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Epitope Mapping of Ligand−Receptor Interactions by Diffusion NMR

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In the pharmaceutical industry, diffusion-based NMR techniques have been incorporated into a variety of methods, such as screening of chemical mixtures,[1] determining the structures of bound ligands without physical separation,[2] and measuring the diffusion coefficient of small metabolites in biofluids.[3] The diffusion coefficient D is obtained by a linear fit of the intensity decay to the gradient strength according to $\ln I = \ln I_0 - \gamma^2\delta^2(\Delta - \delta/3 - \tau/2)Dg^2$, where γ is the gyromagnetic ratio, g is the gradient strength, δ is the width of the gradient pulse, Δ is the diffusion period length, τ is the gradient recovery time, I is the signal intensity, and I_0 is the I when g is zero. The diffusion coefficient enables the ligand−receptor interaction to be monitored by directly observing the ligand signals.[3,4]

In any *s*timulated *e*cho (STE) experiment,[5] the magnetization is stored in the longitudinal direction during the diffusion period. During this time cross-relaxation occurs between ligand and protein.[6] Through the NOE, magnetization is transferred between protons in the protein−ligand complex. The NOE exchange is small relative to the observed intensity particularly when the gradient is weak, but when the gradient is strong the NOE can be a significant contribution. Due to this perturbation the line is curved upward on the ($\ln I - g^2$) plot. Stronger deviations from linearity are obtained with longer diffusion times, but no deviation was observed for the free ligands.[6a] As a result, ^{1}H diffusion experiments of protein−ligand systems can be severely compromised by intermolecular NOE when the diffusion time is long. The concept was investigated using dihydrofolate reductase (DHFR) and its ligand trimethoprim (TMP). The human serum albumin (HSA) and L-tryptophan (Trp) system was also tested (Supporting Information). With a long diffusion time, the observed signal intensities within a single molecule decay with different rates when protein is present. Shown in Figure 1 are expansions of 1D spectra of TMP with DHFR recorded with low (dot) and high (solid) gradient strengths with the peak heights of 7-CH_2 scaled for comparison. When the gradient increases, the methyl signals decay faster than the methylene protons, not due to diffusion, but because its protons have been perturbed more strongly by the protein NOE. As a result, the 7-CH_2 has a larger deviation than methyl signals. As shown in Figure 2, the deviation from linearity for each proton is different and dependent on the distance to the protein. For the free ligand, all the lines are straight and parallel. The ligand proton nearest the protein generates the strongest NOE during the diffusion period and has the largest deviation. Therefore, this diffusion deviation is a novel method for characterizing the ligand epitope. The epitope map of TMP on DHFR generated with this method is in excellent agreement with the structure and dynamic results obtained by X-ray crystallography[7] and NMR.[8]

To build an epitope map with this method, the diffusion data were collected with a long diffusion time to allow the NOE transfer. Using an ($\ln I - g^2$) plot, the signals were fit with the quadratic equation $y = \alpha + \beta x + \kappa x^2$, where the free constant α corresponds to the offset $\ln I_0$, the constant β describes the linearity of the curve, while the constant κ represents the deviation from linearity. Thus, κ can be employed as an epitope value to characterize the binding interactions. For convenience, the largest value is normalized to 100, and the relative κ values are used in the mapping.

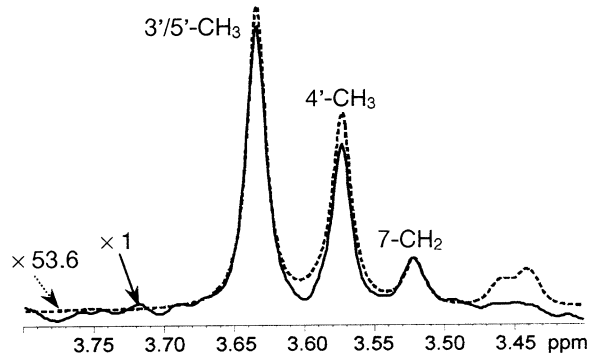

Figure 1. Expanded 1D spin−echo stimulated bipolar pair pulsed field gradient (BPP) STE spectrum of 4.2 mM TMP with 120 uM DHFR. Δ = 800 ms, δ = 1.0 ms, τ = 0.2 ms. The total length of CPMG filter is 49.3 ms with the short delay of 0.50 ms. Gradient strength of 3.98 (dotted-line curve) and 34.1 (solid-line curve) G/cm were applied, respectively.

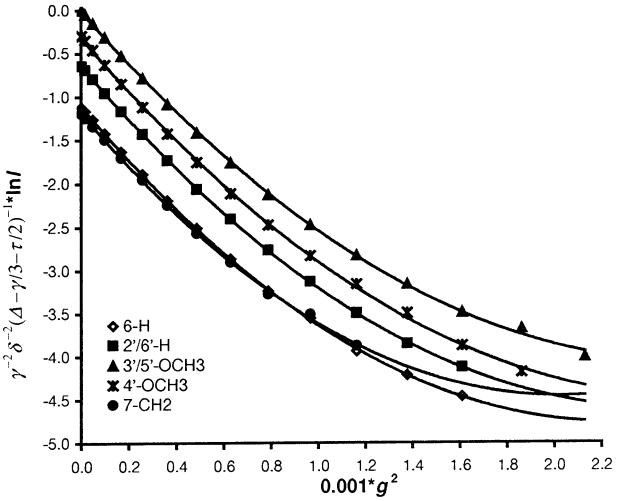

Figure 2. Plot of $\gamma^{-2}\delta^{-2}(\Delta - \delta/3 - \tau/2)^{-1}$ ln I vs g^2 of 4.2 mM TMP in the presence of 120 uM DHFR. Data were recorded by BPPSTE with CPMG filter, Δ = 800 ms, δ = 1.0 ms, the total length of T_2 filter is 49.3 ms with the short delay of 0.50 ms. The polynomial fitting line of each proton is shown; fitting equations are listed in Supporting Information.

