Steady-State Carbon-13 Nuclear Magnetic Resonance Spectra of Acyl-α-chymotrypsin†

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ABSTRACT: When [^{13}C]carbonyl-enriched *p*-nitrophenyl 5-*n*-propyl-2-furoate is incubated with α-chymotrypsin, a new peak appears in the ^{13}C NMR spectrum. On the basis of its position and the fact that it is "chased" with unlabeled substrate, we conclude that this new signal is due to the acyl-enzyme intermediate. In spectra taken during steady-state turnover, the acyl-enzyme ester carbonyl ^{13}C chemical shift displays a pH dependence that fits to a titration curve with an apparent p*K* of 7.1 (0.1). The apparent p*K* of the k_{cat} vs. pH curve for enzyme-catalyzed hydrolysis of the same substrate under conditions differing only in reactant concentration is 7.0 (0.1). We have found no spectral evidence for a tetrahedral intermediate.

A complete enzyme mechanism is unavailable for the serine proteases, although we probably know more about this class of enzyme than about any other. Spectroscopic measurements of enzyme reaction intermediates may offer some of the detailed information needed to understand a catalytic mechanism, and there have been a number of such studies with the serine proteases: ultraviolet spectroscopy (Bernhard & Malhotra, 1974), resonance Raman spectroscopy (Phelps et al., 1981; MacClement et al., 1981; Carey & Phelps, 1983; Argade et al., 1984), ^{13}C NMR [see recent reviews by Steitz & Shulman (1982) and Mackenzie et al. (1984)], ^{15}N NMR (Kanomori & Roberts, 1983), and ^{31}P NMR (Gorenstein & Findlay, 1976; Reeck et al., 1977; Proubcan et al., 1979). Of interest for this paper are the attempts to measure the resonance position of the carbonyl C in the acyl intermediate of proteases. This approach has the following advantages: (i) there are large chemical shifts associated with substitutions at a carbonyl carbon; (ii) the values of the longitudinal relaxation times are reduced, permitting faster data acquisition, since chemical shift anisotropy is the predominant relaxation mechanism at high field strengths; (iii) the line widths of the observed signals are smaller than those for carbons directly bonded to protons, in which case dipolar relaxation predominates. There have been preliminary reports of the ^{13}C spectrum of acetyl-α-chymotrypsin (Niu et al., 1977; Tobias et al., 1984) at low pH and/or low temperatures. Robillard et al. (1974) measured the spectrum of [^{13}C]carbonyl-enriched *N*-acetyltyrosine semicarbazide in equilibrium with chymotrypsin and found no apparent change in the peak position as compared with the substrate alone.

The [(5-*n*-alkyl-2-furoyl)acyl]-α-chymotrypsins deacylate slowly between pH 4 and pH 9 and in the temperature range of 5–50 °C (Baggott & Klapper, 1976; Wang et al., 1981). Because of this slow turnover, and the low K_m associated with the *p*-nitrophenyl ester substrates, the enzyme can be kept fully acylated at neutral pH and room temperature for 1 h or more. In this paper we present ^{13}C NMR spectra of functional [^{13}C]carbonyl-enriched [(5-*n*-propyl-2-furoyl)acyl]-α-chymotrypsin and describe the pH dependence of the acyl-enzyme [^{13}C]carbonyl signal. A preliminary presentation of this work has been made elsewhere (McWhirter et al., 1984).

