Unambiguous Determination of the Ionization State of a Glycoside Hydrolase Active Site Lysine by ^{1}H-^{15}N Heteronuclear Correlation Spectroscopy

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Supporting Information

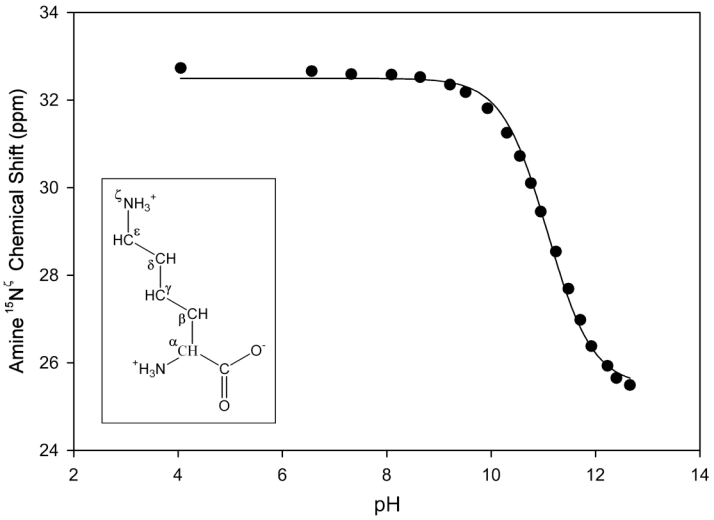

Figure S1: Measurement of the pH-dependence of the $^{15}N^{\zeta}$ chemical shift of ~10 mM DL-[$^{15}N^{\zeta}$, $^{13}C^{\varepsilon}$]-Lysine (Isotec #489042) in 10% D_2O / 90% H_2O by ^{15}N-NMR. Spectra were recorded at 25 ^{o}C with a 10 mm broadband probe in a Varian Unity 500 MHz NMR spectrometer. After a 3 s recycle delay, 1.5 s of ^{1}H waltz-16 decoupling (0.84 kHz) was applied at 6.5 ppm for a NOE signal enhancement, followed by 0.256 s of ^{15}N acquisition, also with ^{1}H decoupling. The ^{15}N transmitter frequency was set at 19.2 ppm and the spectral width was 4000 Hz. The sample pH, measured at room temperature (~21 ^{o}C), was changed from 4.0 to 12.7 by addition of 0.5 M or 1.0 M NaOH. The ^{15}N chemical shifts were referenced indirectly to liquid $^{15}NH_3$ at 0.0 ppm using a γ_{15N}/γ_{1H} ratio of 0.101329118 and an external ^{1}H reference sample of DSS (2,3-dimethyl-2-silapentane-5-sulfonate).[1] Fitting of the data to a single titration yielded a pKa of 11.1 and limiting chemical shifts of 32.5 ppm ($-^{15}NH_3^{+}$) and 25.5 ppm ($-^{15}NH_2$) for the sidechain amine of lysine.

(1) Wishart, D. S.; Bigam, C. G.; Yao, J.; Abildgaard, F.; Dyson, H. J.; Oldfield, E.; Markley, J. L.; and Sykes, B. D. *J. Biomol. NMR* **1995**, *6*, 135-140

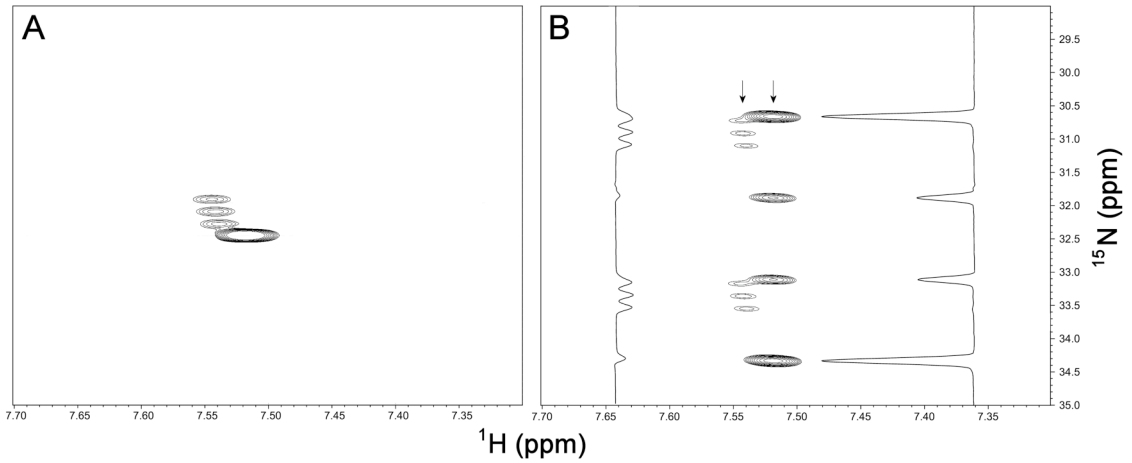

Figure S2: The high-resolution ^{1}H-^{15}N HSQC spectra of ~10 mM DL-[$^{15}N^{\zeta}$, $^{13}C^{\varepsilon}$]-Lysine in 10% D_2O / 90% H_2O with (A) and without (B) ^{1}H decoupling during t_1, recorded at 30 ^{o}C and pH 1.8 using a Varian Inova 600 MHz NMR spectrometer. ^{13}C decoupling was applied in both cases. In (A), the -$^{15}NH_3^+$ yields the strong singlet, whereas the small triplet arises from -$^{15}NH_2D^+$, offset by deuterium isotope shifts of $^{1}\Delta$ = -0.36 ppm for the $^{15}N^{\zeta}$ and $^{2}\Delta \sim +0.025$ ppm for the $^{1}H^{\zeta}$ per deuteron added, and split by $|^{1}J_{ND}|$ = 11.5 Hz and $|^{2}J_{HD}|$ = 1.5 Hz, with the J values having the opposite signs. In (B), the ^{15}N signal from the -$^{15}NH_3^+$ moiety is split by $|^{1}J_{NH}|$ = 74 Hz into a quartet of relative peak intensities ~2.7:1:1:2.7. This value for the highly mobile amine is close to that of 3:1:1:3 expected for four lines of a quartet in the absence of differential relaxation.[2] The ^{15}N signal from the -$^{15}NH_2D^+$ appears as a "triplet" of intensity ratios 1:0:1, with further deuterium couplings and isotope shifts. The arrows indicate the ^{1}H shifts at which the ^{15}N traces were extracted.

