A Conformational Study of 2-Oxanol: Insight into the Role of Ring Distortion on Enzyme-Catalyzed Glycosidic Bond Cleavage

Brian J. Smith

Contribution from the Biomolecular Research Institute, Parkville, Victoria 3052, Australia

Received July 5, 1996. Revised Manuscript Received November 4, 1996[⊗]

Abstract: Ab initio molecular orbital theory at the G2(MP2,SVP) level has been used to study several conformations, defining part of the pseudorotational itinerary, of equatorial 2-oxanol. Half-chair ($^3H_4$) and boat ($B_{1,4}$) transition states lie 23.7 and 14.3 kJ mol^{-1} above the chair conformation ($^1C_4$), respectively, while the twist-boat conformers ($^3S_1$ and $^5S_1$) lie 6.9 and 5.9 kJ mol^{-1} above the chair, respectively. Protonation of the glycosidic oxygen of the chair conformer yields an oxonium ion in the chair conformation. All other conformations collapse to give an oxocarbonium ion−water complex upon protonation. The axial anomer in the chair conformation lies 12.0 kJ mol^{-1} lower than the equatorial anomer. Protonation of the axial anomer in the chair conformation does not yield a chair, but collapses to the oxocarbonium ion. A clear role is shown for ring distortion in enzymes which perform acid-catalyzed hydrolysis of equatorial glycosides. In addition to avoiding high-energy oxonium ion intermediates, distortion of the ring also reduces the glycosidic bond-stretch energy which delays the transition state and reduces the reaction barrier. Enzymes which hydrolyze the axial anomer do not require ring distortion to achieve a concerted pathway to the oxocarbonium ion. These results are discussed in relation to three enzymes, lysozyme, neuraminidase, and β-amylase.

Introduction

The means by which enzymes achieve their high degree of substrate specificity and catalytic rate enhancement is critically dependent upon how the protein binds the substrate. Enzymes achieve their catalytic rate enhancement by binding the transition state more tightly than the substrate.[1] There may then be a predisposition of enzymes toward binding substrates in conformations which in some way resemble the transition state and which are not the lowest energy conformation in the free state.

There are a large number of enzymes which hydrolyze the glycosidic bond, *O*-glycosyl hydrolases (glycosidases, EC 3.2.1.x).[2] These enzymes catalyze the acid-assisted hydrolysis of the glycosidic bond. One of the most widely studied and best understood (mechanistically) is lysozyme. The Phillips mechanism[3] for action of lysozyme employs a distortion of the pyranose ring into the half-chair conformation which closely resembles the oxocarbonium ion transition state. This distortion, it is claimed, relieves the steric interference which would otherwise restrict the binding of the polysaccharide. Structural studies of the influenza virus neuraminidase with its receptor bound in the active site[4,5] have shown that sialic acid (*N*-acetylneuraminic acid) adopts a boat conformation. This presents the carboxylate group in an equatorial position, allowing favorable interactions with several side-chain groups of the protein. Again, the distortion away from the chair geometry produces a conformation of the ring that resembles that of the oxocarbonium ion which is speculated to form the transition state complex in this enzyme. In contrast to both of these, β-amylase appears to bind all glucopyranosyl groups of maltotetraose in the chair conformation.[6] Therefore, geometrical distortion of the substrate by the enzyme is not a general component of catalysis in glycosidases. There is also the view that geometrical distortion is unlikely to be a significant factor in enzyme catalysis.[7,8]

Glycosidases can be classified into four specific classes, depending on whether they retain or invert the configuration at the anomeric center, and whether the leaving group (aglycon) in the substrate is equatorial or axial (in the normal chair conformation).[9] This classification is independent of the chirality at the anomeric center. The general enzymatic hydrolysis of glycosides takes place via acid catalysis and requires two critical residues, a proton donor and a nucleophile.[10] Protonation of the glycosidic oxygen results in cleavage of the glycosidic bond and formation of the oxocarbenium ion. Stabilization of this ion by the nucleophile is believed to play a pivotal role in the rate enhancement of this class of enzyme. There is evidence that this ion forms a covalent link with the nucleophile in some enzymes,[2,11] while in some enzymes the nucleophile is missing.[12] Reversal of this process with a water molecule substituted for the aglycon completes the reaction.

[⊗] Abstract published in *Advance ACS Abstracts,* February 15, 1997.

(1) Wolfenden, R. *Nature (London)* **1969**, *223*, 704.

(2) McCarter, J. D.; Withers, S. G. *Curr. Opin. Struct. Biol.* **1994**, *4*, 885. Sinnott, M. *Chem. Rev.* **1990**, *90*, 1171.

(3) Phillips, D. C. *Sci. Am.* **1966**, *215*, 78.

(4) Varghese, J. N.; McKimmin-Breshkin, J. L.; Caldwell, J. B.; Kortt, A. A.; Colman, P. M. *Proteins* **1992**, *14*, 327.

(5) Burmeister, W. P.; Riugrok, R. W. H.; Cusack, S. *EMBO J.* **1992**, *11*, 49.

(6) Mikami, B.; Degano, M.; Hehre, E. J.; Sacchetini, J. C. *Biochemistry* **1994**, *33*, 7779.

(7) For arguments against a significant role for distortion in enzyme catalysis, see: Page, M. I. In *The Chemistry of Enzyme Action*; Page, M. I., Ed.; Elsevier: New York, 1984.

(8) Levitt, M. In *Peptides, Polypeptides and Proteins*; Blout, E. R., Bovey, F. A., Goodman, M., Lotan, N., Eds.; John Wiley and Sons: New York, 1974; p 99. Warshel, A.; Levitt, M. *J. Mol. Biol.* **1976**, *103*, 227. Pincus, M. R.; Scheraga, H. A. *Macromolecules* **1979**, *12*, 633.

(9) Sinnott, M. L. In *Enzyme Mechanisms*; Page, M. I., Williams, A., Eds.; Royal Society of Chemistry: London, 1987; p 259.

(10) For a recent review, see: Davies, G.; Henrissat, B. *Structure* **1995**, *3*, 853.

(11) White, A.; Tull, D.; Johns, K.; Withers, S. G.; Rose, D. R. *Nature Struct. Biol.* **1996**, *3*, 149.

(12) Weaver, L. H.; Grutter, M. G.; Matthews, B. W. *J. Mol. Biol.* **1995**, *245*, 54.

S0002-7863(96)02302-5 CCC: $14.00

It has long been recognized[13,14] that the relative rate of acid hydrolysis of glycosides can be correlated with the ease with which the glycoside can be converted to the half-chair conformation. Andrews *et al.*[15] recently studied protonated 2-methoxytetrahydropyran with the intention of determining the transition state species for cleavage of the glycosidic C−O bond. The most probable reaction path was determined from the lowest energy stationary points. Here, through studies of 2-oxanol, the effects of distortion of the ring on the acid-catalyzed cleavage of the glycosidic bond are to be investigated. The intention is to apply conventional theories of chemical reactivity to derive some understanding of how enzymes might utilize inherent properties of the pyranose ring to achieve their catalytic rate enhancement.

