Substrate-assisted Leaving Group Activation in Enzyme-catalyzed N-Glycosidic Bond Cleavage*

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Stefan Loverix§, Paul Geerlings§, Michael McNaughton**, Koen Augustyns**, An Vandemeulebroeck‡, Jan Steyaert‡, and Wim Versées‡

From the §Department of Cellular and Molecular Interactions, Vlaams Interuniversitair Instituut voor Biotechnologie and the ‡Department of General Chemistry, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium and the **Department of Medicinal Chemistry, University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium

In enzymatic depurination of nucleosides, the 5′-OH group of the ribose moiety of the substrate is often shown to contribute substantially to catalysis. The purine-specific nucleoside hydrolase from Trypanosoma vivax (TvNH) fixes the 5′-OH group in a gauche,trans orientation about the C4′–C5′ bond, enabling the 5′-oxygen to accept an intramolecular hydrogen bond from the C8-atom of the purine leaving group. High level ab initio quantum chemical calculations indicate that this interaction promotes protonation of the purine at N7. Steady state kinetics comprising engineered substrates confirm that a considerable fraction of the catalytic 5′-OH effect can be attributed to leaving group activation.

The putative transition state for enzymatic cleavage of N-glycosidic bonds is generally highly dissociative with substantial lengthening of the scissile bond but no bond formation to the incoming nucleophile (5, 7). The transition state has a high ribo-oxocarbenium character with sp2 hybridization at C1′ and a C3′-exo pucker. In purine nucleosides, departure of the leaving group is facilitated by protonation at N7 prior to reaching the transition state. In the base aspecific NH of Crithidia fasciculata a histidine residue has been identified as the general acid to accomplish this. However, a remarkable feature of the TvNH enzyme is the apparent lack of an acidic group to protonate the leaving purine. In this enzyme, x-ray crystallography and mutagenic scanning analysis did not reveal a suitable general acid candidate (1). Rather, a tryptophan (Trp-260) was found to be the only catalytic residue in the vicinity of the purine leaving group. A previous study combining quantum chemical calculations, mutagenesis, and pre-steady state kinetics revealed that an aromatic stacking interaction between Trp-260 and the purine ring contributes to catalysis by raising the basicity of the latter (8). Because enhanced basicity facilitates protonation by solvent, it appears that the TvNH enzyme employs specific, rather than general, acid catalysis.

Depurination by TvNH is further catalyzed by interactions with the three hydroxyls of the ribose moiety of the substrate, because the 2′-, 3′-, and 5′-deoxy nucleosides are severely impaired in binding and catalysis (1). The inosine bound in the TvNH active site adopts an unusual C4′-endo ribose pucker, with a H2′-C2′-C1′-N9 dihedral angle near 0°. This eclipsed conformation about the C2′-C1′ bond is indicative of ground state destabilization. Interestingly, the C4′-endo pucker is about 2 kcal/mol higher in energy than the C3′-exo pucker adopted in the transition state (9). The C4′-endo pucker is stabilized by interactions between the 2′-, 3′-, and 5′-OH groups and the enzyme, which could in part explain the necessity of these groups in catalysis. The 5′-OH is tightly bound in a gauche,trans orientation about the C4′–C5′ bond (see Fig. 1a) via hydrogen bonds to Glu-184 (2.57 Å) and Asn-173 (2.94 Å) (Fig. 2). A similar anchoring of the 5′-OH is observed in the homologous and extensively studied C. fasciculata NH (10). In the latter enzyme as well, the 5′-OH was found to be crucial for catalysis (2), and it was suggested that the lone pair electrons of the 5′-oxygen stabilizes the ribo-oxocarbenium transition state electrostatically via a “neighboring group interaction” with O4′ (Fig. 1a, dashed line) (5, 10). Such an interaction would imply that the oxonium-like resonance form of the oxocarbenium predominates (Fig. 1b). In the present study, we set out to investigate the catalytic role of the 5′-OH by theoretical and experimental methods.

**EXPERIMENTAL PROCEDURES**

*Ab Initio Quantum Chemical Calculations—Gas phase energies at the B3LYP/aug-cc-pVDZ level (11–13) for the ribo-oxocarbenium ion were obtained after unrestrained optimizations starting with the orientation about the C4′–C5′ bond either gauche,trans or trans,gauche or gauche–gauche (Fig. 1a). The conformers of purine nucleoside were optimized at the B3LYP–G(d,p) level with the constraint of a C4′-endo ribose pucker, followed by a single point calculation at the B3LYP/aug-cc-pVDZ level. Proton affinities were calculated as the energetic difference between the protonated and unprotonated species. The same approach was applied.

