Mechanistic Diagnoses of N-Ribohydrolases and Purine Nucleoside Phosphorylase

L. John Mazzella,† David W. Parkin,† Peter C. Tyler,‡ Richard H. Furneaux,‡ and Vern L. Schramm*,†

Department of Biochemistry
Albert Einstein College of Medicine
Bronx, New York 10461,
Industrial Research Limited
P.O. Box 31-310, Lower Hutt, New Zealand

Received October 23, 1995

The N-ribohydrolases and transferases represent a class of catalytic activities of increased interest, since they are involved in novel pathways of purine salvage in protozoan parasites,[1] in nucleic acid repair,[2] in the actions of bacterial and plant toxins,[3] and in regulation of calcium ion flux.[4] Transition states for purine N-ribohydrolases and phosphorylases share ribosyl oxycarbonium-ion character.[5] Substrate specificity is conferred by the nature of the enzyme-activated nucleophile and the leaving-group interactions. Among the known N-ribohydrolases, specificity is high for the ribosyl group but varies for the leaving group purine or pyrimidine. The inosine-uridine nucleoside hydrolase (IU-nucleoside hydrolase) from the trypanosome *Crithidia fasciculata* hydrolyzes all of the naturally occurring purine and pyrimidine ribonucleosides with similar catalytic efficiencies, while the guanosine-inosine enzyme (GI-nucleoside hydrolase) from the same organism has a strong preference for the eponymous substrates and is nearly inert with other purine and pyrimidine nucleosides.[6] A nucleoside hydrolase from *Trypanosoma brucei brucei* demonstrates purine nucleoside specificity but includes inosine, adenosine, and guanosine as good substrates (IAG-nucleoside hydrolase).[7] AMP nucleosidase from bacterial sources is highly specific for the adenine base, and the 5′-phosphoryl is required for hydrolysis of the N-ribosidic bond.[8] Purine nucleoside phosphorylase from mammalian sources is specific for inosine and guanosine as substrates and activates phosphate or arsenate anions to attack C1′ of these nucleosides.[9]

The common catalytic feature of these enzymes is the oxycarbonium-ion character of the transition state, in which cleavage of the C−N ribosidic bond occurs by an S_N1-like mechanism. This transition state can be achieved by three distinct reaction pathways: (1) activation of the leaving group, as in the case for the acid-catalyzed solvolysis of purine nucleosides;[10] (2) catalytic site interactions with the ribosyl moiety, to stabilize both the oxycarbonium-ion charge and geometry similar to mechanisms proposed for lysozyme;[11] and (3) ionization of the 2′-hydroxyl to stabilize the developing oxycarbonium, as proposed for base-catalyzed NAD^+ solvolysis and established in chemical models and in calf spleen NAD^+ glycohydrolase.[12] A combination of these features may also occur in some N-ribohydrolases.

We report here that *p*-nitrophenyl β-D-ribofuranoside (nitrophenylriboside) and its 5-phosphate can be used as substrates to distinguish these mechanisms for five members of the family of N-ribohydrolases. The favorable *p*-nitrophenyl leaving group lacks the pyrimidine and purine ring nitrogens which are proton acceptors in acid-catalyzed solvolysis. If substrate activation required protonation (or hydrogen bonds of similar electron-withdrawing strength) at the nitrogen(s) of the leaving group, nitrophenylriboside would be a poor substrate. In contrast, substantial enzymatic activity with this compound would indicate mechanisms in which the enzyme interacts with the ribosyl to stabilize an oxycarbonium-ion transition state, or ionizes a ribosyl hydroxyl to facilitate the unassisted departure of the *p*-nitrophenolate ion,[12d] or protonates the β-oxygen bridge to the *p*-nitrophenyl group,[12c] These mechanistic proposals can be further distinguished by comparing the kinetic parameters for enzyme-catalyzed hydrolysis of the normal substrate and nitrophenylriboside as a function of pH.

