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Characterization of Protein−Ligand Interactions by High-Resolution Solid-State NMR Spectroscopy

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Abstract: A novel approach for detection of ligand binding to a protein in solid samples is described. Hydrated precipitates of the anti-apoptotic protein Bcl-xL show well-resolved $^{13}C-^{13}C$ 2D solid-state NMR spectra that allow site-specific assignment of resonances for many residues in uniformly ^{13}C-enriched samples. Binding of a small peptide or drug-like organic molecule leads to changes in the chemical shift of resonances from multiple residues in the protein that can be monitored to characterize binding. Differential chemical shifts can be used to distinguish between direct protein−ligand contacts and small conformational changes of the protein induced by ligand binding. The agreement with prior solution-state NMR results indicates that the binding pocket in solid and liquid samples is similar for this protein. Advantages of different labeling schemes involving selective ^{13}C enrichment of methyl groups of Ala, Val, Leu, and Ile ($C\delta1$) for characterizing protein−ligand interactions are also discussed. It is demonstrated that high-resolution solid-state NMR spectroscopy on uniformly or extensively ^{13}C-enriched samples has the potential to screen proteins of moderate size (∼20 kDa) for ligand binding as hydrated solids. The results presented here suggest the possibility of using solid-state NMR to study ligand binding in proteins not amenable to solution NMR.

Introduction

The application of NMR as a tool for the characterization of protein−ligand complexes is a powerful methodology that can complement other drug discovery technologies.[1−6] The strength of an NMR-based approach is that it provides a very reliable assay for direct detection of ligand binding. It is widely used as a secondary assay to confirm and characterize mechanism-based inhibition of a target protein, and, for protein−protein interactions, it is often the first assay that is available for evaluation of drug discovery leads. NMR screening has also proven to be very useful in fragment-based approaches to drug design. In this approach, called "structure−activity relationship by NMR" (SAR by NMR), NMR-based screening is used to identify small molecules that can be linked to obtain tight-binding drug leads.[7,8]

Solution-phase NMR is currently limited to low- or medium-molecular-weight, highly soluble proteins. This excludes many potential drug targets from NMR analysis, including membrane-bound proteins. In addition, systems prone to aggregation or with low aqueous solubility are not readily amenable to analysis by solution NMR. Recent advances in solid-state NMR methodology, protein labeling, and sample preparation have opened the opportunity to extend the application of NMR to larger or membrane-bound proteins, although significant methodological developments are needed to make these methods generally applicable. If ligand binding is monitored via chemical shift changes of the protein,[7] a prerequisite for the use of solid-state NMR techniques in NMR-based lead finding is to obtain highly resolved spectra of the protein. The ability to obtain high-resolution spectra of proteins in the solid state has recently been reported for several proteins, including BPTI,[9] the α-spectrin SH3 domain,[10] ubiquitin,[11,12] and Crh.[13]

On the basis of these reports, it is clear that the global topologies of these proteins do not change significantly between the liquid and solid states. However, it is currently unclear whether the methods used to prepare and analyze the protein

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samples might interfere with ligand binding, either by minor disruptions in the protein binding site, unfavorable packing interactions, or radical changes in buffer composition that preclude complex formation. Studies along these lines are critical to assess the applicability of solid-state NMR to the study and characterization of ligand binding.

Here we describe a detailed experimental protocol that uses solid-state NMR experiments to monitor the binding of peptides and small organic molecules to a hydrated precipitate of uniformly or extensively ^{13}C-labeled protein. As a test system, we have used the anti-apoptotic protein Bcl-xL (MW $\approx$ 20 kDa), for which peptide and non-peptide ligands are known.[14] Bcl-xL belongs to an important family of proteins involved in regulation of programmed cell death (apoptosis). This family of proteins controls the threshold whereby "assaults" on a cell, such as chemical or radiation signals, result in apoptosis. Bcl-xL acts as an apoptosis antagonist, while other proteins, such as Bak or Bax, promote cell death, including apoptosis following exposure to chemotherapeutic drugs. Recently, solid-state NMR experiments on Bcl-xL in oriented lipid bilayers have been used to study its membrane association.[15]

In this work we show that high-resolution, highly reproducible spectra can be obtained on Bcl-xL in the presence or in the absence of ligand. Significantly, in analogy to solution-state NMR, multiple cross-peaks can be monitored not only to indicate the presence of a ligand but also to map the ligand binding site. In addition, structural changes in the protein that occur upon ligand binding can be detected, and differential chemical shift changes between ligands can be observed. All of these results strongly suggest that solid-state NMR techniques will be powerful tools in the characterization of ligand binding and the structure determination of protein−ligand complexes.