Materials and Methods

Preparation of [^{13}C]carbonyl-enriched *p*-nitrophenyl 5-*n*-propyl-2-furoate (PNPPF):[1] ^{13}C-labeled dimethylformamide was synthesized by the following procedure. To sodium metal (0.07 g) dissolved in 50 mL of isopropyl alcohol in a 75-mL autoclave we added 4 g of >99% ^{13}C-enriched carbon monoxide (Isotec Inc.) to give an initial pressure of 1800 psi. This mixture was rocked for 2 days at room temperature, at which time the pressure had dropped to approximately 50 psi. To the product mixture, containing ^{13}C-enriched isopropyl formate, isopropyl alcohol, and sodium isopropoxide, we rapidly added dimethylamine with stirring. After reaching 50 °C, the solution was cooled to and stirred for 2 h at room temperature. The mixture was then deionized by addition of 15 mL of anhydrous Dowex 50 (H^+); after 1 h, the slurry was filtered and the [^{13}C]dimethylformamide purified by distillation: 9.4 g, 93% yield (Whaley & Ott, 1975; Larsen et al., 1975). We then synthesized 5-*n*-propyl-2-furfural with the Vilsmeier reaction. Phosphorus oxychloride (6.5 g, 0.042 mol) was added with constant stirring to 3.1 g (0.042 mol) of [^{13}C]dimethylformamide in a flask fitted with a condenser. The reaction mixture was maintained at −10 °C during the addition (~10 min) and then at 5 °C for 2 h. After addition of 4.6 g (0.042 mol) of *n*-propylfuran (ICN Pharmaceuticals) to the reaction slurry over a 5-min period at 5 °C, the mixture was warmed to 50 °C to initiate the exothermic reaction, cooled in an ice bath until the evolution of HCl had subsided, heated to 90 °C to complete the reaction, and poured into ice–water. The solution was then neutralized by slow addition of 10 mL of 4.7 M potassium carbonate, the resultant solution was extracted with ether (8 times 50 mL), and the combined ether fractions were dried over sodium sulfate. The 5-*n*-propyl-2-furfural that remained after removal of the ether was distilled at low pressure in 82% yield, 4.8 g (Traynelis et al., 1957; Arnold & Zemlicka, 1959; Bosshard & Zollinger, 1959). The acid chloride was synthesized by oxidation of the aldehyde with freshly made *tert*-butyl hypochlorite (Chattaway & Backeberg, 1923). To 4.8 g (0.035 mol) of 5-*n*-propyl-2-furfural we added 25 g of *tert*-butyl hypochlorite in 30 mL of CCl_4. After 5 days

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[1] Abbreviations: PNPPF, *p*-nitrophenyl 5-*n*-propyl-2-furoate; Me_2SO, dimethyl sulfoxide; k_{cat}, apparent first-order rate constant for enzyme turnover, which in the case of *p*-nitrophenyl ester substrates is the rate constant for enzyme deacylation; DIP, diisopropyl phosphate.

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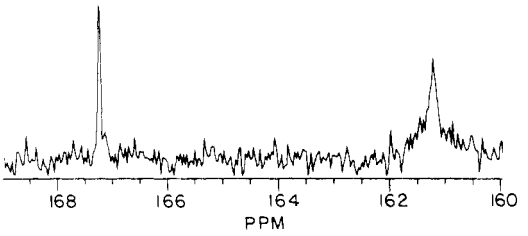

FIGURE 1: Spectrum of [(5-*n*-alkyl-2-furoyl)acyl]-α-chymotrypsin. α-Chymotrypsin (0.5 mM) was mixed with [^{13}C]carbonyl-enriched PNPPF (2 mM) at an apparent pH of 7.0 (0.1 M phosphate buffer) and 27% (v/v) perdeuterated Me_2SO at 25 ± 1 °C. The spectrum is the time-averaged result of 2862 individual scans over a period of 1 h referenced against the ^{13}C shift (assigned at 39.5 ppm) of the internal Me_2SO.

at 5 °C, excess *tert*-butyl hypochlorite, CCl_4, and *tert*-butyl alcohol were removed at low pressure (Ginsberg, 1951; Walling & Mintz, 1967). To the acid chloride, used with no further purification, we added 4.8 g (0.035 mol) of *p*-nitrophenol in 10 mL of ether and 2.73 g (0.035 mol) of pyridine. Upon being cooled to 0 °C, the ester immediately precipitated. The crude PNPPF was washed with water and recrystallized from methanol (mp 68 °C, 40% yield from [^{13}C]carbon monoxide).

