The Use of Differential Chemical Shifts for Determining the Binding Site Location and Orientation of Protein-Bound Ligands

Ales Medek, Philip J. Hajduk, Jamey Mack, and Stephen W. Fesik*

Pharmaceutical Discovery Division
Abbott Laboratories, Abbott Park, Illinois 60064

Received November 5, 1999
Revised Manuscript Received December 14, 1999

Protein−ligand interactions play an important role in a variety of biological processes. To characterize these interactions at the molecular level, nuclear magnetic resonance (NMR) spectroscopy has become a valuable tool. NMR structures of proteins complexed to small organic molecules,[1] nucleic acids,[2] peptides, and other proteins[3] have yielded a wealth of information on the important determinants of molecular recognition. However, high-resolution NMR structure determination of protein/ligand complexes is a tedious and time consuming process due to the large amount of data that must be analyzed. Moreover, structures of many interesting systems cannot be obtained due to solubility limitations, unfavorable dynamics, or signal overlap. In contrast, the approximate binding site location is routinely obtained by NMR from a comparison of the chemical shifts of the free versus complexed protein[4] and can be determined even for large molecular assemblies.[5] Although this chemical shift perturbation approach is useful for quickly identifying general sites of interaction, no information on the orientation of the ligand in the binding site is obtained. Furthermore, the interpretation of the chemical shift changes upon ligand binding can be complicated by ligand-induced conformational changes of the protein.[6−8]

Here we describe a simple chemical shift based technique for rapidly determining the precise location of the ligand binding site and the orientation of the ligand in the binding pocket. This approach involves the comparison of chemical shift changes of a protein induced by a series of closely related ligands. Using this method, the region of the binding site that is proximal to the portion of the ligand that differs within the series is readily identified from localized shielding differences of the protein. By comparing the chemical shift changes caused by ligands with minor chemical alterations in different parts of the molecule, the ligand may be docked into the binding pocket.

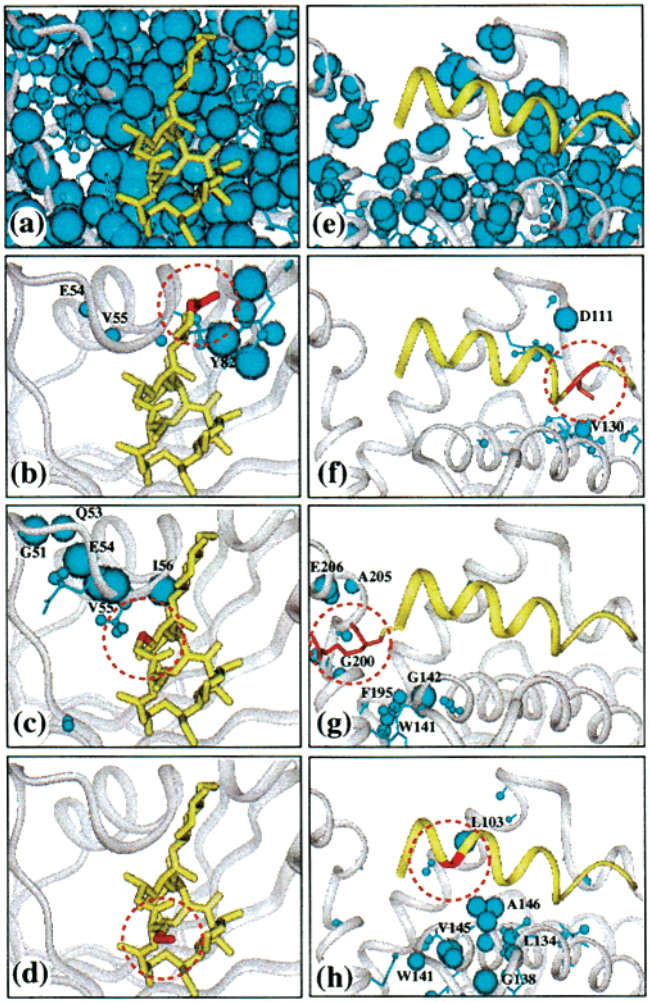

Figure 1. Schematic representation of the protein differential ^{1}H, ^{13}C, ^{15}N NMR chemical shifts for the FKBP−ascomycin complex compared to (a) free FKBP, (b) FKBP/31-keto-32-desoxy-ascomycin, (c) FKBP/24-desoxy-ascomycin, and (d) FKBP/FK506. The differential chemical shifts of the Bcl-x_L/Bak 16mer peptide complex are compared to (e) free Bcl-x_L, (f) Bcl-x_L/V307A Bak mutant, (g) the Bcl-x_L/*R*320A Bak mutant, and (h) Bcl-x_L/G315A Bak mutant. The ligands are shown in yellow with the specific position of the ligand's chemical mutation highlighted in red. Atoms which exhibit significant differential chemical shifts are shown as blue spheres. The diameter of each sphere is directly proportional to the magnitude of the chemical shift differences in the range from 0.15 to 0.65 ppm for ^{13}C and ^{15}N nuclei and from 0.03 to 0.13 ppm for ^{1}H nuclei, respectively. Shifts smaller than the lower limit are not indicated, and those exceeding the upper limit are represented by the same maximum sphere size.

