Notes

Mechanism of Inhibition of Poly(ADP-ribose) Glycohydrolase by Adenosine Diphosphate (Hydroxymethyl)pyrrolidinediol

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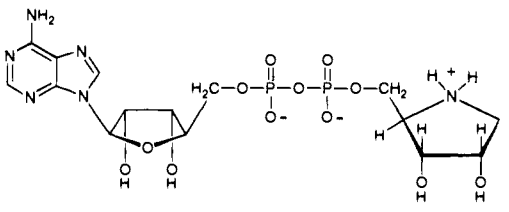
Adenosine diphosphate (hydroxymethyl)pyrrolidinediol (ADP-HPD), a nitrogen-in-the-ring analog of ADP-ribose, was recently shown to be a potent and specific inhibitor of poly(ADP-ribose) glycohydrolase. Analysis of the inhibition kinetics of the hydrolase by ADP-HPD using the method of Lineweaver and Burk yields a noncompetitive double-reciprocal plot. Both the intercept ($1/V$) versus [inhibitor] replot and the slope (K_m/V) versus [inhibitor] replot are hyperbolic, indicating partial noncompetitive inhibition. Inhibitor dissociation constants K_{ii} = 52 nM and K_{is} = 80 nM were determined for ADP-HPD by analysis of the intercept versus [inhibitor] and slope versus [inhibitor] replots. These results show that although ADP-HPD is extremely potent in inhibiting poly(ADP-ribose) glycohydrolase, its effectiveness is limited by its partial inhibition. ADP-HPD was significantly less potent as an inhibitor of the NAD glycohydrolase from *Bungarus fasciatus* venom. Analysis of the inhibition kinetics using the Lineweaver and Burk method indicated that ADP-HPD was a linear-competitive inhibitor of the NAD glycohydrolase with a K_i of 94 μM. The results indicate that at low concentration ADP-HPD will be a selective inhibitor of poly(ADP-ribose) glycohydrolase; however, complete inactivation of the activity will be difficult to obtain.

Introduction

Poly(ADP-ribose) glycohydrolase is the enzyme responsible for the degradation of ADP-ribose polymers in the nucleus of eukaryotic cells (Figure 1).[1] Since the metabolism of ADP-ribose polymers is required for the process of cellular recovery from DNA damage,[2] we proposed that inhibition of poly(ADP-ribose) glycohydrolase will prevent the removal of poly(ADP-ribose) from chromatin, prevent the completion of the cycle of DNA repair, and therefore increase the cytotoxicity of DNA damaging drugs and radiation. We recently reported that *a*denosine *di*phosphate (*h*ydroxymethyl)-*p*yrrolidine*di*ol (ADP-HPD, 1), an –NH– analog of ADP-ribose, was a highly specific inhibitor of poly(ADP-ribose) glycohydrolase, which inhibited the glycohydrolase with an IC_{50} of 0.12 μM.[3] ADP-HPD is therefore a lead structure for developing chemosensitizing agents for cancer chemotherapy.[3] ADP-HPD will further be applied as a tool to study ADP-ribose polymer metabolism



ADP-HPD (1)

