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^{13}C Nuclear Magnetic Resonance Observations on the Interaction between *P*-Amidinophenylpyruvic Acid and Trypsin

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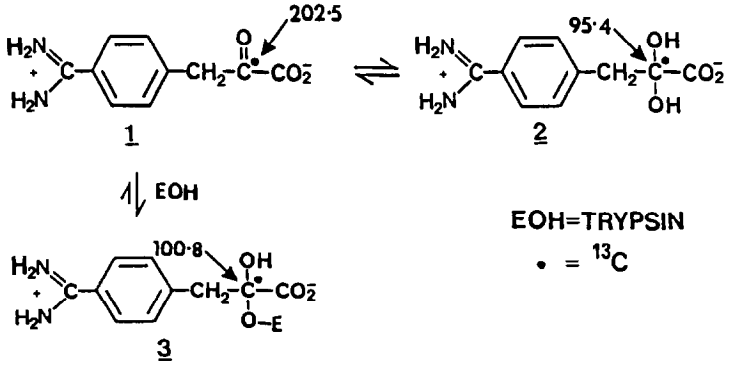
The formation of an enzyme–inhibitor adduct between bovine trypsin and [2-^{13}C]*p*-amidinophenylpyruvic acid has been investigated by ^{13}C NMR spectroscopy. The observation of a resonance at 100.8 ppm demonstrates that the hemiketal formed between the hydroxyl of serine-195 and the 2-^{13}C carbon of *p*-amidinophenylpyruvic acid is sp^3 hybridized with no significant deviation from tetrahedral geometry. It is shown that stabilization of the hemiketal oxyanion if it occurs is less effective than in chloromethylketone inhibitor complexes. The tetrahedral adduct is stable from pH 3 to 8. The mechanisms of breakdown of the tetrahedral adduct at pH extremes are discussed.

INTRODUCTION

The inhibitory effect on trypsin of compounds containing either the amidinophenyl- or pyruvic acid functionalities was noted in 1967 by Geratz (*1*). He demonstrated that the combination of these two functionalities in *p*-amidinophenylpyruvic acid (APPA[2], **1**, Scheme 1) gave a very potent competitive



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[2] Abbreviations used: APPA, *p*-amidinophenylpyruvic acid; DMSO, dimethyl sulfoxide; FID, free induction decay; THA, tetrahedral adduct; Z-Lys-CMK, N^{α}-CBZ-L-lysine chloromethyl ketone; TMS, tetramethylsilane.

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inhibitor (K_i = 0.96 μM) against the enterokinase activation of trypsinogen. This compound has since been shown to have general inhibitory activity against trypsin-like serine proteinases (*2–4*) and is the strongest small molecule inhibitor known (K_i = 0.1–4 μM). X-Ray crystallographic studies of its interaction with trypsin (*5, 6*) have shown that the amidinophenyl portion of the inhibitor is bound into the specificity pocket as an analog of the arginine side chain of a true substrate. The Ser-195–O^{γ} is within bonding distance of the inhibitor C-2 carbonyl carbon, but it was suggested (*5*) that full tetrahedral geometry was not realized. The carboxylate of the inhibitor was also shown to be close enough to the imidazole of His-57 to interact with it.

^{13}C NMR has been used to investigate the formation of a fully tetrahedral protein–inhibitor complex formed between trypsin and N^{α}-CBZ-L-lysine chloromethyl ketone (Z-Lys-CMK) (*7, 8*). The ionic interactions and stabilizations occurring in the complex have been probed by observing the titration shifts of the THA. In view of the controversy concerning whether the trypsin APPA adduct is fully tetrahedral, it was decided to investigate this system by ^{13}C NMR. It was also hoped that we would be able to determine whether the presence of an inhibitor carboxylate-enzyme imidazolium ion-pair interaction would affect hemiketal oxyanion stabilization.