The abbreviations used are: TvNH, Trypanosoma vivax nucleoside hydrolase; NH, nucleoside hydrolase.

1 The term “base aspecific” implies that the enzyme accepts substrates with either a purine or a pyrimidine leaving group.

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¶To whom correspondence should be addressed: Eenheid Algemene Chemie, Vrije Universiteit Brussels, lokaal 10G703, Pleinlaan 2, 1050 Brussels, Belgium. Tel.: 32-2-629-35-16; Fax: 32-2-629-33-17; E-mail: loverix@vub.ac.be.

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on a series of nitrogen bases for which experimentally determined pK_a values are available: allyldimethylamine, imidazole, pyridine, thiazole, oxazole, and pyrazine (14). Atomic charges were derived according to the ChelpG scheme (15). All calculations were carried out using the Gaussian 03 package of programs (16).

Kinetics—Mutagenesis and protein purification were performed as described previously (17). Kinetic measurements were carried out in 50 mM phosphate buffer, pH 7.0, at 35 °C. The kinetic properties were determined spectrophotometrically using the difference in absorption between the nucleoside and the purine base. The ∆A values (mm−1 cm−1) used were: guanosine, −4.0 at 260 nm or 0.5 at 300 nm; 5′-deoxy-2-aminoguanosine, 0.5 at 300 nm; 7-methylguanosine, −3.3 at 258 nm or −0.3 at 310 nm; 5′-deoxy-7-methylguanosine, −4.0 at 258 nm or −0.4 at 310 nm. Guanosine and 7-methylguanosine were purchased from Sigma. 5′-Deoxyguanosine and 5′-deoxy-7-methylguanosine were synthesized as described below.

Synthesis of 5′-Deoxy Compounds—5′-Deoxyguanosine was synthesized according to the procedures of McGee and Martin (18); spectral data were in accord with that reported. 5′-Deoxy-7-methylguanosine was synthesized by the following procedure: 5′-deoxyguanosine (57 mg, 0.21 mmol) and iodomethane (3 equivalents) in N,N-dimethylacetamide (50.5 ml) were stirred at ambient temperature for 24 h. The solution was treated with Celite, followed by EtOH and Et2O, which caused an oil to form. Solvents were decanted and acetone added to the residue, forming a powder. Further washing with acetone and Et2O and drying provided the product (18 mg, 21%) as a white powder.

RESULTS AND DISCUSSION

The 5′-OH Group in TvNH Does Not Contribute to Ribo-oxocarbenium Ion Stabilization—In all NHs, the 5′-OH group of the ribose moiety of the substrate has been shown to play a prominent role in catalysis (1–4). In the NHs (10), as well as in purine nucleoside phosphorylase (19), this 5′-OH catalytic effect has been proposed to originate from a stabilization of the ribo-oxocarbenium transition state via an electrostatic neighboring group interaction with the O4′ atom. This proposal was based on crystal structures of the enzymes in complex with substrate analogues or transition state inhibitors (iminoribitols), invariably showing the 5′-OH group firmly anchored in a gauche,trans orientation about the C4′–C5′ bond. This orientation implies a close proximity between the O5′ and O4′ atoms (N4′ atom in iminoribitols). Allegedly, the 5′ lone pair electrons would electrostatically stabilize the developing positive charge on the O4′ atom. To test this hypothesis, we used high level ab initio methods to calculate the charge distribution in the ribo-oxocarbenium ion. The results indicate that the positive charge resides primarily on the C1′ atom, whereas the O4′ atom remains negatively charged, albeit to a lesser extent than in the nucleoside ground state. Thus, although the ribo-oxocarbenium tends toward an oxonium ion (Fig. 1b), the absence of positive charge on O4′ excludes a favorable electrostatic interaction with the O5′ lone pair electrons, undermining the neighboring group hypothesis. To further scrutinize any effects of the 5′-OH group on the stability of the ribo-oxocarbenium ion, we calculated the gas phase energies of three conformers about the C4′–C5′ bond. Apart from the gauche,trans conformation observed in enzyme-bound species, two other staggered conformers exist (Fig. 1a). The gauche,gauche conformer has the 5′-OH positioned over the ribosyl ring, whereas this group is pointing away in the trans,gauche conformer. Energy optimizations of the three conformers for the ribo-oxocarbenium ion show that the trans,gauche and gauche,trans conformers are energetically equal within the uncertainty on the calculated values, despite the proximity between the O5′ and O4′ atoms in the latter. This observation excludes a stabilizing O5′–O4′ neighboring group interaction. Remarkably, the gauche,gauche ribo-oxocarbenium ion is found to be 5 kcal/mol more stable than the other two conformers, presumably because of the proximity (3.07 Å) between O5′ and the positively charged C1′. It is quite surprising that NHs do not utilize this potential of the 5′-OH to lower the energy of a ribo-oxocarbenium-like transition state, because the gauche,gauche conformer has never been observed.

