DNA Repair xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

DNA Repair

journal homepage: www.elsevier.com/locate/dnarepair

Alteration in substrate specificity of horse liver alcohol dehydrogenase by an acyclic nicotinamide analog of NAD$^+$

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ARTICLE INFO

Article history:
Received 10 April 2014
Received in revised form
10 September 2014
Accepted 13 September 2014
Available online xxx

Keywords:
Alcohol dehydrogenases
NAD$^+$ analogs
α-NADH
Coenzyme specificity

ABSTRACT

A new, acyclic NAD-analog, acycloNAD$^+$ has been synthesized where the nicotinamide ribosyl moiety has been replaced by the nicotinamide (2-hydroxyethoxy)methyl moiety. The chemical properties of this analog are comparable to those of β-NAD$^+$ with a redox potential of −324 mV and a 341 nm λ_{max} for the reduced form. Both yeast alcohol dehydrogenase (YADH) and horse liver alcohol dehydrogenase (HLADH) catalyze the reduction of acycloNAD$^+$ by primary alcohols. With HLADH 1-butanol has the highest V_{max} at 49% that of β-NAD$^+$. The primary deuterium kinetic isotope effect is greater than 3 indicating a significant contribution to the rate limiting step from cleavage of the carbon–hydrogen bond. The stereochemistry of the hydride transfer in the oxidation of stereospecifically deuterium labeled *n*-butanol is identical to that for the reaction with β-NAD$^+$. In contrast to the activity toward primary alcohols there is no detectable reduction of acycloNAD$^+$ by secondary alcohols with HLADH although these alcohols serve as competitive inhibitors. The net effect is that acycloNAD$^+$ has converted horse liver ADH from a broad spectrum alcohol dehydrogenase, capable of utilizing either primary or secondary alcohols, into an exclusively primary alcohol dehydrogenase. This is the first example of an NAD analog that alters the substrate specificity of a dehydrogenase and, like site-directed mutagenesis of proteins, establishes that modifications of the coenzyme distance from the active site can be used to alter enzyme function and substrate specificity. These and other results, including the activity with α-NADH, clearly demonstrate the promiscuity of the binding interactions between dehydrogenases and the riboside phosphate of the nicotinamide moiety, thus greatly expanding the possibilities for the design of analogs and inhibitors of specific dehydrogenases.

1. Background

Analogs of pyridine dinucleotide coenzymes have played an important role in the investigation of the mechanism of action of NAD-dependent enzymes [1]. Previous investigators have focused primarily on analogs containing modified pyridine rings with particular emphasis on alterations in the substituent at the 3-position. In contrast, modifications of the sugar moiety of the pyridine nucleotide have received little systematic attention. Substitution of the nicotinamide ribose with glucose [2] yields an analog with no enzymatic activity. An acyclic NAD-analog where a nicotinamide ring is attached to a C5-alkyl group was also inactive, although the acetylpyridine analog reportedly has coenzyme activity [3]. Finally, many common classes of dehydrogenases function with the potentially naturally occurring alpha anomer of NADH [4], both at significant rates and with the identical stereochemistry of hydride transfer as with the corresponding β-NADH. These studies were extended with the synthesis of the α- and β-arabino analogs of NAD$^+$ [5]. They found that the V_{max} for α-araNADH was 1.7 times that for the natural β-riboNADH with HLADH. These results demonstrate that the active sites of dehydrogenases are able to accommodate major alterations in the nicotinamide-sugar moiety while maintaining coenzyme function.

In this paper we report the synthesis of an acyclic NAD-analog (Structure 1) where the sugar moiety of the nicotinamide ribotide portion of the coenzyme has been replaced with a (2-hydroxyethoxy)methyl functionality. Because of the α-methyl ether structure, the chemical properties of this analog are designed to mimic more closely those of NAD$^+$, in contrast to the previous methylene chain analogs. The bond lengths and electronic properties at the C1′ carbon in acycloNAD$^+$ should be similar to those

Abbreviations: acycloNAD$^+$, oxidized *N*-[(2-hydroxyethoxy)methyl] nicotinamide adenosine dinucleotide; acycloNADH, reduced *N*-[(2-hydroxyethoxy) methyl] nicotinamide adenosine dinucleotide; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; Taps, tris(hydroxymetyl)methylaminopropanesulfonic acid; ADH, alcohol dehydrogenase; EGTA, ethylenebis(oxyethylenenitrilo)-tetraacetic acid.

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http://dx.doi.org/10.1016/j.dnarep.2014.09.005
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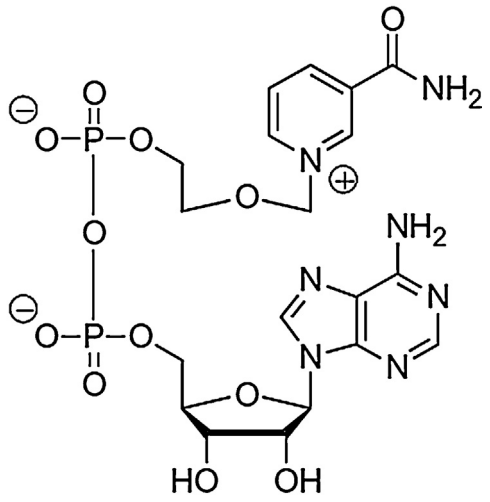


Structure 1.

of β-NAD+, thus the influence of the increased torsional flexibility, lack of stereo centers and decreased steric bulk can be studied directly.

