Cyclic ADP-Ribose: Synthesis and Structural Assignment

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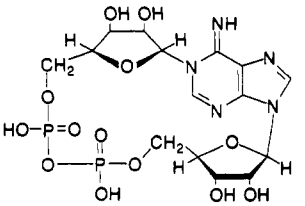
Abstract: Cyclic ADP-ribose (cADPR) is a naturally occurring cyclic nucleotide and a potent mediator of calcium mobilization in many mammalian tissues. Previous studies have shown that cADPR is synthesized from β-NAD^+ via the scission of the nicotinamide–ribose linkage and cyclization by forming a new bond between the ribose and the nitrogen of the adenine ring. However, the position and stereochemistry of this newly formed linkage were not unequivocally determined. In this study we have established that cADPR has the anomeric carbon of the ribose attached onto the N^1-nitrogen of the adenine nucleus via a β-*N*-glycosyl linkage. The structural assignment was made by correlating cADPR to N^1-(5′-phosphoribosyl)AMP, a known intermediate of histidine biosynthesis. This was achieved by cleaving the pyrophosphate bond of cADPR under conditions (DMSO/*tert*-butoxide) not perturbing the integrity of the C–N glycosyl bond. Furthermore, cADPR was successfully synthesized by cyclization of N^1-(5′-phosphoribosyl)ATP catalyzed by NAD^+ pyrophosphorylase in an organic solvent–aqueous medium.

Introduction

Many cellular functions are modulated by the concentration of intracellular calcium ions.[1] Two major mechanisms of calcium mobilization are known that utilize calcium stored in cytoplasmic compartments for signaling. In epithelial and blood cells, the predominant mechanism of Ca^{2+} release is triggered by the interaction of the second messenger, inositol 1,4,5-triphosphate (IP_3),[2] with its receptor, a ligand-activated calcium-selective channel. The binding of IP_3 promotes channel opening, allowing Ca^{2+} to flow into the cytoplasm. A second class of intracellular calcium-releasing channels is the ryanodine receptor.[3] These receptors are present in muscle[4] and the brain[5] and may be activated by the plant alkaloid, ryanodine, and caffeine. Although the physiological activator of this receptor is unknown, it can be activated by Ca^{2+}, causing the so called Ca^{2+}-induced Ca^{2+} release (CICR).[6] One candidate is a metabolite of β-NAD^+, cyclic ADP-ribose (cADPR), which was shown to be as potent as IP_3 in mobilizing intracellular Ca^{2+} stores in sea urchin eggs[7] and in rat pituitary cells.[8] The widespread occurrence of the ryanodine receptor and cADPR in mammalian tissues suggests that cADPR may be an endogenous messenger for ryanodine receptors.[9] Several other recent studies[10] are consistent with this proposition.

Cyclic ADP-ribose (cADPR)[11] is a metabolite of β-NAD^+, and its synthesizing enzyme, ADP-ribosyl cyclase,[12] has been shown to be present in various mammalian and invertebrate tissues. In 1989, Lee and co-workers[13] presented structural evidence indicating that the metabolite is a cyclized ADP-ribose having an *N*-glycosyl linkage between the anomeric carbon of the terminal ribose unit and the N^6-amino group of the adenine moiety. However, the spectral data did not contain enough information to allow for the assignment of the stereochemistry of the newly formed C–N bond. In view of the potential physiological importance of cADPR as a second messenger analogous to IP_3 in Ca^{2+} signaling, we have reexamined the data and found them to be at variance with the proposed structure. In this report, we describe the synthesis and the experiments used in establishing the unique cyclic structure of cADPR as **1**.



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Results and Discussion

A soluble form of the enzyme, ADP-ribosyl cyclase, has been purified[14] and cloned[15] from the ovotestis of the marine mollusk *Aplysia californica*, but unfortunately, these systems are not accessible for general use. Hence, we turned our attention to the use of NAD^+ glycohydrolases (NADases), enzymes that are widely distributed among mammalian tissues and were shown to catalyze the synthesis of cADPR from β-NAD^+.[16,17] The specific activities (nmol/mg of protein/h) of the NADases in most tissues were too low to be synthetically useful. However, we have found that freshly prepared acetone powders of pig brain have good cADPR synthesizing activities of around 300 as compared to the published preparations from sea urchin eggs (0.3)[17] and dog brain (63).[17]

cADPR was prepared by exposure of β-NAD^+ to this acetone powder, and an HPLC elution profile of the reaction mixture is shown in Figure 1. In this system, cADPR has a retention time of 13.5 min, accompanied by the competing hydrolytic products ADP-ribose (ADPR) (20.0 min) and nicotinamide (8.8 min).