MATERIALS AND METHODS

Materials

Bovine β-trypsin (Type III, two times recrystallized, dialyzed, and lyophilized) was obtained from Sigma (Batch 103F 8075). Chromatographic analysis (*9*) determined that this was 85% β-trypsin and 15% α-trypsin. The commercial enzyme was thus used without further purification. SP-Sephadex C-50 was obtained from Pharmacia. [2-^{13}C] Glycine (90 at.%) was obtained from KOR Isotopes (lot ECK-I-89). $^2H_2$-Water (99.8 at.%) was obtained from Aldrich. [$^2H_6$]Dimethyl sulfoxide (99.9 at.%) was obtained from Merck Sharpe & Dohme. Benzamidine was obtained from Aldrich.

Synthesis of [2-^{13}C]APPA

[2-^{13}C]Glycine was converted to [2-^{13}C]APPA following literature procedures (*10–12*).

Trypsin Solutions

Protein concentrations were determined by using ε_{280}(trypsin) = 36,960 M^{-1} cm^{-1} (*8*).

The concentration of fully active trypsin was determined by active site titration with *p*-nitrophenyl *p'*-guanidobenzoate (*13*).

Inhibition of Trypsin with [2-^{13}C]APPA

Varying amounts of APPA in DMSO were added to the enzyme (0.1 μM) in 0.1 M phosphate buffer, pH 7.0, and the initial rate of hydrolysis of the substrate N^{α}-benzoyl-DL-arginine-*p*-nitroanilide (30 μM) was measured at 410 nm. K_i was determined from a plot of the reciprocal of the initial rate versus the inhibitor concentration by the method of Dixon and Webb ((*14*), p. 332 ff).

For ^{13}C NMR experiments, a solution of [2-^{13}C]APPA in DMSO-d_6 was added to 1 mM fully active trypsin in 20 mM acetate buffer, 10% D_2O, pH 5.0. This solution had a final concentration of DMSO of less that 5% and was used without further purification.

pH Titrations and pH Measurement

pH measurements were made with an Altex Model 500 digital pH meter with a Beckman No. 39522 combination electrode. The values given are as read, no correction being made for samples containing 10–12% D_2O.

pH titrations were carried out by careful addition of small volumes of acid (0.875 M HCl, 12.5% D_2O) or base (0.875 M NaOH, 12.5% D_2O) to the rapidly stirring sample. The pH was measured at the beginning and end of each spectral acquisition. Results for samples which varied by more than 0.05 pH units were discarded.

pH data were fitted as described by Malthouse *et al.* (*8*).

NMR Spectra

Spectra were measured on a Bruker WM300 wide-bore spectrometer (7.045 T). Chemical shifts are reported relative to TMS at 0 ppm. Sample size was 8–10 ml in a 20-mm-diameter tube. Spectral conditions were 16 K time-domain data points; 22 μs pulse width ($\equiv$18° pulse); 0.492 s acquisition time with spectral width approx 220 ppm; 5 Hz line broadening (exponential multiplication of FID prior to transformation); low power noise decoupling (0.3–0.5 W).

Linewidths were measured either manually or by an iterative procedure contained in the software package of the Bruker spectrometer.

RESULTS AND DISCUSSION

The synthetic sample of [2-^{13}C]APPA was shown to be a competitive inhibitor of trypsin with a K_i = 2.5 μM which is similar to previously reported values of 1.6 to 6 μM (*2*, *15*, *16*).

APPA by itself has very low solubility in aqueous solution at physiological pH values and requires strong acid or the presence of a large proportion of organic cosolvent (>50% DMSO) to achieve the concentrations necessary for the NMR experiment (~1 mM). The presence of trypsin greatly aided solubility and it was found that 1 mM trypsin solution could solubilize up to 6 mM APPA without recourse to low pH or a large amount of cosolvent (<5% DMSO). These APPA