NMR spectra were obtained with the following solutions. Bovine α-chymotrypsin (lots 102F-8050 and 63F-8030, Sigma Chemical Co.), prepared freshly in 0.001 M HCl, was added to a solution of ^{13}C-labeled ester, perdeuterated dimethyl sulfoxide (Me_2SO), and phosphate buffer. α-Chymotrypsin concentrations were determined from the absorbance of the stock solution at 280 nm ($\epsilon = 5.0 \times 10^4$ M^{-1} cm^{-1}; Laskowski, 1961). The apparent pH of the solution was measured after calibration of the electrode with aqueous phosphate buffer, pH 7. When the apparent pH of the protein/substrate solution in the mixed solvent was at or below 5.5, the solutions were stored to allow complete protein acylation. At higher apparent pH the solutions were immediately placed into the NMR instrument. All NMR spectra were obtained at 25 °C without proton decoupling and at a field strength of 75, 90, 100, or 125 MHz. All peak positions were referenced to internal perdeuterated Me_2SO, which was assigned a resonance value of 39.5 ppm.

The chymotrypsin catalysis of PNPPF hydrolysis was measured under steady-state conditions with the assay procedure described in Wang et al. (1981). All other reagents, the sources of which were not specifically mentioned, were obtained commercially and used with no further purification.

Results

On mixing α-chymotrypsin with [^{13}C]carbonyl-enriched *p*-nitrophenyl 5-*n*-propyl-2-furoate (PNPPF), a new NMR peak appears in the region of 161–162 ppm (Figure 1), with the precise shift dependent on the pH and composition of the solution. The carbonyl resonances of the substrate, product as an acid, and product as a base are approximately 158, 162, and 167 ppm, respectively. Since furoate is the predominant form of the product above pH 5 and since the NMR band of the carbonyl carbon in ethyl-2-furoate is at 161.6 ppm, we conclude that the new peak is associated with the acyl-enzyme. This assignment was verified by mixing chymotrypsin and ^{13}C-labeled PNPPF in approximately equimolar amounts at pH 4.7. At this pH the acyl-enzyme forms slowly and is not hydrolyzed, so we observe the 161 ppm peak but not the 5-*n*-propyl-2-furoate peak (Figure 2a). After addition of unlabeled substrate (2:1 excess) and incubation of the enzyme at pH 7.6, the 161 ppm peak disappears, and the product furoate peak appears at 167 ppm (Figure 2b,c).[2] The time

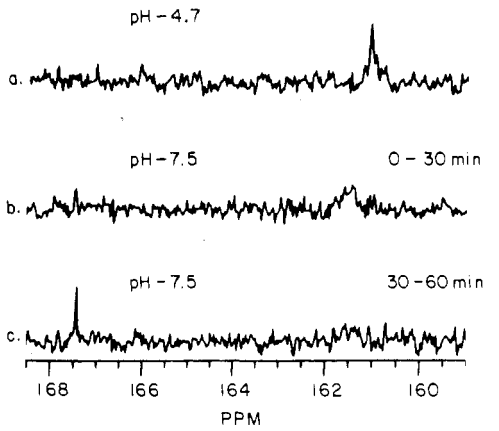

FIGURE 2: pH jump–unlabeled substrate chase experiment. Each spectrum was obtained at 25 °C from 30 min of data accumulation referenced against internal 25% (v/v) perdeuterated Me_2SO: (a) acylchymotrypsin (1 mM) prepared and maintained at pH 4.7 by mixing enzyme and PNPPF at a 1:1 molar ratio; (b) spectrum of the same acylchymotrypsin sample accumulated over the first 30 min after raising the pH to 7.5 in the presence of 2 mM unenriched PNPPF; (c) spectrum of the reaction mixture as in (b) accumulated between 30 and 60 min after raising the pH.