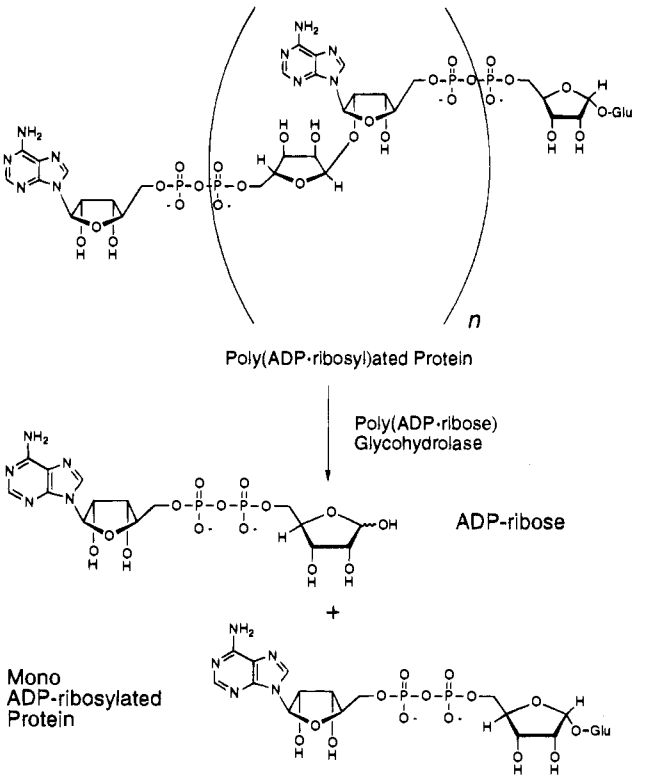


Figure 1. Hydrolysis of ADP-ribose polymers to ADP-ribose and an ADP-ribose–protein core catalyzed by poly(ADP-ribose) glycohydrolase. The final ADP-ribosyl residue is removed from the protein by a second enzyme, ADP-ribosyl protein lyase.

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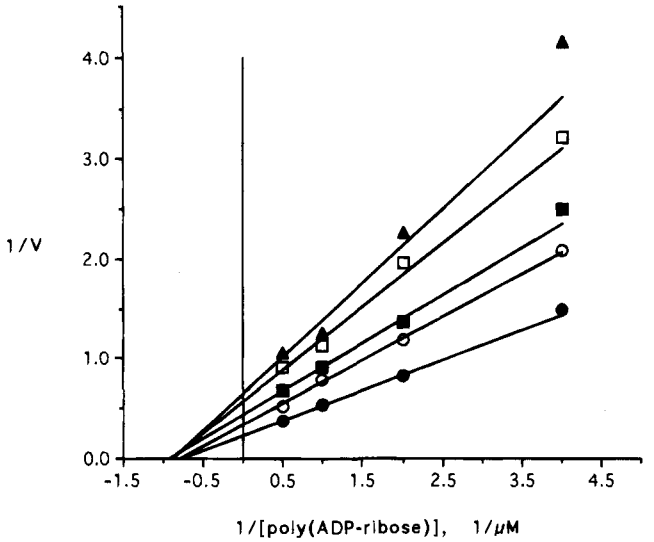

Figure 2. Lineweaver–Burk plot showing the effect of the addition of ADP-HPD (1) on the initial rates of hydrolysis of ADP-ribose polymers catalyzed by poly(ADP-ribose) glycohydrolase. The assay was conducted as described in the Experimental Section. The units for the vertical axis, $1/V$, are $(\text{pmol/min})^{-1}$. The concentration of the substrate poly(ADP-ribose) was varied from 0.25 to 2 μM. Inhibitor concentrations were (●) no inhibitor, (○) 0.50 μM, (■) 0.1 μM, (□) 0.2 μM and (▲) 0.4 μM. The slope (K_m/V) and intercept ($1/V$) for each line defined at each fixed concentration of inhibitor were determined by nonlinear regression fit to eq 1, the Michaelis–Menten equation.

by selectively inhibiting the glycohydrolase[4] and to develop specific photoaffinity labels and affinity absorbents for poly(ADP-ribose) glycohydrolase.[5] The continuing development of ADP-ribose glycohydrolase inhibitors with improved pharmacological properties and the application of ADP-HPD to studies on glycohydrolase inhibition necessitates an understanding of its mechanism of inhibition. In this study we report the elucidation of the kinetic mechanism of inhibition of poly(ADP-ribose) glycohydrolase and the related NAD glycohydrolase by ADP-HPD.

Results

The mechanism of inhibition of poly(ADP-ribose) glycohydrolase by ADP-HPD was determined by analysis of the effect of varying substrate concentrations on the initial rates of enzymatic hydrolysis conducted at several fixed inhibitor concentrations. The results are shown graphically as a double-reciprocal plot in Figure 2, according to the method of Lineweaver and Burk. The convergence of the lines for each fixed inhibitor concentration to a point on the horizontal 1/[substrate] axis indicated that ADP-HPD was a noncompetitive inhibitor of poly(ADP-ribose) glycohydrolase.[6,7]

The change in the magnitude of the intercept values, $1/V_{max}$, as a function of inhibitor concentration is shown in Figure 3A. The relationship is hyperbolic, meaning that the inhibitor is less effective at high concentrations than would be predicted by linear extrapolation of its activity at low concentrations. A similar hyperbolic relationship was observed between the change in the magnitude of the slopes, K_m/V_{max}, and inhibitor concentration as shown in Figure 3B. ADP-HPD is therefore best described as an intercept-hyperbolic, slope-hyperbolic noncompetitive inhibitor of poly(ADP-ribose) glycohydrolase.