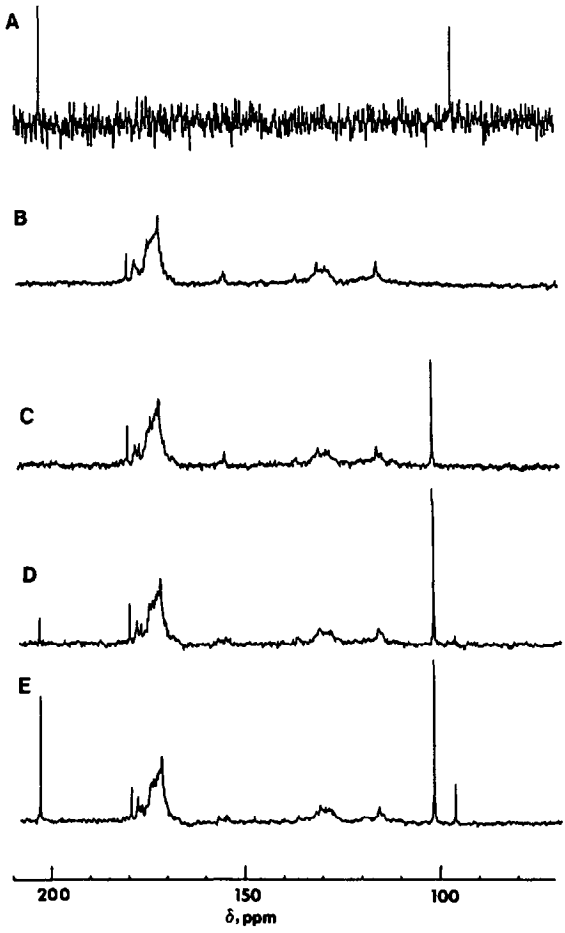

FIG. 1. ^{13}C NMR spectra on addition of [2-^{13}C]APPA to trypsin. (A) 6 mM [2-^{13}C]APPA in 7:3, 1 mM HCl: d_6-DMSO, 73,400 transients. (B)–(C) 1.0 mM fully active trypsin, 20 mM acetate, pH 5.1, 10% D_2O; 1–5% d_6-DMSO. Concentration of [2-^{13}C]APPA and number of transients as follows: (B) 0, 86,400; (C) 0.61 mM, 40,000; (D) 1.21 mM, 40,000 (E) 1.78 mM, 80,000. X = acetate buffer carboxylate carbon.

concentrations were sufficient for the NMR experiments while the amount of DMSO in the final solution was low enough to be disregarded.

Figure 1 shows the addition of [2-^{13}C]APPA to 1 mM fully active trypsin. The inhibitor by itself shows two resonances (Fig. 1A), at 202.5 and 95.4 ppm, assignable to the ^{13}C-enriched ketone (**1**, Scheme 1) and its hydrate (**2**, Scheme 1), respectively. On addition to trypsin these resonances are not observed in the spectrum until more than an equivalent of the inhibitor has been added to the enzyme. Rather, a new signal is observed at 100.8 ppm (Fig. 1C) which is broader (8 Hz) than those of the free inhibitor (4 Hz). The linewidth of this trypsin-bound resonance also compares favorably to that observed for the complex formed between [2-^{13}C]Z-Lys-CMK and trypsin (7 Hz) (*7*, *8*) in which the labeled atom has been shown to exist as a tetrahedral hemiketal by X-ray crystallography (*17*).

The chemical shift observed here for the enzyme-bound species (100.8 ppm) (**3**, Scheme 1) is also similar to that seen for the trypsin/Z-Lys-CMK adduct (98.0 ppm), both being consistent with the formation of a hemiketal. Furthermore, the T_1 value of the enriched atom in the trypsin/APPA complex (4.3 ± 0.1 s) and that in the trypsin/Z-Lys-CMK complex (4.3 ± 0.2 s) are identical (*18*). These results suggest that the environment of the labeled carbon atom in the two systems is similar.

