Probing *Aplysia californica* Adenosine 5′-Diphosphate Ribosyl Cyclase for Substrate Binding Requirements: Design of Potent Inhibitors†

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ABSTRACT: Readily synthesized nicotinamide adenine dinucleotide (NAD^+) analogues have been used to investigate aspects of the cyclization of NAD^+ to cyclic adenosine 5′-*O*-diphosphate ribose (cADPR) catalyzed by the enzyme adenosine 5′-*O*-diphosphate (ADP) ribosyl cyclase and to produce the first potent inhibitors of this enzyme. In all cases, inhibition of *Aplysia californica* cyclase by various substrate analogues was found to be competitive while inhibition by nicotinamide exhibited mixed-behavior characteristics. Nicotinamide hypoxanthine dinucleotide (NHD^+), nicotinamide guanine dinucleotide (NGD^+), C1′-*m*-benzamide adenine dinucleotide (Bp_2A), and C1′-*m*-benzamide nicotinamide dinucleotide (Bp_2N) were found to be nanomolar potency inhibitors with inhibition constants of 70, 143, 189, and 201 nM, respectively. However, NHD^+ and NGD^+ are also known substrates and are slowly converted to cyclic products, thus preventing their further use as inhibitors. The symmetrical bis-nucleotides, bis-adenine dinucleotide (Ap_2A), bis-hypoxanthine dinucleotide (Hp_2H), and bis-nicotinamide dinucleotide (Np_2N), exhibited micromolar competitive inhibition, with Ap_2A displaying the greatest affinity for the enzyme. 2′,3′-Di-*O*-acetyl nicotinamide adenine dinucleotide ($AcONAD^+$) was not a substrate for the *A. californica* cyclase but also displayed some inhibition at a micromolar level. Finally, inhibition of the cyclase by adenosine 5′-*O*-diphosphate ribose (ADPR) and inosine 5′-*O*-diphosphate ribose (IDPR) was observed at millimolar concentration. The nicotinamide aromatic ring appears to be the optimal motif required for enzymatic recognition, while modifications of the 2′- and 3′-hydroxyls of the nicotinamide ribose seem to hamper binding to the enzyme. Stabilizing enzyme/inhibitor interactions and the inability of the enzyme to release unprocessed material are both considered to explain nanomolar inhibition. Recognition of inhibitors by other ADP ribosyl cyclases has also been investigated, and this study now provides the first potent nonhydrolyzable sea urchin ADP ribosyl cyclase and cADPR hydrolase inhibitor Bp_2A, with inhibition observed at the micromolar and nanomolar level, respectively. The benzamide derivatives did not inhibit CD38 cyclase or hydrolase activity when NGD^+ was used as substrate. These results emphasize the difference between CD38 and other enzymes in which the cADPR cyclase activity predominates.

Enzymatic hydrolysis of nicotinamide adenine dinucleotide (NAD^+)[1] to adenosine 5′-*O*-diphosphate ribose (ADPR) has long been thought to mainly involve one type of protein, the NAD^+-glycohydrolases (*1*). These enzymes, studied both from catalytic and from cellular perspectives for many decades, catalyze the cleavage between the terminal ribose of NAD^+ and nicotinamide (*2*). Recently, new NADase-like enzymes, involved in the biosynthesis and catabolism of cyclic adenosine 5′-*O*-diphosphate ribose (cADPR) (*3*), have been identified as ADP ribosyl cyclase (*4*) and cADPR hydrolase (*5*). ADP ribosyl cyclase, first isolated from the mollusk *Aplysia californica* (*6*), stoichiometrically converts NAD^+ to cADPR. The metabolite cADPR is thought to be an endogenous regulator of the calcium-induced calcium release (CICR) process mediated by ryanodine receptors (*7*), and a role as a novel second messenger is supported by an increasing number of experiments (*8*). Recent results have demonstrated a role of cADPR in the induction of hippocampal LTD (*8c*) and also as a regulator of Ca^{2+} signaling in T-lymphocytes (*8d*). cADPR is produced by enzyme-catalyzed displacement of the nicotinamide of NAD^+ and formation of a glycosidic bond between the C1′ of the terminal ribose and the N1 of adenine with overall retention

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[1] Abbreviations: $AcONAD^+$, 2′,3′-di-*O*-acetyl nicotinamide adenine dinucleotide; ADPR, adenosine 5′-*O*-diphosphate ribose; ADP, adenosine 5′-*O*-diphosphate; AMP, adenosine 5′-*O*-monophosphate; ATP, adenosine 5′-*O*-triphosphate; Ap_2A, bis-adenine dinucleotide; Bp_2A, C1′-*m*-benzamide adenine dinucleotide; Bp_2N, C1′-*m*-benzamide nicotinamide dinucleotide; cADPR, cyclic adenosine 5′-*O*-diphosphate ribose; cGDPR, cyclic guanosine 5′-*O*-diphosphate ribose; cIDPR, cyclic inosine 5′-*O*-diphosphate ribose; DCC, *N*,*N*-dicyclohexylcarbodiimide; DMF, dimethylformamide; DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IDPR, inosine 5′-*O*-diphosphate ribose; IMP, inosine 5′-*O*-monophosphate; Hp_2H, bis-hypoxanthine dinucleotide; β-NAD^+, nicotinamide adenine dinucleotide; β-NHD^+, nicotinamide hypoxanthine dinucleotide; β-NGD^+, nicotinamide guanine dinucleotide; NMN, nicotinamide 5′-*O*-monophosphate nucleotide; Np_2N, bis-nicotinamide dinucleotide; SDS, sodium dodecyl sulfate; SUH, sea urchin homogenate; TEAB, triethylamine bicarbonate.

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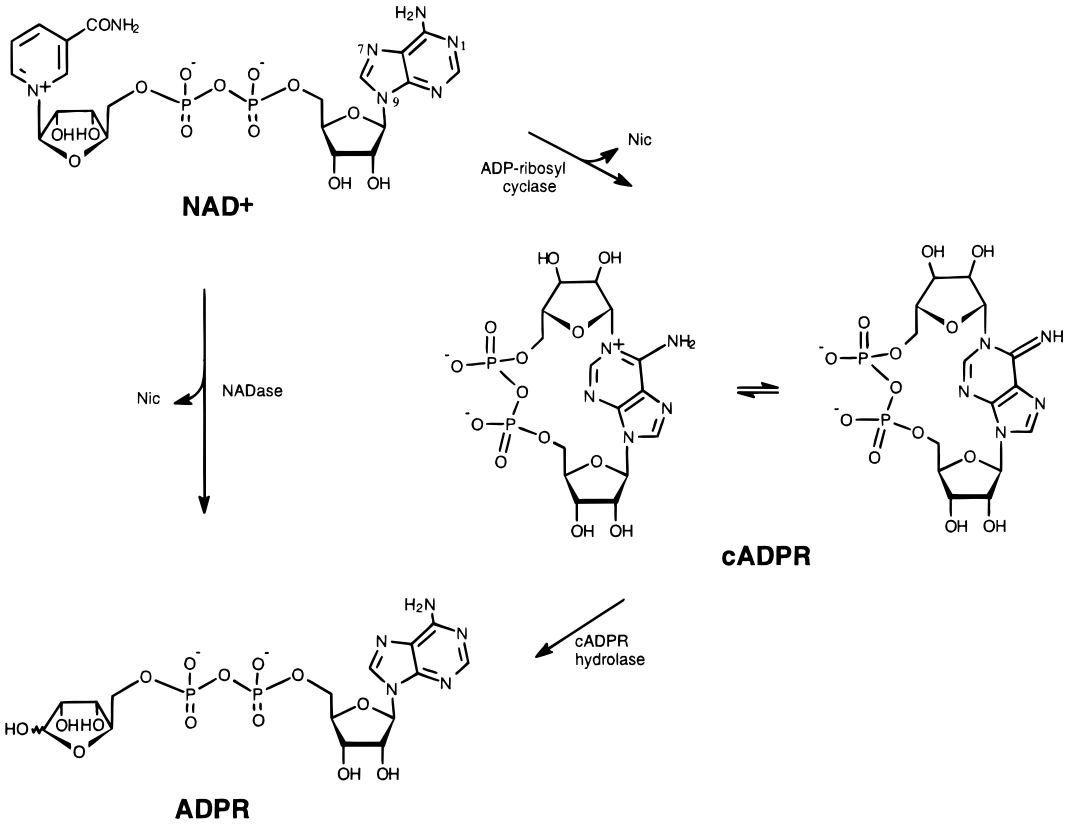
Scheme 1: Enzymatic Cyclization and Hydrolysis of NAD^+



