Diffusion Edited NMR: Screening Compound Mixtures by Affinity NMR to Detect Binding Ligands to Vancomycin

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Affinity NMR can be used to produce an edited NMR spectrum that identifies ligands that bind to vancomycin from solution mixtures containing nonbinding molecules. The Diffusion EnCODEd Spectroscopy (DECODES) experiment performed directly on the same sample can be used to determine the structure of the binding ligands without the need for a physical separation step. The all-D amino acid tetrapeptides DDFA and DDFS, known ligands for vancomycin, were identified in the presence of eight nonbinding tetrapeptides. The bound-ligand signals in the two-dimensional DECODES spectrum are readily identified by comparison with the spectral patterns of the vancomycin cross-peaks in the 2D total correlation spectroscopy and correlation spectroscopy spectra. The screening of solution mixtures of molecules for direct detection of molecular interactions and structural identification of the interacting ligands provides a powerful new tool to complement methods, such as affinity MS, which rely on the physical separation of mixture components to identify molecular interactions. The solution mixtures of compounds for screening by affinity NMR could come from any source where the components are in similar relative amounts, including synthesis by combinatorial chemistry methods.

Introduction

The glycopeptide vancomycin is an important antibiotic agent for the treatment of bacterial infections, especially streptococcal and staphyloccal infections, which have become resistant to β-lactam antibiotics.[1] The recognition of vancomycin-resistant bacterial strains has spurred renewed interest in this class of antibiotics.[2] Development of additional agents which overcome bacterial resistance will benefit from an understanding of the mode of action of vancomycin and its binding interactions. Vancomycin inhibits bacterial cell wall biosynthesis by specifically binding to a carboxy-terminal D-Ala−D-Ala sequence and preventing the cross-linking of peptidoglycan strands. This binding interaction to D-Ala−D-Ala peptides has been studied extensively.[3] It has been proposed that the development of bacterial resistance is the result of a modification from the carboxy terminal D-Ala−D-Ala sequence to a carboxy-terminal D-Ala−D-Lac sequence which no longer binds vancomycin and so fails to inhibit cross-linking.[4] Recent efforts on the synthesis of vancomycin and related molecules[5,6] may ultimately lead to derivatives which overcome bacterial resistance. The ability to study binding interactions of vancomycin and derivatives will play an important role in seeking to overcome resistant bacterial strains. This has been well recognized, as indicated by the new studies involving vancomycin which have recently appeared, some of which are useful to screen for new molecules with interesting binding interactions.[7]

The vancomycin binding interaction to D-Ala−D-Ala peptides has been well studied by NMR spectroscopy.[8] Described below is a new method to evaluate vancomycin binding interactions using NMR diffusion spectroscopy, which we have called affinity NMR.[9] This method relies on pulsed field gradient (PFG)-NMR to spatially encode molecules in solution. The technique is based on the principle that translational diffusion in solution is size dependent. The diffusion coefficient of a small molecule in solution is altered on a time-averaged basis by complexation with another molecule. If the diffusion coefficient of the combined interacting molecules is different enough from molecules in solution which do not interact, PFG conditions can be found where the more rapidly diffusing molecules are not detected in the NMR spectra. This allows mixtures of compounds in solution to be evaluated simultaneously for interactions with a potential binding partner as only those molecules which

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interact would appear in the edited NMR spectrum. In essence, this is a spectroscopic separation of compounds related to the physical separation of compounds by affinity chromatography.

Affinity NMR can be used to spectroscopically separate ligand molecules that bind from those that do not bind, without the need for any physical separation of compound mixtures. The value of affinity NMR is greatly enhanced because the structure of the ligand molecule can also be determined in the same experiment. Because the translational diffusion coefficient value is an intrinsic property of a molecule as a whole, it can be used to distinguish resonances arising from different molecules.[10] Diffusion EnCODEd Spectroscopy (DECODES), which involves PFG-NMR combined with total correlation spectroscopy (TOCSY), allows the structure of the bound substrate to be directly identified.[11]

Presented here are two studies: a rank-ordering experiment for the interaction of vancomycin with three known binding elements of different binding affinities (the dipeptide D-Ala−D-Ala plus two all-D tetrapeptides, DDFS and DDFA)[12] and a DECODES study of a mixture of 10 tetrapeptides in the presence of vancomycin to determine if affinity NMR can be used to correctly detect ligands with low and high micromolar dissociation constants.

