Facile Detection of Protein−Protein Interactions by One-Dimensional NMR Spectroscopy†

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Received November 20, 2002; Revised Manuscript Received January 30, 2003

ABSTRACT: Two methods for detecting protein−protein interactions in solution using one-dimensional (1D) NMR spectroscopy are described. Both methods rely on measurement of the intensity of the strongest methyl resonance (SMR), which for most proteins is observed at 0.8−0.9 ppm. The severe resonance overlap in this region facilitates detection of the SMR at low micromolar and even sub-micromolar protein concentrations. A decreased SMR intensity in the ^{1}H NMR spectrum of a protein mixture compared to the added SMR intensities of the isolated proteins reports that the proteins interact (SMR method). Decreased SMR intensities in 1D ^{13}C-edited ^{1}H NMR spectra of ^{13}C-labeled proteins upon addition of unlabeled proteins or macromolecules also demonstrate binding (SMRC method). Analysis of the interaction between XIAP and Smac, two proteins involved in apoptosis, illustrates both methods. A study showing that phospholipids compete with the neuronal core complex for Ca^{2+}-dependent binding to the presynaptic Ca^{2+}-sensor synaptotagmin 1 illustrates the usefulness of the SMRC method in studying multicomponent systems.

Virtually every cellular process is governed by interactions of proteins with other proteins or macromolecules. A wide variety of methods that have different advantages and drawbacks have been developed to study such interactions (*1*). Two-hybrid assays (*2*, *3*) and mass spectrometry of tagged protein complexes (*4*, *5*), among other techniques, have been used in comprehensive analyses of protein−protein interactions. However, only a small percentage of the interactions described in these studies were supported by more than one method (*6*), emphasizing the importance of verifying their relevance by individualized experiments. Fluorescence resonance energy transfer techniques (*7*) now allow study of interactions in living cells, but it is difficult to demonstrate direct interactions using in vivo experiments alone. Direct protein interactions can be analyzed in vitro by numerous methods, including chromatography, electrophoresis, ultracentrifugation, fluorescence spectroscopy, calorimetry, and techniques based on immobilization of proteins on resins or solid supports (*1*). However, the use of fusion proteins, tags, or chemical modifications required for some of these methods can yield binding artifacts or occlude binding sites, while other methods can only detect complexes with slow off-rates or are limited to the study of binary interactions. Hence, there is a need for techniques that do not suffer from these drawbacks and can be generally used to unambiguously demonstrate direct protein−protein interactions and to analyze multicomponent systems. Here we show that one-dimensional (1D) nuclear magnetic resonance (NMR)[1] spectroscopy provides a simple, general tool for detecting protein−protein interactions in solution that can complement existing techniques.

MATERIALS AND METHODS

Basis for the SMR Method. The line widths of protein resonances increase with molecular mass because the slower tumbling of larger molecules results in increased transverse relaxation rates. The resulting decrease in the intensity of individual resonances has an approximately linear dependence on the molecular mass, although it also depends on the molecular shape (intensity defined as signal height, rather than area). Since the strongest methyl resonance (SMR) in protein ^{1}H NMR spectra results from overlap of the signals from multiple methyl groups, the decrease in SMR intensity due to resonance broadening is compensated by an increase in the number of leucine, valine, and isoleucine residues, which also depends linearly on the molecular mass as long as the protein has an average amino acid sequence composition. The degree of compensation between the increase in resonance broadening and the number of methyl groups is also affected by the dispersion of the methyl resonances. In practice, these parameters are sufficiently similar in most proteins to result in a similar SMR intensity for the same protein concentration (within an approximately ±20−30% margin). This observation arises from NMR studies of more than 50 proteins or protein complexes with molecular masses ranging from 7 to 100 kDa that we have performed over the

† This work was supported by a grant from the Welch Foundation and by NIH Grant NS40944.

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[1] Abbreviations: FID, free induction decay; HSQC, heteronuclear multiple-quantum coherence; NMR, nuclear magnetic resonance; SMR, strongest methyl resonance; SMRC, strongest methyl resonance ^{13}C-edited; SNARE, soluble *N*-ethylmaleimide sensitive factor attachment protein receptor; XIAP, X-linked inhibitor-of-apoptosis protein.

10.1021/bi0272050 CCC: $25.00
Published on Web 02/22/2003

years. Unfolded proteins or proteins with large unstructured regions represent exceptions where the methyl signal is usually stronger because the lack of structure decreases the resonance dispersion, and internal motions result in sharper signals with stronger intensities. It should also be noted that the temperature and ionic strength have substantial effects on the observed SMR intensities and proteins should thus be compared under analogous conditions.

