Journal of Biomolecular NMR (2006) 36: 1–11
DOI 10.1007/s10858-006-9028-7

Article

Identification of individual protein–ligand NOEs in the limit of intermediate exchange

Mikhail Reibarkh[a], Thomas J. Malia[a,b], Brian T. Hopkins[c] & Gerhard Wagner[a,*]
[a]*Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115, USA;* [b]*Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139, USA;* [c]*Infinity Pharmaceuticals, Inc., 780 Memorial Drive, Cambridge, MA 02139, USA*

Received 15 March 2006; Accepted 3 May 2006

Key words: first generation compound, high-throughput screening, intermediate exchange, Nuclear Overhauser Effect

Abstract

Interactions of proteins with small molecules or other macromolecules play key roles in many biological processes and in drug action, and NMR is an excellent tool for their structural characterization. Frequently, however, line broadening due to intermediate exchange completely eliminates the signals needed for measuring specific intermolecular NOEs. This limits the use of NMR for detailed structural studies in such kinetic situations. Here we show that an optimally chosen excess of ligand over protein can reduce the extent of line broadening for both the ligand and the protein. This makes observation of ligand resonances possible but reduces the size of the measurable NOEs due to the residual line broadening and the non-stoichiometric concentrations. Because the solubility of small molecule drug leads are often limited to high micromolar concentrations, protein concentrations are restricted to even lower values in the low micromolar range. At these non-stoichiometric concentrations and in the presence of significant residual line broadening, conventional NOESY experiments very often are not sensitive enough to observe intermolecular NOEs since the signals inverted by the NOESY preparation pulse sequence relax prior to significant NOE build up. Thus, we employ methods related to driven NOE spectroscopy to investigate protein–ligand interactions in the intermediate exchange regime. In this approach, individual protein resonances are selectively irradiated for up to five seconds to build up measurable NOEs at the ligand resonances. To enable saturation of individual protein resonances we prepare deuterated protein samples selectively protonated at a few sites so that the 1D ^{1}H spectrum of the protein is resolved well enough to permit irradiation of individual protein signals, which do not overlap with the ligand spectrum. This approach is suitable for measuring a sufficiently large number of protein–ligand NOEs that allow calculation of initial complex structures, suitable for structure-based optimization of primary drug leads obtained from high-throughput screening. The method was applied to measure individual intermolecular NOEs between the anti-apoptotic protein Bcl-xL at 25 μM and a ''first generation'' small-molecule ligand, for which the spectrum is entirely broadened at stoichiometric concentrations. This approach is general and can also be used to characterize protein–protein or protein–nucleic-acid complexes.

*To whom correspondence should be addressed.
E-mail: gerhard_wagner@hms.harvard.edu

Abbreviations: NMR – nuclear magnetic resonance; NOE – nuclear Overhauser effect; NOESY – 2D NOE spectroscopy; STD – saturation transfer difference spectroscopy; SOS NMR – structural information using Overhauser effects and selective labeling; NMR-DOC – Nuclear Magnetic Resonance Docking of compounds

Introduction

Protein interactions are central for many biological processes and form key elements of molecular switches, such as in gene expression, receptor interactions, signaling, apoptosis, or immune processes. Thus, characterizing the interaction of proteins with other proteins, nucleic acids or small molecules has been a central goal of structural biology and protein biochemistry in general. It has also been recognized for a long time that imbalanced protein interactions may lead to disease. This has motivated searches for small molecule inhibitors of such interactions, both to reveal the significance of particular interactions and to find potential drug leads to fight disease. Thus, discovery and optimization of small molecules that bind proteins to interfere with their function has been a long-standing goal of rational drug design.

Many biologically significant interactions are weak and transient, such as in cellular switches that have to be able to turn on and off. Examples of such weak interactions are found for the recognition of cell surface receptors and counter receptors (Sun et al., 1999), communication between the components of the T-cell receptor of antigens (Sun et al., 2001; Bunnell et al., 2002; Arnett et al., 2004), interactions that initiate or inhibit eukaryotic translation (Gross, et al., 2003; Marintchev and Wagner, 2004; Oberer et al., 2005), protein interactions within electron-transfer complexes (Matsuo et al., 1999) and many interactions in cellular signaling (Roehrl et al., 2004; Barda-Saad et al., 2005). However, structural information is primarily available from tight complexes because these can readily be studied with X-ray crystallography or NMR spectroscopy. Thus, available knowledge is biased towards tight complexes, and atomic-detail structural information about weak interactions is sparse lacking suitable methods for their characterization.

