Leech Sialidase L Cleaves the Glycon–Aglycon Bond with the Substrate in a Normally Disfavored Conformation

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Received December 9, 1992

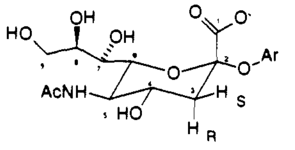
Reaction of enzyme substrates through normally disfavored conformations is a commonly suggested but infrequently demonstrated phenomenon in enzymology.[1] One type of glycoside-cleaving enzyme must, however, invert the pyranose ring conformation of its substrates at some stage, since the products are internal glycosides based on the dioxabicyclo[3.2.1]octane skeleton, rather than reducing sugars. An α-glucosyl transferase from *Aspergillus niger* is reported to yield 2% of 1,6-anhydroglucose,[2] but the best characterized of these enzymes are phage λ lysozyme[3] and the recently discovered leech (*Macrobdella decora*) sialidase II,[4] which converts glycosides of *N*-acetylneuraminic acid (I) to 2,7-anhydro-*N*-acetylneuraminic acid (II) as the only detectable sialic acid product. We now present evidence, based upon the relative sizes of the secondary β-deuterium kinetic isotope effects of the two diastereotopic hydrogens at position 3, that this last enzyme cleaves the glycon–aglycon bond with the pyranose ring in a normally disfavored conformation. β-Deuterium kinetic isotope effects are geometry-dependent, their main, hyperconjugative component varying as the square of the cosine between the C–H or C–D bond and the electron-deficient p orbital on the adjacent carbon.[5] Therefore, if glycon–aglycon fission took place from the $^5C_2$ conformation of the substrate (i.e., from structure III), the effect for the *pro-S* deuterium would be bigger than that for the *pro-R* deuterium, whereas reaction from the normally favored $^2C_5$ conformation (I), if undistorted, would give effects of the same size; any distortion to the $^4H_5$ conformation of the oxocarbonium ion would result in the *pro-R* effect being bigger than the *pro-S*, as is observed in nonenzymatic hydrolysis.[6]

Michaelis–Menten parameters for hydrolysis of seven aryl glycosides[6] are given in Table I. Both first- and second-order rate constants correlate well with aglycon pK_a [$\beta_{lg}(V) = -0.50$, $r = -0.98$; $\beta_{lg}(V/K) = -0.60$, $r = 0.99$], indicating that cleavage of the glycon–aglycon bond is both the first irreversible and the rate-determining step.[7] High K_m values precluded determination of isotope effects on V_{max}, so effects of V_{max}/K_m were measured by direct comparison of first-order rate constants[8] for hydrolysis of labeled and unlabeled *p*-nitrophenyl glycoside, at a concentration of $K_m/25$. Data were taken pairwise in the order H/D, D/H, H/D, DH, and so on to minimize systematic error caused by enzyme denaturation. Effects of $^{D_S}(V/K) = 1.054 \pm 0.017$ ($n = 12$) and $^{D_R}(V/K) = 1.020 \pm 0.012$ ($n = 12$) were obtained. Selective contamination of the deuterio compound by inhibitory impurities, the other possible source of systematic error, is unlikely because the effect for the dideuterio compound [$^{D_2}(V/K) = 1.075 \pm 0.018$ ($n = 8$)] is the product of the effects for the individual monodeuterated compounds (1.075).

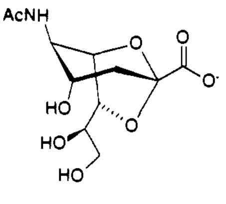
Table I. Michaelis–Menten Parameters for Hydrolysis of Aryl *N*-Acetyl-α-neuraminides by Leech Sialidase L[a]

aglycon	k_{cat} (s^{-1})	K_m (mM)
4-nitrophenol	217	5.7
4-cyanophenol	100	12.5
3-nitrophenol	87	15.8
3,4-dichlorophenol	44	13.3
3-chlorophenol	27.5	13.9
4-chlorophenol	12.9	14.7
phenol	11.8	15.2

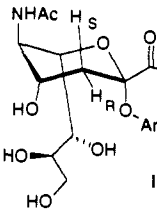
[a] Data refer to 50 mM sodium acetate buffer, pH 5.5, at 37.0 °C. Absolute k_{cat} values were calculated on the assumption that enzyme of specific activity 210 units/mg with the 4-methylumbelliferyl glycoside as substrate was pure, 100% active protein of molecular weight per active site 84 kDa. K_m and relative k_{cat} values are good to ±10%.



