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Examination of the mechanism and energetic contribution of leaving group activation in the purine-specific nucleoside hydrolase from *Trypanosoma vivax* ☆

John N. Barlow *, Jan Steyaert

Department of Molecular and Cellular Interactions, VIB, Free University of Brussels, Pleinlaan 2, 1050 Brussel, Belgium

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Abstract

The mechanism and energetics of the purine-specific nucleoside hydrolase from *Trypanosoma vivax* (*Tv*NH) are examined by stopped-flow at low temperatures. *Tv*NH is shown to follow an ordered uni–bi kinetic mechanism and high forward commitment with inosine as substrate (C_f=1.9±0.6). Measurement of partitioning of the Michaelis complex, which exists at negligible concentrations in the steady state, is achieved using a novel sequential-mixing stopped-flow method. A product burst is observed with *p*-nitrophenyl riboside (pNPR) in the pre-steady state, indicating that a step after chemistry rate determines k_{cat}. Comparison of the kinetics of inosine and pNPR turnover shows that the dominant energetic contribution towards catalysis in *Tv*NH comes from ribosyl and water activation (11 kcal/mol); however, leaving group activation still makes a considerable (8 kcal/mol) contribution. A solvent isotope effect (^{D2O}k=1.7) on the chemistry transient τ1 with guanosine as substrate was observed. Therefore, the leaving group is unlikely to be protonated prior to N-glycosidic bond cleavage. We propose that leaving group protonation is, by itself, unlikely to account for the large energetic contribution of leaving group activation. Instead, we postulate that active site binding interactions to the purine leaving group are required for efficient ribosyl and/or water activation.

Keywords: Transient kinetic; Nucleoside hydrolase; Solvent isotope effect; Forward commitment

1. Introduction

Nucleoside hydrolases (NHs) are a family of structurally related enzymes that catalyse the calcium-dependent hydrolysis of nucleosides (Scheme 1) [1]. Although found in most organisms, their physiological role is only well understood in certain parasitic protozoa, such as trypanosomes, where they are involved in purine salvage pathways. Their absence in higher eukaryotes, including mammals, has led to the proposal that NH may be a suitable target for antibiotic development against trypanosomal diseases. NH has also been proposed for use as pro-drug activators in chemotherapy [2].

NHs are notable for their catalytic power, showing rate enhancements of the order of 10^{12} with respect to the uncatalysed reaction [3]. Extensive studies by the Schramm laboratory have established three catalytic strategies in the base-aspecific NH from *Crithidia fasciculata* (*Cf*NH): (i) stabilisation of a ribooxocarbenium ion-like transition state, (ii) activation of substrate water, and (iii) activation of the leaving group [4]. Purine leaving groups are activated for departure by protonation at N-1 or N-7 using the general acid His 241 [5]. By contrast, general acid catalysis does not play a role in leaving group activation in the purine-specific NH from *Trypanosoma vivax* (*Tv*NH [1]). This is intriguing because leaving group activation is thought to make a greater catalytic contribution in purine-specific NHs. For example, in the purine-specific NH from *Trypanosoma brucei brucei* (*Tbb*NH), it was estimated that leaving group activation is responsible for ~50% of the total catalytic rate enhancement, as opposed to ~25% in *Cf*NH [6].

Abbreviations: NH, nucleoside hydrolase; *Tv*NH, nucleoside hydrolase from *Trypanosoma vivax*; *Cf*NH, nucleoside hydrolase from *Crithidia fasciculata*; TbbNH, nucleoside hydrolase from *Trypanosoma brucei brucei*; pNPR, *p*-nitrophenylriboside; Guo, guanosine; Ino, inosine; NP, nucleoside phosphorylase; XO, xanthine oxidase; 7MG, 7-methylguanosine; τ1, transient

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* Corresponding author. Tel.: +32 2 6291846; fax: +32 2 6291963.
E-mail address: jbarlow@vub.ac.be (J.N. Barlow).

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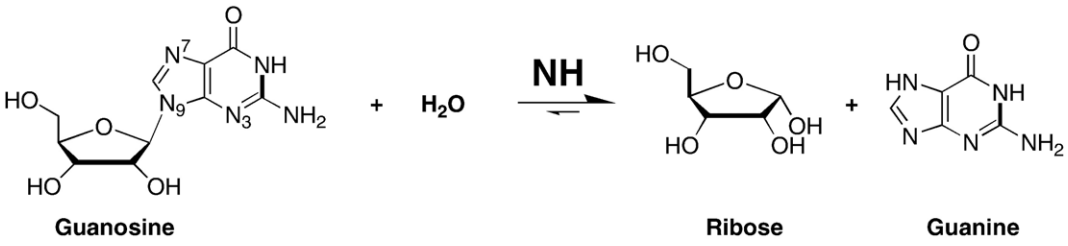


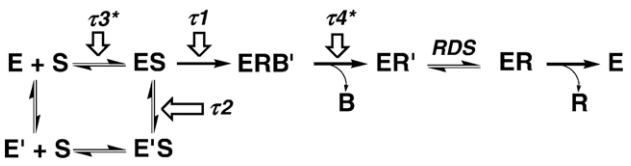
Scheme 1. Reaction catalysed by NH.

Further insight into the mechanism of leaving group activation in *Tv*NH was made possible by the determination of the crystal structure of *Tv*NH with bound substrates and inhibitors [1,7]. These structures show that the leaving group is stacked between two active site tryptophan residues (Trp 83 and Trp 260). Mutagenesis and ab initio computational studies indicate that aromatic stacking can promote protonation by increasing the pK_a at N-7 [8]. Protonation is further facilitated by formation of an intramolecular O5′-H-C8 hydrogen bond between ribose and the leaving group base [9].

