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BCL-2 Protein Family Interaction Analysis by Nuclear Magnetic Resonance Spectroscopy

Thomas P. Garner^{1,2,3,4} and Evripidis Gavathiotis^{5,6,7,8}

¹Department of Biochemistry, Albert Einstein College of Medicine, Bronx, NY, USA.

²Department of Medicine, Albert Einstein College of Medicine, Bronx, NY, USA.

³Albert Einstein Cancer Center, Albert Einstein College of Medicine, Bronx, NY, USA.

⁴Wilf Family Cardiovascular Research Institute, Albert Einstein College of Medicine, Bronx, NY, USA.

⁵Department of Biochemistry, Albert Einstein College of Medicine, Bronx, NY, USA.

⁶Department of Medicine, Albert Einstein College of Medicine, Bronx, NY, USA.

⁷Albert Einstein Cancer Center, Albert Einstein College of Medicine, Bronx, NY, USA.

⁸Wilf Family Cardiovascular Research Institute, Albert Einstein College of Medicine, Bronx, NY, USA.

Abstract

Biomolecular nuclear magnetic resonance (NMR) is a powerful and versatile method for studying both protein–protein interactions (PPIs) and protein–small molecule binding. NMR has been used extensively in the investigation of BCL-2 family proteins revealing the structure of key family members, identifying binding partners and interaction sites, and screening small molecule modulators. In this chapter we discuss the application of NMR to identify interaction sites and structure determination of protein–protein and protein–small molecule complexes using two examples.

Keywords

Nuclear magnetic resonance (NMR); Protein–protein interactions (PPI); Chemical shift perturbation (CSP); Paramagnetic relaxation enhancement (PRE); BCL-2 family; BCL-2; BAX; MCL-1

1 Introduction

Nuclear magnetic resonance (NMR) has developed into a powerful method for the determination of protein structure and dynamics in solution as well as the investigation of protein interactions with other biomolecules and small molecules. One of the unique strengths of NMR is its ability to investigate interactions over a wide range of affinities with

dissociation constants ranging from mM to nM, in many cases allowing the simultaneous identification of both dissociation constants and binding site in a single experiment. In the case of very weak interactions ($K_d > 100 \mu\text{M}$) NMR remains one of the few techniques capable of accurately detecting specific binding and measuring affinity. The importance of weak interactions in biology has become increasingly clear, involved in a variety of key biological processes including focal adhesion, actin filament formation and signal transduction [1]. The identification of weak interactions are also important in any ligand-based drug discovery campaign, where the initial screening hits are typically low affinity (K_d mM– μM) small molecules and where binding site identification offers significant advantage [2].

NMR has been extensively used in the structure determination and interaction analysis of the BCL-2 family proteins. Elucidation of the ever-growing network of interactions of BCL-2 family members with both BCL-2 and non-BCL-2 proteins is central to the understanding of the regulation of cell death. The identification and structural characterization of many of these key interactions has been determined using a combination of chemical shift perturbation (CSP) and paramagnetic relaxation enhancement (PRE) methodology. This has been particularly successful in the case of the multidomain proapoptotic members, identifying many key interactions including those of BAK with the BH3 domain of BID, and of BAX with the BH3 domain of BIM, P53 and PIN1, the viral protein vMIA, and the BH4 domain of BCL-2 [3–7].

In the case of small molecule modulators, NMR has been instrumental in the discovery of small molecules, which target both antiapoptotic and proapoptotic members of the BCL-2 family. The CSP methodology, in conjunction with other complementary NMR techniques, has been used for screening and discovery of small molecule modulators, including BAX activators and inhibitors of antiapoptotic proteins such as BCL-2, BCL-X_L, and MCL-1 [8–13]. Notably, fragment-based drug discovery using NMR led to the discovery of ABT-737, an inhibitor of BCL-2, BCL-X_L, and BCL-w, which was further developed into Venetoclax (ABT-199), the first FDA-approved drug to directly target a BCL-2 family member [11, 14].

Here we will provide experimental procedures to perform CSP and PRE analysis of protein–small molecule and protein–peptide complexes to identify interaction sites and determine their interactions.