Methods

Standard ab initio molecular orbital calculations[16] were carried out using the GAUSSIAN 92 program.[17] Calculations were performed at the G2(MP2,SVP)[18−20] level. This variant of the highly successful G2 theory[21] was developed recently to allow calculations on moderately large systems at a high level of theory and at a reasonable cost. Energies are evaluated using the additivity relationship

$$E(\text{QCISD(T)/6-311+G(3df,2p)}) \approx E(\text{QCISD(T)/6-31G(d)}) + \Delta_{\text{MP2,SVP}}$$

where the basis set extension, $\Delta_{\text{MP2,SVP}}$, is given by

$$\Delta_{\text{MP2,SVP}} = E(\text{MP2/6-311+G(3df,2p)}) - E(\text{MP2/6-31G(d)})$$

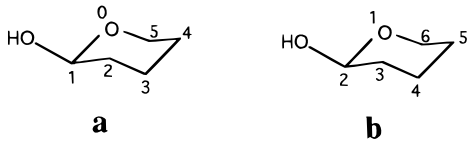
on geometries obtained at the MP2/6-31G(d) level. This method is similar to G2(MP2) theory[22] but with the QCI contribution evaluated using the 6-31G(d) basis in place of the 6-311G(d,p) basis. To these energies are added thermodynamic and higher level corrections (HLC). Harmonic frequencies were calculated at the HF/6-31G(d) level in order to characterize stationary points as either minima or transition states and, after scaling by 0.8929, to evaluate zero-point vibrational energies (ZPVE) and temperature corrections (ΔH_{298-0}, S). Note that, for the energy comparisons relevant to this paper, the empirical isogyric corrections cancel out. Composite theories of this type are known to reproduce gas-phase energies to an accuracy typically better than 10 kJ mol^{-1}.[23] Gibbs free energies at 298 K were evaluated as

$$G_{298} = E(\text{QCISD(T)/6-311+G(3df,2p)}) + \text{ZPVE} + \Delta H_{298-0} + \text{HLC} - TS$$

Results and Discussion

The standard rules for naming heterocyclic systems and those describing the conformation of cyclic systems differ in the way in which ring atoms are numbered. When the conformation of pyranose rings is described, carbon atoms are numbered sequentially following the oxygen atom (the oxygen atom is numbered zero) (Chart 1, structure a).[24,25] When heterocyclic systems are named,[26] however, the position of the heteroatom is used to determine the numbering; i.e., the oxygen atom is labeled atom 1 (Chart 1, structure b). These standard rules are followed in all further discussions.

Chart 1



a **b**

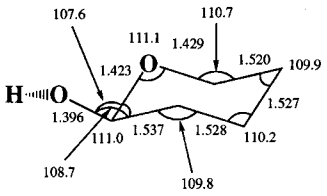
Calculated energies, zero-point vibrational energies, and temperature and higher level corrections for the systems of interest (**1**−**13**) are presented as Supporting Information. Unless otherwise noted, all results refer to G2(MP2,SVP) Gibbs free energies at 298 K. Displayed in Figure 1 are the key geometrical parameters of the MP2/6-31G(d)-optimized structures **1**−**13**.

1. Conformational Analysis. On the pseudorotational potential energy surface of pyranose rings there are only two classes of minima, the chair (*C*) and twist-boat or skew (*S*) conformations. Conversion of chair to twist-boat occurs via a half-chair (*H*), and conversion between twist-boat conformations occurs through the boat (*B*) conformer.[24,25] For equatorial 2-oxanol the $^1C_4$ chair conformation (**1**) converts to the $^3S_1$ twist-boat (**3**) via the $^3H_4$ half-chair transition state (**2**), while the $B_{1,4}$ boat (**4**) connects $^3S_1$ and $^5S_1$ (**5**) twist-boat conformers. In Figure 2 a segment of the polar-coordinate sphere which describes the ring puckering in six-membered rings[27] illustrates the geometrical relationship between each conformation.

In the $^3H_4$ half-chair structure (**2**), the τ_{5612} and τ_{6123} dihedral angles are both small, which results in five of the ring atoms being almost coplanar (with the C_4 atom lying out of the plane). The Cremer and Pople[27] (CP) ring-puckering parameters [Q = 0.558 Å, $\theta = 55.2°$, and $\phi = 15.1°$] indicate a structure which is intermediate between the half-chair and half-boat (the half-boat (sofa or envelope) is intermediate between the chair and boat [$\theta = 45°$ and $\phi = 60°$]). The internal ring angle at oxygen increases from 111.1° in the chair to 124.0° in the half-chair. In boat and twist-boat conformations, this angle varies between 111.1° and 116.2°. The C_2-O_1 length is longest in the chair conformer (1.423 Å) and shortest in the boat (1.410 Å), while the glycosidic C−O length is shortest in the chair conformer (1.396 Å) and longest in the boat (1.420 Å). Such observations are completely consistent with the orbital overlap arguments originally forwarded by Deslongchamps;[28] in the chair conformation, the glycosidic C−O bond is antiperiplanar only to ring bonds, whereas the other conformers have available a lone pair of electrons on the ring oxygen atom, antiperiplanar to the glycosidic C−O bond.

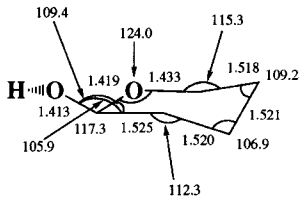
The barrier for ring inversion in cyclohexane[29] (through the half-chair conformation) at the G2(MP2,SVP) level, 46.1 kJ mol^{-1}, is in excellent agreement with the experimentally

(13) Edward, J. T. *Chem. Ind. (London)* **1955**, 1102.
(14) Woods, R. J.; Andrews, C. W.; Bowen, J. P. *J. Am. Chem. Soc.* **1992**, *114*, 859.
(15) Andrews, C. W.; Fraser-Reid, B.; Bowen, J. P. *J. Am. Chem. Soc.* **1991**, *113*, 8293.
(16) Hehre, W.; Radom, L.; Schleyer, P. v. R.; Pople, J. A. *Ab Initio Molecular Orbital Theory*; John Wiley: New York, 1986.
(17) Frisch, M. J.; Trucks, G. W.; Head-Gordon, M.; Gill, P. M. W.; Wong, M. W.; Foresman, J. B.; Johnson, B. J.; Schlegel, H. B.; Robb, M. A.; Replogle, E. S.; Gomperts, R.; Andres, J. L.; Raghavachari, K.; Binkley, J. S.; Gonzalez, C.; Martin, R. L.; Fox, D. J.; DeFrees, D. J.; Baker, J.; Stewart, J. J. P.; Pople, J. A. *GAUSSIAN 92*, Revision C; Gaussian Inc.: Pittsburgh, PA, 1992.
(18) Smith, B. J.; Radom. L. *J. Phys. Chem.*. **1995**, *99*, 6468.
(19) Smith, B. J.; Radom. L. *Chem. Phys. Lett.* **1995**, *245*, 123.
(20) Curtiss, L. A.; Redfern, P. C.; Smith, B. J.; Radom, L. *J. Phys. Chem*. **1996**, *104*, 5148.
(21) Curtiss, L. A.; Raghavachari, K.; Trucks, G. W.; Pople, J. A. *J. Chem. Phys.* **1991**, *94*, 7221.
(22) Curtiss, L. A.; Raghavachari, K.; Pople, J. A. *J. Chem. Phys.* **1993**, *98*, 1293.
(23) Curtiss, L. A.; Raghavachari, K. In *Quantum Mechanical Electronic Structure Calculations with Chemical Accuracy*; Langhoff, S. R., Ed.; Kluwer: Dordrecht, The Netherlands, 1995.
(24) Stoddard, J. F. *Stereochemistry of Carbohydrates*; Wiley-Interscience: New York, 1971.
(25) IUPAC-IUB Joint Commission on Biochemical Nomenclature. *Eur. J. Biochem.* **1980**, *111*, 295.
(26) IUPAC Organic Nomenclature Rules A and B, 1969.
(27) Cremer, D.; Pople, J. A. *J. Am. Chem. Soc.* **1975**, *97*, 1354.
(28) Deslongchamps, P. *Stereoelectronic Effects in Organic Chemistry*; Pergammon Press: Oxford, 1983.
(29) The G2(MP2,SVP) free energies (298 K) for chair and half-chair conformations of cyclohexane are −235.272 45 and −235.254 90 hartrees, respectively.



