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Article

Enhanced signal dispersion in saturation transfer difference experiments by conversion to a 1D-STD-homodecoupled spectrum

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Abstract

The saturation transfer difference (STD) experiment is a rich source of information on topological aspects of ligand binding to a receptor. The epitope mapping is based on a magnetization transfer after signal saturation from the receptor to the ligand, where interproton distances permit this process. Signal overlap in the STD spectrum can cause difficulties to correctly assign and/or quantitate the measured enhancements. To address this issue we report here a modified version of the routine experiment and a processing scheme that provides a 1D-STD homodecoupled spectrum (i.e. an experiment in which all STD signals appear as singlets) with line widths similar to those in original STD spectrum. These refinements contribute to alleviate problems of signal overlap. The experiment is based on 2D-J-resolved spectroscopy, one of the fastest 2D experiments under conventional data sampling in the indirect dimension, and provides excellent sensitivity, a key factor for the difference experiments.

Abbreviations: J-resolved – J-resolved spectroscopy; STD – saturation transfer difference; wg – watergate.

Introduction

Current developments in NMR techniques are aimed to provide and refine tools for drug discovery that allow to probe specificity, affinity and structural aspects of receptor–ligand interaction (Meyer and Peters, 2003; Peng et al., 2004). Among the NMR methods suitable for analyzing ligand binding such as transferred NOE (trNOE) (Meyer et al., 1997; Mayer and Meyer, 2000), inter-ligand NOE (Li et al., 1999; London, 1999; Li et al. 2001), diffusion (Lin and Shapiro, 1996, Lin et al., 1997, Yan et al. 2002), relaxation (Hadjuk et al., 1997, LaPlante et al., 2000), NOE pumping (Chen and Shapiro, 1998, 2000), quantification of the binding affinity by ligand release in competition experiments (Jahnke et al., 2002; Siriwardena et al., 2002) and water-LOGSY (Dalvit et al., 2000,2002). The elegant Saturation Transfer

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Difference (STD) experiment has attained notable popularity (Mayer and Meyer, 1999). This experimental approach is based on magnetization transfer from a receptor after signal saturation to ligand protons in spatial vicinity. Acquisition of on- and off-resonance spectra with subsequent subtraction generates difference spectra that contain only signals of the ligand which are indicative of the contact area. There are several advantages which make the STD experiment attractive (1) it can be run without any labelling or immobilization of either the ligand or the receptor, (2) the size of the receptor does not become a factor limiting applicability, (3) it can be performed in experimental designs close to the physiological conditions using living cells (Claasen et al., 2005; Mari et al., 2005), (4) it can be used as a fast NMR screening method to pinpoint reactive compounds in a mixture (Mayer and Meyer, 1999; Klein et al., 1999), (5) it can define the critical epitope, that is, the portions of the ligand in close contact with the receptor (Mayer and Meyer, 2001), and (6) it can even provide information of the conformation of the bound ligand in the complex if strategically combined with modeling (Siebert et al., 2003; Hajduk et al., 2004; Di Micco et al., 2005; Yuen et al., 2005). A serious limitation is posed by signal overlap. To address this issue, this study has been performed.

Signal overlap in conventional 1D-STD spectra can preclude assignment or quantification of the enhancements. Therefore, modifications of the basic protocol of STD experiments such as 2D-STD-TOCSY (Mayer and Meyer, 1999; Vogtherr and Peters, 2000; Möller et al., 2002) or the selective version 1D-STD-selective TOCSY (Johnson and Pinto, 2002) have already been proposed. We report here a new approach for this type of NMR experiment and its protocol. Based on the emerging significance of protein-carbohydrate interactions for drug design and the regulation of various cellular activities we test our technique with an animal lectin (Reuter and Gabius, 1999; Gabius et al., 2004; Gabius, 2006). The introduced methodological modification enabled us to obtain a 1D-STD Homodecoupled spectrum (i.e. an experiment in which all STD signals appear as singlets) with line widths similar to the original STD spectrum. Our results suggest that the reported protocol adds a new option to deal with signal overlap in STD analysis.