2 Materials

2.1 Reagents

1. BAX NMR Buffer: 20 mM potassium phosphate, pH 6.0, 50 mM NaCl, 10% D₂O.
2. ¹⁵N-enriched BAX (residues 1–192).
3. MCL-1 NMR Buffer: 20 mM Potassium phosphate, pH 7, 50 mM NaCl, 10% D₂O.
4. ¹⁵N-enriched MCL-1 (residues 172–327) C286S.

5. ^{15}N -ammonium chloride.
6. 1.7 mm NMR microtubes.
7. Microtube syringe (<1 mm diameter needle).
8. 1.7 mm adapter for 5 mm spinner.
9. Bruker Avance 600 MHz NMR spectrometer with cryoprobe.
10. Small molecule 1 (SM1), 10 mM in d6-DMSO.
11. BIM-SAHB peptide with an N or C-terminal cysteine labeled with (S-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methanesulfonylthioate) (MTSL).
12. 1 M ascorbic acid.

2.2 Software

1. qMDD (mddNMR).
2. NMRPipe/NMRDraw (Delaglio, NIH).
3. Analysis (CCPNMR).
4. Excel (or other spreadsheet software).
5. Prism (or other data analysis software).

3. Methods

3.1 NMR Sample Preparation

1. Produce in *E. coli* uniform ^{15}N -enriched proteins by inducing protein expression in M9 media. *E. coli* are grown as normal in 1-L LB-media at 37 °C until an OD₆₀₀ of 0.7–0.9 is reached.
2. Harvest cells by centrifugation at 3000 × *g* for 10 min.
3. Resuspend cell pellets in M9 media with 1.5–2 g/L of ^{15}N -ammonium chloride as the sole source of nitrogen.
4. Grow cells for 30–45 min at 37 °C before inducing protein expression using 1 mM IPTG at 18 °C for 16 h (*see* Note 1).
5. Because MCL-1 (residues 172–327) C286S is expressed as a histidine-tagged maltose binding (MBP) fusion protein, purify protein by affinity chromatography followed by proteolytic cleavage using TEV protease to remove the His-MBP tag (*see* Note 2) [15, 16].

¹Expression of proteins in M9 media can result in reduced yields and protein solubility. Optimization of induction temperature, cell line, and IPTG concentration is encouraged. In the case of low yielding proteins a high cell-density approach can be used where 2 L of LB is used for each 1 L of M9 resulting in a doubling of the final OD.

²MCL-1 172–327 represents the BCL-2 family fold but lacks the native disordered region seen in the first 170 residues and the C-terminal trans-membrane helix. Both the N- and C-terminal extensions lead to lower protein stability, in addition the sharp resonances observed for the disordered N-terminal region preclude the recording of quality NMR spectra. Similarly the C286S mutant improves the stability of the protein; however, wild type MCL-1 172–327 also gives good quality spectra and can be used when desirable.

6. Because BAX (residues 1–192) is expressed as a chitin-binding domain fusion protein separated by a self-cleaving intein, purify protein by affinity chromatography using chitin-resin followed by cleavage with 50 mM DTT [5, 17].
7. Purify proteins by size exclusion chromatography using a Superdex 75 increase 10/300 GL column in FPLC buffer consisting of NMR buffer without D₂O and supplemented with additional KCl up to 150 mM.
8. Confirm fractions containing the desired protein by SDS-PAGE; pool and buffer exchange into NMR buffer using a 10-KDa cutoff spin concentrator.
9. Adjust the final concentration of labeled protein between 50 and 100 μ M in NMR buffer. The final volume for each NMR sample is 55 μ L in a 1.7 mm NMR microtube.
10. To prevent dilution of the protein or the sequential addition of DMSO during titrations of peptides or small molecules, each ratio of protein to small molecule or peptide is performed on separate samples with all samples normalized for volume and the addition of DMSO (*see* Note 3).

