Segment Identification of a Ligand Binding with a Protein Receptor Using Multidimensional $T_{1\rho}$ -, Diffusion-Filtered and Diffusion-Ordered NOESY Experiments

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Multidimensional $T_{1\rho}$ -, diffusion-filtered and diffusion-ordered NOESY techniques were applied to identify segments of a ligand binding with a protein receptor. These experiments can easily provide intermolecular NOEs of the complex, which are of great significance for characterizing the binding epitopes of a ligand. This information cannot be obtained by high-throughput 1D NMR experiments, used for determining the binding affinity, although multidimensional NMR experiments require more experiment time. The present results indicate that the current experiments using $T_{1\rho}$ - and diffusion-edited techniques are very suitable for identifying segments of a ligand binding with a protein receptor in the drug discovery process.

(Received May 23, 2003; Accepted July 22, 2003)

Introduction

Protein-ligand, protein-protein and protein-nucleic acid interactions are of primary interest in pharmacology and structural biology, and often provide useful clues for understanding the physiological function of drugs and macromolecules. Specific characterization of direct contact in a complex is currently left largely to X-ray analyses. Because X-ray studies are often prevented by a difficulty to obtain crystals suitable for diffraction, NMR spectroscopy is an attractive alternative for characterizing macromolecular complexes and for determining compounds with binding affinity to macromolecules. There is a growing need for efficient methods capable of reliably identifying compounds with the desired binding activity, and several NMR-based screening methods have been developed using chemical-shift perturbation,¹⁻⁹ transferred NOE¹⁰⁻¹³ and diffusion and relaxation editing.¹⁴⁻¹⁶ In the development of NMR techniques for measuring binding affinity, the main aim is to reduce the experiment time and material usage without a need for isotope labeling. Although a high-resolution structure of the protein-ligand complex is desirable in rational drug design, a map of the interaction sites obtained by NMR techniques often provides valuable insights for identifying lead compounds, or making a judgment as to whether different compounds share a common binding mode.

Recently it was shown that 1D NOE pumping¹⁷ and 1D reverse NOE pumping¹⁸ experiments can be effectively used to distinguish molecules that bind to macromolecules from nonbinding compounds. The 1D saturation transfer difference

NMR technique using a $T_{1\rho}$ -filter was also developed to obtain information about the binding specificity at the atomic level.¹⁹ Although these 1D experiments are very effective for rapidly characterizing binding processes, it is not easy to identify the binding interface in the complex. Here, we present 2D $T_{1\rho}$ - and diffusion-filtered NOESY experiments and 3D DOSY-NOESY experiment to identify segments of ligand binding with a protein receptor without the need for isotope labeling. The ability of these 2D techniques to identify the binding epitopes of a ligand has been demonstrated with the human serum albumin (HSA)-salicylic acid system. HSA is the most abundant protein in serum plasma, and is capable of transporting fatty acids and of binding a variety of metabolites and drugs. It consists of three repeating domains (I, II and III); domain III possesses a high affinity for small anionic aromatic compounds and the primary binding site for medium-long chain fatty acids.

Experimental

All NMR experiments were performed on a JEOL ECA600 spectrometer equipped with a 5-mm inverse triple-resonance probe head at 25°C. All of the spectra were recorded using 10 mM salicylic acid and 0.1 mM HSA in 99.9% $^2\text{H}_2\text{O}$ containing PBS buffer (pH 7.4). HSA and salicylic acid were purchased from Wako (Osaka, Japan). The pulse sequences of the 2D $T_{1\rho}$ - and diffusion-filtered NOESY, and 3D DOSY-NOESY experiments are shown in Fig. 1. The experimental parameters used for the $T_{1\rho}$ -filtered NOESY were $T_{1\rho}$ -filtering spin-lock = 70 ms; NOE mixing (τ_m) = 100 ms; data size in t_1 = 256 complex points; data size in t_2 = 2048; spectral width in f_1 = 7203 Hz; spectral width in f_2 = 9006 Hz; number of transients per increment = 64. The total duration of the experiment was