of configuration (Scheme 1) (*9*). Around physiological pH, two distinct forms of cADPR with a pK_a of 8.2 are possible (Scheme 1) (*3*). cADPR hydrolase catalyzes the cleavage of this newly formed glycosidic bond to form ADPR (*5*).

Cyclase activity and cADPR formation have been detected in a wide range of cells (*10*). Calcium release induced by cADPR is observed in plants, smooth muscle, heart muscle, and other tissues. In human cells, both the cyclase and hydrolase activities are found on the same membrane-bound protein (*11*), and investigations of the biological functionalities of cADPR are very often impaired by this dual activity. Since *A. californica* cyclase does not present noticeable hydrolase activity (*4*) and can easily be purified (*6*), it has become a very important tool in investigating this potential new second messenger. Other than using the *A. californica* cyclase to synthesize cADPR analogues to probe cADPR receptor sites, this cyclase can potentially be used for probing the biological importance of cADPR by using its capacity to detect potential in vivo inhibitors of cADPR cyclases and hydrolases, and for investigating the mechanism of action of cyclase/hydrolase bifunctional enzymes.

A. californica cyclase has already been extensively used in the synthesis of cADPR and cADPR analogues (*12*) as potential antagonists (*13*), partial agonists (*14a*), and non-hydrolyzable full agonists (*14b*) at the cADPR receptor involved in CICR. The large variety of enzymatically synthesized cADPR analogues suggests a lack of substrate selectivity. Modification at the C8 or C7 positions of the adenine ring with either hydrophobic or hydrophilic groups had no apparent effect on the ability of the enzyme to cyclize the NAD^+ analogues (*13*). The main kinetic effect observed when modifications were made to the purine ring was a slower turnover (*15*). Exchange of the C2′ or C3′ hydroxyl of the adenosine ribose moiety for charged groups such as phosphate (*17*) or more hydrophobic moieties such as methoxy (*12a*) was also of little consequence. Nicotinamide adenine 5′-*O*-triphosphate derivatives could also be cyclized by ADP ribosyl cyclase (*18*).

In the present study of *A. californica* ADP ribosyl cyclase, we have investigated the importance of the nicotinamide ribose and the nicotinamide aromatic ring in the enzyme/substrate recognition step, by challenging the enzyme with modified NAD^+ analogues. Until now, the adenosine motif of NAD^+ has been the only moiety modified. We have also explored the importance of the purine ring and its orientation for recognition and binding by the ADP ribosyl cyclase active site. We describe here the identification of potent *A. californica* inhibitors and also explore one of them in a wider inhibition context.

MATERIALS AND METHODS

Materials. β-NAD^+, NHD^+, NGD^+, IDPR, NMN, AMP, and IMP were obtained from Sigma, and their purity was ascertained by HPLC. Nicotinamide, HEPES, SDS, and DTT were also purchased from Sigma. 3-(1-Deoxy-β-D-ribofuranosyl)benzamide, the benzamide analogue of nicotinamide nucleoside (*19*), was kindly donated by Professor K. Krohn, University of Paderborn, Germany. Cation-exchange resin (CM), Fast Flow Q-Sepharose gel, and Sephacryl S-100 HR prepacked size exclusion column were supplied by Pharmacia. HPLC chromatographic analyses were carried out using an anion-exchange Partisil SAX column obtained from HPLC Technology (U.K.), and a Shimadzu HPLC system constituted of two LC-6A Liquid Chromatograph pumps, a SPD-6A UV Spectrophotometric Detector, a SCL-6B System

Controller, and a C-R6A Chromatopac printer. Fluorescence experiments were performed on a Perkin-Elmer LS 50-B spectrofluorometer using Perkin-Elmer FL Win Lab software. ^{1}H NMR and ^{31}P NMR spectra were recorded on a JEOL JNM GX-270 FT NMR spectrometer. Chemical shifts were measured in ppm relative to deuterated water (D_2O) for ^{1}H NMR and to external 85% H_3PO_4 for ^{31}P NMR. For the latter, δ values are positive when downfield from this reference. *J* values are given in Hertz (Hz). Mass spectra were recorded at the University of Bath on a VG Autospec spectrometer. FAB samples were recorded using *m*-nitrobenzyl alcohol as the matrix.

Aplysia ADP Ribosyl Cyclase. Homogeneous enzyme purified from *A. californica* ovotestis extract (*4*) was used for all kinetic experiments. It was purified according to the methods developed by Hellmich and Strumwasser (*6*). The crude enzyme obtained from Dr. A. Galione, Oxford University (0.5 mL, 10 mg/mL), was loaded on a CM column (20 mL) equilibrated at pH 8 with 25 mM HEPES buffer at 4 °C. After elution of the enzyme with an NaCl gradient (250 mL, 0−250 mM), the fractions (1.5 mL) containing cyclase activity were combined. Cyclase activity was detected by incubating for 10 min, 10 μL of each fraction with 190 μL of an NAD^+ solution buffered at pH 8 with 25 mM HEPES. Newly formed cADPR was eluted at 2 mL/min on a Partisil SAX HPLC column equilibrated with a phosphate buffer (KH_2PO_4, pH 3, 5% MeOH). Concentration of the combined fractions via ultrafiltration through an Amicon membrane (YM10) resulted in homogeneous enzyme (5.00 μg/mL) that was stable in storage at 4 °C for 1 week. Homogeneity was confirmed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and fast-flow size-exclusion column chromatography. Polyacrylamide gel electrophoresis of the purified cyclase in the presence of denaturing agent (SDS) was performed according to Laemmli (*20*). Gels were fixed with acetic acid solution and visualized with Coomasie Brilliant Blue. Standards used for molecular weight determination consisted of lysozyme (MW 14 300), β-lactoglobulin (MW 18 400), trypsinogen (MW 24 000), carbonic anhydrase (MW 29 000), pepsin (MW 34 700), egg albumin (MW 45 000), and bovine albumin (MW 66 000). A single band that corresponded to a protein with a molecular weight of 29 000 was detected. Similar results were obtained by fast-flow size-exclusion chromatography on the SDS-denatured enzyme, using water as the eluant and UV spectroscopic analysis at 259 nm for the detection of the protein. The standards used for the molecular weight determination were aprotinin (MW 6500), cytochrome *c* (MW 12 400), carbonic anhydrase (MW 29 000), bovine albumin (MW 66 000), and blue dextran (MW 2 000 000). Under nondenaturing conditions, fast-flow size-exclusion chromatography on freshly purified enzyme indicated that the cyclase was present as a dimer in solution. However, size-exclusion chromatography showed that upon long-term storage at −20 °C the enzyme aggregated. Recovery was possible by thawing and storing the enzyme solution at 4 °C for 24 h. Addition of DTT to the solution resulted in a rapid loss of enzyme activity.