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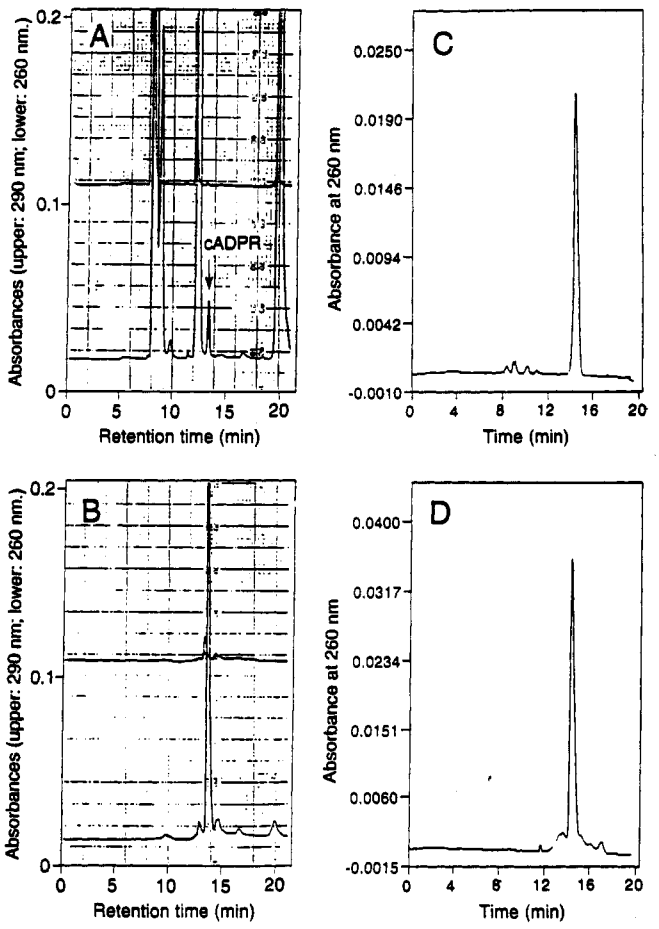
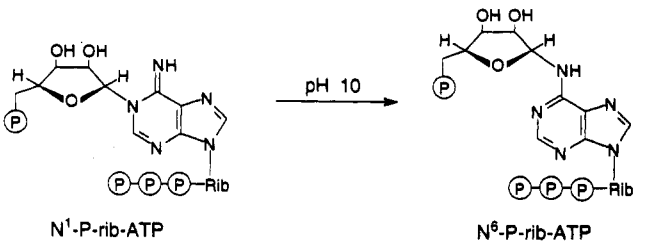


Figure 1. Isolation and characterization of the cADPR synthesized from β-NAD$^+$ using pig brain acetone powder. (A) Anion-exchange HPLC analysis of the reaction mixture on a SynChropak AX-100 column (250-× 7.8-mm i.d., 5 μm). The elution conditions are described in the Experimental Section. (B) Anion-exchange HPLC analysis of standard cADPR (purchased from Amersham). (C) Capillary electrophoresis of the cADPR on an uncoated silica capillary (50 μm × 50 cm) using the BioFocus 3000 CE system (sample loading, electrophoretic, 10 kV for 5 s; running buffer, pH 8.2, 0.1 M sodium phosphate; voltage, 15 kV; 20 °C; 260 nm). (D) Capillary electrophoresis of standard cADPR performed under the same conditions.

The cADPR fractions were combined and further purified by chromatography on a Waters C_{18} Nova-Pak column. The purity of the cADPR preparation was confirmed by capillary electrophoretic analysis using an uncoated silica capillary on a BioFocus 3000 CE system. The yield of cADPR varied between 1.5 and 4% depending on the reaction conditions. The retention times of the biosynthesized cADPR were found to be identical to those of an authentic specimen of cADPR purchased from Amersham using capillary electrophoresis and HPLC (reverse phase and anion exchange). Moreover, the ^{1}H-NMR spectrum was identical to the published spectra of cADPR.[13] The exact mass of the (M − H)$^-$ ion was measured to be 540.0535, corresponding to the calculated (M − 1)$^-$ ion of cADPR with an accuracy of better than 1 ppm. This enzymatic procedure furnished us with adequate quantities of cADPR for our structural investigations.

