Biochemistry

 Volume 27, Number 22 November 1, 1988

Accelerated Publications

Isotope-Edited Proton NMR Study on the Structure of a Pepsin/Inhibitor Complex

Stephen W. Fesik,*,‡ Jay R. Luly,§ John W. Erickson,∥ and Celerino Abad-Zapatero∥

NMR Spectroscopy, Cardiovascular Chemistry, and Protein Crystallography, Pharmaceutical Discovery Division, Abbott Laboratories, Abbott Park, Illinois 60064

Received July 21, 1988; Revised Manuscript Received September 8, 1988

ABSTRACT: A general approach is illustrated for providing detailed structural information on large enzyme/inhibitor complexes using NMR spectroscopy. The method involves the use of isotopically labeled ligands to simplify two-dimensional NOE spectra of large molecular complexes by isotope-editing techniques. With this approach, the backbone and side-chain conformations (at the P_2 and P_3 sites) of a tightly bound inhibitor of porcine pepsin have been determined. In addition, structural information on the active site of pepsin has been obtained. Due to the sequence homology between porcine pepsin and human renin, this structural information may prove useful for modeling renin/inhibitor complexes with the ultimate goal of designing more effective renin inhibitors. Moreover, this general approach can be applied to study other biological systems of interest such as other enzyme/inhibitor complexes, ligands bound to soluble receptors, and enzyme/substrate interactions.

Renin, an aspartic proteinase, plays an important role in the regulation of blood pressure [for a review, see Re (1987)]. Although an experimentally determined structure of renin has not been reported, models of renin have been described (Blundell et al., 1983; Sibanda et al., 1984; Akahane et al., 1985; Carlson et al., 1985; Sham et al., 1988) that have proved useful in designing improved renin inhibitors. These models are based on experimentally determined three-dimensional structures of fungal aspartic proteinases (Bott et al., 1982; James et al., 1982; Pearl & Blundell, 1984) that exhibit sequence homology to human renin (21, 22, and 25% for penicillopepsin, endothiapepsin, and *Rhizopus* pepsin, respectively). Better models of renin/inhibitor complexes could potentially be obtained by examining experimental structures of mammalian aspartic proteinases such as porcine pepsin (Andreeva et al., 1984), which are more homologous (39%) to renin (Sham et al., 1988).

In principle, NMR spectroscopy could be used to provide structural information on enzyme/inhibitor complexes as large as a pepsin/inhibitor complex (MW ~ 35000) from nuclear Overhauser effect (NOE)[1] measurements that indicate the proximity of protons in space (Noggle & Schirmer, 1971). However, conventional two-dimensional NOE spectra of enzyme/inhibitor complexes of this size are extremely complex and too difficult to analyze. Recently, experimental approaches have been proposed (Otting et al., 1986; Bax & Weiss, 1987; McIntosh et al., 1987; Fesik et al., 1987a) to simplify complicated 2D NOE spectra. Using these methods that rely on the selective observation of protons attached to isotopically labeled nuclei (e.g., ^{13}C, ^{15}N) (Freeman et al., 1981; Bendall et al., 1981), it is possible to determine the structures of large molecules and molecular complexes.

In this paper we describe the application of isotope-edited two-dimensional NOE experiments to the structural study of a pepsin/inhibitor (Figure 1) complex. Even though this complex is large from an NMR perspective, isotope editing allowed both the conformation of an enzyme-bound inhibitor and structural information on the active site to be obtained. This system was chosen as part of our ongoing investigations aimed at understanding the subtle structural differences among various inhibitor/aspartic proteinase complexes with the ultimate goal of providing structural information (Sham et al., 1988; Fesik et al., 1987b,c) to aid in the design of improved renin inhibitors.

* Address correspondence to this author.
‡ NMR Spectroscopy.
§ Cardiovascular Chemistry.
∥ Protein Crystallography.

[1] Abbreviation: NOE, nuclear Overhauser effect.

