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Perspective

NMR Studies of Molecular Complexes as a Tool in Drug Design

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For the last 30 years, nuclear magnetic resonance (NMR) has been an important tool in drug research. Its major role has been as an analytical method for the structural elucidation of molecules that have been chemically synthesized or isolated from natural sources. In principle, NMR could also be used to aid in the design of improved pharmaceutical agents. Early attempts have involved the use of NMR to probe the conformations of drug molecules in solution to understand the conformational requirements for biological activity.[1-7] However, most molecules, in the absence of their receptors, are flexible and exist in several rapidly interconverting conformations, making it extremely difficult to correlate conformation and biological activity. A more promising approach involves the determination of the three-dimensional structure of a drug when bound to its biological site of action. In these studies the "bioactive" conformation of a drug molecule is not inferred from the solution conformation but is obtained directly from the NMR studies of the complex. Knowledge of its bound conformation may aid in the design of an analogue with the functional groups necessary for binding held in their experimentally determined spatial orientation by a different molecular framework. The new analogue may be designed to have different physical properties (leading to improved oral activity), to be more metabolically stable, or to be easier to synthesize.[8]

Another important piece of information that could be obtained from NMR studies of drug/receptor complexes is the identification of those portions of the ligand that interact with the target site. This information may help identify the functional groups of the ligand important for binding and determine those portions of the ligand that could be modified without affecting the binding affinity but which may drastically alter the physical properties (e.g., water solubility) of the analogue.[8,9]

The complete three-dimensional structure of drug/receptor complexes could also be obtained by NMR. From these studies the functional groups of the receptor responsible for ligand binding may be determined. In addition, new areas of the target molecule could be identified to aid in the design of analogues that contain additional functional groups to interact with these sites.

Until recently, obtaining detailed structural information on large molecular complexes by NMR was impossible due to the lack of suitable NMR techniques to study large molecular complexes and the inability to obtain large quantities of the biomacromolecules which function as drug

(1) Mirau, P. A.; Shafer, R. H. High-resolution proton nuclear magnetic resonance analysis of conformational properties of biosynthetic actinomycin analogues. *Biochemistry* **1982**, *21*, 2622–2626.
(2) Hruby, V. J.; Mosberg, H. I. Conformational and dynamic considerations in peptide structure-function studies. *Peptides* **1982**, *3*, 329–336.
(3) Kessler, H. Conformation and biological activity of cyclic peptides. *Angew. Chem., Int. Ed. Engl.* **1982**, *21*, 512–523.
(4) DeJong, A. P.; Fesik, S. W.; Makriyannis, A. Conformational requirements for norepinephrine uptake inhibition by phenethylamines in brain synaptosomes. Effects of α-alkyl substitution. *J. Med. Chem.* **1982**, *25*, 1438–1441.
(5) Fesik, S. W.; Armitage, I. M.; Ellestad, G. A.; McGahren, W. J. Nuclear magnetic resonance studies on the antibiotic avoparcin. Conformational properties in relation to antibacterial activity. *Mol. Pharmacol.* **1984**, *25*, 274–280.
(6) Kessler, H.; Klein, M.; Miller, A.; Wagner, K.; Bats, J. W.; Ziegler, K.; Frimmer, M. Conformational prerequisites for the in vitro inhibition of cholate uptake in hepatocytes by cyclic analogues of antamanide and somatostatin. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 997–999.
(7) Baniak, E. L., III; Rivier, J. E.; Struthers, R. S.; Hagler, A. T.; Gierasch, L. M. Nuclear magnetic resonance analysis and conformational characterization of a cyclic decapeptide antagonist of gonadotropin-releasing hormone. *Biochemistry* **1987**, *26*, 2642–2656.
(8) Fesik, S. W. In *Computer-Aided Drug Design. Methods and Applications*; Perun, T. J., Propst, C. L., Eds.; Marcel Dekker: New York, 1989; pp 133–184.
(9) Fesik, S. W. Isotope-edited NMR spectroscopy. *Nature* **1988**, *332*, 865–866.

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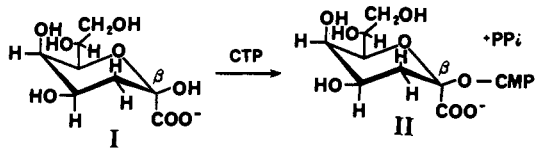

Figure 1. Reaction catalyzed by CMP-KDO synthetase.

target sites. In the last few years, however, through rapid advances in molecular biology, gram quantities of pharmacologically relevant drug "receptors" have become available for structural studies. Furthermore, several new multidimensional NMR techniques have been recently developed which facilitate the spectral interpretation of large molecular complexes containing many overlapping signals.

In this Perspective, some of the recently developed approaches for studying large molecular complexes by NMR are described. The advantages and limitations of the individual approaches for providing structural information useful in drug design are discussed. Finally, the advances in NMR that may be expected in the future are presented. These developments should have a major impact on the manner in which new drugs are designed.

Transferred NOE

For weakly bound ligands, experimental approaches have been developed to circumvent the problems associated with selectively detecting the resonances of the ligand in the presence of the many signals of the macromolecule to which it is bound. These techniques involve the measurement of transferred nuclear Overhauser effects (NOEs) for ligands that exchange rapidly from the bound to the free state.[10-13] Negative NOEs due to the bound state are observed on the free or averaged resonances of the ligand which are narrower than the signals of the macromolecule and easily identified. From an analysis of these NOEs, the conformation of a ligand bound to a macromolecule can be determined. Indeed, from the measurement of transferred nuclear Overhauser effects, the conformation of ligands bound to proteins,[14-19] phospholipids,[20] and nucleic acids[21] have been reported.

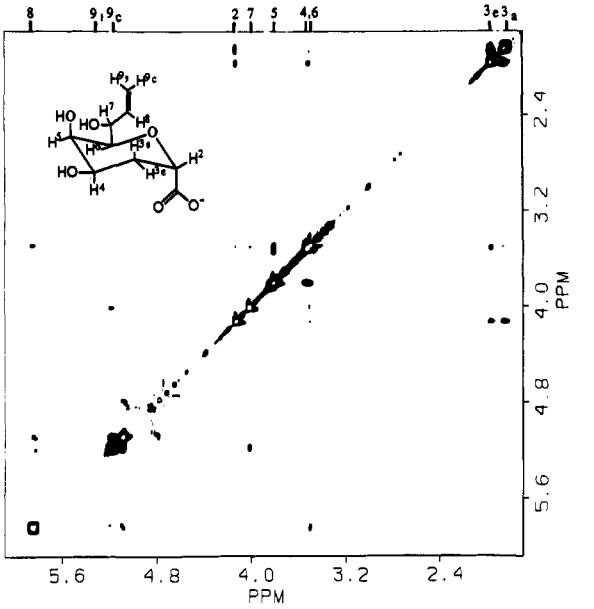

Figure 2. Contour plot of a two-dimensional transferred NOE experiment of CMP-KDO synthetase and an inhibitor (inset) in a 1:10 mole ratio dissolved in a ^2H_2O solution (50 mM PO_4, pD = 7.8). NMR data were acquired on a GN 500 NMR spectrometer at 7 °C. Proton NMR signals of the inhibitor were assigned by a COSY experiment of the free inhibitor.