The proposed position of the *N*-glycosyl linkage in the putative structure of cADPR was between the anomeric carbon of the terminal ribose unit and the N^6-amino group of the adenine moiety.[13] This structural assignment was based largely on the observation that, under their unspecified conditions, cADPR had a UV spectrum similar to those of NAD$^+$ and ADPR. They reasoned that linkage to any of the ring nitrogens such as the N^1-position would introduce a positive charge on the adenine nucleus and that the UV spectra of such compounds would differ

Scheme 1. Base-Catalyzed Transformation of N^1-P-rib-ATP to N^6-P-rib-ATP



from those of NAD$^+$ and ADPR. At pH 7.0, we found that the UV spectrum of cADPR, notably the ratio of absorbances at 260/290, was clearly different from that of ADPR. Since cADPR was hydrolyzed by NADase to yield ADPR,[13] these results suggested that the newly formed *N*-glycosyl linkage in cADPR was with the N^1-nitrogen of the adenine ring. To clarify the spectral properties of cADPR, it is imperative to employ model compounds whose relevant structural features coincide closely with those of cADPR. A careful search of the literature reveals that the compound N^1-(5′-phosphoribosyl)ATP meets the structural requirements. This intermediate of the histidine biosynthetic pathway was synthesized using ATP-phosphoribosyl transferase isolated from the *Salmonella typhimurium* strain TA 453, a his01242hisEIFAN double mutant, following a published procedure.[18] This organism lacks the E, I, F, A, and N genes and also contains a mutation in the histidine operator; it has about 15 times the level of ATP-phosphoribosyl transferase as compared to the repressed wild-type strain. Exposure of phosphoribosylpyrophosphate (P-rib-P-P) and ATP to ATP-phosphoribosyl transferase (partially purified cell extract using a three-step purification procedure) provided N^1-(5′-phosphoribosyl)ATP (N^1-P-rib-ATP) in about 14% yield.

It is well-known that N^1-alkylated adenosine derivatives undergo the Dimroth rearrangement in base to give the N^6-derivative.[19] Numerous mechanistic studies, including ^{15}N-isotope labeling, have shown that this rearrangement proceeds through adenine ring opening without cleavage of the N^1-alkyl bond. In fact, Ames[20] has observed that N^1-P-rib-ATP underwent an analogous rearrangement at pH 10 (Scheme 1). Therefore, using these series of reactions, we prepared both the N^1-, and N^6-isomers of P-rib-ATP for our spectroscopic studies without disturbing the stereochemistry at C-1′.

It is known that N^1-P-rib-ATP has a pK_a of 8.8 and a molar extinction coefficient (ε) at 290 nm (pH 8.5) of 3600, with an A_{260}/A_{290} = 6–7,[20] which is affected by changes in pH. The pH titration curve of cADPR showed that it has a pK_a of 8.2 and ε = 3200 at 290 nm and A_{260}/A_{290} = 3.9 at pH 8.5. The absence of a pK_a at pH 4.0, corresponding to the pK_a of the amino group of ADPR or AMP, indicates that cADPR, like N^1-P-rib-ATP, forms an imino base rather than an amino base. Also, the ratios of the absorbances at 260–290 nm (A_{260}/A_{290}) for N^1-P-rib-ATP, N^6-P-rib-ATP, ADPR, and cADPR were determined and compared at various pH values under the same conditions (Table 1). A very similar pattern (A_{260}/A_{290}) was observed for cADPR and N^1-P-rib-ATP when the pH of the medium varied from basic to acidic. That is, protonation of N^1-P-rib-ATP and cADPR under acidic conditions resulted in a decrease in absorbance at 290 nm, thereby increasing the ratio of A_{260}/A_{290}. This spectral behavior may be explained by the transformation of the imino structure, which has a higher extinction coefficient at 290 nm, to the N-1 cationic form of the amino structure which predominates under more acidic conditions (Scheme 2). In contrast, the

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Table 1. Effect of pH on the Ratio of Absorbances at 260–290 nm (A_{260}/A_{290})[a]

pH	N^1-P-rib-ATP	N^6-P-rib-ATP	cADPR	ADPR
10.5	5	60	2.4	58
8.5	6–7	60	3.9	50
7.0	11	60	6.0	44
4.5	30	60	23	43
3.0	35	60	30	38

[a] A_{260}/A_{290} ratios for N^1-P-rib-ATP, N^6-P-rib-ATP, ADPR, and cADPR were determined at different pH values. Each sample (20 μL, 1 mM) was diluted with 1 mL of 0.1 M of the following buffers: 50 μM glycine, pH 10.5; 50 μM Tris-HCl, pH 8.5; 50 μM sodium phosphate, pH 7.0; 50 μM sodium acetate, pH 4.5; 50 μM sodium pyrophosphate, pH 3.0.

Scheme 2. Tautomerization of cADPR and N^1-P-rib-ATP under Acidic Conditions

A_{260}/A_{290} of N^6-P-rib-ATP was unaffected by changes in pH. These spectral data further confirm that the newly formed *N*-glycosyl bond is attached onto the N^1-nitrogen of the adenine ring rather than to the N^6-nitrogen as proposed. A recent publication from Jacobson's group arrived at the same conclusion.[21]

Further, Huckel molecular orbital calculations reveal that the N^1-site of adenine is negatively charged whereas the N^6-site is positively charged and the most basic ring nitrogen of adenine is that at the N^1-position.[22] Hence, methylation of adenine derivatives with alkylating agents give N^1-alkyladenosine derivatives as main products. These theoretical considerations are consistent with the view that enzymatic cyclization of ADPR proceeds via the union of the C-1′ position of the ribose to the N^1-nitrogen of the adenine ring. To ascertain the structure and the stereochemistry of the newly formed *N*-glycosyl bond in cADPR, we found it necessary to correlate cADPR to intermediates of known absolute stereochemistry via degradation and synthesis.

