2′-Ribose Substituent Effects on the Chemical and Enzymatic Hydrolysis of NAD+

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Received July 5, 1994

The enzyme-catalyzed cleavage of the nicotinamide−ribosyl bond of NAD^+ and the attendant delivery of the ADP ribosyl moiety to acceptors is central to the diverse biological activities of the pyridine nucleotides as cellular effectors and metabolic regulators.[1−3] Previous measurements of secondary deuterium kinetic isotope effects,[4] and leaving group effects for 3- or 4-substituted pyridine nucleotides on the chemical and enzyme-catalyzed cleavage of the nicotinamide−ribosyl bond point to the reaction being dissociative in character. In the latter studies, the rates of hydrolysis correlate with the pK_a of the pyridine moiety, giving a β_{LG} of −1.11 and −0.90, respectively, for data extrapolated to 37 °C (Table 1).[5,6] A dissociative mechanism is further supported by the large 2'-substituent effects observed for the hydrolysis of nicotinamide arabinosides.[7]

A similar dissociative mechanism involving an oxocarbenium-like transition state has been proposed for the acid-catalyzed cleavage of purine nucleoside[8,9] and the enzyme-catalyzed hydrolysis of AMP[10] and inosine.[11] All of these studies have focused primarily on characterizing the transition state and not on the question of how structural elements of the substrate can be used to drive a dissociative reaction toward the transition state.

In this communication we report preliminary results for studies of the effects of 2′-substitution of the ribofuranosyl ring on the chemical and enzyme-catalyzed cleavage of the nicotinamide−ribosyl bond (see Scheme 1) for a series of β-NAD^+ analogues containing H, NH_2, OH, N_3, or F at the 2′-position.[12] A plot of the log of the rate constant of the pH-independent hydrolysis of the nicotinamide−ribosyl bond against the Taft σ_i value of the substituent is shown in Figure 1a. The chemical hydrolysis gives a linear relation with a slope $\varrho = -7.0$. The corresponding plot for the previously reported series of 2′-substituted β-nicotinamide arabinosides[7] gives a $\varrho = -6.7$ (see Figure 1a), thus demonstrating that the configuration of the 2′-substituent has only a minor influence on the value of ϱ for these compounds. The observation that the rates of hydrolysis for the 2′-amino-substituted arabinoside and riboside and the ribose diol anion of NAD^+ all fall on their respective σ_i plots establishes that there is no special rate acceleration in these analogues due to anchimeric assistance.[13]

Table 1. Rates of Enzyme-Catalyzed and Chemical Hydrolysis of NAD^+ analogues at 37 °C

		nonenzymatic	
	enzymatic[a] V_{max} (μmol/min/mg protein)	2′-X-riboside+ [b] (s^{-1})	2′-X-arabinoside[b,c] (s^{-1})
NAD^+	201	n.a.[d]	n.a.[d]
2′-H	23.8	1.45×10^{-4}	1.45×10^{-4}
2′-NH_2	1.76	1.78×10^{-5}	1.07×10^{-5}
2′-N_3	1.90×10^{-3}	2.82×10^{-7}	2.00×10^{-7}
2′-F	4.20×10^{-4}	3.00×10^{-8}	4.20×10^{-8}
neutral diol	n.a.[d]	1.69×10^{-6}	9.71×10^{-7}
diol anion	n.a.[d]	5.01×10^{-3}	

[a] The hydrolysis of the 1,N^6−etheno derivatives of the NAD^+ analogues catalyzed by purified calf spleen NAD^+ glycohydrolase[2] was followed at 37 °C by a continuous fluorometric assay as described previously.[21] Modification of the adenine ring had a negligible effect on the rate of hydrolysis of the nicotinamide−ribosyl bond. [b] The chemical hydrolysis of the less reactive analogues was conducted at elevated temperatures and extrapolated at 37 °C as outlined previously.[7,20] [c] Data from Handlon and Oppenheimer.[7] [d] Not applicable.

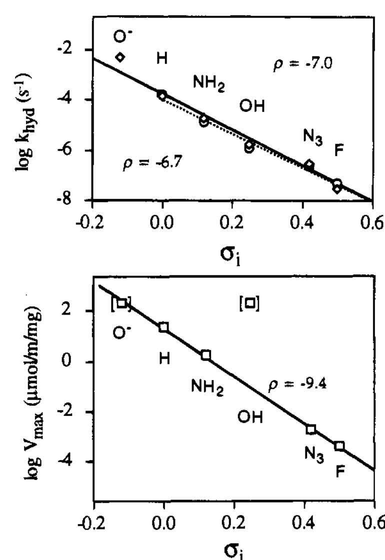
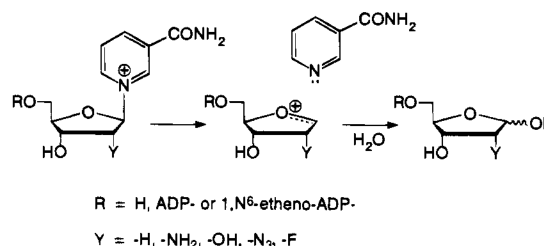


Figure 1. (a, top) Plot of the log of the rate of the pH-independent chemical hydrolysis of 2′-substituted arabino (○) and ribo (◇) β-NAD^+ analogues vs the Taft σ_i constant at 37 °C. (b, bottom) Plot of the log of the V_{max} for the enzyme-catalyzed hydrolysis of the 2′-substituted ribo-NAD^+ analogues vs the Taft σ_i constant at 37 °C. The two points in brackets designate the relative rate for the enzyme-catalyzed hydrolysis of NAD^+ corresponding to σ_i values of an alkoxide and neutral hydroxyl substituent, −0.12[16] and 0.25, respectively.