D-Ribose (**I**) was converted to *p*-nitrophenyl β-D-ribofuranoside (**III**) as previously described[13,14] and phosphorylated at the 5-position (Scheme 1). Kinetic parameters for hydrolysis of ribosides **III** or **V** by several enzymes are compared with those of normal substrates in Table 1. The enzymes with the most stringent substrate specificities, GI-nucleoside hydrolase, AMP nucleosidase, and purine nucleoside phosphorylase, gave k_{cat}/K_m values with nitrophenylriboside (or its 5-phosphate) which are 4×10^{-5}, 3×10^{-7}, and 1×10^{-6} of those for the substrates, guanosine, AMP, and inosine, respectively. In contrast, the same comparison of k_{cat}/K_m values for the nonspecific IU-nucleoside hydrolase favored **III** by a factor of 54. The ribosyl moiety is unchanged between the normal substrates and the nitrophenylribosides. The K_m values for nitrophenylribosides are within a factor of 42 of those for the normal substrates for all of the enzymes in Table 1, implicating the ribosyl in substrate recognition. Catalysis primarily by ribose diol anion formation[12d] or by enforcing ribo-oxycarbonium formation[5a] would give good activity with nitrophenylriboside; therefore, the enzymes with stringent base specificity are likely to involve a substantive component of leaving-group activation. Protonation at N7 of AMP is known to be an important feature of the transition states stabilized by AMP nucleosidase, since formycin 5′-phosphate, which is protonated at this position, is a transition state

* Corresponding author: telephone, (718) 430-2813; FAX, (718) 892-0703; e-mail, vern@aecom.yu.edu.
† Albert Einstein College of Medicine.
‡ Industrial Research Limited.

(1) Hammond, D. J.; Gutteridge, W. E. *Mol. Biochem. Parasitol.* **1984**, *13*, 243.
(2) Sancar, A.; Sancar, G. B. *Annu. Rev. Biochem.* **1988**, *57*, 29.
(3) (a) Endo, Y.; Gluck, A.; Wool, I. G. *J. Mol. Biol.* **1991**, *221*, 193. (b) Aktories, K., Ed. *Current Topics in Microbiology and Immunology. ADP-Ribosylating Toxins*; Springer-Verlag: Berlin, 1992.
(4) Lee, H. C.; Galione, A.; Walseth, T. F. *Vitam. Horm.* **1994**, *48*, 199.
(5) (a) Mentch, F.; Parkin, D. W.; Schramm, V. L. *Biochemistry* **1987**, *26*, 921. (b) Horenstein, B. A.; Parkin, D. W.; Estupinan, B.; Schramm, V. L. *Biochemistry* **1991**, *30*, 10788. (c) Kline, P. C.; Schramm, V. L. *Biochemistry* **1995**, *34*, 1153.
(6) (a) Parkin, D. W.; Horenstein, B. A.; Abdulah, D. R.; Estupinan, B.; Schramm, V. L. *J. Biol. Chem.* **1991**, *266*, 20658. (b) Estupiñán, B.; Schramm, V. L. *J. Biol. Chem.* **1994**, *269*, 23068.
(7) Parkin, D. W. Unpublished observation, manuscript in preparation.
(8) (a) Hurwitz, J.; Heppel, L. A.; Horecker, B. L. *J. Biol. Chem.* **1957**, *226*, 525. (b) DeWolf, W. E., Jr.; Fullin, F. A.; Schramm, V. L. *J. Biol. Chem.* **1979**, *254*, 10868.
(9) (a) Kline, P. C.; Schramm, V. L. *Biochemistry* **1992**, *31*, 5964. (b) Kline, P. C.; Schramm, V. L. *Biochemistry* **1993**, *32*, 13212.
(10) (a) Parkin, D. W.; Leung, H. B.; Schramm, V. L. *J. Biol. Chem.* **1984**, *259*, 9411. (b) Garrett, E. R.; Mehta, P. J. *J. Am. Chem. Soc.* **1972**, *94*, 8532.
(11) Imoto, T.; Johnson, L. M.; North, A. C. T.; Phillips, D. C.; Rupley, J. A. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1972; Vol. 7, p 665.
(12) (a) Oppenheimer, N. J.; Handlon, A. L. in *The Enzymes*, 3rd ed.; Sigman, D. S., Ed.; Academic Press: San Diego, 1992; Vol. 20, p 454. (b) Cherian, X. M.; VanArman, S. A.; Czarnik, A. W. *J. Am. Chem. Soc.* **1990**, *112*, 4490. (c) Rosenberg, S.; Kirsch, J. F. *Biochemistry* **1981**, *20*, 3196. (d) Handlon, A. L.; Xu, C.; Muller-Steffner, H. M.; Schuber, F.; Oppenheimer, N. J. *J. Am. Chem. Soc.* **1994**, *116*, 12087.
(13) Recondo, E. F.; Rinderknecht, H. *Helv. Chim. Acta* **1959**, *42*, 1171.
(14) Honma, K.; Nakazima, K.; Uematsu, T.; Hamada, A. *Chem. Pharm. Bull.* **1976**, *24*, 394. ^{1}H NMR of **III**, (DMSO-d_6): δ 8.20 and 7.17 (2H each, d, Ar), 5.63 (1H, d, J = 0.7 Hz, H-1), 4.07−4.02 (2H, m, H-2,3), 3.96−3.92 (1H, m, H-4), 3.57−3.50 (1H, m, H-5), 3.37−3.29 (1H, m, H-5′). ^{13}C NMR: δ 163.1, 142.7, 127.0, 117.9 (Ar), 106.4 (C-1), 86.4 (C-4), 75.9, 71.6 (C-2,3), 63.6 (C-5). Yield of **IV** from **III** was 20%, and yield for conversion of **IV** to **V** was 56%. ^{1}H NMR of **V** (D_2O): δ 8.12 and 7.08 (2H each, d, Ar), 5.71 (1H, s, H-1), 4.43−4.11 (3H, m), 3.97−3.70 (2H, m). ^{13}C NMR: δ 164.1, 144.9, 128.9, 119.3 (Ar), 107.6 (C-1), 86.1 ($J_{c,p}$ = 8.2 Hz, C-4), 77.3, 73.9 (C-2,3), 67.7 ($J_{c,p}$ = 4.6 Hz, C-5).
(15) (a) Leung, H. B.; Kvalnes-Krick, K. L.; Myer, S. L.; deRiel, J. K.; Schramm, V. L. *Biochemistry* **1989**, *28*, 8726. (b) Leung, H. B.; Schramm, V. L. *J. Biol. Chem.* **1980**, *255*, 10867.