Histidine is biosynthesized by microorganisms from ATP and P-rib-P-P through a series of 10 enzymatic steps.[23] The first two steps of the pathway produce N^1-(5′-phosphoribosyl)ATP and N^1-(5′-phosphoribosyl)AMP (Scheme 3). The first enzyme, ATP-phosphoribosyltransferase, is feedback inhibited by histidine and has been the most extensively studied one of the pathway. It catalyzes the substitution of the C-1 ribose carbon of P-rib-P-P which is in α-linkage to the pyrophosphate. The stereochemistry of this C–N bond forming reaction was extensively investigated by several investigators,[24,25] who proposed a double displacement mechanism involving a β-linked 5-phosphoribosyl enzyme intermediate which would give N^1-P-rib-ATP of α-stereochemistry. However, subsequent investigations by Chelsky and Parsons[26] using optical rotation measurements of model compounds showed that the product of the first reaction, N^1-P-rib-ATP, has the β-stereochemistry at the newly formed bond. From extensive secondary tritium kinetic isotope effect studies, these investigators put forward a carbocation mechanism to account for the exchange reaction, formation of a phosphoribosylated enzyme, and the β-stereochemistry of the product, N^1-P-rib-ATP. This postulate is consistent with other phosphoribosyltransferase reactions that utilize carbocation catalytic mechanisms; all of them result in an inversion of configuration.[27]

With this background in mind, we envisaged that a direct correlation could be made between cADPR and N^1-(5′-phosphoribosyl)AMP (N^1-P-rib-AMP) provided that one could cleave the pyrophosphate bond of cADPR under conditions not affecting the integrity of the C–N glycosyl bond. Many attempts were made to cleave the cyclic structure using pyrophosphatases from various sources including *Crotalus atrox* venom nucleotide pyrophosphatase, which normally cleaves β-NAD^+ to NMN and AMP, but unfortunately, cADPR was found to be resistant to the action of these enzymes. However, treatment of cADPR in DMSO in the presence of 10 mol equiv of *t*-BuOK at 24 °C effectively hydrolyzed the pyrophosphate linkage of cADPR to yield N^1-P-rib-AMP (Scheme 4). The retention times of this product on reverse-phase HPLC and capillary electrophoresis were found to be identical to those of a sample of N^1-P-rib-AMP prepared using the *Salmonella typhimurium* strain I-648. Cell extracts of this organism catalyzed the condensation of P-rib-P-P and ATP to yield N^1-P-rib-AMP directly.[28] N^1-P-rib-AMP also was conveniently synthesized from N^1-P-rib-ATP in high yields using enzymes such as *Crotalus atrox* venom nucleotide pyrophosphatase (90%), apyrase (90%), and dog kidney ATPase (68%). That the A_{260}/A_{290} ratio of P-rib-AMP produced from cADPR and the histidine intermediate N^1-P-rib-AMP were identical at pH 3.0 (31) and pH 8.3 (5.0) clearly indicated that the N^1-glycosyl linkage was not perturbed upon treatment with *t*-BuOK in DMSO.

Final confirmations of the structure and stereochemistry of cADPR were achieved via its synthesis (Scheme 4). Although cADPR was formed when N^1-P-rib-AMP was treated with 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride (EDCI) in 1.5 M Hepes buffer (pH 6.8), the yield was only on the order of 1%. Hence we turned our attention to its synthesis from N^1-P-rib-ATP using the enzyme NAD^+ pyrophosphorylase.[29] While this enzyme normally catalyzes the formation of NAD^+ from nicotinamide mononucleotide (NMN) and ATP, we envisaged that the analog N^1-P-rib-ATP could well serve as an alternate substrate for this enzyme. Our initial experiments in aqueous buffer at pH 7.5 afforded only a trace of cADPR (<0.1%), accompanied by the formation of N^1-P-rib-AMP as the major product. However, this observation suggested that the competing hydrolysis of the adenyl–enzyme intermediate[30] in such an aqueous environment dominated the cyclization pathway. In order to facilitate intramolecular adenylyl transfer leading to the formation of cADPR, we conducted the reaction in a mixture of organic solvents and aqueous buffer with a view to decreasing the activity of water. Of the water-miscible solvents tested, acetonitrile at a concentration of 25% was found to be the most suitable. Under these conditions, approximately 7% of cADPR was obtained by incubating N^1-P-rib-ATP with pig liver NAD^+ pyrophosphorylase for 48 h at 24 °C (Figure 2A). We also attempted the synthesis of cADPR using pyrophosphatases, with the notion that these enzymes may catalyze the cleavage of the pyrophosphate bond, followed by the transfer of the terminal phosphate group onto an acceptor. However, when we incubated snake venom nucleotide pyrophosphatase,[31] an enzyme that converts N^1-P-rib-ATP to N^1-P-rib-AMP in 90% yield, with N^1-P-rib-ATP in the buffer containing 25% acetonitrile or 20% acetone, no cyclization of N^1-P-rib-ATP was noted even after prolonged incubation; instead only N^1-P-rib-AMP was produced. Attempts to synthesize

