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Characterization of the Acetyl-chymotrypsin Intermediate by ^{13}C Nuclear Magnetic Resonance Spectroscopy

Sir:

Carbon-13 NMR is a valuable tool in studies of proteins in solutions at natural abundance of ^{13}C.[1] However, problems of sensitivity and assignment arise in the observation of single carbon atom resonances in proteins. To overcome these problems several applications have been carried out in which ^{13}C enriched chemically modifying groups have been attached to proteins as ^{13}C NMR reporter groups.[2,3] We wish to report the first observation of the resonance of a ^{13}C enriched enzyme-substrate intermediate.

The mechanistic pathway in chymotrypsin catalysis is thought to involve an acyl-enzyme intermediate.[4] The evidence for such intermediates is based upon enzyme activity studies,[5-7] and upon spectrophotometric observations.[8,9] Labeled ^{14}C acetate was used to show the position of acetylation on the active serine 195.[10] In order to study the properties of the active site environment of the enzyme in solution, a ^{13}C NMR study of ^{13}C labeled acyl-chymotrypsin intermediates has been undertaken. We report the characterization of the first member of this series [1-^{13}C]acetyl-α-chymotrypsin ($CH_3{}^{13}CO$-E).

The ^{13}C enriched (90%) *p*-nitrophenyl acetate was prepared by the general method of Okawa and Hase.[11] The compound was recrystallized from hot ethanol, and exhibited a single carbon signal at 175.0 ppm downfield from Me_4Si. The methyl resonance of the acetyl group was split into a doublet (J = 7 Hz) instead of a singlet, as in the unenriched sample.

The purified acetyl-α-chymotrypsin intermediate was prepared by the method of Bender et al.,[12] with an enzyme (0.2 mM) to substrate ratio of 1:10. Phosphate buffer (pH 5.1) and a reaction time of 4 h were employed. Membrane ultrafiltration (Diaflo ultrafilter PM10) was used to purify the intermediate instead of column chromatography. Enzyme activity was determined with the substrate, carbobenzyloxy-L-alanine *p*-nitrophenyl ester, at pH 5.1.[13] In this pilot run approximately 95% of the protein was recovered with a residual enzyme activity of 4-5% of the control. On stirring the protein solution at pH 8.0, the enzyme activity was fully recovered within 30 min.

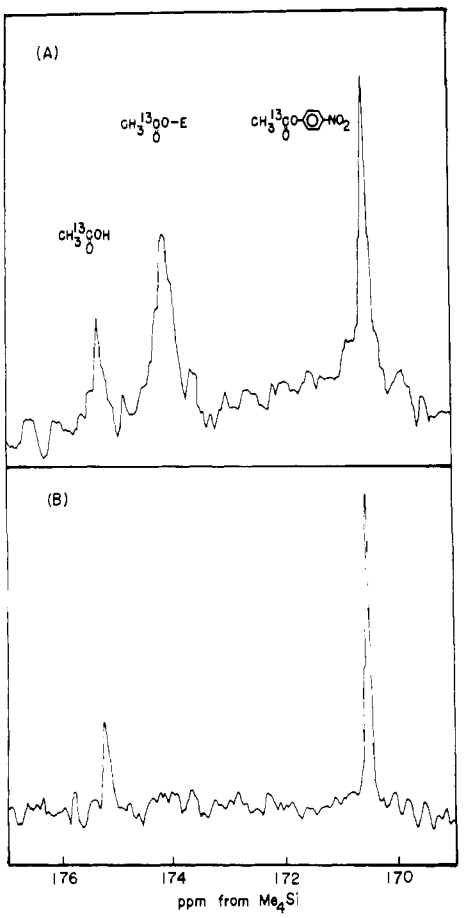

Figure 1. Proton-decoupled 68-MHz ^{13}C NMR spectra: (A) α-chymotrypsin (2.0 mM) plus *p*-nitrophenyl [1-^{13}C]acetate (3.0 mM) at room temperature in 0.2 M phosphate buffer (pH 5.1). (After 2 h, the pH of the solution was adjusted to 3.2.); (B) 3.7 mM *p*-nitrophenyl [1-^{13}C]acetate under the same conditions after 3 days.

With the ^{13}C enriched substrate at a molar ratio of 1:1 with enzyme, the acyl-α-chymotrypsin intermediate was prepared as above, without ultrafiltration. Upon ^{13}C NMR examination of the reaction mixture three major signals were observed (170.4, 174.0, 175.1 ppm downfield from Me_4Si) (Figure 1A). The signals at 170.4 and 175.1 ppm were readily attributed to substrate and acetic acid, respectively (Figure 1B). After 4 h the carbon signal of the substrate had disappeared (Figure 2A). Increasing the pH of a reaction mixture to 8.0 at room temperature eliminated the signal at 174.0 ppm, while the intensity of the signal due to acetic acid was correspondingly increased (Figure 2B). These results were consistent with the enzyme activity studies. In another experiment, in which the purified ^{13}C enriched intermediate was prepared in larger quantity (4 mM) using ultrafiltration, 90% of the protein was recovered with a residual enzyme activity of 15%. Nonetheless, only one major carbon signal at 174.0 ppm was observed. Incubation of this solution at 37 °C and pH 4.5 for 30 min resulted in a dramatic increase in the intensity of the acetic acid carboxyl carbon signal with a concomitant reduction in the signal at 174.0 ppm. Upon reassay of this solution it was found that 80% of the original activity had been recovered.