Experimental Section

Materials. Vancomycin was purchased from Sigma and was used without further purification. The D tetrapeptides YPFV, GLGG, GPRP, RGDS, GRGD, RGFF, KDEL, and DASV were purchased from NOVABIOCHEM and were also used without further purification. D_2O, NaOD, and DCl were obtained from ISOTEC, Inc.

Synthesis of the all-D amino acid tetrapeptides DDFA and DDFS. D amino acids were purchased from NOVABIOCHEM. The two tetrapeptides DDFA and DDFS were synthesized on 2-chlorotrityl resin in manual equipment by standard techniques. One equivalent of the corresponding D-Fmoc amino acid and 4 equiv of *N*-methylmorpholine in DMF were added to 2-chlorotrityl chloride resin. The resin suspension was shaken overnight, filtered, and washed with DMF, and Fmoc deprotection was performed by adding 20% piperidine in DMF for 30 min. Three equivalents of the next D amino acid was activated and coupled by adding it and 3.3 equiv TBTU and 3.3 equiv *N*-methylmorpholine in DMF to the resin. Completion of the coupling was monitored using the Kaiser test. The couplings were repeated to prepare the tetrapeptides. The peptides were cleaved from the resin and deprotected using 95% TFA for 2 h. The resulting solution was filtered and reduced to a third of the original volume, and the peptide was precipitated by adding cold diethyl ether. The resulting white powders were pure by HPLC and had the expected mass by ES/MS.

Sample Preparation. Each peptide was weighed and dissolved in D_2O. The pH was adjusted to pD 8 with NaOD and DCl. The samples were then lyophilized, redissolved in D_2O, and used as stock solutions. The peptide mixture was created by mixing each stock solution so that the final concentration of each individual peptide was 1 mM. Vancomycin was prepared by weighing out the appropriate amount of the sample and dissolving it in D_2O and adjusting the pD to 8.2. The ratio of vancomycin to each peptide used was approximately one to one.

NMR Experiments. All NMR experiments were carried out on a Bruker DMX-500 NMR spectrometer equipped with an Acustar II field gradient accessory. 1D PFG-NMR spectra were acquired using LED pulse sequence[10] with 2 ms gradient pulse and 0.2 s diffusion delay time. TOCSY experiments were performed using mlevtp sequence with a mixing time of 75 ms, 2K data points were collected in F2 , and 256 increments were collected in the F1 dimension. The DECODES spectra were acquired with the use of LEDmlevtp sequence using a 75 ms mixing time. A 2K × 512 matrix was formed after zerofilling in the F1 dimension; 256 scans were used for each increment. The PFG conditions used in DECODES were the same as those used in the 1D PFG experiments.[13]

Binding Constants. Binding constants of D-Ala−D-Ala and DDFS with vancomycin were measured by the PFG-NMR technique.[13] The peptide diffusion coefficients were measured in the absence and presence of vancomycin. Because the free and bound ligands are in fast exchange on the NMR time scale in the presence of vancomycin, the observed diffusion coefficients are the weighted average of the free and bound species. Assuming that the diffusion coefficient of the ligand bound to vancomycin is the same as that of vancomycin alone, the fraction of bound ligand can be calculated as follows:

$$D_{obs} = F_{free}D_{free} + F_{bound}D_{bound}$$

where D_{obs} is the observed diffusion coefficient and D_{free} and D_{bound} are the diffusion coefficients of free and bound peptides, respectively.

Results

Binding Strength of D-Ala−D-Ala, DDFS, and DDFA. The binding site of vancomycin and its interactions with C-terminal D-Ala−D-Ala derivatives (R−D-Ala−D-Ala, where R can be as simple as acetyl) has been extensively studied by NMR spectroscopy.[8] We chose to study the unsubstituted D-Ala−D-Ala dipeptide, which binds more weakly to vancomycin than derivatized C-terminal D-Ala−D-Ala peptides and gives us a wider range of dissociation constants for evaluation.