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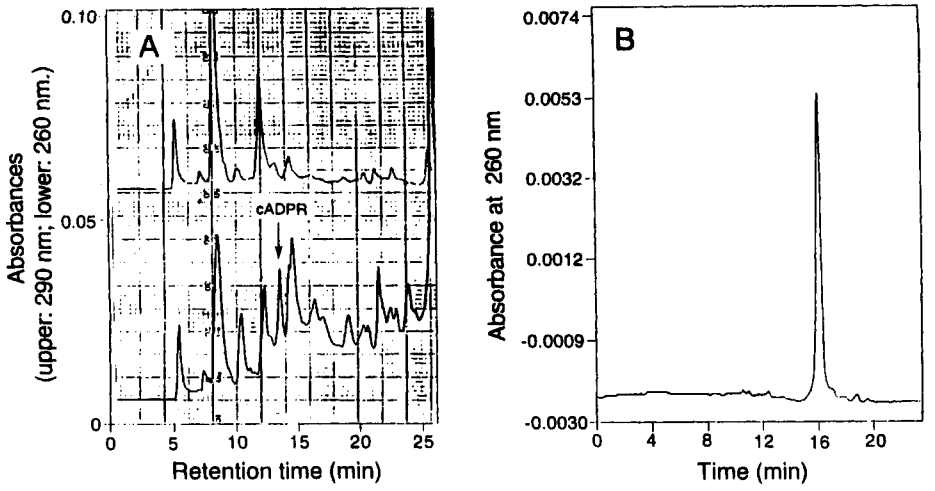
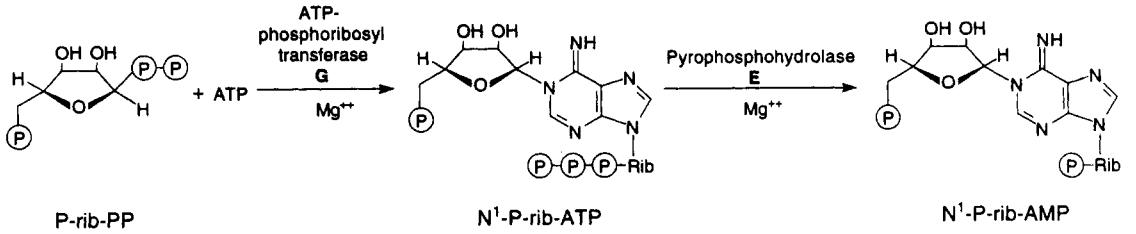
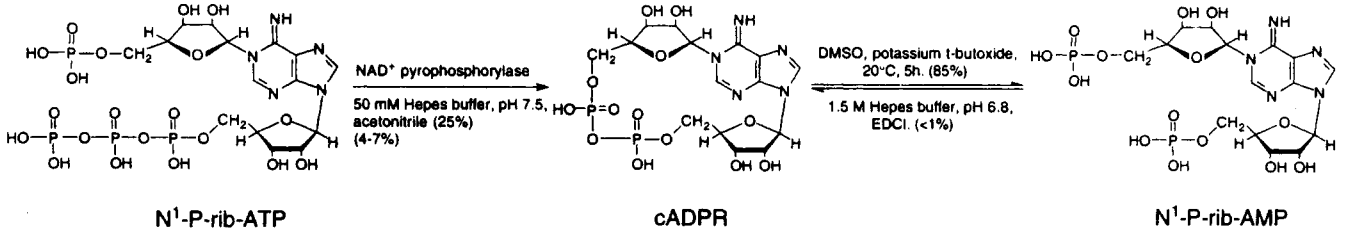


Figure 2. Synthesis of cADPR from N^1-P-rib-ATP using pig liver NAD^+ pyrophosphorylase. (A) Anion-exchange HPLC analysis of the reaction mixture on a SynChropak AX-100 column (250- × 7.8-mm i.d., 5 μm). The elution conditions are described in the Experimental Section, and the newly biosynthesized cADPR had a retention time of 13.5 min. (B) Capillary electrophoresis of a mixture of biosynthesized cADPR and authentic cADPR (1:1) on an uncoated silica capillary (50 μm × 50 cm) using the BioFocus 3000 CE system. The conditions are described in the Experimental Section. A single peak with twice the area was eluted at 16.2 min.