Materials and Methods

Sample Preparation. Uniformly [^{13}C,^{15}N]-labeled Bcl-xL protein was prepared as described previously.[16] A soluble protein construct where the transmembrane helix and the loop between residues 49 and 88 have been truncated[14] was used in all studies. This protein has anti-apoptotic activity similar to that of full-length Bcl-xL.[16] Selective ^{13}C-labeling of the methyl groups of Leu, Val, and Ile (Cδ1) was achieved by supplementing the growth medium with the respective amino acid biosynthesis precursors [3-^{13}C]-α-ketobutyrate and [3,3′-^{13}C]-α-ketoisovalerate.[17−19] Methyl-labeled Ala was incorporated directly by adding [2-^{13}C]-Ala to the growth medium.

Protein samples were concentrated to about 25 mg/mL in a sodium citrate buffer (20 mM, pH7.5). For the precipitant, a stock solution (PEG4600) of 50% (w/w) poly(ethylene glycol) (Aldrich, M_r = 4600) in sodium citrate buffer (20 mM, pH 4.2) was used. About 1000 μL of PEG4600 solution was added slowly to 500 μL of protein solution. The final solution contained about 30−35% PEG at a pH of about 5.6 and was stored overnight at 4 °C before harvesting. Using this protocol, an apparently amorphous precipitate was obtained. No powder diffraction data were obtained for the samples to quantitate the amount of local order. This protocol is different from other studies which have used microcrystalline material that is large enough to be visible under the microscope.[11,12,20] The Bcl-xL/Bak peptide complex was formed by addition of lyophilized Bak peptide to the protein solution with a molar excess of about 1.2:1 (peptide to protein). After a 30 min incubation at 4 °C, the protein was precipitated as described above. For the preparation of the protein/ligand complexes, the organic compounds were dissolved in DMSO to a concentration of 50 mM, before addition to the protein solution in a 1.5 molar excess. The final DMSO concentration did not exceed 4% (v/v). Due to the limited solubility of these organic molecules in aqueous buffers, the incubation time was increased to 2−3 h before addition of the precipitant. The protein/ligand precipitate was centrifuged at 10 000 rpm for 30 min, and most of the supernatant was removed. For MAS experiments, about 40−50 mg of the wet precipitate was transferred into a 4 mm Bruker MAS rotor and confined to the center of the rotor by Teflon spacers. Rotors were stored at −80°C until use.

NMR Experiments. All solid-state NMR experiments were performed on a Bruker Avance DRX-750 spectrometer, operating at 750.22 MHz proton and 188.65 MHz carbon frequency. A double-resonance (H−F/X) widebore MAS probe with 4.0 mm rotor diameter was used. The air inlet temperature was adjusted to 255 K for all experiments, and the spinning frequency was set to 10 kHz. 1D CP-MAS spectra were acquired with a ramped RF field on the proton channel (80−100%), with an RF field strength of 57 kHz on proton and 68 kHz on ^{13}C during the cross-polarization time of 2.0 ms.

For carbon−carbon correlation experiments, the "^{1}H−^{13}C dipolar assisted rotational resonance" (DARR)[21] experiment was used. An ~85 kHz decoupling field strength was applied on the proton channel during evolution periods t_1 and t_2 using the XiX sequence.[22] During the mixing time of 10 ms, the proton RF irradiation field strength was matched with the spinning frequency of 10 kHz. For acquisition of the uniformly labeled samples, the transmitter frequency was set to 75 ppm, covering a spectral width of 265 ppm in ω_2 and 150 ppm in ω_1, respectively. For the ^{13}C-methyl-labeled samples, the spectral width was reduced to 88 ppm in ω_1 with the transmitter at 45 ppm. Typically 80 or 96 scans with 2 s recycling delay were acquired for each of the 350 points in the t_1 dimension using the TPPI method for phase-sensitive detection.[23] The total acquisition time was between 16 and 20 h for the 2D spectra and 5−10 min for the 1D spectra. Both 1D and 2D data sets were processed with NMR Pipe.[24] 1D data sets were zero-filled to 4096 points before Fourier transform and phasing. For the 2D data sets, a phase-shifted sinebell function was used for apodization and zero-filling to 2048 points in t_2 and 1024 points in t_1 prior to FT in each dimension. The magnetic field was referenced externally to DSS,[25] using the ^{13}C methylene peak in solid adamantane (Aldrich). 2D spectra have been analyzed and assigned using Sparky version 3.1 (T. D. Goddard and D. G. Kneller, University of California, San Francisco).