Determination of the Michaelis Constant of NAD^+. Purified *Aplysia* ADP ribosyl cyclase (100 ng/mL) was incubated at 17 °C with various concentrations of substrate (50, 100, 150, 250, and 500 μM) in a 200 μL solution buffered at pH 8 with 25 mM HEPES. Nicotinamide, NAD^+, and cADPR were eluted at 2 mL/min on a Partisil SAX HPLC column equilibrated with a phosphate buffer (KH_2PO_4, pH 3, 5% MeOH). Respective retention times were 0.95, 1.22, and 2.10 min. The progress curves were obtained from plotting the amount of newly formed cADPR detected at 259 nm as a function of time. Time points were taken every 2 min for 12 min, over which the production of cADPR remained linear under the conditions stated. The K_m values were determined by a standard reciprocal plot of rate vs substrate concentration.

Determination of the Michaelis Constants for NHD^+ and NGD^+ by Fluorescence Analysis. The purified ADP ribosyl cyclase was incubated at 17 °C with various concentrations of NHD^+ and NGD^+ in a 200 μL solution buffered at pH 8 with 25 mM HEPES. The formation of cyclic products was monitored by fluorescence (excitation at 300 nm, emission at 410 nm) as described by Graeff et al. (*16*). The progress curves were obtained from plotting the amount of newly formed cIDPR and cGDPR, respectively, as a function of time. The production of cyclic products remained linear throughout the experiment. The K_m values were determined by a standard reciprocal plot of rate vs substrate concentration.

Determination of Inhibition Constants. The purified ADP ribosyl cyclase was incubated with various concentrations of substrates and inhibitors at 17 °C in 25 mM HEPES (pH 8) solution of 200 μL total volume. The concentration of ADP ribosyl cyclase in the reaction mixture was 100 ng/mL. All the above enzymatic reactions were followed as previously described for the determination of the Michaelis constant of NAD^+. Concentration ranges: NAD^+, 50 μM, 100 μM, 150 μM, 250 μM, 500 μM; NHD^+, 0, 10 nM, 50 nM, 100 nM, 500 nM; NGD^+, 0, 10 nM, 50 nM, 100 nM, 500 nM; ADPR, 0, 2.5 μM, 5 μM, 250 μM, 500 μM, 1 mM; IDPR, 0, 2.5 μM, 5 μM, 250 μM, 500 μM, 1 mM; $AcONAD^+$, 100 μM; nicotinamide, 0, 50 μM, 100 μM, 250 μM, 500 μM; Bp_2A, 0, 250 nM, 500 nM, 1 μM, 2.5 μM, 5 μM; Bp_2N, 0, 50 nM, 500 nM, 1 μM, 2.5 μM; Np_2N, 0, 100 nM, 1 μM, 10 μM, 50 μM; Ap_2A, 0, 100 nM, 1 μM, 10 μM, 100 μM; Hp_2H, 0, 100 nM, 500 nM, 5 μM, 10 μM, 100 μM.

Measurements of Cyclase Inhibition in Sea Urchin Homogenate SUH. The eggs of the sea urchin *Lytechinus pictus* were used for this study. *L. pictus* homogenate (2.5%) (kindly provided by Dr. A. Galione, University of Oxford) was incubated at 17 °C in an intracellular-like medium (Im) containing an ATP-regenerating system, mitochondrial inhibitors, and Fluo-3 (3 μM) (*13c*). Extramicrosomal Ca^{2+} was measured by monitoring Fluo-3 fluorescence (excitation 490 nm, emission 535 nm). The SUH (1.6 mL) was placed in cuvettes to stabilize at 17 °C for 1 h prior to use. Addition of benzamide derivatives was then made, and the system was left to equilibrate for 10 min. A control set in which no benzamide derivatives had been added was prepared simultaneously, and left to settle at 17 °C. The Ca^{2+} release resulting from the addition of 5, 10, 20, and 30 μM NAD^+ was then monitored by an increase in fluorescence at 17 °C. The amount of Ca^{2+} was directly calculated from a standard curve (Ca^{2+} concentration vs fluorescence). The maximum additions to the cuvettes were between 5 and 10 μL in Im. The typical fluorescence responses are shown Figure 3a,b.

IC_{50} Measurements for SUH Cyclase by Benzamide Derivatives. The procedure described previously was used to calculate the IC_{50} values of Bp_2A as a SUH cyclase inhibitor. NAD^+ concentrations for the inhibition of the SUH cyclase were maintained at 10 μM, while the concentration of Bp_2A was 0, 10, 25, and 50 μM (Figure 4).

Measurements of Hydrolase Inhibition in SUH. The eggs of the sea urchin *Lytechinus pictus* were used for this study and prepared as previously described. Degradation of cADPR by the SUH hydrolase was determined using the Ca^{2+} release bioassay described above. At appropriate time intervals, 50 μL samples were taken from an SUH sample (1.6 mL) in which cADPR (35 μM) had been incubated, and added to a new SUH sample (1.6 mL). The samples were assayed for remaining cADPR by following the Ca^{2+} release by Fluo-3 fluorescence. cADPR concentrations were obtained from a standard curve. The typical fluorescence responses are shown Figure 5a,b. The data used to calculate the IC_{50} values for Bp_2A as a SUH hydrolase inhibitor are not shown.

CD38 Inhibition. The same incubation conditions as described by DeFlora et al. (*30*) and the same conditions as described under *Determination of the Michaelis Constants for NHD^+ and NGD^+ by Fluorescence Analysis* were used for this experiment. Recombinant CD38 was a kind gift of Professor H. C. Lee, University of Minnesota.

Synthesis of Inhibitors: General Methods for the Synthesis of NAD^+ Analogues. (A) Phosphorylation of Nucleosides (21). Nucleosides (0.10 mmol) dried in vacuo over P_2O_5 were dissolved in hot triethyl phosphate under N_2. After cooling the mixture to 0 °C, phosphorus oxychloride (3 equiv) was added under N_2. The reaction was stirred at 0 °C for 3 h and warmed to room temperature. After 24 h, the reaction was quenched by addition of ice-cold aqueous pyridine (3:1, v/v, pyridine/water) at 0 °C. The solvents were removed under reduced pressure, and the residue was purified by anion-exchange column chromatography using Fast Flow Q-Sepharose gel (50 mL) and a gradient of triethylammonium bicarbonate (TEAB) buffer (900 mL, 0−500 mM). Fractions containing the required material (>95% HPLC purity) were combined and concentrated. Excess TEAB was removed by coevaporating with 2-propanol. The phosphorylated species were quantified using the assay developed by Ames (*22*). Yields ranged from 40 to 80%. The monophosphorylated species were then dried in vacuo by coevaporating with DMF.