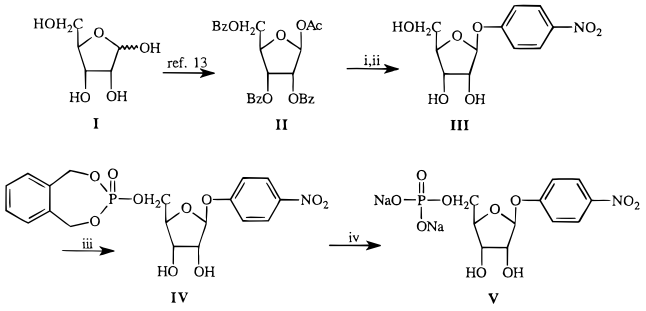
0002-7863/96/1518-2111$12.00/0

Table 1. Kinetic Parameters for N-Ribohydrolases and Purine Nucleoside Phosphorylase with Nucleosides and Nitrophenyl Riboside[a]

nitrophenyl riboside substrate				purine substrate[b]				
enzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat}/K_m ratio[g]
IU-nucleoside hydrolase[R,c]	239 ± 32	58 ± 23	4.1×10^6	inosine	28	380	7.6×10^4	54
IAG-nucleoside hydrolase[R,d]	0.82 ± 0.03	560 ±50	1.5×10^3	inosine	34	18	1.9×10^6	8×10^{-4}
GI-nucleoside hydrolase[R,c]	0.07 ± 0.01	468 ± 130	1.4×10^2	guanosine	231	77	3.2×10^6	4×10^{-5}
AMP nucleosidase[P,e]	0.0004 ± 0.0001	6250 ± 1700	6.2×10^{-2}	AMP	27	150	1.8×10^5	3×10^{-7}
purine nucleoside phosphorylase[R,f]	0.00020 ± 0.00004	224 ± 76	8.9×10^{-1}	inosine	12	19	6.3×10^5	1×10^{-6}

[a] The enzymes were highly purified samples from *C. fasciculata*,[c,6] *T. brucei brucei*,[d,18] *Escherichia coli*,[e,15] and bovine spleen.[f,9a] Assays were at pH 8.0 in 50 mM HEPES, 30 °C. For the allosteric AMP nucleosidase, MgATP was present at 100 μM. Purine nucleoside phosphorylase was assayed for phosphorolysis by including 3 mM phosphate in assay mixtures. The superscripts R and P refer to nitrophenyl riboside and nitrophenyl riboside 5-phosphate, respectively, as substrates. [b] Substrate specificity, for purine substrates, the kinetic constants, and their standard errors are available in refs 6a,b, 7, 15b, and 9. [g] The k_{cat}/K_m ratio compares the k_{cat}/K_m for nitrophenyl riboside to that for the indicated purine substrate.

Scheme 1. Synthesis of Nitrophenylribose (**III**) and Nitrophenylribose 5-Phosphate (**V**)[a]



[a] Reagents: i, *p*-nitrophenol, $BF_3 \cdot OEt_2$; ii, NaOH, MeOH; iii, *N*,*N*-diethyl-1,5-dihydro-2,4,3-benzodioxaphosphepin-3-amine, tetrazole, then MCPBA; iv, H_2, Pd/C, EtOH, then NaOH.