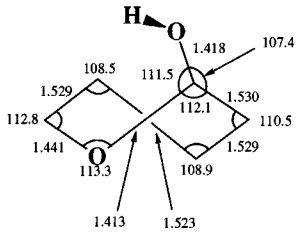
τ_{1234} -57.4
τ_{2345} 52.4
τ_{3456} -52.3
τ_{4561} 56.9
τ_{5612} -62.4
τ_{6123} 62.8

(1)



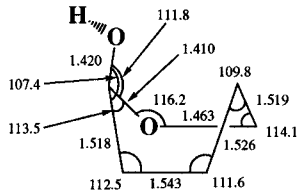
τ_{1234} -21.4
τ_{2345} 56.6
τ_{3456} -66.3
τ_{4561} 40.6
τ_{5612} -4.1
τ_{6123} -6.0

(2)



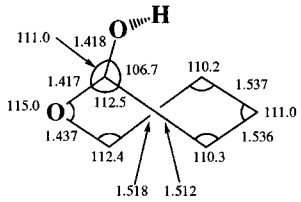
τ_{1234} 23.3
τ_{2345} 38.6
τ_{3456} -65.0
τ_{4561} 27.6
τ_{5612} 36.0
τ_{6123} -64.3

(3)



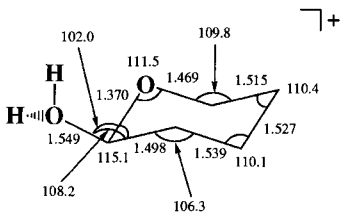
τ_{1234} 47.0
τ_{2345} 4.5
τ_{3456} -51.1
τ_{4561} 51.2
τ_{5612} -0.1
τ_{6123} -50.1

(4)



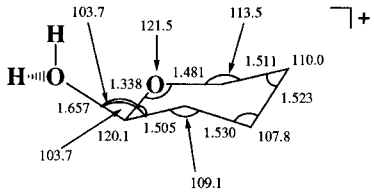
τ_{1234} 63.0
τ_{2345} -31.5
τ_{3456} -25.8
τ_{4561} 61.9
τ_{5612} -32.9
τ_{6123} -28.7

(5)



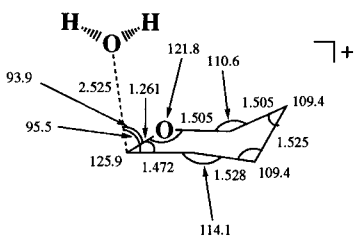
τ_{1234} -59.5
τ_{2345} 54.1
τ_{3456} -55.3
τ_{4561} 54.9
τ_{5612} -57.7
τ_{6123} 62.3

(6)



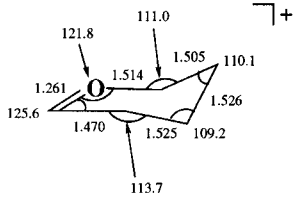
τ_{1234} -40.8
τ_{2345} 58.3
τ_{3456} -62.7
τ_{4561} 45.9
τ_{5612} -27.3
τ_{6123} 25.4

(7)



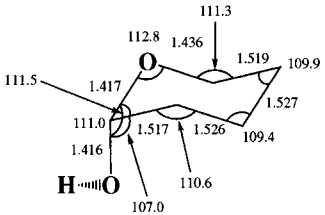
τ_{1234} 1.5
τ_{2345} 31.9
τ_{3456} -59.8
τ_{4561} 54.9
τ_{5612} -22.1
τ_{6123} -7.1

(8)



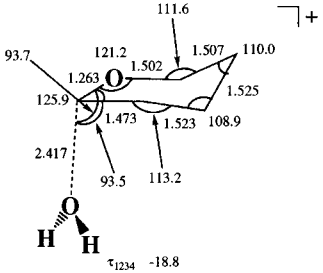
τ_{1234} -8.8
τ_{2345} 38.4
τ_{3456} -60.2
τ_{4561} 51.3
τ_{5612} -21.5
τ_{6123} -0.4

(9)



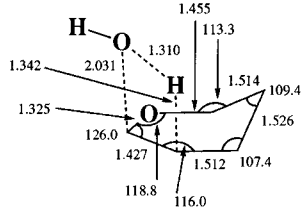
τ_{1234} -54.5
τ_{2345} 52.9
τ_{3456} -54.4
τ_{4561} 57.0
τ_{5612} -58.7
τ_{6123} 57.4

(10)



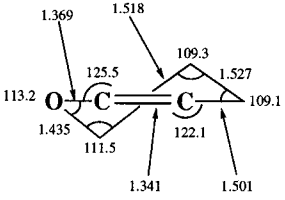
τ_{1234} -18.8
τ_{2345} 42.2
τ_{3456} -59.8
τ_{4561} 51.3
τ_{5612} -26.7
τ_{6123} 10.3

(11)



τ_{1234} 1.6
τ_{2345} 34.1
τ_{3456} -59.9
τ_{4561} 53.4
τ_{5612} -17.5
τ_{6123} -11.1

(12)



τ_{1234} 4.1
τ_{2345} 12.9
τ_{3456} -43.9
τ_{4561} -46.7
τ_{5612} 63.6
τ_{6123} 13.2

(13)

Figure 1. Key geometrical features of the MP2/6-31G(d)-optimized structures. Torsional parameters (τ) are numbered according to Chart 1, structure b.