Weak protein–ligand interactions are also typically observed at initial stages of drug-discovery efforts when small-molecule ligands of proteins are obtained from high-throughput screens of chemical libraries. Inhibitors of protein interactions found in screens of moderate-size libraries (10,000 to 100,000 compounds) typically have affinities in the low micromolar range (Degterev et al., 2001; Lugovskoy et al., 2002; Roehrl et al., 2004; Roehrl et al., 2004a, b). If crystals of the protein targets are available structures of such complexes can be solved with X-ray methods. However, soaking of crystals or co-crystallization may fail if the ligands bind surface areas involved in crystal contacts (Roehrl et al., 2004) or cause structural changes. Ligands with low-micromolar dissociation constants and diffusion-controlled on-rates typically cause NMR line broadening due to intermediate exchange and inhibit measurements of specific protein–ligand NOEs. This hampers determination of structures of protein–ligand complexes when using traditional methods.

NMR is frequently used for identifying binding faces by mapping ligand-induced chemical shift changes onto protein surfaces (Chen et al., 1993; Markus et al., 1994; Shuker et al., 1996). Similarly, binding sites can be identified from line broadening of specific residues upon ligand titration (Matsuo et al., 1999; Degterev et al., 2001; Lugovskoy et al., 2002). However, these methods monitor both direct and allosteric effects, and the exact mapping of the binding site may not always be possible. Chemical shift perturbation data have been used as ambiguous constraints to calculate models of protein–ligand complexes (Schieborr et al., 2005). A more precise mapping of protein–protein interfaces can be obtained with cross saturation techniques where the mapping is based on dipole–dipole interactions (Takahashi et al., 2000; Shimada, 2005).

Saturation transfer difference (STD) spectroscopy has recently been described focusing at the spectra of small-molecule ligands of proteins. It was originally introduced to detect in a mixture of compounds those molecules that interact with a macromolecule (Mayer and Meyer, 1999). Subsequently, it has also been employed to identify the

binding faces on the small-molecule ligands by comparing the relative amounts of saturation transfer (Mayer and Meyer, 2001). However, STD is not designed to determine individual intermolecular ^{1}H–^{1}H contacts that could be used for determining structures of protein–ligand complexes.

A crude characterization of structures of protein–ligand complexes can be achieved by analyzing differential chemical shift changes between different ligand analogs. A slight modification of the ligand may cause different chemical shift changes of a subset of resonances in the binding pocket and indicate the direction where the modified ligand site is pointing to in the protein (Medek et al., 2000; Lugovskoy et al., 2002; Pellecchia et al., 2002). This requires that analogs of a parent ligand are available and bind similarly. The structural information obtainable is qualitative but has been used in combination with computer docking programs to produce models for protein–ligand complexes (Medek et al., 2000; Lugovskoy et al., 2002; Fahmy and Wagner, 2002).

Recently, a method based on the STD approach has been presented that uses deuterated proteins that are selectively protonated at the methyl groups of isoleucines, leucines, valines or methionines. Saturating the methyl resonances in the different samples yields different saturation patterns in the spectrum of the ligand. If the structure of the protein is known it is possible to use docking programs to determine the structure of the complex without the need to assign the protein spectrum. This approach was termed SOS NMR (structural information using Overhauser effects and selective labeling) and has been applied for characterizing protein–ligand complexes in fast exchange using excess of ligand (Hajduk et al., 2004). Contacts between ILV methyls and the ligands were used as ambiguous constrains in the DOCK (Kuntz et al., 1994) software package. A related approach termed NMR-DOC (Nuclear Magnetic Resonance Docking of Compounds) has been proposed and demonstrated for complexes in fast exchange (Pellecchia et al., 2002). It is tailored towards targets with known structures and ligand binding sites. Perdeuterated protein samples with $^{13}C^{1}H$ methyl groups of Met, Thr and Ile are prepared, and methyl resonances lining the known ligand binding site are assigned by comparing differential chemical shift changes due to ligand analogs. Similar to the SOS method, the whole methyl spectrum of the selectively labeled protein is saturated and the saturation transfer to the ligand spectrum is interpreted based on the known protein structure to determine the structure of the protein–ligand complex. This method requires that both protein and ligand spectra exhibit sharp resonances, and the complexes studied were in fast exchange, which is usually the case for very weak binders.

In the limit of slow exchange, and when protein resonance assignments are known, various NMR methods are available to determine the structures of protein–ligand complexes. This includes isotope-filter methods (Otting et al., 1986; Ikura and Bax, 1992), isotope labeling schemes that facilitates distinction of inter-and intra-molecular NOEs (Walters et al., 1996; Matsuo et al., 1997; Gross et al., 2003), as well as the use of a combination of residual dipolar couplings and NOEs (Clore, 2000). Methods for this situation are well established, and numerous structures of complexes in slow exchange have been reported.

However, weak interactions often exhibit the kinetic limits of intermediate exchange, which causes severe NMR line broadening of interface resonances of the protein and the ligand. If protein and/or ligand resonances are severely broadened identification of individual protein–ligand NOEs is difficult or even impossible with conventional approaches. This is particularly limiting in structure-based drug design where structural information of protein–compound complexes is highly desired to guide a rational improvement of initial drug leads.

