Biochemical Pharmacology, Vol. 40, No. 1, pp. 161–167, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

NMR METHODS FOR DETERMINING THE STRUCTURES OF ENZYME/INHIBITOR COMPLEXES AS AN AID IN DRUG DESIGN

STEPHEN W. FESIK,* ERIK R. P. ZUIDERWEG, EDWARD T. OLEJNICZAK and ROBERT T. GAMPE, JR.

Pharmaceutical Discovery Division, Abbott Laboratories, Abbott Park, IL 60064, U.S.A.

Abstract—Four approaches are described for providing detailed structural information on large enzyme/inhibitor complexes to aid in the design of improved enzyme inhibitors. In one approach, proton NMR spectra are simplified by isotope-editing procedures in which only those protons that are attached to isotopically labeled nuclei (e.g. ^{13}C or ^{15}N) and their scalar or dipolar coupled partners are observed. Using this strategy, the conformation of an inhibitor bound to porcine pepsin can be determined and structural information on the active site obtained. In another approach, two-dimensional nuclear Overhauser effect (2D NOE) difference spectra are obtained by subtracting NOE spectra of two enzyme/inhibitor complexes prepared with either a protonated or a deuterated inhibitor. Only NOEs arising from protons of the inhibitor substituted with deuterium appear in the 2D NOE difference spectra as illustrated for a pepsin/inhibitor complex. In a third strategy, deuterated enzymes are employed to eliminate the many proton NMR signals of the enzyme and allow the selective detection of the resonances corresponding to the bound ligand as demonstrated for CTP bound to CMP-3-deoxy D-manno-octulosonic acid (KDO) synthetase. Finally, a fourth approach is described using heteronuclear three-dimensional NMR spectroscopy in which homonuclear 2D NMR spectra are edited with respect to the heteronuclear chemical shifts. Using these methods the complete three-dimensional structures of large enzyme/inhibitor complexes can potentially be obtained. Examples of the spectral simplification that can be achieved using 3D NMR are given for ^{15}N-labeled CMP-KDO synthetase complexed with an inhibitor and CTP.

The development of enzyme inhibitors that are clinically useful is an extremely difficult task. Although compounds may be found that inhibit the enzyme, these initial leads may possess serious drawbacks (e.g. toxicity, metabolic instability, poor absorption) that preclude their use in the clinic. Structural studies on the binding of these lead compounds to the enzyme could provide important information to aid in the design of improved inhibitors [1]. For example, the experimentally determined conformation of the bound ligand could help in the design of rigid analogs with higher affinity and selectivity for the enzyme or analogs with metabolically stable structural frameworks that have the important functional groups in the same spatial orientation as the lead inhibitor. In addition, knowledge of the structure of the active site could suggest ways to modify the inhibitor to enhance the binding to the enzyme or ways to change the physical properties of the analog without altering its binding affinity.

In principle, this type of structural information on enzyme/inhibitor complexes could be obtained by NMR spectroscopy. However, enzyme/inhibitor complexes are large and difficult to study by conventional NMR techniques due to the vast number of overlapping signals. In this paper, several approaches are described for simplifying the proton NMR spectra of enzyme/inhibitor complexes which greatly facilitate the spectral interpretations. Examples of the type of detailed structural information that can be obtained using these techniques are presented along with a comparison of the different methods.

^{13}C, ^{15}N-*Edited* ^{1}H *NMR*

One approach to simplify complicated proton NMR spectra is by isotope-editing procedures in which only those protons which are attached to isotopically labeled nuclei and their scalar or dipolar coupled partners are observed [2–9]. Using these techniques the proton NMR spectra of enzyme/inhibitor complexes can be simplified markedly as illustrated in a one-dimensional ^{15}N-edited proton NMR spectrum (Fig. 1) of a ^{15}N-labeled inhibitor (Fig. 2) complexed with porcine pepsin [10]). Only the three NH signals of the bound pepsin inhibitor are observed. The ability to selectively observe the amide signals of the bound inhibitor allowed measurement of the NH exchange rates of the pepsin inhibitor when bound to the enzyme. These NH exchange rates were used to define the relative solvent accessibility of the amide protons of the bound inhibitor [10].