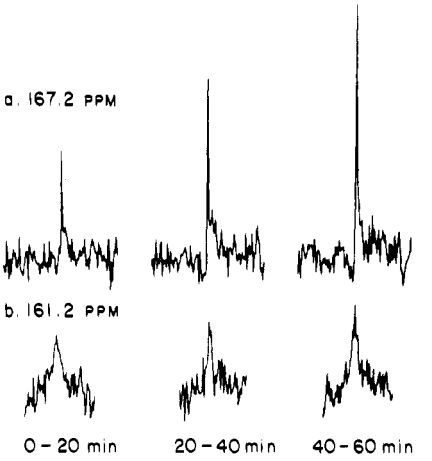

FIGURE 3: Steady-state product turnover. Product (a) and acyl-chymotrypsin (b) signals were obtained during steady-state substrate turnover. Each spectrum represents 20 min of data accumulation under experimental conditions identical with those described in the legend of Figure 1.

required for complete disappearance of the acyl peak—approximately 60 min—is consistent with the deacylation half-life of approximately 30 min, determined independently under conditions differing only in reactant concentrations. Thus, the 161 ppm peak behaves as would be expected of the acyl-enzyme.

With substrate in a 5:1 molar excess over enzyme at an apparent pH of 7.0, there is an initial steady-state turnover as seen by the linear increase of the product signal height with time (Figure 3a). We are able to obtain the spectrum of the acyl-enzyme during this steady-state period (Figure 3b), because the substrate turnover is relatively slow. When product release slows and then stops, the 161 ppm peak of the acyl-enzyme becomes more complex. We believe that this quali-

[2] With Me_2SO present at 20–25 v/v % we have observed an approximately 50% decrease in V_{max}. Similar inhibition observed previously has been explained by a decrease in the concentration of H_2O (Fink, 1973). If the "pH-jump" experiment is done in the absence of unlabeled substrate, the 161 ppm peak does not disappear, and its shape becomes more complex. We believe that this stabilized spectrum is due to denaturation of the acyl-enzyme catalyzed by that fraction of enzyme that has already lost the acyl group.

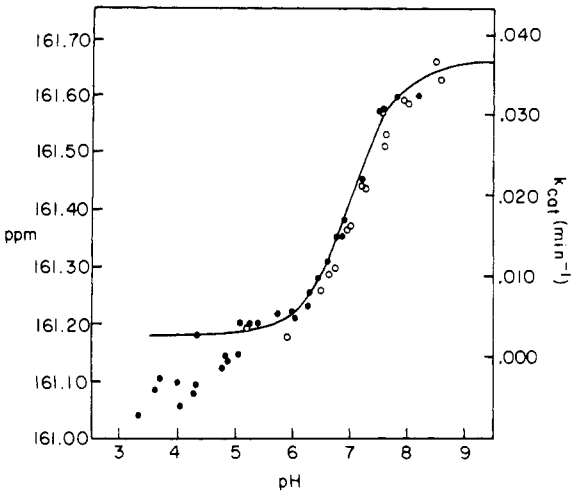

FIGURE 4: pH dependence of acylchymotrypsin chemical shift and of enzyme activity. All spectra were obtained at 25 °C referenced against internal 22% (v/v) perdeuterated Me_2SO. The pH was obtained as described under Materials and Methods. The solid line represents the best fit titration curve to the NMR data with an apparent pK_a of 7.0 (0.1). Enzymatic activity under conditions closely similar to the NMR experiments was measured as described in the text. (●) Chemical shift; (○) turnover rate constant.

tative change in the 161 ppm peak is due to proteolysis of the acyl-enzyme catalyzed by the fraction of the enzyme no longer acylated when substrate is depleted. Thus, all of our experiments were performed under conditions of substrate excess with spectra collected while the intensity of the product peak continued to rise linearly.