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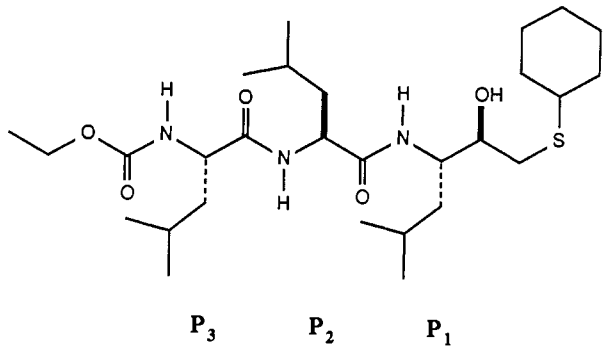

FIGURE 1: Structure of the pepsin inhibitor used in this study.

Experimental Procedures

Porcine pepsin A obtained from Sigma Chemical Co. was purified on DE-52 cellulose equilibrated with 0.1 M sodium acetate (pH = 5.6), dialyzed against 50 mM ammonium acetate (pH = 5.0), and lyophilized. The labeled tripeptide pepsin inhibitor was synthesized by using isotopically labeled (^{15}N, ^{13}C, or ^{2}H) leucine (Cambridge Isotopes) by methods previously described for the preparation of related inhibitors (Luly et al., 1987a).

Sample Preparation. A 1 mM solution of the pepsin/inhibitor (1/1) complex was prepared for the NMR experiments by rapidly mixing a DMSO-d_6 solution (0.025 mL) of the isotopically labeled inhibitor into a $H_2O/^2H_2O$ (9/1) or a ^2H_2O solution of pepsin at pH = 3.7. The small amount (5%) of DMSO used to solubilize the inhibitor did not have any deleterious effect on the ability of the tripeptide to inhibit pepsin ($IC_{50} = 1.7 \times 10^{-7}$ M) as determined in a porcine pepsin assay (Luly et al., 1987b).

NMR. All NMR experiments were performed at 40 °C on a General Electric GN-500 NMR spectrometer. For the ^{15}N-isotope-edited 2D NOE experiments, a proton probe with a broad-band coil on the outside tuned to ^{15}N (50.7 MHz) was employed, with a proton and ^{15}N 90° pulse of 10.5 and 88 μs, respectively. The ^{13}C-isotope-edited NMR experiments were conducted with a $^{13}C/^{1}H$ dual probe with a 21-μs proton 90° pulse and a 18-μs ^{13}C 90° pulse. The sequence used to acquire the data (Fesik et al., 1987a) consisted of a conventional 2D NOE experiment in which a proton spin–echo pulse sequence (90°–τ–180°–τ) was substituted for the last proton pulse. In addition, a 180° pulse was applied on alternate scans at the frequency of the X-nucleus (^{13}C or ^{15}N) concurrent with the proton 180° refocusing pulse of the spin–echo part of the experiment. By subtraction of the data collected with or without a 180° pulse applied to the X-nucleus, only those protons attached to the isotopically labeled nuclei (ω_2) and their dipolar coupled partners (ω_1) were observed. Decoupling of the X-nucleus was achieved during the t_1 and acquisition (t_2) periods with a MLEV-64 pulse sequence (Levitt et al., 1982) using a 90° pulse of 58 μs (^{13}C) and 184 μs (^{15}N).

The NOE experiments were recorded with a mixing time of 30 or 50 ms, a sweep width of 7812 Hz, and a 1.4-s delay between scans. Typically, 512 scans were recorded for 2 × 110 t_1 increments for a total time of 54 h. A spin–echo delay of τ = 3.0 and 3.5 ms was employed for the ^{13}C- and ^{15}N-isotope-edited 2D NOE experiments, respectively.

