Identification by NMR Spectroscopy of Residues at Contact Surfaces in Large, Slowly Exchanging Macromolecular Complexes

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Received June 7, 1999

Identification of the specific interactions at protein−protein or protein−oligonucleotide binding sites is often crucial for understanding biological function.[1−3] Technical difficulties can render the structure determination of large protein complexes to be a difficult and time-consuming process, however. Mutational screening of surface residues, together with a functional or binding assay, can provide indirect information on a binding epitope.[4] More directly, when the time regime of chemical exchange is fast, binding sites can be mapped by using NMR spectroscopy with chemical shift perturbation studies.[5] When exchange between the bound and free states is slow or intermediate, however, the analysis is less trivial, particularly in large complexes. We have found by using line shape simulations of NMR signals that, under certain conditions, the binding epitopes on small proteins contacting much larger molecules can be identified from the differential line broadening of their NMR signals in the free state. The validity of this approach is demonstrated with the 16 kDa regulatory protein B (MMOB) in slow exchange with a complexed form containing the 251 kDa hydroxylase (MMOH) of the soluble methane monooxygenase system from *Methylococcus capsulatus* (Bath).

Whereas interactions of high affinity and specificity are generally believed to originate from large contact surfaces involving 10−30 side chains from each protein component,[1,2] protein−protein interactions are energetically driven by only a small subset of residues or hot spots, localized at the contact interface.[3] NMR spectroscopy provides an approach to probe protein interactions with small ligands, oligonucleotides, and other proteins by monitoring differential line broadening[6−8] and chemical shift changes[5,9] upon binding in [^{15}N,^{1}H]-HSQC spectra. Chemical exchange between two states has been well studied by using NMR, and the line shapes during exchange can be simulated with eq 1, which is derived from principles previously outlined:[10,11]

$$\mathbf{I}(\omega) = \mathrm{re}\int_0^\infty \mathbf{W}\exp\{i(\Omega - \omega\mathbf{E})t + \mathbf{K}t + \mathbf{R}t\}\,\mathbf{1}\,\mathrm{d}t \quad (1)$$

Matrix **R** contains the transverse relaxation rates in s^{-1}, where relaxation is described by a single exponential, whereas matrices **K** and Ω contain the chemical exchange rates and chemical shifts, respectively. Matrix **W** contains the probability of occurrence at each frequency, and **E** and **1** are the identity matrix and unity vector, respectively. In cases where the protein of interest is binding to a large macromolecule, having a molecular weight greater than 150 kDa, the bound state is not readily observable by NMR due to its long rotational correlation time.[12] We have performed simulations by using eq 1 for a two-state system where the bound state is unobservable by NMR and only the free state can be used to characterize binding.

The effect on the unbound state of the increased molecular weight of the bound state was explored by using eq 1 for a system undergoing exchange (Figure 1 A,B). For chemical shift differences of 500 Hz, line broadening of the free state is dominated by the exchange effect, and variations in the transverse relaxation rate of the bound state do not make significant contributions to free-state resonance broadening (Figure 1B). When the chemical shifts in the free and bound states are the same, however, exchange broadening due to chemical shift differences is absent, and the signal is very sensitive to the relaxation rate of the bound state (Figure 1A).

Simulations of eq 1 indicate that resonance line widths at the frequency of the free state are highly sensitive to differences in chemical shift perturbations upon binding, even when exchange occurs with a large molecule having a transverse relaxation rate of 250 s^{-1} (Figure 1C). Chemical exchange causes significant broadening, and resonances having chemical shifts that are unaffected by binding have considerably larger peak heights (red) than those which are shifted.

The effect of variations in k_{off} values on the resonance line shape has been well studied and is highly dependent on the magnitude of chemical shift perturbations upon binding.[10,11] Figure 2 indicates that in large, slowly exchanging complexes, chemical shift perturbations upon binding can be studied by using differential line broadening of resonances in the observable free state. We confirmed the presence of such differential line broadening in a system undergoing slow exchange. The soluble methane monooxygenase system from *M. capsulatus* (Bath), in which the regulatory protein B (MMOB) is in slow exchange between a free 16 kDa state and a large 267 kDa complex state with the hydroxylase protein (MMOH), was used to assess the applicability of our simulations. The method used for sample preparation and data analysis was reported previously.[13] ^{15}N-Labeled MMOB at a 0.2 mM concentration was used with increasing concentrations of hydroxylase to produce ratios of 10:1, 6:1, 3:1, and 2:1 for MMOB/hydroxylase. Differential line broadening of the cross-peaks corresponding to unbound MMOB residues was observed in [^{15}N,^{1}H]-HSQC spectra, and peak height reductions consistent

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10.1021/ja991881g CCC: $18.00
Published on Web 10/06/1999