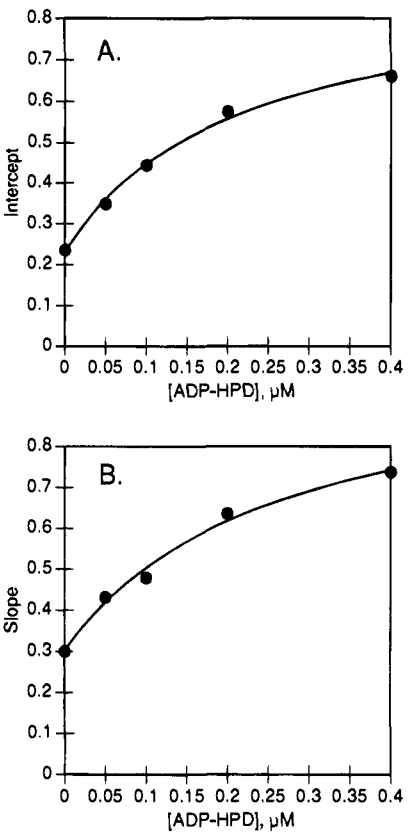

Figure 3. (A) Replot of the intercepts $1/V$ $[(\text{pmol/min})^{-1}]$ versus [I] (μM). The solid line was calculated using eq 2, and the experimental parameters determined for the $1/V$ vs [I] replot are as listed in Table 1. (B) Replot of the slope K/V [(μM)/(pmol/min)] versus [I] (μM). The solid line was calculated using eq 2, and the experimental parameters determined for the K_m/V vs [I] replot are as listed in Table 1.

To determine the magnitude of the inhibition constants, the replot data were fit to the equation describing a hyperbola using a nonlinear least-squares method (see the Experimental Section), and the results are presented in Table 1. K_{ii}, the dissociation constant for the release of the inhibitor from the enzyme–substrate–inhibitor complex, was determined from the $1/V$ versus [I] replot to be 52 ± 10 nM. The dissociation constant for the inhibitor from the enzyme–inhibitor complex, K_{is}, was similarly determined by analysis of the K/V (slope) versus [I] replot to be 80 ± 20 nM.

Effect of ADP-HPD on NAD Glycohydrolases. ADP-HPD inhibited the hydrolysis of NAD to ADP-ribose and nicotinamide catalyzed by the *Bungarus fasciatus* venom NAD glycohydrolase[8] at high concentrations. When the glycohydrolase assay was conducted at pH 7.5 using 50 μM substrate, the IC_{50} for inhibition of the venom enzyme was 260 μM. The mechanism of inhibition of the venom NAD glycohydrolase by ADP-HPD was of interest because this enzyme catalyzes a hydrolytic reaction at an ADP-ribose linkage but possesses an active site capable of accommodating only a single ADP-ribose residue. The mechanism of its inhibition by ADP-HPD was established by measuring the initial rates of NAD hydrolysis as a function of substrate concentration at several fixed concentrations of inhibitor. The results are shown in a Lineweaver–Burk plot in Figure 4, where the individual lines converge to a point on the vertical $1/V$ axis, a result indicative of competitive inhibition. ADP-HPD was further shown

Table 1. Kinetic Constants for Inhibition of Poly(ADP-ribose) Glycohydrolase by ADP-HPD

replot data source[a]	a[b]	K_{in}[c] (μM)	K_{id}[d] (μM)	$a(K_{id}/K_{in})$[e]
$1/V$ vs [I] replot	0.23 ± 0.05 (pmol/min)$^{-1}$	0.052 ± 0.01 (K_{ii})	0.21 ± 0.05	0.885 (pmol/min)$^{-1}$
Km/V vs [I] replot	0.301 ± 0.02 (μM)(pmol/min)$^{-1}$	0.08 ± 0.02 (K_{is})	0.26 ± 0.09	0.98 (μM)(pmol/min)$^{-1}$

[a] Data are fit to eq 2 as described in the Experimental Section. [b] Represents either $1/V_{max}$ or K_m/V_{max} for the uninhibited enzyme. [c] Represents the inhibitor dissociation constant. [d] Describes the approach of the hyperbolic function to its new limiting value as [I] increases. [e] Limiting value of replot as [I] increases.