NMR data were processed either on a VAX 780 computer using software obtained from Dr. Dennis Hare or on a slave CSPI Minimap array processor using software written by Dr. Erik Zuiderweg. The complex time domain data in the t_1 dimension were assembled from the extracted real parts of the two sets of Fourier-transformed spectra as previously described (States et al., 1982). For the ^{15}N-isotope-edited 2D NOE spectra, free induction decays were exponentially multiplied in both dimensions by using a line broadening of 30 Hz before being Fourier transformed. For these data sets, base-line correction was performed in both ω_1 and ω_2 with a fifth-order polynomial. Gaussian multiplication was applied to the free induction decays obtained in the ^{13}C-isotope-edited experiments by using a negative line broadening of 20 Hz.

Results and Discussion

Figure 2a depicts a contour map of an isotope-edited two-dimensional NOE experiment of the tri-^{15}N-labeled inhibitor complexed with porcine pepsin. As shown in the figure, the spectrum is markedly simplified compared to unedited 2D NOE spectra of the complex, which would contain well over 1000 cross-peaks in the same spectral region. Only diagonal peaks corresponding to the labeled P_1, P_2, and P_3 amide protons (assigned by using single ^{15}N-labeled inhibitors; Fesik et al., 1987b) and NOE cross-peaks between the amide protons and other protons of the enzyme and inhibitor are observed. These NOE cross-peaks identify protons that are close in space (<3.0 Å) to the "labeled" protons.

In order to determine which proton pairs correspond to the NOE cross-peaks, additional experiments were performed with ^{15}N-labeled inhibitors that were also deuterium labeled. Those NOEs involving protons of the inhibitor that have been substituted with a deuterium should disappear from the NOE spectra (Figure 2b,c), allowing the NOE cross-peaks to be assigned from comparisons of the spectra. For example, from the missing cross-peak (Figure 2b, indicated by the arrow) in the NOE spectra obtained with the inhibitor deuteriated at the P_2 site and ^{15}N labeled at P_1 and P_3, the NOE observed at this location in Figure 2a could be assigned to an NOE between P_1NH and one of the protons of the P_2 residue. On the basis of its resonance position, the NOE is assigned to P_1NH/P_2H^{α}. Analogously, from the NOE cross-peak absent in Figure 2c, a NOE between P_2NH and P_3H^{α} was defined.

These intense NOEs observed between the amide and α protons of adjacent amino acid residues of the enzyme-bound inhibitor in conjunction with the absence of NOEs between the amide protons are characteristic of an extended conformation (Wüthrich et al., 1984) of the main chain. These results are in agreement with the main-chain conformations of different inhibitors bound to homologous aspartic proteinases (James et al., 1982; Bott et al., 1982; Foundling et al., 1987) as well as to pepsin (unpublished data) determined by X-ray crystallography.

The side-chain conformations of the P_3 and P_2 sites were also examined by isotope-filtered NMR methods using ^{15}N- and ^{13}C-labeled inhibitors. The proton signals of the P_2 and P_3 side chains were assigned from ^{1}H–^{13}C correlation experiments (Bax et al., 1983) on the basis of the number of cross-peaks correlating the ^{1}H and ^{13}C NMR signals (two H^{β}/C^{β} and one H^{γ}/C^{γ}) and the characteristic differences in the β and γ ^{13}C NMR chemical shifts (Wüthrich, 1976). Figure 3A depicts a contour map of an isotope-edited 2D NOE experiment of a pepsin/inhibitor complex containing the inhibitor that was uniformly ^{13}C-labeled (85%) at the P_3 site. The larger NOE in Figure 3A between P_3H^{α} and $P_3H^{\beta 3}$ compared to P_3H^{α} and $P_3H^{\beta 2}$ and the intense NOE observed between P_3NH and P_3H^{γ} (parts a and b of Figure 2) suggest a χ^1 angle (ca. −60°) consistent with a g^+ side-chain conformation in which the C^{γ} is trans to the carbonyl group (Janin et al., 1978). Additional NOEs ($P_3NH/H^{\beta 2}$ and $P_3H^{\alpha}/H^{\delta 2}$) and the absence of an NOE between P_3H^{α} and $P_3H^{\delta 1}$ confirmed the conformation of the P_3 side chain depicted in Figure 4.