Results and Discussion

NMR is a powerful technique to monitor protein−ligand interactions. By detecting spectral changes, residue-specific details of the protein−ligand interaction can be obtained. For example, binding of a ligand to a protein will perturb the environment of the interacting amino acids and thus alter the

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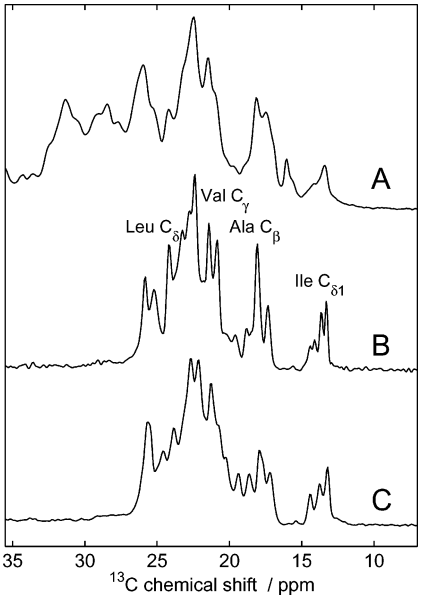

Figure 1. (A) Aliphatic region of the $^{13}C-CP-MAS$ spectra of uniformly [^{13}C,^{15}N]-labeled Bcl-xL precipitated with PEG4600. (B) Spectrum for the Bcl-xL protein with selectively ^{13}C-labeled methyl groups of Ala, Leu, Val, and Ile (Cδ1). (C) Spectrum of the ^{13}C-methyl-labeled Bcl-xL protein ligated by the Bak peptide. The approximate positions of methyl resonances are indicated for each amino acid.

chemical shift of the nuclei close to the binding site. The spectral changes that are most easily observed are for well-resolved resonances. Resolution can be achieved by combining multidimensional NMR techniques with either uniformly labeled protein samples with NMR-active isotopes or specific labeling schemes of certain residues and/or functional groups in the side chains.[26,27] In principle, residue-specific assignments can be obtained from labeling, by reference to known ligands,[28] or by using recently developed assignment experiments for the solid state.[9−12] However, Bcl-xL (20 kDa) is relatively large compared to proteins for which full assignment of spectra has presently been achieved on the basis of solid-state NMR data alone. Furthermore, the all-α-helical structure of Bcl-xL provides only limited chemical shift dispersion and makes this protein a challenging target for the application of solid-state NMR techniques.

Ligand Binding Detected by 1D ^{13}C CP-MAS Experiments. In Figure 1A, we show the aliphatic region of the ^{13}C CP-MAS spectrum obtained for the precipitates of the uniformly [^{13}C,^{15}N]-labeled sample of Bcl-xL. Because of the large number of resonances, the 1D spectra of the protein have very few well-resolved features. In Figure 1B, we show the improvement that can be obtained by using selective ^{13}C-labeling of the methyl groups of Ala, Val, Leu, and Ile (Cδ1). Both samples have been prepared using the same precipitation protocol. The better resolution in Figure 1B is due to a reduction in the number of resonances and to an improvement in the resonance line width due to the removal of homonuclear ^{13}C *J* couplings as well as any relaxation broadening from directly bonded ^{13}C.

