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Evidence for a Strong Hydrogen Bond in the Catalytic Dyad of Transition-State Analogue Inhibitor Complexes of Chymotrypsin from Proton−Triton NMR Isotope Shifts

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Investigations of small molecules have shown that NMR chemical shift differences (isotope shifts) between heavy and light isotopes of hydrogen (deuteron vs proton or triton vs proton) are indicative of the strengths of hydrogen bonds. Hibbert and Emsley have reported that the isotope shift is negligibly small for a weak hydrogen bond and becomes positive for a strong hydrogen bond. As the bond becomes still stronger and approaches a single-well potential, the isotope shift returns to zero and then becomes negative.[1] We present here the first accurate measurements of ^{1}H (H) versus ^{3}H (T) isotope shifts ($\Delta\delta_{T-H} = \delta_T - \delta_H$) for resonances from a protein. This approach was used to investigate the strength of the hydrogen bond between His^{57} and Asp^{102} in the catalytic dyad of chymotrypsin in three transition state analogue inhibited complexes: *N*-acetyl-L-phenylalanyl trifluoromethyl ketone (N-AcF-CF$_3$), *N*-acetyl-L-valyl-L-phenylalanyl trifluoromethyl ketone (N-AcVF-CF$_3$), and *N*-acetyl-L-leucyl-L-phenylalanyl trifluoromethyl-ketone (N-AcLF-CF$_3$). The measured $\Delta\delta_{T-H}$ values for His^{57} H$^{\delta 1}$ in these complexes were between −0.63 and −0.68 ppm. These values are consistent with a “strong” hydrogen bond in each of these complexes, but not with a “very strong” hydrogen bond, which is expected to have a $\Delta\delta_{T-H}$ value near or greater than zero.[1]

Serine proteinases are one of the most studied families of enzymes. While the general mechanism of peptide hydrolysis has been understood for decades, the details of the chemistry that takes place during catalysis are still a subject of controversy and continued investigation.[2−8] Although evidence indicates that the transition state for peptide bond hydrolysis resembles a tetrahedral intermediate,[9] the strength of the hydrogen bond between the N$^{\delta 1}$ of His^{57} and the O$^{\delta 1}$ of Asp^{102} in this state remains a key issue. It has been suggested that the increase in the strength of this hydrogen bond, from the resting state of the enzyme (or Michaelis complex with substrate) to the tetrahedral complex formed between the active site Ser^{195} O$^{\gamma}$ and the carbonyl of the scissile peptide bond of the substrate, is a key mechanism for lowering the energy of the transition state for peptide hydrolysis.[3,10−12] Robillard and Shulman[13,14] observed a proton NMR signal in the 15−18 ppm chemical shift range and assigned it to H$^{\delta 1}$ of the His^{57} imidazole ring. The chemical shift of this resonance, which is unusually far downfield when the imidazole is protonated, has been interpreted as indicating that a low-barrier hydrogen bond (LBHB) is formed between His^{57} N$^{\delta 1}$ and Asp^{102} O$^{\delta 1}$.[10,15] In the nomenclature of Hibbert and Emsley,[1] an LBHB is a strong but not a very strong hydrogen bond. Others have presented arguments, however, that the catalytic apparatus of serine proteinases does not support a LBHB during catalysis and that the chemical shift of the histidyl proton is merely the result of a normal, charged hydrogen bond.[6,8] If the His-Asp dyad at the active site of the serine proteinases forms a strong hydrogen bond in the transition state, the His^{57} H$^{\delta 1}$ should exhibit a large, negative $\Delta\delta_{T-H}$ as a consequence of the anharmonicity of the strong hydrogen-bond potential.