In the presence of vancomycin and an excess of D-Ala−D-Ala, no separate signals were observed for the free and bound peptide, indicating fast exchange of free and bound D-Ala−D-Ala on the NMR time scale and allowing the binding constants of this dipeptide to be measured by PFG-NMR. The diffusion coefficients of vancomycin and D-Ala−D-Ala were each determined in aqueous solution at 300 K and found to be $(3.64 \pm 0.08) \times 10^{-6}$ cm^2/s and $(8.73 \pm 0.1) \times 10^{-6}$ cm^2/s, respectively. In the presence of vancomycin, the observed diffusion coefficient of D-Ala−D-Ala is $(6.06 \pm 0.05) \times 10^{-6}$ cm^2/s, and the diffusion coefficient of the vancomycin in the same solution remains the same, within experimental error, as that measured in the absence of D-Ala−D-Ala, $(3.61 \pm 0.07) \times 10^{-6}$ cm^2/s. According to these results, the dissociation constant for D-Ala−D-Ala was estimated as 1 mM from the fraction of bound calculated using the above equation. In the same manner, the binding of DDFS with vancomycin was also measured and gave a dissociation constant of 200 μM. DDFA, in contrast, interacts more strongly under the conditions used here

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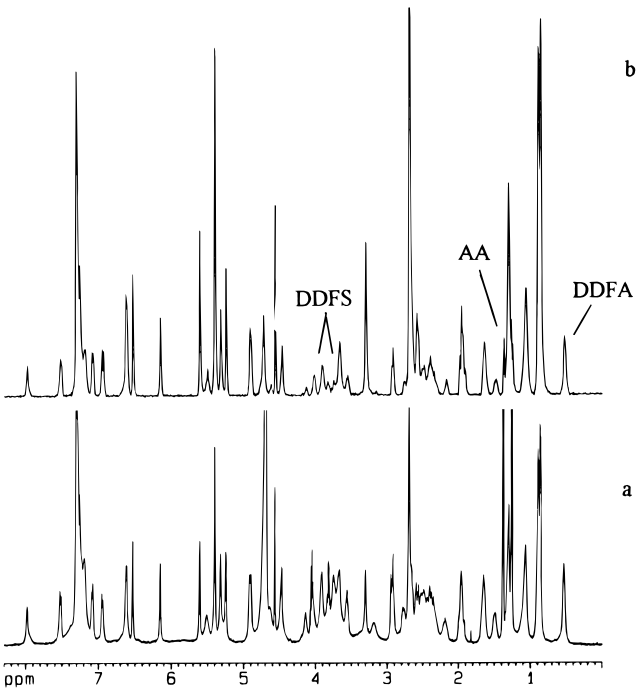

Figure 1. Comparison of the ^{1}H NMR spectrum for DDFA, DDFS, and D-Ala−D-Ala in the presence of vancomycin: (a) normal and (b) with PFG indicating key resonance positions where resonances for DDFA, DDFS, and AA appear.

and shows separate NMR signals for free and bound ligand, so the binding constant can be directly calculated to be 7 μM. This is consistent with the previously reported 6.8 μM for DDFA and with the rank order for DDFA and DDFS.[12]

Affinity NMR. The affinity NMR experiment was first conducted on a simple mixture of vancomycin with three peptides, DDFA, DDFS, and D-Ala−D-Ala, which exhibit different binding affinities from micromolar to millimolar, as determined above. To perform the affinity NMR experiment, the PFG conditions at which no NMR signals are observed for the three-peptide mixture in the absence of vancomycin were first determined. This is a requirement of the affinity NMR experiment because these same PFG conditions will be used on the mixture after the addition of vancomycin. Approximately 1 equiv of vancomycin was then added to this peptide mixture, and a PFG-NMR spectrum was obtained under the same conditions as determined for the peptides alone. Figure 1 shows a comparison of a normal ^{1}H NMR spectrum of this peptide/vancomycin mixture with that obtained using PFG-NMR. In the 1D ^{1}H NMR spectrum, Figure 1a, the methyl group of the bound DDFA can be readily observed at 0.53 ppm. The resonances of the alanine methyl groups of D-Ala−D-Ala are also readily observed in the normal 1D spectrum at 1.26 and 1.38 ppm, respectively. In contrast to the methyl resonance of DDFA, the two methyl signals of D-Ala−D-Ala are very sharp and exhibit no significant chemical shift changes upon the addition of vancomycin, giving the first indication that there is a difference in binding affinity for these peptides.