Isotope-edited two-dimensional NMR experiments have also been described [6, 8, 9]. Figure 3 depicts three ^{15}N-edited two-dimensional proton NMR spectra of ^{15}N-labeled inhibitors (top) complexed with pepsin [11]. Only diagonal peaks corresponding to the amide protons of the inhibitor that are attached to ^{15}N-labeled nuclei and nuclear Overhauser effect (NOE) cross-peaks between these amides and other nearby protons of the enzyme and inhibitor are observed. As shown in Fig. 3, the assignments for some of the NOE cross-peaks were made by identifying those NOEs that disappeared in

* Author to whom correspondence should be addressed.

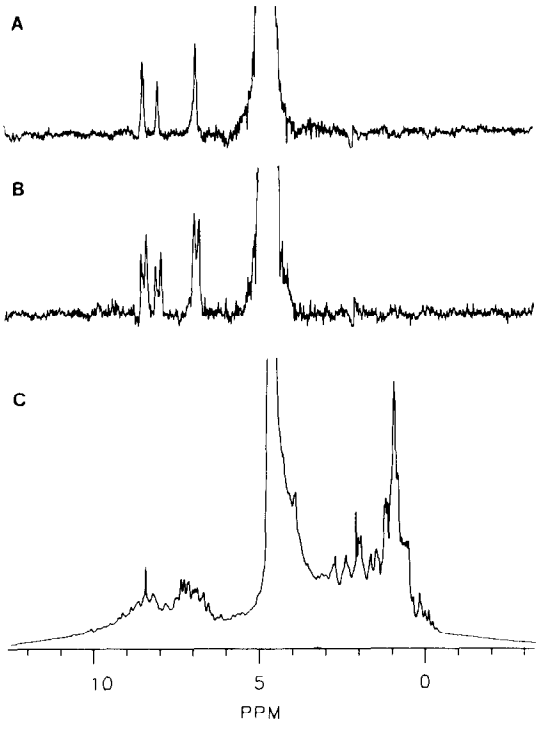

Fig. 1. Proton NMR spectra of a H_2O solution (1 mM) on a ^{15}N-labeled inhibitor complexed to porcine pepsin. (A) ^{15}N-decoupled isotope-edited, (B) ^{15}N-coupled isotope-edited and (C) conventional proton NMR spectrum. Reprinted with permission from *Biochem Biophys Res Commun* **147**: 892–898, 1987 [Ref. 10].

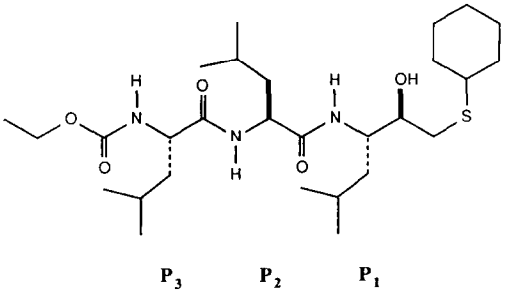

Fig. 2. Structure of the pepsin inhibitor ($IC_{50} = 1.7 \times 10^{-7}$ M) used in the isotope-edited and 2D NOE difference experiments.

NOE spectra of ^{15}N-labeled inhibitors that were also deuterium labeled. Using this strategy, for example, intense NOEs between the amide and α protons of the adjacent amino acid residues were unambiguously assigned. These NOEs suggest that the inhibitor adopts an extended backbone conformation when bound to porcine pepsin [11].