Materials and methods

All spectra were acquired at 15°C on a Varian INOVA 750 MHz spectrometer equipped with a triple gradient shielded probe and processed with MestRe-C v 3.x software (Cobas and Sardina, 2004) (*Mestrelab research*). The test samples containing 12 mg of lactose and 3 mg of chicken galectin CG-14 was prepared in 600 µl $NaCl/P_i$ (buffer concentration 20 mM, H_2O/D_2O = 9:1, 0.05 % NaN_3) at pH 7.0. The ligand to protein ratio was about ~200:1. Furthermore, the pulse sequence was also successfully tested using a second ligand, a complex tetrasaccharide:methyl 4-O-(3-O-(2-acetamido-2-deoxy-4-O-β-D-galactopyranosyl-β-D-glucopyranosyl)-β-*D*-galactopyranosyl)-β-D-glucopyranoside. The molar ratio tetrasaccharide:lectin was also ~200:1.

Two 2D-STD-$T_{1\rho}$-Jres-wg (2D-STD-$T_{1\rho}$-J-resolved-watergate) spectra were acquired with the protocol steps given in Figure 1. The total saturation time was 1s (and d_1 = 1s) and 2s (and d_1 = 0 s), respectively, while all other parameters to record and process the spectra were the same. The selective on-resonance/off-resonance saturation was placed at ~7.5 ppm (signals CG-14)/20 ppm (empty region). Saturation was accomplished by a series of Gaussian pulses of 50 ms pulse length and a 32 Hz field separated by a delay of 1 ms. The $T_{1\rho}$ filter used to suppress protein signals is applied as a continuous-wave spin-lock pulse of 60 ms with a 2 kHz field. The selective 90° pulses used for solvent suppression with the Watergate method (Piotto et al., 1992) were set to the centre of the on-resonance water signal, shaped as *sinc* pulses with a pulse width of about 1.1 ms, and the power was adjusted to obtain optimal solvent suppression. Gradients were of rectangular shape, and their duration of 1 ms was followed by a stabilization delay period of 0.2 ms. The strength of G_w was adjusted to 30 G/cm, a value that was found to provide a convenient degree of water suppression. The spectral width in the acquisition (proton) and J-resolved dimensions was 9500 and 65 Hz, respectively. The spectrum was recorded with 128 scans and 48 increments in the J-resolved dimension. The total completion time of the experiment was ~4 h.

The processing of the 2D-STD-$T_{1\rho}$-Jres-wg esperiment to obtain the 1D-STD-homodecoupled experiment is based on the method proposed by Guenneau et al. (1999). It starts with remov-