In the PFG-NMR spectrum, Figure 1b, the intensities of these sharp methyl signals of the D-Ala−D-Ala dipeptide are dramatically decreased as compared to the signals of the other two components in the mixture. This reduction in signal intensity indicates that D-Ala−D-Ala has a substantially faster diffusion coefficient than the other peptides.

A better comparison of the binding affinity of these three peptides with vancomycin can be obtained if we extend the 1D PFG-NMR spectrum into two dimensions. To better observe the individual peptides, we obtained a 2D DECODES spectrum of this peptide mixture, shown in Figure 2a. The PFG conditions used in the DECODES spectrum were the same as those used in the 1D PFG experiment shown in Figure 1. A non-diffusion-weighted TOCSY spectrum is shown for comparison, Figure 2b. In the DECODES spectrum, the cross-peaks of vancomycin and DDFA can be easily identified by the chemical shifts of the alanine methyl/α proton cross-peaks at $\delta = 0.53$ and 3.92. In addition, the cross-peaks arising from DDFS are observed at $\delta = 2.15$, 3.90 and 3.75, 4.1. The cross-peaks of the D-Ala−D-Ala dipeptide can be found, albeit at a much weaker intensity. The expanded region of the DECODES spectrum is seen in Figure 3. The intensities of the cross-peaks are found to be concordant with the relative binding strengths of these three peptides to vancomycin, indicating that rank-order binding can be determined using the DECODES method. This is consistent with similar rank ordering results reported previously using affinity NMR on a system of nonpeptide molecules binding to a small molecule receptor.[9b]

To extend the affinity NMR technique to a larger and more complicated mixture, we conducted a similar experiment on a mixture of 10 tetrapeptides (DDFA, YPFV, GLGG, GPRP, RGDS, GRGD, RGFF, KDEL, DASV, and DDFS), which included the two studied above plus eight additional tetrapeptides that contain the same amino acids. The normal proton spectrum of this mixture in the presence of vancomycin is shown in Figure 4a. Figure 4b shows the 1D PFG-NMR spectrum, acquired under the appropriate PFG conditions determined as above, whereby the signals for the nonbinding ligands are edited from the spectrum. As was the case for the three-peptide mixture, an alanine methyl group resonance can be easily observed at 0.53 ppm, suggesting DDFA or DASV is binding to vancomycin. Most of the signals from the other tetrapeptides have disappeared from the spectrum. However, since the spectrum is still complex it is not easy to evaluate which of the other peptides show binding affinity.

A subtraction method recently described by Fesik et al.[14] that could potentially solve this problem does not work in our hands for this mixture because there are significant chemical shift changes in both the vancomycin and the peptides upon mixing, which result in significant subtraction artifacts.

A non-PFG edited TOCSY spectrum of this 10-peptide mixture in the presence of 1 equiv of vancomycin was obtained and is shown in Figure 5a. The DECODES spectrum of the mixture in Figure 5b contains considerably fewer peaks. The vancomycin signals can be clearly identified in the DECODES spectrum and the cross-peak of $\alpha CH-CH_3$ from alanine of DDFA or DASV can be readily identified at 0.53 ppm. The cross-peaks of aspartic acid and phenylalanine can also be observed in the spectrum, a result similar to that obtained for the simple three-peptide mixture. In addition, we also observed the cross-peaks indicative of a serine residue.