The presence of $^{13}C-^{13}C$ homonuclear *J* couplings (35−55 Hz) and residual dipolar couplings limits the spectral resolution in samples with uniform ^{13}C enrichment. We estimate that the ^{13}C line widths in the uniformly labeled sample are in the 0.5−0.7 ppm range. In the selectively labeled sample, all four Ile (Cδ1) resonances of the protein are resolved in the 1D spectrum, and we estimate that the line widths are in the 0.2−0.3 ppm range. When the (isotopically unenriched) 16-residue Bak peptide is bound to the ^{13}C-methyl-labeled protein prior to precipitation of the sample, many peaks show a significant change in their chemical shift (Figure 1C). This simple experiment shows that binding of a ligand can be detected qualitatively in a simple 1D NMR experiment with an accumulation time of about 10 min. This suggests that screening of ligand binding using 1D NMR experiments would be feasible in the solid state. In particular, 1D spectra could be useful for the characterization of leads that come from high-throughput screening or the analysis of other small libraries of compounds.

Ligand Binding Detected by 2D $^{13}C-^{13}C$ Correlation Experiments. To obtain a detailed site-specific mapping of a ligand binding site, it is important to obtain sufficient resolution to resolve a large number of protein resonances. Figure 2 shows the aliphatic region of the $^{13}C-^{13}C$ correlation spectra for the uniformly ^{13}C-enriched Bcl-xL apo protein (green) overlayed with the Bcl-xL/Bak complex (red). Some amino acid types, such as Ile, are fully resolved in the 2D spectrum, while other regions (e.g., Ala $C_\alpha-C_\beta$) are still very congested. Several resolved peaks have been assigned on the basis of the chemical shifts obtained previously by solution NMR.[14] As an example, we discuss Ile and Ala residues, for which a significant shift upon binding of the Bak peptide has been observed. In particular, peaks assigned to the Cδ1 methyl groups of Ile118 and Ile144 shift significantly upon addition of the peptide, while those of Ile170 and Ile186 are affected much less.

This is consistent with the NMR structure of the Bcl-xL/Bak complex[14] (PDB entry 1bxl), where the Cδ1 methyls of residues Ile118 and Ile144 are closer to the Bcl-xL's binding groove (9.2 and 10.5 Å, respectively) than Ile170 and Ile186 (12.3 and 15.4 Å, closest distance measured between the carbon atoms of the protein residue and a residue of the Bak peptide). Changes in the protein structure upon peptide binding result in an altered position and orientation for Ala123 and might be the primary reason for the large peak shift (Figure 2, middle). Differences in chemical shifts observed here on solid precipitates correlate very well with those observed in solution by $^{1}H-^{13}C$ HSQC spectra obtained from the same protein preparation (data not shown).

Figure 2 reveals also that the shifts observed are more significant for the carbon atoms far away from the backbone. While methyl groups of Ile and Leu show large shifts upon peptide binding, the C_α and C_β atoms of the same amino acids are only slightly affected. Hence, significant effects on the chemical shift pattern would be expected for a $^{1}H-^{13}C$ correlation experiment involving the methyl groups.

Figure 3A shows the $^{13}C-^{13}C$ spectra of the uniformly labeled Bcl-xL incubated with an organic molecule of nanomolar affinity. While the shifts are smaller than those for the Bak peptide, they are still significant and reproducible. It has been often observed in the past that binding of ligands results in multiple changes in the spectrum, some of which are long-range

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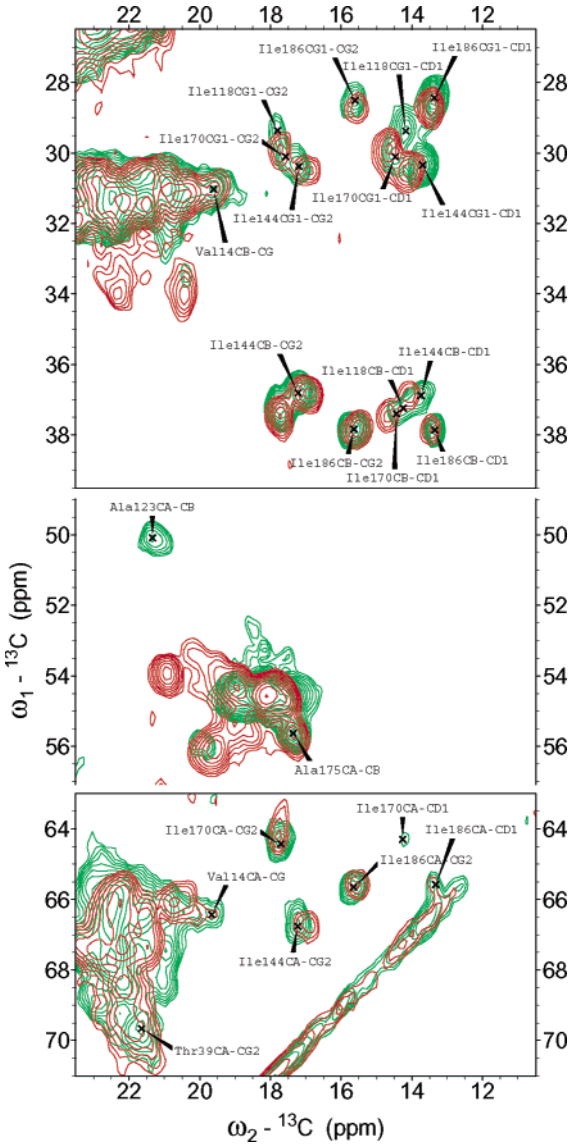

