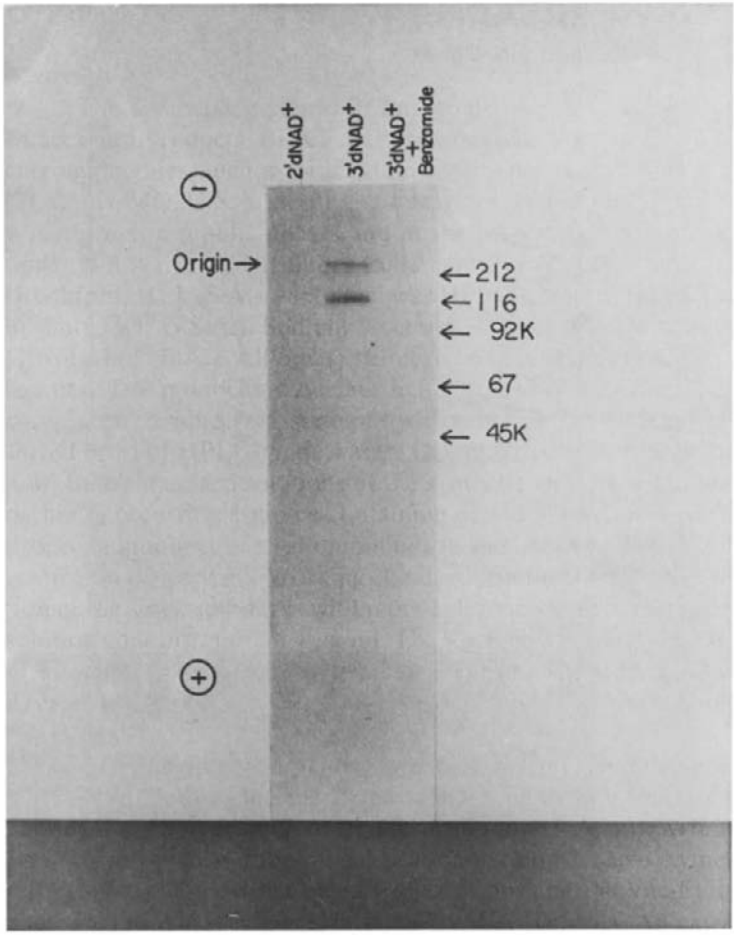

Fig. 1. Polyacrylamide gel electrophoresis of poly(ADP-ribose) polymerase following incubation with [^{32}P]radiolabeled 2'-dNAD$^+$ and 3'-dNAD$^+$ in the absence and presdence of benzamide. The molecular weight markers used were: myosin (212 kilodalton); β-galactosidase (116 kilodalton); phosphorylase *b* (92 kilodalton); bovine serum albumin (67 kilodalton) and ovalbumin (45 kilodalton).

electrophoresed on a 10% polyacrylamide gel. Fig. 1 shows that a ^{32}P-labeled band migrates at the position of the 116-kilodalton marker, following incubation of the polymerase with ^{32}P-labeled 3'-dNAD$^+$ which correlates with the migration of automodified enzyme as previously shown by others[17,18]. Again, the presence of benzamide in the incubation mixture totally prevented the labelling of the enzyme (Fig. 1). Incubation of the enzyme with [^{32}P]2'-dNAD$^+$ under identical conditions did not result in the appearance of a labeled protein migrating with the enzyme. This result, further demonstrates that 2'-dNAD$^+$ is not a substrate for poly(ADP-ribose) polymerase.