The side chain conformations at the P_3 and P_2 sites of the bound inhibitor were also examined by isotope-edited 2D NOE experiments. In Fig. 4. two ^{13}C-edited 2D NOE spectra are shown of pepsin/inhibitor complexes prepared with inhibitors that were uniformly ^{13}C-labeled (85%) at P_3 (Fig. 4A) or P_2 (Fig. 4B). From an analysis of the NOES observed in these experiments ($P_3H^{\alpha}/P_3H^{\beta 3}$, $P_3H^{\alpha}/P_3H^{\delta 2}$, $P_2H^{\alpha}/P_2H^{\beta 3}$, $P_2H^{\alpha}/P_2H^{\gamma}$) and those obtained from the ^{15}N-edited 2D NOE spectra ($P_3NH/P_3H^{\beta 2}$, P_3NH/P_3H^{γ}, $P_2NH/P_2H^{\beta 2}$, P_2NH/P_2H^{δ}), the side-chain conformations of the P_3 and P_2 sites were determined [11].

In addition to the information that can be derived on the conformation of bound ligands, isotope-edited 2D NOE spectra can also help characterize the structure of the active site. As shown in Fig. 4, NOEs between the inhibitor and enzyme (boxed NOE cross-peaks) can be identified in these experiments which are characteristic of the type of amino acids present in the different enzyme subsites. For example, NOEs were observed (Fig. 4A) between the P_3 leucine methyl protons and an aromatic proton of the enzyme. Conversely, no NOEs were observed between enzyme aromatic protons and the P_2 methyl groups of the inhibitor. These characteristic inhibitor/enzyme NOEs (even when unassigned) can be of value to test whether different inhibitors bind to the same site on the enzyme. Moreover, in those cases in which the NOEs can be assigned either by performing additional NMR experiments or by considering structural information from other sources, the NMR data can be used to define in detail the structure of the active site [11].

2D NOE difference using deuterated ligands

Another approach for simplifying the 2D NOE spectra of enzyme/inhibitor complexes involves the subtraction of 2D NOE spectra of two enzyme/inhibitor complexes prepared with either a protonated or a deuterated inhibitor [12]. In the 2D NOE difference spectrum, only NOEs arising from protons of the ligand that have been substituted with deuterium should appear, facilitating the interpretation of the spectra.

At first glance, the reliable subtraction of two 2D NOE data sets acquired on different samples would appear to be very difficult. In fact, as described in detail elsewhere [12], the manner in which the data is collected and processed is very important to obtain a difference spectrum that is relatively free from artifacts.

Figure 5 depicts 2D NOE difference spectra obtained by subtracting a 2D NOE spectrum of a pepsin/inhibitor complex prepared with an inhibitor perdeuterated at P_3 (Fig. 5A) or P_2 (Fig. 5B) from a pepsin/inhibitor complex prepared with pronotated inhibitor [12]. As illustrated in Fig. 5, the 2D NOE difference spectra are greatly simplified which allowed the interpretation of the data in terms of the conformation of the bound inhibitor and structure of the active site. The only artifacts that are present can be attributed to the relatively poor subtraction of the diagonal peaks which limit the utility of the experiment for identifying NOEs close to the diagonal.

Perdeuterated enzymes

Another approach to facilitate the NMR study of enzyme-bound ligands is to use deuterated enzymes. Perdeuteration of the enzyme may be accomplished