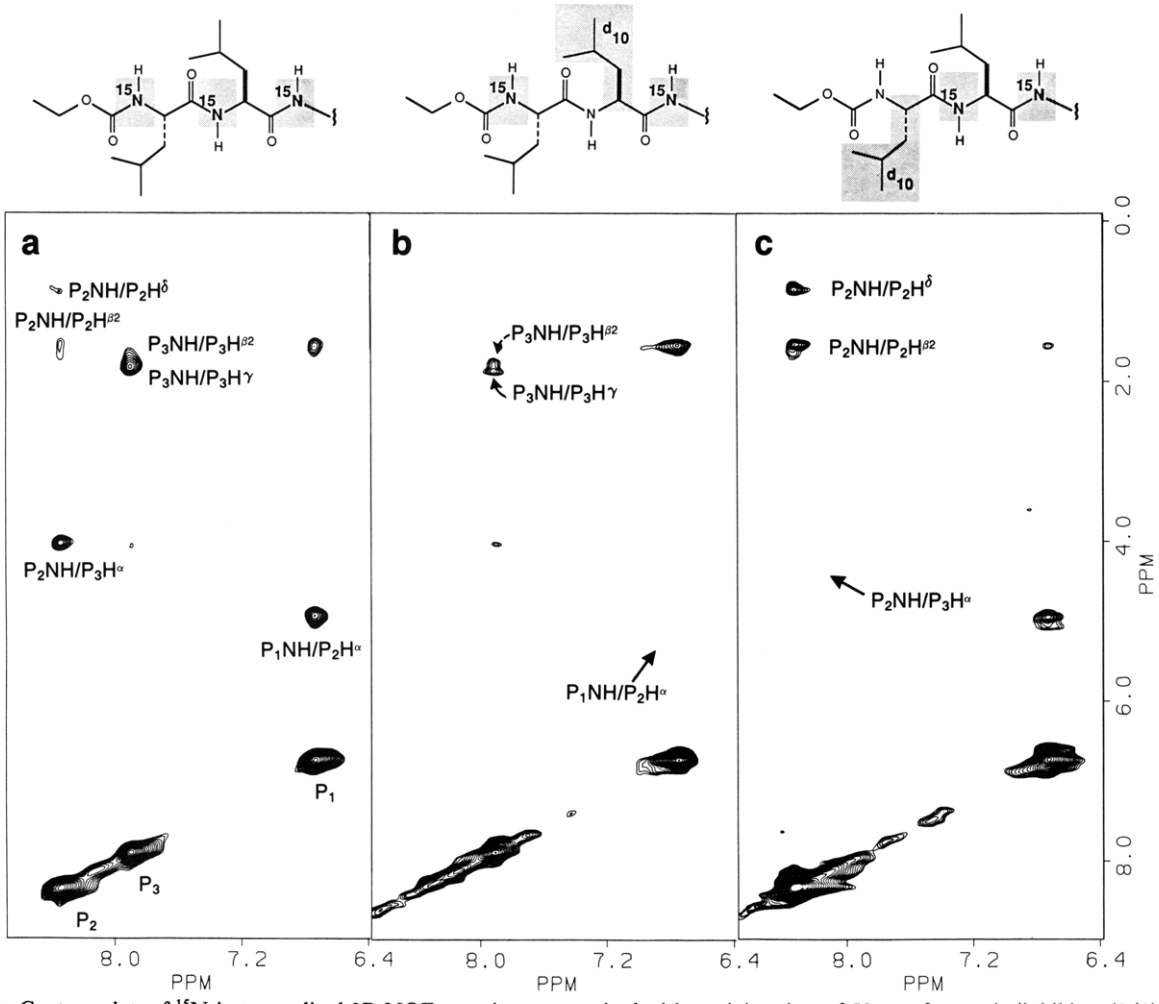

FIGURE 2: Contour plots of ^{15}N-isotope-edited 2D NOE experiments acquired with a mixing time of 50 ms of a pepsin/inhibitor (1/1) complex by using the labeled inhibitors shown at the top of the spectra. The plots were obtained with the graphics software of Dr. D. Hare. In (a), diagonal peaks corresponding to the P_1–P_3 amide protons are labeled, and in (b) and (c), arrows indicate the location in the spectra where cross-peaks are absent due to deuterium labeling.