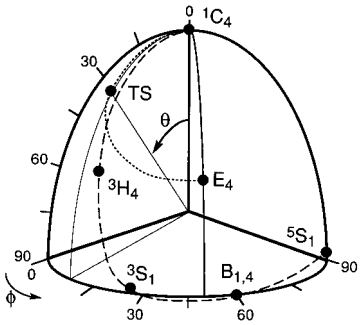


Figure 2. Segment of a sphere describing ring puckering in pyranose ring systems. Each conformation of the ring is described by a spherical polar coordinate set (Q, θ, ϕ) (the amplitude, Q, is constant in the diagram). In the ring puckering coordinates for six-membered rings, the chair conformation corresponds to angles $\theta = 0°$ and $\phi = 0°$. Equatorial positions on the sphere ($\theta = 90°$) correspond to boat ($\phi = n\pi/3$) and twist-boat ($\phi = (2n + 1)\pi/6$) conformations. Half-boat and half-chair conformations have $\theta = 45°$ and $\phi = n\pi/3$ or $\phi = (2n + 1)\pi/3$, respectively. The dashed line illustrates the connectivity defining part of the pseudorotational itinerary of the neutral pyranose. The dotted line indicates the connection between stationary points for the protonated pyranose.

determined value[30] of 43 kJ mol^{-1} and confirms the reliability of this computational procedure for calculating these energies. For equatorial 2-oxanol the $^3H_4$ half-chair lies 23.7 kJ mol^{-1} above the $^1C_4$ chair and 16.8 kJ mol^{-1} above the $^3S_1$ twist-boat. The $B_{1,4}$ boat lies 7.4 and 8.4 kJ mol^{-1} above $^3S_1$ and $^5S_1$ twist-boats, respectively.

Protonation of $^1C_4$ yields an oxonium ion in the chair conformation (**6**). The glycosidic C−O bond length is 0.15 Å longer and the C_2-O_1 and C_2-C_3 bonds are 0.05 and 0.04 Å shorter, respectively, than found in the neutral $^1C_4$. The gas-phase basicity for the chair conformation is 787.2 kJ mol^{-1}, in good agreement with a recent experimental[31] estimate of 787 ± 13 kJ mol^{-1} for glucose. The proton affinity (ΔH_{298}) is 790.2 kJ mol^{-1}, which is significantly greater than the proton affinity of methanol calculated at this level[19] of 754.4 kJ mol^{-1}.

Conversion of the protonated chair (**6**) to the oxocarbonium−water complex (**8**) occurs through transition structure **7** (TS) which lies just 3.4 kJ mol^{-1} above **6**. Structure **8** has a half-boat conformation [$Q = 0.515$ Å, $\theta = 56.0°$, and $\phi = 51.7°$]. The ion−water complex lies 75.3 kJ mol^{-1} lower in energy than **6**. The glycosidic C−O link in this complex is calculated to be 2.525 Å at the MP2/6-31G(d) level, compared with 1.549 and 1.657 in the oxonium chair and transition state structures, respectively. In both structures **7** and **8** the internal ring angle at oxygen, 121.5° and 121.8°, respectively, is considerably larger than in the oxonium ion **6** (111.5°). Dissociation of this complex to the oxocarbenium ion **9** requires 3.7 kJ mol^{-1}. In Figure 3 is presented the schematic free energy profile illustrating the connection among structures **1**−**9**. Kinetic isotope effect experiments[32] on the acid-catalyzed hydrolysis of equatorial methyl glucopyranosides suggest the most probable transition structure to be a chair flattened somewhat toward the half-chair, which corresponds closely with the transition structure **7** in these calculations. The proton is believed to be totally transferred to the glycosidic oxygen in the transition state, with the C−O bond "largely cleaved". The glycosidic C−O length in the transition structure **7** (1.657 Å) is 0.26 Å longer than the neutral chair and 0.87 Å shorter than in the oxocarbonium ion water complex. The C_2-O_1 length in **7** is 0.09 Å shorter than the neutral chair, indicating considerable double bond character.

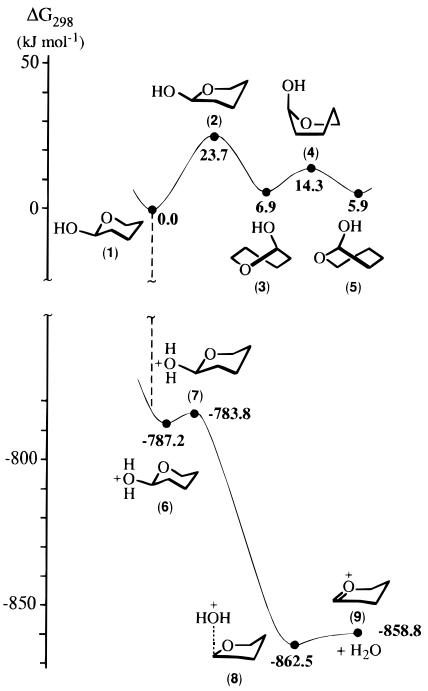


Figure 3. Schematic free energy profile (G2(MP2,SVP) level, kJ mol^{-1}) for neutral and protonated equatorial 2-oxanol.

Andrews, Bowen, and Fraser-Reid[33] have previously studied protonated dimethoxymethane ($CH_3OCH_2OH^+CH_3$) as a glycoside model. Many of the structural effects they observed in the acetal could be adequately accounted for using the anti-periplanar lone pair hypothesis.[34] Conformations of the acetal which correspond to boat and half-chair conformations of 2-oxanol showed appreciably longer C−O lengths (∼0.1 Å) between the CH_3OH leaving group and the oxocarbenium ion ($CH_3OCH_2^+$), and shorter C−O lengths within the ion, than conformations corresponding to the chair. The energy of the protonated acetal was lowest for a gauche arrangement (corresponding to the $^5S_1$ conformation in 2-oxanol), although the energy was found to differ by no more than 10 kJ mol^{-1} among all of the conformations considered.

Protonation of conformations of equatorial 2-oxanol other than the chair *all* lead to the oxocarbonium ion complex (**8**); no oxonium ion with a structure resembling the half-chair, boat, or twist-boat could be found. Why protonation of these conformations should lead to **8** and not **6** is clear from inspection of Figure 2. These conformations all have CP ring puckering angles greater than the protonated chair transition structure (**7**). Protonation of these conformations is unlikely to cause them to collapse to the chair conformation (**6**). In contrast, Andrews *et al.*[15] found that several conformations of equatorial 2-methoxytetrahydropyran (including the chair and twist-boat) all proceeded to the oxocarbenium ion through the same transition state following protonation.

Two distinctly different paths exist for the acid-catalyzed glycosidic cleavage of equatorial 2-oxanol. For the chair conformation a stepwise path exists, characterized by a stable oxonium ion following protonation, which subsequently dissociates to the oxocarbonium ion. In contrast, protonation of any of the half-chair, twist-boat, or boat conformations (on this part of the pseudorotational itinerary) will result in a concerted dissociation to the oxocarbonium ion, thereby avoiding formation of the high-energy intermediate oxonium ion.

Determining which of the concerted or stepwise paths is of lower energy requires calculation of the proton transfer energy.

(30) Anet, F. A. L.; Bourn, A. J. R. *J. Am. Chem. Soc.* **1967**, *89*, 760.
(31) Jebber, K. A.; Zhang, K.; Cassady, C. J.; Chung-Phillips, A. *J. Am. Chem. Soc.*
(32) Bennet, A. J.; Sinnot, M. L. *J. Am. Chem. Soc.* **1986**, *108*, 7287.
(33) Andrews, C. W.; Bowen, J. P.; Fraser-Reid, B. *J. Chem. Soc., Chem. Commun.* **1989**, 1913.
(34) Kirby, A. J. *CRC Crit. Rev. Biochem.* **1987**, *22*, 282.

