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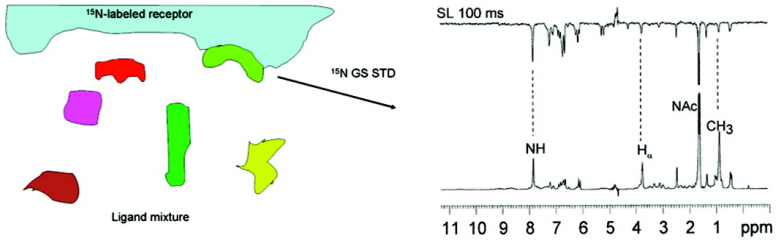

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Molecular Recognition and Screening Using a ^{15}N Group Selective STD NMR Method

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Abstract: We present a novel saturation transfer difference (STD) experiment where group selective (GS) saturation of amide protons in ^{15}N labeled hosts is achieved. It is demonstrated that a train of BIRD^d pulses that inverts only protons attached to ^{15}N indeed results in saturation of the amide protons, while the background proton magnetization is much less affected. The undesired effect of partial saturation of the unlabeled protons can be completely cancelled out in difference spectra by switching the ^{15}N carrier between the on- and the off-resonance frequencies. As a result, clean and artifact-free STD spectra are obtained without the need of time-consuming optimization of experimental parameters and acquiring control spectra in the absence of the host. The use of the ^{15}N-GS STD experiment is demonstrated for the case of a glycopeptide antibiotic (dimeric eremomycin)−cell-wall analogue peptide (N−Ac−D-Ala) model system where the host and guest ^1H signals overlap. The application seems feasible for ligand screening against proteins without the prerequisite of a clean on-resonance frequency or defined ligand library. The new experiment can be used as the basis for studying intermolecular interactions where the standard STD experiment is difficult to optimize.

Introduction

Molecular recognition is at the heart of key biomedical and biological events. Thus, detecting interactions between molecules is of paramount importance for drug development. From the chemical, and particularly from a NMR viewpoint, saturation transfer difference spectroscopy (STD) is the key method to identify and characterize ligand−receptor interactions.[1,2] STD experiments generally start by a few seconds of semiselective irradiation of protons in the receptor whose magnetization is then transferred to ligand protons by NOE and spin-diffusion through a relay of close contacts. Comparison to a reference spectrum reveals which ligand resonances make intimate contacts with the receptor. Not surprisingly, the STD technique has found widespread applications in carbohydrate−protein recognition phenomena.[3,4] The STD signals are typically in the 0−10% range when compared to reference intensities.[5] As nonbinding ligands provide no STD signals, STD is used as a screening technology to identify small molecules whose moieties can be incorporated into rationally designed drugs.[6] The semiselective irradiation of the receptor protein is usually performed at ca. −1 or −2 ppm[7] (or at ca. 7 ppm, provided that the potential ligands do not have aromatic protons). However, for many polypeptide systems, especially those including unfolded, partly folded, or molten globule proteins, the perturbation at −2 ppm might be very inefficiently transferred along the polypeptide chain, thus compromising the success of the experiment. Moreover, ligand peptides might also show signals in this spectral region. In addition, when STD is applied to the screening of defined combinatorial libraries with unknown NMR properties, such as heterogeneous, undefined samples (e.g., plant or cell extracts), it can produce high incidences of false positive STD signals, due to the presence of ligand signals along many different frequencies. STD screening frequently utilizes large volumes of low-concentration recombinant protein, for which cost-effective ^{15}N labeling is often possible, and thus, the method presented herein may be of general use, even when no assignment of the $^1H-^{15}N$ HSQC spectrum has been performed yet. The amide region of the proteins can overlap with aromatic and amide signals from small molecules whose ^1H signals should not be subjected to any irradiation. However, the ligand will contain a 0.36% natural abundance of ^{15}N, and this could result in artifacts, particularly

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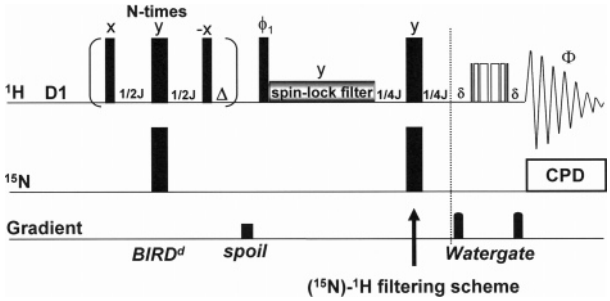