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Fig. 1 The 2D (a) $T_{1\rho}$ -filtered NOESY, (b) diffusion-filtered NOESY and 3D DOSY-NOESY pulse sequences. The thin and thick bars represent 90 and 180 degree pulses, respectively. All pulses were along x, unless otherwise shown. Phases in (a) are $\phi_1 = 4(x, y), 4(-x, -y); \phi_2 = 4(y, -x), 4(-y, x); \phi_3 = 4x, 4y, 4(-x), 4(-y), 4y, 4(-x), 4(-y), 4(x); \phi_r = 2(-x), 2x, 2(-y), 2y, 2x, 2(-x), 2y, 2(-y), 2(-y), 2y, 2x, 2(-x), 2y, 2(-y), 2(-x), 2x, 2x, 2(-x), y, 2(-y), 2(-x), 2x, 2(-y), 2y, 2y, 2(-y), 2(-x), 2x, 2(-y), 2y, 2x, 2(-x)$. Phases in (b) for diffusion-filtered NOESY and DOSY-NOESY are $\phi_1 = 4(x, y), 4(-x, -y); \phi_2 = 16x, 16y, 16(-x), 16(-y); \phi_3 = 4x, 4y, 4(-x), 4(-y), 4y, 4(-x), 4(-y), 4(x); \phi_r = 2(-x), 2x, 2(-y), 2y, 2x, 2(-x), 2y, 2(-y), 2(-y), 2y, 2x, 2(-x), 2y, 2(-y), 2(-x), 2x, 2x, 2(-x), y, 2(-y), 2(-x), 2x, 2(-y), 2y, 2y, 2(-y), 2(-x), 2x, 2(-y), 2y, 2x, 2(-x)$. In the DOSY-NOESY experiment, gradient amplitudes ranged 2 – 20 G/cm as described in the text.

approximately 18 h. In the diffusion-filtered NOESY measurement, the experimental parameters used were $\tau = 2.1$ ms; $T = 150$ ms; NOE mixing (τ_m) = 100 ms; $G = 20.0$ g/cm; the number of transients per increment was 64. In the DOSY-NOESY measurement, the experimental parameters were τ , 2.1 ms; T , 150 ms; NOE mixing (τ_m), 300 ms; data size in t_1 , 128 complex points; data size in t_2 , 1024; spectral width in f_1 , 7203 Hz; spectral width in f_2 , 9006 Hz; number of transients per increment, 40. The gradient amplitudes ranged from 2 to 20 G/cm in ten equal steps. The total duration of the experiment was approximately 60 h. All other experimental parameters were identical in these measurements.

Results and Discussion

The pulse sequence of the 2D $T_{1\rho}$ -filtered NOESY (Fig. 1a) is conceptually similar to that presented by Scherf and Anglister.²⁰ The $T_{1\rho}$ -filtered NOESY results in a spectrum with binding ligand signals in the first dimension and protein signals in the second dimension. The technique is based on $T_{1\rho}$ -filtering using a spin-lock pulse before the evolution period (t_1), such that the broad resonances of the protein protons decay before the evolution period. Therefore, no protein magnetization is labeled during the evolution period. During the mixing time, the magnetization of the protein protons partially relax due to cross-relaxation with the ligand and other protein protons, and the partially recovered longitudinal magnetization is detected during the acquisition period. In the diffusion-filtered NOESY experiment (Fig. 1b), NOE-pumping is used to transfer the signal from the protein (receptor) to the bound ligand. The magnetization of the ligand is destroyed by the diffusion filter; the protein signals, which are to be transferred to the ligand, are only preserved during the evolution period. This experiment results in a spectrum with protein signals in the first dimension and binding ligand signals in the second dimension, which is

Fig. 2 2D (a) $T_{1\rho}$ -filtered NOESY, (b) diffusion-filtered NOESY and (c) 3D DOSY-NOESY spectra of 0.1 mM human serum albumin and 10 mM salicylic acid acquired at 25°C. In (c), a NOESY plane with the diffusion-coefficient of 4.8×10^{-10} (m^2/s) is shown. An expansion of each spectrum shows interactions of salicylic acid with human serum albumin. The assigned protons of salicylic acid are marked in each spectrum.

opposite to the $T_{1\rho}$ -filtered NOESY experiment. In the 3D DOSY-NOESY experiment, the gradient amplitudes were ranged linearly using the pulse sequence of 2D diffusion-filtered NOESY. It yields NOESY planes with diffusion-coefficient-labeled peaks; also, diffusion labeling provided by the DOSY portion of the experiment is retained in the NOE cross peaks of the NOESY planes.²¹ The DOSY-NOESY experiment uses the effects of diffusion to enhance the study of complex mixtures or molecules in dynamic equilibrium with bound and free states.