Figure 2. DARR experiment on precipitated samples of uniformly [^{13}C,^{15}N]-enriched Bcl-xL protein obtained with 10 ms mixing time. The aliphatic region of the ^{13}C−^{13}C correlation spectrum of the Bcl-xL apo protein (green) is shown overlayed with the spectrum of the Bcl-xL/Bak complex (red). The assignment is largely based on the chemical shift obtained by solution NMR.[14] Regions with large spectral overlap have not been assigned.

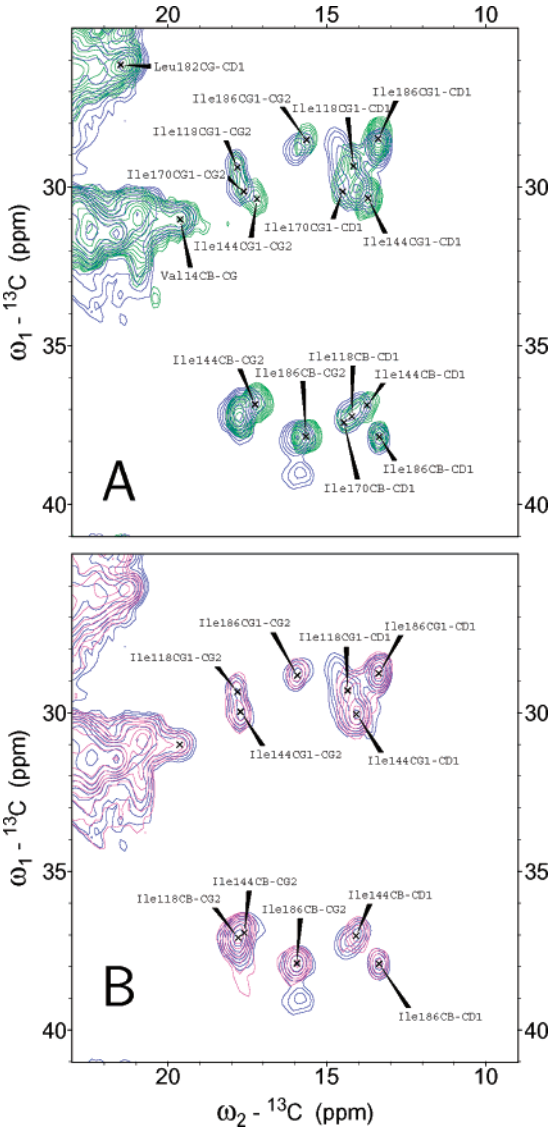

Figure 3. (A) DARR spectrum of the Bcl-xL apo protein (green) overlayed with the spectrum protein after binding compound **1** (blue). The shifts are different than observed for the Bak peptide, indicating a different influence on the structure of the protein than for Bak. (B) Comparison of the spectra obtained for uniformly labeled Bcl-xL bound to compound **1** (blue) or compound **2** (magenta). The shifts are almost identical for both ligands, indicating that they give rise to very similar conformational changes of the protein. By comparing the spectra of the protein bound to a variety of structurally similar compounds, shifts due to ligand contact and remote structural changes can be distinguished.

