Journal of Magnetic Resonance 202 (2010) 245–249

Contents lists available at ScienceDirect

Journal of Magnetic Resonance

journal homepage: www.elsevier.com/locate/jmr

STD-DOSY: A new NMR method to analyze multi-component enzyme/substrate systems

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ARTICLE INFO

Article history:
Received 8 May 2009
Revised 21 October 2009
Available online 6 December 2009

Keywords:
NMR
STD-DOSY
Protein/substrate interaction
Diffusion coefficient
WGA

ABSTRACT

A new approach to analyze multi-component Saturation Transfer Difference (STD) NMR spectra by combining the STD and the DOSY experiment is proposed. The resulting pulse sequence was successfully used to simplify an exemplary multi-component protein/substrate system by means of standard DOSY processing methods. Furthermore, the same experiment could be applied to calculate the ratio of saturated substrate molecules and its saturation rate in the case of competitive interactions. This ratio depends on the strength of this interaction between the substrates and the protein, so that this kind of information could be extracted from the results of our experiment.

1. Introduction

For dealing with protein/ligand interactions employing the NMR spectroscopy, the *S*aturation *T*ransfer *D*ifference (STD) experiment [1] proves to be a great tool for screening different substrates in terms of their affinity to a protein. If it is necessary to analyze mixtures of substrates for competitive interaction with one enzyme, the STD spectra of these samples are often overcrowded and difficult to interpret. On the other side, the *D*iffusion *O*rdered NMR *S*pectroscop*Y* (DOSY), developed by Johnson et al. [2–5], is a useful NMR experiment, which can be applied for the simplification of spectra of multi-component mixtures by dividing them into several sub-spectra taking into account the diffusion coefficients of the components. In this paper a new method to separate different STD data sets of multiple protein-binding species by their diffusion coefficients is presented.

2. Results and discussion

The pulse sequence for this NMR experiment is a combination of the standard *B*ipolar *P*ulse *P*air incorporating a *L*ongitudinal *E*ddy current *D*elay (BPP-LED) [2] and the STD pulse sequence (Fig. 1). The resulting 2D spectrum contains only those signals, which experience a reduction in signal intensity caused by the saturation transfer from the protein to the substrate.

We exemplarily used a mixture composed of two substrates (Fig. 2) and *W*heat *G*erm *A*gglutinin (WGA) with a molar ratio of 50:50:1 (S1:S2:WGA).

In a first step we utilize the presented pulse sequence to gain a DOSY-like spectrum with a chemical shift scale in F2 direction and a diffusion coefficient scale in the second dimension.

The STD-DOSY spectrum resulting from the processing method *Exponential* is shown in Fig. 3. Four separated sets of peaks (Enzyme (E), S1, S2 and water) and one set of overlapping signals of the ring protons of the substrates (S1 + S2) are revealed. These overlapping signals could not be separated with the methods available with TopSpin (Exponential, CONTIN [6]).

With this method it is possible to discriminate between STD signals arising from different substrates, provided that these substrates obtain unequal diffusion coefficients in the mixture investigated.

In a second step the same pulse program was used to perform self diffusion measurements and to quantify the transfer of saturation by employing diffusion coefficients of overlapped signals.

When comparing the separate STD spectra of S1 and S2 (Fig. 4), the chemical shifts of the strong signals of the NHAc groups are very similar and overlap in the STD-DOSY spectrum.

In the following it will be shown that it is possible to calculate the ratio of the partly saturated substrates for different saturation times from these overlapping signals. Normally, this result is obtained by employing the integrals of two signals of an STD spectrum each belonging to a different substance and comparing it to the integrals of the corresponding signals in the ^{1}H NMR spectrum of the mixture. The disadvantage of the latter method is that non-overlapping STD signals of carbohydrates are often low in intensity

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doi:10.1016/j.jmr.2009.11.007

