Punching Holes in an Enzyme

John P. Richard[1,*]
[1]Department of Chemistry, The State University of New York at Buffalo, Buffalo, NY 14260-3000, USA
*Correspondence: jrichard@buffalo.edu
DOI 10.1016/j.chembiol.2009.09.003

Schramm and coworkers have punched holes into human purine nucleoside phosphorylase by substitution of glycine for aromatic amino acid residues at a protein lid. The results of studies on the enzymes with holes illuminate hidden chemistry that occurs at the enzyme active site.

For many years, a large group of chemists and biologists chose to study nonenzymatic models for enzyme-catalyzed reactions in water in order to better understand the mechanism of enzyme catalysis of these reactions. This work was driven by the notion that enzymes, which operate in aqueous solution, will most often choose to stabilize the transition state that forms most readily in the nonenzymatic reaction in water. The determination of the structure and stability of these transition states has provided a good measure of the burden borne by many enzyme catalysts (Wolfenden and Snider, 2001). This serves as a starting point for the evaluation of the enzyme-ligand interactions responsible for transition-state stabilization.

A large number of enzymes catalyze transfer of the glycosyl group to water and other acceptor nucleophiles. Amyes and Jencks (1989) showed, in a study of nonenzymatic hydrolysis of sugar derivatives, that glycosyl transfer proceeds by a stepwise mechanism through a glycosyl cation intermediate that has a short but significant lifetime of 10^{-12} s in water ($k_s \approx 10^{12}$ s^{-1}) (Figure 1). This work raises two fundamental questions about enzyme-catalyzed nucleophilic substitution reactions at sugars: (1) what is the mechanism for the stabilization of the high-energy transition state at the enzyme active site; and (2) what is the lifetime (if any) for the enzyme-bound oxocarbenium ion intermediate?

Enzymologists now have a good understanding of the mechanism for the stabilization of the oxocarbenium ion-like transition states for enzyme-catalyzed glycosyl transfer through formal nucleophilic catalysis and electrostatic stabilization of the cationic glycosyl carbon (Zechel and Withers, 1999), and through protonation of the oxygen leaving group (Richard, 1998). However, the oxocarbenium ion intermediates and/or oxocarbenium ion-like transition states that form within the confines of enzyme-active sites are effectively sequestered from reaction with bulk solvent, and their lifetimes are far too short to allow detection by X-ray crystallographic analysis. In recent work, Ghanem et al. (2009) adopted the brutal approach of punching holes into human purine nucleoside phosphorylase (PNP) in order to increase the accessibility of the active site to bulk solvent.

Purine nucleoside phosphorylases catalyze the phosphorolysis of 6-oxypurine nucleosides to the corresponding purine base and α-D-ribose 1-phosphate (pathway A, Figure 2). The transition state for the reaction catalyzed by human PNP has been characterized as having a structure similar to that for the fully dissociated ribocation, with leaving group departure