Scheme 3. The First Two Steps of Histidine Biosynthesis



Scheme 4. Conversion of cADPR to N^1-P-rib-AMP and Its Synthesis from N^1-P-rib-ATP



cADPR were also unsuccessful using the enzymes apyrase and ATPase, both of which have strong pyrophosphorolytic splitting activities and hydrolyze N^1-P-rib-ATP to N^1-P-rib-AMP in high yields. It is worthy of note that the protonated N^1-P-rib-ATP (a major form of the substrate at pH 7.5) with its β-N-glycosidic linkage resembles the NAD-like cation and, thus, may be accepted by NAD^+ pyrophosphorylase as a substrate to allow cyclization via a facile intramolecular adenylyl transfer.

Table 2 summarizes the reaction conditions and the yields of the products. The optimal concentration of the substrate N^1-P-rib-ATP was around 1–1.2 mM. The yield of cADPR decreased to 1.5% when the concentration of acetonitrile increased to 35%. This could be explained by the inhibition of NAD^+ pyrophosphorylase activity at high concentrations of the organic solvent, for the formation of N^1-P-rib-AMP was also decreased significantly (6.8%). Purification of cADPR was achieved using anion-exchange HPLC on a SynChropak AX-100 column followed by reverse-phase HPLC on a Waters Nova-Pak C_{18} column. As shown in Table 3, the retention times of this newly biosynthesized cADPR on two different HPLC columns were found to be identical to those of an authentic sample. The purity of this cADPR preparation was verified to be more than 95% by capillary electrophoretic analysis on an uncoated silica capillary (50 μm × 50 cm), and its behavior was indistinguishable from standard cADPR shown in Figure 2B. Its UV spectral properties were also found to be identical to those of authentic cADPR (Table 3). Its ^{1}H-NMR spectrum and negative ion fast atom bombard-

Table 2. Synthesis of cADPR from N^1-P-rib-ATP with Pig Liver NAD^+ Pyrophosphorylase[a]

organic solvents (%)	temp (°C)	time (h)	yield (%)[b] cADPR	yield (%)[b] N^1-P-rib-AMP
none	24	48	<0.1	51
acetonitrile (25)	24	24	4.2	15.8
acetonitrile (25)	24	48	7.2	20.1
acetonitrile (35)	24	48	1.5	6.8
acetone (25)	24	24	0.8	28.5
DMF (20)	24	24	<0.1	15.5

[a] The reactions were performed in a mixture of N^1-P-rib-ATP (1.16 mM), varying amounts of organic solvents, 50 mM Hepes buffer (pH 7.5) containing 20 mM $MgCl_2$, pig liver NAD^+ pyrophosphorylase (0.3 U/mL), and yeast inorganic pyrophosphatase (0.1 U/mL) in a total volume of 1 mL. Proteins were precipitated by the addition of 2% acetic acid and 50% acetonitrile and removed by centrifugation. The supernatant was analyzed by anion-exchange HPLC. [b] The yields of cADPR and N^1-P-rib-AMP were calculated from their molar extinction coefficients at 260 nm (pH 3.0) of 13 700 and 14 400, respectively, and their peak areas.

Table 3. Comparison of the HPLC Retention Times and the Spectral Properties of the Newly-Biosynthesized cADPR to Those of Authentic cADPR

sources	HPLC retention time (min)[a] Waters Nova-pak C_{18} (100- × 8-mm i.d.)	SynChropak AX-100 (250- × 7.8-mm i.d.)	SynChropak AX-100 (250- × 4.6-mm i.d.)	capillary electrophoresis[b] (50 μm × 50 mm, uncoated silica) coinjected with the standard	UV (A_{260}/A_{290}) pH 3.0	pH 7.0	pH 8.5
cADPR from β-NAD^+ by pig brain acetone powder	3.6	13.5	6.7	one single peak	30.5	6.2	3.9
cADPR from N^1-P-rib-ATP using pig liver NAD^+ pyrophosphorylase	3.6	13.5		one single peak	30.5	6.0	4.0
standard cADPR	3.6	13.5	6.7		30	6.0	4.0

[a] Waters Nova-Pak C_{18} column (100- × 8-mm i.d., 4 μm) was eluted isocratically with 4 mM formic acid at a flow rate of 1 mL/min. SynChropak AX-100 anion-exchange columns were eluted using the same procedure described in the Experimental Section. [b] Capillary electrophoretic analyses were performed on an uncoated silica capillary (50 μm × 50 cm) using the BioFocus 3000 CE system. The conditions are described in the Experimental Section.

ment mass spectral analysis exhibited a $(M - H)^-$ ion at 540 and an exact mass of 540.0533 for $(M - H)^-$, all consistent with reported values for cADPR.[13]

In conclusion, all of these experimental data confirm that the *N*-glycosyl bond in cADPR is attached onto the N^1-position of the adenine nucleus with a β-stereochemical configuration as shown in **1**. We are now exploring the non-enzymatic synthesis of cADPR from β-NAD^+. The results of these studies will be reported in future accounts.