We have applied the transferred NOE experiment in a study of a series of inhibitors bound to cytidine-5′-monophosphate-3-deoxy-D-*manno*-octulosonate (CMP-KDO) synthetase[22] (EC 2.7.7.38). This bacterial enzyme (27.5 kDa, 248 amino acids) catalyzes the reaction shown in Figure 1. CMP-KDO (II) that is formed in this reaction is required for the incorporation of the eight-carbon sugar, KDO (I), into lipopolysaccharides, an important component of Gram-negative bacteria. From ^{31}P and ^{13}C NMR studies of this enzymatic reaction,[23] it was found that

(10) Albrand, J. P.; Birdsall, B.; Feeney, J.; Roberts, G. C. K.; Burgen, A. S. V. The use of transferred nuclear Overhauser effects in the study of the conformations of small molecules bound to proteins. *Int. J. Biol. Macromol.* **1979**, *1*, 37–41.
(11) Clore, G. M.; Roberts, G. C. K.; Gronenborn, A.; Birdsall, B.; Feeney, J. Transfer-of-saturation NMR studies of protein–ligand complexes. Three-site exchange. *J. Magn. Reson.* **1981**, *45*, 151–161.
(12) Clore, G. M.; Gronenborn, A. M. Theory of the time dependent transferred uclear Overhauser effect: Applications to structural analysis of ligand–protein complexes in solution. *J. Magn. Reson.* **1983**, *53*, 423–442.
(13) Clore, G. M.; Gronenborn, A. M. Theory and applications of the transferred nuclear Overhauser effect to the study of the conformations of small ligands bound to proteins. *J. Magn. Reson.* **1982**, *48*, 402–417.
(14) Levy, H. R.; Ejchart, A.; Levy, G. C. Conformations of nicotinamide coenzymes bound to dehydrogenases determined by transferred nuclear Overhauser effects. *Biochemistry* **1983**, *22*, 2792–2796.
(15) Gronenborn, A. M.; Clore, G. M.; Brunori, M.; Giardina, B.; Falcioni, G.; Perutz, M. Stereochemistry of ATP and GTP bound to fish haemoglobins, A transferred nuclear Overhauser enhancement ^{31}P-nuclear magnetic resonance, oxygen equilibrium and molecular modelling study. *J. Mol. Biol.* **1984**, *178*, 731–742.
(16) Clore, G. M.; Gronenborn, A. M.; Greipel, J.; Maass, G. Conformation of the DNA undecamer 5′d(A-A-G-T-G-T-G-A-T-A-T) bound to the single-stranded DNA binding protein of Escherichia coli. A time-dependent transferred nuclear Overhauser enhancement study. *J. Mol. Biol.* **1986**, *187*, 119–124.
(17) Banerjee, A.; Levy, H. R.; Levy, G. C.; Chan, W. W. C. Conformations of bound nucleoside triphosphate effectors in aspartate transcarbamylase. Evidence for the London-Schmidt model by transferred nuclear Overhauser effects. *Biochemistry* **1985**, *24*, 1594–1598.
(18) Glasel, J. A.; Borer, P. N. NMR studies of flexible opiate conformations at monclonal antibody binding sites. I. Transferred nuclear Overhauser effects show bound conformations. *Biochem. Biophys. Res. Commun.* **1986**, *141*, 1267–1273.
(19) Clore, G. M.; Gronenborn, A. M.; Carlson, G.; Meyer, E. F. Stereochemistry of binding of the tetrapeptide acetyl-Pro-Ala-Pro-Tyr-NH_2 to porcine pancreatic elastase. Combined use of two-dimensional transferred nuclear Overhauser enhancement measurements, restrained molecular dynamics, x-ray crystallography and molecular modelling. *J. Mol. Biol.* **1986**, *190*, 259–267.
(20) Wakamatsu, K.; Okada, A.; Migazawa, T.; Masui, Y.; Sakakibara, S.; Higashijima, T. Conformations of yeast α-mating factor and analog peptides as bound to phospholipid bilayer. Correlation of membrane-bound conformation with physiological activity. *Eur. J. Biochem.* **1987**, *163*, 331–338.
(21) Gronenborn, A. M.; Clore, G. M.; McLaughlin, L. W.; Graeser, E.; Lorber, B.; Giege, R. Yeast $tRNA^{Asp}$: Codon and wobble condon–anticodon interactions. A transferred nuclear Overhauser enhancement study. *Eur. J. Biochem.* **1984**, *145*, 359–364.
(22) Ghalambor, M. A.; Heath, E. C. The enzymatic synthesis of cytidine monophospho-2-keto-3-deoxy-octonate. *Biochem. Biophys. Res. Commun.* **1963**, *10*, 346–351.
(23) Kohlbrenner, W. E.; Nuss, M. M.; Fesik, S. W. ^{31}P and ^{13}C NMR studies of oxygen transfer during catalysis by 3-deoxy-D-mannooctulosonate cytidyl-transferase from Escherichia coli. *J. Biol. Chem.* **1987**, *262*, 4534–4537.

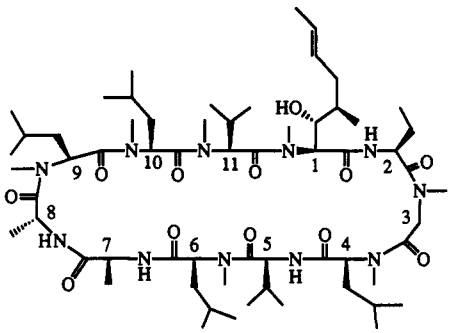

Figure 3. Structure of cyclosporin A.

CMP-KDO formation occurs with retention of configuration at the anomeric center, suggesting a mechanism involving the nucleophilic displacement of the pyrophosphoryl group of CTP by the anomeric hydroxyl of KDO (Figure 1). In addition, ^{13}C NMR studies revealed that the β-pyranose form of KDO, the least abundant tautomeric form in solution, is the actual substrate in the reaction.[24] On the basis of these results, KDO analogues were synthesized that were locked in the β-pyranose configuration to mimic the active tautomeric form of KDO and which lacked the anomeric hydroxyl group to eliminate its ability to act as a substrate.[25] Although these compounds were shown to inhibit CMP-KDO synthetase, more potent inhibitors with better transport properties were sought. Conformational studies of these lead inhibitors when bound to CMP-KDO synthetase were undertaken to provide structural information to aid in the design of improved inhibitors of CMP-KDO synthetase which could be of clinical utility as antibiotics against Gram-negative bacteria.

Figure 2 depicts a contour plot of a two-dimensional transferred NOE experiment of an inhibitor (inset) bound to CMP-KDO synthetase obtained in the absence of CTP. NOEs of equal intensity were observed between proton pairs 2/3a and 2/3e. These NOEs in conjunction with the NOE observed between 4 and 3e suggest that this inhibitor adopts a $^5C_2$ ring conformation when bound to CMP-KDO synthetase. The side-chain conformation of the inhibitor was defined by NOEs between the protons 8/6 and 9t/7 and the relative low intensity or absence of NOEs between 7/6, 7/5, 8/7, and 9c/7. On the basis of additional 2D transferred NOE experiments, the bound conformations of several inhibitors of CMP-KDO synthetase were determined. For all of the inhibitors, the bound conformation of the sugar ring was found to be the same; however, the side-chain conformation differed for the inhibitors, depending on the hydrophobicity of the side chain.

Unfortunately, the 2D transferred NOE studies of the CMP-KDO inhibitors could only be performed in the absence of CTP. When CTP is present, the inhibitors bind more tightly to the enzyme, eliminating the applicability of the transferred NOE experiment which can only be applied to the study of weakly bound ligands that rapidly exchange on and off the enzyme. This is a severe limitation of the transferred NOE experiment, since most ligands of interest bind tightly ($K_a > 10^7$) to their receptors.