The epitope map of TMP, obtained from the measurement shown in Figure 2, was presented in Figure 3 (bold print). The methylene protons have the largest κ value, which indicates proximity to DHFR. The next highest κ value of 91 is associated with H6 of the pyrimidine ring. When TMP binds to DHFR ($K_a = 2 \times 10^7$ M^{-1}),[8a]

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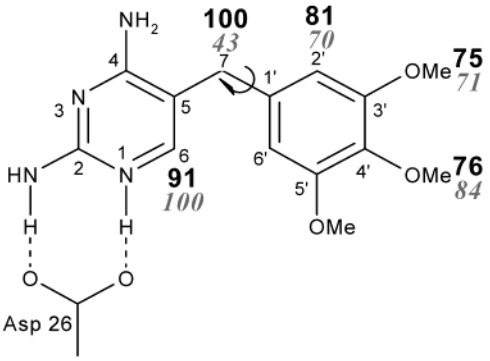

Figure 3. Epitope mapping of trimethoprim binding on DHFR by diffusion NMR (bold numbers) and by STD (gray italic numbers). Diffusion epitope resulted from the measurement shown in Figure 2. The presaturation time of STD experiment is 2.0 s with a total delay of 4.0 s. The protein was irradiated with a Gaussian-shaped pulse at 0.5 ppm. The length of the selective pulse is 8.5 ms followed by a delay of 4.0 ms. The intensity of the Gaussian pulse corresponds to a strength of 59 Hz. A CPMG T_2 filter of 32.9 ms was used to clean the protein background. The reference spectrum was recorded by irradiating at −9.5 ppm at the same condition. The small numbers are atom ID.

the pyrimidine N1 is protonated, and 1-NH and 2-NH_2 are involved in hydrogen bonds.[8c,e] The X-ray structure shows that TMP binds in the active site of DHFR with its pyrimidine ring and C7 methylene being held in the interior of a deep cleft, while the trimethoxy-benzyl side chain extends out toward the entrance of the binding pocket.[7] In all 12 restrained minimized structures of DHFR-TMP reported by Feeney and co-workers,[8c] three amino acids have intermolecular H−H distances smaller than 3.5 Å to the H6 of TMP. There are two residues within the 3.5 Å range to one of the H7 protons, and parts of another residue are close to another H7. Some DHFR residues are close to other protons of TMP, but the short distances necessary for NOE only show up in 75% of the structures. The ring-flipping of the trimethoxy-benzyl ring of the TMP in the binary and ternary complexes of DHFR-TMP has also been characterized,[8e] suggesting a lack of consistent contact with DHFR at the trimethoxy-benzyl ring.

It is interesting that only weak NOE was observed for the methylene protons in the NMR studies,[8c] although it is in close contact with the protein. The spin−lattice relaxation time T_1 of H7 (0.49 s) is very short when DHFR is present, 3.8 times shorter than that of pyrimidine H6 (1.89 s), and 2.3 times shorter than the average T_1 of other three proton signals. The short T_1 relates to the intramolecular dynamics of the trimethoxy-benzyl ring and makes it hard to measure the related intermolecular NOEs by the standard NOE methods, such as *n*uclear *O*verhauser *e*nhancement *s*pectroscop*y* (NOESY),[9] *r*otating-frame nuclear *O*verhauser *e*nhancement *s*pectroscop*y* (ROESY)[10] or saturation transfer difference (STD).[11] The T_1 relaxation reduces the signal intensities in the diffusion experiments but should not affect the deviation due to the NOE buildup nor the epitope mapping from the diffusion measurement since the diffusion delays are fixed. No correlations were observed between T_1 and the epitope value κ (Supporting Information). The epitope map obtained by the STD[11b] experiment measured with presaturation time of 2.0 s is also presented in Figure 3 (gray italic numbers). It is not surprising that the epitope results from STD and diffusion are in general agreement except for the methylene protons H7 where the difference arises from its very short T_1 relaxation time.

The goal of ligand epitope mapping is to provide detailed information on the binding of ligand to target and to lead the SAR of a potential compound. From the diffusion epitope map, the modification of TMP should be carried out on the trimethoxy-benzyl side chain, since the pyrimidine ring and C7 methylene of TMP are involved in direct contacts to DHFR. This agrees with the early study of receptor-based design of DHFR inhibitors.[12]

The intermolecular NOE builds up during long diffusion periods and creates a deviation from the linear ($\ln I - g^2$) plot, interfering with the diffusion measurement of ligands when protein is present, but this interference can be used to map out the parts of the ligand involved in the protein interaction. The diffusion map of TMP on DHFR is in excellent agreement with the structural and dynamic studies by crystallography and NMR, as well as the medicinal chemistry results. When the off-rate is much faster than cross-relaxation, the epitope map most clearly reflects the ligand's interactions with the target. However, when those rates are comparable, the spin diffusion will equalize the deviations. In the example of Trp and HSA, different κ were obtained for two geminal protons (Supporting Information), suggesting that the cross relaxation is not as fast as the off rate because the epitope values are still distinct. Because the diffusion epitope mapping is not affected by T_1 relaxation, it is useful when ligand protons have different T_1 values. Since a series of 1D diffusion experiments is usually necessary, epitope mapping by diffusion NMR may not be a rapid method. Efforts are ongoing to improve the efficiency of this experiment and avoid the collection of an entire diffusion series.

Supporting Information Available: Additional data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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