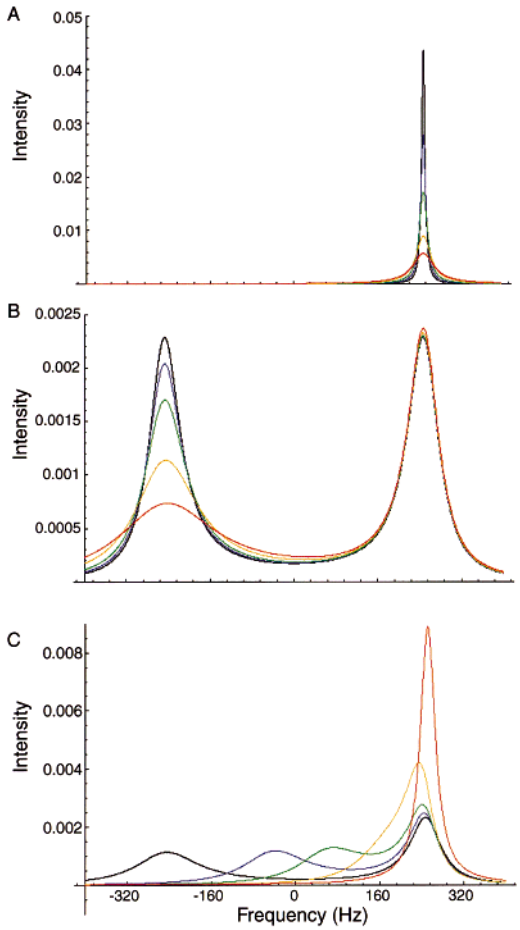

Figure 1. Line shape simulations generated by using eq 1. The transverse relaxation rate of the bound state, R_b, was varied with (A) no and (B) a 500-Hz change in chemical shift upon binding. In C, R_b is 250 s^{-1}, and the change in chemical shift that occurs upon binding is varied. In all cases, the fraction of free protein is 0.5, and the transverse relaxation rate of the free state (R_f) and k_{off} are 23 and 200 s^{-1}, respectively. The resonance position of the free state, ω_1, is 250 Hz. In A and B, R_b values of 500, 250, 100, 50, and 23 s^{-1} are indicated in red, orange, green, purple, and black, respectively. In C, chemical shift changes upon binding of 0, 100, 200, 300, and 500 Hz are indicated in red, orange, green, purple, and black, respectively.

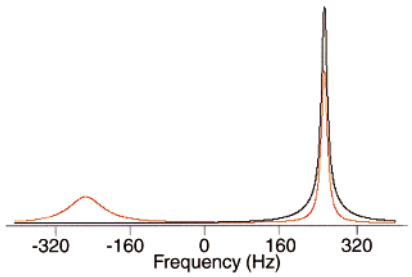

Figure 2. Line shape simulation by using eq 1 of a system in slow exchange with a bound state having a large molecular weight. In these simulations, the fraction of free protein is 0.5. R_f, R_b, and k_{off} are 23, 250, and 25 s^{-1}, respectively. Chemical shift differences of 0 (black) and 500 Hz (red) are used. The resonance position of the free state, ω_1, is 250 Hz.

with the simulations of eq 1 were observed. Figure 3A,B displays representative 1D slices from [^{15}N,^{1}H]-HSQC spectra of resonances originating from residues with average and greater than average broadening in the presence of hydroxylase, respectively. The data indicate that the residue with the greater resonance broadening, L96, interacts with the hydroxylase, whereas that with

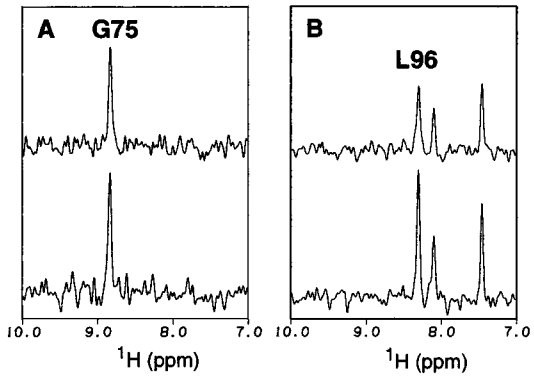

Figure 3. Selected 1D slices of the [^{15}N,^{1}H]-HSQC spectra of (A) G75 and (B) L96 of MMOB in the absence of hydroxylase (bottom) and at a ratio of 10:1 MMOB/hydroxylase (top).

less broadening, G75, does not form a direct contact. By using this approach, we were able to identify a cluster of conserved residues on the MMOB surface which appears to form direct contacts with the hydroxylase and to make a model of the MMOB/hydroxylase complex.[13] Although the structure of the complex has not yet been determined, our technique was validated in part by the high correlation between residues that were most broadened upon binding and those that are conserved among different hydroxylase regulatory proteins.[13]

Furthermore, if we assume that the majority of the amide proton resonances are not significantly perturbed upon binding, the transverse relaxation rate of the MMOB bound state can be estimated. To carry out this analysis, resonances experiencing average peak height reductions at a ratio of 10:1 MMOB/hydroxylase were assumed not to undergo changes in their chemical shifts upon binding. Solving eq 1 as a ratio between MMOB in the presence and absence of hydroxylase allowed an estimate of the transverse relaxation rate of the MMOB bound state to be obtained. Using the known value for k_{off}[14] and assuming from the binding affinity of MMOB for hydroxylase that all of the hydroxylase is bound,[14] a rate of 270 s^{-1} was obtained. This value is reasonable for a molecule of this size and within the error range of the molecular weight-based empirically determined value of 250 s^{-1}.[15]

In summary, much interest has been directed toward overcoming the NMR size limitation to study large biological systems. We have applied well-known formalisms to show that differential line broadening upon binding of the free state of residues of small proteins, which are in slow exchange with a large complex, can be used to identify binding sites using NMR. The predicted differential line broadening of the free state of a protein in slow exchange with a much larger state was observed in the MMO system. Although the 251 kDa hydroxylase of the MMO system is too large to be observed directly using NMR, we were able to apply the technique described in this paper to obtain information on the binding interactions formed between the regulatory protein B and hydroxylase. With this approach, models of large complexes can be generated for systems awaiting structural determination.

Acknowledgment. This work was supported by grants from the National Institute of General Medical Sciences, (GM32134 to S.J.L. and GM47467 to G.W.). G.T.G. is a NIH postdoctoral fellow. H.M. and K.J.W. contributed equally to this work.

Supporting Information Available: A more detailed description of the equations used for the simulations presented in this publication (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA991881G

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