Direct ^{13}C NMR Evidence for a Tetrahedral Intermediate in the Binding of a Pepstatin Analogue to Porcine Pepsin

Daniel H. Rich,*† Michael S. Bernatowicz,† and Paul G. Schmidt‡

School of Pharmacy, University of Wisconsin—Madison Madison, Wisconsin 53706
Oklahoma Medical Research Foundation and Department of Biochemistry and Molecular Biology University of Oklahoma Health Sciences Center Oklahoma City, Oklahoma 73104

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Pepstatin (1)[1] (see Chart I) is a specific and potent inhibitor[2] of acid (carboxyl) proteases[3] including pepsin, renin, cathepsin D, and several microbial enzymes. The peptide binds to the active site of these enzymes with an unusually small dissociation constant[4,5] (5×10^{-11} M for the porcine pepsin–pepstatin interaction). Steady-state kinetic inhibition studies using a wide variety of synthetic pepstatin analogues[6] demonstrate the importance of the C_3 OH group of statine (4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoic acid (Sta)) at position 3 with the proper stereochemistry (*S*) needed for strongest binding. In line with this a recent X-ray diffraction analysis of a crystal with pepstatin bound to the acid protease from *Rhizopus chinensis*[7] shows the hydroxy group hydrogen bonded to one of the protein active-site aspartyl side-chain carboxyl groups.

The statine-3 residue in pepstatin was earlier proposed as a transition-state analogue[8,9] because of the tight binding and specificity for acid proteases of certain Sta-containing peptides and because of its structural similarity to the tetrahedral intermediate for amide bond hydrolysis. If this were the case one would expect that the *ketone* analogue of Sta at position 3 would resemble a substrate and could be used to probe for events in the catalytic process. One would then predict that hybridization of C_3 would tend toward tetrahedral in the enzyme-inhibitor complex.

We have synthesized a ketone analogue of pepstatin and shown by steady-state kinetic inhibition studies that it is an effective inhibitor of pepsin.[10] In the present communication we used ^{13}C NMR to determine hybridization at C_3 when the inhibitor is bound to pepsin. Analogous earlier ^{13}C NMR studies of trypsin plus soybean trypsin inhibitor[11] or bovine pancreatic trypsin inhibitor[12] showed that these complexes do not contain covalent, fully tetrahedral intermediates, because the chemical shift of the ^{13}C-labeled reactive carbonyl in the inhibitor is not appreciably affected by binding to the enzyme. We obtain a very different result in the present study for the binding of a keto Sta peptide to pepsin.

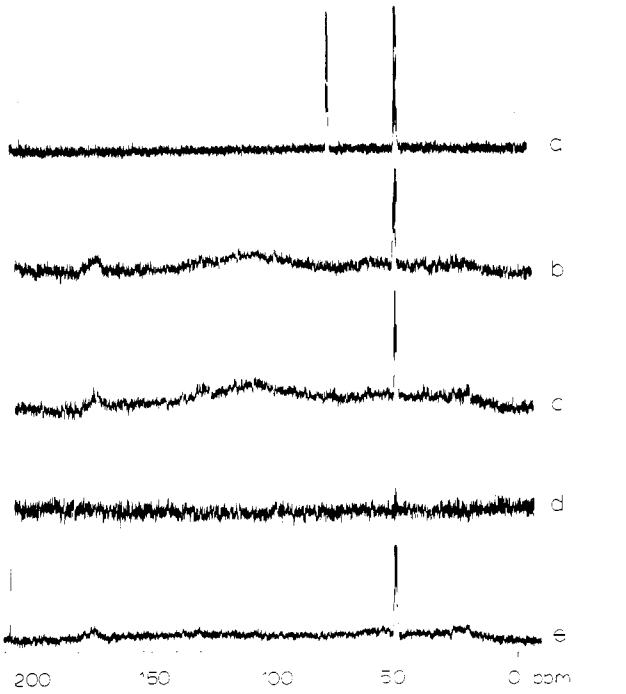
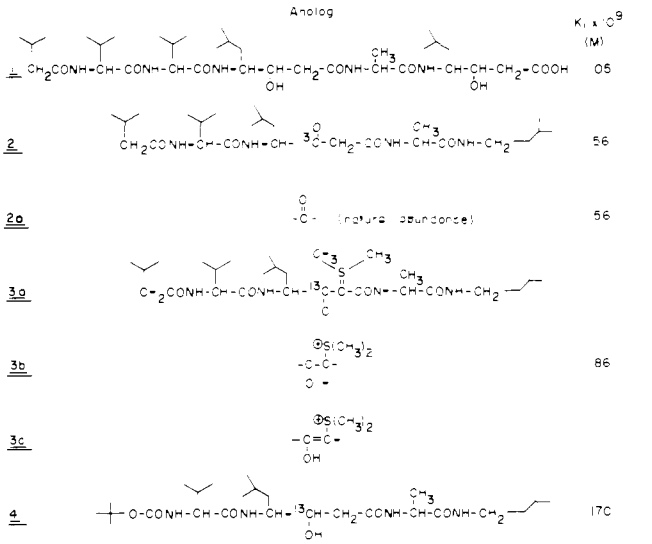