Modulation of Purine Basicity via a C8H–O5′ Hydrogen Bond—Because the catalytic function of the 5′-OH in TvNH cannot be attributed to ribo-oxocarbenium ion stabilization, another mechanism should be envisioned. From the crystal structure of the slow D10A mutant complexed with the substrate inosine (1), it appears that the gauche,trans conformation about the C4′–C5′ bond combined with the anti conformation of the nucleobase about the C1′–N9 bond allows for the formation of a hydrogen bond between C8-H of the purine and the 5′-oxygen (Fig. 2). Because of the low electronegativity of carbon, a CH–O hydrogen bond is a weak interaction and its importance is generally ignored. Yet, ab initio studies have shown that the strength of a CH–O bond may surpass that of a conventional uncharged OH–O hydrogen bond when the donor molecule is positively charged (20). It turns out that the carbon atom between the nitrogens in the imidazolium ion is an extremely potent hydrogen bond donor. Inversely, one could argue that the formation of a CH–O hydrogen bond between imidazole and an acceptor molecule should increase the nitrogen basicity of the former. Likewise in TVNH, the 5′-oxygen could promote the protonation of the nucleobase at N7 by preferentially accepting a hydrogen bond from the C8 of a positively charged purine ring. To corroborate this hypothesis, we compared the gas phase N7 proton affinities of C4′-endo puckered purine nucleoside in the trans,gauche (no C8–O5′ hydrogen bond) and gauche,trans (C8H–O5′ hydrogen bond) conformations. It turns out that upon hydrogen bond formation, the gas phase N7 proton affinity increases by about 5 kcal/mol. The existence of a genuine CH–O hydrogen bond in the calculated protonated species is further supported by a C–O distance of 2.92 Å and a 108 cm−1 red shift of the C8-H stretching frequency upon going from the trans,gauche to the gauche,trans conformer. To translate the calculated change in proton affinity to changes in the acid dissociation constant (pK_a), we calculated proton affinities for a series of amine bases. Plotting...
these values against their experimental pK_a values yields a linear curve with a correlation coefficient of 0.99 (data not shown) that was used to estimate the difference in pK_a between the various conformers of purine nucleoside from their calculated proton affinities. Via this approach, an increase in the pK_a of purine nucleoside (at N7) by about 1.9 units is found going from the gauche,trans to the trans,gauche conformer. Considering the low pK_a range (1–4) of purine nucleosides (21), this pK_a shift raises the concentration of the reactive (protonated) species by more than an order of magnitude at physiological pH. These calculations thus imply that the 5’-OH in TvNH could establish a near 100-fold rate enhancement via leaving group activation.