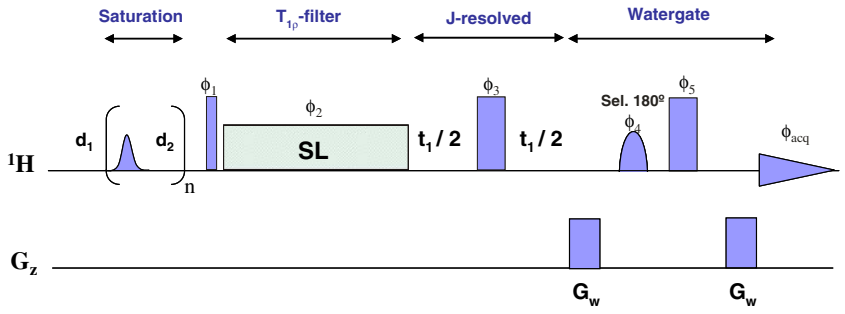

Figure 1. Scheme of the 2D-STD-$T_{1\rho}$-Jres-wg (2D-STD-$T_{1\rho}$-J-resolved-watergate) experiment. Filled and open rectangles correspond to 90° and 180° hard pulses, d_1 is an optional relaxation delay. The selective saturation of the protein is accomplished as a series of soft Gaussian pulses separated by a short delay (d_2). The $T_{1\rho}$ filter is operated as a continuous-wave spin-lock pulse. In the Watergate module, the gradient power level, G_w and the selective 180° pulse should be calibrated to suppress the strong solvent signals. On alternate control scans, saturation is tested outside the range of protein resonances, and subtraction is attained by phase cycling, i.e. ϕ_1: $x, x, -x, -x, y, y, -y, -y$, ϕ_2: $y, y, -y, -y, -x, -x, x, x$, ϕ_3: x, x, x, x, y, y, y, y, ϕ_4: $x, -x, -x, x, y, -y, -y, y$, ϕ_5: $-x, x, x, -x, -y, y, y, -y$, and ϕ_{acq}: $x, -x, -x, x, y, -y, -y, y$. Pulses whose phase is not indicated correspond to the x-axis.

ing the residual solvent signal using a high-pass filter function. The spectrum was then Fourier-transformed along the acquisition (proton) and J-resolved dimensions with a 45° and 90° shifted sinebell apodization in F2 and F1, respectively, and zero filling was applied in both dimensions to extend the data to 4096 and 128 complex points in F2 and F1, respectively. The spectrum was then tilted by 45° along the acquisition dimension, and a derivative algorithm was applied along F1 to enhance the resolution in this dimension. In this respect, instead of using the common discrete derivative method, a continuous wavelet derivative method (Wu et al., 2002) was applied with a factor of 10 for the dilation filter which provided an improved signal-to-noise ratio. This processing provided a 2D-STD-J-resolved spectrum that was represented in the power mode. The 1D STD-homodecoupled spectrum was generated by calculating the projection-sum along all the F2 rows (Guennenau et al., 1999; Cobas and Martín-Pastor, 2004) from the already processed 2D-STD-$T_{1\rho}$-Jres-wg spectrum. A 1D-STD-$T_{1\rho}$-wg (Wang et al., 2004) experiment was performed using conditions analogous to those described for the 2D-STD-$T_{1\rho}$-Jres-wg experiment. For this purpose the same series as given in Figure 1 was used but omitting the t_1 evolution period from the protocol. The spectrum was recorded with 512 scans in 21 min. A 2D-HSQC-wg experiment was carried out under standard conditions. This spectrum was used as reference for the proton chemical shifts.

Results and discussion

Our aim was to design a new method to tackle the problem of severe signal overlap in STD experiments. In the 2D-STD-$T_{1\rho}$-Jres-wg (2D-STD-$T_{1\rho}$-J-resolved-watergate) sequence given in detail in Figure 1 the STD–NOE signals are spread along an indirect J-resolved dimension. Hereby, potential problems due to signal overlap which – when undetected – could cause errors in assignment or quantification of the STD–NOEs can be sorted out. The given procedure is suitable for binding studies with biomolecules. Subtraction errors in STD–NOEs caused by temperature fluctuations or sample aggregation during the acquisition are minimized by means of the phase cycling, attaining subtraction in consecutive scans during the on-resonance and off-resonance experiments. The duration of the $T_{1\rho}$-filter element can be adjusted to destroy comparatively rapidly relaxing signals of a protein while preserving those of the small ligand under study (Hajduk et al., 1997). A strong degree of solvent suppression can be achieved by means of the Watergate element (grad, selective 180°, 180°, grad) (Piotto et al., 1992) which has been added after the t_1 incremented delay of the J-resolved element. Since our sequence extends the original 1D-STD protocol by a J-modulation period, which consists of a t_1 incremented delay and a 180° spin echo pulse by means of the Watergate element, its sensitivity per scan should not be compromised. In fact, we expect that the quality of this parameter is maintained. Because

the spectral width in Hz of the J-resolved dimension is relatively modest, the number of points required to digitize this dimension can even be relatively small. Typically, the J-resolved dimension can be acquired with ~64 points in the magnitude mode (i.e. not required to double the number of increments to obtain a pure phase-sensitive spectrum), which drastically reduces the total acquisition time in comparison to other 2D-STD spectroscopy (Mayer and Meyer, 1999; Vogtherr and Peters, 2000; Möller et al., 2002).