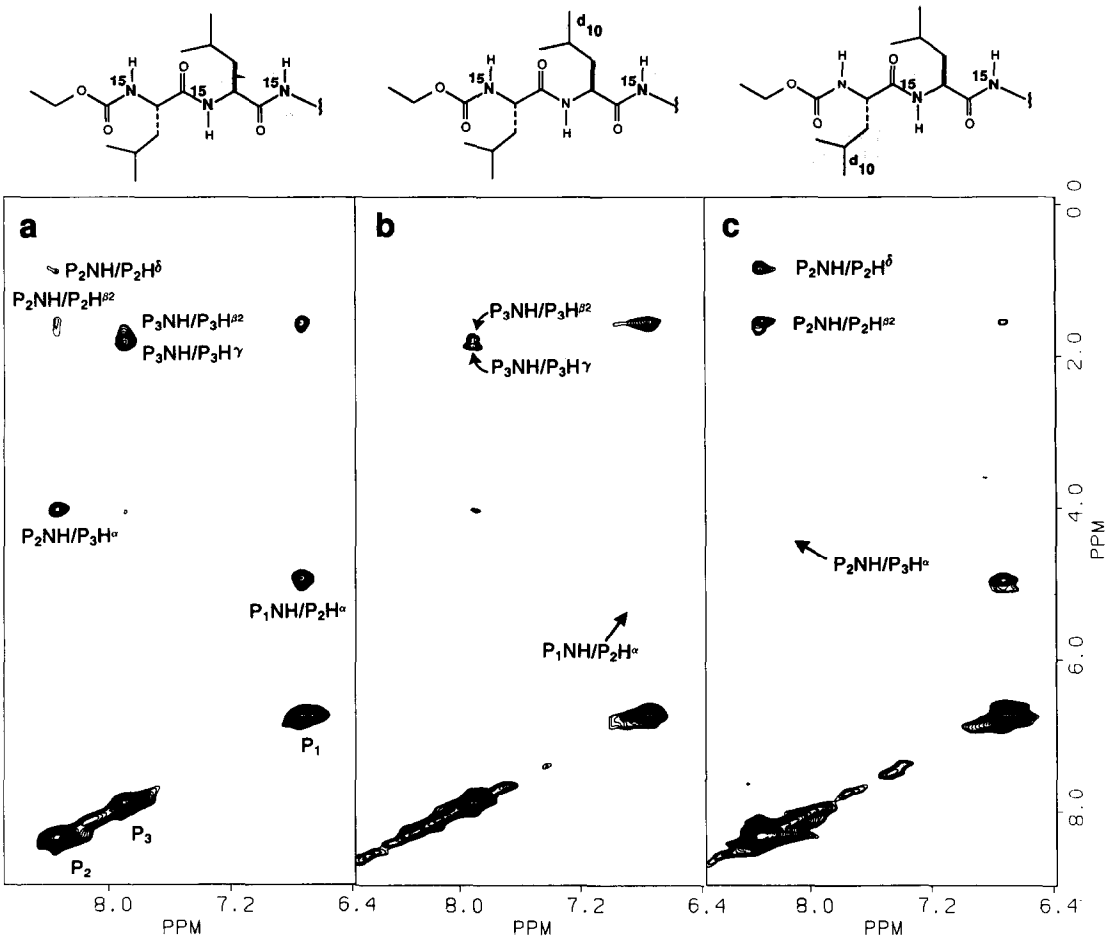

Fig. 3. Contour plots of ^{15}N-isotope-edited 2D NOE experiments of a pepsin/inhibitor (1/1) complex using the labeled inhibitors shown at the top of the spectra. The diagnonal peaks corresponding to the P_1–P_3 amide protons are labeled in (a), and in (b) and (c) arrows indicate the location in the spectra where cross-peaks are absent due to deuterium labeling. Reprinted with permission from *Biochemistry* **27**: 8297–8301, 1988. Copyright (1988) American Chemical Society. [Ref. 11].

by expressing the enzyme of interest into a bacterial host that can be grown on deuterated minimal media (e.g. [^{2}H]succinate and ^2H_2O) or rich media prepared from perdeuterated bacterial extracts. Once the deuterated enzyme is obtained, the binding of protonated substrates or inhibitors may be studied directly using conventional ^{1}H NMR techniques.