The 2D $T_{1\rho}$ - and diffusion-filtered NOESY and 3D DOSY-NOESY spectra are shown in Fig. 2. In the 2D $T_{1\rho}$ -filtered NOESY spectrum (Fig. 2a), intense intermolecular NOE cross peaks are observed between the H3/5 (6.9 ppm) and H6 (7.8 ppm) protons of salicylic acid and methyl and some aliphatic protons (0.6 – 1.8 ppm) of HSA, and almost no NOE cross peaks are observed for the H4 (7.3 ppm) proton of salicylic acid. In the 2D diffusion-filtered NOESY spectrum (Fig. 2b), however, most of the intermolecular NOE cross peaks are observed between the H3/5 protons of salicylic acid and

aliphatic protons of HSA, and fewer peaks are observed for the H4 and H6 protons. In the course of detecting signals that are pumped from the protein to the binding ligands in the NOE pumping experiment, rapid relaxation, especially T_2 relaxation, generally associated with macromolecules, could easily reduce the protein signals, resulting in a reduction of the sensitivity of the experiment. Strong NOE cross peaks are only observed in the diffusion-filtered NOESY spectrum. In the 3D DOSY-NOESY spectrum (Fig. 2c), more NOE cross peaks are observed for the H4 proton of salicylic acid, and NOE cross peaks for the H3/5 and H6 protons are not streaked as observed in the $T_{1\rho}$ -filtered NOESY spectrum (Fig. 2a). The NOESY plane with a diffusion-coefficient of 4.8×10^{-10} (m^2/s), as shown in Fig. 2c, provides very clear and sufficient intermolecular NOEs in the HSA-salicylic acid system. The diffusion of salicylic acid is expected to be near 4.8×10^{-10} (m^2/s). As signals that are pumped from HSA to salicylic acid (molar ratio = 1:100) are detected, intermolecular NOEs are observed in the plane with the diffusion-coefficient close to salicylic acid.

Recently, NMR-derived structural and binding studies of domain III of HSA complexed to the salicylic acid moiety of diflunisal have been performed.²² Diflunisal binds to the hydrophobic cavity, comprising five helices within subdomain IIIA. The observed intermolecular NOEs between diflunisal and methyl groups of valine, leucine and isoleucine of domain III of HSA, reveal that the two aromatic rings of diflunisal penetrate into a deep hydrophobic cavity, while the carboxyl and hydroxyl groups point toward the hydrophilic entrance. The pattern of intermolecular NOEs is quite similar to that observed in salicylic acid complexed to HSA. Structure-affinity studies of diflunisal analogues using NMR indicate that the addition of a phenyl ring or an aliphatic group at the H5 position of salicylic acid resulted in nearly a 4–17 fold increase in the binding affinity.²² This result indicates that an additional hydrophobic group at the H5 position is required for better contact with the binding cavity of domain III of HSA, because salicylic acid is incapable of filling the cavity sufficiently, which could be related to the observation of a few intermolecular NOEs at the H4 position.

In conclusion, these NOESY experiments can easily provide intermolecular NOEs of the complex, which are of great significance for characterizing binding epitopes of a ligand. This information cannot be obtained in the high-throughput 1D NMR experiments used for determining the binding affinity. The present results indicate that NOESY experiments using $T_{1\rho}$ - and diffusion-editing are very suitable techniques for identifying segments of a ligand binding with a protein receptor in the drug discovery process.

Acknowledgements

We would like to thank Dr. William S. Price (Tokyo Metropolitan University) for useful discussions and for critical reading of the manuscript.

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