Scheme 1



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0002-7863/94/1516-12087$04.50/0 © 1994 American Chemical Society

Our previous work has established that steric factors do not represent a major influence in catalysis. The values of K_m and K_i for the hydrolysis of NAD^+ analogues by the calf spleen NAD^+ glycohydrolase are remarkably insensitive to the size or position of the substituent on the pyridine ring[5,6] as are the values of V_{max}, with the exception that 3-carbonyl substituents are hydrolyzed up to 14 times faster, e.g., in the case of NAD^+, than expected from extrapolation of the Brønsted plot.[6] We likewise find that substitutions at the 2′-position with a ribo configuration are well-tolerated by the enzyme and that all ribo analogues tested serve as substrates, whereas the 2′-hydroxy- and 2′-fluoro-*arabino* analogues are not hydrolyzed and instead are potent, slow binding inhibitors of this enzyme.[14]

In the enzyme-catalyzed hydrolysis of the 2′-substituted ribo analogues, the plot of the log of V_{max} vs the Taft σ_i values is also linear with a slope $\varrho = -9.4$ (see Figure 1b). Even the slowly hydrolyzed analogues (2′-azido and 2′-fluoro) show no evidence for the formation of covalently-bound intermediates, i.e., they are simple competitive inhibitors relative to NAD^+. In contrast, 2-fluoropyranosides yield inhibitory covalent complexes with β-glucosidase.[15]

A crucial issue is the appropriate value to use for the σ_i of NAD^+ in the plot. As shown in Figure 1b, if the σ_i of a neutral hydroxyl is used (0.25), then the point for NAD^+ lies far above the line defined by the other analogues. Alternatively, if the σ_i of an alkoxide is used (−0.12),[16] then the point falls directly on that line. This is the first experimental evidence that the functional equivalent of a diol anion participates in the enzyme-catalyzed cleavage of the ribosyl bond.[17] Such a participation would account for a *ca.* 10^4 rate enhancement of the enzyme-catalyzed hydrolysis of NAD^+ relative to that anticipated for the neutral ribose diol. The proposed inductive stabilization could come from interactions in the active site with bases, such as carboxylates, directed at either or both of the ribose hydroxyls. Indeed, interactions directed primarily at the 3′-position would explain the insensitivity to the presence of the various 2′-substituents.

The obligatory participation of a diol anion in the mechanism of other ribonucleosidases would also explain the observed slowness of purine deoxynucleosidases relative to the comparable ribonucleosidase[18] in spite of the *ca.* 10^3 greater rate of the acid-catalyzed hydrolysis of deoxypurines relative to ribopurines.[8,9,19] We propose that the valid comparison would be between a 2′-H substituent and the equivalent of a ribose diol anion, and therefore ribonucleosidases operating via a dissociative mechanism would be intrinsically much faster than the corresponding deoxynucleosidases. Finally, the mechanistic involvement of a diol anion would have far-ranging consequences for the design of inhibitors of such nucleosidases.

Acknowledgment. Supported in part by NIH Grant GM-22982 (N.J.O.) and by the CNRS (H.M.S. and F.S.). The UCSF Magnetic Resonance Laboratory is supported in part by grants from the NSF (DMB 840626) and NIH (RR-01668 and RR-4789), and the UCSF Mass Spectrometry Facility is supported in part by grants from the NSF (DIR 8700766) and NIH (RR-01614). We thank Neil Buckley and Dr. Rachel Ta-Shma for helpful discussions.

(13) Anchimeric assistance and formation of 1,2-anhydro sugar intermediates have been widely invoked in the alkaline hydrolysis of glycosyl bonds and nucleosides. See, for example: Sinnott, M. L. In *Enzyme Mechanisms*; Page, M. I., Williams, A., Eds.; Burlington House: London, 1987; pp 259−297.

(14) Nicotinamide arabinoside adenine dinucleotide ($araNAD^+$) and nicotinamide 2′-deoxy-2′-fluoroarabinoside adenine dinucleotide (2′-F-$araNAD^+$) are not hydrolyzed enzymatically at measurable rates ($<10^{-6}$ the rate for NAD^+) and are the first documented examples of slow binding inhibitors for this class of enzyme (ref 21).

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(17) A fully formed diol anion ($pK_a \sim 12$) need not be invoked in the active site, but only that the inductive polarization at the 2′-position must approximate an anion. The same semantics have been used regarding the charge state of the active site serine hydroxyl of proteases (Bachovchin, W. W. *Biochemistry* **1986**, *25*, 7751−7759.)

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