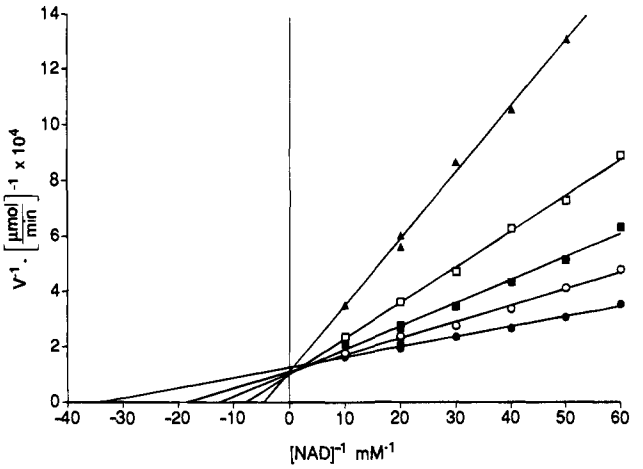

Figure 4. Lineweaver–Burk plot showing the effect of the addition of ADP-HPD (1) on the initial rates of hydrolysis of NAD catalyzed by the *B. fasciatus* venom NAD glycohydrolase. The assay was conducted as described in the Experimental Section. The concentration of NAD was varied from 16.7 to 100 μM. Inhibitor concentrations were (●) no inhibitor, (○) 48.7 μM, (■) 103.4 μM, (□) 200.8 μM, and (▲) 499 μM. The slope and intercept for each line defined by each fixed concentration of inhibitor were determined by nonlinear regression fit to eq 1, the Michaelis–Menten equation. Replots of the intercepts or the slopes determined for the family of lines shown in the figure were linear (data not shown).

to be a linear competitive inhibitor of the NADase. The data were fit to the equation describing competitive inhibition using the Cleland procedure[9] to determine that the inhibitor dissociation constant was 94 ± 4 μM.

Discussion

Sugar analogs in which the ring oxygen is replaced by nitrogen frequently inhibit glycosidases and nucleotidases. These nitrogen-in-the-ring sugar derivatives are often envisioned as electronic transition state analogs, mimicking the positive charge development of an oxocarbonium ion-like intermediate. The results of this study show that ADP-HPD is unlikely to function as a transition state analog. ADP-HPD inhibits poly(ADP-ribose) glycohydrolase noncompetitively, lowering V_{max}. For single substrate enzymes in rapid equilibrium with substrate and products, noncompetitive inhibition means that inhibitor binds both to free enzyme and to the enzyme–substrate complex. Therefore a noncompetitive inhibitor and the substrate often bind at separate sites on the enzyme surface allowing the formation of a ternary enzyme–substrate–inhibitor (E–S–I) complex. Although it is possible that ADP-HPD interacts with an undiscovered regulatory site, ADP-HPD may occupy part of the substrate-binding site since poly(ADP-ribose) hydrolyzes a polymeric substrate and likely posssesses an extended substrate-binding domain capable of interacting with multiple ADP-ribosyl residues at several subsites. This is experimentally supported by the observation that long ADP-ribose polymers exhibit a much higher affinity for the enzyme than do ADP-ribose oligomers.[10,11] In such a case, noncompetitive inhibitors could occupy a binding subsite while still permitting the substrate at a remote site to undero hydrolysis. ADP-HPD in binding within such a subsite lowers the rate of catalysis and therefore reduces V_{max}. The observed hyperbolic relationship between $1/V_{max}$ and K_m/V_m with respect to inhibitor concentration, [I] (Figure 3), however, requires that the inhibitor-saturated enzyme be capable of producing product, albeit at a rate less than the normal rate of product formation. Thus, poly(ADP-ribose) glycohydrolase is still partially active, even in the presence of saturating concentrations of ADP-HPD. The relative activity of the enzyme will depend both on the concentration of inhibitor and upon the concentration of substrate. The V_{max} of the enzyme in the presence of saturating inhibitor can be determined using data in Table 1. At infinite inhibitor concentration, the $1/V$ versus [inhibitor] replot reaches a limiting value of $(1/V)(K_{id}/K_{in}) = 0.885$. The reciprocal of this value, 1.1 pmol/min, is the $V_{max\text{-}app}$ of the inhibited enzyme, a value representing 25% of the activity of the uninhibited enzyme when assayed using saturating levels of substrate.