3.2 NMR Spectroscopy

1. For all NMR experiments use a 600-MHz or higher NMR magnet equipped with a 5 mm H/F-TCI CryoProbe at 25 °C.
2. Load 1.7 mm tubes into the 5 mm probe using a 1.7 mm tube adapted spinner.
3. Record ¹H–¹⁵N heteronuclear single quantum coherence (HSQC) experiments [18].
4. Acquire data with 2048 × 256 complex data points, a ¹H spectral width of 13 ppm and ¹⁵N spectral width of 30 ppm in Topspin3.0 using a standard nonuniform sampling regime with 50% sampling [19].
5. Process spectra with qMDD (mddnmr v2.0) using the “compression sensing” algorithm, followed by further processing using NMRPIPE and spectra analysis using Analysis (CCPNMR) [19–21].

3.3 Chemical Shift Perturbation (CSP): Small Molecule Binding to MCL-1

CSP studies rely on changes in the chemical shifts as observed by 2D ¹H–¹⁵N HSQC analyses. ¹H–¹⁵N HSQC spectra provide information on all ¹⁵N atoms bound to a ¹H, in the

³Traditionally titrations are performed on a single 600 μ L NMR sample recorded in a standard 5 mm tube, with experiments acquired after the sequential addition of increasing amount of small molecule or peptide. However the use of smaller 55 μ L individual samples recorded in microtubes affords several significant advantages. Individual samples can be DMSO matched for each concentration of titrant, this removes potential DMSO-induced chemical shift changes improving the sensitivity of CSP measurements. In the case of unstable proteins the ability to rerecord spectra of the same apo-protein sample at the beginning and end of the experiment allows for the confirmation of molecule- or peptide-induced changes compared to sample degradation or spontaneous changes. Proapoptotic BCL-2 family members are known to undergo stimulus driven conformational changes; these conformational changes can have slow kinetics and can mask ligand binding chemical shift changes when the same sample is used for multiple concentration titrations. The ability to add each concentration of peptide or molecule immediately prior to data acquisition can minimize the contributions of slow conformational changes improving binding site identification.

case of proteins this includes one backbone amide NH peak for each protein residue except for proline, providing a site-specific probe for each residue. Interactions that alter the local environment of these probes, are observed as changes in chemical shifts or a “CSP” for that residue. By monitoring these CSPs we can identify binding sites and measure affinities (*see* Notes 4 and 5).

1. Prepare 340 μL ($(6 \times 55 \mu\text{L}) + 10 \mu\text{L}$) stock of 100 μM ^{15}N -MCL-1 in MCL-1 NMR Buffer.
2. Split the stock into six samples of 55 μL in 1.5 mL centrifuge tubes on ice.
3. Dissolve SM1 in d6-DMSO to a concentration of 20 mM and vortex, if SM1 does not immediately dissolve, gently sonicate the sample in a sonication waterbath for 5 min, vortex and repeat as necessary.
4. Prepare five dilutions of SM1 in d6-DMSO to create final concentrations of 20, 15, 10, 5, and 2.5 mM.
5. Add 0.55 μL from each of the five dilutions of SM1 to separate samples, creating final concentrations of 200, 150, 100, 50, and 25 μM , add 0.55 μL of d6-DMSO to the sixth sample.
6. Centrifuge each sample at $4000 \times g$ for 5 min.
7. Load each sample into separate 1.7 mm NMR tubes and record ^1H - ^{15}N HSQC spectra on each sample.
8. Process all spectra and overlay them in Analysis. The resonance assignments obtained from the BMRB (BMRB entry 19654) are imported into the apo-MCL-1 NMR spectrum (0 μM SM1).
9. Determine binding by concentration dependent, residue specific changes in some (but not all) cross-peaks (Fig. 1a). For cross-peaks that do not change the resonance assignments are simply transferred from the apo-protein spectra to the SM1 bound protein. In cases where cross-peaks do change the cross-peaks are followed through the titration series from the apo-spectra to the bound state (Fig. 1b) (*see* Note 6).

⁴. Resonance assignments of MCL-1 C286S were obtained from the Biological Magnetic Resonance Bank (BMRB), entry 19,654. In order to identify the binding site of small molecule 1 (SM1) on MCL-1 and estimate the K_d a simple 6-point titration experiment was performed.

In this example we use a concentration range for SM1 consistent with the expected weak affinity. For other molecules the concentration range would need to be optimized for the K_d , typically a highest concentration $2-5 \times K_d$ is a good starting point.