Following these methological considerations, we document application of this type of 2D-STD-$T_{1\rho}$-Jres-wg experiment to the study of the recognition of lactose by an animal lectin, belonging to the family of galectins (β-galactoside binding lectins) (Leonidas et al., 1998). This class of adhesion/growth-regulatory effectors influencing growth and invasion behavior of tumor cells by virtue of glycan binding is gaining increasing attention as target for drug design and as potential pharmaceuticals (Gabius, 1997; Gabius, 2001; Solís et al., 2001). As role model, we selected chicken galectin CG-14, one of the few monomeric galectins which had been tested previously in functional assays on tumor proliferation and neuronal differentiation (Kopitz et al., 2004; André et al., 2005). The binding affinity of galectins is typically in the micro- and millimolar range, satisfying the essential requirement for transferred-NOE and STD experiments (Meyer and Peters, 2003; Dam et al., 2005). In fact, STD experiments are a very good alternative to the use of synthetically engineered carbohydrate ligands to deduce the binding epitope to lectin receptors (Rivera et al., 1992; Solis et al., 1993).

An essential step for the NOE study was to determine the exact positions of the proton resonances of lactose under the given experimental conditions. Thus, a $^{1}H/^{13}C$ 2D-HSQC-wg correlation experiment was instrumental to separate the proton resonances, which were then completely assigned based on earlier reports (Breg et al., 1988; Platzer et al., 1989).

For the interaction study, a ratio of ~200:1 with lactose and the lectin was prepared. The 2D-STD-$T_{1\rho}$-Jres-wg difference spectrum obtained upon saturation at the aromatic region of CG-14 (sat. time 2 s) is shown in Figure 2. For comparison, the ^{1}H-$T_{1\rho}$-filtered spectrum which shows all signals of lactose is added in the top part of this figure. The peaks observed in the 2D J-resolved spectrum are STD–NOEs generated through dynamic interaction of lactose with the lectin. At each proton chemical shift, the peaks are dispersed along the J-resolved dimension according to the respective scalar coupling multiplicity, aiding to identify any instance of signal overlap and to contribute to the reliability of signal assignment.

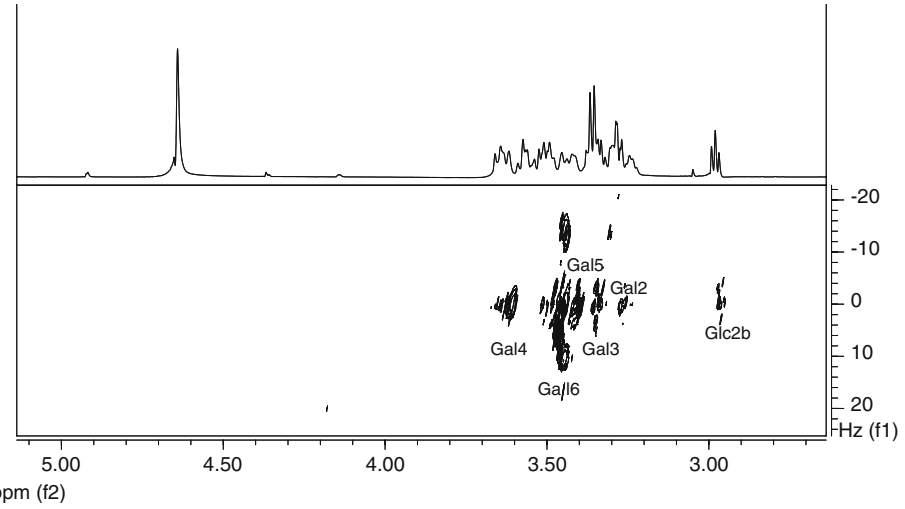

Figure 2. 2D-STD-$T_{1\rho}$-Jres-wg difference spectrum of lactose and the chicken galectin CG-14 upon saturation of the aromatic signals of the protein (sat. time 2 s). The 1D ^{1}H-$t_{1\rho}$-filtered spectrum of the sample is given for comparison (top panel). Protein signals were effectively filtered out in both spectra by means of the $T_{1\rho}$ filter. Proton labelled with b refer to the beta anomer of the glucose moiety of lactose.