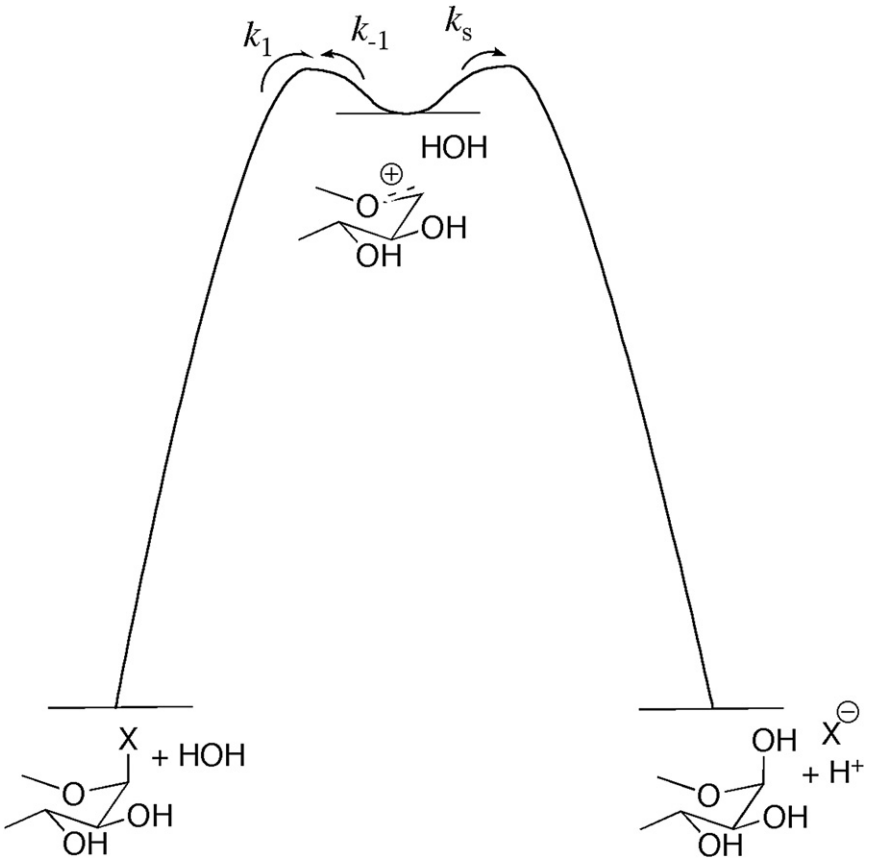

Figure 1. Free Energy Profile for Glycoside Hydrolysis
Free energy reaction profile for cleavage of a glycoside derivative (k_1) to form a glycosyl oxocarbenium ion that partitions between return to reactant (k_{-1}) and addition of solvent ($k_s \approx 10^{12}$ s^{-1}) (Amyes and Jencks, 1989).

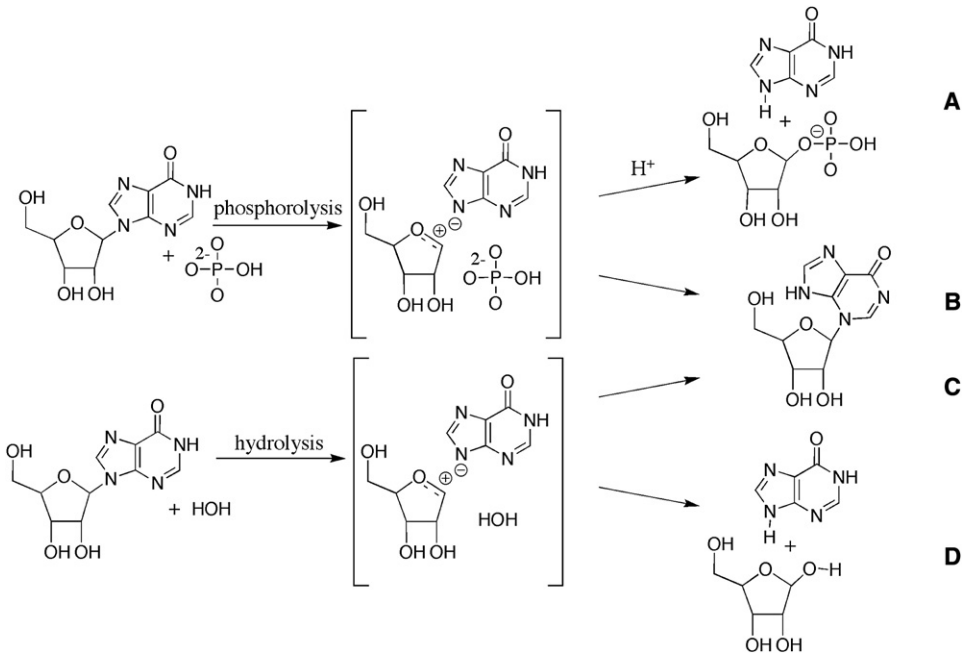

Figure 2. Human PNP Catalyzed Phosphorolysis and Hydrolysis Reactions of Inosine through Oxocarbenium Ion Intermediates of Unknown Lifetime

The intermediate of the enzyme-catalyzed phosphorolysis reaction is shown to partition between addition of phosphate anion (pathway A) and N-9 to N-3 isomerization (pathway B). The intermediate of the enzyme-catalyzed hydrolysis reaction is shown to partition between N-9 to N-3 isomerization (pathway C) and addition of water (pathway D).

occurring prior to migration of the ribosyl cation to the phosphate nucleophile (Lewandowicz and Schramm, 2004). Oxocarbenium ions exist for several bond vibrations or longer in water (Amyes and Jencks, 1989), and an enzyme bound ribocation would be strongly stabilized by interactions with the protein catalyst. However, the possibility that the reaction proceeds through an oxocarbenium ion-like transition state that exists at a flat energy maximum (Carpenter, 2004), by a mechanism that avoids formation of a intermediate with a vibrational lifetime, is difficult to exclude.