Experimental Section

^{1}H-NMR spectra were obtained on a Bruker AM-300 spectrometer operating at 300 MHz in deuterium oxide. UV absorbances were measured using a Perkin-Elmer 559A UV/vis spectrophotometer. FAB mass spectral analyses of cADPR were carried out at the University of Nebraska, Department of Chemistry, Midwest Center for Mass Spectrometry, using a Kratos MS-50TC mass spectrometer. HPLC analyses were performed using a Waters system consisting of a 712 WISP autoinjector, two model 510 pumps, a Digital Professional 310 computer, and a 490 programmable multiwavelength detector.

Standard cADPR was purchased from Amersham Life Science. β-NAD^+, phosphoribosylpyrophosphate (P-rib-PP), nicotinamide mononucleotide (NMN), ATP, ADP, AMP, apyrase (3.3 units/mg of protein), *Crotalus atrox* venom nucleotide pyrophosphatase (4.9 units/mg of protein), and ATPase (1.1 units/mg of protein) were products of Sigma Chemical Co. Potassium *tert*-butoxide and dimethyl sulfoxide (DMSO) were products of Aldrich Chemical Co. and were of analytical grade. YM ultrafiltration membranes were products of Amicon. All other reagents were purchased from commercial sources and were of analytical grade.

Salmonella typhimurium strain TA 453 (hisO1242hisEIFAN double mutant) was obtained from Professor Hartman of Johns Hopkins University, and *Salmonella typhimurium* strain I-648 (hisI mutant) was obtained from Professor Ames of the University of California–Berkeley. N^1-P-rib-ATP was prepared by following a published procedure.[18] Rearrangement of N^1-P-rib-ATP to N^6-P-rib-ATP was accomplished by incubating N^1-P-rib-ATP (7 μmol) in 0.5 mL of 50 mM glycine buffer (pH 10.0) at 45 °C for 10 h. A Sephadex G-15 column (60 × 2.8 cm) was used for the purification of N^6-P-rib-ATP, and the column was eluted with H_2O. N^1-P-rib-AMP was biosynthesized according to Ames' procedure.[28] Pig brain acetone powder (22 g) was prepared from 150 g of pig brain by a known procedure[32] and possessed an NADase activity of 0.03–0.04 U/mg. The cell nuclei of pig liver were prepared as described by Ferro and Kuehl.[33] NAD^+ pyrophosphorylase was partially purified from washed pig liver nuclei as described by Atkinson et al.[34] About 40 units of NAD^+ pyrophosphorylase were obtained from 1.6 kg of pig liver with a specific activity of 0.15 U/mg of protein, measured by RP-HPLC quantitation of the reaction product β-NAD^+ using β-NMN and ATP as substrates.

(32) Windmueller, H. G.; Kaplan, N. O. *Biochim. Biophys. Acta* **1952**, *15*, 388–394.
(33) Ferro, A. M.; Kuehl, L. *Biochim. Biophys. Acta* **1975**, *410*, 285–289.
(34) Atkinson, M. R.; Jackson, J. F.; Morton, R. K. *Biochem. J.* **1961**, *80*, 318–323.

Anion-Exchange HPLC. cADPR was analyzed and purified by anion-exchange HPLC. The sample was injected onto a SynChropak AX-100 column (250- × 7.8-mm i.d., 5 μm) equilibrated with 0.1 M KH_2PO_4 (pH 3.0) (solvent A). The column was eluted using a linear gradient of 0.1 M KH_2PO_4 and 1.5 M NaCl (pH 3.0) (solvent B) to 70% for 14 min, followed by elution using 70% solvent B until 20 min, and another linear gradient was employed using solvent B from 70% to 100% until 24 min and finally isocratic elution using solvent B to 30 min, at a flow rate of 1 mL/min. This elution procedure gave a complete separation of adenine (8.2 min), nicotinamide (8.8 min), β-NAD^+ (12.3 min), α-NAD^+ (13.2 min), cADPR (13.5 min), N^1-P-rib-AMP (14.2 min), N^6-P-rib-AMP (15.3 min), AMP (16.1 min), N^1-P-rib-ADP (17.1 min), N^6-P-rib-ADP (17.9 min), ADPR (20.0 min), ADP (22.5 min), N^1-P-rib-ATP (26.2 min), and N^6-P-rib-ATP (27.2 min).