We have used this approach in our studies of the enzyme CMP–KDO synthetase (CTP:3-deoxy-D-manno-octulosonate cytidylyl transferase; EC 2.7.7.38). This bacterial enzyme catalyzes the formation of CMP–KDO from CTP and 3-deoxy D-manno-octulosonic acid (KDO) [13]. This reaction is required for the incorporation of KDO into lipopolysaccharide, an important component of the outer membrane of Gram-negative bacteria. Information about this reaction and enzyme was sought from NMR to aid in the design of inhibitors of CMP–KDO synthetase which could be of clinical utility as antibiotics against Gram-negative bacteria [14].

In Fig. 6 a ^{1}H NMR spectrum is shown of the substrate CTP bound to perdeuterated CMP–KDO synthetase isolated from bacteria grown on deuterated succinate (>99%) and ^2H_2O (>99%). The free and bound CTP signals, which are in slow exchange on the NMR time scale, can easily be observed in the presence of the residual proton NMR signals of the deuterated enzyme. The ability to selectively observe the proton NMR signals of bound ligands has allowed us to: (1) measure the relative binding affinities of CTP and a series of nucleotide analogs, probing the structural specificity of the nucleotide binding site, (2) determine the bound conformation of CTP and CTP analogs, and (3) study the binding of KDO analogs that inhibit CMP–KDO synthetase.

Heteronuclear 3D NMR

A limitation of all of the NMR experiments described above is that they can only provide structural information on the bound ligand and active site. To determine the complete three-dimensional structure of an enzyme/inhibitor complex, other approaches are needed. As previously demonstrated