Figure 1. Pulse sequence of the ^{15}N-GS STD experiment. Hard 90 and 180° pulses are marked by solid bars. $T_{1\rho}$ relaxation filter SL pulse is applied at reduced power, ca. 70−80 μs duration for ^{1}H 90° pulse. BIRD^d delay is set to $1/(2J_{NH})$, and the phase setting of ^{1}H pulses as indicated leads to inversion of ^{1}H−^{15}N while non-labeled protons are left unaffected. Duration of the interpulse delay, Δ, within the BIRD^d saturation pulse train varies between 100 and 300 ms, depending upon the size of the host molecule. After the relaxation filter, a heteronuclear spin−echo sequence was included to create antiphase ^{1}H−^{15}N magnetization, which can be eliminated with ^{15}N decoupling during acquisition. For solvent suppression, a gradient enhanced Watergate sequence is applied. Between on- and off-resonance experiments, only the ^{15}N carrier frequency is changed. Phase cycling: $\phi_1 = x, -x$ and $\Phi = x, -x$ for on-resonance and $\Phi = -x, x$ for off-resonance, respectively. After four transients, the phases of all pulses subsequent to the saturation train as well as that of the receiver are incremented by 90°. Sine bell shaped gradients of 2 ms duration were used, followed by a recovery delay of 200 μs. Gradient strengths for purging: 37% and for Watergate: 20%, given as a percentage of the absolute gradient strength of 50 G/cm.

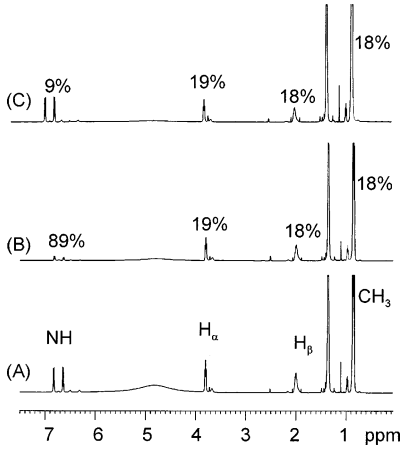

Figure 2. Saturation efficiency achieved with BIRD^d pulse train demonstrated on ^{15}N labeled L-Val. (A) ^{1}H NMR spectrum recorded without ^{15}N decoupling during acquisition. (B) ^{1}H−^{15}N saturation with BIRD^d pulse train, where $D1 = 4$ s, $\Delta = 240$ ms, $N = 30$, and number of scans is 8. ^{15}N carrier frequency is set to amide ^{15}N. Numbers in percentages indicate the efficiency of saturation of ^{1}H−^{15}N and partial saturation of background ^{1}H magnetization, respectively. (C) Experimental setup is the same as in spectrum B, except that the ^{15}N carrier frequency is offset. Note that the partial saturation of background ^{1}H magnetization remains the same as in spectrum B; correspondingly, this undesired effect is cancelled out in the ^{15}N-GS STD difference experiment.

if present in 100-fold excess over the receptor concentration. These potential artifacts will be reduced if ligand HN proton exchange with bulk solvent is fast as compared to the duration of the group selective (GS) saturation pulse train and because magnetization transfer within a small molecule is less efficient. Therefore, we decided to design and test a ^{15}N-GS STD NMR experiment that could achieve the exclusive excitation of receptor amide protons in the presence of an undefined, and even heterogeneous, ligand library. We think that this kind of multiple site saturation can compensate for sensitivity losses due to partial saturation and leakage inherent with this technique and can potentially disclose binding sites that are hidden to selective methods.

