ASSIGNMENT OF TRYPTOPHAN INDOLE NH PROTON RESONANCES OF LYSOZYME

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Summary

Resonances of the indole NH protons for five of the six tryptophan residues of hen egg white lysozyme are resolved when the PMR spectrum is observed in H_2O. Assignments of the resonances to specific tryptophan residues were accomplished by differential deuterium exchange rates, inhibitor perturbation and chemical modification. Indole NH resonances from all five tryptophan residues of human lysozyme also are observable.

A major requirement for the successful application of proton magnetic resonance (PMR) spectroscopy to determination of protein structure is identification of observed resonances with specific protons of protein amino acid residues. Certain high-field resonances of hen egg white (HEW) lysozyme have been attributed to methyl groups of component leucine, isoleucine, methionine, and valine residues.[1,2,3,4] The resonances of the C-2 protons of the four histidine residues of ribonuclease have been resolved and assigned.[5] For certain heme proteins contact interactions displace resonances into otherwise unoccupied regions of the spectrum permitting at least partial assignment of these resonances to specific protons of the heme moiety.[6,7]

Hen egg white (HEW) lysozyme possesses six tryptophan residues at positions 28, 62, 63, 108, 111, and 123.[8,9,10] Try-62, try-63, and try-108 are at the substrate binding cleft containing the active site, and the first two residues are believed to participate in binding the substrate.[11,12] Here we report the resolution in the PMR spectrum of HEW lysozyme of five of the six

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tryptophan indole NH resonances and their assignment to specific tryptophan residues. These resonances can be used to monitor in a highly specific fashion interactions at and structural perturbations on the active site of lysozyme. Extension of the approach to other proteins containing tryptophan should be possible.

The PMR spectra of proteins normally are obtained using D_2O as solvent to reduce the intensity of the H_2O absorption. In D_2O, the NH and other exchangeable protons are replaced by deuterium and, therefore, become nonobservable in PMR spectroscopy. In H_2O, however, those protons which exchange with water protons with an apparent first order rate constant less than about 10 sec^{-1} should be observable. At pH's of less than 7, indole and amide NH proton resonances are separately resolvable from those of H_2O for aqueous solutions of proteins.

The low field portion of the PMR spectrum of thermally denatured HEW lysozyme dissolved in H_2O is shown in Figure 1a. Resonance absorption in the region 1540-1700 Hz originates from the aromatic CH protons.[1,4] The 1700-2140 Hz region of absorption derives from the amide NH protons. Similarly, the peak at 2194 Hz is assigned to indole NH protons of the six tryptophan residues. This assignment is supported by the PMR of a variety of indoles, integrated resonance intensities and deuterium exchange studies. The 2194 Hz peak is missing in RNase under similar denaturing and solvent conditions; RNase does not contain tryptophan.

Upon refolding into the biologically active, native conformation, the 2194 Hz resonance breaks up into six peaks, each of unit intensity, reflecting the nonequivalent local environments of the six tryptophan residues in the folded conformation (Figure 1b). Five of these (labeled I-V) are clearly resolved; the sixth (referred to as VI) presumably is shifted further to high field into the complex amide NH region of resonance absorption and has not yet been uniquely located.

A spectrum taken under the same conditions as the spectrum in Figure 1b of HEW lysozyme oxidized with one equivalent of N-bromosuccinimide was very similar to that of Figure 1b except that resonance III was absent from its normal position.[13] Since the indole group of tryptophan 62 is known to be selectively oxidized to an oxindole,[14] resonance III can be unequivocally assigned to tryptophan 62. PMR studies of oxindole suggest that the NH resonance of 62-oxindole tryptophan probably occurs in the complex amide NH region of resonance absorption.

Binding of the inhibitors N-acetylglucosamine (NAG) and N,N'-diacetylglucosamine $(NAG)_2$ to HEW lysozyme perturbs resonances III and V of Figure 1b but not the other indole NH proton resonances. Since the indole NH protons of tryptophans 62 and 63 are believed to be hydrogen bonded to oxygen atoms of the inhibitors,[12] resonances III and V can be assigned to tryptophans 62 and 63. Since III has already been assigned to tryptophan 62, V must derive from tryptophan 63.

A sample of protonated HEW lysozyme was dissolved in D_2O at about 5°C. The spectrum of Fig. 1c was obtained on this sample 55 min. later when partial replacement by deuterium of the indole NH protons had occurred. It is clear that resonances III, IV, and V originate from protons which undergo deuterium exchange much more rapidly than the protons associated with resonances I and II. The first order rate constants for the disappearance of peaks I and II were $(1.81 \pm 0.44) \times 10^{-2}$ and $(0.72 \pm 0.15) \times 10^{-2}$ min^{-1} respectively, whereas III, IV, and V disappeared with rate constants greater than 4×10^{-2} min^{-1}. All rate constants were measured at 35°C and pD 3.3. Blake *et al.*[11] report that tryptophans 62, 63, and 123 are on the surface of HEW lysozyme and should be readily accessible to solvent. Since these residues probably correspond to the rapidly exchanging indole NH protons (III, IV, and V), and since III and V have already been assigned to tryptophans 62 and 63 respectively, IV can be assigned to try-123.