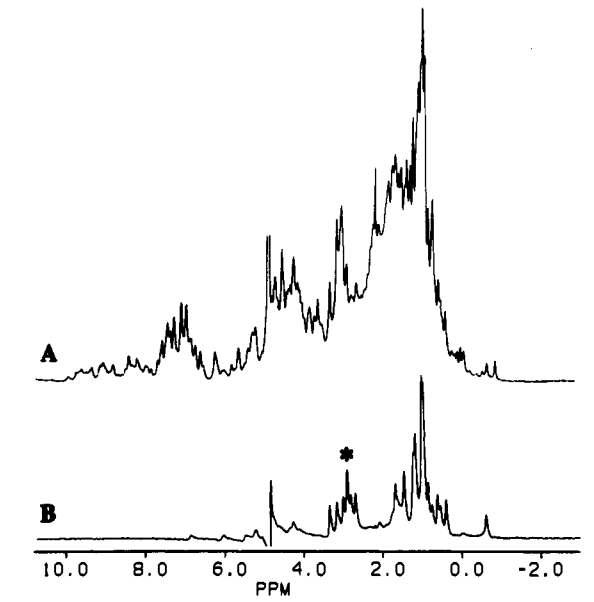

Figure 4. (A) Conventional and (B) isotope-edited 1D NMR spectrum of a 1.3 mM solution of a [U-^{13}C]CsA/cyclophilin complex in which only the proton NMR signals of ^{13}C-labeled (>95%) CsA are observed. The asterisk indicates two overlapping (MeLeu^4, MeLeu^{10}) NCH_3 signals of cyclosporin A bound to cyclophilin.

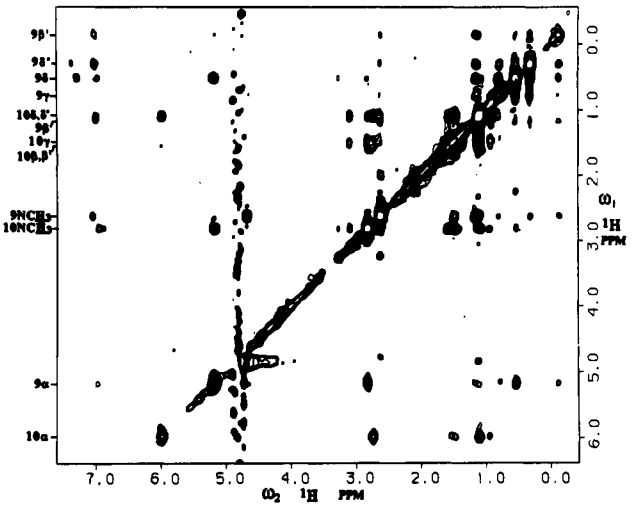

Figure 5. Contour plot of an isotope-edited 2D NOE spectrum of cyclophilin and CsA in which the MeLeu residues in the 9 and 10 positions were uniformly labeled (>95%) with ^{13}C. The NMR spectrum was acquired at 20 °C on a Bruker AM 500 NMR spectrometer as previously described[38] (reprinted with permission from ref 38. Copyright 1990 American Association for the Advancement of Science).

Isotope-Edited Proton NMR

One method for simplifying complicated proton NMR spectra that does not depend on the rapid exchange of ligands involves the selective detection of protons attached to isotopically labeled nuclei.[26-34] Using these isotope-

(24) Kohlbrenner, W. E.; Fesik, S. W. Determination of the anomeric specificity of the Escherichia coli CTP:CMP-3-deoxy-D-manno-octulosonate cytidyl-transferase by ^{13}C NMR spectroscopy. *J. Biol. Chem.* **1985**, *260*, 14695–14700.

(25) Goldman, R.; Kohlbrenner, W.; Lartey, P.; Pernet, A. Antibacterial agents specifically inhibiting lipopolysaccharide synthesis. *Nature* **1987**, *329*, 162–164.

(26) Freeman, R.; Mareci, T. H.; Morris, G. A. Weak satellite signals in high resolution NMR spectra: Separating the wheat from the chaff. *J. Magn. Reson.* **1981**, *42*, 341–345.

(27) Bendall, M. R.; Pegg, D. T.; Doddrell, D. M.; Field, J. NMR of protons coupled to ^{13}C nuclei only. *J. Am. Chem. Soc.* **1981**, *103*, 934–936.

(28) Griffey, R. H.; Redfield, A. G. Identification of isotope-labeled resonances in two-dimensional proton-proton correlation and exchange spectroscopy with gated heteronuclear decoupling. *J. Magn. Reson.* **1985**, *65*, 344–347.

editing techniques, the proton signals of an isotopically labeled ligand can be selectively observed in the presence of the many signals of its target site.[9,35-38] The spectral simplification that can be obtained is illustrated in our NMR studies[38] of cyclosporin A (CsA) (Figure 3) bound to its putative target protein, cyclophilin (17.7 kDa, 165 amino acids). The individual proton NMR signals of cyclosporin A cannot be identified in the conventional proton NMR spectrum (Figure 4A) of the CsA/cyclophilin complex; however, the CsA signals are readily identified in a ^{13}C-edited proton NMR spectrum of [U-^{13}C]CsA bound to cyclophilin (Figure 4B).

A ^{13}C-edited two-dimensional NOE spectrum of [U-^{13}C-MeLeu^{9,10}]CsA bound to recombinant human cyclophilin is shown in Figure 5. In the ω_1 dimension, only those protons attached to the ^{13}C-labeled nuclei of CsA are detected. In the ω_2 dimension, NOE crosspeaks between these "labeled" protons and other nearby protons of CsA and cyclophilin are observed. The intense NOE observed between MeLeu^{10} NCH_3 and MeLeu^9 H^α of CsA (Figure 5) and the lack of an NOE between the α protons of the adjacent amino acids indicated an extended conformation of the peptide backbone with a trans 9,10 amide bond.[38] The observation of a trans 9,10 peptide bond suggested that the conformation of CsA when bound to cyclophilin is markedly different from the reported crystalline and solution conformation of CsA that contains a cis 9,10 amide bond.

Isotope-edited 2D NOE experiments can also provide structural information about the enzyme binding site by means of the NOEs between "labeled" protons of the ligand and receptor. For example, as shown in Figure 5, NOEs were observed[38] between several protons of the MeLeu^9 residue of CsA and aromatic protons of cyclophilin that were assigned to Trp and Phe residues. These results

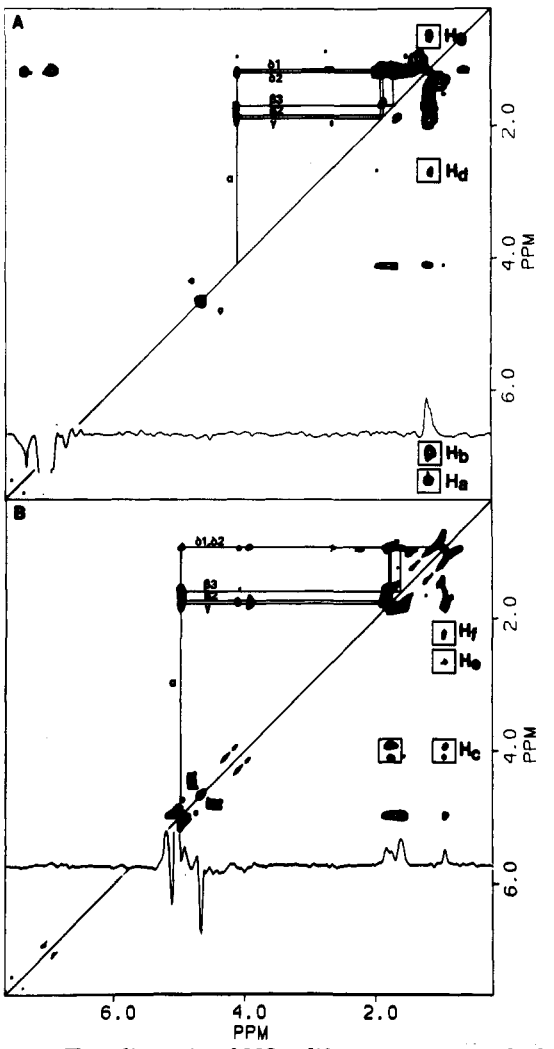

Figure 6. Two-dimensional NOE difference spectra calculated from a 2D NOE spectrum of a protonated inhibitor (Figure 7) complexed to pepsin minus a 2D NOE spectrum of a pepsin inhibitor bound to an inhibitor perdeuterated (>98% at A) P_3 or (B) P_2. NOEs between ligand protons are connected by solid lines. Inhibitor/pepsin NOEs (Ha–Hg) are indicated by boxes and were found to be similar to the NOEs observed in isotope-edited 2D NOE spectra[35] (reprinted with permission from ref 40. Copyright 1989 American Chemical Society).