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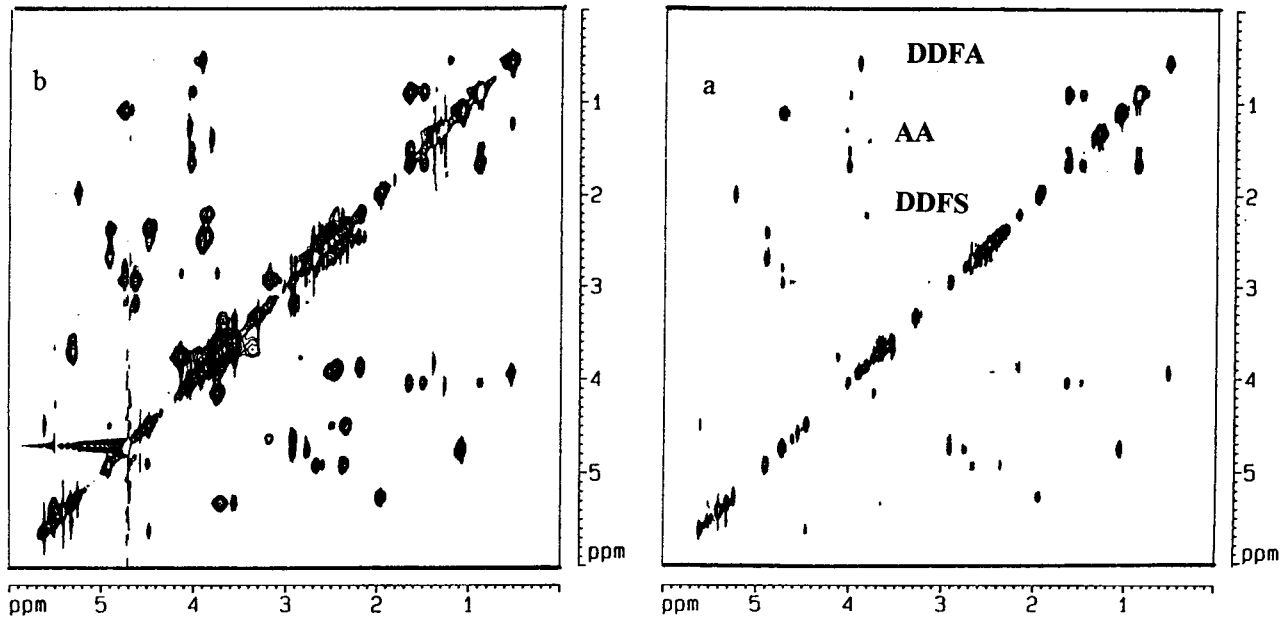

Figure 2. Comparison of the (a) DECODES spectrum with (b) a normal TOCSY spectrum for peptides DDFA, DDFS, and D-Ala–D-Ala.

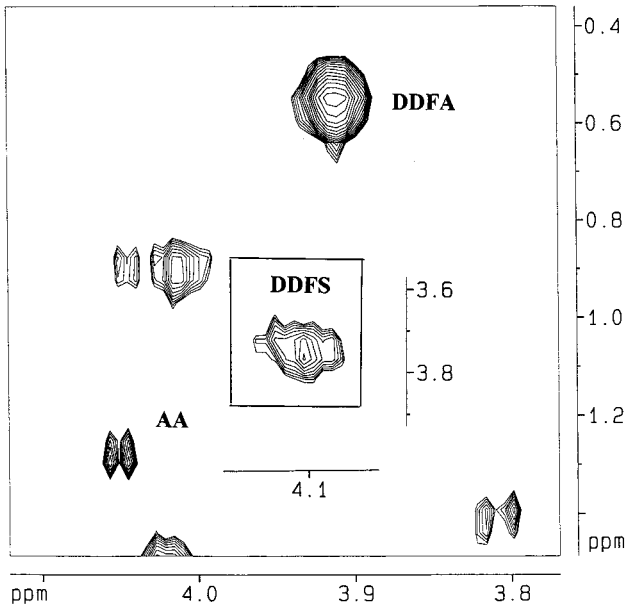

Figure 3. Expanded region of the DECODES spectrum for peptides DDFA and D-Ala–D-Ala. The insert contains the cross-peak for DDFS. The intensities of the cross-peaks indicate the relative binding affinities: DDFA > DDFS > AA.