Human PNP is a homotrimer with active sites located near the subunit-subunit interfaces. Residues H257, F200, and Y88, from a given subunit, and F159′, from the adjacent one, form a hydrophobic lid to the catalytic site and act to shield the enzyme-bound reactants from bulk solvent (Saen-oon et al., 2008). Ghanem et al. (2009) have prepared glycine mutants of each of these four lid residues in an attempt to punch holes in human PNP and introduce a solvent leak.

Wild-type human PNP catalyzes phosphorolysis of inosine with $(k_{cat})_{phos}$ = 44 s^{-1} (pathway A, Figure 2) and hydrolysis of inosine with k_{hyd} = 7.5 × 10^{-4} s^{-1} (pathway D, Figure 2). The rate constant ratio $(k_{cat})_{phos}/k_{hyd}$ = 59,000 shows that the transition state for phosphorolysis is stabilized by 6.5 kcal/mol relative to that for hydrolysis. Much or all of this stabilization is from direct electrostatic interactions between the cationic oxocarbenium ion and the phosphate anion. Mutations of the lid residues cause up to a 44-fold decrease in $(k_{cat})_{phos}$ to 1.0 s^{-1} and a 5-fold increase in k_{hyd} to 3.6 × 10^{-3} s^{-1} for the reaction catalyzed by F200G human PNP. The change in the rate constant ratio to $(k_{cat})_{phos}/k_{hyd}$ = 280 for the F200G mutant shows that the mutation causes a 3.2 kcal/mol increase in the energy of the transition state for the phosphorolysis compared with the hydrolysis reaction. A simple interpretation of these results is that punching holes in human PNP allows additional water to enter the active site and causes an increase in the effective dielectric constant of the active site to a value closer to that for water. This then leads to a reduction in the direct electrostatic stabilization of the transition state by interactions between the cationic oxocarbenium ion and the phosphate dianion, and a decrease in $(k_{cat})_{phos}$ for formation of this transition state. The modest ≤ 5-fold increases in k_{hyd} observed for 3 of the 4 mutant enzyme-catalyzed reactions show that the overall rate of the hydrolysis reaction is rather insensitive to an increase in the accessibility of the active site to solvent.

Wild-type human PNP catalyzes the novel N-9 to N-3 isomerization reaction of inosine in the absence of an added phosphate (pathway C, Figure 2) with a rate constant k_{iso} = 1 × 10^{-3} s^{-1}. This shows that the intermediate, which forms in the cloistered environment of the wild-type enzyme active site, partitions at similar rates to form isomerization and hydrolysis (k_{hyd} = 7.5 × 10^{-4} s^{-1}, pathway D) reaction products. No isomerization products are observed during the hydrolysis of inosine catalyzed by the lid mutants of human PNP. This suggests that the dominant effect of punching holes in human PNP is to slow isomerization by facilitating the escape of the hypoxanthine product to solvent.

No products of N-9 to N-3 isomerization are observed during wild-type human PNP-catalyzed phosphorolysis of inosine. This shows that the reaction along

pathway A of Figure 2 dominates over pathway B for the wild-type enzyme. Both H257G and F200G mutant PNPs catalyze N-9 to N-3 isomerization of inosine at a rate that is detectably competitive with the rate of phosphorolysis. For example $(k_{cat})_{phos}/k_{iso}$ = 240 was determined for the reaction catalyzed by F200G mutant human PNP. The N-9 to N-3 isomerization reaction is only observed when phosphate anion is bound to the oxocarbenium ion intermediate of these lid mutants. Therefore, the presence of bound phosphate anion causes an increase in the selectivity of the oxocarbenium ion intermediate for trapping by N-3 of hypoxanthine. The explanation for this effect of phosphate anion is unclear.

