THE STEREOSPECIFICITY OF PIG BRAIN NAD-GLYCOHYDROLASE-CATALYZED METHANOLYSIS OF NAD^+

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1. Introduction

Cells contain high concentrations of enzymic activity that cleaves the nicotinamide–glycosyl bond [1]. This activity can be classified into two general types; NAD-glycohydrolases, where the primary acceptor of the ADP-ribose moiety is water, and ADP-ribosyl transferases [2]. To date, the stereospecificity has been determined for three such enzymes. Calf spleen NAD-glycohydrolase [3] has been shown to catalyze β-methanolysis of NAD^+, whereas both poly(ADP-ribose) synthase [4] and choleragen [5] catalyze α-ADP-ribosylation. The difference in stereochemistry found for these enzymes has prompted the initiation of a general investigation into the stereospecificity of enzymes that cleave the nicotinamide–glycosyl bond. This report presents results on the determination of the stereospecificity of pig brain NAD-glycohydrolase and provides a detailed ^1H NMR analysis of the spectra of the products, β-methyl-ribose-5-phosphate and β-methyl-ADP-ribose.

2. Experimental

2.1. *Materials*

NAD^+, NMN^+, calf spleen NAD-glycohydrolase and pig brain NAD-glycohydrolase were obtained from Sigma biochemicals and used without further purification.

2.2. *Synthesis of methyl-ADP-ribose*

NAD^+ or NMN^+ (30 μmol) was dissolved in 10 ml of 0.1 M ammonium bicarbonate, 5 M methanol, then 100 mg (~1.6 units) of the acetone powder preparation of NAD-glycohydrolase (pre-washed twice with buffer) was added. The course of the reaction was monitored by the standard cyanide assay [6] and terminated when the amount of NAD^+ became <5% of the initial value. The mixture was then centrifuged and the supernatant filtered to remove particulate debris. The filtrate was lyophilized and applied to a DEAE cellulose, anion exchange column and eluted with a linear 0–0.5 M ammonium bicarbonate gradient. Under these reaction conditions the ratio of methanolysis to hydrolysis was about 20:1, as determined from the ^1H NMR spectra using the relative integrated areas of the ribosyl anomeric protons. The adenyl and ribosyl moieties will be designated with an A and R, respectively.

2.3. *NMR parameters*

Spectra were obtained at 360 MHz on a Bruker HXS-360 NMR spectrometer using quadrature detection and equipped with a Nicolet Technologies 1180 computer/Fourier transform system and a computer controlled homonuclear decoupling accessory. Pulse angle and time between acquisition of transients was adjusted to yield equilibrium intensities for all resonances. Samples were prepared by twice lyophilizing from 99.8% D_2O then dissolving in 50 mM potassium phosphate buffer (pD 7.1 in 100% D_2O). The concentration of β-methyl-ADP-ribose was determined spectrophotometrically. The internal standard, 0.5 mM 3-trimethylsilyl-2,2,3,3-d_4-sodium propionate (TSP) was used and 0.5 mM EDTA was added to suppress any effects from paramagnetic ions.

3. Results and discussion

3.1. *Spectral analysis*

The ^{1}H NMR spectrum at 360 MHz of the product isolated from incubation of NAD^+ with pig brain NAD-glycohydrolase gives a well resolved spectrum. The ribose region (4.9–3.9 ppm) is shown in fig.1. The assignments of the resonances are based on sequentially spin decoupling the resonances, starting at the anomeric 1′ protons. These latter protons are assigned by comparison with the chemical shift for the A1′ proton in ADP-ribose [7] and the R1′ proton of β-methyl ribofuranoside [3]. The spectrum can be simplified by taking advantage of the differences in longitudinal relaxation times of the methylene and methine proton resonances. Thus, by using the standard 180°–τ–90° pulse sequence [8] and careful selection of τ, spectra can be obtained where either the methine, or alternatively the methylene proton resonances are nulled (see fig.1). This technique also serves to confirm the assignment of methylene and methine proton resonances.

The chemical shifts for β-methyl-ADP-ribose and related compounds are listed in table 1 and the coupling constants in table 2. The values have been derived by an iterative fitting of the observed spectra using the Nicolet Technologies program, ITRCAL.