In order to show that APPA is indeed bound to the active site of trypsin, the competitive inhibitor benzamidine was added to a stoichiometric mixture of [2-^{13}C]APPA and trypsin. The two inhibitors compete for the active site of the enzyme and when a sufficiently large concentration of benzamidine is employed, ^{13}C NMR signals for unbound [2-^{13}C]APPA can be seen at 202.5 and 95.4 ppm. Benzamidine is known to bind into the specificity pocket of trypsin (*19*) and the decrease in intensity of the resonance at 100.8 ppm confirms that this peak is due to a complex in which APPA itself is bound into this pocket. If it is assumed that when the concentration of benzamidine is zero, all of the APPA is bound to the enzyme, we can use the intensity of the peak at 100.8 ppm as a direct measure of the amount of APPA bound to the enzyme during the course of the addition of benzamidine. The intensity of this resonance (δ = 100.8 ppm) decreased with increasing benzamidine concentrations. Data were analyzed by Eq. 1 (see Fig. 2).

$$\frac{[E_{\text{total}}]}{[E.\text{APPA}]} = \frac{[\text{APPA}] + K_A}{[\text{APPA}]} + \frac{K_A}{K_B\,[\text{APPA}]}\,[\text{benzamidine}] \qquad [1]$$

A plot of $[E_{\text{total}}]/[E.\text{APPA}]$ versus [benzamidine] gives a straight line whose slope is proportional to K_A, the binding constant of APPA to trypsin. [APPA] = 1 mM

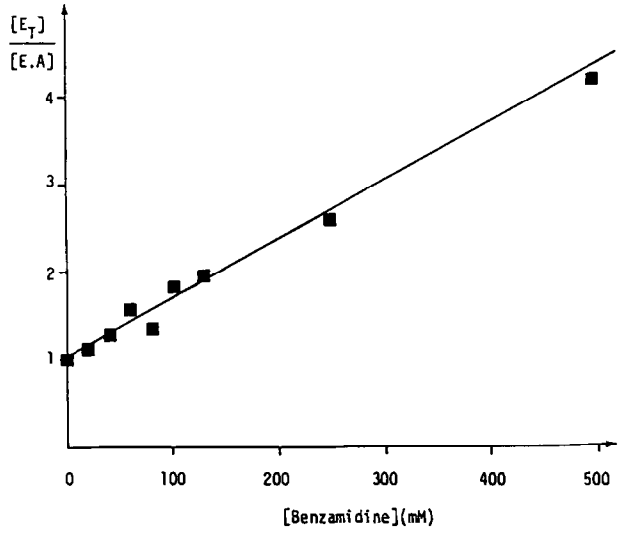

FIG. 2. Data from displacement of APPA from trypsin by benzamidine. Effect of benzamidine concentration on intensity of the trypsin/APPA adduct peak at 102.8 ppm. Plot of $[E_{\text{total}}]/[E.\text{APPA}$ adduct] versus [benzamidine]. Straight line fitted by least-squares linear regression. Slope, 6.37 M^{-1}.

and K_B = 16.8 μM (*16*), from which a value of K_A = 0.11 μM can be deduced. This is an order of magnitude less than was measured by kinetic methods (2.5 μM, see Materials and Methods). Although this discrepancy is large, it could arise from many factors, e.g., the K_I values were obtained at different pH values, enzyme concentrations, buffers, and ionic strengths. It should also be noted that in order to calculate the binding constant (K_A) for APPA the binding constant for benzamidine (K_B) obtained under very different conditions were used.

In previous studies of trypsin–inhibitor adducts by ^{13}C NMR (*7, 8*), it has been shown that hydroxyl group of the hemiketal formed between Ser-195–O^{γ} and the carbonyl of the inhibitor (Z-Lys-CMK) can be deprotonated at alkaline pH values to yield an oxyanion, which is stabilized by interaction with the imidazolium cation of His-57. Similar pH studies were therefore undertaken with the [2-