Figure 1. ^{13}C NMR spectra at 75.5 MHz of isotopically enriched (99% ^{13}C at C_3) keto Sta analogue **2**: (a) 1.5 mM **2** in $CHCl_3$-*d* (15% CH_3OH-d_4) (Me_4Si = 0 ppm), solvent peaks at 76.9 ($CHCl_3$-*d*) and 49.2 ppm (CH_3OH-d_4); (b) 0.5 mM **2** plus 0.5 mM porcine pepsin in ^2H_2O, pH* 4.7, 28 °C, MeOH-d_4 (5%) = 49.2 ppm, broad band ^1H decoupling, 19 408 scans, 50° pulse, 1.25 s repetition period, 5-Hz line broadening due to exponential multiplication of free induction decay; (c) 0.5 mM **2a** (natural abundance analogue of **2**) plus 0.5 mM porcine pepsin in ^2H_2O, pH* 4.7 28 °C. (experimental conditions as in b). (d) difference spectrum, b – c. (e) 0.5 mM pepstatin A (**1**) added to 0.5 mM pepsin plus 0.5 mM **2**, ^2H_2O, pH* 4.5, 25 °C, 5436 scans (other parameters as in b or c. In all spectra the large multiplet at 49.2 ppm (off scale) is from CH_3OH-d_4, due to addition of peptide stock solutions.

Chart I



Pepstatin A and the synthetic analogues[13] used are illustrated in Chart I, where their kinetic inhibition constants are also listed. A ^{13}C NMR spectrum[14] of ketone **2** in $CHCl_3$-*d* reveals the

† University of Wisconsin—Madison.
‡ University of Oklahoma Health Sciences Center.

(1) Abbreviations: Sta, 4(S)-amino-3(*S*)-hydroxy-6-methylheptanoic acid; keto Sta, 4(*S*)-amino-3-oxo-6-methylheptanoic acid, pH*, uncorrected pH reading in ^2H_2O.

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(13) Analogues **2**, **3**, and **4** were synthesized as previously described[10] by using precursors specifically enriched in ^{13}C. L-[1-^{13}C]Leu (99% ^{13}C) was obtained from Merck Sharp and Dohme and converted to (3*S*,4*S*)-statine (99% 3-^{13}C) and related peptides by established methods[5,6] and oxidized to the ketone.[10] Oxidation of **4** with pyridine–SO_3 complex followed by deprotection and acylation gave **3a** in 50% yield. Reduction of **3a** with zinc in acetic acid gave **2**. Synthetic details will be published separately.

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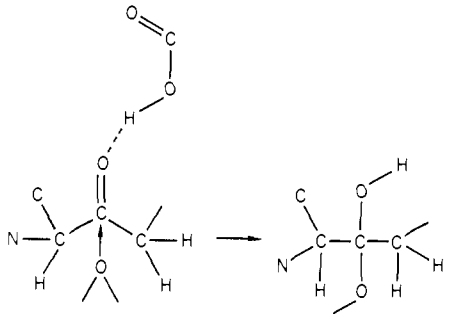
Scheme 1



isotopically enriched carbonyl peak at 204.2 ppm (Figure 1a). When 1 equiv of ketone **2** is added to a solution of porcine pepsin in ^2H_2O, the low-field signal disappears and a new peak appears at 99 ppm (Figure 1b). With the natural abundance peptide **2a** instead of **2** the spectrum appears to be missing only the peak at 99 ppm (Figure 1c), This is confirmed by the difference spectrum 1b minus 1c (Figure 1d), which contains only the 99-ppm peak, Addition of pepstatin A (**1**) to a sample containing ketone **2** plus pepsin yielded a spectrum with the inhibitor signal at 207.2 ppm (Figure 1e). Pepstatin A, which binds to pepsin 1000-fold tighter than ketone **2**,[10] displaces the keto Sta analogue, which is then observed as the free species in solution.

A shift of 105–108 ppm upfield for the C_3 resonance when ketone **2** binds to pepsin is direct evidence that the carbonyl carbon is converted from trigonal to tetrahedral geometry or very close to it, due to nucleophilic addition to the carbonyl. An analogous shift occurs for fructose, whose ketone carbon chemical shift of 217.4 ppm in pyridine-d_6 (Me_4Si) moves to 107.5 and 105.0 in the α- and β-furanose forms or 99.8 and 100.6 ppm in the α- and β-pyranose structures.[15] Baillargeon et al.[11] examined chemical shifts in a series of constrained model compounds where a carbonyl was undergoing partial nucleophilic attack. They found that even, for a nitrogen–carbonyl carbon bond distance of only 1.6 Å (compared to 1.48 Å for a covalent bond) the carbonyl chemical shift remained at about 200 ppm, These data would suggest that nucleophilic addition to form a tetrahedral intermediate has occurred when **2** is bound to pepsin. Oxygen is the most likely nucleophile since only O ligands or water would be close to the inhibitor carbonyl group in the pepsin active site.[7]