Results and Discussion

The scheme of the proposed ^{15}N-GS STD difference experiment is depicted in Figure 1. GS saturation of the amide protons attached to ^{15}N is achieved by a BIRD^d pulse train. The BIRD^d pulse inverts the ^{1}H−^{15}N magnetization, while the background proton magnetization is left unaffected. As a result, the BIRD^d pulse train with a Δ interpulse delay will efficiently saturate the ^{1}H−^{15}N magnetization and cause only a partial saturation of the non-labeled proton magnetization, due to pulse imperfections and incomplete relaxation. It is important to note that the effect of this partial saturation is entirely eliminated in the difference experiment, yielding a clean and artifact-free saturation transfer difference spectrum. The perfect suppression of any undesired signals is based on the following: the on- and off-resonance experiments are accomplished with exactly the same pulse sequence, as shown in Figure 1. Only the ^{15}N carrier frequency is switched between on-resonance (center of amide ^{15}N region) and off-resonance frequencies for the corresponding experiment. Since the proton carrier frequency remains the same throughout the measurement, the background proton magnetization is affected by the same pulse train in both the on- and the off-resonance experiment, thus resulting in its perfect elimination in the difference spectrum. The signals that appear in the difference spectrum belong to the BIRD^d saturated ^{1}H−^{15}N resonances of the host molecule (e.g., protein and glycopeptide) and to those resonances of the host, as well as to those ligand molecules. These are affected by spin-diffusion or saturation transfer during the saturation BIRD^d pulse train of a typically 2−4 s duration. The signals of the host molecule can be removed, or at least significantly reduced, by applying a $T_{1\rho}$ relaxation filter after the 90° read pulse, as indicated in the pulse scheme of Figure 1.[8] In the case of relatively small hosts−such as dimeric eremomycin, ∼3 kDa in our example−the suppression of the ^{1}H−^{15}N magnetization of the host molecule can be further enhanced with the use of a heteronuclear spin−echo sequence of $1/2J_{NH}$ duration and applying ^{15}N decoupling during acquisition to cancel out ^{1}H−^{15}N antiphase proton signals. As a result, in the case of an efficient saturation transfer mechanism, the ^{15}N-GS STD ^{1}H difference spectrum contains the ligand STD signals, and some attenuated signals of the host molecule, which pass the filters due to inefficient relaxation.

The performance of the BIRD^d saturation pulse train was tested on a simple model compound, ^{15}N labeled Boc-protected amino acid, (^{15}N)-N^α-*t*-Boc-L-valine. The efficiency of saturation of the ^{1}H−^{15}N magnetization was ca. 89% under the experimental conditions given in the figure legend of Figure 2. Note that the non-labeled H_α and side chain protons were also affected ca. 18−19% due to pulse imperfections. However, as mentioned previously, this undesired effect is perfectly cancelled out in the difference experiment. This fact is verified by the spectrum of Figure 2C, which was recorded under the same experimental conditions as Figure 2B except that the ^{15}N irradiation frequency was set away. The partial saturation of the background proton magnetization was not affected by the ^{15}N frequency switch.

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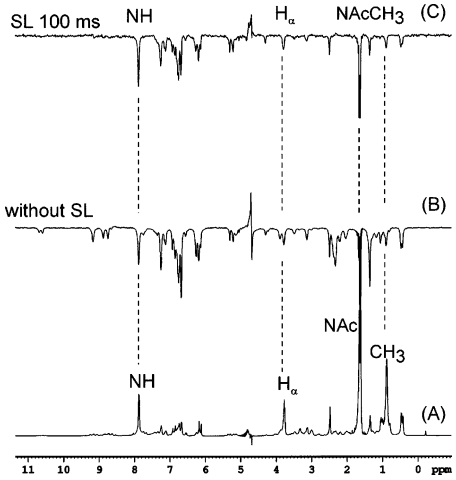
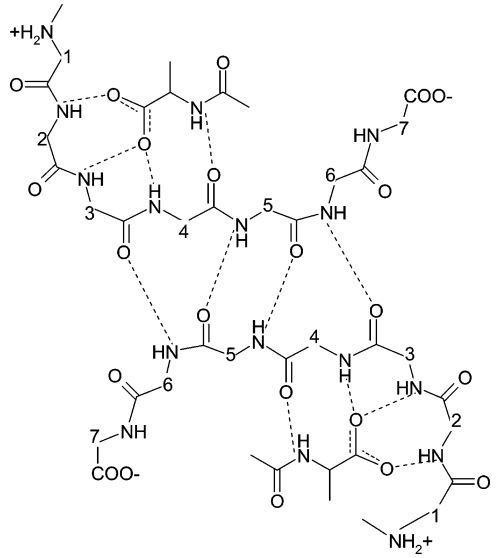