(2) Tugarinov, V.; Hwang, P. M.; Ollerenshaw, J. E., Kay, L. E. *J. Am. Chem. Soc*. **2003**, *125*, *10420-10428.*

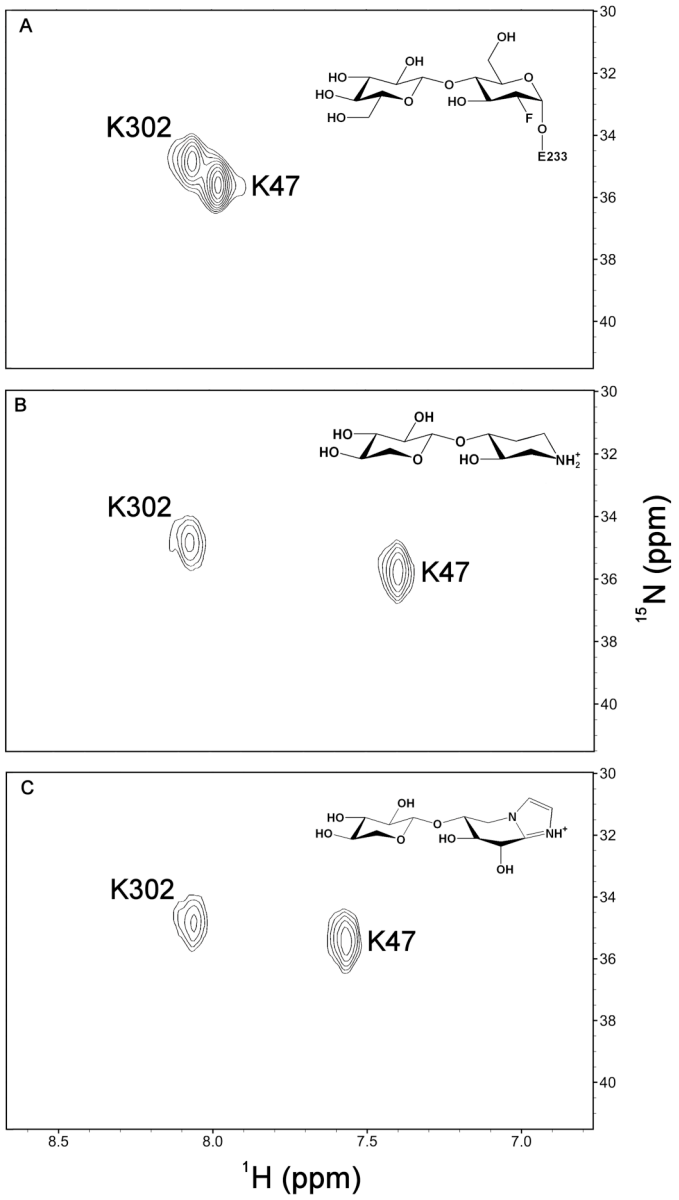

***Figure S3*:** Selected regions of the ^{1}H-^{15}N HMQC spectra of CexCD in its (A) trapped glycosyl-enzyme intermediate form (2FCb-CexCD), and in its non-covalent complexes with the high-affinity competitive inhibitors (B) xylobiose-derived isofagomine and (C) xylobiose-derived imidazole.[3,4] In all three complexes, the amines of K47 and K302 are positively-charged (-NH_3^+) as evident by their $^{15}N^{\zeta}$ chemical shifts (Figure S1), and by the appearance of their $^{15}N^{\zeta}$ signals as quartets in ^{1}H-coupled ^{1}H-^{15}N HSQC spectra (not shown). X-ray crystallographic studies[4] revealed very similar hydrogen bonding interactions between the sidechain amine of K47 and distal sugar and proximal aza-sugar moieties of the xylobiose-derived inhibitors as those seen with the covalently-bonded 2-deoxy-2-fluoro-cellobioside (Figure 2). Therefore, the variation in the $^{1}H^{\zeta}$ shifts of K47

must reflect subtle structural or electrostatic differences between the three complexes. In contrast, the $^{15}N^{\zeta}$–$^{1}H^{\zeta}$ signal from K302 is invariant in the spectra of apo-CexCD, 2FCb-CexCD, and CexCD with the two non-covalent inhibitors. Thus this lysine, which is distant from the active site of the enzyme, is not perturbed upon complex formation. The spectra were recorded at 30 ^{o}C using a Varian Inova 600 MHz spectrometer, and the ^{15}N-labeled proteins were in 20 mM potassium phosphate, pH 6.5, and 0.02% NaN_3.

(3) Williams, S. J.; Hoos, R.; Withers, S. G. *J. Am. Chem. Soc.* **2000**, *122*, *2223-2235*.
(4) Notenboom, V.; Williams, S. J.; Hoos, R.; Withers, S. G.; Rose, D. R.; *Biochemistry* **2000**, *39*, *11553-11563*.