(B) Synthesis of the Pyrophosphate Nucleotides (23). To a 2 mL solution of aqueous pyridine (3:1,v/v, pyridine/water) containing 2 equiv of β-nicotinamide mononucleotide was added the required phosphorylated nucleoside. A large excess of DCC (2 g) was then added and the heterogeneous mixture stirred for 5 days at room temperature under N_2. Water was added and the solution stirred for 2 h. Dicyclohexylurea was then extracted with ether while the newly formed pyrophosphate remained in the aqueous layer. Pyridine and water were removed under reduced pressure at 20 °C. To simplify the purification of the symmetrical dinucleotide, the residue obtained after concentration was dissolved in 5 mL of 50 mM diethanolamine solution buffered at pH 9.8 containing 0.5 mM $MgCl_2$. One unit of alkaline phosphatase was added. The reaction was incubated at 17 °C for 30 min followed by HPLC. The reaction was terminated by diluting the reaction solution with 100 mL of water. The solution was then loaded on a Q-Sepharose anion-exchange chromatography column and the dinucleotide purified using similar conditions as described previously. Nonsymmetrical dinucleotides were directly purified by Q-Sepharose anion exchange chromatography under similar conditions as described previously. Yields ranged from 10% for the benzamide series to 80% for Np_2N. ADPR was prepared by incubating 50 mg of NAD^+ at 30 °C in 5 mL of a 25 mM HEPES solution in the presence of 0.5 unit of NADase for 12 h. The reaction was monitored by HPLC. The solution was diluted to 100 mL with water, filtered, and loaded on an anion-exchange chromatography column and the product purified as described previously.

(C) Spectroscopic Data. Bis-nicotinamide dinucleotide (Np_2N): ^{1}H NMR (D_2O) δ 9.43 (Hnic2, s), 9.25 (Hnic6, d, J = 6 Hz), 8.95 (Hnic4, d, J = 8 Hz), 8.26 (Hnic5, dd, J = 6, 8 Hz), 6.17 (H1′, d, J = 5.5 Hz), 4.60 (H4′, m), 4.53 (H2′, dd, J = 5, 5.5 Hz), 4.26 (H5′, ddd, J = 2.5, 5, 12 Hz), 4.11(H5′, ddd, J = 1.8, 5, 10 Hz); ^{31}P NMR (D_2O) δ −11.5 (s); MS *m*/*z* rel int (FAB^+) 97 (100), 420 (80), 651 ($M+H^+$, 75); HRMS (FAB^+) calculated for $C_{22}H_{29}O_{15}N_4P_2$ ($M+H^+$) 651.110469, found 651.109665; λ_{max} 249 nm, ϵ 3600 M^{-1} cm^{-1}.

2′,3′-Di-*O*-acetyl nicotinamide adenine dinucleotide (AcONAD$^+$) (*24*): ^{1}H NMR (D_2O) δ 9.22 (Hnic2, s), 9.03 (Hnic6, d, J = 6.4 Hz), 8.76 (Hnic4, d, J = 8.0 Hz), 8.32 (Hade2, s), 8.14 (Hnic5, s), 8.06 (Hade8, s), 5.97 (Hnic1′, d, J = 5 Hz), 5.92 (Hade1′, d, J = 6.0 Hz), 5.23 (Hnic2′, m), 4.61−4.03 (9H, m), 2.89 (3H, s), 2.73 (3H, s); ^{31}P NMR (D_2O) δ −11.3 (d, J = 15 Hz), −11.9 (d, J = 15 Hz); MS *m*/*z* rel int (FAB^+) 749 ($M+H^+$, 1); λ_{max} 259 nm, ϵ 17 300 M^{-1} cm^{-1}.

Bis-hypoxanthine dinucleotide (Hp_2H) (*25*): ^{1}H NMR (D_2O) δ 8.17 (Hino2, s), 7.95 (Hino8, s), 5.83 (H1′, d, J = 5 Hz), 4.50 (H2′, dd, J = 5 Hz), 4.31 (H3′, dd, J = 4, 4.5 Hz), 4.17 (H4′, m), 4.08 (2xH5′, m); ^{31}P NMR (D_2O) δ −11.5 (s); MS *m*/*z* rel int (FAB^+) 102 (100), 679 ($M+H^+$, 1), (FAB^-), 188 (100), 677 ($M-H^+$, 90); HRMS (FAB^+) calculated for $C_{20}H_{25}O_{15}N_8P_2$ ($M+H^+$) 679.09146, found 679.09334; λ_{max} 249 nm, ϵ 7100 M^{-1} cm^{-1}.

Bis-adenine dinucleotide (Ap_2A) (*26*): ^{1}H NMR (D_2O) δ 8.05 (Hade2, s), 7.89 (Hade8, s), 5.78 (H1′, d, J = 5 Hz), 4.41 (H2′, dd, J = 5, 5.2 Hz), 4.27 (H3′, dd, J = 4.2, 4.4 Hz), 4.18 (H4′, m), 4.08 (2xH5′, m); ^{31}P NMR (D_2O) δ −11.5 (s); λ_{max} 256 nm, ϵ 16 500 M^{-1} cm^{-1}.

Benzamide adenine dinucleotide (Bp_2A) (*27*):^{1}H NMR (D_2O) δ 8.07 (Hade2, s), 7.89 (Hade8, s), 7.92 (Hben2, s), 7.82 (Hben4, d, J = 7.6 Hz), 7.72 (Hben6, d, J = 7.6 Hz), 7.55 (Hben5, dd, J = 7.6 Hz), 5.77 (H1′, d, J = 5 Hz), 3.80−4.50 (11H, m); ^{31}P NMR (D_2O) δ −10.5 (d, J = 20 Hz), −10.8 (d, J = 20 Hz); λ_{max} 256 nm, ϵ 16 000 M^{-1} cm^{-1}.

Benzamide nicotinamide dinucleotide (Bp_2N): ^{1}H NMR (D_2O) δ 9.44 (Hnic2, s), 9.30 (Hnic6, d, J = 6.1 Hz), 8.93 (Hnic4, d, J = 8 Hz), 8.30 (Hnic5, dd, J = 6, 8 Hz), 7.90 (Hben2, s), 7.83 (Hben4, d, J = 7.6 Hz), 7.72 (Hben6, d, J = 7.6 Hz), 7.58 (Hben5, dd, J = 7.6 Hz), 5.99 (Hnic1′, d, J = 5.5 Hz), 3.91−4.43 (11H, m); ^{31}P NMR (D_2O) δ −10.5 (d, J = 20 Hz), −10.8 (d, J = 20 Hz); MS *m*/*z* rel int (electrospray), 648 ($M+2H^+$, 75), 237; λ_{max} 264 nm, ϵ 2000 M^{-1} cm^{-1}.

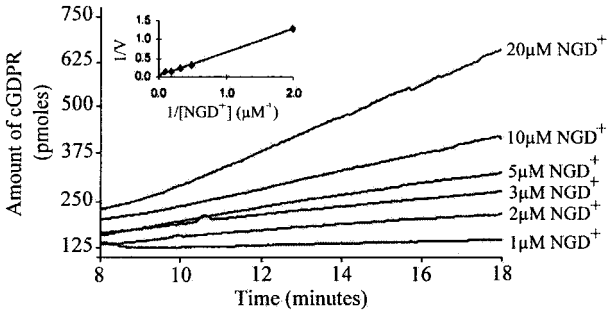


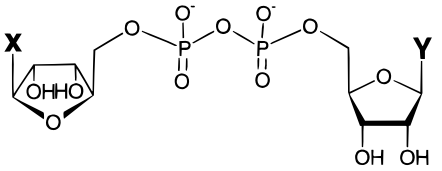
FIGURE 1: Kinetic data for NGD^+. The fluorimetric assay developed by Lee et al. (*16*) was used to determine the kinetic data of fluorescence-yielding compounds. Picomoles of cGDPR were calculated for the total volume of the assay mixture. The inset shows the double-reciprocal plot of the reaction rate (μmol min^{-1} mg^{-1}) vs NGD^+ concentration (μM).