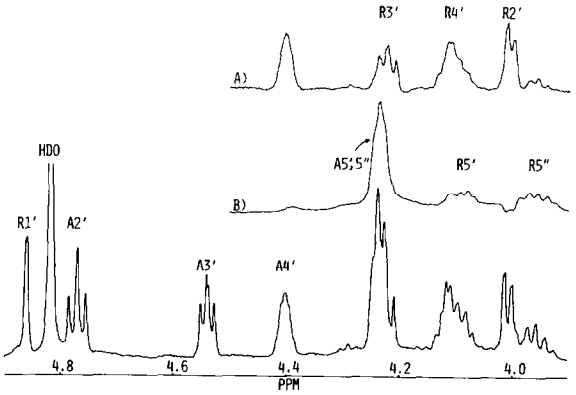

Fig.1. ^{1}H NMR spectrum of the ribosyl proton region of 5 mM β-methyl-ADP-ribose in D_2O. Subspectrum A is a partially relaxed Fourier transform spectrum with a τ value of 0.21 s which nulls the methylene proton resonances, allowing direct observation of the methine resonances. Subspectrum B has a τ value of 0.55 s. which nulls the methine protons, allowing direct observation of the methylene resonances.

Table 1
Chemical shifts[a]

	β-Methyl-ADP-ribose		β-Methyl-ribose-5-P
	Adenyl	Ribosyl	
A8	8.492		
A2	8.225		
1′	6.164	4.886	4.922
2′	4.795	4.029	4.083
3′	4.563	4.249	4.258
4′	4.424	4.136	4.128
5′	(4.265)	4.120	3.947
5″	(4.265)	3.980	3.846
Methyl		3.343	3.428

[a] Chemical shifts are reported in ppm from the internal reference TSP and are accurate to within 0.001 ppm

The coupling constant $J_{1'-2'}$ is critical for the determination of the anomeric configuration since if its value is > 7 Hz or < 3 Hz, then the configuration must be β. This is a consequence of the geometric constraints that limit α-ribofuranosides to coupling constants with values from 3–7 Hz. Thus the value of 1.2 Hz observed for the methyl-ADP-ribose clearly demonstrates a β-linkage. Note that the coupling constants for the model compounds (table 2) are all

Table 2
Coupling constants[a]

	β-Methyl-ADP-ribose		β-Methyl-ribose-5-P
	Adenyl	Ribosyl	
$J_{1'-2'}$	5.8	1.2	1.6
$J_{2'-3'}$	5.0	4.9	4.9
$J_{3'-4'}$	3.9	6.3 ± 0.2	6.3
$J_{4'-5'}$	(2.8)	4.0 ± 0.2	4.1
$J_{4'-5''}$	(2.8)	5.9	5.8
$J_{5'-5''}$	–[b]	−11.3	−11.3
$J_{4'-P}$	2.5	<0.8	<0.5
$J_{5'-P}$	(5.0)	5.4 ± 0.3	5.2
$J_{5''-P}$	(5.0	5.7	5.6

[a] Coupling constants are reported to within 0.1 Hz except as noted

[b] This value can not be determined because of the chemical shift equivalence of the A5′ methylene protons. Coupling constants reported in parentheses represent averaged values because of this equivalence

similar, indicative of the relative insensitivity of $J_{R1'-R2'}$ to the 5′ substituent for these compounds. Finally, the ^1H NMR spectrum (not shown) of methyl-ADP-ribose synthesized by calf spleen NAD-glycohydrolase in 5 M methanol is identical, thus confirming the stereochemical assignments made in [3].

3.2. *Stereospecificity of cleavage*

The pig brain NAD-glycohydrolase has been shown to catalyze the β methanolysis of NAD^+, further evidence for the similarity between the enzymes from pig brain and calf spleen. In contrast the cleavage of the nicotinamide–glycosyl bond by poly(ADP-ribose) synthase [4] and choleragen [5] occurs with the formation of an α-linkage, with the latter enzyme also yielding no detectable methanolysis. The results indicate a possible difference in the mechanism of these proteins compared to the NAD-glycohydrolases like the pig brain enzyme. Finally these experiments provide further evidence for the correlation, albeit speculative, of enzymes that catalyze ADP-ribosylation as being α-specific and those that catalyze hydrolysis as β-specific. It remains to be determined whether this correlation as to α- or β-specificity merely reflects a difference in mechanism between these enzymes or is inherent to their function as well.

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