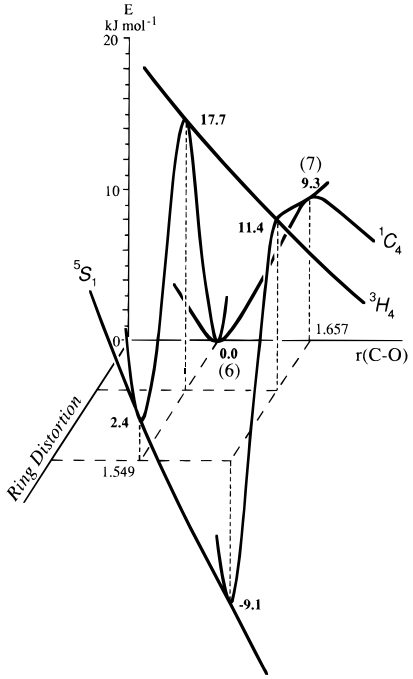


Figure 4. Schematic potential energy surface (MP2/6-31G(d) level, kJ mol^{-1}) for protonated equatorial 2-oxanol. Geometries are fully optimized at the MP2/6-31G(d) level with the glycosidic C−O length fixed at either 1.549 or 1.657 Å.

Calculation of these energies is, however, beyond the scope of the methods used here. An indication of the relative energies for the two paths can be obtained by constraining the glycosidic C−O length in the various conformations of the oxonium ion. In this way an estimate of the energy of the oxonium ion on the (fictitious) stepwise path of the distorted ring system can be obtained. The energy of the transition state for the concerted reaction must lie lower in energy than this constrained oxonium ion; otherwise, a stepwise path would be preferred.

Displayed in Figure 4 is a schematic potential energy surface obtained at the MP2/6-31G(d) level, illustrating the relationship between various ring conformations of the protonated ring system at fixed C−O lengths. The chair oxonium ion (**6**) has a C−O length of 1.549 Å. At this distance the only other conformational minimum found was the $^5S_1$ twist-boat, lying just 2.4 kJ mol^{-1} above **6**. The half-chair conformation connecting the $^5S_1$ conformation and **6** lies 17.7 kJ mol^{-1} above **6**. With the C−O length fixed at 1.657 Å, the distance found in the transition structure **7**, the $^5S_1$ conformation lies 9.1 kJ mol^{-1} lower than **6** and 18.4 kJ mol^{-1} lower than **7**. The half-chair lies just 2.1 kJ mol^{-1} higher than **7**. For both the twist-boat and half-chair conformations there exists no stable oxonium ion stationary point. Since the transition state for the concerted reaction must lie lower in energy than any likely (constrained) oxonium ion derived from protonation of a distorted ring system, it is clear it must also lie lower in energy than **7** and probably lower than **6**, and must therefore provide a lower energy pathway to dissociation than the stepwise path.

Axial 2-oxanol (**10**) lies 12.0 kJ mol^{-1} lower in energy than the equatorial anomer. The axial anomer of 2-methoxytetrahydropyran is also believed to be favored over the equatorial anomer.[35] The preference in aqueous solution for equatorial conformations of glycosides is due to solvation effects.[36] The C−O lengths of the hemiacetal are very similar to those in the equatorial twist-boat conformations, as might be expected from the orbital overlap arguments (all have the glycosidic bond antiperiplanar to the lone pair of the ring oxygen). Protonation of the axial chair produces the oxocarbonium ion−water complex **11** directly; no oxonium ion in the chair conformation could be located. Therefore, hydrolysis of the axial chair conformation is likely to proceed through a concerted path without the need for distortion of the ring. The ion−water complex (**11**) lies 1.2 kJ mol^{-1} higher than the equatorial conformer (**8**). The ring geometries of axial and equatorial oxocarbonium ion−water complexes are very similar; dissociation of both leads to the oxocarbenium ion **9**.

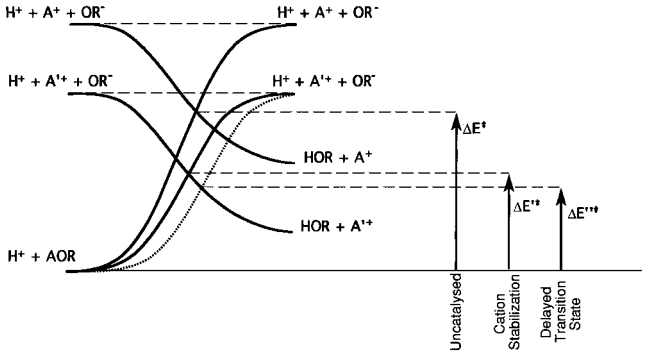


Figure 5. Bell−Evans−Polanyi diagram showing the effect of stabilization of the oxocarbenium cation and delay of the transition state on the reaction barrier.

2. Qualitative Glycosidase Activity. An enzyme which binds a substrate in a particular conformation (through specific interactions between the protein and substrate) may require a significant amount of energy to distort the substrate into a different conformation. The preference for any one conformation of the substrate upon binding the enzyme may control what conformations are accessible in the transition state. Indeed, preorganization of the reaction environment by an enzyme significantly enhances the rate of catalysis.[37] Protonation of equatorial glycosides bound in the chair conformation by an enzyme may result in an oxonium ion intermediate like **6**, unable to proceed directly to the oxocarbonium ion (despite the small energy difference between the oxonium ion and transition state, 3.4 kJ mol^{-1}). By binding the substrate in a ring conformation other than the chair, such high-energy intermediates may be avoided.

It is generally accepted that an enzyme achieves its catalytic rate enhancement by preferentially stabilizing the transition state, thereby reducing the free energy of reaction. Intermediate states, it is thought, must not be excessively stabilized. Consider the Bell−Evens−Polanyi diagram[38] in Figure 5 which characterizes the general-acid-catalyzed cleavage of the glycosidic bond (A−OR) and the effect of stabilization of the oxocarbenium ion intermediate. Provided the reaction is concerted, such diagrams enable a qualitative explanation for the role of the enzyme.

The energy curves for the glycosidic bond dissociation and the formation of a bond (H−OR) between a proton and the aglycon are superimposed. Dissociation of the glycosidic bond yields the oxocarbenium ion A^+ and aglycon RO^-. If the enzyme stabilizes the cation (to yield A'^+ and RO^-) proportionately to the amount of charge on the fragment, then the energy along the dissociation path in the presence of the enzyme will be lower than in the absence of the enzyme. We assume formation of the H−OR bond occurs independently of the enzyme; therefore, the paths are identical whether the cation is stabilized or not. It can be seen that the transition energies,

(35) Wiberg, K. B.; Murcko, M. A. *J. Am. Chem. Soc.* **1989**, *111*, 4821 and references therein.

(36) Schmidt, R. K.; Karplus, M.; Brady, J. W. *J. Am. Chem. Soc.* **1996**, *118*, 541 and references therein.

(37) Cannon, W. R.; Singleton, S. F.; Benkovic, S. J. *Nature Struct. Biol.* **1996**, *3*, 821.

(38) Pross, A. *Theoretical and Physical Principles of Organic Reactivity*; Wiley: New York, 1995.