A Catalytic Role for the 5’-OH in Leaving Group Activation—To experimentally assess the role of the 5’-OH in leaving group activation in TvNH, a double-substrate engineering approach was employed. The contribution of 5’-OH to catalysis was measured by comparing the kinetic parameters for guanosine and 5’-deoxyguanosine. To filter out the fraction originating from leaving group activation, the kinetic effect of removing the 5’-OH group was also gauged in the presence of an activated leaving group (i.e. comparison of 7-methylguanosine with 5’-deoxy-7-methylguanosine). Similar to protonation, N7-methylation of guanosine induces a positive charge in the purine ring, facilitating leaving group departure. Because TvnH follows a complex mechanism with rate-limiting ribose release (22), the kinetic analysis was performed on the second order rate constant k_cat/K_m, which is independent of product release (23) (Table I). Removal of the 5’-OH group in guanosine causes a 6,000-fold reduction in k_cat/K_m (corresponding to 5.4 kcal/mol). However, with 7-methylguanine as a leaving group, removing the 5’-OH group decreases k_cat/K_m by merely a factor of 50 (corresponding to 2.4 kcal/mol). From the thermodynamic cycle in Fig. 3, it can be deduced that upon removing 5’-OH, a 125-fold reduction in k_cat/K_m comes from weakened leaving group activation. These results, combined with the ab initio calculations, indicate that the 5’-OH group in TvnH contributes 3.0 kcal/mol to leaving group activation by accepting a hydrogen bond from the C8 of the purine. The remaining part of the 5’-deoxy effect (2.4 kcal/mol) could originate from the impossibility of the enzyme to bind 5’-deoxyribose in a C4’-endo pucker, which would result in impaired ground state destabilization. Truncation of the side chains of Glu-184 and Asn-173, which make up the 5’-OH binding pocket in TvnH (Fig. 2), affects k_cat/K_m for guanosine by a factor of 20,000 (Table I). This is the same order of magnitude as the 5’-deoxy effect for the wild type enzyme. However, k_cat/K_m for the substrate 5’-deoxyguanosine is lowered only 40-fold by the mutations. The kinetic coupling between the N173A/E184A double mutant and the presence of the 5’-OH group demonstrate that the catalytic function of the latter depends on correct anchoring and/or polarization by Glu-184 and Asn-173. Furthermore, comparison of the kinetic implications of the double mutant for guanosine and 7-methylguanosine confirms that these side chains have a role in leaving group activation via the 5’OH group.

CONCLUSIONS

Hydrolysis/phosphorolysis of the N-glycosidic bond in (deoxy-) nucleosides and nucleotides commonly involves the protonation of the leaving nucleobase by a general acid, concomitant with nucleophilic attack. Although TvnH lacks a general acid, it is equally catalytically proficient to nucleoside hydrolyses from other sources (17). We have previously shown that, instead of employing general acid catalysis, TvnH activates the leaving purine base by increasing the N7 basicity (hence favoring its protonation) via a parallel aromatic stacking interaction with the indole side chain of Trp-260 (8, 24). The high level ab initio calculations presented here rule out a previously presumed stabilizing catalytic interaction between 5’O and 4’O. Rather, they suggest that a crystallographically observed intramolecular C8H–O5’ hydrogen bond in the purine nucleoside

![Figure 2. The 5’-OH binding pocket in TvnH. Shown is the D10A mutant in complex with the substrate inosine (Protein Data Bank accession number 1KIC). O5’, C8, and N7 are represented as spheres. Note the C4’-endo puckering of the ribose moiety.](Image 96x617 to 268x738)

![Figure 3. Thermodynamic cycle measuring the energetic coupling between the 5’-OH group and leaving group activation in wild type TvnH.](Image 320x395 to 560x653)

### Table I

| Species                  | k_cat/K_m (M⁻¹s⁻¹) for wild type and N173A/E184A TvnH |
|-------------------------|---------------------------------|
| Guanosine               | (1.0 ± 0.2) 10⁶                  | (52 ± 8) 10⁵                  |
| 7-Methylguanosine       | (1.7 ± 0.2) 10⁶                  | (1.5 ± 0.3) 10⁴              |
| 5’-Deoxyguanosine       | 159 ± 20                         | 4 ± 1                         |
| 5’-Deoxy-7-methylguanosine | (3.4 ± 0.6) 10⁴                  | (7 ± 3) 10³                  |

4 The latter rate reduction can be attributed to a structural perturbation of the active site in the N173A/E184A double mutant, as observed in the crystal structure (x-ray data not shown). These structural effects complicate a direct quantitative comparison of the kinetics for the double mutant and the wild type enzyme, but they cancel out in the kinetic coupling between different substrates.
increases the basicity of the leaving group. A double-substrate engineering kinetic analysis confirms the computational results and unambiguously implicates the 5'-OH group in leaving group activation, aided by the side chains of Glu-184 and Asn-173.

Combined, aromatic stacking with Trp-260 and intramolecular C8H–O5/H11032 hydrogen bond formation should raise the pKa of a purine nucleoside sufficiently to allow direct protonation by the solvent. The enzyme thus exemplifies a rare case of specific acid catalysis, in which the catalytic proton is recruited directly from a solvent molecule.

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