Capillary Electrophoretic Analyses. Capillary electrophoresis was performed on an uncoated silica capillary (50-μm i.d. × 50 cm) using a Biofocus 3000 CE system (Bio-Rad) monitored at 260 and 290 nm. Samples (50 μg/100 μL, 10 mM sodium phosphate buffer, pH 8.2) were injected onto the capillary by the electrophoretic method at 10 kV for 5 s and run at a constant voltage of 12.5 kV at 20 °C using 0.1 M sodium phosphate (pH 8.2) as the running buffer.

Synthesis of cADPR from β-NAD^+ Using Pig Brain Acetone Powder. To the pig brain acetone powder (16 mg), suspended in 7.2 mL of 0.2 M sodium phosphate buffer (pH 7.5), was added β-NAD^+ (64 mg) in 0.8 mL of the same buffer. The mixture was incubated with stirring at 37 °C for 30 min. After the reaction was terminated by the addition of 8 mL of acetone, proteins were removed by centrifugation. The supernatant was filtered through an Amicon YM-10 membrane, and acetone was evaporated by a stream of nitrogen. cADPR was purified by anion-exchange HPLC on a SynChropak AX-100 column (250- × 7.8-mm i.d., 5 μm) as described. The cADPR fractions were pooled, reduced in volume in a rotary evaporator under reduced pressure, and further purified on a Waters Nova-Pak C_{18} column (100- × 8-mm i.d., 4 μm). The column was eluted isocratically using 4 mM formic acid at a flow rate of 1 mL/min. The retention time of cADPR was 3.6 min, identical to that of authentic cADPR. The yield of cADPR was determined to be 2.5% on the basis of its molar extinction coefficient at 260 nm (pH 3.0) of 13 700. ^{1}H-NMR (D_2O) exhibited the following signals: δ 9.02 (1H, s, H at C-8 of Ad), 8.39 (1H, s, H at C-2 of Ad), 6.15 (1H, d, J = 7 Hz), 6.07 (1H, d, J = 5 Hz), 5.35 (1H, t, J = 5 Hz), 4.1–4.7 (9H, m). FAB-MS $(M - H)/z$: 540.

Conversion of cADPR to N^1-P-*rib*-AMP. cADPR (20 μg) was incubated with 400 μg of *t*-BuOK in 1 mL of DMSO (containing 2 μL of H_2O) at 24 °C for 5 h. DMSO was evaporated by a stream of nitrogen, and the residue was dissolved in 400 μL of 0.2 M sodium acetate buffer (pH 4.5), which was directly applied to HPLC. The resulting N^1-P-rib-AMP was purified by anion-exchange HPLC on a SynChropak AX-100 column (250- × 7.8-mm i.d., 5 μm) using the same elution conditions as described before. Fractions containing N^1-P-rib-AMP were further purified on a Waters Nova-Pak C_{18} column (100- × 8-mm i.d., 4 μm), which were eluted isocratically using 4 mM formic acid at a flow rate of 1 mL/min. The retention time of N^1-P-rib-AMP was 4.8 min. The yield of N^1-P-rib-AMP was determined to be 85% on the basis of a molar extinction coefficient at 260 nm (pH 3.0) of 14 400.

Synthesis of cADPR from N^1-P-rib-ATP Using Pig Liver NAD^+ Pyrophosphorylase. To N^1-P-rib-ATP (23.2 μmol, 17.8 mg), dissolved

in 15 mL of 50 mM Hepes buffer (pH 7.5) containing 20 mM $MgCl_2$, were added 5 mL of acetonitrile, NAD^+ pyrophosphorylase (40 mg, 6 units), and yeast inorganic pyrophosphatase (2 units). The mixture was incubated with stirring at 24 °C, and the reaction was terminated after 48 h by the addition of 2% acetic acid and 50% (v/v) acetonitrile. Proteins were removed by centrifugation, and acetonitrile was evaporated by a stream of nitrogen. The pH of the supernatant was adjusted to 6.5 by the addition of 2 M Tris base. The newly synthesized cADPR was purified by anion-exchange HPLC on a SynChropak AX-100 column followed by reverse-phase HPLC on a Waters Nova-Pak C_{18} column as described previously. The yield of cADPR was determined to be 6.5% on the basis of its molar extinction coefficient (ϵ = 13 700) at 260 nm (pH 3.0). ^{1}H-NMR (D_2O) δ: 9.02 (1H, s, H at C-8 of Ad), 8.40 (1H, s, H at C-2 of Ad), 6.16 (1H, d, J = 7 Hz), 6.07 (1H, d, J = 5 Hz), 5.36 (1H, t, J = 5 Hz), 4.1–4.7 (9H, m). FAB-MS (M – H)/z: 540.

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