From these observations, it follows that if two proteins form a stable complex, the SMR intensity of the complex will be similar to that of each individual protein, i.e., ~50% of the addition of the SMR intensities from the individual proteins. This percentage assumes average properties as outlined above, and will depend on the dispersion of the methyl resonances, the methyl group content, the oligomerization state and the molecular shape of the individual proteins and the complex formed between them. In most cases, complex formation should lead to a substantial, detectable decrease in the SMR intensity compared to that of the added spectra of the two proteins (an at least 20−30% decrease). When one or both proteins contain unstructured regions that contribute substantially to their SMR intensity, the ratio between the intensity of the mixture and the added spectra is expected to be substantially smaller than 50% if the unstructured regions participate in the interaction, and substantially larger if they remain unstructured.

Measurement of Dissociation Constants Using the SMRC and SMR Methods. The dissociation constant for dissociation of a ^{13}C-labeled protein from an unlabeled protein can be measured using the SMRC method by acquiring a series of 1D ^{13}C-edited ^{1}H NMR spectra of the ^{13}C-labeled protein at a constant concentration (P_1) while changing the concentration of the unlabeled protein (P_2). The SMRC intensity of the ^{13}C-labeled protein (I) can then be expressed as a function of P_2 by the following equation, assuming a simple 1:1 equilibrium binding model:

$$I = I_F + (I_B - I_F)[P_1P_2K_D - \sqrt{(P_1P_2K_D)^2 - 4P_1P_2}]/(2P_1) \quad (1)$$

where I_F and I_B represent the SMRC intensities of the free and bound ^{13}C-labeled protein, respectively, and K_D is the dissociation constant. The data of Figure 3C were fit with this equation using a constant P_1 value of 3 μM. For measurement of the dissociation constant for dissociation of two unlabeled proteins using the SMR method, both protein concentrations should be kept equal and changed simultaneously during a titration. The signal intensity can then be expressed as a function of protein concentration using the equation described above, but with P_1 being equal to P_2.

Sample Preparation. All proteins were expressed in bacteria with a His_6 tag (BIR3 and Smac) or as GST fusions (all others), isolated by affinity chromatography, and purified by gel filtration or ion exchange chromatography (after cleaving the GST moiety in GST fusion proteins). The syntaxin 1A−munc18-1 complex was prepared by adding purified syntaxin 1A to GST-munc18-1 cell supernatants, isolation on glutathione−agarose beads, thrombin cleavage, and purification by gel filtration. The core complex was prepared by mixing equimolar amounts of the four purified SNARE motifs and purified by gel filtration after overnight incubation. NMR samples were prepared in the corresponding buffers (see figure legends) by dilution from concentrated stocks or by dialysis. Using the SMR method at submicromolar concentrations requires careful washing of all materials used for sample handling to avoid contaminants that contain methyl groups and hence contribute to the SMR. Obtaining reliable signal intensities at these concentrations is also difficult for some proteins because of their tendency to bind to glass surfaces. The SMRC method helps to solve both problems because the signals from impurities or from glass-coating reagents that minimize binding of the proteins being studied to the glass do not appear in 1D ^{13}C-edited ^{1}H NMR spectra (e.g., 10 μM bovine serum albumin was used for the titration of Figure 3C).

NMR Spectroscopy. All NMR spectra were acquired at 27 °C on Varian INOVA500 or INOVA600 NMR spectrometers equipped with PFG triple-resonance probes. ^{1}H NMR spectra were acquired with a standard 1D pulse sequence using very low-power presaturation and watergate (3-9-19) for water suppression. Prior to Fourier transformation, the FIDs were multiplied by a strong Gaussian function with maximum at 0 ms and half-height at 30 ms to improve the sensitivity. All 1D ^{13}C-edited ^{1}H NMR spectra were obtained by acquiring the first increment of a gradient-enhanced ^{1}H−^{13}C heteronuclear single-quantum coherence (HSQC) spectrum. The SMRC intensities observed in these spectra are ~85% of the SMR intensities observed in 1D ^{13}C-decoupled ^{1}H NMR spectra of the same samples for proteins in the 20−30 kDa range.

RESULTS

SMR Method. NMR spectroscopy is already a well-established technique for structure determination of protein complexes at atomic resolution (*8*), and heteronuclear two-dimensional (2D) and three-dimensional (3D) NMR spectra can be used to quickly map binding sites in proteins of known structure (*9*−*11*). However, these experiments require high protein concentrations (usually more than 100 μM and often close to 1 mM) that put stringent demands on the solubility of the proteins under study and can promote nonspecific interactions. The method that we have developed to use NMR spectroscopy as a more general biochemical tool is based on two observations. First, 1D ^{1}H NMR spectra have much higher sensitivity than multidimensional NMR spectra because the resonances are not spread in multiple dimensions. The strongest resonance in 1D ^{1}H NMR spectra of proteins is usually observed in the region between 0.8 and 0.9 ppm, where most of the resonances from leucine, valine, and isoleucine methyl groups are overlapped. This resonance, which we will call the SMR (strongest methyl resonance), can be detected at low micromolar and even sub-micromolar concentrations (see below). Second, the SMR has a similar intensity for most monomeric proteins at the same concentration (in micromolar units) independent of their molecular mass, since the increase in the number of methyl groups with molecular mass is compensated by a corresponding increase in resonance broadening (see Materials and Methods for further details). This observation is exemplified by the SMRs of 10 μM solutions of the third BIR domain (BIR3, 14 kDa) of the X-linked inhibitor-of-apoptosis protein (XIAP) (*12*) (Figure 1A) and the double C_2 domain region (C_2AB, 33

