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NMR Localization of Protons in Critical Enzyme Hydrogen Bonds

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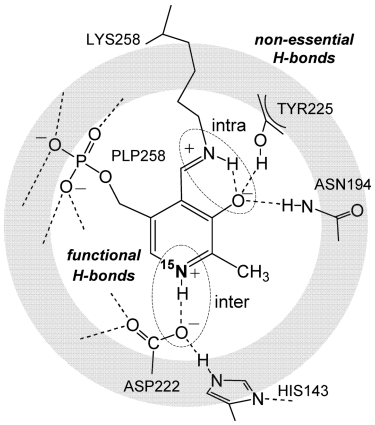
Knowledge of proton positions in mechanistically critical H-bonds in enzyme active sites is essential for understanding their great catalytic powers. Unfortunately, it is difficult to localize protons using X-ray diffraction. Neutron diffraction has been rarely applied and only to proteins below ~30 kDa.[1] The main drawback of using neutron diffraction in protein structure determination has been the requirement for relatively large protein single crystals.[2] NMR methods exploiting dipolar couplings are also limited to smaller biopolymers.[3] In this work, we demonstrate that a mechanistically critical proton in a large enzyme can be localized by combining NMR studies of enzyme and model systems, a stratagem that does not require single crystals.

We studied *E. coli* aspartate aminotransferase (AspAT, ~88 kDa), a pyridoxal-5′-phosphate (PLP, vitamin B_6)-dependent enzyme that catalyzes reactions of various amino acids.[4] The PLP cofactor is covalently bound to LYS258 as an "internal" aldimine in the active site of AspAT (Scheme 1).[5] Its pyridine nitrogen forms an OHN hydrogen bond to the side-chain carboxylate oxygen of ASP222. An O···N distance of 2.64 Å was determined, but the proton was not localized.[5b]

It has been postulated that the proton must be located on the pyridine nitrogen to give a zwitterionic structure of the *intramolecular* OHN hydrogen bond between the phenolic O and the aldimine N (Scheme 1) to enable AspAT catalytic activity.[4] Indeed, removal of the carboxylate group from the position 222 side chain by the D222A mutation indeed produces an inactive enzyme.[6] Recent X-ray diffraction as well as solid and liquid state NMR studies on model systems have confirmed this cooperative H-bond coupling,[7] which is suppressed in aqueous solution.[7c] Moreover, it was shown for pyridine rings in aldimine model systems[7,8] by dipolar NMR that their ^{15}N chemical shifts can be used to estimate the OHN hydrogen bond geometries, which in one case was in excellent agreement with neutron diffraction.[7b] Here, we extend this method to estimate the geometry of the critical intermolecular OHN hydrogen bond in the active site of AspAT and show that acid—base behavior in enzymes is better modeled using aprotic polar rather than aqueous solutions.

Figure 1a depicts the solid state ^{15}N CPMAS NMR spectrum of microcrystalline holo-AspAT containing ^{15}N in the pyridine ring of PLP synthesized as described previously.[7b] Its ^{15}N signal resonates at 175 ppm, whereas the signals of the nitrogen atoms of the AspAT backbone appear around 82 ppm (confirmed by removal of PLP-^{15}N). Using the ^{15}N chemical shift distance correlation for PLP models,[7] we find an estimated N—H distance of 1.09 Å and a H···O distance of 1.54 Å. This clearly indicates a zwitterionic

Scheme 1. ^{15}N-Labeled Cofactor ^{15}N-PLP (internal aldimine in the active site of AspAT, structure adapted from ref 5b)



structure for the intermolecular OHN hydrogen bond in AspAT. The estimated errors are given in Table 1 and contain a contribution from the signal width and another from the neglect of the difference between the mean average distances and the inverse cubic distances measured by dipolar NMR. The sum of the OH and HN distance estimated is in excellent agreement with the crystallographic O···N distance of 2.64 Å.[5b] The distance was reported to shorten to 2.58 Å when maleate (an inhibitor) binds to AspAT, leading to the closed enzyme conformation.[5b] This result is confirmed here by NMR (Table 1).

The ^{15}N signal of holo-AspAT in aqueous solution at pH 7.5 (Figure 1c) exhibits a small shift to 167 ppm, consistent with a slight shortening of the N···H distance and a slight increase of the H···O distance. Thus, the pyridine nitrogen remains protonated under physiological conditions. However, the pK_a of the pyridine N of the methylamine-PLP aldimine in water is 5.8;[7c] that is, this molecule is deprotonated at pH 7.6, leading to a signal at 262 ppm (Figure 1d). This indicates a weak hydrogen bond to surrounding water molecules, with an estimated H···N distance of 1.73 Å. Comparison with Figure 1c begs the following question: *What properties of the AspAT active site enforce protonation of the pyridine N of PLP?*

The answer comes from model studies of aldimines in polar aprotic solvents. Figure 1e depicts the ^{15}N spectrum of a model aldimine in the freon mixture[8d] CDF_3/$CDClF_2$. The chemical shift of 277 ppm is typical for the free base. The formation of a 1:1 complex with the ASP222 model Boc-Asp-O*t*Bu gives a signal at 238 ppm (Figure 1f), corresponding to a H···N distance of 1.43 Å. The 2:1 complex (Figure 1f) resonates at 181 ppm, characterized by a scalar ^{1}H—^{15}N coupling constant of −76 Hz. Thus, a zwitterionic structure with a H···N distance of 1.11 Å requires the increased acidity of the aspartic acid dimer.