Here we present an approach for identification of individual intermolecular ^{1}H–^{1}H NOEs in the intermediate exchange limit. We use high excess of ligand over protein to limit but not eliminate exchange broadening. Given the poor solubility of the ligand we have to work at low protein concentration, as low as 25 μM. Since conventional NOESY experiments are not sensitive enough at such low concentrations and ligand line broadening we use 1D NOE-difference experiments where a single or a group of resonance(s) is saturated for up to several seconds, a technique known as driven NOE (Wagner and Wüthrich, 1979) and related to the STD technique (Mayer and Meyer, 1999). This experiment is an order of magnitude more sensitive than NOESY, which relies on the transient NOE principle (Solomon,

1955). To allow selective irradiation of protein resonances we produce a perdeuterated protein that is protonated at select methyl groups of leucine, valine and/or isoleucine as produced with established methods (Rosen et al., 1996; Goto et al., 1999; Medek et al., 2000; Gross et al., 2003). The approach is demonstrated in a characterization of the interaction of a small chemical with the anti-apoptotic protein Bcl-xL.

Materials and methods

All NMR experiments were carried out with a form of Bcl-xL described previously lacking a long unstructured loop (residues 45–84) and a putative transmembrane region (Sattler et al., 1997; Degterev et al., 2001; Lugovskoy et al., 2002). Deuterated Bcl-xL with protonated and ^{13}C labeled methyl groups of isoleucine, leucine and

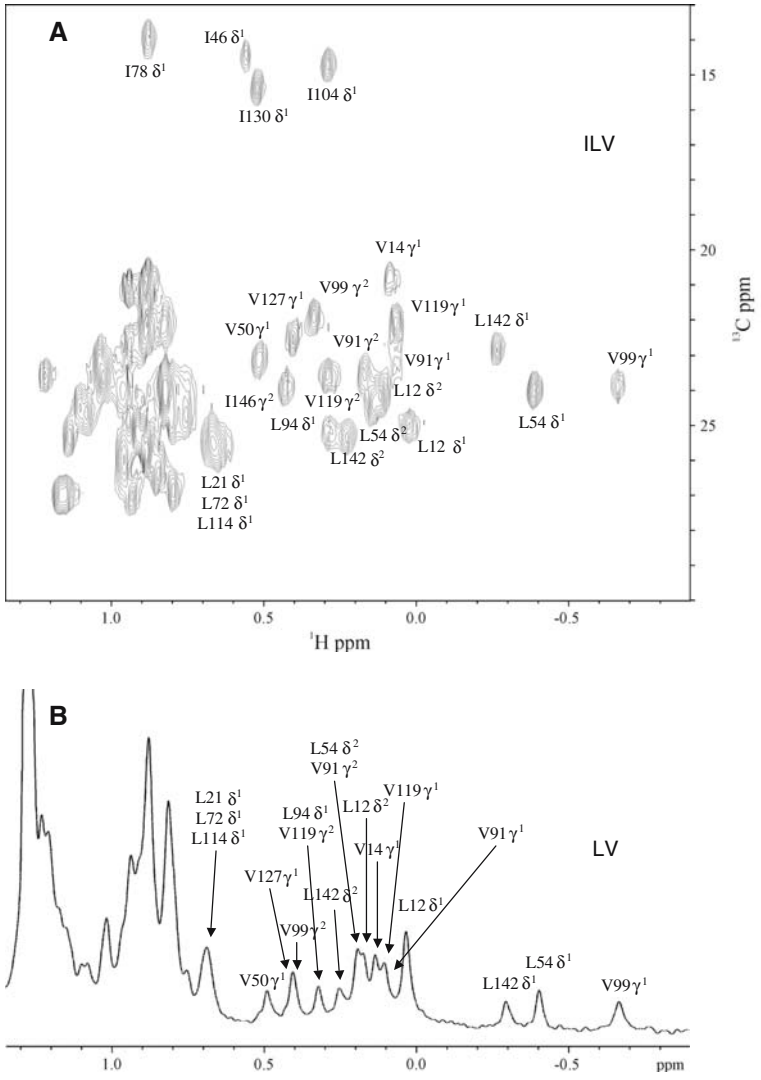

Figure 1. (A) HSQC spectra of ^{13}C ILV labeled Bcl-xL, 25μM in $^{2}H_2O$, pH 7.2, at a compound concentration of 250μM, and 5% DMSO. The total measurement time was 3 h 12 min. (B) Methyl region of the 1D ^{1}H NMR spectrum of perdeuterated Bcl-xL that is protonated at the methyl groups of leucine and valine residues but not ^{13}C labeled. Bcl-xL is at 25μM with 250μM compound, other conditions are identical to A. The positions of CW irradiations are indicated with arrows. The large signal at 1.3 ppm is from methyl 18 of the compound, which is at 10-fold excess.

valine as a group (Figure 1A) was prepared following procedures described previously (Gross et al., 2003). The sample used for Figure 1B was produced similarly but only the valine and leucine but not the isoleucine methyl groups were protonated (Goto et al., 1999; Gross, Gelev et al., 2003), and no ^{13}C labels were introduced. The latter was desirable to avoid the ^{13}C splitting during the selective saturation, and ^{13}C decoupling simultaneously with proton saturation. All NMR experiments were carried out on a Varian INOVA500 instrument equipped with a cryogenic probe. Protein concentrations were 25 μM at pH7.2 with 5% DMSO.