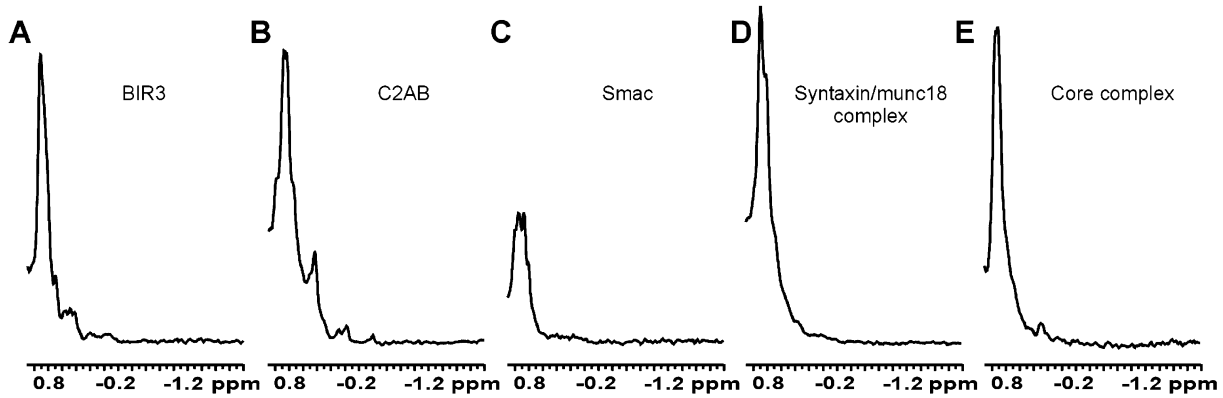

FIGURE 1: Most proteins and protein complexes have similar SMR intensities independent of their molecular mass. Expansions including the SMR of 500 MHz ^{1}H NMR spectra of 10 μM XIAP BIR3 (residues 238−358) (A), 10 μM synaptotagmin 1 C_2AB region (residues 140−421) (B), 10 μM Smac (monomer concentration, residues 1−162) (C), 10 μM complex formed by the cytoplasmic region of syntaxin 1A (residues 2−253) and munc18-1 (D), and 10 μM core complex formed by the four soluble *N*-ethylmaleimide sensitive factor attachment protein receptor (SNARE) motifs of syntaxin 1A (residues 180−264), synaptobrevin (residues 29−93), and SNAP-25 (residues 11−82 and 141−203) (E). All spectra are shown at the same vertical scale. Samples were prepared in 20 mM Tris-d_{11} buffer at pH 7.7 (A and C) or pH 7.4 (B, D, and E) containing 150 mM NaCl (A−C) or 200 mM NaCl (D and E), 0.5 mM EDTA and 5% D_2O. The spectra resulted from 1000−2000 transients, for total acquisition times of 26−53 min (1.6 s recycling delay).

kDa) of the presynaptic Ca^{2+}-sensor synaptotagmin 1 (*13*) (Figure 1B).

For proteins that form stable dimers, the relevant concentration that determines the intensity of the SMR is the dimer concentration. Correspondingly, the SMR intensity of the dimeric apoptotic protein Smac (20 kDa monomer and 40 kDa dimer) (*14*) dissolved at a monomer concentration of 10 μM (Figure 1C) is approximately half of that observed for 10 μM BIR3 and C_2AB. Similarly, the SMR intensities observed for stable protein complexes correspond to those expected for the concentration of the complex independent of the size and the number of proteins involved, as shown for the neuronal syntaxin 1A−munc18-1 complex (95 kDa) (*15*) (Figure 1D) and for the neuronal core complex formed by four protein fragments (33 kDa) (*16*) (Figure 1E). These observations lead naturally to the method of detecting protein−protein interactions summarized in Figure 2A, which we call the SMR method. When two proteins that do not interact are mixed, the intensity of the SMR should correspond to the intensity resulting after adding the spectra of the two separate proteins. On the opposite end, full binding between two proteins should result in resonance broadening and thus in a substantially lower SMR intensity, similar to the intensity of each protein and ~50% from the intensity of the added spectra (see Materials and Methods for more details).