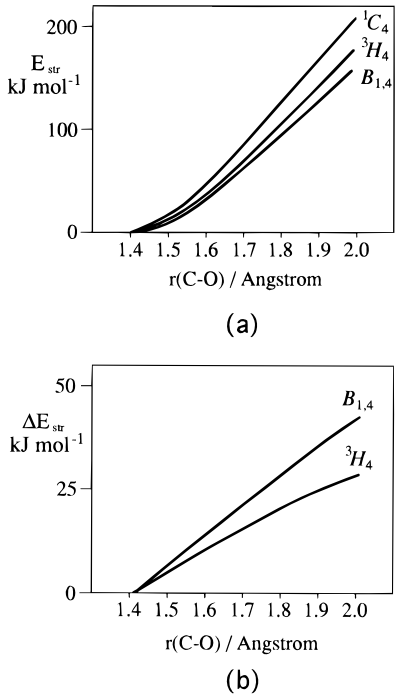


Figure 6. (a) Bond-stretch energy (E_{str}) profiles for the glycosidic bond of chair ($^1C_4$), half-chair ($^3H_4$), and boat ($B_{1,4}$) conformations of 2-oxanol. (b) Difference in bond-stretch energy (ΔE_{str}) between chair and half-chair ($^3H_4$) or boat ($B_{1,4}$) conformations.

reflected in the crossing of the two paths, are reduced by the stabilization of the cation; the reaction barrier of the uncatalyzed reaction $\Delta E^{\ddagger}$ is greater than the cation-stabilized reaction $\Delta E'^{\ddagger}$. This also leads to an *earlier* transition state and a higher reverse barrier. An increase in the reverse barrier will result in an increase in the lifetimes of intermediates which may reduce the efficiency of the enzyme.[39] The amount of energy by which the transition state is lowered is always less than the total stabilization energy of the cation.

Further reduction of the transition energy and of the reverse barrier can be achieved if the transition state is *delayed*. Delaying the transition state requires reduction of the A−OR bond stretch energy (dotted line in Figure 5); the reaction barriers of both the uncatalyzed ($\Delta E^{\ddagger}$) and cation-stabilized ($\Delta E'^{\ddagger}$) reactions are greater than for the reaction in which the transition state is delayed ($\Delta E''^{\ddagger}$). Note, this is not a requirement that the dissociation energy be decreased. Both forward and reverse barriers are reduced beyond that of the uncatalyzed reaction by delaying the transition state later than the uncatalyzed reaction. Notably, the scission of the glycosidic bond has been shown to be far advanced in the transition states for both lysozyme and β-glucosidase A.[40]

Displayed in Figure 6a is the energy profile of lengthening the glycosidic C−O bond for chair, half-chair, and boat conformations, calculated at the MP2/6-31G(d) level.[41] The glycosidic C−O bond-stretch energy (E_{str}) is significantly less for the half-chair and boat conformations than for the chair. In Figure 6b is shown the difference in the bond-stretch energy (ΔE_{str}) between the chair and half-chair or boat conformations. Stretching the C−O bond to 2.0 Å requires 31.6 kJ mol^{-1} less in the half-chair than in the chair conformation, and 46.3 kJ mol^{-1} less for the boat. Therefore, distortion of the ring will result in a delay of the transition state.

(39) Page, M. I., In *Enzyme Mechanisms*; Page, M. I., Williams, A., Eds.; Royal Society of Chemistry: London, 1987; p 1.
(40) Rosenberg, S.; Kirsch, J. F. *Biochemistry* **1981**, *20*, 3196.
(41) Geometries are fully optimized (the MP2/6-31G(d) level) at fixed glycosidic C−O bond lengths.

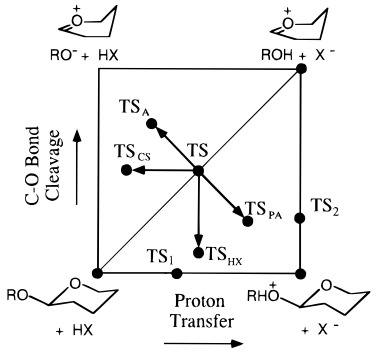


Figure 7. General PES diagram for acid-catalyzed glycosidic cleavage showing displacement of the concerted reaction transition state (TS) through stabilization of the oxocarbenium cation (TS_{CS}), the acidity of the aglycon ROH (TS_A) and HX (TS_{HX}), and the proton affinity of the glycosidic oxygen (TS_{PA}). The stepwise path proceeds along the axes through the transition states TS_1 and TS_2.

Insight into the role of the aglycon and acid on the different paths for glycosidic cleavage can be obtained by considering the potential energy surface (PES) diagram[38,42] in Figure 7. The vertical axis corresponds to changes in the glycosidic bond length, and the horizontal axis to changes in the H−X bond distance of the acid. Movement from left to right increases the X−H distance and decreases the H−O separation. Acid-catalyzed cleavage of the glycosidic linkage takes a path from the bottom left-hand corner to the top right-hand corner. The top left-hand corner corresponds to complete glycosidic bond dissociation without protonation, while the bottom right-hand corner represents the oxonium ion (**6**). The precise location of the pathway on the PES will depend on how glycosidic bond breaking and H−O bond formation are synchronized.

The stepwise pathway for cleavage of the chair conformation combines a horizontal movement to the right through the transition state species TS_1, followed by a vertical movement through TS_2. Distortion of the pyranose ring into conformations other than the chair conformation ensures a concerted reaction through a single transition state TS, represented ideally by the diagonal line in the figure. Any effect which lowers the relative energy of either the upper right-hand corner or lower left-hand corner moves the transition state away from that corner according to the Hammond postulate. Stabilization of either of the other corners moves the transition state toward that corner (anti-Hammond effect).[38]

The transition state is moved in the direction of the lower right-hand corner by an R substituent which increases the proton affinity of the glycosidic oxygen (TS_{PA}), but toward the upper left-hand corner by a stronger acid ROH (TS_A). Stabilization of the oxocarbenium cation lowers the energy of both upper left and right corners, and moves the transition state horizontally toward the left (TS_{CS}). Notably, an earlier transition state is predicted with no change in the C−O length, in contrast to what is predicted from the Bell−Evans−Polyani diagram (Figure 5). Reducing the bond-stretch energy, however, is predicted to reduce the energy of the transition state, moving it toward the upper left corner. An increase in the acidity of HX stabilizes the upper and lower right-hand corners and moves the transition state vertically downward (TS_{HX}). Therefore, the strength of the acid HX is not predicted to change significantly the degree of proton transfer in the transition state. However, the glycosidic C−O separation should be longer the weaker the acid HX.

The calculations here indicate that distortion of equatorial 2-oxanol has a dramatic effect upon the acid-catalyzed cleavage of the glycosidic bond. The high-energy oxonium ion intermediate is avoided through a concerted protonation and cleavage

(42) Jencks, W. P. *Chem. Rev.* **1972**, *72*, 705.

reaction. The glycosidic bond-stretch energy is significantly reduced which is likely to manifest itself in a delayed transition state with a reduced transition state energy. Cleavage of the glycosidic bond in the axial anomer proceeds through a concerted reaction without distortion. These properties of this, the simplest of pyranose ring systems, should be general for all such systems, and enzymes which catalyze these reactions may be able to take advantage of these properties.