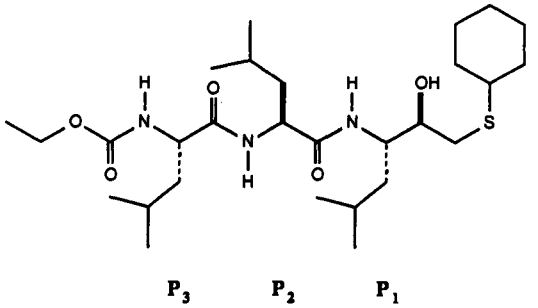

Figure 7. Structure of the pepsin inhibitor used in the 2D NOE difference experiments.

indicate that the MeLeu^9 residue of CsA is in close proximity to cyclophilin and contradict earlier conclusions based on structure/activity relationships which suggested[39]

(29) Griffey, R. H.; Jarema, M. A.; Kunz, S.; Rosevear, P. R.; Redfield, A. G. Isotopic-label-directed observation of the nuclear Overhauser effect in poorly resolved proton NMR spectra. *J. Am. Chem. Soc.* **1985**, *107*, 711–712.
(30) Wilde, J. A.; Bolton, P. H.; Stolowich, N. J.; Gerlt, J. A. A method for the observation of selected proton NMR resonances of proteins. *J. Magn. Reson.* **1986**, *68*, 168–171.
(31) Otting, G.; Senn, H.; Wagner, G.; Wuthrich, K. Editing of 2D ^1H NMR spectra using X half-filters. Combined use with residue-selective ^{15}N labeling of proteins. *J. Magn. Reson.* **1986**, *70*, 500–505.
(32) Rance, M.; Wright, P. E.; Messerle, B. A.; Field, L. D. Site-selective observation of nuclear Overhauser effects in proteins via isotopic labeling. *J. Am. Chem. Soc.* **1987**, *109*, 1591–1593.
(33) Bax, A.; Weiss, M. A. Simplification of two-dimensional NOE spectra of proteins by ^{13}C labeling. *J. Magn. Reson.* **1987**, *71*, 571–575.
(34) Fesik, S. W.; Gampe, R. T., Jr.; Rockway, T. W. Application of isotope-filtered 2D NOE experiments in the conformational analysis of atrial natriuretic factor (7–23). *J. Magn. Reson.* **1987**, *74*, 366–371.
(35) Fesik, S. W.; Luly, J. R.; Erickson, J. W.; Abad-Zapatero, C. Isotope-edited proton NMR study on the structure of a pepsin/inhibitor complex. *Biochemistry* **1988**, *27*, 8297–8301.
(36) Wider, G.; Weber, C.; Traber, R.; Widmer, H.; Wuthrich, K. Use of a double-half-filter in two-dimensional ^1H nuclear magnetic resonance studies of receptor-bound cyclosporin. *J. Am. Chem. Soc.* **1990**, *112*, 9015–9016.
(37) Weber, C.; Wider, G.; von Freyberg, B.; Traber, R.; Braun, W.; Widmer, H.; Wuthrich, K. The NMR structure of cyclosporin A bound to cyclophilin in aqueous solution. *Biochemistry* **1991**, *30*, 6563–6574.
(38) Fesik, S. W.; Gampe, R. T., Jr.; Holzman, T. F.; Egan, D. A.; Edalji, R.; Luly, J. R.; Simmer, R.; Helfrich, R.; Kishore, V.; Rich, D. H. Isotope-edited NMR of cyclosporin A bound to cyclophilin: Evidence for a trans 9,10 amide bond. *Science* **1990**, *250*, 1406–1409.
(39) Quesniaux, V. F. J.; Schreier, M. H.; Wenger, R. M.; Hiestand, P. C.; Harding, M. W.; VanRegenmortel, M. H. V. Cyclophilin binds to the region of cyclosporine involved in its immunosuppressive activity. *Eur. J. Immunol.* **1987**, *17*, 1359–1365.

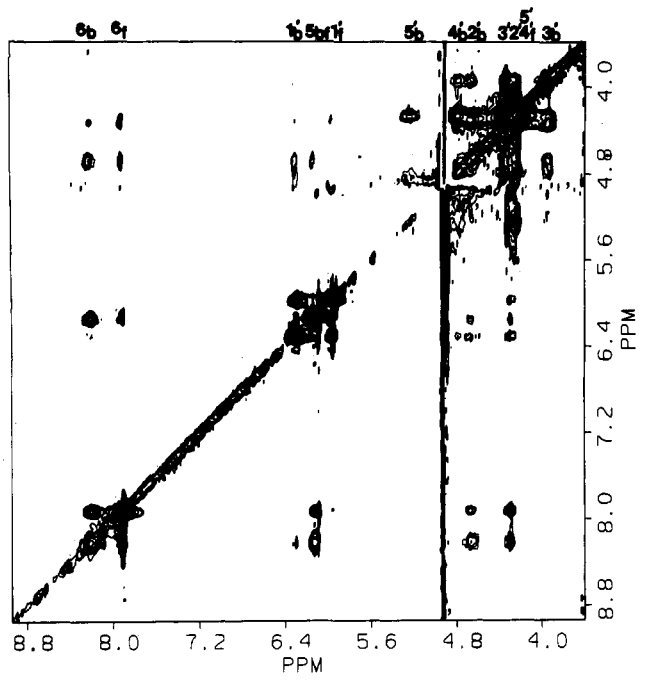

Figure 8. Contour plot of a conventional 2D NOE spectrum of CTP and perdeuterated (>98%) CMP-KDO synthetase (1.1 mM) in a mode ratio of 4:1. NMR data were acquired at 10 °C in 15 h on a Bruker AM 500 NMR spectrometer using a mixing time of 35 ms. Assignments of free (f) and bound (b) CTP are given at the top of the spectrum.

that this CsA residue was not involved in binding to cyclophilin.