The position of the 161 ppm peak is dependent upon the pH of the solution, shifting downfield as the pH rises (Figure 4). Between the apparent pH values of 5.25 and 8.25, this dependence fits well to the equation for a simple titration curve:

$$\delta = (K_a\delta_A + [H^+]\delta_{HA})/([H^+] + K_a) \quad (1)$$

where δ_{HA} and δ_A are the chemical shifts of the protonated and unprotonated acyl-enzyme species A and HA, respectively, and the equation has been derived with the assumption of a rapid interconversion between both forms, characterized by the equilibrium constant K_a. The values of the three constants (and estimates of associated standard deviations) that were extracted from the data of Figure 4 with a BASIC version of Bevington's (1969) GRIDL algorithm are as follows: pK_a, 7.0 (0.1); δ_A, 161.66 (0.03) ppm; δ_{HA}, 161.18 (0.02) ppm. The apparent pK_a compares well with the pK of 7.1 (0.1) obtained from kinetic measurements of the steady-state PNPPF turnover under similar experimental conditions.

When the apparent pH is greater than 6.7, a poorly defined shoulder is observed on the downfield side of the major acyl peak (Figure 1). At higher pH this shoulder is resolved into a small peak (Figure 5) with a pH-dependent shift qualitatively similar to that of the main acyl peak. Below the apparent pH of 5.25 a transition, slow on the NMR time scale, is also observed. Because of the nature of the data, no pK could be assigned to this transition. Similar acid transitions have been observed in the ^{1}H NMR titrations of native and DIP-inhibited trypsinogen (Porubcan et al., 1978) and trypsin (Markley & Porubcan, 1976). The nature of this process remains to be elucidated.

Discussion

On mixing the [^{13}C]carbonyl-enriched substrate PNPPF with α-chymotrypsin, a peak appears near 161 ppm in the NMR spectrum. On the basis of the following facts, we conclude that this signal is due to the [(5-*n*-propyl-2-furoyl)acyl]chymotrypsin intermediate: (i) it has a chemical shift similar to that of ethyl-2-furoate; (ii) after formation at low pH, it is "chased out" at neutral pH by unenriched substrate; (iii) it is present throughout the steady-state release of product; (iv) its shift has a pH dependence described by a titration curve with an apparent pK of 7. There have been previously reported NMR spectra of acylprotease intermediates (Niu et al., 1977; Malthouse et al., 1982; Mackenzie et al., 1984; Tobias et al., 1984), but in all these cases the intermediate has been stabilized in an inactive form by low pH, low temperature, or both. We believe that this is the first reported ^{13}C spectrum of a fully functional acyl intermediate, a spectrum we were able to obtain because of the slow deacylation of [(5-*n*-alkyl-2-furoyl)acyl]chymotrypsins near room temperature and neutrality. Even though the furoyl substrate is poor, the ability to study the acyl-enzyme during turnover holds out the hope of obtaining data pertinent to the catalytic mechanism.

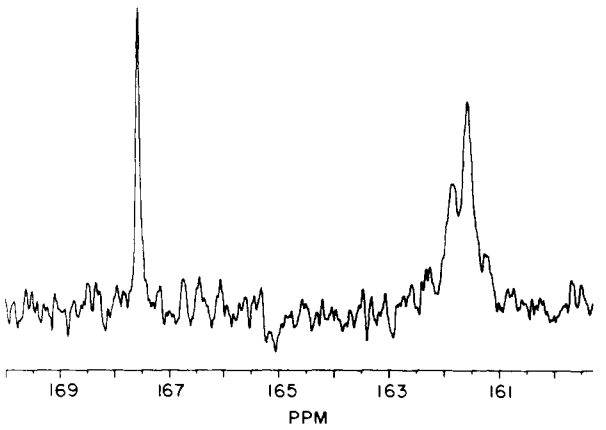

FIGURE 5: Steady-state spectrum of acylchymotrypsin at an apparent pH of 7.5. The spectrum is the time-averaged result in 100 min of accumulation with a 0.6 mM enzyme concentration at 25 °C. Substrate saturation over the entire time of data accumulation was inferred from the linear increase of the product signal height at 167.6 ppm. Spectra were referenced against 22% (v/v) internal perdeuterated Me_2SO.