To demonstrate the utility of this approach for characterizing the structure of protein/drug complexes, the chemical shift changes of the FK506 binding protein (FKBP) were analyzed upon the addition of a series of potent FK506 analogues.[9] The chemical shift mapping typically employed to examine such interactions would involve a comparison of the shifts for free and bound FKBP. As shown in Figure 1a, most (>90%) of the FKBP signals change upon the addition of an FK506 analogue, ascomycin,

* To whom correspondence should be addressed.

(1) Stockman, B. J. *Prog. NMR Spectrosc.* **1998**, *33*, 109−151.
(2) Wijmenga, S. S.; Vanbuuren, B. N. M. *Prog. NMR Spectrosc.* **1998**, *32*, 287−387.
(3) Clore, G. M.; Gronenborn, A. M. *Curr. Opin. Chem. Biol.* **1998**, *2*, 564−570.
(4) Shuker, S. B.; Hajduk, P. J.; Meadows, R. P.; Fesik, S. W. *Science* **1996**, *274*, 1531−1534.
(5) Sun, C.; Cai, M.; Gunasekera, A. H.; Meadows, R. P.; Wang, H.; Chen, J.; Zhang, H.; Wu, W.; Xu, N.; Ng, S. C.; Fesik, S. W. *Nature* **1999**, *401*, 818−822.
(6) Spassov, V. Z.; Bashford, D. *J. Comput. Chem.* **1999**, *20*, 1091−1111.
(7) Johnson, E. R.; McKay, D. B. *Biochemistry* **1999**, *38*, 10823−10830.
(8) Ozarowski, A.; Barry, J. K.; Matthews, K. S.; Maki, A. H. *Biochemistry* **1999**, *38*, 6715−6722.

(9) FKBP was recloned into the pET-29 vector (Novagen) and purified in a single affinity chromatography step using Ni^{2+} resin (Invitrogen) and eluting with 500 mM imidazole. The ascomycin analogues were predissolved to 100 mM in DMSO-d_6 and added to a solution of 0.9 mM FKBP in aqueous buffer (40 mM phosphate, 5 mM DTT, pH 6.5) to a final compound concentration of 2 mM. The samples of FKBP complexes were allowed to stand at room temperature for 24 h before data acquisition. Chemical shifts for the free protein as well as the parent FKBP/ascomycin complex were obtained from 3D ^{13}C-edited $^{1}H-^{1}H$ NOESY spectra (^{13}C carrier at 72 ppm, 16 scans, 32 × 128 × 512 points, 89 h) using previously reported assignments.[11] Assignments for the remaining complexes were based on the acquisition of two constant time $^{1}H-^{13}C$ HSQC spectra (aliphatic: ^{13}C carrier at 40 ppm, constant time period of 28 ms, 8 scans, 300 × 512 points, 1.5 h; aromatic: ^{13}C carrier at 130 ppm, constant time period of 18 ms, 16 scans, 128 × 512 points, 1.5 h) and an $^{1}H-^{15}N$ HSQC spectrum (^{15}N carrier at 117 ppm, 8 scans, 175 × 512 points, 1 h). Nearly complete aromatic and amide assignment was accomplished from 2D HSQC spectra for all complexes whereas only a partial assignment of the aliphatic region was achieved due to a larger signal overlap. All spectra were acquired on a Bruker DRX 800 spectrometer.

10.1021/ja993921m CCC: $19.00
Published on Web 01/29/2000

making it difficult to even identify the binding pocket and impossible to orient the ligand.[10] In contrast, by analyzing the differential chemical shifts observed for a series of closely related ascomycin analogues, the region of the binding pocket that is near the altered portion of the ligand is easily identified (Figure 1b,c). For example, by comparing the chemical shifts of the FKBP/ascomycin complex to those of FKBP/32-desoxy-31-keto-ascomycin, the cyclohexyl ring of ascomycin can be shown to bind in the vicinity of Y82 (Figure 1b). Similarly, the localized chemical shift differences of residues 51−56 indicate the binding site location of the hydroxyl group at carbon 24 of ascomycin from a comparison with 24-desoxy-ascomycin (Figure 1c). As shown in Figure 1d, no chemical shift perturbations are observed when the ethyl group of ascomycin is replaced by an allyl moiety (FK506). These results suggest that this group is pointing away from the binding pocket, consistent with the previously determined structures of FKBP/ascomycin and FKBP/FK506[11] complexes. This information is useful for identifying regions of small molecules that may be altered to improve their pharmacokinetic properties without affecting binding affinity.