To test the SMR method with an established protein−protein interaction, we analyzed the complex formed by Smac and BIR3 of XIAP. This interaction is involved in regulating the activity of a caspase that mediates the apoptotic cascade (*14*), has been studied at atomic resolution (*17*, *18*), and has a dissociation constant of ca. 0.8 μM as measured by a fluorescence-based competition assay (*19*). Acquisition of ^{1}H NMR spectra of separate 2 μM samples of BIR3 and Smac, and of a 2 μM mixture of both proteins, revealed that the SMR intensity of the mixture (Figure 2B, mixture) is 40% smaller than that of the added spectra of the two separate proteins (Figure 2B, add). An additional decrease in the relative SMR intensity of the mixture with respect to that of the added spectra was observed when analogous experiments were performed at a protein concentration of 5 μM (Figure 2C), but no further significant decrease was observed at higher protein concentrations (data not shown). These results indicate that BIR3 and Smac interact partially at 2 μM and that the interaction is largely saturated at 5 μM, as expected for a dissociation constant of 0.8 μM. A control experiment with BIR3 and an unrelated protein (the synaptotagmin 1 C_2AB region) yielded the same intensity for the spectrum of the mixture and the addition of the spectra of the separate proteins (Figure 2D), showing that BIR3 and C_2AB do not interact.

SMRC Method. The results presented above illustrate how 1D ^{1}H NMR spectra can be used to detect protein−protein interactions at low micromolar protein concentrations. Indeed, the signal-to-noise ratios observed with a concentration of 2 μM (e.g., Figure 2B) show that the sensitivity is sufficient to apply this method well below this concentration, and semiquantitative measurements of dissociation constants can in principle be obtained with titrations in which the concentration of both proteins is changed simultaneously (note that if one protein is in substantial excess over the other, it is difficult to quantitate binding because the SMR intensity is dominated by the excess free protein). In practice, quantitative measurements of dissociation constants are more easily obtained by the method described in the legend of Figure 3A, which is based on the same principles and involves isotopic labeling of one of the proteins with ^{13}C. In this method, the SMR of the ^{13}C-labeled protein is monitored with 1D ^{13}C-edited ^{1}H NMR spectra and will thus be called the SMRC. The signals from the unlabeled protein do not contribute to these spectra. Hence, addition of the unlabeled protein should not affect the SMRC of the ^{13}C-labeled protein if there is no interaction, but binding should result in substantial resonance broadening and a consequent decrease in the SMRC intensity. If the two proteins have a similar molecular mass, a decrease of ~50% in SMRC intensity is expected assuming an approximately linear relationship between line width and molecular mass. If one of the two proteins is much larger than the other, the method is much more sensitive when the small protein is labeled with ^{13}C since its resonances should experience more severe broadening upon complex formation.