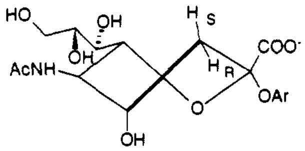
I



II



III



IV

The effects preclude enzyme glycon–aglycon fission taking place from conformations derived from the ground-state $^2C_5$ conformation of the substrate and require it to occur from the $^5C_2$ conformation of the product, or the $^{3,6}B \leftrightarrow {}^6S_2$ (IV) $\leftrightarrow B_{2,5}$ portion of the skew-boat itinerary. The question then arises as to whether the enzyme binds the small proportion of the free substrate populating the disfavored conformation directly, or first binds the substrate in its preferred conformation, which is then changed in a subsequent step.

The similar, steep dependence of both k_{cat} and k_{cat}/K_m on leaving group acidity requires any initial change in the conformation of the ES complex to be rapid and reversible. On the other hand, for the enzyme to pick out that fraction of the substrate already in a disfavored conformation, that conformation must be suffi-

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(5) Melander, L.; Saunders, W. H. *Reaction Rates of Isotopic Molecules*; John Wiley: New York, 1980; p 174.

(6) Ashwell, M; Guo, X.; Sinnott, M. L. *J. Am. Chem. Soc.* **1992**, *114*, 10158. Substrate characterization data are given in this paper.

(7) The negative β_{lg} values preclude any ring-opening mechanism, for which chemical realism and the experimental direct β-deuterium kinetic isotope effects would require accumulation of charge on the aglycon and hence positive β_{lg} values.

(8) Calculated by least-squares fitting experimental time courses of absorbance to $A = A_0 + B(1 - e^{-kt})$ using *Kaleidagraph* (Synergy Software, Reading, PA).

0002-7863/93/1515-3334$04.00/0

ciently abundant that absolute values of k_{cat}/K_m (e.g., 4×10^4 M^{-1} s^{-1} for the *p*-nitrophenyl compound) are compatible with known rates of glycosidase–glycoside diffusional combination (e.g., $1-2 \times 10^7$ M^{-1} s^{-1} for *Agrobacterium* β-glucosidase[9]). While nonchair conformers are not detectable by conventional ^1H NMR ($^3J_{proRH3-H4} = 12$ Hz)[6], an empirical estimate of the conformational energy difference between I and IV (3 kcal mol^{-1})[10] is bracketed by two theoretical estimates. Molecular mechanics (MM2) indicates that IV is 6.1 kcal mol^{-1} higher in energy than I, whereas MOPAC/AM1 suggests that IV is slightly more stable than I (by 0.6 kcal mol^{-1}). Therefore, the balance of evidence suggests that the enzyme binds that proportion of the substrate already in the disfavored conformation directly, even though it is not possible to rule out completely a "kinetically silent" conformational change.

Acknowledgment. Financial support from NIH Grants GM 42469 (to M.L.S.) and NS 09626 (to Y.-T.L) and NSF Grant MCB 9213894 (to Y.-T.L) is gratefully acknowledged. We also thank very particularly Dr. George L. Gould, UIC, for MOPAC and MM2 calculations.

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(9) Kempton, J. B.; Withers, S. G. *Biochemistry* **1992**, *31*, 9961.

(10) The $^{2}C_{5} \leftrightarrow {}^{6}S_{2}$ energy difference is estimated as 3 kcal mol^{-1} from the chair–skew difference in cyclohexane (5.5 kcal mol^{-1}), reduced by the favorable axial/equatorial interchange at C2: the anomeric equilibrium of *N*-acetylneuraminic acid (Friebolin, H.; Supp, M.; Brossmer, R.; Keilich, G.; Ziegler, D. *Angew. Chem., Int. Ed. Engl.* **1980**, *19*, 208) was adjusted for the hydroxy–*p*-nitrophenoxy change by the difference between the anomeric equilibrium of glucose and the conformational equilibrium of 2-(*p*-nitrophenoxy)tetrahydropyran (Ouedraogo, A.; Lessard, J. *Can. J. Chem.* **1991**, *69*, 474).