There was no evidence for any peak near 100 ppm, the approximate shift expected for the carbon of a tetrahedral intermediate on the basis of the ^{13}C NMR spectra of ortho esters. Of peripheral interest, albeit of some importance, is our finding that acylchymotrypsin appears to be sensitive to inactivation under the experimental conditions of our experiments when excess substrate is not available. This inactivation was evidenced by a stable, more complex 161 ppm peak that was observed when jumping from low to neutral pH in the absence of excess substrate, or when steady-state turnover ceased. We suggest that this inactivation is due to chymotrypsin catalysis of acylchymotrypsin proteolysis. Thus, at the relatively high protein and Me_2SO concentrations used in these and other reported ^{13}C NMR experiments, it is important to ensure that no free enzyme is permitted in the reaction mixture in order to avoid possible artifacts.

We were encouraged to find that the acyl-enzyme [^{13}C]-carbonyl shift has a pH dependence that can be fit to a simple titration curve with a pK_a of 7.0. This value is in good agreement with the independently determined kinetic pK for the steady-state turnover of the *p*-nitrophenyl substrate. Several NMR titrations have been reported for various nuclei attached to the active site serine residue (Porubcan et al., 1979) and/or attached to the imidazole side chain of serine protease [see reviews by Steitz & Shulman (1982) and Kanamori & Roberts (1983)]. Our pK_a determination agrees approximately

with the results of these other experiments but, unlike them, pertains directly to the active acylated protein. From the fit of the titration data to eq 1 we obtained a chemical shift for the high-pH form of the acyl-enzyme, in which the active-site histidine is presumably unprotonated, that is similar to the chemical shift of ethylfuroate. While this suggests that the carbonyl carbon atom of the active acyl-enzyme may be unperturbed by its environment, we cannot rule out a fortuitous resemblance without fully understanding the physical basis of the pH dependence.

Above pH 6.6 we observed a shoulder or small peak on the downfield side of the major acyl peak. The chemical shift of this downfield component may have a pH dependence similar to that of the major peak, but better data will be required to establish this point quantitatively. The observation of this downfield feature is interesting because of the suggestion that acylchymotrypsin may have more than one active form (Wedler et al., 1975; Baggott & Klapper, 1976; Ando et al., 1980; MacClement et al., 1981). Shah and co-workers (1984) have recently reported that the NMR spectrum of the hemiacetal formed between *N*-acetyl-L-[1-^{13}C]phenylalaninal and chymotrypsin displays two signals above pH 7, but not at lower pH. They gave three possible suggestions in explanation: the simultaneous presence of neutral and anionic hemiacetals, slowly interconverting conformational isomers, or an interaction with an active site titratable group such as histidine-57. In our case, the first does not apply, and the last is unlikely in view of the smooth change in chemical shift with pH. Whether the shoulder we observe does represent a second structural form of the active acyl-enzyme must await further results.

Our success in finding a ^{13}C signal associated with an active acylchymotrypsin suggests that more information about the acyl form of the enzyme may be available from ^{13}C NMR experiments. Specifically, it should now be possible to determine with relaxation studies the acyl group mobility in the catalytically functional active site.

ACKNOWLEDGMENTS

We thank Professors T. Kaiser and D. Mueller, who graciously arranged access for us to a Nicolet 360-MHz spectrometer and a Bruker 400-MHz spectrometer, respectively. Spectra were obtained at Ohio State with a 500-MHz Nicolet and a 300-MHz Bruker located in the Campus Chemical Instrument Center.

Registry No. PNPPF, 59212-59-8; [^{13}C]PNPPF, 96055-97-9; isopropyl alcohol, 67-63-0; [^{13}C]carbon monoxide, 1641-69-6; dimethylamine, 124-40-3; [^{13}C]dimethylformamide, 32488-43-0; *n*-propylfuran, 4229-91-8; [^{13}C]-5-*n*-propyl-2-furfural, 96055-98-0; *p*-nitrophenol, 100-02-7.

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