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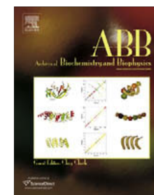

The base exchange reaction of NAD$^+$ glycohydrolase: Identification of novel heterocyclic alternative substrates

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ABSTRACT

ADP-ribosyl cyclase and NAD$^+$ glycohydrolase (CD38, E.C.3.2.2.5) efficiently catalyze the exchange of the nicotinamidyl moiety of NAD$^+$, nicotinamide adenine dinucleotide phosphate (NADP$^+$) or nicotinamide mononucleotide (NMN$^+$) with an alternative base. 4′-Pyridinyl drugs (amrinone, milrinone, dismerinone and pinacidil) were efficient alternative substrates (k_{cat}/K_M = 0.9–10 $\mu M^{-1}\ s^{-1}$) in the exchange reaction with ADP-ribosyl cyclase. When CD38 was used as a catalyst the k_{cat}/K_M values for the exchange reaction were reduced two or more orders of magnitude (0.015–0.15 $\mu M^{-1}\ s^{-1}$). The products of this reaction were novel dinucleotides. The values of the equilibrium constants for dinucleotide formation were determined for several drugs. These enzymes also efficiently catalyze the formation of novel mononucleotides in an exchange reaction with NMN$^+$, k_{cat}/K_M = 0.05–0.4 $\mu M^{-1}\ s^{-1}$. The k_{cat}/K_M values for the exchange reaction with NMN$^+$ were generally similar (0.04–0.12 $\mu M^{-1}\ s^{-1}$) with CD38 and ADP-ribosyl cyclase as catalysts. Several novel heterocyclic alternative substrates were identified as 2-isoquinolines, 1,6-naphthyridines and tricyclic bases. The k_{cat}/K_M values for the exchange reaction with these substrates varied over five orders of magnitude and approached the limit of diffusion with 1,6-naphthyridines. The exchange reaction could be used to synthesize novel mononucleotides or to identify novel reversible inhibitors of CD38.
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Members of the ADP-ribosyl cyclases/NAD$^+$ glycohydrolase (E.C.3.2.2.5) enzyme family are found in both prokaryotic and in eukaryotic systems. These enzymes catalyze the equilibration of nicotinamide adenine dinucleotide (NAD$^+$) with cyclic ADP-ribose (cADPR) and free nicotinamide. They also catalyze hydrolysis of NAD$^+$ to ADP-ribose (ADPR) and nicotinamide [1–5]. Two nucleotide products of the enzymatic reaction, cADPR and ADPR, are implicated as second messengers in a number of important biological processes and disease states [6,7]. For example, cADPR stimulates the release of calcium from ryanodine-sensitive intracellular stores [6] and ADPR positively modulates a membrane-bound calcium channel (LTRPC2), resulting in a net influx of calcium [8]. Rising levels of intracellular calcium may enhance insulin or cytokine secretion, alter neutrophil chemotaxis or inhibit bone resorption [9–11].

Aplysia ADP-ribosyl cyclase has 27–35% primary sequence identity with the human membrane-bound glycoproteins CD38 and CD157/BST-1. Both CD157/BST-1 and the *Aplysia* enzyme crystallize as homodimers and have a high degree of structural similarity. Comparison of the crystal structures of these enzymes indicated that each subunit contains a binding pocket for NAD$^+$. The high degree of structural and functional similarity has justified using ADP-ribosyl cyclase as a biochemical model for CD38 [11–13]. The catalytically relevant residues are functionally conserved between the *Aplysia* and human enzymes [12].

Site-directed mutagenesis and mechanism-based inhibitor studies with CD38 demonstrated that a glutamyl residue displaces the nicotinamidyl moiety of NAD$^+$ to yield a covalent ester-linked ADP-ribosyl intermediate. The glutamyl residue is subsequently displaced by an incoming nucleophile to yield product and regenerate enzyme [14]. For example, nucleophilic attack by the endocyclic N1 residue of adenine results in the formation of the hormone, cADPR (*Aplysia*). If the attacking nucleophile is hydroxide the products of the reaction are ADP-ribose and nicotinamide (CD38), resulting in the net hydrolysis of NAD$^+$. With both enzymes nicotinamide analogs can displace the glutamyl residue to form novel dinucleotide analogs (XDN). In this case the overall reaction is a nicotinamide exchange (transglycosidation) reaction [15].