One difficulty with this model is that the observed rate of N-glycosidic bond cleavage is relatively independent of pH between pH 4.5 and 8.0 [10]. If the N-7 protonated [*Tv*NH.substrate] complex is an intermediate on the catalytic pathway, then the rate of chemistry should decrease at pH values above the pK_a of the leaving group. One possible explanation for the absence of this effect is that the pK_a of the leaving group is increased from 2 to at least 8, some 6 pK_a units (~8 kcal/mol). Alternatively, the measured rate of N-glycosidic bond cleavage may actually be rate determined by a step prior to chemistry (e.g., a conformation change). This is a distinct possibility given that the active site is known to exist in at least two conformation states (termed the open and closed forms) [7].

A second, related issue that needs to be addressed is to determine the energetic contribution of leaving group activation in the *Tv*NH reaction. Estimates of the energetic contribution of leaving group activation in *Cf*NH and *Tbb*NH have been made previously based on steady-state rates with the mechanistic probe substrate *p*-nitrophenyl riboside (pNPR) [11,6]. The hydrolysis of pNPR cannot be acid catalysed above pH 7 because protonation of both substrate and product *p*-nitrophenylate become thermodynamically unfavourable [12,13]. The rate enhancement observed with pNPR (enzymatic rate minus the non-enzymatic rate) is therefore attributed to the capacity of the NH to stabilise the ribosyl moiety as it proceeds through a ribooxocarbenium transition state. However, the complex kinetic mechanism of *Tv*NH makes comparisons of steady-state rates difficult to assess (Scheme 2). While k_{cat} represents the intrinsic rate of chemistry for *Cf*NH [3], k_{cat} for *Tv*NH corresponds to the rate of isomerisation of the [*Tv*NH.ribose] product complex for purine nucleoside substrates [14]. k_{cat} for *Tv*NH with pNPR is smaller than with purine nucleoside substrates, suggesting that it represents a step upstream of the [*Tv*NH.ribose] isomerisation step [15]. Comparisons of k_{cat}/K_m are also difficult because the forward commitments (C_f) for purine nucleosides and pNPR are unknown for *Tv*NH ($C_f \sim 0$ in *Cf*NH).

Here we develop kinetic schemes for both inosine and pNPR at 5 °C that allow the relative energetic contributions of ribosyl and leaving group activation to be calculated. We show that product release is ordered for inosine and calculate the forward commitments for both inosine and pNPR. We also examine solvent isotope effects on the transient kinetics of guanosine in order to probe the mechanism of leaving group protonation.



Scheme 2. Kinetic mechanism of *Tv*NH with guanosine as substrate. Also indicated are the steps that determine the rate of observed stopped-flow transients (τ1–τ4*) reported in this work and the overall rate-determining step (RDS). *E*=free *Tv*NH, *E′*=isoform of TvNH, *ES*=[*Tv*NH.substrate] complex, *EBR′*=[*Tv*NH.base.ribose] complex, *ER′*=[*Tv*NH.ribose] complex, *ER*=isoform of *ER′*, *R*=ribose, *B*=nucleobase.

2. Experimental procedures

2.1. General procedures

The concentrations of enzyme, nucleoside and base stock solutions were determined using the following extinction coefficients [16]: *Tv*NH, ε_{280} (47.75 mM^{-1} cm^{-1}, pH 7.0); guanosine, ε_{253} (13.6 mM^{-1} cm^{-1}, pH 6.0); inosine, $\varepsilon_{248.5}$ (12.3 mM^{-1} cm^{-1}, pH 6.0); 7-methyl guanosine, ε_{258} (8.5 mM^{-1} cm^{-1}, pH 7.0), ε_{281} (7.4 mM^{-1} cm^{-1}, pH 7.0); hypoxanthine, $\varepsilon_{249.5}$ (10.7 mM^{-1} cm^{-1}, pH 6.0). The concentration of *p*-nitrophenyl riboside was determined by measuring the absorbance change upon complete hydrolysis in 2.5 M NaOH at 15 °C ($\Delta\varepsilon_{405\ nm}$=18.3 mM^{-1} cm^{-1}). Nucleosides and purine solutions were made in deionised water and used freshly except for 7-methyl guanosine and *p*-nitrophenyl riboside which were stored as frozen stock solutions. *p*-Nitrophenyl riboside was kindly provided by Annelies Goeminne (University of Antwerp). The purification and storage of recombinant *Tv*NH were described previously [10]. Bovine xanthine oxidase was purchased as a suspension (28 mg/ml) in 2.3 M ammonium sulphate.

2.2. Stopped-flow absorbance and fluorescence

The experimental procedures and data analysis for single mixing experiments were described previously [10]. Progress curves were corrected by subtracting control progress curves (substrate mixed with buffer only). $\Delta\varepsilon$ values for substrate hydrolysis were determined by measuring the absorbance change upon complete hydrolysis of substrate. Some selected values are: pNPR, $\Delta\varepsilon_{400\ nm}$ (15.5 mM^{-1} cm^{-1}, pH 8.0); Ino, $\Delta\varepsilon_{258\ nm}$ (0.45 mM^{-1} cm^{-1}, pH 8.5); Guo, $\Delta\varepsilon_{260\ nm}$ (−4.5 mM^{-1} cm^{-1}, pH 7.0). A pathlength of 1 cm was used for the