2D NOE Difference Using Deuterated Ligands

Another approach for simplifying the 2D NOE spectra of drug/receptor complexes involves the subtraction of 2D NOE spectra of two drug/receptor complexes: one prepared with a protonated ligand and the other with a deuterated ligand.[40] In Figure 6, 2D NOE difference spectra are shown that were obtained by subtracting a 2D NOE spectrum of a pepsin/inhibitor complex prepared with an inhibitor (Figure 7) perdeuterated at P_3 (Figure 6A) or P_2 (Figure 6B) from a 2D NOE spectrum of a pepsin/inhibitor complex prepared with protonated inhibitor. From the spectral simplification that is achieved, NOEs between inhibitor protons which define the conformation of the inhibitor can be identified as well as NOEs between the inhibitor and pepsin which provide structural information on the active site. Thus, the approach using 2D NOE difference spectroscopy and deuterated ligands can provide similar structural information as isotope-edited NMR experiments that utilize ^{13}C- or ^{15}N-labeled ligands. However, the 2D NOE difference method is more limited, since clean subtraction of NOE data sets acquired on two different samples is difficult to achieve.[40]

Perdeuterated Receptors

Another approach to facilitate the NMR study of receptor-bound ligands is to use deuterated receptors.[41,42]

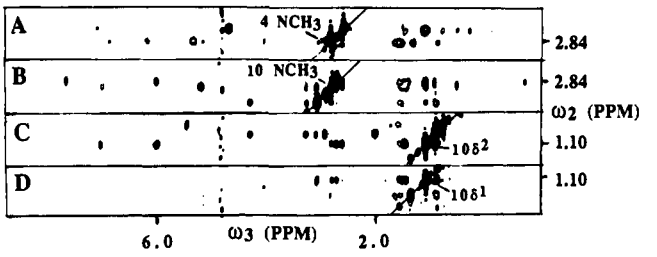

Figure 9. Cross sections (ω2,ω3) from a 3D HMQC-NOESY data set of a 1.3 mM [U-^{13}C]CsA/cyclophilin complex acquired with a mixing time of 70 ms. The individual $^1H,^1H$ planes were extracted at the ^{13}C chemical shifts (ω_1): (A) 29.3, (B) 32.2, (C) 19.1, (D) 23.5 ppm.

By deuterating the receptor and eliminating many proton NMR signals in the spectra, the proton NMR resonances of substrates or inhibitors can be selectively observed using conventional ^1H NMR techniques.

This approach is illustrated in our studies of CMP-KDO synthetase. In Figure 8 a 2D NOE 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 NOEs observed in the spectrum between the 6 and 2′ protons are consistent with a C(2′)-endo-anti conformation for CTP when bound to CMP-KDO synthetase.

This method has the advantage that isotopically labeled ligands are not required. Thus, providing that the receptor can be perdeuterated, the bound conformations of a large number of unlabeled compounds can be rapidly investigated using conventional proton NMR techniques. These studies may allow a better understanding of structure/activity relationships. Unfortunately, a severe limitation of this approach using perdeuterated proteins is that NOEs between the ligand and enzyme are not observed, eliminating the possibility of obtaining structural information on the active site.[42]

Heteronuclear 3D NMR

The approaches that have been described above involve the simplification of proton NMR spectra of large ligand/receptor complexes either by taking advantage of rapid ligand exchange rates in 2D transferred NOE experiments, by detecting only those protons attached to isotopically labeled nuclei, or by eliminating proton NMR signals by deuterating the ligand or receptor. However, even when the proton NMR signals of the ligand can be selectively observed, analysis of the spectra may still be difficult due to the overlap of the remaining proton NMR signals of the ligand. One approach for further simplifying the spectra is by editing the proton resonances of the ligand in a third dimension by the ^{13}C or ^{15}N frequencies of the ligand in a heteronuclear three-dimensional NMR experiment.[43] The utility of heteronuclear 3D NMR spectroscopy for resolving spectral overlap is illustrated in our studies[44] of uniformly ^{13}C-labeled cyclosporin A

(40) Fesik, S. W.; Zuiderweg, E. R. P. An approach for studying the active site of enzyme/inhibitor complexes using deuterated ligands and 2D NOE spectroscopy. *J. Am. Chem. Soc.* **1989**, *111*, 5013–5015.

(41) Seeholzer, S. H.; Cohn, M.; Putkey, J. A.; Means, A. R.; Crespi, H. L. NMR studies of a complex of deuterated calmodulin with melittin. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 3634–3638.

(42) Fesik, S. W.; Zuiderweg, E. R. P.; Olejniczak, E. T.; Gampe, R. T., Jr. NMR methods for determining the structures of enzyme/inhibitor complexes as an aid in drug design. *Biochem. Pharmacol.* **1990**, *40*, 161–167.

(43) Fesik, S. W.; Zuiderweg, E. R. P. Heteronuclear three dimensional NMR spectroscopy. A strategy for the simplification of homonuclear two dimensional NMR spectra. *J. Magn. Reson.* **1988**, *78*, 588–593.

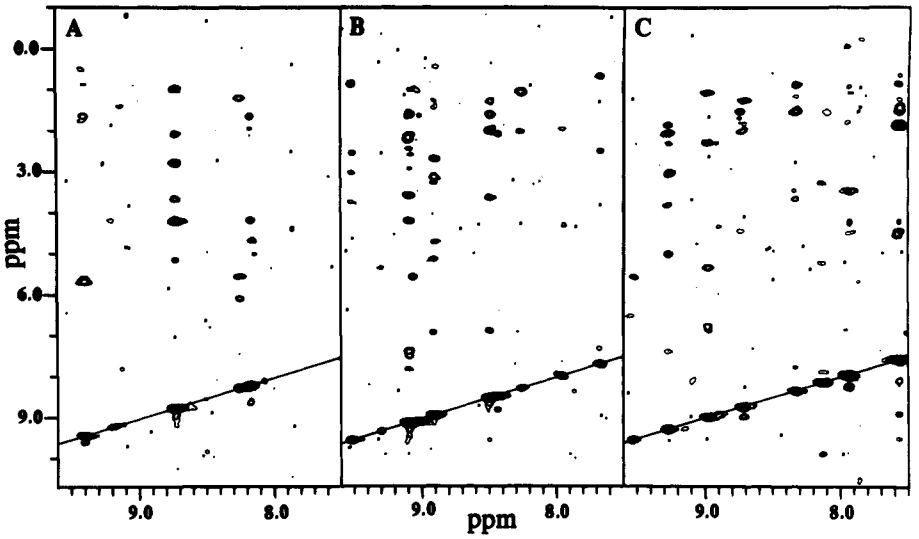

Figure 10. Cross sections (ω_1,ω_3) extracted from a 3D NOESY-HMQC data set of a 1.3 mM CsA/[U-^{15}N] cyclophilin complex in H_2O at three different ^{15}N chemical shifts (ω_2). The 3D NMR data (128 complex t_1 by 39 complex t_2 FIDs) were acquired on a Bruker AM 500 NMR spectrometer in 6 days using a mixing time of 100 ms.

bound to cyclophilin. Despite the fact that the MeLeu^4 and MeLeu^{10} NMe protons of CsA resonate at the same frequency (Figure 4B, asterisk), NOEs involving these protons can be resolved in a heteronuclear 3D NOE experiment. As shown in Figure 9A,B, NOEs from MeLeu^4 and MeLeu^{10} NMe protons of CsA can be distinguished from one another since they are observed in different planes corresponding to different ^{13}C frequencies of the NMe groups in the 3D NOE spectrum. Analogously, NOEs involving the MeLeu^{10} methyl groups of CsA that resonate at exactly the same proton frequencies appear on different planes in the 3D NOE spectrum corresponding to the different ^{13}C chemical shifts of their attached carbons (Figure 9C,D). Thus, the NOEs observed (Figure 9C) along ω_3 between δ-methyl protons of MeLeu^{10} and an α-proton (6.00 ppm) of CsA and a cyclophilin proton at 6.99 ppm arise from the δ-methyl with a ^{13}C chemical shift of 19.1 ppm which was assigned[44] to the *pro-S* (δ^2) methyl group of MeLeu^{10}. The MeLeu^{10} H^{δ^2}/H^{α} NOE was important for determining the side-chain conformation of this CsA residue when bound to cyclophilin, and the NOE between the MeLeu^{10} *pro-S* methyl group of CsA and cyclophilin identified this methyl group as being close to the protein.[44] From a quantitative analysis of the 3D NOE data, three-dimensional structures of CsA were calculated using a distance geometry/dynamical simulated annealing protocol.[45,46] The conformation of CsA when bound to cyclophilin was found to be very different from the conformation of uncomplexed CsA determined by X-ray crystallography[47] and NMR spectroscopy.[47] In addition to the three-dimensional structure of CsA when bound to cyclophilin, those portions of CsA that interact with cyclophilin were identified in the NMR studies[44] and interpreted in terms of structure/activity relationships.[39,48-52]