RESULTS

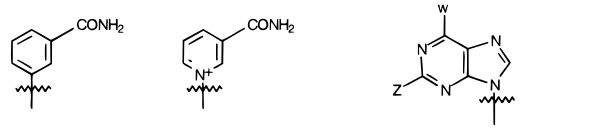
The synthesis of each of the NAD^+ analogues generally followed published procedures. In general, when aqueous pyridine was used to quench the phosphorylation, the nucleotides were obtained in high yield with good recovery of unreacted material. However, when pyridine or water alone was used, lower yields were observed as a result of formation of polyphosphorylated species. Unsymmetrical coupling reactions between nucleotides catalyzed by DCC were low yielding (typically less than 15%). However, symmetrical dinucleotides were obtained in reasonable yield, with 30% and 60% for inosine and adenosine, respectively, and up to 80% for the bis-nicotinamide dinucleotide.

Kinetic studies on NAD^+, NHD^+, and NGD^+ were conducted and compared to literature data. Michaelis constant (K_m) and maximum velocity (V_{max}) values differed by 10-fold from the values obtained by Lee and co-workers (*16*) for these substrates. We determined the respective K_m values as 125, 8.8, and 15.5 μM [lit. 39, 0.5, and 1.7 μM (*16*)]. Kinetic data for NAD^+ were obtained from progress curves acquired by monitoring the production of HPLC-purified cADPR detected by UV spectroscopy. The kinetic data for NHD^+ and NGD^+ cyclization were obtained by fluorescence assay. The V_{max} values for NHD^+ and NGD^+ were 44.5 and 24.8 μmol/min, respectively. Kinetic data for NGD^+ are given in Figure 1. These results indicate that the enzyme shows high affinity for NHD^+ and NGD^+ (low K_m) and yet cannot easily convert them to product (low V_{max}). Such characteristics make NHD^+ and NGD^+ ideal starting molecules for the design of cADP-ribosyl cyclase inhibitors. When NHD^+ and NGD^+ were tested against NAD^+ for inhibition of the enzyme, we found efficient inhibition at nanomolar levels (Table 1). Kinetic data for enzymatic inhibition by NGD^+ are given in Figure 2. The respective inhibition constants of NHD^+ and NGD^+ for this enzyme were calculated as 70 and 143 nM. Lineweaver−Burke, Dixon, and Hanes−Woolf plots provided confirmation of the competitive behavior of these two inhibitors (*28*). However, a replot of the Lineweaver−Burke plots leads to a nonlinear curve when micromolar concentrations of NHD^+ were used as inhibitor (data not shown). The concentration of NHD^+ in the presence of enzyme does not remain constant during the course of the experiment, perhaps explaining the nonlinearity of some of the kinetic data at high concentration.

Table 1: Michaelis Constants for NAD^+ and Its Analogues as Substrates for ADP-Ribosyl Cyclase and Inhibition Constants for NAD^+ Analogues as Inhibitors of the Cyclase



	X	Y	K_m	K_i	K_m/K_i
NAD^+	nicotinamide	adenine	135μM		
NHD^+	nicotinamide	hypoxanthine	8.80μM	70.0nM	1929
NGD^+	nicotinamide	guanine	15.5μM	143nM	944
ADPR[a]	OH	adenine		0.80mM	0.17
IDPR[a]	OH	inosine		1.30mM	0.10
nicotinamide				500μM	0.27
				1.10mM	0.12
Bp_2A	benzamide	adenine		189nM	714
Bp_2N	benzamide	nicotinamide		201nM	672
Np_2N	nicotinamide	nicotinamide		23.0μM	5.90
Ap_2A	adenine	adenine		900nM	150
Ip_2I	hypoxanthine	hypoxanthine		12.0μM	11.3



[a] Value based on IC_{50} (SD = ±0.1 mM).

Similar curvature was also observed for NGD^+ when tested for inhibition at high concentration.

When ADPR was tested for inhibition of *A. californica* cyclase against NAD^+ at millimolar level, decreased enzyme activity was detected (Table 1). Since NHD^+ was proven to be a better active-site binding species than NAD^+ (lower K_m), the hydrolyzed derivative of NHD^+, IDPR, was also tested. Inhibition of the cyclase was only observed at a millimolar level of IDPR (Table 1). Studies by Inageda and co-workers showed no inhibition of *Aplysia kurodai* cyclase by ADPR when tested at a concentration of 10 mM (*29*). Our results also slightly differed from those of Inageda et al. in the K_m of NAD^+. When we tested nicotinamide on *A. californica* cyclase, we observed mixed inhibition of the cyclase with inhibition constants at 500 μM and 1.1 mM. Inageda et al. also found nicotinamide to be a mixed-inhibitor of the *A. kurodai* cyclase at a micromolar level.

The terminal nicotinamide moiety seems to have a substantial influence on the efficacy with which the substrate binds to the enzyme. The recognition step of the terminal nicotinamide moiety by the enzyme could be due to the aromaticity of the nicotinamide and/or to its positively charged structure. The benzamide derivative of NAD^+, Bp_2A, and the benzamide analogue of the bis-nicotinamide dinucleotide Np_2N, Bp_2N (Table 1), were prepared to elucidate this aspect of the binding. Both benzamides were found to be good competitive inhibitors of the cyclase at a nanomolar

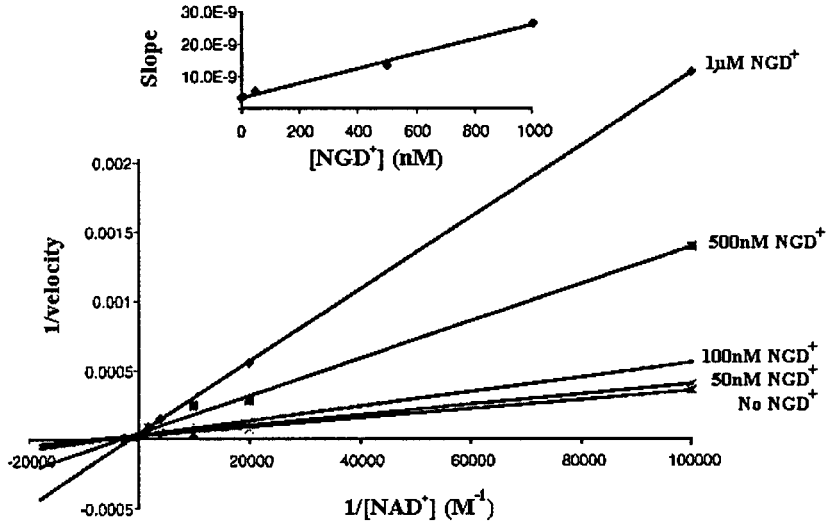


FIGURE 2: Inhibition data for NGD^+. The variation in cADPR production catalyzed by ADP ribosyl cyclase as a function of inhibitor concentration was followed by HPLC. The present assay uses the true substrate for the determination of the inhibition constant.

level. The respective inhibition constants were 189 and 201 nM. When the nanomolar K_i of Bp_2N is compared to the micromolar K_i obtained for Np_2N (Table 1), it becomes clear that enzyme recognition is highly dependent on the charge distribution on the substrate. No release of nicotinamide could be detected by UV spectroscopy (data not shown) when Bp_2N and Np_2N were incubated with the *A. californica* enzyme in the absence of NAD^+. Bp_2N and Np_2N are therefore not hydrolyzed by this cyclase.