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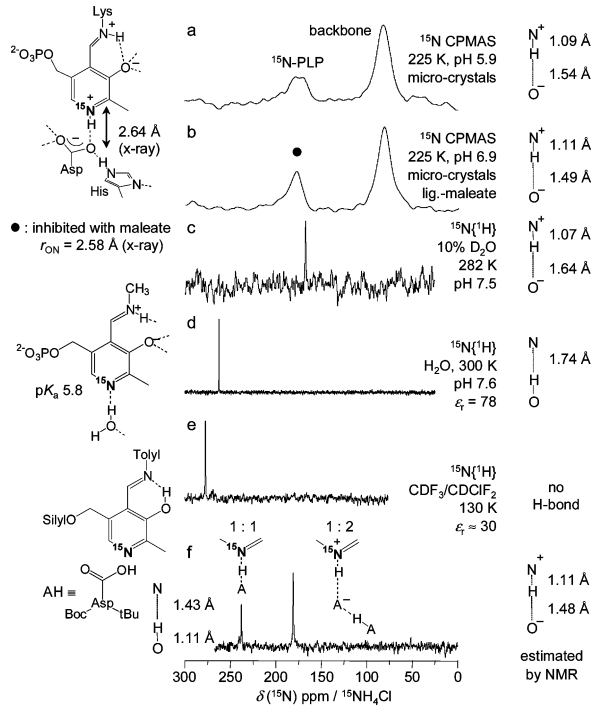

Figure 1. ^{15}N NMR spectra of ^{15}N-PLP samples: (a) microcrystalline holo-AspAT, (b) microcrystalline maleate-liganded holo-AspAT, and (c) holo-AspAT in aqueous solution, (d) aldimine model in aqueous solution from ref 7c. (e and f) Aldimine model in polar aprotic liquid in the absence and presence of protected aspartic acid (Boc-Asp-O*t*Bu). Distances estimated by NMR are listed in Table 1. For the values of ϵ_r, see ref 7e.

Table 1. ^{15}N NMR Parameters and Geometries of the Intermolecular OHN Hydrogen Bonds of the Systems in Figure 1

system	solvent	$\delta(^{15}N)$/ppm[a]	r_{NH}/Å[b]	r_{OH}/Å[b]	r_{ON}/Å[c]
holoenzyme[d]	microcryst.	174 ± 8.0	1.09 ± 0.05	1.54 ± 0.05	2.63 ± 0.1
maleate-liganded[e]	microcryst.	180 ± 8.0	1.11 ± 0.05	1.49 ± 0.05	2.60 ± 0.1
holoenzyme	10% D_2O	167.4 ± 0.6	1.07 ± 0.01	1.64 ± 0.01	2.71 ± 0.02
methyl-aldimine	H_2O	262.5± 0.1	1.74 ± 0.02	1.01 ± 0.02	2.75 ± 0.04
tolyl-aldenamine	freon[f]	277.4 ± 0.1	–	–	–
1:1 complex[g]	freon[f]	237.7 ± 0.1	1.43 ± 0.01	1.11 ± 0.01	2.54 ± 0.02
1:2 complex[g]	freon[f]	180.8 ± 0.1	1.11 ± 0.01	1.48 ± 0.01	2.59 ± 0.02

[a] Ref external solid $^{15}NH_4Cl$. [b] Average distances estimated from $\delta(^{15}N)$ according to refs 7a and 7b; possible systematic errors are not included. [c] Calculated assuming linear H-bonds. [d] X-ray:[5b] r_{ON} = 2.64(74) Å. [e] X-ray:[5b] r_{ON} = 2.58(68) Å. [f] Freon mixture CDF_3/CDF_2Cl, dielectric constant $\epsilon_r \approx 30$.[8d] [g] Tolylaldenamine complexes with Boc-Asp-O*t*Bu.

It follows that the pyridine ring and the ASP222 in the active site of the enzyme behave as in polar organic media: when they lose their water shell and come in direct contact, their combined basicity leads to a high pK_a for the binuclear base. By contrast, the pK_a values of the pyridine N and the aspartate carboxylic acid in water are not appropriate for determination of the position of the proton in the intermolecular ASP222/pyridine N OHN hydrogen bond. The enzyme must provide additional interactions to allow proton transfer to the pyridine N. The hydrogen bonds from ASP222 to HIS143 and two conserved water molecules in the AspAT active site are, therefore, probably the most important secondary interactions required to shorten the H···N distance and produce active enzyme. This work also demonstrates that the active site environment is better modeled using proton donors in polar aprotic solvents than in water. This is a satisfying result since highly structured hydrogen bonding within a polar aprotic milieu lacking bulk water is observed in enzyme active site structures.

In conclusion, we have shown that liquid and solid state ^{15}N NMR of enzymes in conjunction with model studies provides a powerful tool for localizing mechanistically critical protons in enzyme active sites.

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Supporting Information Available: Material and methods are presented. Shown are the ^{1}H NMR spectra corresponding to Figure 1f. This material is available free of charge via the Internet at http://pubs.acs.org.

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