⁵. In this example we are interested in a single molecule with suspected binding to MCL-1, however this method is also applicable to small molecule screening. While in this case each sample contains a different concentration of SM1, each sample could instead contain a different compound. In the case of high-throughput screening, it is common for each sample to contain up to five different screening compounds. In those instances those samples, which show CSP are further investigated to identify the active molecule and determine its binding site.

⁶. Exchange between the bound and free states of a protein takes place in one of three NMR time scales: fast, intermediate, or slow. SM1 binding is in fast exchange, this is the most favorable exchange regime. In fast exchange a single peak is observed for each residue, the observed chemical shifts are a population weighted average between the free and bound chemical shifts. In the case of slow exchange the cross-peaks of the apo-protein reduce in intensity as the binding partner of interest is added, these peaks are replaced with those of the bound protein with both being observed simultaneously. In the case of slow exchange the bound chemical shifts often require reassigning. If the interaction is somewhere between these scenarios it is in intermediate exchange, the signal from the bound state will reduce in amplitude and broaden in linewidth, resulting in a disappearance of the signal as the population shifts to the bound state. This is the least optimal exchange regime for studying protein interactions by NMR, as it makes quantification of the

10. Once the resonance assignments have been transferred from the apo-MCL-1 spectrum to the SM1 bound spectrum, export the peak tables from Analysis and import them into Excel to determine the CSP values.
11. Calculate the CSP for each residue. The CSP is calculated from the difference in the free and bound chemical shifts of each residue using the equation.

$$\text{CSP} = \sqrt{\left(\left(H_{\text{apo}} - H_{\text{bound}}\right)^2 + \left(\left(N_{\text{apo}} - N_{\text{bound}}\right)/4\right)^2\right)}$$

where H_{apo} and H_{bound} are the ^1H chemical shifts of the apo and bound residues and N_{apo} and N_{bound} are the ^{15}N chemical shifts of the apo and bound residues.

12. Consider residues having significant CSP if their CSP is above a threshold defined as

$$\text{Th} = \text{CSP}_{\text{av}} + \text{CSP}_{\text{stdev}}$$

where Th is the threshold and CSP_{av} and $\text{CSP}_{\text{stdev}}$ are the proteins average CSP over all residues and the standard deviation of that average respectively. Typically CSP as a function of residue number is plotted as a bar graph with the residues above the threshold indicated by color (Fig. 1d).

13. Plot residues showing significant CSP onto the structure of MCL-1 (Fig. 1e). If significant CSPs are observed to cluster into a patch or patches on the 3D structure of the protein, this indicates that SM1 binds to this region, in this case the “canonical groove” of MCL-1.
14. To determine the affinity of SM1 the CSP can be plotted as a function of SM1 concentration for each residue showing significant CSP, the resulting binding isotherm can be fitted using Prism to extract a K_d (see Note 7) (Fig. 1c).
15. Use the CSP and the region identified as the small molecule binding site to guide molecular docking software such as Glide, AutoDock, or Gold to estimate protein–small molecule interactions [22–24].

3.4 Interaction of BAX with BIM-BH3 Domain Investigated by PRE

PRE studies rely on changes in the intensities of observed cross-peaks in 2D ^1H – ^{15}N HSQC caused by the presence of unpaired electrons from either a paramagnetic metal center or a radical trap, typically in the form of MTSL (*S*-(1-oxy-2,2,5,5-tetramethyl-2,5-dihydro-1H-

relative populations difficult. Changes in temperature, buffer conditions, or the affinity of the ligand can push the interaction into the fast or slow regimes. Although it is an oversimplification generally, low affinity (K_d in mM– μM) interactions display fast exchange while high affinity (K_d in nM) results in slow exchange. Molecules which induce large scale conformational changes may also be in slow exchange due to the slow time scale of those changes.