Although it is certainly possible to analyze the 2D-STD-J-resolved spectrum in the common way to obtain the signal assignment as shown in Figure 2, an arising possibility described for J-resolved type spectra is to process the data to yield a 1D-Homodecoupled spectrum (i.e. a 1D spectrum in which all proton signals appears as singlets). Such a data presentation might improve the analysis.

From the different processing methods described in the literature to obtain a 1D-homodecoupled spectrum (Guenneau et al., 1999, and references therein), a particularly simple and robust method is given by Guenneau et al. (1999). In this method, a 2D J-resolved spectrum is apodized and processed with a Fourier transform along both dimensions, then the spectrum is tilted by 45° along the acquisition dimension and displayed in power mode to remove the phase-twist problem. The projection sum of all the F2 rows of the proton dimension directly provides the corresponding 1D-homodecoupled spectrum. The line widths achievable in this spectrum are similar to that in the original 1D proton spectrum and, of note since the 1D-homodecoupled spectrum is built from a sum of 1D proton spectra, there is an important gain in terms of sensitivity. This parameter is important for experiments based on difference spectroscopy such as the STD. We therefore present the comparison regarding this factor next.

Figures 3a and b show the conventional 1D-STD-$T_{1\rho}$-wg experiment (512 scans) and the 1D-STD-homodecoupled experiment (128 scans per increment), respectively, using a saturation time of 2 s. Apart from the obvious gain in terms of signal to noise ratio in the spectrum of Figure 3b, it can be seen that the complex multiplet signal at ~3.45 ppm in the 1D-STD spectrum of Figure 3a is separated into two partially overlapping singlets in the 1D-STD-homodecoupled spectrum of Figure 3b, while other the signals appear unaltered. Using a 2D-HSQC-wg spectrum as reference, it was possible to identify these two singlets as arising from Gal-6 and Gal-5 (Figure 3b). Other signals in the 1D-STD-homodecoupled spectrum do not show problems of overlap and were assigned without difficulties