The results of these preliminary experiments are consistent with the proposal that the signal at 174.0 ppm arises from the [1-^{13}C]acetyl-chymotrypsin intermediate. This provides further direct evidence for the formation of acyl-chymotrypsin intermediates during the hydrolysis of esters by chymotrypsin.

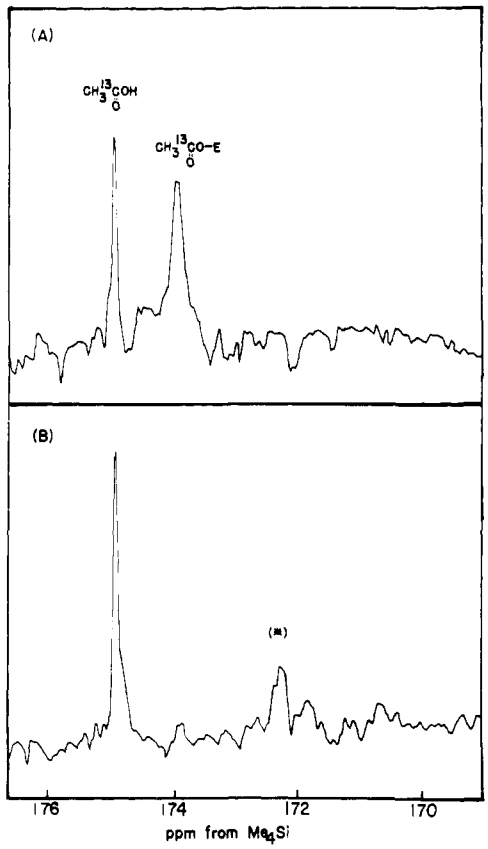

Figure 2. Proton-decoupled 68-MHz ^{13}C NMR spectra: (A) α-chymotrypsin (2.0 mM) plus *p*-nitrophenyl (2.6 mM) prepared as in Figure 1, except the pH of solution was adjusted to 3.2 after 4 h; (B) this solution was adjusted to pH 8.0 at room temperature. After 30 min, the pH of solution was adjusted to 3.2 (the carbon signal marked with an *asterisk* is of unknown origin, but might be residual tetrahedral intermediate of the enzyme).

By measurement of the relative areas of the carbon signals reported here we anticipate obtaining detailed information on the quantity of intermediate present under different experimental conditions.

In order to study the mobility of the acetyl group in the active site environment of the enzyme, the spin-lattice relaxation time (T_1) of the carbonyl carbon of the [1-^{13}C]acetyl-chymotrypsin intermediate was determined. At the same time an average T_1 value for the carbonyl signals of the enzyme backbone was measured by the progressive saturation method. The T_1 value of the carbonyl carbon of the acetyl intermediate was 2.6 s, while the value for the enzyme backbone carbonyls was 2.9 s, under the same experimental conditions. From these results, it seems that the carbonyl of the acetyl group is restricted in motion in the intermediate. This may result from the hydrogen bond between the carbonyl oxygen of the acetyl group and either the NH of glycine-193 or serine-195, which has been suggested by the x-ray analysis of the complex of chymotrypsin with formyl-L-tryptophan by Steitz et al.[14] This explanation is also consistent with spectrophotometric studies of cinnamoyl-chymotrypsin[9] and cinnamoyl-elastase[15] in which a hydrogen bond between the carbonyl group of the acyl derivative and the enzyme backbone has also been proposed.

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Metal Atoms as Superbases: the Gas Phase Proton Affinity of Uranium

Sir:

The ability to determine the strengths of acids and bases in the gas phase makes possible the search for extremes in behavior for these properties. Discovery of a species having an unusually high acidity or basicity sheds light on the factors which determine these properties and suggests other studies and applications which derive advantage from the exceptional behavior. With this in mind, we would like to report that metal atoms can be very strong bases in the gas phase. This conclusion is based on the determination of the proton affinity of the technologically important uranium atom, for which we have measured PA(U) = 238 ± 4 kcal/mol.

The endothermic reactions of uranium ions with D_2, process 1, and CD_4, process 2, have been observed in an ion beam-collision chamber apparatus, previously described.[1] The uranium ion source comprises a tubular stainless steel tube, operated at approximately 700 K, which vaporizes solid UF_4 onto a resistively heated rhenium ribbon at ~2300 K, where dissociation and surface ionization occurs.[2] Uranium ions are accelerated to a selected energy and allowed to interact with the target gas in a collision chamber at 400 K. Product ions scattered in the forward direction are detected using a quadrupole mass spectrometer.[3]

$$U^+ + D_2 \rightarrow UD^+ + D \quad (1)$$

$$U^+ + CD_4 \rightarrow UD^+ + CD_3 \quad (2)$$

Ion product abundance was measured as a function of target gas pressure to yield the cross section for reaction. The variation in cross section with relative kinetic energy for reaction 1 is shown in Figure 1. Significant Doppler broadening due to thermal motion of the target gas is evident in the threshold region. Using the analysis of Chantry[4] for a cross section which increases linearly with energy above threshold, an extrapolation of the straight line portion of the curve gives a threshold too low by $3\gamma kT$ where T is the temperature of the target gas and $\gamma = m_U/(m_U + m_{AB})$, m_U and m_{AB} being the incident particle