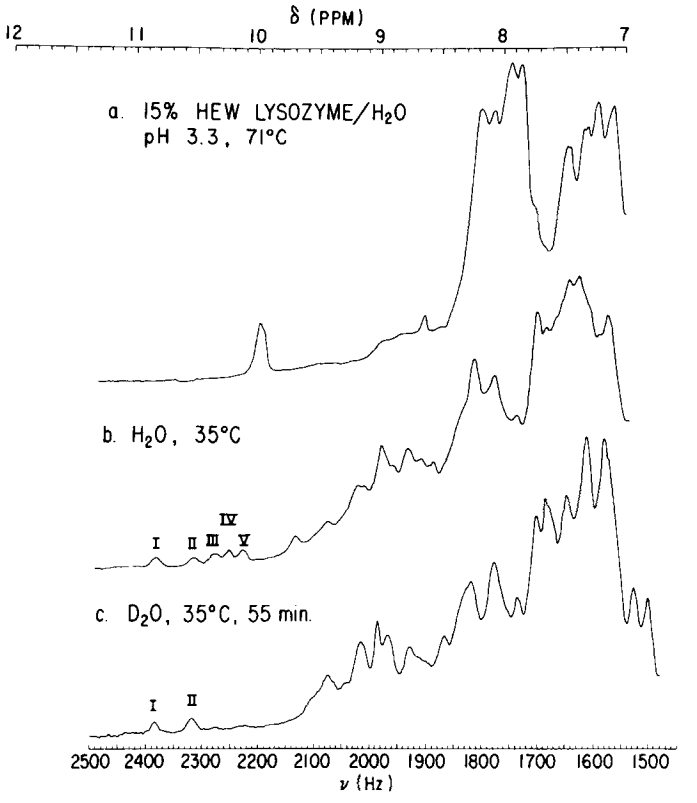

Figure 1. 220 MHz PMR Spectra of HEW Lysozyme: (a) Thermally denatured HEW lysozyme 15% (w/v) in H_2O at pH 3.3 and 71°C; (b) Native lysozyme 15% (w/v) in H_2O at pH 3.3 and 35°C; (c) Native lysozyme 15% (w/v) in D_2O at pD 3.3 and 35°C, 55 minutes after dissolution at 5°C. Chemical shifts were measured relative to 2,2-dimethyl-2-silapentane-5-sulfonate as internal standard. The pD (unbuffered) was the observed pH meter reading plus 0.40.

In a 0.01 M lysozyme/D_2O solution containing 0.2 M NAG the first order rate constant for deuterium exchange obtained from the disappearance of resonance I was $(1.02 \pm 0.34) \times 10^{-2}$ min^{-1} (essentially the same as in the absence of NAG), whereas the proton associated with resonance II exchanged at a rate too

slow to follow. Blake et al.[12] have shown that the indole NH protons of tryptophans 28 and 108 are hydrogen bonded to the amide carbonyl groups of tyr-23 and leu-56, and that try-111 is located in a relatively interior position of the lysozyme molecule. Hence, these three tryptophans should possess slowly exchanging indole NH protons. Of these three residues, X-ray studies indicate that access to the solvent of only try-108 should be further obstructed by inhibitor binding.[12] Therefore, resonance II can be assigned to try-108 and resonance I to trypotphan 28 or 111; not enough data are yet available to distinguish which of the latter tryptophans corresponds to resonance I and which to the as yet unidentified resonance VI.

In the presence of excess NAG, resonances III and V shift downfield and upfield respectively and overlap resonance IV. Of the three overlapping peaks, one exhibited a rate constant for deuterium exchange of at least 4×10^{-2} min^{-1} (i.e., the rate of one proton was unperturbed by inhibitor binding), whereas the other two protons exchanged with an apparent rate constant of $(0.82 \pm 0.28) \times 10^{-2}$ min^{-1} (considerably slower than in the absence of NAG). The slower rate can be ascribed to indole NH protons of tryptophans 62 and 63 that hydrogen bond to the inhibitor.[12]

The assignments are summarized in Table 1.

Table 1

Assignment of HEW Lysozyme Tryptophan Indole NH Resonances

Resonance[a]	I	II	III	IV	V	VI[b]
Residue	28 or 111	108	62	123	63	111 or 28

a. See Figure 1b.

b. Not definitely identified in the spectrum of HEW lysozyme.

Studies of human lysozyme[15] have been pursued concurrently with those of HEW lysozyme. All five of the tryptophans of the latter protein yielded well-resolved indole NH resonances at 2259, 2233, 2173, 2161, and 2141 Hz (10% human lysozyme, H_2O, pH 5.0, 55°C). Binding of $(NAG)_2$ perturbed the 2259, 2173, and 2161 Hz resonances indicating that at least three of the five tryptophan residues of human lysozyme are affected by inhibitor binding.

A more detailed description of these studies is in preparation.

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