Results

Studies of protein interactions with weak ligands often suffer from severe line broadening both of protein and ligand resonances. We encountered this problem in several projects that had the goal to determine the structures of protein complexes with small molecule inhibitors identified through screening of large compound libraries. Often initial screening hits that have affinities in the low micromolar range experience such NMR line broadening when used at stoichiometric concentrations (Degterev et al., 2001; Lugovskoy et al., 2002; Roehrl et al., 2004). Line broadening or disappearance of signals due to chemical exchange makes it difficult or even impossible to measure protein–ligand NOEs. Figure 2B shows an example of the aromatic spectrum of a compound (Figure 2A) that has been identified as an inhibitor of the interaction between the anti-apoptotic protein Bcl-xL and the pro-apoptotoic protein Bak. The compound binds Bcl-xL with an apparent Kd of 80 μM as estimated with fluorescence spectroscopy. While the free compound exhibits a well resolved and sharp spectrum, addition of increasing amounts of Bcl-xL broadens the spectrum, and already at a compound: protein concentration of 3:1, the spectrum of the compound is completely broadened and not suitable for measuring any NOEs (Figure 2B). The line broadening appears to be due to intermediate exchange on the NMR time scale, which is observed if the lifetime of the bound compound is comparable to the time it takes to record a free-induction decay. An additional contribution to the line broadening can be due to exchange between multiple conformations of the bound compound. This is indeed the case for some regions of the interface between Bcl-xL and the inhibitor discussed here (see below) (Reibarkh et al., 2006). The appearance of the spectra in Figure 2B indicates that at least a tenfold excess of compound over protein is necessary to observe a ligand spectrum sufficiently resolved for measurement of NOEs. However, there is significant line broadening at these conditions that makes measurement of NOEs non trivial.

The spectrum of the protein, on the other hand, may experience ligand dependent chemical shift changes and line broadening. Line-broadening due to the time scale of the dissociation rate can be overcome by providing excess ligand because then all protein molecules are associated with a ligand, and the population of free protein is very low (Reibarkh et al., 2006). Thus, using conditions of excess ligand is best for observing resolved protein and ligand resonances.

When pursuing measurements of intermolecular NOEs at tenfold excess of ligand we have encountered solubility problems. The compound studied here is limited to 0.25 mM, which is not unusual for small molecule inhibitors of protein interactions. With a tenfold excess of ligand the protein concentration is limited to 25 μM. Traditional 2D NOESY experiments at these protein concentrations, and observing broadenend ligand resonances, are not sensitive enough even with modern high-field spectrometers and cryogenic probes. The NOESY experiment relies on the transient NOE principle (Solomon, 1955) where the magnetization of selectively inverted signals is transferred to nearby spins. The size of NOEs is limited by the amount of the inverted magnetization, which is transferred to all nearby spins. The amount of non-equilibrium magnetization to be distributed to nearby protons can be dramatically increased by continuously saturating a single resonance instead of just inverting it. This is used in the driven NOE (Wagner and Wüthrich, 1979) and also in the STD experiment (Mayer and Meyer, 1999). In order to identify individual intermolecular NOEs with this approach it is necessary to be able to selectively irradiate single protein resonances; however, this is usually not straightforward in a crowded protein spectrum.

To enable selective irradiation of individual protein resonances we decided to prepare