The ADP-ribose polymers that are synthesized *in vivo* as well as the poly(ADP-ribose) used as a substrate for our assay of poly(ADP-ribose) glycohydrolase are heterogeneous in size.[12] The polymers used in this study consisted mainly of a high molecular weight, highly branched material.[12] Since the glycohydrolase shows preference for hydrolysis of high molecular weight substrate,[10] and since this is the type of ADP-ribose polymer thought to be important physiologically, our assay measuring the initial rates of hydrolysis of the high molecular weight branched polymer should give an accurate indication of the ability of ADP-HPD to inhibit polymer degradation *in vivo*. A detailed interpretation of the mechanism of inhibition of poly(ADP-ribose) glycohydrolase by ADP-HPD can not yet be done because the kinetic mechanism of the uninhibited enzyme itself is not known. Poly(ADP-ribose) glycohydrolase is known to catalyze exoglycosidic cleavage, endoglycosidic cleavage, and polymer debranching. It is possible that ADP-HPD inhibits these reactions differently. The interpretation of the noncompetitive inhibition would be further complicated if the polymeric substrate was hydrolyzed processively. In such a case, the inhibitor could affect the processive nature of the reaction, thereby slowing the overall rate of hydrolysis as we observed. Noncompetitive inhibition might therefore only indicate that the inhibitor binds to a form of the enzyme different from that form of the enzyme which binds substrate and that the rate of breakdown of this ternary complex is slow compared to that of the E–S complex. Interpretation of the detailed mechanism of inhibition must therefore await the investigation of the kinetic mechanism of poly(ADP-ribose) glycohydrolase.

Certain NAD glycohydrolases were previously shown to be sensitive to inhibition by relatively high concentrations of ADP-HPD.[3] We selected the ADP-HPD sensitive NADase from *B. fasciatus* venom for further study because this enzyme was shown to be soluble and stable and could be purified to homogenity using a short, straightforward procedure.[8] The *B. fasciatus* venom enzyme was carefully studied with respect to its sensitivity to inhibition[13] and its ability to catalyze pyridine base exchange reactions[14] and in all respects was found to closely resemble the mammalian NAD glycohydrolase. The mechanism for inhibition of the *B. fasciatus* venom NAD glycohydrolase by ADP-HPD is linear-competitive. The inhibitor dissociation constant for ADP-HPD and the venom NAD glycohydrolase was 94 μM, a value smaller than the K_i of the product ADP-ribose (360 μM) but greater than the K_m of the substrate NAD (15 μM).[8,13] Thus for the single-substrate, hydrolytic enzyme NAD glycohydrolase, ADP-HPD behaves as an ordinary product analog. A practical consequence of the relatively high K_d for inhibition of the NAD glycohydrolase is that low concentrations of ADP-HPD will selectively inhibit poly(ADP-ribose) glycohydrolase activity even in the presence of a NADase.

The potency and high specificity of ADP-HPD suggest that it will become an important tool for the study of ADP-ribose polymer metabolism. Interpretations of the results of such inhibition studies must however recognize that, because of the mechanism of inhibition, residual enzymatic activity will always be present in cell or cell free extracts even in the presence of saturating concentrations of ADP-HPD.