The number of detailed structures of enzyme−substrate complexes for glycosidases is few. Many structures with substrate mimics exist; however, since they do not undergo cleavage, information on conformational requirements for cleavage may be concealed. We examine here three glycosidases, to determine the possible role of these properties in their mechanism.

3. Lysozyme. The detailed mechanism of lysozyme, despite being the mechanistically best understood of all enzymes, is not yet totally resolved. The Phillips mechanism[3] suggests that the pyranose ring of the substrate is distorted from the chair into the half-chair conformation to relieve bad contacts between the substrate and enzyme. This distortion in turn reduces the energy difference between the substrate and transition state, facilitating cleavage of the glycosidic linkage. While there is both experimental[7] and computational[8,43] evidence that distortion is unlikely to play an important role in catalysis, recent X-ray crystallographic studies[44] of substrate bound in the active site (D subsite) of lysozyme indicate that the substrate adopts a sofa-like conformation [$Q = 0.440$ Å, $\theta = 32.6°$, and $\phi = 27.0°$],[45] intermediate in its structure to the transition state **7** and oxocarbenium ion **9** structures of 2-oxanol.

The general properties of pyranose rings shown here now place the role of ring distortion in lysozyme in clear context. The important catalytic functions of the enzyme would appear to be (1) geometrical distortion, (2) protonation of the glycosidic bond, and (3) stabilization of the oxocarbenium ion through electrostatic interactions.

Lysozyme also hydrolyzes chitin (poly-NAG), but with much less efficiency. The recent structural determination of goose egg-white lysozyme in complex with chitin fragments shows all pyranose rings in the chair conformation.[12] This supports those calculations[8] which have focused on the geometry of the pyranose ring in the active site of lysozyme and have indicated a chair conformation on the basis of calculations involving poly-NAG fragments. It may well be that the slow rate of hydrolysis of chitin, however, is a direct result of this substrate binding without distortion (into a conformation other than the chair), unable to avoid the high-energy oxonium intermediate.

Recently, the structure of a mutant of T4 lysozyme has been solved in which the substrate forms a covalent bond with the nucleophile.[46] The substrate adopts a half-chair conformation and may further suggest a role for ring distortion.

4. Neuraminidase. Neuraminidases are a class of enzymes whose function is to cleave the glycosidic bond between the penultimate sugar and terminal sialic acid of oligosaccharides. Neuraminidase from the influenza virus is one of two glycoproteins found on the surface of this virus. Its role is to catalyze the cleavage of terminal sialic acid (*N*-acetylneuraminic acid) from cell surface glycoconjugates, an event which appears to relate to the release of progeny virions from infected cells.[47]

The acid hydrolysis of α-glucosides of *N*-acetylneuraminic acid occurs through a "flattened chair" transition state in which the angle between the glycosidic bond and an electron deficient p-orbital on the carbon atom adjacent to the glycosidic carbon is 30°.[48] This angle is close to the dihedral angle found in the transition state **7** of 2-oxanol between the glycosidic bond and the *pro-R* hydrogen at C_2 calculated at the MP2/6-31G(d) level of 37.4°.

While there is clear evidence for the participation of an oxocarbenium (sialosyl) ion intermediate in the neuraminidase reaction,[49] details of the pathway are unclear. One proposal[50] suggests that binding of the substrate first induces formation of the *strained* oxocarbonium ion followed by cleavage of the glycosidic bond, simply by enforcing conformational changes. The leaving group is subsequently protonated by the solvent, and by maintaining planarity at the glycosidic carbon through multiple interactions with functional groups in the active site, the cation is stabilized. If strain energy is to be responsible for bond cleavage, then this energy must be realized in the binding energy of the substrate to the enzyme. Binding energies of substrates to their receptors are, however, unlikely to be as large as the energies required to cleave covalent bonds. Stabilization of an intermediate requires that its energy be reduced below that found in an enzyme-free environment. The oxocarbonium ion is planar at the glycosidic carbon in its minimum energy conformation, and therefore a displacement away from planarity would be destabilizing; however, no stabilization of the oxocarbonium ion can be achieved by maintaining planarity.

An alternative mechanism whose general features have been proposed by several researchers involves binding of the substrate in the boat conformation.[49,51,52] This is followed by protonation of the glycosidic oxygen, yielding the oxocarbonium ion intermediate, similar to the Phillips mechanism for lysozyme. In contrast to lysozyme, however, there is no likely source of a proton in the active site of neuraminidase. An aspartic acid group conserved in all neuraminidases[53] (Asp151 in the N2 strain of viral influenza) which is observed to lie within hydrogen bonding distance of the glycosidic oxygen[4,5] is not necessarily protonated. A nonspecific acid, perhaps a water molecule, an activated hydroxonium ion (H_3O^+), and the aspartic acid at low pH have all been forwarded as possible sources of a proton in these mechanisms.

In neuraminidase from influenza virus the substrate, sialic acid, binds in a conformation which lies between the boat and twist-boat[4] [$Q = 0.680$ Å, $\theta = 85.6°$, and $\phi = 46.5°$] (PDB[54] entry 2BAT). Binding in this conformation ensures a concerted reaction, and delays the transition state. In 2-oxanol, reduction of the bond-stretch energy is roughly 45% greater in the boat conformation than the half-chair (Figure 6b), and therefore the amount of energy by which the transition barrier is reduced through delaying the transition state is also greater in the boat than the half-chair conformation. Stabilization of the oxocarbenium ion is likely to be achieved through interaction with several anionic species in the active site (Asp151, Glu277) and the carboxylate of the substrate.

There exist several possibilities for the mechanism of hydrolysis in neuraminidase. On the basis of the predictions from the PES diagram (Figure 7), if Asp151 is protonated, we

(43) Pincus, M. R.; Scheraga, H. A. *Biochemistry* **1981**, *20*, 3960.
(44) Strynadka, N. C. J.; James, M. N. G. *J. Mol. Biol.* **1991**, *220*, 401.
(45) Strynadka, N. C. J. Personal communication.
(46) Kuroki, R.; Weaver, L. H.; Matthews, B. W. *Science* **1993**, *262*, 2030.
(47) Palese, P.; Jobita, K.; Ueda, M.; Compans, R. W. *Virology* **1974**, *61*, 397.
(48) Ashwell, M.; Guo, X.; Sinnot, M. L. *J. Am. Chem. Soc.* **1992**, *114*, 10158.
(49) Chong, A. K. J.; Pegg, M. S.; Taylor, N. R.; von Itzstein, M. *Eur. J. Biochem.* **1992**, *207*, 335.
(50) Janakiraman, M. N.; White, C. L.; Laver, W. G.; Air, G. M.; Luo, M. *Biochemistry* **1994**, *33*, 8172.
(51) Taylor, N. R.; von Itzstein, M. *J. Med. Chem.* **1994**, *37*, 616.
(52) Burmeister, W. P.; Henrissat, B.; Bosso, C.; Cusack, S.; Ruigrok, R. W. H. *Structure* **1993**, *1*, 19.
(53) Varghese, J. N.; Colman, P. M. *J. Mol. Biol.* **1991**, *221*, 473.
(54) Bernstein, F. C.; Koetzle, T. F.; Williams, G. J. B.; Meyer, E. F.; Brice, M. D.; Rodgers, J. R.; Kennard, O.; Shimanouchi, T.; Tasumi, M. *J. Mol. Biol.* **1977**, *112*, 535.