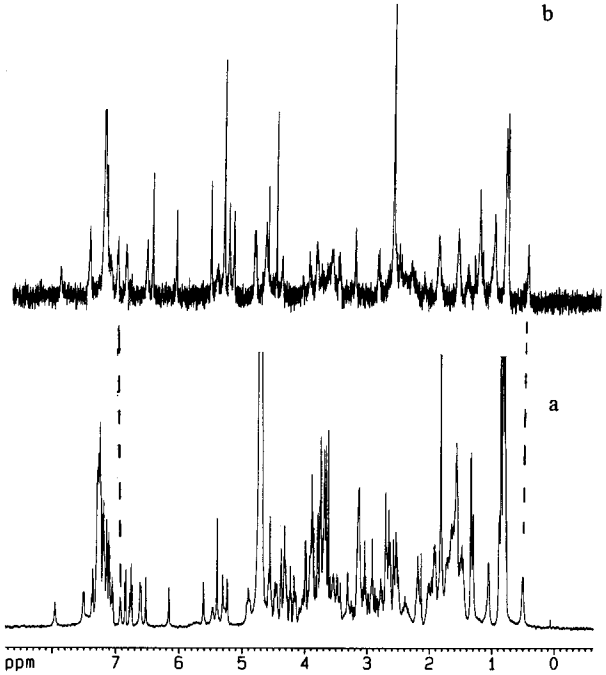

Figure 4. (a) The normal proton spectrum of the mixture of 10 tetrapeptides (DDFA, YPFV, GLGG, GPRP, RGDS, GRGD, RGFF, KDEL, DASV, and DDFS) in the presence of vancomycin. (b) The PFG proton spectrum of the same mixture of 10 tetrapeptides in the presence of vancomycin. The decrease in signal intensity is reflective of the gradient field strength and the loss of signal due to diffusion editing.

In this 2D NMR spectrum, signals arising from amino acids L, R, K, P, and G are not observed. This rules out the tetrapeptides RGDS, GRGD, KDEL, YPFV, GPRP, RGFF, and GLGG as peptides that bind to vancomycin which leaves DDFA, DDFS, and DASV as candidate structures which bind. Because there are two candidates, DDFA and DASV, containing an Ala residue, the observed NMR signals do not allow a conclusion as to which one or if both of these two peptides are binding. However, we did not observe the valine methyl signals in the DECODES experiment, making it unlikely that DASV was binding. In a separate experiment to confirm this conclusion, a mixture of DASV and vancomycin was prepared and the 1D spectrum of this mixture did not show the upfield alanine methyl resonance at 0.53 ppm observed in the mixture, which eliminates DASV as a candidate structure for binding. In conclusion, DDFS and DDFA were successfully detected as binding ligands to vancomycin from a solution mixture of 10 tetrapeptides using affinity NMR, and the structures could be deduced despite the closely related tetrapeptides in the mixture.

Discussion

The goal of affinity NMR is to detect and identify the structure of ligands which have binding affinity to a