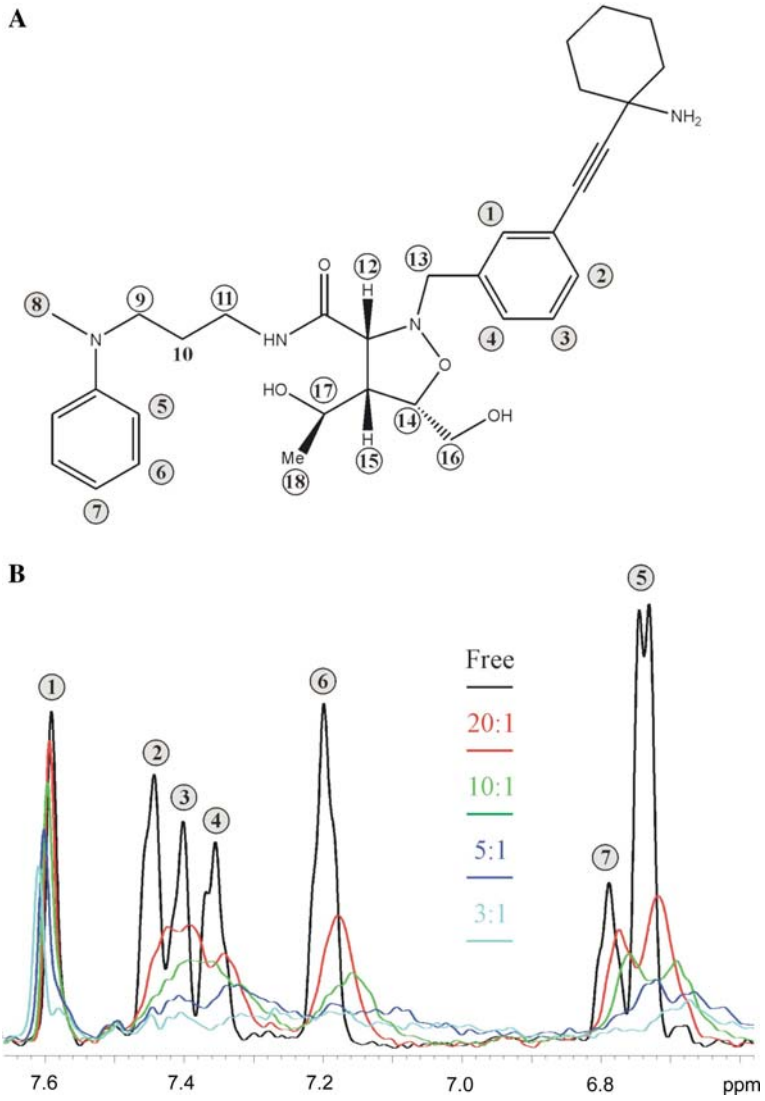

Figure 2. Structure and NMR spectra of the Bcl-xL inhibitor discussed in the text. (A) Chemical structure of the Bcl-xL inhibitor discussed here. Aliphatic groups of interest are numbered and the aromatic protons and one methyl group are labeled with encircled numbers. The molecule binds Bcl-xL with an approximate K_D of 80 μM as estimated with fluorescence spectroscopy. (B) Proton spectra of the compound in a complex with deuterated Bcl-xL (25 μM, pH 7.2, 25 C, 20 mM Tris buffer, 5 mM DTT, 1 mM EDTA, 5% DMSO) at different compound/protein ratios. The resonances of aromatic protons are marked with the same numbers as above.

perdeuterated protein that contains protonated methyl groups for single or groups of residue types. Production of deuterated proteins with protonated methyls of isoleucines, leucines and valines (ILV) has been described (Rosen et al., 1996; Goto et al., 1999), refined (Medek et al., 2000; Gross et al., 2003) and extensively applied so that a relatively large variety of selectively labeled samples can be made. Figure 1A shows a ^{1}H–^{13}C HSQC spectrum of a 25 μM solution of ILV-labeled Bcl-xL. A 1D spectrum of a similar sample that is deuterated elsewhere, and protonated without ^{13}C labeling at the Leu and Val methyls only is shown in Figure 1B. A large number of the methyl resonances are separated well enough so that they can be saturated selectively. To simplify the spectra even further, perdeuterated samples can be prepared that contain protonated methyls for only Leu, Val or Ile (Goto et al., 1999), and other labeling methods should be possible with cell-free expression methods (Yokoyama, 2003).