We were interested in exploring the possibility that potent nicotinamide analogs (heterocycles) could potentially perturb cADPR or ADPR-dependent signaling pathways. Heterocycles can potentially inhibit the formation of cADPR by functioning as alternative substrates in the exchange reaction [16]. Inhibition of cADPR-dependent signaling pathways may prove to be pharmacologically relevant in several disease states [9,10]. Imidazole, thiadazole, indazole, benzimidazole and benzotriazole analogs have been used as substrates for the exchange reaction to create novel (mono-) and dinucleotides [17–19]. Several of these heterocyclic mono- or dinucleotide analogs are known antimetabolites, that inhibit pyridine-

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dependent dehydrogenases [20]. Synthesis of novel dinucleotides usually requires high concentrations of heterocyclic bases because the equilibrium constant for dinucleotide formation is typically large (>1 mM).

Isoniazid (isonicotinic hydrazide) is a 4′-pyridinyl drug that is used in the therapy of *Bacillus tuberculosis* infections. Isoniazid is also a substrate in the exchange reaction [21]. The product of the exchange reaction of isoniazid with NAD^+ glycohydrolase is a novel dinucleotide, with a isoniazidyl moiety replacing the nicotinamidyl moiety. The dinucleotide product has been detected in lung tissue treated with drug [22].

Pinacidil, ((±) *N*-cyano-*N*′-4-pyridinyl-*N*″-[1,2,2-trimethylpropyl]-guanidine) is a 4′-pyridinyl drug that is used in the therapy of hypertension. Pinacidil is a K^+ channel opener (KCOs). The drug is believed to act by preferentially binding to the SUR2 subunits of vascular K_{ATP} channels. Tight binding ($K_D < 1$ μM) of pinacidil to SUR2 apparently occurs only in the presence of Mg^{2+}ATP. Binding causes activation of the K_{ATP} channel leading to hyperpolarization of the vascular endothelium, vasorelaxation and reduced blood pressure [23]. This unique mechanism of drug action differentiates KCOs from peripheral vasorelaxants like amrinone and milrinone, which are type III phosphodiesterase inhibitors.

Amrinone, (5-amino-[3,4′-bipyridine]-6-(1H)-one, inamrinone, Inocor^R) and milrinone (1,6-dihydro-2-methyl-6-oxo-[3,4′-bipyridine]-5-carbonitrile, Primacor^R) are bipyridinyl drugs that are used in the therapy of congestive heart failure [24]. Milrinone is a potent peak III cyclic AMP phosphodiesterase (PDE III) inhibitor that increases the contractile force of cardiac muscle [24]. The steady-state blood concentrations of milrinone (0.45–1.2 μM) are consistent with the inhibition of PDE III causing the observed vasodilator effects. In contrast to milrinone, the mechanism of amrinone's vasodilator and inotropic effects is less well understood.

Herein we show that amrinone, milrinone, dismerinone (desmethylmilrinone) and pinacidil are efficient alternative substrates of the ADP-ribosylcyclase/$NAD(P)^+$ glycohydrolase family of enzymes. We measured the catalytic efficiencies (k_{cat}/K_M values) with ADP-ribosyl cyclase and CD38, a prototypical NAD^+ glycohydrolase. We measured the value of the equilibrium constant for formation of the dinucleotide products from amrinone, dismerinone, pinacidil and cADPR.

In an effort to develop a novel screening technology for isolation of NAD glycohydrolase inhibitors, several novel heterocyclic bases were identified as efficient alternative substrates. The NAD^+ glycohydrolase-catalyzed reaction between 2-isoquinolines, 1,6-naphthyridines, tricyclic bases and NAD^+/NMN^+ were characterized by absorbance and fluorometric methods. Substrate (NMN^+, NAD^+) and species-specific results were obtained using CD38 and *Aplysia* ADP-ribosyl cyclase.