effects resulting from small conformational changes induced by the ligand.[29] These "remote" conformational effects can make the interpretation of chemical shift changes difficult, especially if site-specific assignments are not available. An efficient solution to this problem can be the differential chemical-shift perturbation method. Here, the spectra of the protein when it is bound to two slightly different ligands are compared.[29] Figure 3B compares the Bcl-xL spectra obtained for two similar but slightly different ligands which are assumed to cause similar global conformational changes. Differences in the spectra are now expected, particularly for residues which are in contact with the modified part of the ligand. This not only confirms the ligand binding but also proves the reproducibility of the spectra. Furthermore, a detailed analysis of the differential chemical shifts for several structurally analogous molecules may provide direct information on the orientation of the ligands and may identify contacts between certain residues and specific parts of the ligand molecule, as previously done for the Bcl-xL/Bak complex.[29]

Spectral Reproducibility. The reproducibility of the solid-state NMR spectra in terms of line width and peak position has been assessed by repeating the measurements on precipitates prepared and measured independently but using the same protocol and experimental conditions. Replicate 2D data sets that exhibited chemical shifts from well-resolved peaks agreed within less than 0.05 ppm (RMS deviation). This is significant, as it suggests that differential shifts in the order of 0.1 ppm are meaningful and can be used to confirm ligand binding.

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Long-Range Correlations between Methyl Groups. Structural changes that occur upon ligand binding have been probed in more detail using the DARR experiment recoupling distant spins. For the sample with selective ^{13}C enrichment in the methyl groups of Ala, Val, Leu, and Ile (Cδ1), $^{13}C-^{13}C$ correlation experiments with short mixing times are restricted to the detection of intraresidue methyl groups in Val and Leu. However, those methyl groups usually show a very similar chemical shift, thereby generating cross-peaks close to the diagonal which are difficult to resolve and assign. Nonetheless, with spin diffusion or DARR type experiments, correlations between very distant carbon atoms can be obtained using longer mixing times.[30] This requires isolated spin systems where the chemically bonded carbons are not simultaneously labeled and hence the dominant one-bond dipolar couplings are suppressed. The selective ^{13}C enrichment in the methyl groups of Val, Leu, and Ile (Cδ1) satisfies this and is well suited for detection of long-range correlations.

Figure 4 shows carbon−carbon long-range correlation spectra obtained with a DARR experiment using a mixing time of 500 ms for the Bcl-xL apo protein (green) and the Bcl-xL/Bak complex (red). On the basis of the chemical shifts obtained from solution NMR and the three-dimensional structure,[14,16] several interresidue contacts have been identified with distances of up to 6 Å. This method is time-consuming; however, it provides the possibility to obtain structural constraints as well as chemical shift mapping. Such structural information might be used to discriminate between conformational changes induces by the ligand and a direct protein−ligand contact. For example, several long-range correlations are observed for Ile118 in the apo protein which disappear in the Bcl-xL/Bak complex. As illustrated in Figure 4B, these changes are a result of a reorientation of the Ile118 side chain following peptide binding. In a similar way, Ala123 shows a drastic change in position and orientation of the methyl group upon Bak binding, which correlates well with the observed chemical shift differences (Figure 2).

Conclusion

The strategies described in this work can potentially be used to extend NMR-based ligand binding studies to proteins not amenable for study using solution NMR techniques. In this work we have demonstrated the feasibility and reliability of ligand binding detected by solid-state NMR methods using uniformly ^{13}C-enriched samples or samples selectively ^{13}C-enriched at the methyl groups. We have also demonstrated the possibility of obtaining structural data on the protein−ligand complex using carbon−carbon correlation experiments and differential chemical shift analyses.

In principle, the methodology outlined in the preceding paragraphs can be readily extended toward drug screening by solid-state NMR. However, any drug screening project should also involve a statistical analysis of a larger family of compounds, as well as a thoughtful consideration of time efficiency. Weak or nonbinding compounds, added in large quantities, raise particular concerns for solid-state NMR, different from the situation for solution NMR, including possible effects on crystallization or crystal contacts. Of course, the effect of the precipitation and the solid-state formulation on the drug affinity must be considered as well; the method is most suited for cases where the solid-state formulation is an acceptable facsimile of the in vivo conditions.