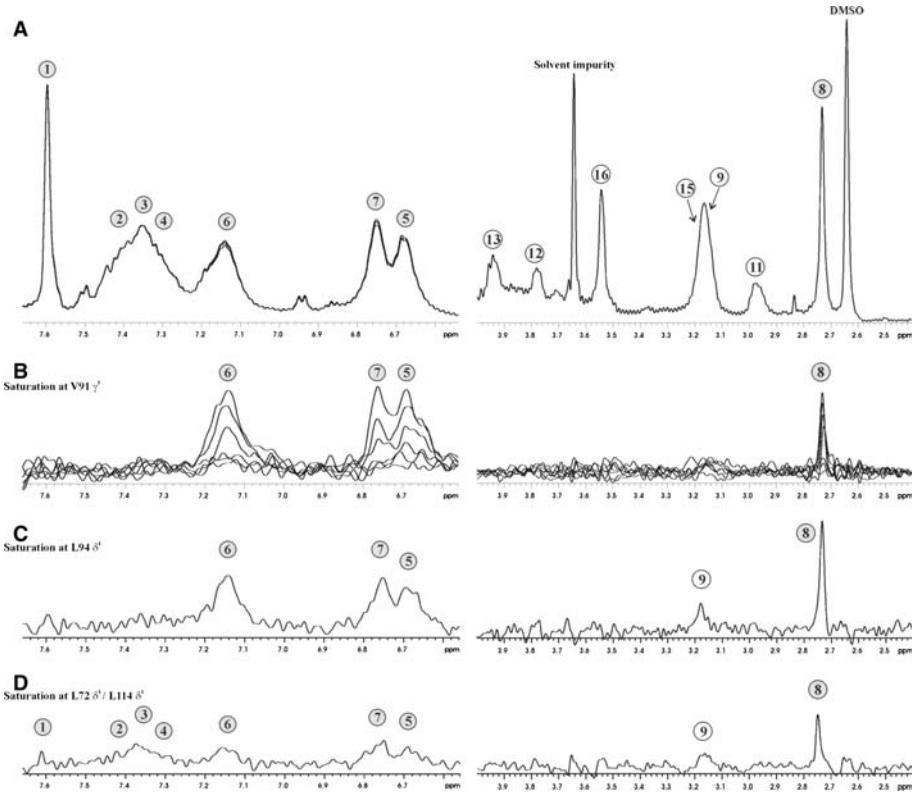

Figure 3. Driven NOEs from selective irradiation of individual methyl groups in perdeuterated Bcl-xL. (A) NOEs from Val 91 γ^1 methyl to aromatic (left) and methyl (right) resonances with irradiation times of 0, 0.1, 0.2, 0.5, 1.0, 2 s. Spectra with different irradiation times are plotted on top of each other. (B) Same as A but plotted as NOE difference spectra where the experiment with irradiation is subtracted from a reference spectrum without irradiation. (C) NOE difference spectra obtained upon irradiation of Leu 94 δ^1 with an irradiation time of 2 s. (D) Same as C with irradiation of the methyl resonances of Leu 72 δ^1 and Leu 114 δ^1. Each spectrum was acquired with 2048 transients. Resonances are numbered as in Figure 2.

Figure 3 shows the aromatic and a segment of the aliphatic region of the driven NOE spectra of the sample used for Figure 1B. The spectral regions contain only ligand signals. To identify NOE contacts, several methyl resonances were irradiated. In Figure 3A, the γ^1 methyl resonance of Val 91 (0.1 ppm) was irradiated for up to 2.0 sec, and the spectra were plotted on top of each other. Clearly a subset of the resonances (aromatic resonances 5, 6 and 7, and the methyl resonance 8) decreases significantly in intensity (Figure 3A). This is best seen in the traces of the NOE difference spectrum (Figure 3 B). The NOEs are selective for the left-hand side of the compound as displayed in Figure 2A. Irradiating Leu 94 δ^1 (Figure 3C) produces essentially the same NOE pattern except that now a small NOE is also seen at proton 9. Irradiating simultaneously Leu 72 δ1 and Leu 114 δ^1 produces a similar NOE pattern; however, now the resonances 1, 2, 3 and 4 also show weak NOEs (Figure 3D). These patterns of NOEs establish an approximate docked conformation of the compound on the protein.

Figure 4A shows the NOE build-up curves for irradiating Val 91 γ^1 up to 2 s. Resonances 6, 7 and 8 exhibit classical build-up curves whereas signals of the right-hand aromatic ring (Figure 2A) show no NOE at all, and the values for proton 3 are shown Figure 4A as a reference. There is no further increase of NOEs at longer irradiation times, there is no indication of significant spin diffusion, and the maximum nuclear Overhauser effect is less than 7%. For comparison, we also measured the build-up curves of intramolecular NOEs (Figure 4B). Irradiating Val 99 γ^1 causes NOE build-up at Val 99 γ^2 reaching around 65%, and at the nearby Leu142 δ^1 leveling off at about 30%.

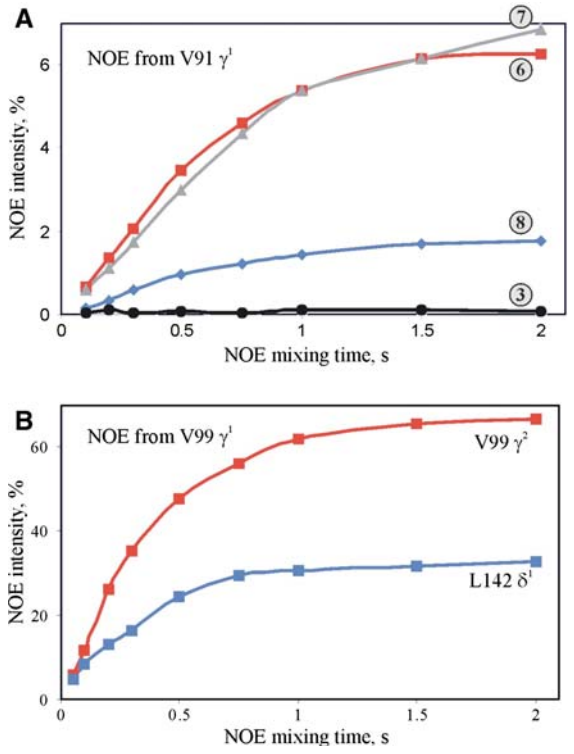

Figure 4. (A) Time-dependence of driven NOEs from Val 91 γ^1 to proton resonances of the compound. Peaks 1 and 3 are not affected at all, while peaks 5, 6, and 7 exhibit NOE of a small magnitude. (B) Build-up curves for intra-protein NOEs from V99 γ^1. These NOEs provide internal referencing for distance quantification.