When an α-dimethyl sulfonium derivative of ketone **2** was examined, the results were quite different. Sulfonium ylide **3a** displays a single peak for the isotopically enriched carbon at 188 ppm in $CHCl_3$-*d* (data not shown). Bound to pepsin, the peak splits into two very broad resonances near 191 and 192 ppm. No other inhibitor peaks were found in the spectrum of the complex. Preliminary experiments showed that the peaks become narrower at lower temperature, so it is most likely that the two broad peaks represent two forms of bound **3**, probably the keto and enol forms (**3b** and **3c**), exchanging at a rate on the order of the chemical shift difference between them. In either case the carbon remains trigonal in the complex.

Conclusion. The carbonyl group in keto Sta of peptide **2** undergoes a major chemical change when the peptide binds to the pepsin active site. A tetrahedral species is formed whose properties are consistent with Scheme I. Further experiments are aimed at determining the nature of the nucleophile and the constraints on peptide structure leading to development of the intermediate.

(14) ^{13}C NMR spectra were taken at 75.5 MHz with a Cryomagnet Systems CMS 300/50 magnet and 10 mM probe (Cryomagnet Systems, Indianapolis, IN). In-house rf electronics and a Nicolet 1180 computer complete the system. Temperatures were maintained with a thermostated N_2 stream and were measured by insertion of a thermocouple directly in the NMR sample. Porcine pepsin was prepared by activation of pepsinogen as previously described (Schmidt, P. G. In "Frontiers in Protein Chemistry"; Liu et al., Eds.; Elsevier: New York, 1980; pp 63–87). Peptides were added to protein solutions as 10 mM stock solutions in CH_3OH-d_4. Generally this resulted in a 5% solution of the alcohol, a level that does not affect binding of pepstatin analogues to pepsin.

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Picosecond Dynamics of Solution-Phase Photofragmentation of $[Mn_2(CO)_{10}]$

Lewis J. Rothberg, N. John Cooper, Kevin S. Peters,* and Veronica Vaida*†

Department of Chemistry, Harvard University Cambridge, Massachusetts 02138

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The identification and characterization of primary photochemical pathways are central to understanding the photochemistry of organo–transition metal complexes. Picosecond flash photolysis allows direct spectroscopic observation of transients following photoexcitation and is therefore ideal for this purpose. We report the first application of this technique to the study of an important photochemical organo–transition metal reaction, the solution photolysis of $[Mn_2(CO)_{10}]$. These experiments provide the first direct evidence for two primary chemical pathways following optical excitation of $[Mn_2(CO)_{10}]$.

The picosecond absorption spectrometer has been described previously.[1] $[Mn_2(CO)_{10}]$, purchased from Alfa Products, was purified by sublimation and added to the sample cell as a 0.05 M solution in ethanol, which was flowed through the cell to avoid buildup of photoproducts. Previous solution flash photolysis studies[2-4] of $[Mn_2(CO)_{10}]$ and $[Re_2(CO)_{10}]$ were carried out on longer time scales on which secondary bimolecular processes are frequently rapid, and the results had to be analyzed in terms of a complex pattern of photochemical and thermal reactions. Picosecond experiments allow direct observation of primary photoproducts under conditions where subsequent bimolecular reactions do not occur. Photolysis employed a 355-nm, 1-mJ, 25-ps excitation pulse and absorption spectra of the products were recorded between 400 and 850 nm with probe delays of 0–250 ns. There is no change in the spectra within this delay range.

A typical spectrum (Figure 1) shows two absorption maxima with λ_{max} 780 and 480 nm. The first of these can be assigned to $[Mn(CO)_5]\cdot$, presumably produced by homolysis, by comparison with the spectrum of $[Mn(CO)_5]\cdot$ observed in a pulse radiolysis[5] as well as a recent matrix isolation experiment.[6] The species generated in ethanol by pulse radiolysis is reported[5] to have a red absorption between 680 and 1030 nm, while $[Mn(CO)_5]\cdot$ synthesized[6] by UV photolysis of $[HMn(CO)_5]$ in solid CO matrices at 10–20 K displays a spectrum with λ_{max} 798 nm and a continuous UV absorption at λ <340 nm. In the present experiment, as in the previous pulse radiolysis work, the UV region (λ <400 nm) is obscured by absorptions from the parent and other species, and the continuous UV absorption at λ <340 nm[6] could not be recorded. The spectrum presently reported for $[Mn(CO)_5]\cdot$ constitutes the first direct observation of this species in solution flash photolysis. No band similar to the 480-nm one observed in the present experiment has been reported for $[Mn(CO)_5]\cdot$ and the

† A. P. Sloan Fellow.

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