In contrast to the results described above, 2'-dNAD$^+$ was found to be a potent non-competitive inhibitor of NAD$^+$ in the automodification reaction catalyzed by poly(ADP-ribose) polymerase. Fig. 2 shows the Dixon plot from which an apparent

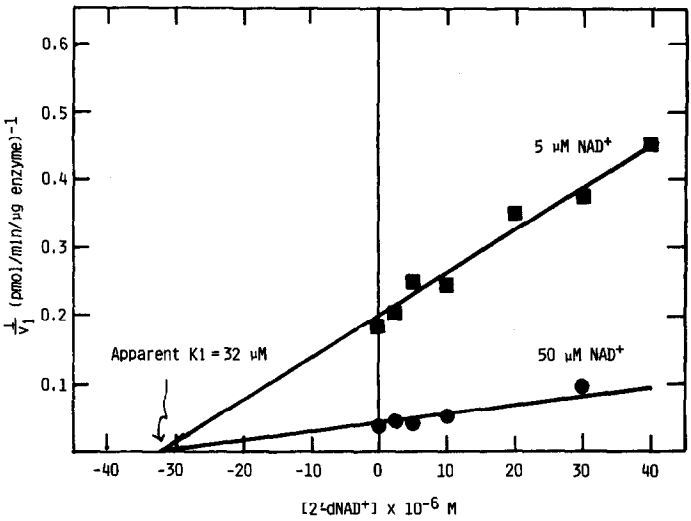

Fig. 2. Determination of the apparent inhibition constant for 2'-dNAD$^+$ in the automodification reaction of poly(ADP-ribose) polymerase by the Dixon plot.

inhibition constant (K_i) of 32 μM was calculated for 2'-dNAD$^+$ as an inhibitor of poly(ADP-ribose) elongation.

DISCUSSION

A chemical analogue of NAD$^+$, containing 2'-deoxyribose proximal to the adenine ring, has previously been synthesized by Suhadolnik *et al.*[19]. However, a simple and reproducible purification procedure for this and other dNAD$^+$ analogues has not been reported. The complete purification of 2'-dNAD$^+$ and other NAD$^+$-related compounds is essential for their characterization as substrates of NAD$^+$ utilizing enzymes. One NAD$^+$ utilizing enzyme activity is poly(ADP-ribose) polymerase, which forms the 2'-1"-O-glycosidic linkage between ADP-ribose units during the $(ADP\text{-}ribose)_n$ covalent modification of chromatin proteins. Potent inhibitors of this enzyme activity include several nicotinamide-containing compounds such as nicotinamide itself, nicotinamide ribose and NMN$^+$ (refs. 1–3). Therefore, it is necessary to quantitatively remove these compounds from dNAD$^+$ preparations obtained via NAD$^+$ pyrophosphorylase, where a large amount of unreacted NMN$^+$ may be present. In this study, the successful purification of both 2'-dNAD$^+$ and 3'-dNAD$^+$ by affinity chromatography on a boronate resin followed by strong-anion-exchange HPLC under isocratic conditions, with a low-salt buffer system, is reported. Moreover, the separation of either one of the two dNAD$^+$ isomers from NAD$^+$ by strong-anion-exchange HPLC, eliminates small amounts of contaminating NAD$^+$ thay might be endogenously bound to the enzyme NAD$^+$ pyrophosphorylase. Here, it is concluded that purified 2'-dNAD$^+$ is not substrate for homogeneous poly(ADP-ribose) polymerase, based on the lack of incorporation of radiolabeled 2'-dNAD$^+$ into acid-insoluble material (Table III), and the absence of the characteristic labeled band

of the automodified enzyme following polyacrylamide gel electrophoresis (Fig. 1). This result is not totally unexpected because the 2'-hydroxyl group of the non-reducing end of the polymer functions as the acceptor for the ADP-ribose residue produced during poly(ADP-ribose) elongation[12]. Furthermore, the non-competitive inhibition of poly(ADP-ribose) biosynthesis by 2'-dNAD$^+$, indicates that this dNAD$^+$ analogue binds to an enzyme site that does not correspond to the NAD$^+$ binding site. It is tempting to speculate that 2'-dNAD$^+$ is a competitive inhibitor of the second substrate of the elongation reaction, *i.e.*, the non-reducing end of the growing polymer. Further experiments are in progress to answer this specific question. Finally, the utilization of 3'-dNAD$^+$ as a substrate by poly(ADP-ribose) polymerase should prove useful in determining the kinetic and chemical enzyme mechanisms by which the (ADP-ribose)$_n$ covalent modification of specific chromatin proteins takes place.

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