Heteronuclear 3D NMR experiments have also been applied to the study of isotopically labeled proteins[53-62]

(44) Fesik, S. W.; Gampe, R. T., Jr.; Eaton, H. L.; Gemmecker, G.; Olejniczak, E. T.; Neri, P.; Holzman, T. F.; Egan, D. A.; Edalji, R.; Simmer, R.; Helfrich, R.; Hochlowski, J.; Jackson, M. NMR studies of [U-^{13}C]cyclosporin A bound to cyclophilin. Bound conformation and portions of cyclosporin involved in binding. *Biochemistry* **1991**, *30*, 6574–6583.

(45) Nilges, M.; Clore, G. M.; Gronenborn, A. M. Determination of three-dimensional structures of proteins from interproton distance data by hybrid distance geometry-dynamical simulated annealing calculations. *FEBS Lett.* **1988**, *229*, 317–324.

(46) Clore, G. M.; Apella, E.; Yamada, M.; Matsushima, K.; Gronenborn, A. M. Three-dimensional structure of interleukin 8 in solution. *Biochemistry* **1990**, *29*, 1689–1696.

(47) Loosli, H. R.; Kessler, H.; Oschkinat, H.; Weber, H. P.; Petcher, T. J.; Widmer, T. J. The conformation of cyclosporin A in the crystal and in solution. *Helv. Chim. Acta* **1985**, *68*, 682–704.

(48) Wenger, R. M. Synthesis of ciclosporin and analogues: Structural and conformational requirements for immunosuppressive activity. *Prog. Allergy* **1986**, *38*, 46–64.

(49) Wenger, R. M. Cyclosporine and analogues: Structural requirements for immunosuppressive activity. *Transplant. Proc.* **1986**, *18*, 213–218.

(50) Durette, P. L.; Boger, J.; Dumont, F.; Firestone, R.; Frankshun, R. A.; Koprak, S. L.; Lin, C. S.; Melino, M. R.; Pessolano, A. A.; Pisano, J.; Schmidt, J. A.; Sigal, N. H.; Staruch, M. J.; Witzel, B. E. A study of the correlation between cyclophilin binding and in vitro immunosuppressive activity of cyclosporine A and analogues. *Transplant. Proc.* **1988**, *20*, 51–57.

(51) Wenger, R. M.; Payne, T. G.; Schreier, M. H. Cyclosporine: Chemistry, structure–activity relationships and mode of action. *Prog. Clin. Biochem. Med.* **1986**, *3*, 157–191.

(52) Rich, D. H.; Sun, C.-Q.; Guillaume, D.; Dunlap, B.; Evans, D. A.; Weber, A. Synthesis, biological activity, and conformational analysis of (2S,3R,4S)-MeBmt^1-cyclosporin, a novel 1-position epimer of cyclosporin A. *J. Med. Chem.* **1989**, *32*, 1982–1987.

(53) Marion, D.; Kay, L. E.; Sparks, S. W.; Torchia, D. A.; Bax, A. Three-dimensional heteronuclear NMR of ^{15}N-labeled proteins. *J. Am. Chem. Soc.* **1989**, *111*, 1515–1517.

(54) Marion, D.; Driscoll, P. C.; Kay, L. E.; Wingfield, P. T.; Bax, A.; Gronenborn, A. M.; Clore, G. M. Overcoming the overlap problem in the assignment of ^{1}H NMR spectra of larger proteins by use of three-dimensional heteronuclear ^{1}H–^{15}N Hartmann–Hahn multiple quantum coherence and nuclear Overhauser–multiple quantum coherence spectroscopy. *Biochemistry* **1989**, *28*, 6150–6156.

(55) Zuiderweg, E. R. P.; Fesik, S. W. Heteronuclear three-dimensional NMR spectroscopy of the inflammatory protein C5a. *Biochemistry* **1989**, *28*, 2387–2391.

(56) Fesik, S. W.; Gampe, R. T., Jr.; Zuiderweg, E. R. P.; Kohlbrenner, W. E.; Weigl, D. Heteronuclear three-dimensional NMR spectroscopy applied to CMP-KDO sythetase (27.5 kD). *Biochem. Biophys. Res. Commun.* **1989**, *159*, 842–847.

(57) Ikura, M.; Kay, L. E.; Tschudin, R.; Bax, A. Three-dimensional NOESY-HMQC spectroscopy of a ^{13}C-labeled protein. *J. Magn. Reson.* **1990**, *86*, 204–209.

(58) Zuiderweg, E. R. P.; McIntosh, L. P.; Dahlquist, F. W.; Fesik, S. W. Three dimensional ^{13}C-resolved proton NOE spectroscopy of uniformly ^{13}C labelled proteins for the NMR assignment and structure determination of larger molecules. *J. Magn. Reson.* **1990**, *86*, 210–216.

and, in principle, could be used to determine the complete three-dimensional structure of a drug/receptor complex. These studies are important for determining the 3D structure of the receptor and identifying the interactions that stabilize the binding to the ligand.

An illustration of how 3D NMR can be used to simplify the analysis of the NMR spectra of a drug/receptor complex is shown in Figure 10. Three slices from a ^{15}N-resolved 3D NOE spectrum of CsA bound to uniformly ^{15}N-labeled cyclophilin are shown. NOEs between the amide protons of cyclophilin appearing on the diagonal (solid line) and other protons of cyclophilin and cyclosporin (vertical axis) are cleanly resolved in different planes of the 3D NOE spectrum by editing with respect to the ^{15}N chemical shifts of the amides. From the spectral simplification that is achieved, the assignment of the NMR signals is facilitated, and the interpretation of the NOE data becomes feasible.

It is clear that heteronuclear 3D NMR methods offer advantages over their 2D analogues, but are these experiments practical? The sensitivity of 3D NMR experiments is determined by a number of factors, including the relaxation behavior of the resonances and the efficiency of coherence (polarization) transfer between the different time domains. Compared to 2D NMR, some loss in sensitivity occurs depending on the additional delays in the 3D experiment; however, most 3D experiments can be performed on modern 500- or 600-MHz NMR spectrometers in 2–4 days using a sample concentration of ≥1 mM in a volume of 0.5 mL. Therefore, for a protein with a molecular weight of 20 kDa, 10 mg of protein is required.

The hardware and software necessary for acquiring the 3D NMR data sets are similar to that required for heteronuclear 2D NMR experiments and are available on modern NMR spectrometers. However, an important difference between 2D and 3D NMR experiments is the amount of disk space necessary for the storage of the 3D data. For example, in a typical 3D HMQC-NOESY experiment, 64 complex 2D data sets of 128 × 1024 complex points are acquired which require 268 Mbytes of mass storage.

Large amounts of disk space is also needed for processing 3D NMR data. Typically, 3D data sets are composed of 128 × 256 × 1024 points (134 Mbytes). In addition fast computers are useful to efficiently process the 3D NMR data. Fortunately, the computers of today can easily meet these demands. For example, on a Silicon Graphics 4D 320 VGX (with 16 Mbytes of memory), a 3D data set of 40 × 192 × 1024 complex points can be processed to a final data set size of 128 × 512 × 1024 real points in 40 min of computer time.

