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Demonstration of Structural Integrity of an Enzyme in Organic Solvents by Solid-State NMR

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Recently, enzymes have been shown to function as catalysts in anhydrous organic solvents,[1] where they exhibit such novel properties as enhanced thermostability[2] and altered specificity,[3] and can catalyze reactions impossible in water.[4] Despite the immense potential of nonaqueous enzymology,[1,4] our understanding of this phenomenon remains in its infancy largely because of the insolubility of proteins in nearly all organic solvents, which precludes the use of most classical biophysical techniques.

High-resolution solid-state NMR spectroscopy[5] with magic angle spinning (MAS) permits the measurement of isotropic chemical shifts in samples exhibiting anisotropic spin interactions, making it attractive for study of heterogeneous systems, e.g., membrane and crystalline proteins.[6] We now report a solid-state MAS ^{15}N NMR investigation of an enzyme suspended in organic solvents, which reveals that the catalytic site remains intact under these unnatural conditions.

In order to assess enzyme structural integrity, we examined the tautomeric structure and hydrogen bonding of His 36 in a serine protease, α-lytic protease (α-LP), as reflected by the isotropic ^{15}N chemical shifts of the imidazole nitrogens.[7] In aqueous solution, His 36 is a part of the catalytic triad of the active center and, at high pH, exists solely in the $N^{\delta 1}$–H tautomeric form (while the $N^{\epsilon 2}$–H tautomer predominates in peptides and proteins[8]), due to a hydrogen bond with the carboxylate of Asp 63.[9] This hydrogen bond, also present at low pH, results in a characteristic 8–12-ppm separation of the two ^{15}N peaks of the protonated imidazole.[9] This interaction is crucial for enzymatic activity in serine proteases; replacement of the participating Asp with Asn[10] drastically lowers k_{cat}, whereas K_m is affected only slightly.

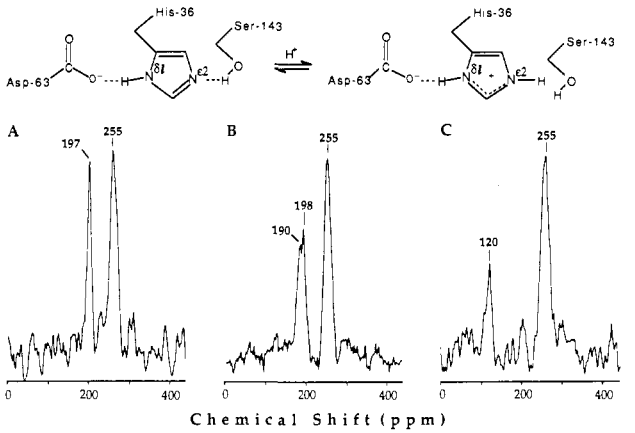

Figure 1. MAS ^{15}N NMR spectra of $^{15}N^{\delta 1}$-His α-lytic protease, "pH 8.6",[11] (A) and $^{15}N^{\delta 1}$,$^{15}N^{\epsilon 2}$-His α-lytic protease, "pH 5.4",[11] (B) in acetone and $^{15}N^{\delta 1}$-His α-lytic protease, "pH 8.6",[11] reclaimed from dimethyl sulfoxide[17] (C). Chemical shifts (±2 ppm) are referenced to the peptide backbone and reported relative to 1 M $H^{15}NO_3$ in D_2O with positive shifts being upfield. Spectra were recorded on a Bruker MSL spectrometer with ^1H and ^{15}N frequencies of 200.1 and 20.27 MHz, respectively. The ^1H $\pi/2$ pulse was 6 μs, and the cross polarization mixing time was 2 ms. All spectra were recorded at room temperature. All samples consisted of 40–60 mg of protein and, in A and B, contained at least 65% w/w of the solvent. Each spectrum represents approximately 50 000 scans, with 100 Hz line broadening applied.