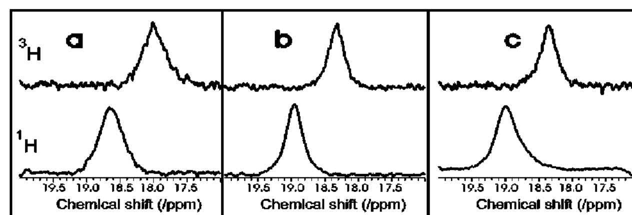

Figure 1. ^{1}H (bottom) and ^{3}H (top) NMR spectra of the high-frequency resonance assigned to His^{57} H$^{\delta 1}$ of bovine chymotrypsin in samples inhibited by three different trifluoromethyl ketone inhibitors:[3] (a) N-AcF-CF$_3$, (b) N-AcVF-CF$_3$, and (c) N-AcLF-CF$_3$. The spectrometer frequencies were 500 MHz for ^{1}H and 533 MHz for ^{3}H. The inhibited chymotrypsin complexes were prepared as described previously.[3] The samples were lyophilized and dissolved in water containing 1.6% ^{3}H at the National Tritium Facility. One-dimensional ^{1}H and ^{3}H NMR spectra were accumulated from the same sample with a probe double-tuned to the two frequencies (500 and 533 MHz for ^{1}H and ^{3}H, respectively). The solvent resonance was suppressed by only exciting a narrow region around 18 ppm with a selective Gaussian-shaped pulse, which was generated with the spectrometer software. ^{1}H NMR peaks were referenced to the ^1H_2O signal, and ^{3}H NMR peaks were referenced to the ^{3}HO^{1}H signal.

Figure 1 shows ^{1}H and ^{3}H NMR spectra of complexes between chymotrypsin and each of the three trifluoromethyl ketone inhibitors that serve as models for the transition state for catalysis.[16] The inhibition constants for the three inhibitors differ by about 1 order of magnitude[17,18] (Table 1); the spectrum of the weakest complex (N-AcF-CF$_3$) is at the left and the strongest (N-AcLF-CF$_3$) at the right in Figure 1. The spectral region shown is the extreme high frequency (low field) region of the ^{1}H and ^{3}H NMR spectra. The single peak in this region has been assigned to the H$^{\delta 1}$ of His^{57} of chymotrypsin.[13,14,19,20]

The ^{1}H and ^{3}H chemical shifts and the ^{3}H−^{1}H isotope shifts for the three complexes are presented in Table 1. Tritium was used in this experiment, because the line width of the spin one-half ^{3}H nucleus is comparable to that of ^{1}H. By contrast, quadrupolar line-broadening of deuterium (spin 1) limits the accuracy of $\Delta\delta_{D-H}$ measurements in this system.[22]

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Table 1. Experimental Results for the Three Transition-State Analogue Inhibitor Complexes of Chymotrypsin Studied Here

inhibitor[a]	K_i (/μM)[b]	His^{57} ^{1}H$^{\delta1}$ (/ppm)[c]	His^{57} H$^{\delta1}$$\Delta\delta_{T-H}$ (/ppm)[d]
N-AcF-CF_3	20	18.61	−0.63
N-AcVF-CF_3	2.8	18.91	−0.65
N-AcLF-CF_3	1.2	18.95	−0.68

[a] Abbreviations: N-AcF-CF_3, *N*-acetyl-L-phenylalanyltrifluoromethylketone; N-AcVF-CF_3, *N*-acetyl-L-valyl-L-phenylalanyltrifluoromethylketone; N-AcLF-CF_3, *N*-acetyl-L-leucyl-L-phenylalanyltrifluoromethylketone. [b] References from 16−18. [c] From reference 21. [d] $\Delta\delta_{T-H}$ is the chemical shift of ^{3}H at this site minus the chemical shift of ^{1}H at this site. See text for details on the measurement of these values.

The present results for the chymotrypsin Asp^{102}-His^{57} dyad differ from those for model compounds containing symmetric-well hydrogen bonds (SWHBs),[23] also referred to as very strong hydrogen bonds:[1] hydrogen maleate (^{1}H chemical shift of 20.2 ppm) has an experimental $\Delta\delta_{T-H} = 0.07$,[24] and hydrogen phthalate (^{1}H chemical shift of 21.0 ppm) has an experimental $\Delta\delta_{T-H} = 0.25$.[24]

The $\Delta\delta_{T-H}$ values reported here are consistent with stabilization of the transition state by a strong hydrogen bond in agreement with the N−O distance of 2.6 Å observed in the 1.5 Å resolution crystal structure of the N-AcLF-CF_3 complex with chymotrypsin.[25] The ^{3}H−^{1}H isotope shift is a new experimental parameter that can be utilized to validate theoretical calculations of the reaction coordinate for catalysis by serine proteinases.

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