PROTON NMR SPECTROSCOPY OF THE ACTIVE SITE HISTIDINE OF α-LYTIC PROTEINASE

Effects of adjacent ^{13}C and ^{15}N labels

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1. Introduction

A histidine auxotroph of *Lysobacter enzymogenes* (ATC 29847) was grown on media containing either isotopically labeled [90% $^{13}C^{\epsilon}$]*L*- or [90% $^{15}N^{\delta}$, 90% $^{15}N^{\epsilon}$]*D,L*-histidine. The enzyme, α-lytic proteinase (EC 3.4.21.12), was isolated from these cultures as well as from cultures of wild-type bacteria grown on unlabeled medium. ^{1}H NMR spectra at 360 MHz obtained with all 3 purified enzymes revealed that the only important differences concerned a resonance previously assigned to the C^{ϵ}–H proton of the active site histidine [1]. α-Lytic proteinase has only a single histidine residue [2] located at position 57 in the chymotrypsinogen numbering scheme [2,3]. Presence of the adjacent ^{15}N labels broadened the histidine C^{ϵ}–H peak by about a factor of 2 by unresolved scalar coupling. Presence of a directly bonded ^{13}C led to disappearance of the histidine C^{ϵ}–H peak by a combination of scalar coupling and dipolar broadening. These effects should be useful for the cross-assignment of ^{1}H NMR peaks of ^{13}C and ^{15}N enriched proteins. The ^{13}C and ^{15}N labeled proteins were found to undergo the reversible *a–b* conformational transition [4] which changes the pK'_a of His^{57} from 6.5–5.9.

2. Experimental procedures

The *Lysobacter enzymogenes* culture was a gift from Dr F. D. Cook. Preparation of the histidine auxotroph was as in [5]. Cultures were grown, and α-lytic proteinase was isolated by minor modification [5] of the procedures in [6]. The [$^{15}N^{\delta}$,$^{15}N^{\epsilon}$]His^{57}-α-lytic proteinase used was that in [5]. 90% Isotopically labeled [$^{13}C^{\epsilon}$]*L*-histidine was purchased from KOR Isotopes (Cambridge MA). The purity and degree of enrichment were verified by ^{1}H NMR and ^{13}C NMR spectroscopy. 360 MHz ^{1}H NMR spectra were obtained with a Nicolet NT-360 spectrometer located in the Purdue University Biochemical Magnetic Resonance Laboratory. Usual procedures were used in handling the NMR samples [7]. Chemical shifts are reported in ppm from internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). α-Lytic proteinase can exist in two conformational forms: (i) that obtained by dialysis against water and then lyophilization and which is stable at low pH (conformer *a*); (ii) that arising after incubation of conformer *a* for ~14 h at neutral pH (conformer *b*) [4,8]. The *b*-conformer of the enzyme can be reconverted to the *a*-conformer by dialysis and lyophilization. The 2 forms are not the result of dimerization, and both forms are catalytically active [4]. All 3 enzymes studied (^{13}C- or ^{15}N-labeled and unlabeled) exhibited the conformational transition which can be followed by ^{1}H-NMR. Spectra of the *a*-conformer are reported here; however, the *b*-conformer gives His C^{ϵ}–H spectra very similar to those shown. The *a–b* transition results in extensive changes in the ^{1}H NMR spectrum of the enzyme; the change can be monitored most conveniently by following the intensity of a methionine methyl peak which shifts from 2.09 ppm in the *a*-form to 2.12 ppm in the *b*-form [4].

3. Results and discussion

The aliphatic and aromatic regions of the 360 MHz

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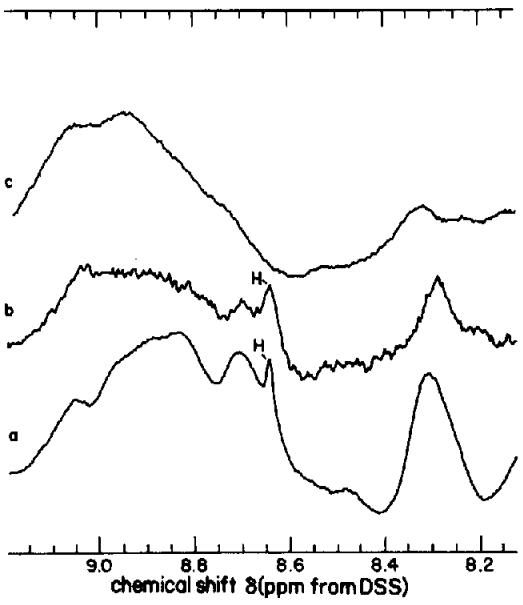

Fig.1. 360 MHz ^{1}H NMR spectra of: (a) unlabeled α-lytic proteinase, pH* 3.5; (b) [$^{15}N^{\delta}$,$^{15}N^{\epsilon}$]His^{57}-α-lytic proteinase, pH* 4.0; and (c) [$^{13}C^{\epsilon}$]His^{57}-α-lytic proteinase, pH* 4.0. The symbol H in (a) and (b) designates the resonance assigned to the C^{ϵ}–H of His^{57}. Experimental conditions: protein was 2 mM in ^2H_2O containing 0.2 M KCl; probe temp. 25°C; pulse sequence, standard 90° pulse (10 μs) followed by an aquisition time 0.819 s and a delay of 3 s; number of points digitized, 8192; exponential filtering, 1 Hz; number of transients, 512; sweepwidth, ±2500 with detection in quadrature. Glass electrode pH meter readings (pH*) are uncorrected for the deuterium isotope effect.