The side-chain conformation at the P_2 site was determined from NOEs observed between P_2H^{α} and $P_2H^{\beta 3}$ and between P_2H^{α} and P_2H^{γ} in the ^{13}C-isotope-edited 2D NOE experiment (Figure 3B) along with NOEs involving P_2NH ($P_2NH/H^{\beta 2}$ and P_2NH/H^{δ}) depicted in parts a and c of Figure 2. Analogous to the P_3 site, the P_2 side chain adopts a g^+ conformation that is of low energy and regularly found in X-ray crystal structures of proteins (Janin et al., 1978). However, as shown in Figure 4, the conformations of P_2 and P_3 were found to be different about the C^{β}–C^{γ} bond (P_2, $\chi^2 \sim 60°$; P_3, $\chi^2 \sim 180°$).

In addition to the bound conformation of the pepsin inhibitor, information was also obtained on the structure of the active site from NOEs between the inhibitor and pepsin (boxed NOE cross-peaks in parts A and B of Figure 3). NOEs were observed (Figure 3A) between both methyl groups of the P_3 side chain and protons of the enzyme resonating at 0.56 (Hg) and 6.91 (Hb) ppm. As shown schematically in Figure 4, these NOEs suggest that an aromatic proton of pepsin (6.91 ppm) and a proton at 0.56 ppm of pepsin are positioned between both P_3 methyl groups of the inhibitor. Other NOEs were observed between protons of the enzyme and only one of the P_3 methyl groups of the inhibitor. The NOE between $P_3H^{\delta 2}$ and a proton at 7.34 (Ha) indicates the close proximity of this methyl group to another aromatic proton on the enzyme. On the basis of an additional NOE, the other P_3 methyl group ($P_3H^{\delta 1}$) must be near a proton of pepsin that resonates at 2.65 ppm (Hd).

From the inspection of a model of this pepsin/inhibitor complex built from a partially refined X-ray crystal structure of a similar inhibitor complexed with porcine pepsin,[2] the inhibitor/pepsin NOE data were rationalized. NOEs to Ha (7.34 ppm) and Hd (2.65 ppm) could easily be accounted for by Phe 111 H^{ϵ} and Glu 13 H^{γ}, respectively, which lie on opposite sides of the P_3 side chain close to different P_3 methyl groups in the model based on the crystal structure. The NOEs to Hg (0.56 ppm) could be explained by the presence of an upfield-shifted methyl group (Thr 77 H^{γ} in the model), which is shielded by an aromatic ring in the enzyme. Finally, the NOE between both P_3 methyl groups and Hb (6.91 ppm) can be accounted for by Tyr 114 H^{ϵ}. In the X-ray crystal structure of a similar pepsin/inhibitor complex[2], Tyr 114 is pointing up and away from the inhibitor. However, the inhibitor used in the X-ray studies contains an iodo-Phe at the P_3 site. With a smaller residue at P_3 (e.g., Leu), the phenyl ring of Tyr 114 can easily be rotated into the active-site pocket close to the two methyl groups of P_3 Leu.

For the P_2 side chain of the inhibitor, NOEs were observed (Figure 3B) between the P_2 methyl groups and protons of the

[2] This crystal structure was solved by using the inhibitor (2*S*,3*R*,4*S*)-2-(EtOC-p-I-Phe-Leu-amino)-1-cyclohexyl-3,4-dihydroxy-6-methylheptane complexed with porcine pepsin. A preliminary account of this work was presented by J. Erickson, C. Abad-Zapatero, T. J. Rydel, and J. Luly at the 14th International Congress of Crystallography, Perth, Australia, 1987.