Figure 3. ^{15}N-GS STD spectra of eremomycin and N−Ac−D-Ala complex recorded with the pulse sequence of Figure 1. (A) Watergate spectrum of the host−guest mixture. (B) ^{15}N-GS STD spectrum recorded in the absence of the SL relaxation filter. Other parameters were set as follows: $D1 = 5$ s, $\Delta = 300$ ms, $N = 8$, number of transients 2×1440 for the difference spectrum. (C) ^{15}N-GS STD spectrum recorded with a 100 ms relaxation filter otherwise using the same experimental parameters as in spectrum B.

Scheme 1. Hydrogen-Bond Network (Dotted Lines) Formed by the Dimeric Eremomycin N−Ac−D-Ala Complex



Finally, a well-described model system was used to prove the utility of the ^{15}N-GS STD experiment. We were able to highlight the intermolecular interactions within the ^{15}N labeled (ca. 60% abundance of ^{15}N) dimeric eremomycin and N−Ac−D-Ala cell-wall analogue peptide complex (Scheme 1).[9−11] A comparison with the traditional STD experiment[1] concerning the sensitivity/insensitivity upon the applied experimental conditions is also discussed. The ^{1}H Watergate and ^{15}N-GS STD spectra of the eremomycin and N−Ac−D-Ala mixture (in ca. 1:6 molar ratio) are shown in Figure 3. The GS STD spectra, Figure 3B,C, were acquired with the pulse sequence of Figure 1, without and with using a spin-lock (SL) relaxation filter pulse, respectively. The ligand signals appearing in the difference

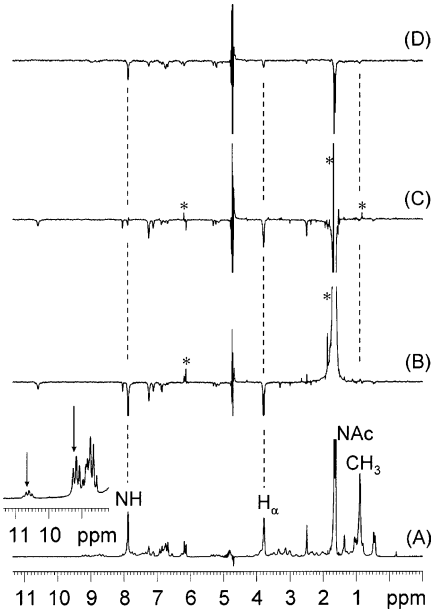


Figure 4. ^{1}H STD spectra of eremomycin and N−Ac−D-Ala complex recorded with the standard pulse sequence. (A) ^{1}H Watergate spectrum: irradiation frequencies, 9.1 and 10.6 ppm, respectively, are indicated by arrows in the inset. (B) STD spectrum obtained with on-resonance irradiation at 10.6 ppm. Train of Gaussian pulses of 50 ms applied for 2.4 s was used for selective saturation. $D1 = 5$ s, $\Delta = 100$ ms, and number of transients 2×800 were used for the difference spectrum. For the off-resonance experiment, the proton carrier frequency was set to −29 ppm. (C) STD spectrum obtained with on-resonance irradiation at 10.6 ppm and off-resonance irradiation at −30 ppm, respectively. Otherwise, the experimental condition was applied as stated previously. (D) STD spectrum obtained with on-resonance irradiation at 9.1 ppm and off-resonance irradiation at −29 ppm.

spectrum−such as NH, H_α, N−Ac, and CH_3 as indicated by the dotted lines−unambiguously confirm the intermolecular interaction with the eremomycin host. In Figure 3C, the host signals are significantly attenuated with the use of a SL filter, rendering the assignment of ligand signals even more straightforward. In our case, the use of a high host concentration (∼4.1 mM for the dimer) did not allow the perfect elimination of all host signals. Under these conditions, the additional use of an even longer SL pulse (>100 ms) would further diminish the ligand signals, compromising the aim of the experiment.