^{1}H NMR spectra (not shown) of the ^{13}C- and ^{15}N-labeled α-lytic proteinase isolated from the histidine auxotroph were virtually identical to that of the enzyme isolated from the wild-type strain. Fig.1 shows a comparison of 360 ^{1}H NMR spectra of the histidine C^{ϵ}–H and amide N–H chemical shift region of: (a) unlabeled α-lytic proteinase; (b) [$^{15}N^{\delta}$,$^{15}N^{\epsilon}$]His^{57}-α-lytic proteinase; and (c) [$^{13}C^{\epsilon}$]His^{57}-α-lytic proteinase. The differences in the baseline of broad resonances in spectra (a), (b) and (c) are attributed to varying amounts of deuterium exchange from the ^2H_2O solvent into backbone amide groups. The samples had been lyophilized from ^2H_2O and were exposed to ^2H_2O for different periods of time. The peak H in spectrum (a) is that assigned to the C^{ϵ}–H of His^{57} in previous studies; in the *a*-conformer of α-lytic proteinase it yields a pK'_a of 6.5 [4]. Peak H in fig.1b also shifts with pH and gives a pK'_a of 6.5. This result along with similarity of the aliphatic and aromatic spectral regions suggests that the enzyme produced by the wild-type strain and auxotroph have equivalent structures. Hence, peak H in spectrum (b) is assigned to the C^{ϵ}–H resonance of His^{57} in [$^{15}N^{\delta}$,$^{15}N^{\epsilon}$]His^{57}-α-lytic proteinase. The linewidth of peak H in the ^{15}N-labeled enzyme ($\Delta\nu_{1/2} \sim 13$ Hz) is about twice that of unlabeled enzyme ($\Delta\nu_{1/2} \sim 6$ Hz). This broadening can be attributed to unresolved 2 bond coupling between the C^{ϵ}–H and the labeled ring nitrogens of ~5–6 Hz [9]. The dipolar interaction between the labeled ring nitrogens and the C^{ϵ}–H is negligible as a contribution to the proton linewidth. The broadening does not result from magnet inhomogeneity or from the presence of paramagnetic ions since the linewidths of the much sharper internal reference DSS peaks were approximately equal for the unlabeled and ^{15}N-labeled samples.

Peak H is not present in spectra of [$^{13}C^{\epsilon}$]His^{57}-α-lytic proteinase (fig.1c). The absence of the ^{1}H NMR peak is not the result of deuterium exchange at this position as demonstrated by the fact that the ^{13}C NMR spectrum of the same sample exhibited the normal ^{1}H–^{13}C coupling constant. In those 90% of the molecules that are ^{13}C-labeled, the histidine C^{ϵ}–^{1}H will be split by scalar coupling to the ^{13}C. The one bond coupling constant $J_{^{13}C\,^1H}$ is expected to be between 205–220 Hz [6]. The signal-to-noise ratio is not sufficient to resolve the histidine peak associated with the 10% population of unlabeled protein. One should be able to resolve the doublet; however, attempts to do so with the present sample have been unsuccessful. The reason apparently is that the dipolar interaction between the histidine $^{13}C^{\epsilon}$ and C^{ϵ}–^{1}H broadens the proton resonances so that they blend with the background of broad, slowly exchangeable, N–H resonances. Resolution of the doublet awaits a method to eliminate the broad amide resonances by deuterium exchange. Several attempts to exchange these groups have been unsuccessful [4]. The excess broadening resulting from the ^{1}H–^{13}C interaction is calculated [10,11] to be 10 Hz, assuming a ^{13}C–^{1}H internuclear distance of 1.089 Å and a correlation time of 6.8×10^{-9} s. The experimental linewidth of the C^{ϵ}–H of His^{57} resonance in unlabeled α-lytic proteinase at low pH is 6 Hz. The extra broadening caused by interaction with the ^{13}C nucleus will cause the total linewidth to be 6 Hz + 10 Hz ≃ 16 Hz for the two ^{1}H-NMR peaks of the C^{ϵ}–H doublet. These broad peaks, which individually have the intensity of 0.5

protons, are in a region containing many other broad resonances and therefore are difficult to resolve.

The results in fig.1 are in agreement with the previous assignment of peak H to His^{57} which was based on the pH dependence of its chemical shift [1] and on the perturbation of its associated pK'_a in the di-isopropylphosphoryl derivative [12]. The present results provide a more rigorous assignment since they rely on specific, non-perturbing isotopic modifications.

There has been some confusion concerning the pK'_a value of His^{57} in α-lytic proteinase. Two values have been reported based on ^1H NMR results [1,8]. It was thought that the discrepancy resulted from improper calibration of a pH meter [13], but later experiments revealed the existence of 2 conformational forms of α-lytic proteinase that have different pK'_a values: the pK'_a of His^{57} is 6.5 in the *a*-form and 5.9 in the *b*-form [4,8]. Differences in experimental conditions and accuracy of pK'_a determination probably account for the difference between these values and the $pK'_a = 7.0$ obtained by ^{15}N NMR [5] and the $pK'_a = 6.8$ obtained in a recent ^{13}C NMR experiment [14]. The report of a low pK'_a (~3.3) for His^{57} of α-lytic proteinase and high pK'_a for Asp^{102} (6.9) based on ^{13}C NMR evidence [6] has been retracted [14]. All NMR experiments now are in agreement that there is no operational 'charge relay system' [15] in any of the serine proteinases studied [8,13]. The existence of 2 conformational forms of α-lytic proteinase having distinct pK'_a values was not detected in the ^{15}N NMR [5] and ^{13}C NMR [14] experiments, but the present ^1H NMR results show that samples equivalent to those used in the ^{15}N and ^{13}C NMR experiments undergo the *a*–*b* transition.

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