4D NMR

As illustrated in Figures 9 and 10, many NOEs can be resolved by editing with respect to the chemical shift of a heteronucleus attached to one of the protons in a 3D NOE experiment. However, for an unambiguous interpretation of the NOEs, it would be advantageous to distinguish *both* protons by the frequencies of the heteronuclei to which they are attached in a 4D NMR experiment.[63-66] In Figure 11 the utility of a 4D NOE experiment is schematically illustrated. In a 2D NOESY experiment, NOEs between overlapping protons Ha/Hb and Hc/Hd cannot be identified, since the f_1 and f_2 frequencies of the NOE crosspeaks are identical. By editing with respect to f_1 by the ^{13}C frequencies of the attached carbons in a 3D NOE experiment, NOEs involving Ha and Hb can be resolved in different planes (Ca and Cb). However, in f_2 the NOE crosspeak is only defined by its proton frequency and cannot be uniquely defined. By further editing

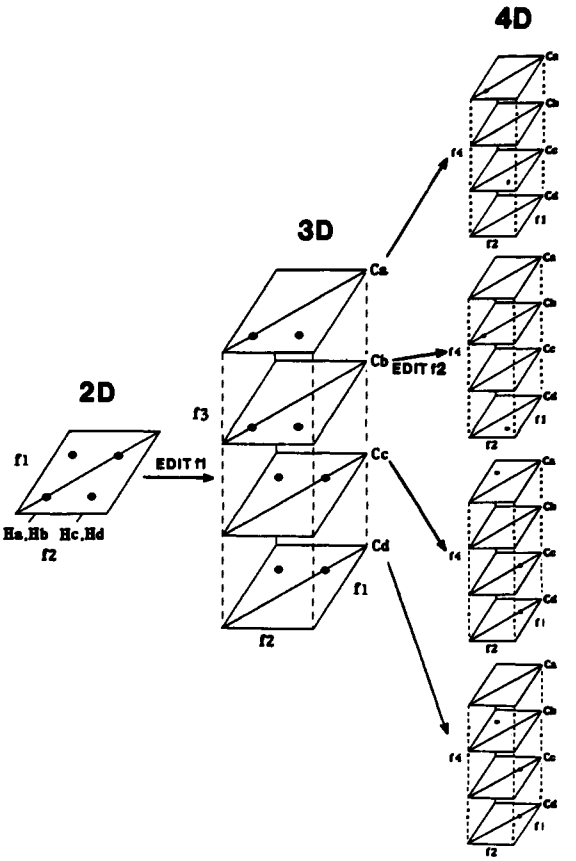

Figure 11. Schematic illustration of 2D, 3D, and 4D NOE spectra of protons with NOEs Ha/Hc, Hb/Hd, and Hc/Hd. The 2D $^1H/^1H$ NOE data are edited in f_1 by the ^{13}C frequencies (f_3) in a 3D NOE spectrum and further edited in f_2 by the ^{13}C frequencies (f_4) in a 4D NMR experiment.

(59) Fesik, S. W.; Zuiderweg, E. R. P. Heteronuclear three-dimensional NMR spectroscopy of isotopically labelled biological macromolecules. *Q. Rev. Biophys.* **1990**, *23*, 97–131.

(60) Fesik, S. W.; Eaton, H. L.; Olejniczak, E. T.; Zuiderweg, E. R. P.; McIntosh, L. P.; Dahlquist, F. W. 2D and 3D NMR spectroscopy employing ^{13}C-^{13}C magnetization transfer by isotropic mixing. Spin system identification in large proteins. *J. Am. Chem. Soc.* **1990**, *112*, 886–888.

(61) Kay, L. E.; Ikura, M.; Bax, A. Proton–proton correlation via carbon–carbon couplings: A three-dimensional NMR approach for the assignment of aliphatic resonances in proteins labeled with carbon-13. *J. Am. Chem. Soc.* **1990**, *112*, 888–889.

(62) Kay, L. E.; Ikura, M.; Tschudin, R.; Bax, A. Three-dimensional triple-resonance NMR spectroscopy of isotopically enriched proteins. *J. Magn. Reson.* **1990**, *89*, 496–514.

(63) Fesik, S. W.; Eaton, H. L.; Olejniczak, E. T.; Gampe, R. T., Jr. Pseudo-four-dimensional NMR by off-resonance decoupling. An approach for distinguishing coupled proton pairs by the frequencies of their attached heteronuclei. *J. Am. Chem. Soc.* **1990**, *112*, 5370–5371.

(64) Kay, L. E.; Clore, G. M.; Bax, A.; Gronenborn, A. M. Four-dimensional heteronuclear triple-resonance NMR spectroscopy of interleukin-1β in solution. *Science* **1990**, *249*, 411–414.

(65) Zuiderweg, E. R. P.; Petros, A. M.; Fesik, S. W.; Olejniczak, E. T. Four-dimensional [^{13}C,^1H,^{13}C,^1H] HMQC-NOE-HMQC NMR spectroscopy: Resolving tertiary NOE distance constraints in the spectra of larger proteins. *J. Am. Chem. Soc.* **1991**, *113*, 370–372.

(66) Clore, G. M.; Kay, L. E.; Bax, A.; Gronenborn, A. M. Four-dimensional $^{13}C/^{13}C$-edited nuclear Overhauser enhancement spectroscopy of a protein in solution: Application to interleukin 1β. *Biochemistry* **1991**, *30*, 12–18.

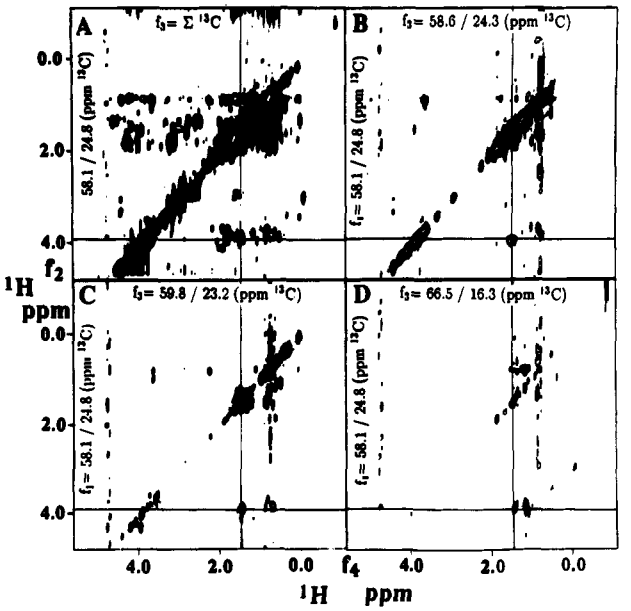

Figure 12. Contour plots from a 4D [^{13}C,^{1}H,^{13}C,^{1}H] HMQC-NOE-HMQC experiment of uniformly ^{13}C-labeled (>95%) T4 lysozyme (3.8 mM). The data was acquired as previously described[65] on a Bruker AM-500 NMR spectrometer in a total time of 216 h and processed with in-house written software. (A) ^{1}H,^{1}H (f_2,f_4) projection of all the f_3 planes at the same f_1 correspondint to ^{13}C frequencies of 58.1 and 24.8 ppm (folded). (B) Individual ^{1}H,^{1}H (f_2,f_4) cross sections at the same f_1 plane but different f_3 frequencies. The cross hairs were placed in the same location in each of the panels and indicate the different NOE cross-peaks (Adapted from Zuiderweg et al. *J. Am. Chem. Soc.* 1991, *113*, 371).[65]

of the proton signals in f_2 by the ^{13}C chemical shifts of their attached carbons in a 4D NOE experiment, the NOEs Ha/Hc and Hb/Hd can be unambiguously identified. In addition, as illustrated in Figure 11, NOEs between protons that resonate at exactly the same frequency (e.g., Hc and Hd) can be identified in the 4D experiment as long as their attached carbons resonate at different frequencies.[65,66]