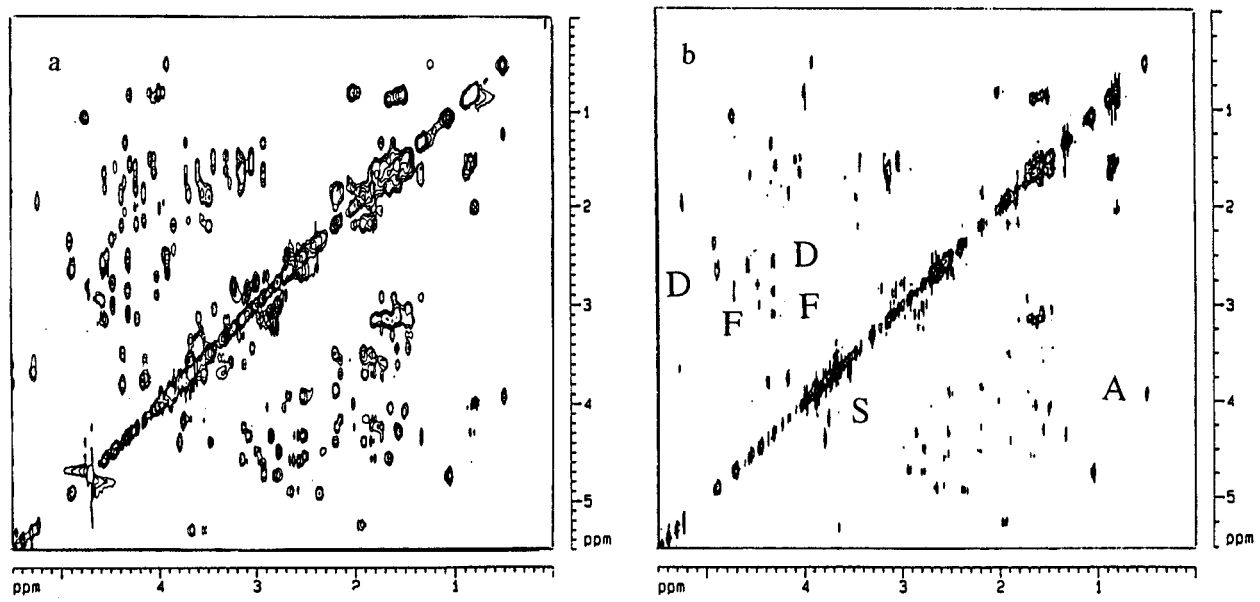

Figure 5. (a) TOCSY NMR spectrum for the peptide mixture DDFA, YPFV, GLGG, GPRP, RGDS, GRGD, RGFF, KDEL, DASV, and DDFS in the presence of vancomycin. (b) The corresponding DECODES spectrum noting the amino acids that remain, D, F, S, and A.

receptor. Usually the ligands are small molecules, whereas the receptors are much larger. This size difference determines the translational diffusion difference in solution. Although small molecules generally diffuse faster than large molecules, once a small molecule binds to a larger one the translational diffusion of the small molecule appears altered on a time-averaged basis. The stronger the binding, the greater the difference in apparent diffusion of the ligand in solution.

We previously introduced affinity NMR using a small molecule ligand−receptor system.[9] In this paper, we have selected the important antibiotic vancomycin as a biologically relevant receptor and studied the binding of a solution mixture of tetrapeptides as ligands. Here we challenge the limits of affinity NMR by (1) selecting a receptor which is only three times larger than the ligands (vancomycin is a medium-size molecule with a molecular weight of 1475, whereas the typical molecular weight of a tetrapeptide is around 500), which minimizes the differences in diffusion; (2) selecting as the ligand mixture 10 tetrapeptides all containing amino acids present in multiple examples, which results in a complex case for structure identification; and (3) selecting both low micromolar and high micromolar binding examples in the same mixture to maximize the editing challenge. The relatively small size differences between the ligands and vancomycin result in diffusion coefficients that are also not very different and limit the choice of the gradient strength for PFG to edit out nonbinders. This is especially true for ligands that have relatively weak binding affinities like DDFS. The difference in binding affinity to vancomycin between DDFA and DDFS is about 30-fold, so under the experimental conditions where the signals for vancomycin are still observed, the NMR signals arising from DDFS cannot be completely eliminated from the DDFA spectrum and complicate the DECODES spectrum

Under particular experimental conditions where the concentration of one component is much greater than all others, the resonance of that component may still be observed under editing conditions. This is especially true when the component has sharp, strong signals as is the case for the D-Ala−D-Ala peptide. In this case, the observation of the NMR signal may not be due to binding affinity but due to the experimental limitations of the gradient field strength typically available. In this instance, a comparison of the 1D PFG spectrum with weak and strong gradients can be very useful. By comparing the PFG-NMR spectrum with the strong gradient to that with weaker gradient strength, the rate of signal decrease can be estimated. Those signals that decrease at the same rate as the receptor, in our case vancomycin, come from the ligands that have strong binding affinity. Those resonances in which signal intensity is decreased more rapidly than vancomycin signals belong to ligands that have low affinity. When the receptor is much larger than the ligand, it is easier to choose the PFG conditions under which the nonbinding ligand signals will not be observed. It can be expected that application of affinity NMR to larger protein receptors, especially when screening for high affinity small molecule binding, will not suffer the same difficulties seen herein.

Despite the challenges presented by this system, we have shown that affinity NMR can be used to produce an edited NMR spectrum that identifies binding ligands to vancomycin from solution mixtures of 10 tetrapeptides containing nonbinding molecules. The DECODES experiment performed directly on the same sample can be used to determine the structure of the binding ligands without the need for a physical separation step. Within the sensitivity limitations of any NMR-based method, the screening of solution mixtures of molecules for direct detection of molecular interactions and structural identification of the interacting ligands provides a powerful new tool to complement methods, such as affinity MS, which rely on physical separation of mixture components to identify molecular interactions.

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