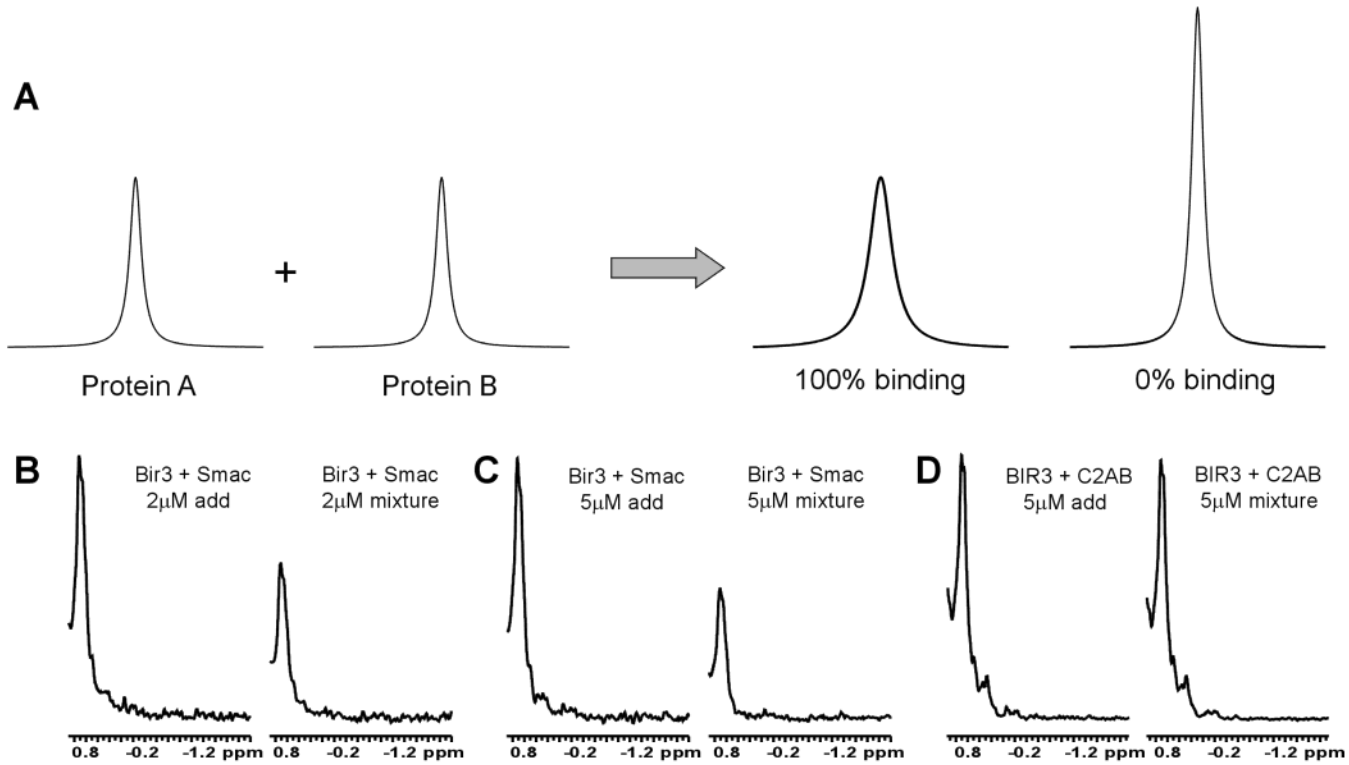

FIGURE 2: SMR method for detection of protein−protein interactions. (A) Scheme summarizing the method. The SMRs of 1D ^{1}H NMR spectra of arbitrary proteins are represented on the left, and the SMRs expected for 100% binding or 0% binding after mixing the two proteins are represented on the right. Full binding yields an SMR intensity comparable to those of the individual proteins since the SMR increases in width rather than intensity (note that the increased width may not be obvious in the actual spectra because the SMRs represent the envelope resulting from addition of multiple broader resonances). The SMR intensity is approximately equal to the addition of the SMR intensities from the individual proteins if there is no binding, since no broadening occurs. (B) Comparison of the SMR resulting from addition of 1D ^{1}H NMR spectra of 2 μM BIR3 and 2 μM Smac (left) and the SMR of a mixture of 2 μM BIR3 and 2 μM Smac (right). (C) Analogous comparison for 5 μM BIR3 and 5 μM Smac. (D) Analogous comparison for 5 μM BIR3 and 5 μM synaptotagmin 1 C_2AB region. For each comparison, the added spectrum and the spectrum of the mixture are plotted at the same vertical scale. All samples were prepared in 20 mM imidazole-d_3 (pH 7.7), 150 mM NaCl, and 0.5 mM EDTA, using D_2O as the solvent. The spectra were acquired at 500 MHz (B and C) or 600 MHz (D) with 1000 (C and D) or 2000 (B) transients, for total acquision times of 26 or 53 min, respectively.

Figure 3B illustrates the application of the SMRC method to the BIR3−Smac interaction. Addition of increasing amounts of unlabeled Smac results in a progressive decrease in the intensity of the SMRC of ^{13}C-labeled BIR3. A complete titration of the intensity changes as a function of Smac concentration (Figure 3C) shows that binding is saturable, and the data fit well to a 1:1 binding model with a dissociation constant of 0.6 ± 0.3 μM (see Materials and Methods), which is comparable to the value measured previously [0.8 μM (*19*)].

Application to the Study of Synaptotagmin Function. The SMRC method (Figure 3A) is also advantageous in analyzing multicomponent systems or competition between different targets of a given protein, as illustrated by the analysis of interactions involving the C_2AB region of synaptotagmin 1 shown in Figure 4. Synaptotagmin 1 acts as a Ca^{2+} sensor in neurotransmitter release (*20*), a role that can be ascribed to the two C_2 domains that form most of its cytoplasmic region (named the C_2A and C_2B domains). Both C_2 domains bind phospholipids in a Ca^{2+}-dependent manner (*20*, *21*). In addition, multiple Ca^{2+}-dependent interactions between synaptotagmin 1 and the neuronal core complex or its components [syntaxin 1, SNAP-25, and synaptobrevin (reviewed in refs *22*−*24*)] have been described. This complex brings the synaptic vesicle and plasma membranes together and is central for membrane fusion. Ca^{2+}-dependent binding to phospholipids and/or the core complex is widely believed to underly the function of synaptotagmin 1 in Ca^{2+} triggering of neurotransmitter release, but it is unclear whether these interactions can occur simultaneously or compete with each other. Thus, answering this question is crucial to understanding the mechanism of release. Co-immunoprecipitation experiments suggested that the C_2AB region of synaptotagmin 1 can bind to phospholipids and the core complex simultaneously (*25*). In GST pulldown assays, we did not observe quantitative binding of the core complex to the C_2AB region, and ultracentrifugation experiments suggested that the core complex cannot bind to the C_2AB region in the presence of phospholipids (data not shown). However, it is possible that these results arise because the off-rates of the C_2AB−core complex interactions are too fast for the time scales of these assays. Application of the SMRC method provided clear-cut results for unraveling this key question.