⁷In most cases extracting binding isotherms from fast exchange titrations should yield a single affinity independent of which residue is fitted. Averaging the value can be achieved in a number of ways, each residue can be fitted individually and the K_d reported as an average over each residue. Alternatively, each binding isotherm can be normalized to the largest CSP each residue reaches, the normalized CSP for all usable residues can then be averaged and the K_d determined from the average. However the most appropriate is to globally fit all residues to the same K_d using global fitting software such as IgorPro (Wavemetrics).

pyrrol-3-yl)methyl methanesulfonothioate). The presence of an unpaired electron causes an increase in the relaxation rate of NMR active nuclei, the strength of this relaxation has an r^{-6} distance dependence. The outcome of this is that NMR signals close to the electron lose intensity; the degree of intensity reduces the further away they are from the unpaired electron. By monitoring the loss of peak intensities we can identify the approximate location of the unpaired electron, and by association, whatever protein/peptide it is attached to. By strategically placing PRE agents on peptides or proteins, one PRE experiment provides important distance restraints information for guiding docking results [25]. In this example, CSP analysis had identified a binding site on BAX for a stapled peptide of the BIM-BH3 domain, BIM SAHB, however CSPs alone were not sufficient to orientate the peptide within the newly discovered binding site. PRE experiments were performed using two BIM SAHB peptides, one with MTSL labeled close to its N-terminus and another with MTSL labeled close to its C-terminus (From here referred to as “BIM SAHB-N” and “BIM SAHB-C” respectively).

1. Prepare a stock of 285 μL of 100 μM BAX in BAX NMR Buffer ($5 \times 55 \mu\text{L} + 10 \mu\text{L}$).
2. Split the stock into five samples of 55 μL in a 1.5 mL centrifuge tube on ice.
3. Prepare a 10 mM stock of BIM SAHB-N and BIM SAHB-C in d_6 -DMSO, and vortex until they are fully dissolved, avoid prolonged exposure of the MTSL labeled peptides to the light (*see* Notes 8 and 9). These will be the “paramagnetic peptide” samples.
4. To generate the “diamagnetic peptide” samples; take a 10 μL sample of both BIM SAHB-N and BIM SAHB-C, to each add 0.5 μL 1 M sodium ascorbate (1:5 molar ratio) and incubate for 1 h at room temperature.
5. Add 0.55 μL of paramagnetic BIM SAHB-N to one BAX sample to generate a 1:1 molar ratio BAX:BIM.
6. Centrifuge the sample at $4000 \times g$ for 5 min.
7. Load the sample into a 1.7 mm NMR tubes and record ^1H - ^{15}N HSQC spectra. In this case the ^1H - ^{15}N HSQC must be modified to lengthen the relaxation delay (D1) to 5 s to allow for full relaxation between scans.
8. Repeat **steps 5–7** replacing paramagnetic BIM SAHB-N with diamagnetic BIM SAHB-N, paramagnetic BIM SAHB-C, diamagnetic BIM SAHB-C, and DMSO as separate samples. In each case the peptide/DMSO should be added

⁸In this case the peptides were provided as MTSL labeled peptides. In many cases this option is not available, the simplest way to generate an MTSL labeled peptide is to generate a mutant containing a single cysteine in the desired position of the unpaired electron. This cysteine can be reacted with 2–3 molar equivalents of MTSL for 2 h at room temperature in the dark followed by removal of the MTSL by buffer exchange or size-exclusion chromatography in the case of proteins or HPLC in the case of peptides or ether precipitation (*see* Note 9).

⁹Cysteine containing peptides can be reacted with MTSL in DMSO to generate MTSL labeled peptides. Where HPLC is not available or not desirable, MTSL can be removed by ether-precipitation. Cool diethyl-ether on ice for 20 min. Mix the peptide reaction at 1:100 with cold ether, vortex and incubate for 30 min on ice followed by centrifugation to pellet the peptide. Remove the ether and wash the pellet with 1 mL of cold ether, centrifuge, remove the ether, and dry under vacuum overnight in the dark.

immediately prior to sample loading and acquisition, as this reduces the risk of signal loss by BAX activation (*see* Note 3).