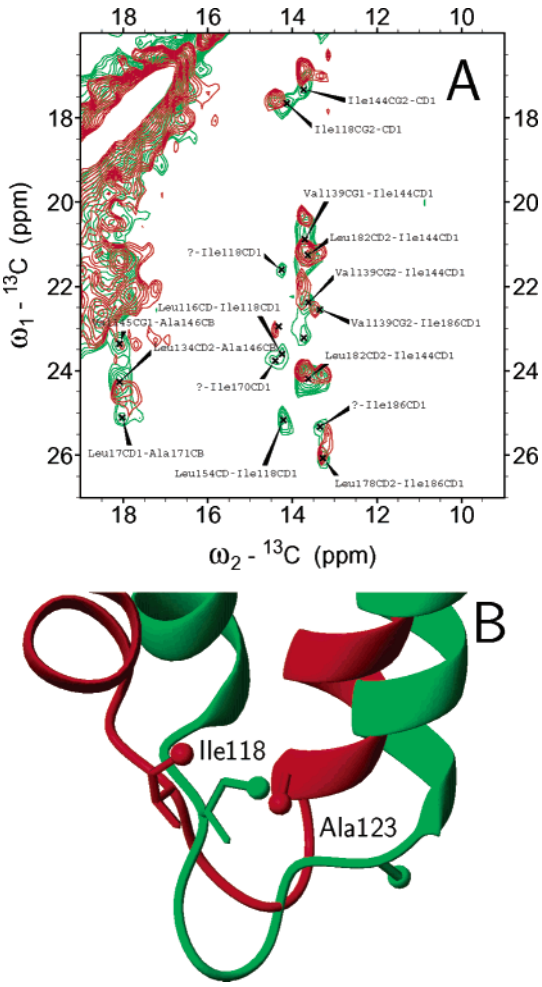

Figure 4. (A) $^{13}C-^{13}C$ correlation spectrum obtained for the ^{13}C-methyl-labeled sample for the apo protein (green) and the Bcl-xL/Bak complex (red). A mixing time of 500 ms has been used to recouple distant carbon atoms. The assignments are based on the chemical shift database obtained from solution NMR and the respective NMR structures. (B) Superposition of the structures for the Bcl-xL apo protein (green, PDB entry 1lxl) and the Bak bound protein (red, PDB entry 1bxl). The backbone atoms for all residues (except for the ones in the unstructured loop between amino acids 43−86) have been fitted to obtain maximum overlap using the program MOLMOL 2K.1.[31] The side chains of Ile118 and Ala123 have been highlighted with their ^{13}C-enriched carbons shown as spheres.

We are currently testing different labeling schemes and experimental approaches to optimize both the sensitivity and resolution of solid-state NMR experiments. These include $^{15}N-^{13}C$ correlation experiments in ^{13}C-methyl-enriched samples, since both backbone nitrogen and methyl group chemical shifts are very sensitive to ligand binding. However, because of the small dipolar coupling between the backbone nitrogen and the methyl groups, such types of experiments are far more demanding on spectrometer stability. In principle, direct detection of the protons could increase the sensitivity of $^{1}H-^{13}C$ or $^{1}H-^{15}N$ experiments by a factor of $(\gamma_H/\gamma_X)^{3/2} \approx 8$ or 30, respectively, thus significantly lowering the amount of sample required. However, for solid-state NMR experiments, specific and more expensive ^{2}H labeling schemes which suppress the network of

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proton−proton couplings are required to obtain a sufficient resolution in the proton dimension. A cost-efficient alternative could be an intraresidue homonuclear correlation for methyl-group-containing amino acids (Val, Leu, Ile), i.e., a carbon correlation between the methyl group and Cα or Cβ atoms in the same amino acid residue. This approach can combine high resolution of $^{13}C-^{13}C$ spectra for sparsely labeled samples (due to the absence of *J* couplings) with reasonable sample cost. Experiments along those lines are currently in progress in our laboratory.

In summary, this study offers clear evidence of the ability of solid-state NMR to detect protein binding of compounds with high affinity, and to identify ligand binding sites. The use of extensive or uniform enrichment with respect to ^{13}C is crucial since a global search for binding sites need not be hypothesis driven when extensive labels are included. To our knowledge, this is the first demonstration of using solid-state NMR to study ligand binding to a uniformly labeled protein that is a potential target for drug design. This study has, therefore, potential long-term implications for the use of solid-state NMR to study ligand binding phenomena.

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