Figure 12B–D depicts different ^{1}H–^{1}H NOESY planes from a four-dimensional [^{13}C,^{1}H,^{13}C,^{1}H] NOE experiment of T4 lysozyme (18.7 kDa, 164 amino acids) extracted at the same ^{13}C f_1 frequency but different ^{13}C f_3 frequencies.[65] Compared to the projection of the 4D spectrum (Figure 12A) which is equivalent to a [^{13}C,^{1}H,^{1}H] 3D NOE spectrum, a marked simplification is achieved by further editing the proton signals observed in f_4 by the frequencies of their attached carbons (f_3). The additional resolution achievable in the 4D experiment allows more NOEs to be unambiguously identified. For example, as shown in Figure 12, the NOEs indicated by the cross hairs which could not be resolved in the 3D NOE experiment (Figure 12A) are clearly distinguished in the 4D NOE spectra (Figure 12B–D).

The additional resolution gained in 4D compared to 3D NMR experiments is clearly important, especially in NOE experiments involving medium-size (10–30 kDa) proteins. Although more disk space is required for the 4D NMR experiments and more time is needed to collect and process the data, the additional spectral information that is obtained outweighs the higher demands of 4D NMR.

Conclusions and Future Perspectives

A variety of approaches have emerged for studying large molecular complexes by NMR. These recently developed methods rely on the use of isotopically labeled ligands or receptors for simplifying complicated proton NMR spectra which facilitates the data analysis and allows detailed structural information to be obtained. A practical limit on the size of the molecules that will be amenable to study by NMR is about 40 kDa. This limitation is mainly due to the broad NMR signals of this size molecules and the corresponding loss of signal intensity observed in multidimensional NMR experiments. Another limitation is the requirement for isotopically labeled receptors or ligands. However, as a result of recent advances in molecular biology, receptor proteins can be produced in large quantities and readily labeled by growing the organism that produces the protein on an isotopically labeled medium. Isotopically labeled ligands may be biosynthetically produced in a similar manner or chemically synthesized. In the best case, both labeled ligands and receptors would be available for NMR studies. The ideal protocol would involve an initial study of labeled ligands bound to their receptors to determine the bound conformation of the ligand and to identify those portions of the ligand that interact with the receptor. This information could be rapidly obtained (1–2 weeks) using isotope-edited 2D or heteronuclear 3D NMR methods and could quickly provide a start for the design of new analogues. The next phase would involve the complete three-dimensional structure determination of the drug/receptor complex using isotopically labeled receptor proteins and multidimensional NMR methods. Although these studies would require more time to complete (6–12 months), these studies are necessary for determining the manner in which the ligand is binding to the active site and identifying those functional groups of the ligand important for binding to the target site. Once an initial structure is determined, additional three-dimensional structures of the same receptor with different inhibitors could be rapidly obtained, facilitating the design of new analogues in an interactive manner.

The key to whether or not the structural information determined by NMR will be of value in drug design greatly depends on the speed and accuracy in which the information can be obtained. The *accuracy* of the structures, important for designing analogues with precisely the correct geometry for tight binding to the receptor, could be improved by obtaining more constraints, including additional distance constraints from 4D NOE experiments[64-66] and dihedral angle constraints from three-bond ^{1}H–^{1}H, ^{1}H–^{15}N, and ^{1}H–^{13}C J couplings measured from E.COSY-type spectra.[67-72] Further improvements could be achieved by obtaining more accurate distance constraints by using an approach involving a full relaxation-rate matrix treat-

(67) Griesinger, C.; Sorensen, O. W.; Ernst, R. R. Two-dimensional correlation of connected NMR transitions. *J. Am. Chem. Soc.* 1985, *107*, 6394–6396.
(68) Griesinger, C.; Sorensen, O. W.; Ernst, R. R. Correlation of connected transitions by two-dimensional NMR spectroscopy. *J. Chem. Phys.* 1986, *85*, 6837–6852.
(69) Griesinger, C.; Sorensen, O. W.; Ernst, R. R. Practical aspects of the E.COSY technique. Measurement of scalar spin–spin coupling constants in peptides. *J. Magn. Reson.* 1987, *75*, 474–492.
(70) Montelione, G. T.; Wagner, G. Accurate measurements of homonuclear H^N–H^α coupling constants in polypeptides using heteronuclear 2D NMR experiments. *J. Am. Chem. Soc.* 1989, *111*, 5474–5475.
(71) Montelione, G. T.; Winkler, M. E.; Rauenbuehler, P.; Wagner, G. Accurate measurements of long-range heteronuclear coupling constants from homonuclear 2D NMR spectra of isotope-enriched proteins. *J. Magn. Reson.* 1989, *82*, 198–204.
(72) Zuiderweg, E. R. P.; Fesik, S. W. Band-selective heteronuclear decoupling using shaped pulses as an aid in measuring long-range heteronuclear J-couplings. *J. Magn. Reson.* 1991, *93*, 653–658.

ment[73-77] of the NOE data or by taking into account molecular motion in the data analysis.

Three-dimensional structures could be obtained more *rapidly* by reducing the time required for assigning the NMR signals through new heteronuclear multidimensional NMR experiments[62] and by the development of software tools for the automated assignment of the NMR data.[78,79]

Further decreases in the time required for determining 3D structures from NMR could be achieved using computer programs which (1) automatically pick and integrate NOE crosspeaks from multidimensional NOE spectra, (2) convert the NOE data into ^1H–^1H distance constraints (if not using the NOE data directly), (3) extract J couplings and analyze them in terms of dihedral angles, and (4) rapidly refine structures on the basis of the experimentally derived constraints. These new developments in NMR should play an important role in improving the utility of NMR as a tool in drug research.

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(73) Keepers, J. W.; James, T. L. A theoretical study of distance determinations from NMR. Two-dimensional nuclear Overhauser effect spectra. *J. Magn. Reson.* **1984**, *57*, 404–426.
(74) Olejniczak, E. T.; Gampe, R. T., Jr.; Fesik, S. W. Accounting for spin diffusion in the analysis of 2D NOE data. *J. Magn. Reson.* **1986**, *67*, 28–41.
(75) Macura, S.; Farmer, B. T., II; Brown, L. R. An improved method for the determination of cross-relaxation rates from NOE data. *J. Magn. Reson.* **1986**, *70*, 493–499.
(76) Fesik, S. W.; O'Donnell, T. J.; Gampe, R. T., Jr.; Olejniczak, E. T. Determining the structure of a glycopeptide-Ac_2-Lys-D-Ala-D-Ala complex using NMR parameters and molecular modeling. *J. Am. Chem. Soc.* **1986**, *108*, 3165–3170.
(77) Fesik, S. W.; Bolis, G.; Sham, H. L.; Olejniczak, E. T. Structure refinement of a cyclic peptide from 2D NMR data and molecular modeling. *Biochemistry* **1987**, *26*, 1851–1859.
(78) Eads, C. D.; Kuntz, I. D. Programs for computer-assisted sequential assignment of proteins. *J. Magn. Reson.* **1989**, *82*, 467–482.
(79) Kleywegt, G. J.; Lamerichs, R. M. J. N.; Boelens, R.; Kaptein, R. Toward automatic assignment of protein ^1H NMR spectra. *J. Magn. Reson.* **1989**, *85*, 186–197.