Addition of 3 μM unlabeled core complex to 2 μM ^{13}C-labeled C_2AB in the presence of 1 mM Ca^{2+} caused a more than 50% decrease in the SMRC intensity of C2AB (Figure 4A,B), and the same result was obtained by adding excess core complex (data not shown). Hence, binding of the core complex to C_2AB is largely quantitative under these conditions. When large unilamellar phospholipid vesicles (LUVs), instead of the core complex, were added to ^{13}C-labeled C_2AB, its NMR signals disappeared completely (Figure 4C), revealing quantitative binding to the vesicles. Note that this is an expected result since, due to the large size of the

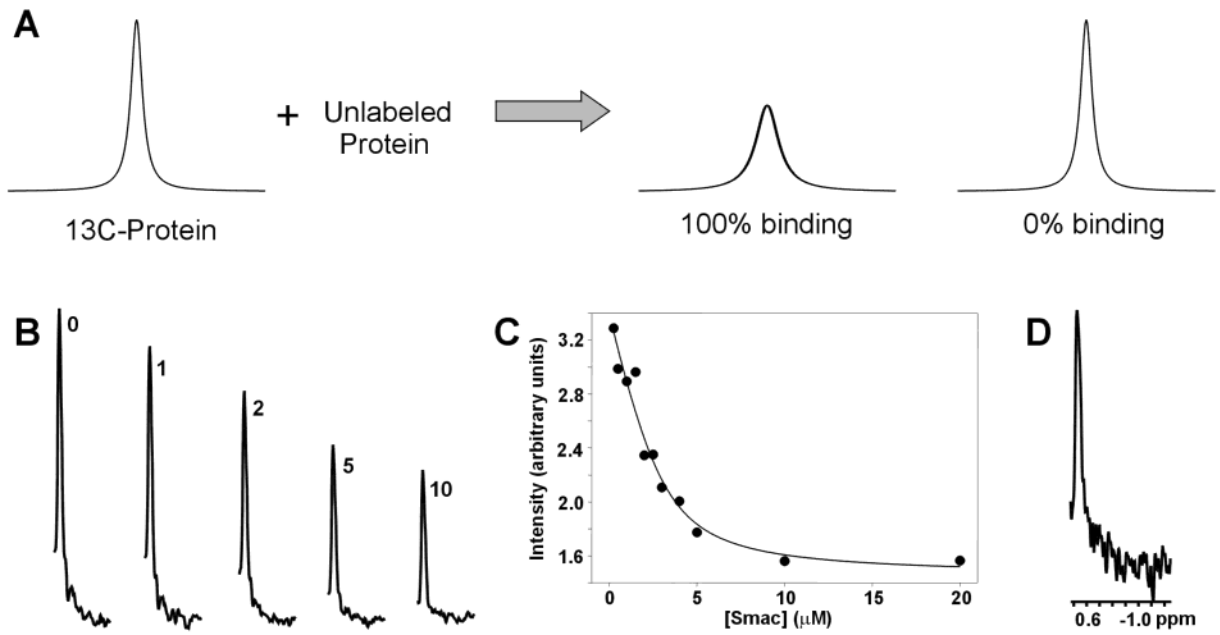

FIGURE 3: SMRC method for studying protein−protein interactions. (A) Scheme summarizing the method. The SMRC observed in the 1D ^{13}C-edited ^{1}H NMR spectrum of a ^{13}C-labeled protein (left) decreases in intensity due to resonance broadening upon binding to an unlabeled protein, while it remains unaffected if there is no binding (right). (B) SMRCs of a series of 1D ^{13}C-edited ^{1}H NMR spectra of 3 μM ^{13}C-labeled BIR3 in the presence of the indicated concentrations of unlabeled Smac in micromolar (all plotted at the same vertical scale). All spectra were acquired at 500 MHz with 2000 transients (53 min total acquisition time). (C) Plot of the SMRC intensity observed in 1D ^{13}C-edited ^{1}H NMR spectra of 3 μM ^{13}C-labeled BIR3 as a function of unlabeled Smac concentration. The data were fit to an equilibrium 1:1 binding model (—) (see Materials and Methods), yielding a dissociation constant of 0.6 ± 0.3 μM. All samples used for panels B and C were dissolved in 20 mM Tris-d_{11} (pH 7.7), 150 mM NaCl, 0.5 mM EDTA, and 5% D_2O containing 10 μM unlabeled bovine serum albumin to prevent binding of Smac to the glass, which becomes significant at sub-micromolar concentrations. (D) SMRC of a 500 MHz 1D ^{13}C-edited ^{1}H NMR spectrum of 250 nM ^{13}C-labeled BIR3 (20000 transients, 8 h total acquisition time).

vesicles (100 nm diameter), binding should dramatically broaden the C_2AB resonances. Analogous results were obtained when both the core complex and LUVs were added to ^{13}C-labeled C_2AB (Figure 4D). Thus, C_2AB remains bound to the lipids in the presence of the core complex, although these experiments do not reveal whether the core complex is still bound to C_2AB under these conditions.