To further investigate the importance of the nicotinamide ribose nucleoside motif in enzyme binding, the 2′,3′-*O*-diacetyl derivative of the nicotinamide ribose of NAD^+, $AcONAD^+$, was prepared. Initially thought to be a potential substrate, $AcONAD^+$ was incubated with *A. californica* cyclase. No cyclic product could be detected by HPLC. This NAD^+ analogue was found not to be a substrate for the enzyme. Initial tests for inhibition showed that more than 80% activity is conserved when 100 μM NAD^+ is incubated with *A. californica* cyclase in the presence of 100 μM $AcONAD^+$. This NAD^+ analogue, like ADPR, is therefore a weak inhibitor.

Because of the difference in binding affinity between NHD^+ and NAD^+, we have also investigated the importance of the purine moiety by synthesizing bis-adenine dinucleotide (Ap_2A) (*26*) and bis-hypoxanthine dinucleotide (Hp_2H) (*25*). Each of these compounds was found to be a competitive inhibitor with micromolar affinity. Bis-adenine dinucleotide was the most potent of all the symmetrical species, and had 150-fold more affinity for the enzyme active site than the natural substrate (Table 1). However, the exchange of adenine for nicotinamide or hypoxanthine resulted in a clear loss of affinity for the enzyme active site, and this can be recognized by comparing the inhibition constants of the symmetrical species (Table 1). In contrast, the difference between the inhibition constant of Ap_2A and ADPR, or Hp_2H and IDPR, implies that the introduction of a second aromatic group at the ribosyl extremity results in a very drastic increase in inhibition.

We have now established that *A. californica* can be efficiently inhibited by a new class of compounds that are not substrates, i.e., the benzamide dinucleotide derivatives. We decided to establish whether these new inhibitors were also potent in other systems. Sea urchins were the first species in which cADPR was detected. We decided to test our new inhibitors on this system. Both cyclase and hydrolase activities are present in this system, and both were individually tested for inhibition. While all the inhibitors of *A. californica* cyclase were preliminary screened for modification of Ca^{2+} release in SUH, only Bp_2A displayed noticeable activity. The other benzamide as well as NGD^+ and NHD^+ were readily hydrolyzed by the homogenate hydrolase and NADases. Further studies of Bp_2A inhibition showed that the SUH cyclase was inhibited by Bp_2A at micromolar levels. However, an accurate value cannot be given since the hydrolase responsible for the degradation of cADPR is also inhibited by Bp_2A which results in a counter-effect on the decrease of Ca^{2+} release in the assay. We unsuccessfully attempted to follow the cADPR formation by HPLC to determine the IC_{50} value accurately. The difficulty resulted from compounds absorbing at the cADPR λ_{max} and present in high concentration in the Im medium. We also unsuccessfully tried to use NGD^+ as substrate and cGDPR formation as a detection method to assay for SUH cyclase inhibition. Fluorometric detection of cGDPR could not be achieved. It is thought that NGD^+ is rapidly hydrolyzed by SUH hydrolase activity instead of being cyclized by the cyclase activity. We then tested for the inhibition of the hydrolase activity in SUH by Bp_2A. Total inhibition of this activity was obtained at the 100 μM level, and the IC_{50} was found to be 300 nM.

Studies on the inhibition of *A. californica* cyclase allowed us to detect a very potent hydrolase inhibitor in SUH. We therefore decided to test this compound also on CD38 to examine its interaction with a mammalian-derived NAD^+ binding site. When we tested Bp_2A on recombinant CD38 using NGD^+ as substrate and the fluorometric detection of cGDPR as the assaying method, we could not observe any effect on the cyclase activity even at 100 μM concentration.

DISCUSSION

A recent X-ray crystallographic analysis of the *A. californica* cyclase has furnished useful information on what the enzyme binding site could be and on the type of interactions

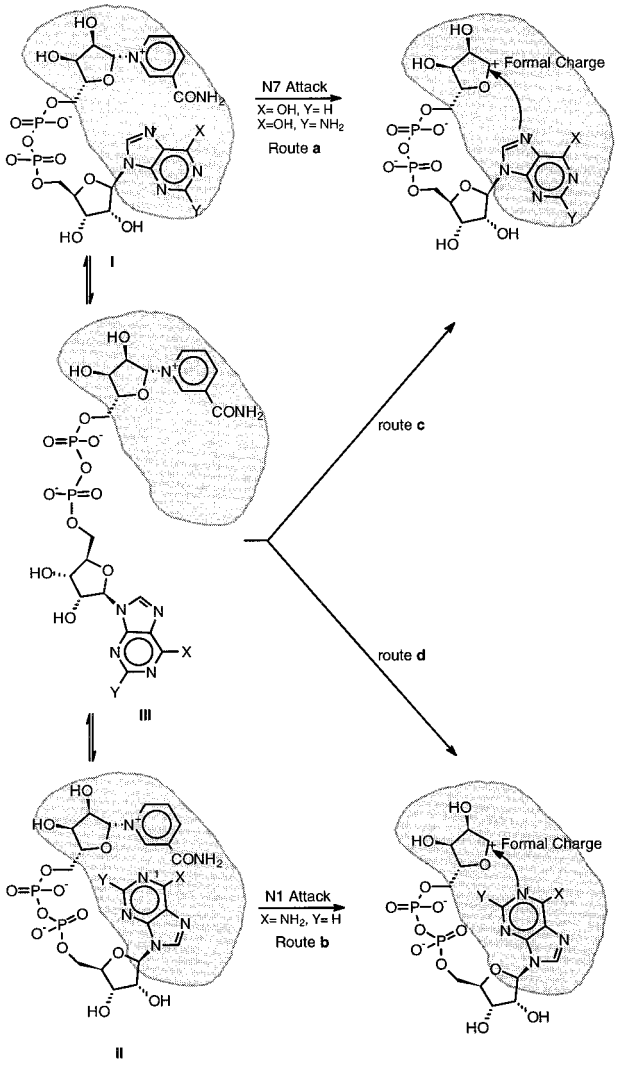
the enzyme might provide for its substrate (*31*). A dimeric structure of this cyclase has been proposed, and we have observed by size-exclusion chromatography under nondenaturing conditions that *A. californica* cyclase could be dimeric. Two aromatic-rich domains have been detected on the surface of the monomer (*31*). These areas would be facing each other in a dimeric structure providing two highly hydrophobic pockets. Most of the adenine-modified NAD^+ derivatives prepared by our group for the synthesis of cADPR analogues contained the two aromatic moieties that granted them tight binding to the enzyme. Exchange of the adenine ring in NAD^+ for another purine such as guanine or hypoxanthine results in the formation of cyclic ADP-ribose analogues in which the newly formed glycosidic bond lies between the terminal C1′ of the ribose ring, which carried the nicotinamide, and the N7 of the purine ring (*16*). However, cyclization of NAD^+ occurs between the terminal C1′ of the nicotinamide ribose ring and the N1 of the adenine ring (*3*). This difference in cyclization site was attributed to the variation in nucleophilicity between the nitrogens of the different purines. Cyclization at N7 results in compounds that are fluorescent and are of considerable use for cADPR cyclase detection in vivo (*16*). High rates of cyclization were observed when cyclization occurred at the N1 of the purine (NAD^+). High affinity and a slow rate of cyclization were observed when the cyclization occurred at N7 (NHD^+ and NGD^+).