When the cell-wall analogue N−Ac−D-Ala sits in the carboxylate anion binding pocket, the nearby amide protons of eremomycin shift to a clean low-field spectral region, which allows the acquisition of control, semiselective STD experiments without affecting the ligand resonances. (Note that in our model, the ligand binding affinity is ca. 0.5−1 mM, while the host dimerizes with a submicromolar affinity. Under our experimental conditions, more than 95% of the host is bound to the ligand.) For comparison, conventional STD experiments were undertaken separately with irradiation at two of the low-field NH frequencies: 9.1 (3-NH) and 10.6 (2-NH) ppm. The difference spectra are depicted in Figure 4B,D, respectively. The artifacts due to accidental excitation with the DANTE[12] side-bands of the Gaussian pulse train and spillover[13] are marked by asterisks. Again, a conventional selective STD spectrum of Figure 4C was recorded with a slightly different off-resonance reference. Upon comparison of the spectra in panels B−D of Figure 4, it

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is apparent that the intensities and phase properties of the artifact signals were strongly affected by the position of the on- and off-resonance frequencies in every regular STD experiment but not in the experiment described herein (Figure 3).

Conclusion

The advantages of the proposed ^{15}N-GS STD difference experiment are as follows: (1) it is potentially applicable to the study of intermolecular interactions of all types of biomolecular systems exhibiting STD effects (provided that isotope labeling is included), such as glycopeptide antibiotic−cell-wall analogue peptide complexes and others in which the ligand and protein or glycopeptide proton signals overlap, leaving no appropriate spectral region for selective saturation of the host proton resonances. We have also recently applied the sequence to a protein−peptide complex.[14] (2) From a technical viewpoint, in contrast to the conventional ^{1}H STD difference experiment, the proposed experiment does not require the recording of a control spectrum on a sample containing the ligand alone. Here, due to the ^{15}N frequency jump between the on- and the off-resonance experiments with a constant proton carrier frequency, any undesired partial saturation is canceled out in the difference spectrum. (3) A further technical advantage of the ^{15}N frequency switching is that careful checking and time-consuming optimization of the on- and off-resonance frequencies−to avoid partial saturation[13] of ligand resonances with the DANTE[12] side-bands of irradiation pulse train−becomes needless. (4) The duration of the interpulse delay between consecutive BIRD^{d} saturation pulse trains may be varied between 100 and 300 ms depending upon the size of the host. Note that, to minimize the partial saturation of ligand resonances due to imperfect BIRD^{d} pulses, longer delays are preferred to allow for relaxation recovery of ligand proton magnetization. (5) The present study with the eremomycin dimer (3 kDa) demonstrates that it is possible to observe ligand binding to small receptors by STD. Furthermore, since the global correlation time of our dimer was found to be 4 ns at 278 K, the method is probably transferable at least to small proteins. However, to increase the sensitivity, we recommend checking the saturation efficiency−this is fast and simple, just omitting the $T_{1\rho}$ relaxation and ^{15}N−^{1}H filtering blocks as well as the difference part of the experiment−before running the complete experiments. For example, in our case, the saturation efficiency was increased from 30 to 80% when the interpulse delay was increased from 100 to 300 ms between repeated BIRD trains.

To summarize, the ^{15}N-GS STD experiment and concept described herein is a robust experiment that can be applied to a variety of interacting molecules that also displays fewer artifacts than the standard ^{1}H selective STD experiments and will be especially useful (and unique) for screening heterogeneous ligand libraries that exhibit overlapped ^{1}H spectra.

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Materials and Methods

Samples. ^{15}N labeled N^{α}-*t*-Boc-L-valine was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA) and used without further purification. A total of 100 mg of sample was dissolved in 0.5 mL of DMSO-d_6. The sample of ^{15}N labeled (60%) eremomycin[10] and N−Ac−D-Ala was prepared by dissolving 6.5 and 3.3 mg, respectively, in 0.5 mL of a H_2O/D_2O (9:1) solvent mixture.

NMR Spectroscopy. All NMR experiments were performed on a Bruker Avance DRX 500 spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a 5 mm *z*-gradient multinuclear proton detected (bbi) probehead. All spectra were processed with XWINNMR 2.6. The duration of the ^{1}H 90° pulse was 12.5 μs and that of the ^{15}N 90° pulse was 23 μs. The WALTZ-16 scheme applied for ^{15}N decoupling was used at reduced power (i.e., the 90° pulse duration for nitrogen was 200 μs).

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