To answer this question, we performed additional experiments where we monitored the SMRC of ^{13}C-labeled core complex. Addition of 2 μM unlabeled C_2AB to 1.5 μM ^{13}C-labeled core complex caused a 33% decrease in the SMRC intensity, confirming the interaction (Figure 4E,F). However, addition of both LUVs and C_2AB yielded the same SMRC intensity observed for the free core complex (Figure 4E,G), instead of broadening beyond detection as would be expected if the core complex were bound to the C_2AB−LUVs complex (i.e., a spectrum similar to that in Figure 4D). These results unambiguously show that phospholipid binding to the synaptotagmin 1 C_2AB region competes with core complex binding, and add to a growing body of evidence that suggests that Ca^{2+}-dependent phospholipid binding is indeed the primary activity that underlies the function of synaptotagmin 1 in neurotransmitter release (*20*, *21*, *26*).

DISCUSSION

Our results show that the SMR and SMRC methods (Figures 2A and 3A, respectively) are powerful tools for studying protein−protein interactions that will complement other existing techniques. The most attractive features of these assays are that they are performed in solution with purified proteins and that they do not require tags or chemical modifications. These methods are based on simple physical properties that directly reflect the populations of the free and bound states of the proteins under study, and hence do not suffer from the drawbacks of techniques such as chromatography that can yield false negative results for interactions with fast off-rates. The methods described here are thus largely free of artifacts and can be used to unambiguously establish whether two or more proteins interact in vitro. In addition, appropriate controls can be used to study the specificity of the observed interactions, and titrations can be performed to show that binding is stoichiometric and saturable, as well as to measure dissociation constants above ca. 0.5 μM. Both methods involve standard NMR experiments that can be performed on most commercial NMR spectrometers with a ^{1}H frequency of ≥400 MHz, making these methods generally accessible to non-NMR experts.

The SMR method is generally applicable to both recombinant proteins and proteins isolated from natural sources. For the SMRC method, at least one of the proteins needs to be expressed by recombinant methods and labeled with ^{13}C. However, this feature represents an advantage for studying multicomponent systems and interactions with other macromolecules, as shown for the analysis of interactions involving the synaptotagmin 1 C_2AB region (Figure 4). In addition, many proteins can be easily labeled with ^{13}C by expression in bacteria in minimal media containing [$^{13}C_6$]-glucose as the sole carbon source, and different types of ^{13}C-enriched media for expression in bacteria, CHO cells, and insect cells can now be obtained from major vendors of isotopically labeled compounds. Note also that the cost of ^{13}C labeling is dramatically lower than is common for