would expect the strong acid to produce a short glycosidic C−O length in the transition state. Addition of a molecule of water to the oxocarbenium ion would regenerate the acid and the acetal. Similarly, a hydroxonium ion could provide the proton. Alternatively, for a weak acid such as water, we expect a long glycosidic C−O length in the transition state. Transfer of a proton to the aglycon oxygen would yield a hydroxide ion ($X^- = OH^-$, Figure 7). This hydroxide ion could then bind to the anomeric carbon to complete the reaction. Alternatively, addition of a molecule of water to the oxocarbenium ion may be followed by abstraction of a proton by the hydroxide ion. The aspartic acid (Asp151) could also act as a base in abstracting the proton, subsequently losing the proton to the hydroxide ion. The glutamic acid Glu172 in *Bacillus circulans xylanase* has been shown recently[55] to act with the duel role of both general acid and general base in the hydrolysis of xylan.

There are two major features that distinguish between these possibilities; the length of the glycosidic C−O bond in the transition state and whether there are some special requirements for proton abstraction. In addition to hydrolyzing glycoconjugates of sialic acid, neuraminidase from influenza also produces the 2-deoxy-3-dehydro derivative of sialic acid (DANA).[52] Formation of DANA can be achieved through deprotonation of the oxocarbenium (sialosyl) ion, or alternatively, it can be formed through direct elimination of a water molecule from sialic acid. Elimination of a water molecule from 2-oxanol (**1**) through the transition state **12** produces 3,4-dihydropyran (**13**) in a half-chair conformation $^5H_4$ [$Q = 0.498$ Å, $\theta = 52.9°$, and $\phi = 88.4°$], lying 12.1 kJ mol^{-1} lower than the boat conformation but with a barrier of 247.0 kJ mol^{-1}. The structure of the transition state for elimination bears a striking resemblance to the oxonium ion; dihedral angles differ by no more than a few degrees. The excessively large barrier in 2-oxanol would suggest, however, that direct elimination from sialic acid is an unlikely pathway for the formation of DANA in this enzyme. By inference then, proton abstraction, although by what means is unclear, is the most likely mechanism for formation of DANA. This process of proton abstraction, rather than being totally unrelated to the hydrolysis, is then the most likely mechanism by which hydrolysis of the oxocarbenium ion proceeds.

In a recent combined QM/MM study[56] of neuraminidases from influenza B and *Salmonella typhimurium*, transition state structures were determined in which the glycosidic bond is very long; however, no indication of the role or extent of proton transfer was provided.

Kinetic isotope effect experiments show for influenza virus neuraminidase that the reactive substrate conformation is the boat.[57] The transition state in leech sialidase L also appears to adopt a boat or twist-boat conformation,[58] whereas in *Vibrio cholerae*[59] the transition state adopts a chair conformation, although the measured effects are consistent with distortion toward the half-chair. In contrast, *S. typhimurium*[57] appears to adopt the chair conformation in the transition state, although the possibility of a slightly flattened boat cannot be excluded. There is some evidence, then, in each of these enzymes of distortion of the substrate which may be a common feature in the mechanism of them all.

5. β-Amylase. β-Amylase, an anomeric inverting enzyme, cleaves successive maltose units from the nonreducing end of α(1→4)glucans. The glycosidic linkage of α(1→4)glucans has the aglycon in the axial position. Maltose binds in tandem to four of the glucose subsites; condensation of these maltose units produces maltotetraose. All four D-glucopyranosyl units of either maltose or maltotetraose appear[60] to be in the chair conformation.[6]

It is expected that axial glycosides will undergo acid-catalyzed cleavage of the glycosidic bond without the requirement for distortion of the ring. A significant part of the role of the enzyme must be to stabilize the oxocarbonium ion intermediate. Without delaying the transition state, however, this leads to an increase in the reverse barrier. In anomeric retaining glycosidases this effectively amounts to increasing the barrier to hydrolysis of the intermediate. In general, though, the reverse barrier will only exceed the forward barrier when the intermediate has its energy reduced below that of the initial enzyme−substrate complex. Thus, while the reverse barrier may be increased by stabilization of the oxocarbonium ion intermediate, the rate at which substrates can be processed by the enzyme is unlikely to be significantly affected unless the energy of the oxocarbonium ion is reduced below that of the product.

Concluding Remarks

Ring distortion of equatorial glycosides has a dramatic effect upon the mechanism of their hydrolysis. Protonation of the $^1C_4$ chair conformation of 2-oxanol yields a stable oxonium ion. All other conformations studied here collapse to the oxocarbonium ion−water complex upon protonation. These properties of the simplest of pyranose systems should be general for all such systems. Thus, enzymes which distort the pyranose ring of glycoside substrates may avoid the formation of the high-energy oxonium ion. Ring distortion also reduces the glycosidic bond-stretch energy which reduces the transition state energy for bond cleavage. Protonation of the chair conformer of the axial anomer collapses to the oxocarbonium ion−water complex. Enzymes which bind axial glycosides do not require distortion of the ring to avoid the oxonium ion intermediate.

Enzymes which hydrolyze equatorial glycosides with retention of configuration about the anomeric center can avoid formation of the high-energy oxonium ion intermediate through ring distortion of both the substrate and the product. With enzymes that bind equatorial glycosides and invert anomerization or form a covalent bond with the nucleophile, an axial intermediate is formed which does not form the oxonium ion. Enzymes which hydrolyze axial glycosides with retention of configuration about the anomeric center also avoid the oxonium ion, although if a covalent link with the nucleophile is formed, the equatorial-linked glycoside would require distortion of the ring to avoid formation of an oxonium ion. Similarly, enzymes which invert the anomerization of axial glycosides would be required to distort the equatorial glycoside formed in the reaction.

Supporting Information Available: Table giving calculated energies, ZPVE, and temperature and higher level corrections for **1**−**13** (1 page). See any current masthead page for ordering and Internet access instructions.

JA9623020

(55) McIntosh, L. P.; Hand, G.; Johnson, P. E.; Joshi, M. D.; Körner, M.; Plesniak, L. A.; Ziser, L.; Warkarchuk, W. W.; Withers, S. G. *Biochemistry* **1996**, *35*, 9958.
(56) Barnes, J. A.; Williams, I. H. *Biochem. Soc. Trans.* **1996**, *24*, 263.
(57) Guo, X.; Laver, W. G.; Vimr, E.; Sinnott, M. L *J. Am. Chem. Soc.* **1994**, *116*, 5572.
(58) Sinnott, M. L.; Guo, X.; Li, S-C.; Li, Y-T. *J. Am. Chem. Soc.* **1993**, *115*, 3334.
(59) Guo, X.; Sinnott, M. L. *Biochem. J.* **1993**, *294*, 653.

(60) While the ligands are modeled in the chair conformation, subtle distortions away from the classic chair could easily be accommodated.