In summary, the innovative and clever approach adopted in this work of punching holes into human PNP shows that this dramatic change in enzyme structure has subtle and fascinating effects on the catalytic reaction mechanism. The results provide a snapshot of the enzyme-bound intermediate that falls short of crystal clarity, and they serve to emphasize the magnitude of the challenge of determining absolute rate constants for the reaction of unstable carbocation and carbanion intermediates within the restricted confines of an enzyme active site.

REFERENCES

Amyes, T.L., and Jencks, W.P. (1989). J. Am. Chem. Soc. *111*, 7888–7900.

Carpenter, B.K. (2004). In Reactive Intermediate Chemistry, R.A. Moss, M.S. Platz, and M.J. Jr, eds. (Hoboken, NJ: John Wiley & Sons), pp. 925–960.

Ghanem, M., Murkin, A.S., and Schramm, V.L. (2009). Chem. Biol. *16*, this issue, 971–979.

Lewandowicz, A., and Schramm, V.L. (2004). Biochemistry *43*, 1458–1468.

Richard, J.P. (1998). Biochemistry *37*, 4305–4309.

Saen-oon, S., Quaytman-Machleder, S., Schramm, V.L., and Schwartz, S.D. (2008). Proc. Natl. Acad. Sci. USA *105*, 16543–16548.

Wolfenden, R., and Snider, M.J. (2001). Acc. Chem. Res. *34*, 938–945.

Zechel, D.L., and Withers, S.G. (1999). In Comprehensive Natural Products Chemistry, C.D. Poulter, ed. (New York: Elsevier), pp. 279–314.

Reassembling Biological Machinery In Vitro

Henry Hess[1,*]
[1]Department of Biomedical Engineering, Columbia University, MC8904, New York, NY 10027, USA
*Correspondence: hh2374@columbia.edu
DOI 10.1016/j.chembiol.2009.09.004

Inspired by the specialized glycolytic system of flagella of mammalian sperm, Mukai et al. (2009) describe the controlled immobilization of two enzymes constituting the first steps in the glycolytic pathway. Extension of this work may provide "power converters" for bionanodevices, which transduce chemical energy from glucose to ATP.

Biomolecules are engineering marvels not only individually, but also in their collective operation within larger structures with complex functions. Research in nanobiotechnology aims to capitalize on biological structures directly by creating hybrid devices that merge biomolecules with artificial building blocks, and, indirectly, by investigating the engineering challenges faced by evolution as it improves molecular biosystems. The potential rewards of this approach include nanodevices with functionalities currently unattainable by manmade building blocks, and synthetic biology informed by engineering understanding.

An example of a biomolecular system redesigned and reassembled for engineering purposes is the motor protein-powered molecular shuttle (Hess et al., 2004). One design for such a nanoscale transport system with applications (e.g., in biosensing (Fischer et al., 2009)) relies on antibody-functionalized microtubules gliding on surface-adhered kinesin motors to capture and transport specific analyte molecules. These molecular shuttles are fueled by ATP, and the controlled and efficient provision of this fuel is a matter of ongoing interest.

Biological systems have evolved many mechanisms to secure the timely delivery of ATP at different locations throughout the cell. The glycolytic cycle couples ATP creation to a high energy fuel source—glucose, for example. To an engineer, the process resembles the conversion of AC high voltage at the wall outlet into DC low voltage used by electronic devices by one of the ubiquitous power supplies.

In this issue, Mukai and colleagues (2009) describe how they have been inspired by the controlled arrangement of glycolytic enzymes on the flagellar principal piece of mammalian sperm to pursue the engineering of a glycolytic ATP supply on a synthetic surface. The starting point of their investigation is the insight that the glycolytic enzymes in the flagellum are attached to the fibrous sheath via specific anchoring domains, and that these domains can be replaced by a His-tag via genetic engineering. These modified enzymes will then bind in specific orientations to synthetic surfaces rendered resistant to nonspecific protein adsorption but presenting Ni-NTA functional groups. By immobilizing at first two and then later all the enzymes of the glycolytic pathway, the ATP fuel can be