2. Experimental

2.1. Chemicals

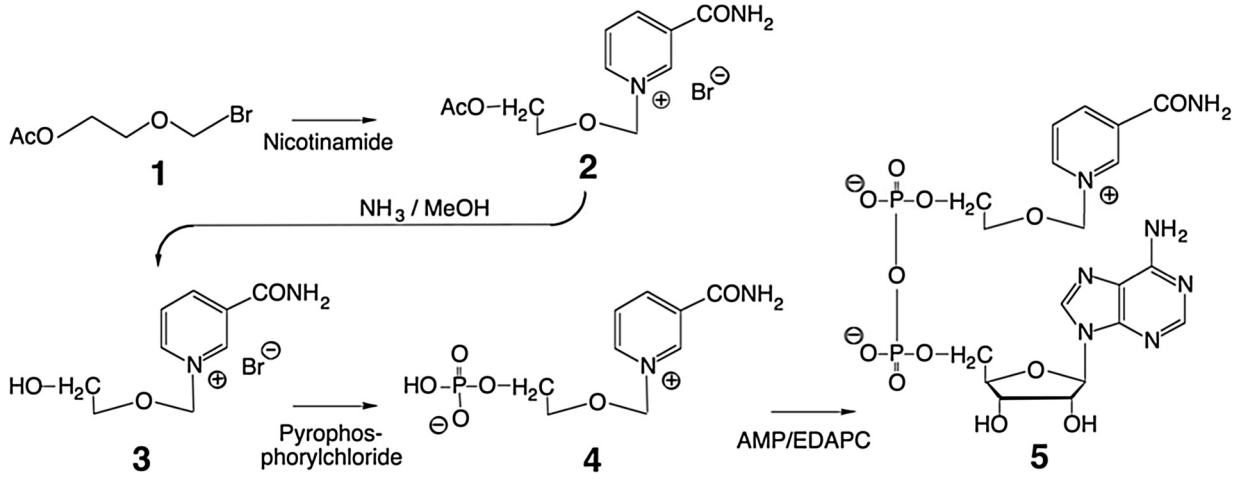
β-NAD+, yeast ADH, horse liver alcohol dehydrogenase, Hepes, Taps and Pipes buffers and semicarbazide hydrochloride were purchased from Sigma (note, the HLADH used in this study was derived from actual horse liver, recently Sigma has replaced it with a recombinant HLADH which we have found to be contaminated with a significant NADH oxidase activity, ca. 10–15%, that cannot be removed by either crystallization or column chromatography). Chemicals and substrates were purchased from Aldrich unless otherwise noted. Ethanol-d6, 1-propanol(1,1-d2) and 1-butanol(1,1-d2) (all 98 atom%) were purchased from ICN Biomedicals. Solvents were either spectrograde or freshly distilled prior to use. All synthetic reagents were of the highest quality commercially available and used without further purification. Labeled 1-[1,1-d2]hexanol (98 atom%) was prepared by reduction of hexanoic anhydride with $LiAlD_4$ as described by Schrumpf and Schlenker [6].

(S)-1-butanol-1-d was synthesized by dissolving 195 mg butanal (2.7 mmol) and 621 mg ethanol-d6 (13.6 mmol) in 15 ml 25 mM pyrophosphate buffer, pH 8.0. The reaction mixture consisted of 10 mg of β-NAD+ (14 μmol) and 10 mg yeast ADH and was left to equilibrate for 15 h at 25 °C. The aqueous solution was extracted with five portions of 10 ml of ether and the organic phase was dried over $MgSO_4$. After filtration the ether was carefully removed by distillation on a rotary evaporator at 100 mmHg and 15 °C. Final purification was achieved by preparative gas–liquid chromatography on a 6 foot glass column packed with 10% Carbowax 20 M on 120/140 mesh Chrom Q. The column temperature was kept at 50 °C. The 1-butanol-1-d was >99% pure based on analytical gas–liquid chromatography and ^1H NMR and the configuration of the chiral alcohol was (R) as described by Levy et al. [7].

2.2. Synthesis of acycloNAD+

Thin layer chromatography used precoated silica gel plastic sheets with *n*-butanol/H_2O/Acetic acid = 5/3/2 (v/v). Compounds were detected under short wave UV light. Pyridinium nucleotides and nucleosides were identified using long wave UV light as fluorescent spots by spraying with NH_3/acetone and heating the plastic strips with a heat gun. Proton nuclear magnetic resonance spectra were acquired at 500 MHz on a General Electric GN-500 instrument. The samples were 1 mM in nucleotide and were lyophilized twice from 99.8% D_2O and then dissolved in 100.0% D_2O [8]. The probe temperature was maintained at 20 °C and samples were run in 5 mm NMR tubes with sodium 3-(trimethylsilyl)propionate-[2,2,3,3-^{2}H4], TSP, used as an internal reference. Assignments of the proton resonances were made by comparison with the spectra of β-NAD+ and confirmed, by spin decoupling experiments. Solvents were either spectrograde or freshly distilled before use. All synthetic reagents were commercially available and were used without further purification. Chemical yields were based on the cyanide addition essay [9]. Aliquots of a sample were dissolved in 0.1 N $NaHCO_3$ and 0.9 ml 1.0 M KCN was added. The increase of the absorption at 327 nm corresponded to the λ_{max} of the 4-cyano-1,4-dihydronicotinamide adduct of the acyclic analog. The concentration of nucleoside was calculated using an extinction coefficient of $\varepsilon_{327} = 5900$.

N-[(2-hydroxyethoxy)methyl]nicotinamide adenine dinucleotide 5 was synthesized by the method outlined in Scheme 1. Acetolysis of 1,3-dioxalane with acetylbromide 6 yielded



Scheme 1.

Please cite this article in press as: O. Malver, et al., Alteration in substrate specificity of horse liver alcohol dehydrogenase by an acyclic nicotinamide analog of NAD+, DNA Repair (2014), http://dx.doi.org/10.1016/j.dnarep.2014.09.005