The ligand-binding site on Bcl-xL is shown in Figure 5 using the coordinates 1BXL from the structure of the Bcl-xL–Bak complex (Sattler et al., 1997) but the Bak peptide is not shown. The surface-exposed isoleucines, leucines and valines are colored in blue, white and yellow, respectively. The locations of the residues irradiated in the experiments shown in Figure 3 are labeled. The effects seen upon irradiating Val 91 γ^1 place the left-hand side of the compound (protons 5, 6, 7 and 8) near toVal91. Irradiating Leu 94 δ^1 produces the additional NOE at proton 9. This indicates that the associated methylene group traces upwards in the orientation of Figure 5. Irradiating L72 and L114, which are both close in space and have overlapping resonances, showed weak NOEs to both aromatic groups indicating that both arms of the compound curl up and bind to the pocket lined by L91, L72, and L114 (Figure 5). The methyl resonances of L76 are very broad in the free protein already, and adding the compound makes them disappear indicating that there is intermediate conformational exchange in the complex structure. Irradiating V99 γ^1 did not cause any ligand NOEs indicating that the compound does not reach up to that site. The central group of the compound (protons 12–18) did not show NOEs in any of the irradiation experiments. We conclude that this

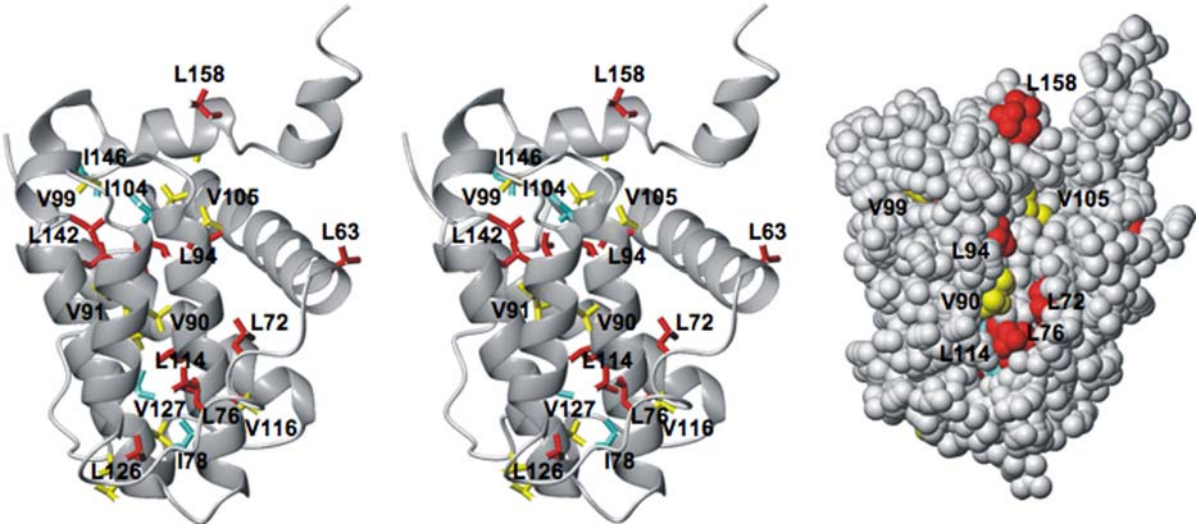

Figure 5. Structure of Bcl-xL, accessible surface area representation (PDB code 1BXL). (Left) Ribbon diagram in stereo. Side chains of Ile (blue), Leu (red) and Val (yellow) in or adjacent to the binding site are labeled. (Right) Space-filled representation of the protein in the same orientation. Only surface-exposed residues are labeled.

group is either not close to the protein surface or is just not nearby any of the ILV methyl groups used here. In summary, the NOEs observed here place the compound at a site that is occupied by the N-terminal end of the Bak peptide in the complex structure of (Sattler et al., 1997).

Discussion

We have developed a procedure for measuring protein–ligand NOEs in the limit of intermediate exchange. In this limit all ligand resonances and many protein resonances are broadened at stoichiometric concentrations. Using large excess of ligand (10-fold in our case) leaves its resonances sufficiently sharp so that they can be observed in NOE difference experiments. By the same means, the protein is fully loaded with ligand, which eliminates exchange broadening of protein resonances due to ligand dissociation because the population of free protein is very low (Reibarkh et al., 2006). Since small-molecule ligands often have poor solubility the protein concentration has to be kept very low at such conditions, which is not suitable for conventional NOESY experiments that rely on the transient NOE concept (Solomon, 1955). Thus, we use driven NOE difference experiments, which employ long selective saturation of individual resonances (Wagner and Wüthrich, 1979). Deuteration and selective labeling of methyl groups is used to produce a relatively sparse spectrum where individual methyl resonances can be saturated selectively. The combination of these experiments enables us to define the approximate orientation of the bound compound on the protein, which is expected to be sufficient for a rational improvement of the compound's affinity.