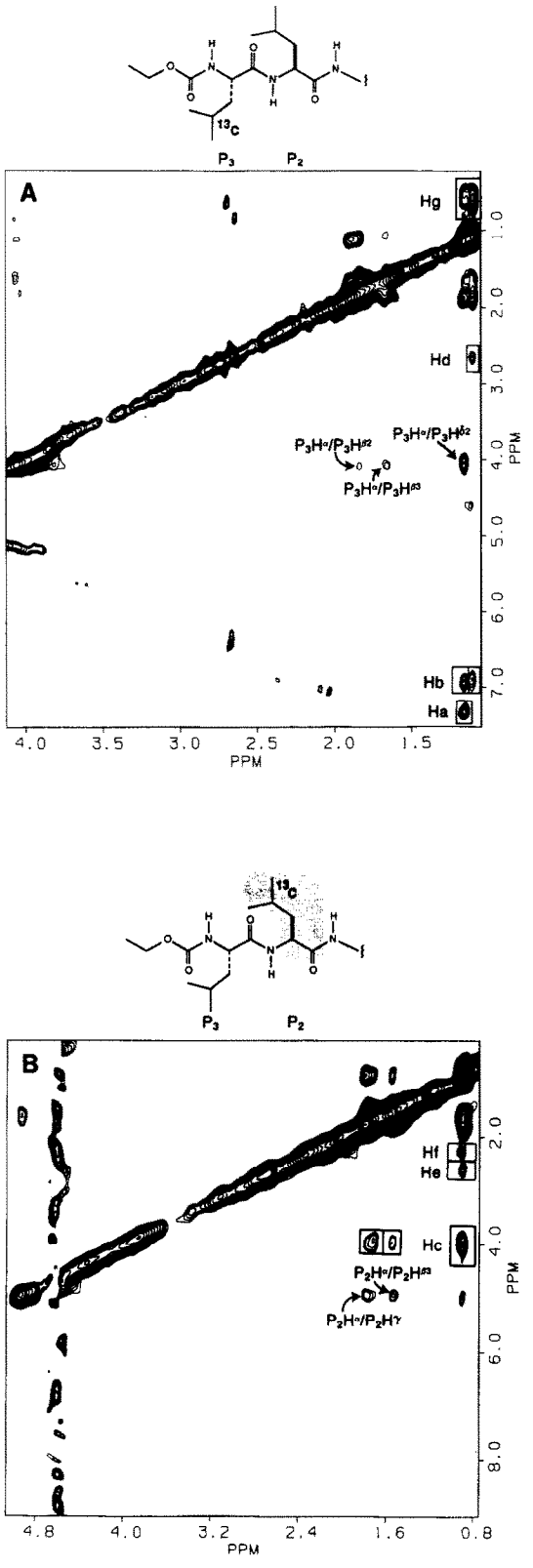

Fig. 4. Contour plots of ^{13}C-isotope-edited 2D NOE experiments using inhibitors uniformly ^{13}C-labeled (85%) at (A) P_3 and (B) P_2. The boxes indicate NOEs between labeled inhibitor and pepsin. Reprinted with permission from *Biochemistry* **27**: 8297–8301, 1988. Copyright (1988) American Chemical Society. [Ref. 11].

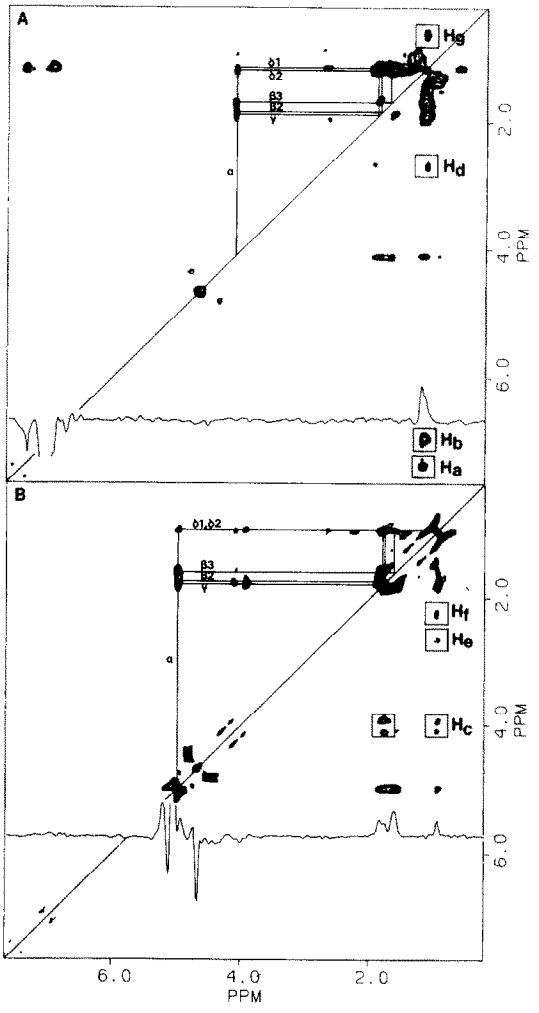

Fig. 5. Two-dimensional NOE difference spectra calculated from a 2D NOE spectrum of a protonated inhibitor complexed to pepsin minus a 2D NOE spectrum of pepsin bound to an inhibitor perdeuterated at (A) P_3 or (B) P_2. Reprinted with permission from *J Am Chem Soc* **111**: 5013–5015, 1989. Copyright (1989) American Chemical Society. [Ref. 12].

[15, 16], one approach is to perform several isotope-edited proton NMR experiments on proteins that have been selectively labeled by residue type with ^{15}N or ^{13}C. The disadvantage with this method is that many NMR samples containing selectively labeled proteins are required. Another possibility is to employ heteronuclear three-dimensional NMR [17] experiments on proteins that have been uniformly labeled with ^{15}N or ^{13}C. In these 3D experiments, homonuclear 2D NMR spectra (e.g. NOESY) are edited with respect to the heteronuclear chemical shifts [17]. Recently, heteronuclear 3D NMR techniques have been applied in the study of staphylococcal nuclease [18], the inflammatory protein C5a [19], T_4 lysozyme [20], and CMP–KDO synthetase [21] uniformly labeled with ^{15}N.