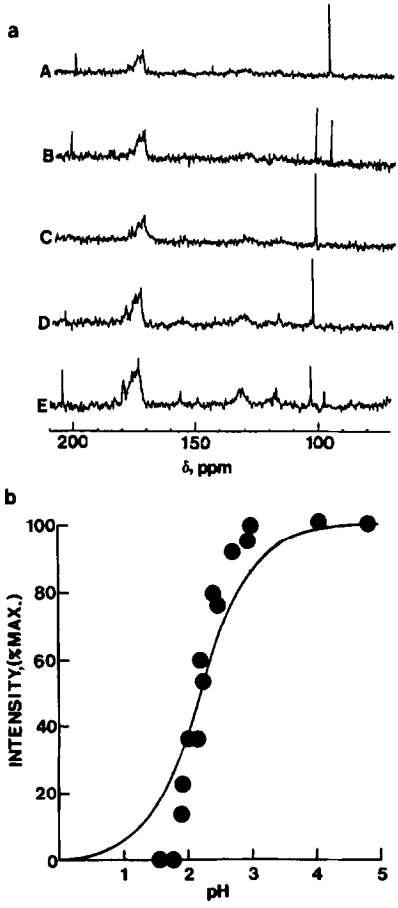

FIG. 3. (a) Effect of pH on the ^{13}C NMR spectrum of a 1 : 1 mixture of [2-^{13}C]APPA and trypsin. Spectra (A)–(B) 1.0 mM fully active trypsin, 1 mM [2-^{13}C]APPA, 10% D_2O, 3% d_6-DMSO. pH as follows: (A) 1.75; (B) 2.21; (C) 4.82; (D) 7.57; (E) 8.60. (b) Intensity of peak at 102.8 ppm versus pH (relative to intensity at pH 5.1 of 100) at pH <5. The solid line was calculated assuming that the decrease in intensity was due to a single ionizing group (pK_a = 2.2).

^{13}C]APPA–trypsin THA in order to determine whether similar stabilization of the hemiketal oxyanion occurs. The effect of pH on a 1:1 trypsin: [2-^{13}C]APPA mixture is illustrated in Fig. 3a. There is a decrease in the intensity of the signal at 100.8 ppm at the extremes of this range with a concomitant appearance of two peaks at ~200 and ~95 ppm, characteristic of free, unbound inhibitor.

The decrease in intensity of the bound APPA resonance (δ = 100.8 ppm) with decreasing pH (Fig. 3b) was reversible at least to pH 2.0. This process was essentially complete within 1 pH unit (pH 2 to 3) and could not be dependent on the ionization of only one group (see Fig. 3b). The X-ray crystallographic data show that the inhibitor carboxylate is in close proximity to the imidazole ring of histidine-57 (*5, 6*) and this interaction plus the ion-pair interaction of the inhibitor guanido group with Asp-189 would be expected to be the predominant factors involved in the tight binding of the inhibitor to trypsin. The protonation and consequent disruption of other ion-pairs such as the Asp-194-N-terminal isoleucine ion-pair (*20*) would also cause conformational changes which would result in the displacement of APPA from trypsin at low pH values. The pK_a values for the inhibitor carboxylate were 1.55 ± 0.06 and 2.90 ± 0.09 in the keto and hydrated forms, respectively (Fig. 4). In the absence of any interaction with the imidazole of His-57, the carboxylate of the inhibitor in the THA would be expected to have a pK_a similar to that of the hydrate. Ion-pair interaction between the inhibitor carboxylate and the imidazolium ion of His-57 would be expected to result in the ion-pair being characterized by two molecular pK_a values (pK_I < 3 and pK_{II} > 7), e.g., if the ion-pair form predominates, pK$_I$ and pK_{II} are due to deprotonation of the neutral carboxyl group and cationic imidazolium ion, respectively. We therefore conclude that the low pH decrease in THA must result from at least two protonation processes.

At alkaline pH values (Fig. 3a) the intensity of the [2-^{13}C]APPA resonance of the THA (δ = 100.8 ppm) decreased with a concomitant increase in the intensities of the resonances due to the free inhibitor (δ = 202.5) and its hydrate (95.4). This process was, however, essentially irreversible due to autolysis. Therefore, only a lower limit can be placed on the pK_a or pK_a's of the group or groups responsible for this process (p$K_a \geq 9.0$).