Protein-binding of small molecules, discovered with high-throughput screening, often results in multiple conformations of the complex. This is not unexpected for ligands that have not yet been refined for binding. It is different from complexes with cofactors or cognate ligands where the binding site has been optimized by evolution. Multiple conformations in the intermediate exchange regime can cause broadening of protein resonances, which cannot be eliminated by providing excess ligand (Reibarkh et al., 2006). Indeed, the very compound used here causes line broadening of some cross peaks in the ^{1}H–^{13}C HSQC spectrum shown in Figure 1A, and excess ligand does not sharpen these lines (Reibarkh et al., 2006). Thus, there is clear evidence for multiple conformations in some parts of the protein–compound complex. Even in this case, the experiments discussed here provide distance information that coarsely defines the conformational space sampled by the ligand, and the approximate positions of the different moieties of the compound can be modeled.

Crucial for this technique to work is a labeling strategy that results in a protein spectrum with fully or partially separated lines that can be saturated selectively. It requires that most of the protein is deuterated and NMR silent while a few groups are selectively protonated. The methyl-labeling technology (Rosen et al., 1996; Goto et al., 1999; Medek et al., 2000; Gross et al., 2003) is ideally suited for this type of experiment. This strategy makes it possible to record spectra in $^{2}H_2O$ and to observe aromatic resonances of the ligands in spectral regions that are otherwise obscured by signals from exchangeable protons. Cell-free expression methods may be used to further extend the variety of selectively labeled samples (Yokoyama, 2003; Torizawa et al., 2004; Vinarov et al., 2004; Kainosho et al., 2006).

Ligand binding often causes changes of the protein structure. This makes it difficult to predict the structure of a complex with docking software. In the case of Bcl-xL, the binding site for the BH3 peptide of Bak is closed in the free protein but opens up upon binding the Bak BH3 domain (Sattler et al., 1997) or small-molecule ligands (Lugovskoy et al., 2002). The methyl labeling strategy used here enables monitoring such conformational changes through methyl–methyl NOEs as shown in Figure 4B. Due to the deuteration of most of the protein, spin diffusion is low and NOEs can be observed over long distances. Thus, the experiments described here can simultaneously be used to characterize ligand-induced changes of the overall protein structure.

Structures of protein–ligand complexes have been solved previously for numerous cases. However, all of previously described studies were for the limits of fast or slow exchange, which avoided the problem of dealing with severe line broadening of the ligand resonances and the signals in binding site on the protein. The experiments described here outline a straightforward method for measuring

individual intermolecular NOEs in the limit of intermediate exchange and in the presence of other causes of line broadening, such as multiple bound conformations. Their interpretation does not require prior knowledge of the protein structure and they can be readily measured and analyzed even if the protein conformation changes upon ligand binding. The individual intermolecular distance constraints obtained from these experiments can be used together with intramolecular constraint for calculating the structures of the protein–ligand complexes.

The experiments described here can readily be applied for characterization of protein–protein or protein–nucleic acid interactions. NMR line broadening upon forming protein–protein complexes at stoichiometric concentrations is a common observation. Following the principles described here, one protein can be deuterated and protonated at ILV methyls; it would be used at low concentration. The other protein would be deuterated and ^{15}N labeled. Selective irradiation of methyl resonances of the first protein should cause NOEs of amide protons in the second protein that is present at high concentration. These NOEs can be detected in ^{1}H–^{15}N HSQC-difference spectra. Experiments along these lines are in progress.

The approach described here is also suitable for studies of interactions between proteins and nucleic acids, in particular with RNA. Since RNA does not contain signals where the ILV methyl groups appear, these resonances can be saturated selectively in samples where the RNA is in excess, and a perdeuterated protein with protonated methyl groups of ILV, LV, L, V or I is at low concentration. Any 1D or 2D spectrum of RNA can be recorded with and without methyl irradiation, and the difference spectra are expected to identify the RNA protons near the irradiated methyl groups. The use of this approach for studies of DNA complexes is less straightforward since there is the potential overlap with thymine methyl groups. Other selective labeling methods may be needed for an extended use of this approach.

Conclusion

Line broadening of NMR resonances in protein/small-molecule, protein–protein or protein–nucleic-acid interactions is a common problem. It is particularly hampering in the process of structure-based drug design. The approach presented here, which makes use of driven NOE and methods of selective protein labeling has the potential of alleviating many of these problems. It can be applied to characterizing protein complexes with cognate small-molecule ligands, or chemical compounds discovered with high-throughput screening. It is also suitable for defining weak and transient complexes between proteins, or of proteins with nucleic acids.

Acknowledgment

We thank Dr. Vladimir Gelev for providing the precursors for the methyl-labeling experiments. This research was supported by NIH (Grants GM47467, GM075879 and EB002026).

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