Materials and methods

Materials

Aplysia ADP-ribosyl cyclase, NAD^+, $NADP^+$, cADPR, nicotinamide mononucleotide (NMN^+), nicotinic acid mononucleotide ($NAMN^+$), AICAR monophosphate, adenosine 5′-diphosphoribose ribose (ADPR), nicotinamide 1,N^6-ethenoadenine dinucleotide (EAD^+), nicotinamide 1,N^6-ethenoadenine dinucleotide phosphate ($EADP^+$), nicotinamide guanine dinucleotide (NGD^+), nicotinamide hypoxanthine dinucleotide (NHD^+), nicotinamide hypoxanthine dinucleotide phosphate ($NHDP^+$), nicotinic acid adenine dinucleotide ($NAAD^+$), nicotinic acid adenine dinucleotide phosphate ($NAADP^+$), 3-acetylpyridine adenine dinucleotide ($APAD^+$), 3-acetylpyridine adenine dinucleotide phosphate ($APADP^+$), 3-pyridinealdehyde adenine dinucleotide ($PAAD^+$), thionicotinamide adenine dinucleotide ($TNAD^+$), nicotinamide, amrinone, milrinone, pinacidil, norharmane, harmane, Hepes and $MgCl_2$ were from Sigma Chemical Co. Dismerinone (WIN 37582), AH-, SKF-, GR-, GW-compounds were obtained from the GlaxoSmithKline compound registry. All GSK compounds were dissolved in 100% DMSO and concentrations were determined gravimetrically. Human CD38 was genetically engineered to remove four potential glycosylation sites and a membrane-spanning segment. CD38 was purified from *Pichia pastoris* cell culture supernatants essentially as described [25], with the exception of an additional heparin sulfate chromatography step before the final P10 desalting step.

Assay of ribosyl cyclase activity

The conversion of drugs into their respective di- or mononucleotides was monitored spectrophotometrically, using either fluorescence (dismerinone, pinacidil) or absorbance methods (dismerinone, milrinone, pinacidil and amrinone). Rates were calculated from the time-course of the absorbance changes using $\Delta\varepsilon_{380} = 28.2\ \text{mM}^{-1}\,\text{cm}^{-1}$ for dismerinone dinucleotide, $\Delta\varepsilon_{318} = 9.5\ \text{mM}^{-1}\,\text{cm}^{-1}$ for pinacidil dinucleotide and $\Delta\varepsilon_{390} = 11\ \text{mM}^{-1}\,\text{cm}^{-1}$ for amrinone dinucleotide (ADN).[1] The extinction coefficient values for the dinucleotides were determined from the fitted maximal absorbance values (using a fixed concentration of drug) obtained in the determination of K_{eq} values (see below). Alternatively, consumption of dismerinone or pinacidil was monitored by the decrease in fluorescence associated with dinucleotide formation. A standard curve was generated for each photomultiplier voltage and slit width setting to determine the number of nanomoles per unit of fluorescence. The standard buffer was 0.05 M Hepes (Na^+), 5 mM $MgCl_2$ at pH 7.0 and 25 °C (buffer A).

K_{eq} determination

The equilibrium concentrations of dinucleotides were determined in solutions that initially contained varying concentrations of drug or nicotinamide with a constant concentration of cADPR in buffer A. Alternatively, varying concentrations of cADPR were mixed with a constant concentration of drug. The reactions were initiated with 15 nM enzyme. Equilibrium was reached after 30 min at room temperature (absorbance stability).

The equilibration of cADPR ($[NA]_t$) with drug or nicotinamide ($[X]_t$) was described by Eq. (1).

$$K_{eq} = \frac{[\text{cADPR}][\text{X}]}{[\text{XDN}]} \quad (1)$$

Titration data for conversion of drug to the corresponding dinucleotide were analyzed by Eq. (2).

$$A([\text{NA}]) = A_0 + A_F\left(\frac{[\text{NA}]_t + [\text{X}]_t + K_{eq}}{2[\text{NA}]_t} - \frac{1}{2[\text{NA}]_t}\sqrt{([\text{NA}]_t + [\text{X}]_t + K_{eq})^2 - 4[\text{X}]_t[\text{NA}]_t}\right) \quad (2)$$

The K_{eq} value for the equilibration of cADPR with drug was calculated from these data with the quadratic equation (the concentration of drug ($[X]_t$) was similar to K_{eq}). Where $[NA]_t$ represents the concentration of cADPR used, $A([NA])$ is the absorbance of the solution, A_0 is the absorbance with $[NA]_t = 0$ and $A_F + A_0$ is the absorbance at saturating [NA]. Quenching of dismerinone fluorescence was also used to monitor the reaction.

Substrate efficiency (k_{cat}/K_M) for dinucleotide substrates was

[1] *Abbreviations used:* ADN, amrinone dinucleotide; DDN, dismerinone dinucleotide; PDN, pinacidil dinucleotide; PMN, pinacidil mononucleotide, E, enzyme, ADPR, ADP-ribose, E-ADPR, covalent complex between E and ADPR, GSK, GlaxoSmithKline.

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