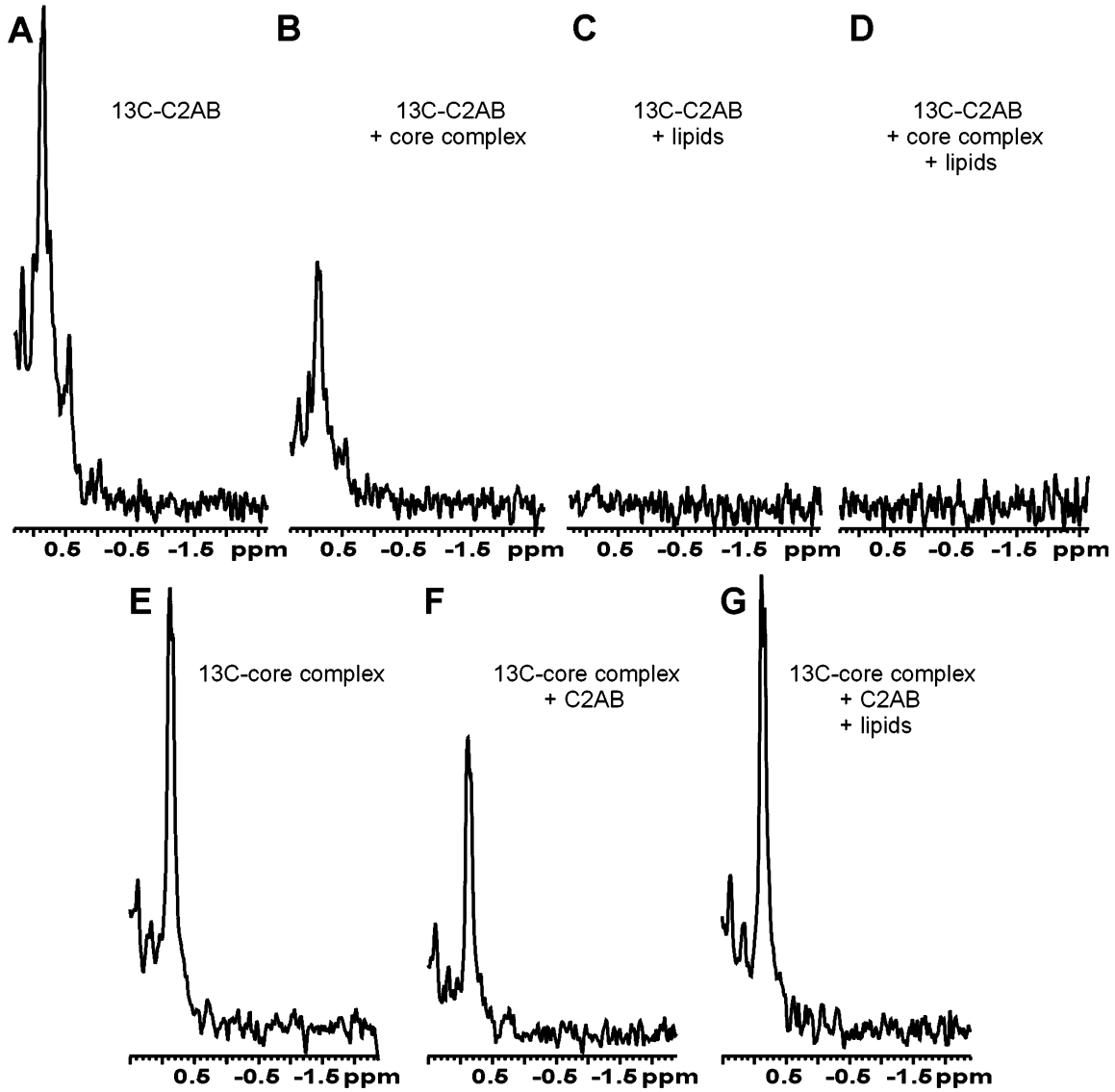

FIGURE 4: Application of the SMRC method to the study of synaptotagmin 1 function. (A−D) SMRCs observed in 1D ^{13}C-edited ^{1}H NMR spectra of 2 μM ^{13}C-labeled synaptotagmin 1 C_2AB region in 20 mM imidazole-d_3 (pH 7.2), 100 mM NaCl, 1 mM Ca^{2+}, and 5% D_2O in isolation (A) and after adding 3 μM unlabeled core complex (B), 0.3 mg/mL LUVs (C), or 3 μM unlabeled core complex and 0.3 mg/mL LUVs (D). (E−G) SMRCs of 1D ^{13}C-edited ^{1}H NMR spectra of 1.5 μM ^{13}C-labeled core complex (all four SNARE motifs labeled with ^{13}C) in the same buffer in isolation (E) and after adding 2 μM synaptotagmin 1 C_2AB region (F) or 2 μM synaptotagmin 1 C_2AB region and 0.3 mg/mL LUVs (G). LUVs (100 nm) contained 30% brain phosphatidylserine and 70% brain phosphatidylcholine, and were prepared using an extruder system from Avanti Polar Lipids according to the procedure recommended by the manufacturer. All spectra were acquired at 600 MHz with 2000−3000 transients (53−79 min total acquisition time). The smaller decrease in SMRC intensity upon binding observed for the core complex, compared to the decrease observed for C_2AB, arises because the correlation time of the core complex is somewhat slower due to its elongated shape, and because of contributions from unstructured tails to the SMRC of the core complex. All observed binding interactions were Ca^{2+}-dependent and reversible, as assessed from spectra recorded after adding 1 mM excess EDTA (data not shown). In multiple experiments that were performed, no decrease or only slight decreases in SMRC intensity were observed for core complex/C_2AB mixtures in the absence of Ca^{2+} compared to the added spectra of separate core complex and C_2AB samples, indicating that the core complex and the synaptotagmin 1 C_2AB region do not bind or bind weakly in the absence of Ca^{2+}.

structural studies since the amounts of protein required for one sample are between 2 and 3 orders of magnitude smaller.

In principle, the SMR and SMRC methods have no fundamental molecular mass limitations, although they are not appropriate for studying protein−small molecule interactions as implemented in this study. However, it is worth noting that a method that relies on resonance broadening effects to screen for small compounds that bind to target proteins using 1D NMR spectroscopy has been described (*27*). The primary advantage that the SMR and SMRC methods offer over 2D NMR techniques in studying protein−protein interactions is the ability to work at low micromolar concentrations, which minimizes the probability of nonspecific interactions and puts less stringent requirements on the solubility of the proteins under study. It is in fact possible to obtain spectra at sub-micromolar protein concentrations, as illustrated by the 500 MHz 1D ^{13}C-edited ^{1}H NMR spectrum of 250 nM ^{13}C-labeled BIR3 shown in Figure 3D, but these experiments are more technically demanding (see Materials and Methods) and require acquisition times of several hours. The sensitivity gains associated with the use of higher magnetic fields and cryoprobe technology should allow one to substantially shorten these acquisition times or to apply these methods at even lower protein concentrations.

Variations of the methods proposed here, including the use of other labeling schemes (e.g., protein perdeuteration) and isotope-filtered experiments, are also expected to further expand the usefulness of 1D NMR spectroscopy in studying protein interactions.

ACKNOWLEDGMENT

We thank Oleg Guryev and Xiaodong Wang for fruitful discussions and for providing vectors for expressing BIR3 and Smac, Xiaocheng Chen and Jun Lu for providing samples of the core complex and the syntaxin 1A−munc18-1 complex, Lewis Kay for providing the HSQC pulse sequence used for the SMRC method, and Hongato Yu, Kevin Gardner, Xiaodong Wang, and Thomas C. Südhof for useful comments on the manuscript.

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BI0272050