Figure 7 (top) depicts a $^1H/^{15}N$ correlation map of CMP–KDO synthetase uniformly labeled with ^{15}N

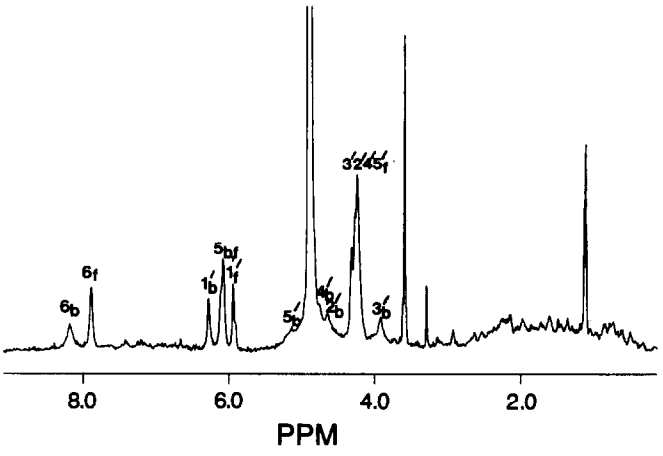

Fig. 6. Proton NMR spectrum of CTP bound to perdeuterated CMP–KDO synthetase. The free (f) bound (b) signals of CTP were assigned by a 2D exchange experiment.

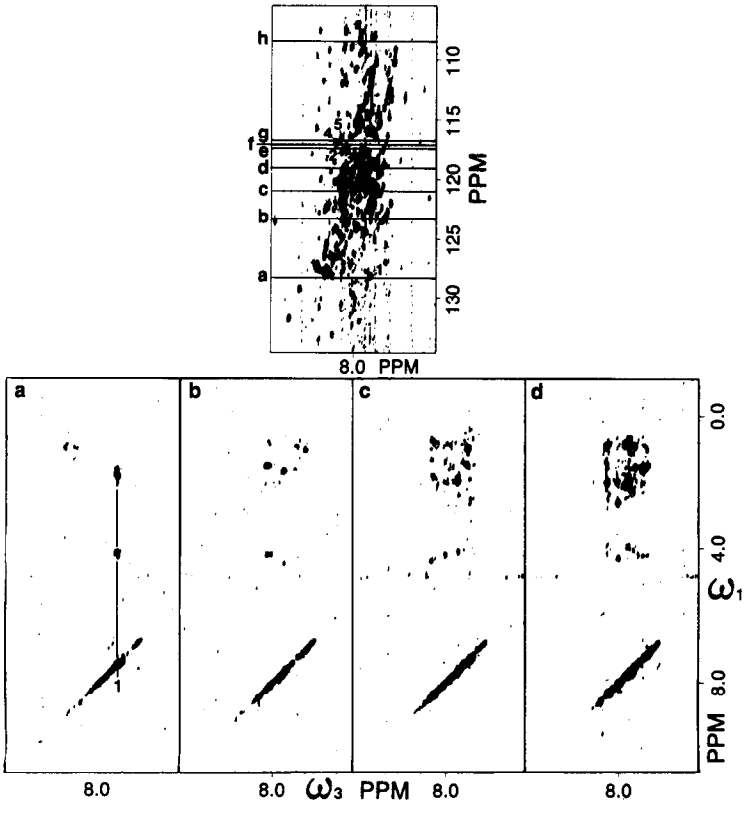

Fig. 7. $^{1}H/^{15}N$ Heteronuclear multiple quantum correlation map (top) of a [^{15}N] CMP–KDO synthetase/inhibitor/CTP complex. (a–d): Individual ω_1, ω_3 planes selected from the 3D data set at various ^{15}N frequencies (a–d) indicated in the $^{1}H/^{15}N$ correlation map. Reprinted with permission from *Biochem Biophys Res Commun* **159**: 842–847, 1989 [Ref. 21].