The differential chemical shift method can also be used to characterize protein/peptide and protein/protein interactions. This is illustrated for the binding of the anti-apoptotic protein Bcl-x_L to a 16mer peptide derived from the pro-apoptotic protein Bak.[12,13] As in the case with FKBP, information on the binding topology cannot be obtained from an analysis of the chemical shift differences between free and bound Bcl-x_L (Figure 1e). However, specific and spatially localized chemical shift perturbations were observed with different Bcl-x_L/Bak complexes formed with alanine mutant Bak peptides. For example, an alanine mutation near the N-terminal portion of the peptide (V307A) caused significant changes only in the spatially proximal residues V130 and D111 of Bcl-x_L when compared with the Bcl-x_L/BAK complex (Figure 1f). Conversely, the R320A mutant located at the C-terminal end of the peptide caused significant differences for residues E206, A205, F195, W141, and G142 on the opposite side of the binding pocket (Figure 1g). A mutation in the middle of the Bak peptide (G315A) caused changes in the middle of the binding site (Figure 1h). These results suggest that using only the differential chemical shift information, the binding site and binding orientation of the Bak 16mer can be reliably determined.

Protein differential chemical shift observations between a series of related ligands allow the binding pocket to be reliably mapped and the orientation of the ligand to be defined. Thus, this method can be used to rapidly determine how a series of small organic compounds bind to proteins. This information will be useful for structure-based drug design, especially in cases where conventional methods for structure determination fail. In addition, the differential chemical shift technique may be used to determine how proteins dock together with other proteins to form molecular assemblies using a strategy of comparing the chemical shifts caused by a series of alanine mutations to pinpoint the binding site locations of the altered residues.[14] Due to the recent advances in isotope labeling[15,16] and NMR methods for studying large biomolecules,[17−19] this approach could be used to characterize the quaternary structure of even very large (>100 kDa) molecular assemblies.

JA993921M

(10) Upon binding of ascomycin, more than 90% of FKBP amide signals display nitrogen shifts larger than 0.15 ppm or proton shifts larger than 0.03 ppm.

(11) Meadows, R. P.; Nettesheim, D.; Xu, X. R.; Olejniczak, E. T.; Petros, A. M.; Holzman, T. F.; Severin, J.; Gubbins, E.; Smith, H.; Fesik, S. W. *Biochemistry* **1993**, *32*, 754−765.

(12) Sattler, M.; Liang, H.; Nettesheim, D.; Meadows, R. P.; Harlan, J. E.; Eberstadt, M.; Yoon, H. S.; Shuker, S. B.; Chang, B. S.; Minn, A. J.; Thompson, C. B.; Fesik, S. W. *Science* **1997**, *275*, 983−986.

(13) Uniformly $^{15}N/^{13}C$ labeled Bcl-x_L and the Bak peptides were prepared as previously described.[12] The peptides were dissolved in aqueous buffer (20 mM phosphate, 5 mM DTT, pH 6.8) and added to a solution of 1 mM Bcl-x_L in the same buffer. The final concentration of Bcl-x_L and peptide was 0.4 and 0.6 mM, respectively. The chemical shift assignment was based on the same experimental strategy as for FKBP complexes except that the number of scans and the total acquisition times were doubled for the HSQC experiments to make up for the lower concentration of the Bcl-x_L protein.

(14) Using differential chemical shifts, it is possible to orient the ligands into the protein binding pocket when the series of ligands bind in a similar conformation and induce the same conformational changes in the protein. In those cases in which the ligands bind differently, chemical shift differences in a larger region of the protein would be expected, allowing these ligands to be quickly identified.

(15) Goto, N. K.; Gardner, K. H.; Mueller, G. A.; Willis, R. C.; Kay, L. E. *J. Biomol. NMR* **1999**, *13* (4), 369−374.

(16) Xu, R.; Ayers, B.; Cowburn, D.; Muir, T. W. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 388−393.

(17) Wand, A. J.; Ehrhardt, M. R.; Flynn, P. F. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 15303−15308.

(18) Pervushin, K.; Riek, R.; Wider, G.; Wüthrich, K. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 12336−12371.

(19) Salzmann, M.; Pervushin, K.; Wider, G.; Senn, H.; Wüthrich, K. *J. Biomol. NMR* **1999**, *14*, 85−88.