No titration shift was observed for the [2-^{13}C]APPA resonance (δ = 100.8 ppm) of the THA over the pH range 1.75 to 8.6. Therefore, there is no evidence for stabilization of the THA hemiketal oxyanion as is observed for chloromethyl ketone inhibitors (*7, 8*). If stabilization does occur then it must be less effective than with THA formed by chloromethyl ketone inhibitors (oxyanion p$K_a \approx 8$ (*7, 8*)) with the pK_a of the oxyanion being greater than 9. The X-ray crystallographic studies (*5*) have indicated that the ion-pair interaction between the inhibitor carboxylate and the imidazolium ion of histidine-57 results in movement of the imidazolium ion to optimize the ion-pair interaction. Thus, the stabilization of the THA hemiketal oxyanion may be preempted by imidazolium-carboxylate ion-pair interaction. We may conclude that oxyanion stabilization is definitely less effective in the APPA–THA with the pK_a of the oxyanion being greater than 9.

Based on their X-ray crystallographic data, Walter and Bode (*5*) have suggested that at least in the crystalline state the geometry at the [2-^{13}C]APPA carbon of the

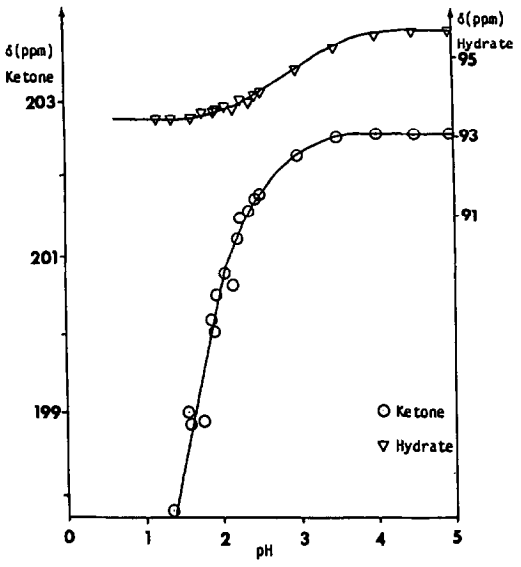

FIG. 4. Titration behavior of [2-^{13}C]APPA. Variation of ^{13}C NMR chemical shift of enriched carbon in [2-^{13}C]APPA (ketone and hydrate forms) with pH. 2 mM [2-^{13}C]APPA, 1.0 mM fully active trypsin, 10% D_2O, 5% d_6-DMSO. No signal was observed for the ketone form of the molecule below pH = 1.3. The solid lines were calculated as described in Materials and Methods. The fitted parameters were as follows: ketone; $\delta_0 = 196.7 \pm 0.4$, $\bar{\delta} = 204.5 \pm 0.1$, $pK_a = 1.53 \pm 0.06$: hydrate; $\delta_0 = 96.27$, $\bar{\delta} = 97.37 \pm 0.07$, $pK_a = 2.92 \pm 0.05$.

trypsin adduct is intermediate between trigonal and tetrahedral. Model compound studies have suggested that for nucleophilic attack at a carbonyl carbon by nitrogen there is a logarithmic relationship between the nitrogen–carbonyl carbon distance and the ^{13}C chemical shift (*21–23*). Consequently, carbonyl chemical shifts (≈210 ppm) only decrease by approximately 10–20 ppm when the N—C distance is decreased from ∞ to 1.64 Å. However, a decrease in N—C from 1.64 to 1.48 Å gives a 100 ppm decrease (*21*). The length of the N—C carbon bond can be related therefore exponentially to the carbonyl bond order (*22*). Because of the lower nucleophilicity of neutral oxygen, similar studies for oxygen attack on a carbonyl are more difficult (*23, 24*) but there is no reason to expect that results (if obtainable) will be any different. Therefore, the chemical shift value at 100.8 ppm indicates the absence of any significant double-bond character at the ^{13}C-enriched carbon of the [2-^{13}C]APPA–trypsin inhibitor adduct.

For enzyme adducts, small differences (±10 ppm) may be observed due to differently charged states of inhibitor or enzyme active site residues. The chemical shift of the [2-^{13}C]APPA–trypsin THA (100.8 ppm) is close to that of the trypsin–Z-Lys-CMK complex (98 to 102 ppm). The chemical shifts of both these trypsin adducts imply that there is negligible distortion of the tetrahedral geometry of these sp^3-hybridized adducts.

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