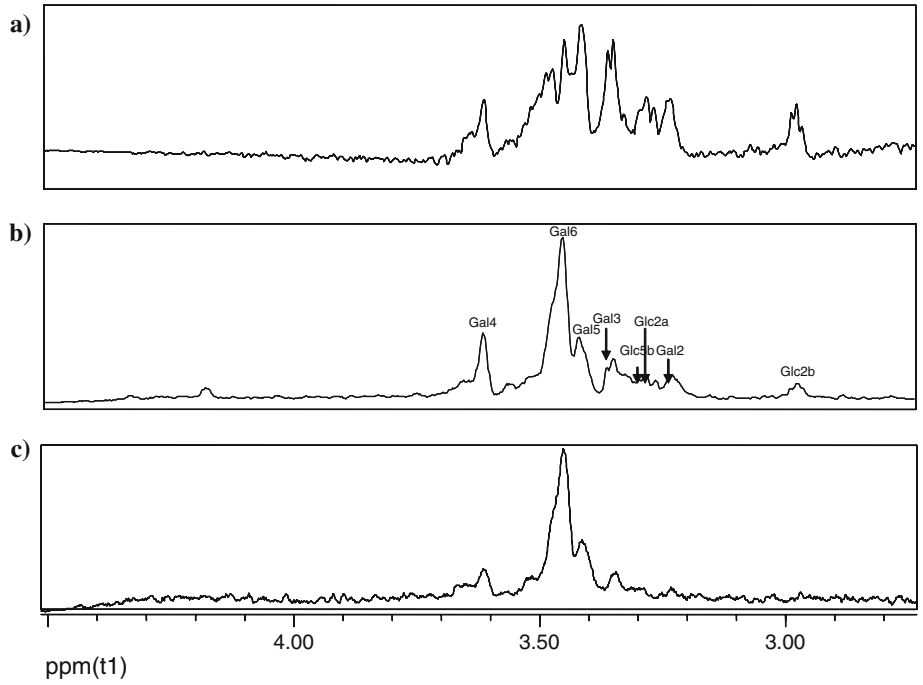

Figure 3. Comparison of STD spectra of the mixture of the chicken galectin CG-14 and its ligand lactose: (a) conventional 1D-STD experiment (sat time 2 s), (b) 1D-STD-Homodecoupled (sat. time 2 s), and (c) 1D-STD-Homodecoupled (sat. time 1 s). Protein signals were effectively filtered out in spectra by means of the $T_{1\rho}$ filter. Protons labelled a and b refer to the alpha and beta anomers of the glucose moiety of lactose, respectively.

(Figure 3b). One interesting aspect of homodecoupled spectra generated with the method of Guenneau et al. (1999) is that despite the fact that the intensities within the spectrum can not be compared because the information of the relative signal intensity is lost due to the use of the power mode during the processing stage of the spectrum, however it is still possible to determine quantitatively the modulation of the signal intensity with respect to a change in a certain acquisition parameter such as mixing time (Guenneau et al., 1999) or gradient strength (Cobas and Martín-Pastor, 2004), being equal the rest of the acquisition and processing parameters. Thus, for the present case of the 1D STD-homodecoupled spectrum, the saturation time can be changed along two or more spectra, and the modulation of any signal be calculated from the square-root of the intensity at the maximum peak height (Guennenau et al., 1999; Cobas and Martín-Pastor, 2004). This quantitative analysis of the 1D-STD-homodecoupled spectrum can be applied for the determination of STD build-up curves what can prove useful for the quantitative interpretation of the STD-NOE (Jayalakshmi and Krishna, 2004, 2005; Jayalakshmi et al., 2004) effect.

In analogy with the conventional 1D-STD experiment, there are situations in which the simple qualitative inspection of the intensities of 1D-homodecoupled experiment acquired with different saturation times allows to distinguish direct ligand-protein STD-NOEs involved in the binding epitope (Mayer and Meyer, 2001) from those NOEs mediated by spin-diffusion effects. This situation is exemplified in the two 1D-STD-homodecoupled spectra of Figures 3b and c acquired with sat. time of 2s and 1s, respectively. In Figure 3b, the extended saturation time generates STD–NOEs with the protons of both the galactose and glucose moieties of the disaccharide, the STD–NOEs with the galactose moiety being clearly more intense than those with the glucose moiety. On the other hand, in the spectrum of Figure 3c which was acquired with the shorter sat. time, there are STD–NOEs exclusively with the galactose moiety (Gal-3, Gal-4, Gal-5 and Gal-6), and their intensities are almost as strong as given in the spectrum of Figure 3b. These observations indicate that the relatively small STD–NOEs observed with the glucose moiety are likely caused by spin diffusion effects, while the relative strong STD–NOEs of Gal-3, Gal-4, Gal-5 and Gal-6 observed in both spectra should correspond to direct STD-NOEs between lactose and the site of interaction with the lectin. Evidently, the non-reducing-end sugar unit comes into close vicinity to the lectin's binding site, as observed in studies using chemical mapping or crystallographic analysis. (Solís et al., 1996; Asensio et al., 1999; Varela et al., 1999; Alonso-Plaza et al., 2001; López-Lucedo et al., 2004) This confirmation attests the validity of the presented results of interaction analysis and thus the given protocol. Moreover, the pulse sequence was also applied to a more complex tetrasaccaride, methyl 4-O-(3-O-(2-acetamido-2-deoxy-4-O-β-D-galactopyranosyl-β-D-glucopyranosyl)-β-D-galactopyranosyl)-β-D-glucopyranoside. The application of the pulse sequence of Figure 1 also indicated the recognition of the non reducing end residue, since only signals for this moiety were indeed observed in the 2D-STD-J-resolved spectrum (Fig. in the supporting information).

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