First, we examined (singly labeled) $^{15}N^{\delta 1}$-His α-LP, "pH 8.6",[11] in anhydrous acetone. The spectrum consists of two peaks (Figure 1A). The peak at 255 ppm arises from the 197 amide nitrogens of the peptide backbone containing ^{15}N at its natural abundance. The peak at 197 ppm arises from $N^{\delta 1}$ of His 36. It indicates that this nitrogen is exclusively in the pyrrole (>NH) form and that the $N^{\delta 1}$ proton is engaged in a hydrogen bond.[9] (In both solids[12] and liquids,[9] a pyrrole-type nitrogen not serving as a hydrogen bond donor resonates at 210 ppm.[13]) The same result was obtained for α-LP in anhydrous octane.

Next, we examined (doubly labeled) $^{15}N^{\delta 1}$,$^{15}N^{\epsilon 2}$-His α-LP at "pH 5.4"[11] in acetone. Since at this "pH" the imidazole ring is fully protonated, both nitrogens exist in the same form (≥NH^+). In addition to the 255 ppm peptide backbone peak, there are peaks at 190 and 198 ppm (Figure 1B). $^{15}N^{\delta 1}$-His α-LP prepared and examined in the same fashion revealed that the low field peak arises from $N^{\delta 1}$; its position indicates that the $N^{\delta 1}$ proton is engaged in a hydrogen bond.[9] In contrast, the shift of 198 ppm for $N^{\epsilon 2}$ shows that its proton is not involved in hydrogen bonding.[9]

The ^{15}N spectra of the α-LP His 36 demonstrate that the unique tautomeric structure and hydrogen bonding interactions in the active center of this enzyme are identical in acetone, octane, and water. Because the catalytic triad comprises residues remote from

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(11) "pH" refers to that of the aqueous enzyme solution prior to lyophilization. There is no difference in the ionization state of the protein in the solid state and in the solution from which it was prepared.[12] In addition, the catalytic triad of α-LP is not disrupted by lyophilization (Huang, T.-H.; Bachovchin, W. W.; Griffin, R. G.; Dobson, C. M. *Biochemistry* **1984**, *23*, 5933).
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(13) Pyridine-type nitrogens (≥N) resonate at 115–138 ppm, depending upon the solvent and hydrogen bonding (Schuster, I. I.; Roberts, J. D. *J. Org. Chem.* **1979**, *44*, 3864). Tautomerization of imidazole in solids is slow on the NMR time scale.[12]

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one another in the polypeptide chain, its intactness indicates that no major distortion of the protein's native, folded structure occurs when the dry enzyme is suspended in anhydrous acetone or octane. Furthermore, there is no evidence of appreciable structural heterogeneity in the population of enzyme active centers in these solvents. Thus, the vast difference in the catalytic efficiency of α-LP in water, acetone, and octane (k_{cat}/K_m = 5800, 3.3, and 5400 M^{-1} min^{-1}, respectively)[14] is not due to disruption of conformation but rather to another effect of the solvent, e.g., a change in the binding affinities of substrates or in protein dynamics (perhaps by stripping bound water[1,15]).

In contrast to the above results, dimethyl sulfoxide (DMSO, in which proteins are soluble[16]) was found to disrupt the catalytic triad. The MAS spectrum of $^{15}N^{\delta 1}$-His α-LP ("pH 8.6") reclaimed from DMSO[17] displayed (Figure 1C), in addition to the peptide backbone peak, a single resonance at 120 ppm, indicating the exclusive existence of the $N^{\epsilon 2}$–H tautomer.[9] This is consistent with the observations that (i) DMSO was the only solvent of seven tested[14] in which α-LP was inactive and (ii) α-LP lyophilized from DMSO (as opposed to water) was inactive when suspended in octane.

In summary, solid-state MAS NMR spectroscopy has been used to show that the catalytic triad of α-LP is intact in acetone and octane, thus providing the first direct physical evidence that enzymes do not denature when suspended in neat organic solvents.

Registry No. His, 71-00-1; α-lytic proteinase, 37288-76-9; acetone, 67-64-1; octane, 111-65-9; dimethyl sulfoxide, 67-68-5.

(14) α-LP was assayed in organic solvents by following transesterification of N-Ac-L-Ala-OCH_2CH_2Cl with ethanol as described for subtilisin[15] and in water by following hydrolysis of the same substrate using a pH-stat. α-LP preinhibited with phenylmethanesulfonyl fluoride was inactive in organic solvents.