A binding mechanism has been proposed by Lee (*16*), who suggested that the enzyme active site has a low recognition specificity for the purine ring. Lee and co-workers proposed that the adenine of NAD^+ was loosely bound to the enzyme, pointing toward the outside of a binding pocket. Results obtained from the synthesis of adenosine ribose-modified cADPR analogues such as 2′-*O*-phosphoro-cADPR (*17*), 3′-*O*-methoxy-cADPR, or 2′- and 3′-deoxy-cADPR (*12*) suggest that modifications of the ribose ring have generally only a small effect on the ability of the enzyme to cyclize the modified nicotinamide adenine dinucleotides. These observations would suggest that the cyclase binding pocket also has low binding requirements for the adenosine ribose. The ribose could even be envisaged as lying outside the enzyme binding pocket. A base-modified nicotinamide adenine triphosphate nucleotide has also been cyclized under similar conditions to a cATPR analogue (*32*), indicating that the pyrophosphate bridge is unlikely to be critically involved in the binding between substrate and enzyme. Adenosine, AMP, and ADP did not inhibit the cyclase at millimolar level (data not shown), implying that recognition and binding to the enzyme active site require more than the phosphorylated adenine motif (*16*, *29*). Many NADases and other NAD^+-dependent enzymes (*32*) are efficiently inhibited by ADPR. In the case of NADases, the ability of ADPR to inhibit nicotinamide cleavage is attributed to the shared structural similarity with the oxycarbonium reactive intermediate (*2*). The cyclization mechanism has been proposed to involve an enzyme-stabilized carboxonium reaction intermediate. A number of experiments which support this proposal have been carried out on various NADases that display some cyclase activity (*34*). Hydroxyl and methoxyl groups and other nucleophiles were introduced onto the stabilized adenosine diphosphate ribosyl reaction intermediate with retention of configuration at the C1′ of the ribose relative to the starting material. A recent study on the CD38 mechanism (*37*) has shown that cyclization and hydrolysis processes go via a partition mechanism in which a stabilized active-site-bound intermediate is capable of behaving as a ribosylating electrophile. However, none of these studies has been performed on *A. californica* cyclase, and this latest conclusion might not be applicable to this cyclase. The enzyme active site and reaction intermediates seem inaccessible to nucleophilic species such as H_2O (no ADPR formation) other than those present in the enzyme active site after initial binding. Inageda et al. have shown that the cyclization was fully reversible only when nicotinamide and cADPR were incubated in the presence of the cyclase (*29*). When all these aspects are considered, the motifs that are the most likely to exhibit tight interactions with the enzymatic binding site are the two aromatic moieties (nicotinamide and purines) and potentially the nicotinamide ribose.

Nicotinamide is more readily bound than ADPR and IDPR although it lacks all the other features of NAD^+. This observation emphasizes the importance of the nicotinamide moiety in the initial interactions with the enzyme catalytic site. Since nicotinamide has pK_a values of 10.6 and 13.5, binding of nicotinamide to the cyclase at physiological pH probably involves the charged species. However, the charged nicotinamide ring of the NAD^+ analogues does not seem to be involved in this first recognition and binding step as shown by the nanomolar K_i of Bp_2A. The low affinity of the enzyme for $AcONAD^+$ indicates that modification of the nicotinamide ribose hydroxyls reduces substrate binding affinity. It would seem that merely the presence of an aromatic ring in the location of the nicotinamide binding site leads to binding. Purine binding to the nicotinamide enzyme binding site was observed when Ap_2A and Hp_2H were tested as competitive inhibitors. The observed difference in behavior between the two symmetrical species Ap_2A and Hp_2H indicates that the preferred binding is between enzyme and adenine. Ap_2A is more tightly bound than Hp_2H, indicating that the enzyme shares more stabilizing interactions with adenine than with hypoxanthine. It is also possible that the hypoxanthine is not readily bound by the nicotinamide binding pocket, which results in a loss of inhibition. Lee et al. observed that a very small percentage of 2ADPR was formed by incubating *A. californica* with NAD^+ and ADPR (*30*). It is, therefore, possible that the adenine moiety of NAD^+ dissociates from the enzyme binding site before cyclization occurs (Scheme 2) and that the enzyme binds another aromatic nucleophilic species present in higher concentration. Ap_2A also binds more tightly to the enzyme active site than Np_2N by approximately 25-fold. The gain from introducing the nicotinamide of Np_2N, native to its pocket, might be negatively outweighed by placing the second charged nicotinamide in the cleft typically occupied by the adenine of NAD^+. This would indicate that this latter cleft would preferably bind neutral moieties.

While the nicotinamide seems to be required for initial recognition, adenine would be involved in subsequent tighter binding. The nanomolar level competitive inhibition observed for both benzamide derivatives of NAD^+ is consistent with tight binding between the enzyme catalytic site and these inhibitors. Benzamide would mimic nicotinamide geometrically in Bp_2A and adenine in Bp_2N by binding the native cleft, respectively. The absence of either a labile glycosidic

Scheme 2: Model of the Binding of Substrates or Inhibitors by ADP-Ribosyl Cyclase Depending on the Purine Substituents[a]



[a] Dinucleotides and analogues can theoretically bind to the enzyme active site (shaded area) in conformation I, II, or III. Binding in I and II would involve simultaneous or rapid stepwise binding of the nicotinamide and the purine to the enzyme active site. In III, the nicotinamide binds initially to the active site, allowing appropriate alignment of the purine for cyclization (routes c and d). If I or II describes how the purine binds to the enzyme, then III could be considered as an intermediate, allowing rotation of the purine ring and providing the correct orientation for cyclization. While route a would lead to cADPR analogues where the glycosidic bond is formed between ribosyl C1′ and N1 of the purine, route b would result in the formation of cGDPR-type compounds where the glycosidic bond is formed between ribosyl C1′ and N7 of the purine.

bond (in Bp_2A) or a nucleophile (in Bp_2N) prevents cyclization. It would seem that the labile glycosidic linkage between the nicotinamide and the ribose ring is not required to provide stabilizing interactions since Bp_2A and Bp_2N display almost identical inhibition characteristics. The first stabilized species on the reaction coordinate must be reached without cleavage of the nicotinamide ribose bond. This proposal suggests that the low K_m and K_i values observed for NGD^+ and NHD^+ are not due to an increase in the stabilizing interactions between the purine and the binding site but rather the inability of the enzyme to release them after they have reached a certain acyclic transition state.

Additionally, from these various observations the possibility of having two distinct binding sites on *A. californica* cyclase for each end of NAD^+ can be postulated (Scheme 2). Simultaneous or sequential binding can be proposed. On one hand, nicotinamide and the purine ring of NAD^+ folded in the proper conformation could bind to the aromatic-rich enzyme domains, activating the catalytic process by, for example, provoking conformational change in the enzyme. On the other hand, substrate (or inhibitor)−enzyme interactions could involve strong binding interactions, such as $\pi-\pi$ stacking, between one of the aromatic-rich domains of the cyclase and the nicotinamide aromatic ring. The nicotinamide moiety of NAD^+ could act as an anchor and allow the other aromatic moiety of NAD^+(or other nucleotide derivatives) to fold and appropriately bind the cyclase active site. The final binding might cause some conformational change in the enzyme or be a result of some conformational change induced by nicotinamide.

Our studies in designing potent inhibitors of *A. californica* cyclase have led to a range of compounds with very distinct affinity for the enzyme. While known enzyme inhibitors such as ADPR and nicotinamide inhibit the enzyme at millimolar level, recently synthesized benzamide derivatives yield nanomolar inhibitors. The importance of the unsubstituted nicotinamide ribose for initial binding is emphasized by the lack of affinity that the acetylated NAD^+ derivative displays for the enzyme. The results from the benzamide derivatives of NAD^+, both potent inhibitors of the *A. californica* cyclase, indicate that a labile bond at the C1′ of the nicotinamide ribose is not required for tight interaction with the enzyme active site. However, only competitive inhibition can be expected from inhibitors such as Bp_2A and Bp_2N.