Experimental Section

Materials. ADP-HPD was synthesized from (2*R*,3*R*,4*S*)-2-(hydroxymethyl)pyrrolidine-3,4-diol[15] according to the procedure of Slama et al.[3] Poly(ADP-ribose) glycohydrolase from bovine thymus was purified and characterized as described by Thomassin et al.[16]

Preparation of [^{32}P]Poly(ADP-ribose) Polymers. [^{32}P]-Poly(ADP-ribose) for glycohydrolase assay was synthesized at high specific activity from [^{32}P]NAD and poly(ADP-ribose) polymerase using the procedure described by Keihlbauch et al.,[12] except that core histones (histone type IIA) were omitted from the incubation. After trichloroacetic acid precipitation, poly(ADP-ribose) was released from protein by treatment with 0.5 M KOH and 50 mM EDTA at 30 °C for 30 min and purified by chromatography on a DHB BioRex-70 column as previously described by Aboul-Ela et al.[17] [^{32}P]Poly(ADP-ribose) prepared according to this procedure, when analyzed by anion exchange HPLC,[12] consisted of high molecular weight, multiply branched polymer, with relatively little unbranched ADP-ribose oligomers present. The elution profile of material typically employed as a substrate resembles that described by Keihlbauch et al. in Figure 4, profile C.[12]

Poly(ADP-ribose) glycohydrolase was assayed at 37 °C and pH 7.5 by measuring the release of [^{32}P]ADP-ribose from [^{32}P]poly(ADP-ribose) (10 μM ADP-ribose residues) as described previously by Menard and Poirier.[18] The substrate [^{32}P]poly(ADP-ribose) was present at a monomer concentration at 10 μM. [^{32}P]ADP-ribose which was liberated was separated from the polymer by TLC on poly(ethylenimine)-impregnated cellulose sheets (0.3 M LiCl–0.9 M acetic acid, the spot excised and quantitated radiometrically). One unit of enzyme is the amount that liberates 1 nmol of ADP-ribose/min at 37 °C.

NAD glycohydrolase from *B. fasciatus* venom was purified using the procedure of Yost and Anderson.[8] The NAD glycohydrolase was assayed at 37 °C and pH 7.5 by measuring the release of [*carbonyl*-^{14}C]nicotinamide from [*carbonyl*-^{14}C]NAD as described by Slama and Simmons.[19] The standard assay was conducted for 30 min at 37 °C and contained 33 mM potassium phosphate, pH 7.5, 50 μM [*carbonyl*-^{14}C]NAD (Amersham; 50 000 dpm), and enzyme (0.3 munit) in a total volume of 0.3 mL. A unit of NADase activity is that quantity of enzyme which catalyzes the hydrolysis of 1 μmol of NAD/min.

Analysis of Enzyme Inhibition Data. The effect of varying substrate concentration on initial rates of enzyme-catalyzed hydrolysis was determined in the presence of several fixed inhibitor concentrations. The data for each fixed concentration of inhibitor were fit by a nonlinear regression procedure to the hyperbolic form of the Michaelis–Menten equation:

$$v = V_{max}[S]/(K_m + [S]) \quad (1)$$

and the line so determined was displayed on a double-reciprocal plot. Replots of slopes and intercepts from the double-reciprocal plot were constructed to determine, through analysis of the shape of the line (straight, concave upward, or concave downward), whether the inhibition was linear, parabolic, or hyperbolic.

Where linear inhibition was encountered, kinetic parameters were determined by using the nonlinear regression analysis developed by Cleland[9] using a translation of the program for the IBM PC workstation and compatibles (obtained from Dr. R. E. Viola, Department of Chemistry, University of Akron, Akron, OH). Each data set was fit to equations describing linear-competitive, noncompetitive, and uncompetitive inhibition. The best description of the data was chosen according to the criteria set forth by Cleland for the evaluation of a successful fit.

Where hyperbolic inhibition was encountered, the data from the slope or the intercept replot were fit to the equation:

$$y = a(1 + [I]/K_{i\text{-numerator}})/(1 + [I]/K_{i\text{-denomenator}}) \quad (2)$$

as described by Cleland[9] to determine the inhibitor dissociation constant $K_{i\text{-numerator}}$ using a commercial nonlinear regression program (Scientist version 2.0, Micromath Software, Salt Lake City, UT).

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