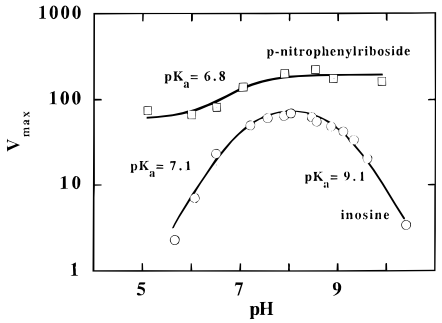


Figure 1. Hydrolysis of nitrophenylriboside and inosine by IU-nucleoside hydrolase as a function of pH. The data for inosine is replotted from ref 18. The data for hydrolysis of nitrophenylriboside was fitted to the equation $\log V_{max} = \log[(V_1+V_2(K_a/[H^+]))/(1+K_a/[H^+])]$.

analogue.[8b,16] Tight binding of hypoxanthine by purine nucleoside phosphorylase[9a] and N7 protonation deduced from transition state analysis[5c] also support leaving-group activation.

Nitrophenylriboside, like its hexose counterparts,[12b,17] undergoes specific acid- and base-catalyzed solvolysis. The riboside is relatively stable from pH 4 to 10 pH with the solvolytic rate increasing rapidly at more extreme pH values, with a near-symmetric response to acid and base. Acid-catalyzed O-glycoside solvolysis occurs via protonation of the glycosidic oxygen while base-catalyzed solvolysis is thought to occur via internal attack of ionized O2 on the anomeric carbon.[12b] These protonic interactions can be detected by the pH dependence of V_{max}.

Hydrolysis of inosine by IU-nucleoside hydrolase requires one proton donor, pK_a 9.1, and one proton acceptor, pK_a 7.1 (Figure 1). These groups are proposed to stabilize the ribooxycarbonium ion formed at the transition state and assist the leaving group by N7 protonation.[18] Hydrolysis of nitrophenylriboside under the same conditions reveals no acid or base groups in the enzyme that are essential for catalysis. Acid- and/or base-catalyzed solvolysis would be expected to show an essential group at pH extremes, with unit slope(s). Instead, the group (pK_a 6.8) which has been proposed to stabilize the oxycarbonium is shown to cause a 3-fold increase in reaction rate but is not essential.

The results demonstrate that the ionizable groups which are required for acid−base solvolysis of inosine by IU-nucleoside hydrolase are not required for the facile solvolysis of nitrophenylriboside. Solvolysis is therefore proposed to occur primarily through activation of the ribosyl toward the oxycarbonium ion. The oxycarbonium-stabilizing group provides weak (3-fold) catalytic assistance, but the leaving-group proton donor is not required for solvolysis of the nitrophenylriboside. The enzymes which are poor catalysts for this substrate have mechanisms which differ, despite similarities in the nature of the ribosyl at the transition state. Activation of the leaving group by protonation and/or other electrostatic interactions contributes more significantly in lowering the transition state barrier for the enzymes which are inefficient in solvolysis of nitrophenylriboside.

Nitrophenylribosides are mechanistic tools to identify enzymes which catalyze N-riboside hydrolysis by forming the ribooxycarbonium ion. The extent of leaving-group activation can then be evaluated. In the test group of five N-ribosidases, the nonspecific IU-nucleoside hydrolase is most efficient in ribooxycarbonium-ion formation to achieve the transition state. Leaving-group activation plays more important roles for the other enzymes. The (1.8×10^8)-fold range in catalytic efficiencies (substrate/nitrophenylriboside) for these enzymes indicates considerable mechanistic diversity among the N-ribosidases.

Acknowledgment. The work was supported by the National Institutes of Health, the United States Army Medical Research and Development Command under Contract No. DAMD 17-93-C-3051, and the New Zealand Foundation for Research, Science and Technology. The views, opinions, and/or findings contained in this report are those of the authors and should not be construed as an official Department of Army position, policy, or decision unless so designated by other documentation.

JA953537Z

(16) (a) Ehrlich, J.; Schramm, V. L. *Biochemistry* **1994**, *33*, 8890. (b) Giranda, V. L.; Berman, H. M.; Schramm, V. L. *Biochemistry* **1988**, *27*, 5813.
(17) (a) Snyder, J. A.; Link, K. P. *J. Am. Chem. Soc.* **1952**, *74*, 1883. (b) Sinnott, M. L.; Jencks, W. P. *Ibid.* **1980**, *102*, 2026.
(18) Parkin, D. W.; Schramm, V. L. *Biochemistry* **1995**, *34*, 13961.