9. Process all spectra and overlay them in Analysis.
10. Import the resonance assignments obtained from the BMRB (BMRB entry 4632) into the apo-BAX NMR spectrum (d6-DMSO only).
11. Transfer the resonance assignments of BIM-bound BAX from the apo-BAX spectra in an analogous method to that described in the CSP section above. Though a number of peaks may be missing in the paramagnetic spectra, the peak position can be identified in the diamagnetic spectra for the same peptide; the cross-peak position should be placed in the same location in both paramagnetic and diamagnetic spectra.
12. Once the resonance assignments have been identified for each spectrum, export the peak tables including both the peak position and peak intensity from Analysis and import them into Excel to determine the CSP (*see* Note 10).
13. Calculate PRE for each residue using the equation.

$$\text{PRE} = I_{\text{Para}}/I_{\text{Dia}}$$

where I_{Para} and I_{Dia} are the cross-peak intensities observed in the paramagnetic and diamagnetic peptide samples respectively.

14. Consider residues having significant PRE if their PRE is below a threshold defined as

$$\text{Th} = \text{PRE}_{\text{av}} - \text{PRE}_{\text{stdev}}$$

where Th is the threshold and PRE_{av} and $\text{PRE}_{\text{stdev}}$ are the proteins average PRE over all residues and the standard deviation of that average respectively. Typically PRE as a function of residue number is plotted as a bar graph with the residues below the threshold indicated by color (Fig. 2a).

15. Plot residues showing significant PRE onto the structure of BAX (Fig. 2b). If significant PREs cluster into a patch on the 3D structure of the protein, this indicates approximate location of the MTSL and therefore the residue to which it was attached.
16. Compare the clustering of the observed PREs with BIM SAHB-N and BIM SAHB-C, and the N- and C-termini of the BIM SAHB are orientated within the newly identified binding site (Fig. 2b) [25].

¹⁰PRE measurements are most commonly reported as simply the ratio of peak intensities, these can be measured either by the area under each cross-peak, or by the height of the peak both of which are reported by the Analysis software. In cases where all peaks are well resolved peak area is typically best, however in highly overlapped spectra peak height may be better. The adjacent peak will influence both of these, and this should be taken into account during analysis.

17. Combine analysis of the binding site identification from CSP analysis and orientation restraints based on distances between the MTSL-labeled residue of the peptide and BAX based on the strength of the PRE.
18. Incorporate CSP and PRE restraints into a protein–protein structure calculation or docking software such as HADDOCK, ROSETTA, or Glide to generate structural models of the protein–peptide complex [26–29].

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Fig. 1. Small molecule binding to MCL-1 investigated by CSP. **(a)** Comparison of the ^1H - ^{15}N HSQC spectra of ^{15}N -MCL-1 apo (green cross-peaks) and ^{15}N -MCL-1 bound to SM1 (red cross-peaks). **(b)** Section of the ^1H - ^{15}N HSQC spectra of ^{15}N -MCL-1 upon titration of SM1 at 0, 1, 2, 4:1 ratio of SM1:MCL-1. **(c)** Titration curve of the normalized CSP for all usable residues averaged in the corresponding ^1H - ^{15}N HSQC spectra in **(b)** versus SM1 concentration. The dissociation constant (K_d) were obtained by fitting the experimental data. **(d)** Chemical shift perturbations (CSP) are plotted as a function of the residue number of MCL-1. Residues with significant backbone amide chemical shift change are colored in red, orange, and yellow. The absence of a bar indicates the presence of a proline or a residue that is overlapped. **(e)** Surface representation of MCL-1 structure (grey color) showing the residues undergoing significant chemical shift changes (red, orange, and yellow color) upon SM1 titration, which are localized on the BH3 binding groove

Fig. 2.

Interaction of BAX with BIM SHAB investigated by PRE. (a) Ratios of ^{15}N -BAX cross-peak intensities in ^1H - ^{15}N HSQC spectra in the presence of oxidized or reduced ($I_{\text{ox}}/I_{\text{red}}$) BIM SAHB-C (A164C-MTSL) (top) and BIM SAHB-N (W147C-MTSL) (bottom) versus residue number. BAX residue cross-peak intensities reduced below 0.6 (dashed green horizontal line) are colored in red (BIM SAHB-C) or in blue (BIM SAHB-N). (b) Orientation of BIM SAHB at the BAX site. On the basis of an $I_{\text{ox}}/I_{\text{red}}$ threshold of 0.6, BAX residues affected by BIM SAHB-C (A164C-MTSL), BIM SAHB-N (W147C-MTSL) or both are colored in red, blue, or purple, respectively