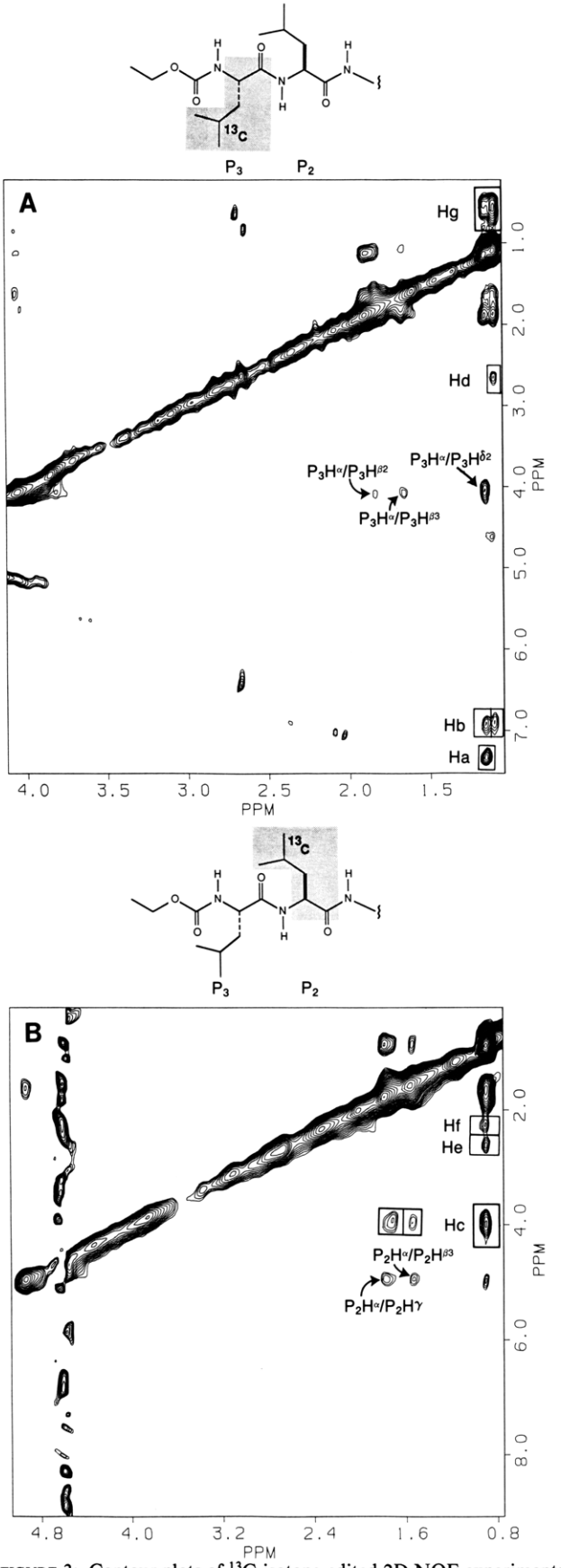

FIGURE 3: Contour plots of ^{13}C-isotope-edited 2D NOE experiments using inhibitors uniformly ^{13}C-labeled (85%) at (A) P_3 and (B) P_2. The boxes indicate NOEs between labeled inhibitor and pepsin.

enzyme at 3.95 (Hc), 2.62 (He), and 2.26 (Hf) ppm and between both P_2H^{β} protons and a proton at 3.95 (Hc) ppm. In contrast to the P_3 side chain, no NOEs were observed from