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Coupling of the Anthracycline Antitumor Drug Menogaril to 2′-Deoxyguanosine through Reductive Activation[1]

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The concept of bioreductive activation as applied by Moore and Czerniak to the anthracycline antitumor drugs, most notably daunomycin and adriamycin, proposes formation of a reactive quinone methide from a hydroquinone state.[2,3] The hydroquinone state is formed through bioreduction, and the quinone methide reacts with a nucleophilic site in a biological macromolecule. The tumor cell toxicity is proposed to result from the reaction of the quinone methide with a nucleophilic site in DNA such as the 2-amino group of 2′-deoxyguanosine. Although such a coupling in itself may not be cytotoxic, it attaches an effective catalyst for the production of reactive oxygen species to DNA. Subsequent catalytic production and reaction of reactive oxygen species may damage the DNA beyond repair.[4] Reductive activation of the quinone antitumor drug mitomycin C leads to coupling to guanine moieties,[5] and the elegant work of Tomasz and Nakanishi has further established that reductively activated mitomycin C cross-links duplex DNA through reaction with two guanine bases.[6] However, Fisher and Aristoff have recently stated in their extensive review of the chemistry of DNA modification by antitumor antibiotics with regard to the quinone methide from reductive activation of anthracyclines: "in principle this quinone methide may be thought of as a DNA-reactive entity, but its behavior with DNA on this score in vitro is ambiguous and numerous efforts to obtain mononucleotide adducts have been unsuccessful" and further "there is no in vitro experiment to provide even the suggestion of the chemistry leading to adduct formation".[7]

We have recently reported that reduction of the anthracycline antitumor drug menogaril (**1**) with substoichiometric quantities of the one-electron-reducing agent bi(3,5-dimethyl-5-(hydroxymethyl)-2-oxomorpholin-3-yl) (**2**, DHM-3 dimer) in an aqueous medium yields a long-lived quinone methide transient, which can be trapped with the sulfur and nitrogen nucleophiles *N*-acetylcysteine[8] and imidazole,[9] respectively. These results suggest that the quinone methide can react with cysteine and histidine residues in peptides and proteins. We now describe the successful reductive coupling of menogaril at the 7-position to 2′-deoxyguanosine at the 2-amino position.

Menogaril (18 mg, 2.0×10^{-4} M) was reacted anaerobically with 25 mol % of DHM-3 dimer (**2**) in 10% dimethylformamide–90% pH 8.2, aqueous, Tris buffer (v/v) in the presence of a 100-fold excess of 2′-deoxyguanosine for 6 days at ambient temperature. Reverse-phase HPLC analysis of the reaction mixture eluting with a gradient consisting of a formate buffer and methanol and detecting at 480 nm showed peaks with retention times of 1.7 min with a shoulder at 2.0 min (17%, 7-(2″-*N*-(2‴-deoxyguanosyl))-7-deoxynogarol stereoisomers,[10] **3**), 5.3 min (17%, menogaril, **1**), 5.6 min (30%, 7-deoxynogarol, **4**), 6.8 min (23%, bi(7-deoxynogarol-7-yl), **5**), and 9.7 min (10%, probably a stereoisomer of **5**). Minor unidentified product peaks appeared at 2.5 min (2%) and 2.8 min (1%). Menogaril, 7-deoxynogarol, and the major stereoisomer of bi(7-deoxynogarol-7-yl) were identified by coinjection onto the HPLC column with authentic samples[8] and purity evaluation of the peaks with a Hewlett-Packard diode array detector. The deoxynogarol dimer **5** was further characterized by isolation from a chloroform extract of the reaction mixture and FAB mass spectral analysis, which showed a characteristic molecular ion at m/z 1021.[8] The major stereoisomer of the deoxyguanosyl–deoxynogarol adduct **3** was isolated from the aqueous phase after the chloroform extraction by evaporation of the water followed by a series of ethanol extractions of the solid material to remove excess deoxyguanosine and chloroform extractions of aqueous solutions to remove byproducts. Extraction was followed by reverse-phase flash chro-

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