complexed with an inhibitor (α-C-1,5-anhydro-7-amino-2,7-dideoxy-D-manno-heptopyranosyl-carboxylate) and CTP [21]. Many of the amides that have identical proton chemical shifts have nitrogens that resonate at different ^{15}N frequencies. Thus, editing by the ^{15}N chemical shifts in a three-dimensional nuclear Overhauser effect spectroscopy-heteronuclear multiple quantum correlation (3D NOESY-HMQC) experiment should simplify the 2D NOE spectrum of the enzyme/inhibitor/CTP complex. As shown in Fig. 7 (bottom, a–d), the ω_1, ω_3 NOE containing planes extracted from 3D NOESY-HMQC data set are markedly simplified compared to the 2D NOE spectrum of the ternary

complex [21]. The increase in resolution offered by this method suggests that 3D NMR will be useful for assigning NMR spectra and determining the three-dimensional structures of biomacromolecules and large molecular complexes.

Conclusions

Several approaches have been described for providing detailed structural information on enzyme/inhibitor complexes using various NMR techniques and isotope-labeling. As outlined in the introduction, the information that can be derived from these experiments on enzyme/inhibitor complexes should be a valuable aid in the design of improved inhibitors. The particular approach that is chosen to obtain this structural information will depend on the question of interest as well as the availability of labeled enzyme and/or inhibitors. To help the reader choose which approach is most suitable, a comparison of the different methods is presented in Table 1. As shown in the table, each method has its own advantages and disadvantages. In the ^{13}C, ^{15}N-edited ^{1}H NMR experiments, the conformation of bound ligands can be defined and structural information on the active site can be obtained. This method has the advantage of producing very clean spectra. The approach using 2D NOE difference spectroscopy can provide the same type of structural information as the isotope-edited NMR experiments. However, subtraction of the data sets is much more difficult since two different samples are employed. On the other hand, unlike the isotope-edited experiments, this method does not require additional hardware for X-nucleus decoupling, fixed delays in the experiment which decrease the sensitivity, or carbon-13 labeling of ligands which is typically more difficult and expensive than deuterium labeling [12]. The third approach which makes use of perdeuterated enzymes to study the conformations of protonated bound ligands suffers from the disadvantage of not being able to probe the active site environment of the bound ligand. Furthermore, this approach requires perdeuterated enzyme which may be difficult to obtain. Nevertheless, since isotopically labeled inhibitors are not required, this method is ideally suited for correlating the conformations of enzyme-bound ligands with their biological activities which may allow a better understanding of structure/activity relationships. The fourth approach, heteronuclear 3D NMR, appears to be the most promising of all of the methods. Unlike the other techniques, it may be possible to determine the complete three-dimensional structures of large enzyme/inhibitor complexes using these experiments. Currently, 3D NMR is in its infancy, but as the 3D techniques mature and improved software is developed for the automated and graphical analysis of the data, this approach will become a powerful tool for determining the three-dimensional structures of biomacromolecules and macromolecular complexes.

Table 1. Comparison of the different methods for studying enzyme/inhibitor complexes by NMR spectroscopy

Method	Use	Advantages	Disadvantages
^{13}C, ^{15}N-edited ^{1}H NMR	Conformation of bound ligands Active site structure	Good subtraction/clean editing	Only obtain information on vicinity of labels Needs ^{13}C- or ^{15}N-labeled ligands
2D NOE difference using ^{2}H ligands	Conformation of bound ligands Active site structure	Sensitive experiment ^{2}H Labeling cheaper and easier	Only obtain information on vicinity of labels Needs ^{2}H ligands Good subtraction difficult
Perdeuterated enzymes	Conformation of bound ligands	No labeling of ligands required Ideal for studying structure/activity relationships	No structural information on the active site Needs ^{2}H-labeled enzyme
Heteronuclear 3D NMR	Complete 3D structure determination of enzyme/inhibitor complex	Does not require the selective labeling of the proton	Needs $^{15}N/^{13}C$-labeled enzyme Needs special software to analyze 3D NMR data Requires considerable amount of NMR time

Acknowledgements—This work was supported in part by NIH Grant U01 A127220-01.

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