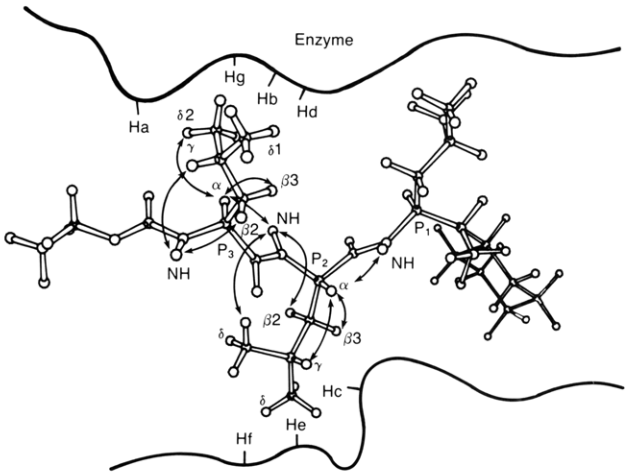

FIGURE 4: Computer-generated model of the bound pepsin inhibitor that is consistent with the NMR data. The arrows indicate NOEs observed between proton pairs of the ligand. Inhibitor/enzyme NOEs are designated by letters corresponding to the chemical shifts of the enzyme protons and have been interpreted on the basis of a model built from a partially refined X-ray crystal structure of a similar inhibitor complexed with pepsin (Ha, 7.34 ppm, Phe 111 H^{ϵ}; Hb, 6.91 ppm, Tyr 114 H^{ϵ}; Hc, 3.95 ppm, Thr 218 H^{α}; Hd, 2.65 ppm, Glu 13 H^{γ}; He, 2.62 ppm, Met 290 H^{γ}; Hf, 2.26 ppm, Met 290 H^{ϵ}; Hg, 0.56 ppm, Thr 77 H^{γ}).

the P_2 side chain to any aromatic protons. This is consistent with the lack of aromatic amino acids in the vicinity of the P_2 site in the model of the complex based on an X-ray crystal structure. The closest amino acid residue to the P_2 site in the model is Met 290, which could account for the NOEs observed at 2.62 (Met H^{γ}) and 2.26 ppm (Met H^{ϵ}). Furthermore, the close proximity of Thr 218 H^{α} to the P_2 side chain is consistent with the NOEs observed to the proton (Hc) on the enzyme which resonates at 3.95 ppm.

In this paper we have demonstrated that, even for a large enzyme/inhibitor complex, NMR spectroscopy can be used to determine the conformation of a tightly bound ligand and help define its active-site environment. In contrast to the complete three-dimensional structures that can be obtained by X-ray crystallography, this approach using isotopically labeled ligands only provides structural information about the inhibitor and its immediate vicinity. However, for the purpose of designing more effective inhibitors, this information may be sufficient (Fesik, 1988). For example, on the basis of the conformation of enzyme-bound inhibitors determined solely from NMR data, analogues could be designed that possess the important functional groups held in their proper orientation by drastically different structural frameworks. Such analogues may increase the metabolic stability, absorption, or selectivity of the compound while retaining biological activity. In addition, NOEs between the inhibitor and enzyme, even if unassigned, can be of value to test whether different inhibitors are binding to the same site on the enzyme as evidenced by the observation of the same characteristic inhibitor/enzyme NOEs. These observations are important to help rationalize the structure/activity relationships. Moreover, in those cases in which the inhibitor/enzyme NOEs can be assigned either by performing additional NMR experiments with isotopically labeled enzyme and/or by considering structural information from other sources (e.g., X-ray crystallography, model building) such as in the study described here, the NMR data become even more powerful. When combined with a three-dimensional structure of a similar enzyme/inhibitor complex or even the enzyme alone, the NMR-determined conformation of the inhibitor and the inhibitor/enzyme NOEs can be used

to dock the inhibitor within the active-site pocket.

In this study we have shown that isotope-edited proton NMR spectroscopy is a valuable method for obtaining detailed structural information on large enzyme/inhibitor complexes. In this case, due to the structural similarity between porcine pepsin and human renin, the structural information may aid in the modeling of renin/inhibitor complexes to help accomplish the ultimate goal of designing more effective renin inhibitors.

ACKNOWLEDGMENTS

We thank N. BaMaung for technical assistance in the preparation of labeled inhibitors; R. Edalji and D. Winn for the purification of porcine pepsin; T. Perun for his support and encouragement; and H. Stein, E. R. P. Zuiderweg, G. Bolis, and J. Greer for helpful discussions.

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