It was also important to examine the wider specificity of inhibitors for other ADP ribosyl cyclases and hydrolases in a preliminary fashion. The design of *A. californica* cyclase inhibitors not only provides further information on the mechanism of this enzyme but also identifies a new potent inhibitor of the SUH hydrolase. ADPR has previously been examined as an inhibitor of the SUH hydrolase. However, potentiation of Ca^{2+} release was only observed at concentrations above millomolar level (*35*). At a low micromolar level of Bp_2A, the SUH hydrolase activity was completely eradicated (Figure 3a,b), while cyclization of NAD^+ to cADPR still occurred (Figure 4). Nicotinamide has been shown to inhibit the SUH cyclase at high millimolar concentrations without inhibiting the hydrolase activity (*36*). Bp_2A displays the alternate behavior. Increasing or maintaining constant the level of cADPR in assays is now possible with almost no perturbation on the rate of cyclization of NAD^+ to cADPR. The cyclase activity was only partially abolished at micromolar levels (Figure 4). This observation could infer that NAD^+ is a better substrate for the SUH bifunctional enzyme than cADPR since it appears that Bp_2A (structural analogue of NAD^+) competes more efficiently with cADPR for the enzyme binding site than with NAD^+. Yet, it is very likely that the level of inhibition observed for the cyclase does not represent the true effectiveness of Bp_2A as an SUH ADP ribosyl cyclase inhibitor. While lower levels of cADPR are produced from NAD^+ by the cyclase in the presence of Bp_2A, its hydrolysis by the hydrolase activity is also inhibited. It is also important to notice that the inhibition of the SUH cyclase by Bp_2A not only delays the Ca^{2+} release

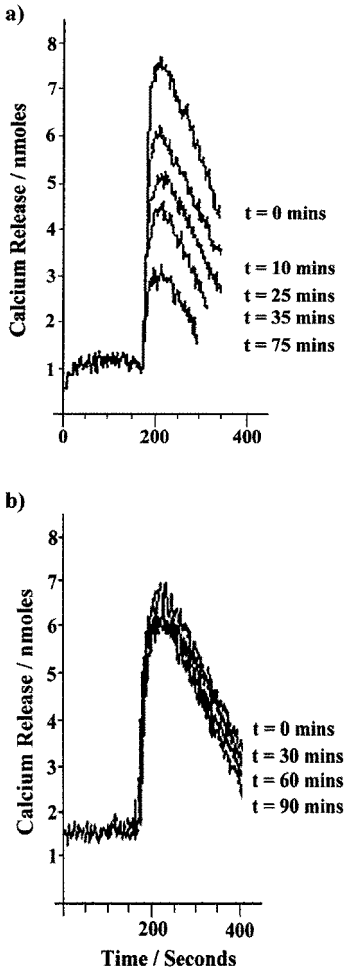


FIGURE 3: Inhibition of sea urchin homogenate cADPR hydrolase by Bp_2A. The depletion of cADPR due to the SUH cADPR hydrolase is monitored by assaying cADPR-induced Ca^{2+} release (a). Inhibition of the hydrolase (b) is shown by a maintained level of released Ca^{2+}. The initial concentrations of cADPR and Bp_2A were 35 and 100 μM, respectively.

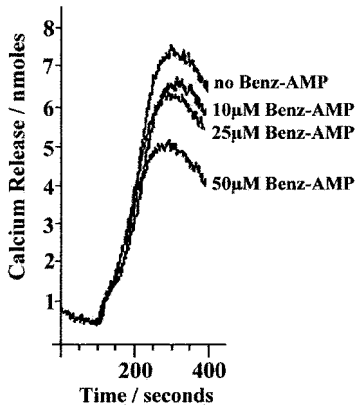


FIGURE 4: Inhibition of the sea urchin homogenate ADP ribosyl cyclase by Bp_2A. The depletion of cADPR due to the inhibition of the SUH ADP ribosyl cyclase by Bp_2A is monitored by assaying the cADPR-induced Ca^{2+} release. The assay was performed on SUH in which 10 μM NAD^+ was incubated with the indicated concentrations of Bp_2A.

response (Figure 5a) but also results in an overall lower level of released Ca^{2+}. These results indicate that Bp_2A is a potent SUH ADP ribosyl cyclase inhibitor.

Attempts (results not shown) to inhibit the CD38 cyclase by the benzamide derivatives showed that these NAD^+

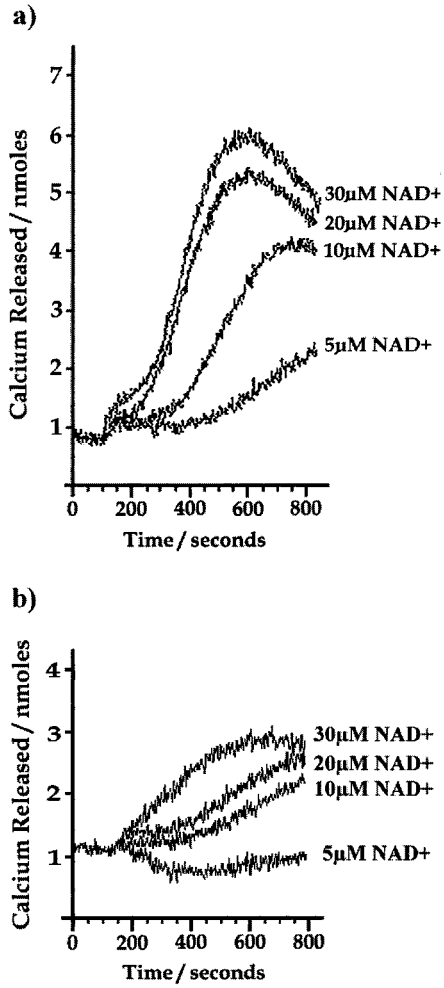


FIGURE 5: Inhibition of the sea urchin homogenate ADP ribosyl cyclase by Bp_2A. Comparison of Ca^{2+} release induced by cADPR from NAD^+ (various concentrations) in the absence (a) and in the presence of Bp_2A (b).

analogues were not able to compete effectively at a micromolar level against NGD^+ for the enzyme active site. Caution should be taken in asserting that the benzamide derivatives are not inhibitors of the CD38 cyclase since NGD^+ is not the true substrate. However, it is possible that the first stable reaction intermediate in CD38 cyclization requires a charged species which cannot be reached by the uncharged Bp_2A. This first reaction intermediate has been proposed by Schramm et al. (*37*). The present result therefore emphasizes the difference in mechanism between *A. californica* cyclase and CD38 since this first uncharged reaction intermediate seems accessible by *A. californica* cyclase bound Bp_2A. Therefore, CD38 appears to be a less suitable system to assay for cyclase and hydrolase inhibitors than *A. californica* cyclase. Also, the assay methods used to test inhibitors on the latter enzyme are more reliable since they employ the true substrate. *A. californica* cyclase also appeared to be inhibited by a wider range of inhibitors than CD38, making it a better tool for screening inhibition.

In summary, we have shown that nanomolar competitive inhibition of *A. californica* cyclase can be obtained with compounds possessing a nonhydrolyzable nicotinamide-related motif (Bp_2A, Bp_2N). These inhibitors may exhibit interesting in vivo properties since Bp_2A is found to be a nanomolar inhibitor of the hydrolase activity in the SUH.

Improving enzyme binding might be achieved by considering noncyclizable NHD^+ analogues such as Bp_2H, the hypoxanthine analogue of Bp_2A. These data represent some first steps in the design of ADP-ribosyl cyclase inhibitors, which may ultimately lead to more desirable inhibitors of mammalian cyclases to intervene pharmacologically in this new cell signaling pathway.

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