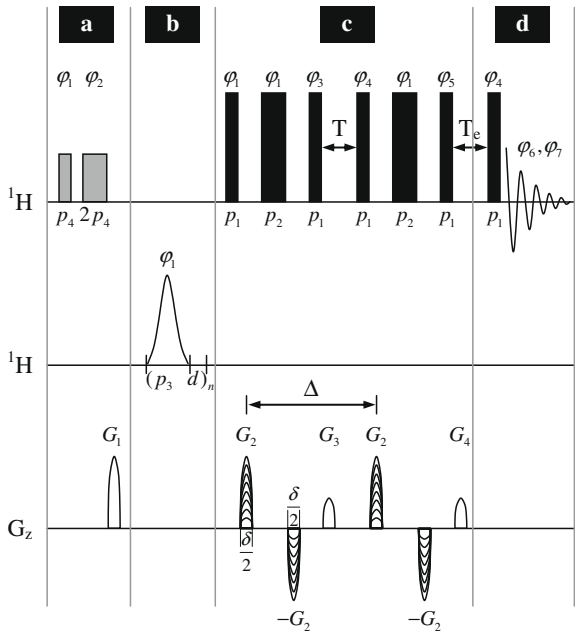

Fig. 1. Pulse sequence for the STD-DOSY experiment (p_1 corresponds to a 90°, p_2 to a 180° high power pulse, p_3 to a selective gaussian pulse and p_4 to a trim pulse. Phase cycles: $\varphi_1 = x$; $\varphi_2 = y$; $\varphi_3 = x, x, -x, -x$; $\varphi_4 = x, x, x, x, -x, -x, -x, -x, y, y, y, y, -y, -y, -y, -y$; $\varphi_5 = x, -x, x, -x, -x, x, -x, x, y, -y, y, -y, -y, y, -y, y$; $\varphi_6 = -x, x, x, -x, x, -x, -x, x, y, -y, -y, y, -y, y, y, -y$; $\varphi_7 = x, -x, -x, x, -x, x, x, -x, -y, y, y, -y, y, -y, -y, y$. Gradient strengths: G_1:G_3:G_4 = 40%:−17.13%:−13.17%; G_2 = 2%..95% (for more details see "Section 4").

and difficult to integrate. Our substrates binding to WGA are all derivatives of *N*-acetylglucosamine, which is one of the specific substrates of WGA [7] and the strongest STD signals are produced by the methyl group of the NHAc residues. For both substrates S1 and S2 of equal concentration and the same saturation times the saturation transfer from WGA to the substrates is nearly the same

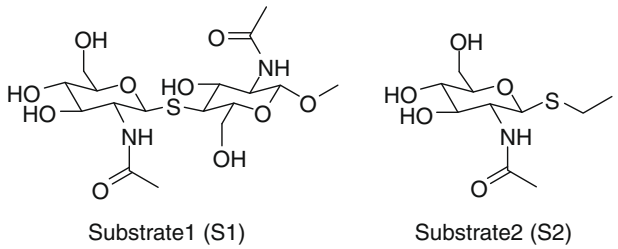

Fig. 2. Structure of the substrates.

(Fig. 4). Furthermore, in the case of S1 only one of the NHAc groups (the one linked to the glucose residue not containing the OMe group) is saturated. But due to overlapping no direct comparison of their integrals in the mixture is possible.

As the standard BPP-LED pulse sequence can be used to get diffusion coefficients of different substances in a mixture [8], we tried to do the same with the STD-DOSY pulse sequence. In a first step we recorded five data sets with different saturation times ranging from 0.5 to 5 s and one data set without saturation, which corresponds to a normal pulsed field gradient self diffusion experiment. For the latter data set, we changed the STD-DOSY sequence in that manner, that the same receiver phases were employed ($\varphi_6 = \varphi_7$) which corresponds to a summation of the signals. Furthermore, the protons of the protein were left unsaturated, which was realized by two off-resonance frequencies at 40 ppm. This procedure secures, that the same pulse program could be used and errors due to incomparable experiments are negligible.

The diffusion coefficients calculated from the overlapping methyl signals of the NHAc groups at ca. 2 ppm increases with increasing saturation time as shown in Fig. 5. This can be explained by different saturation transfer due to unequal binding behavior of S1 and S2 in the mixture.

For overlapping signals the diffusion coefficient obtained from these signals calculates to Eq. (1):

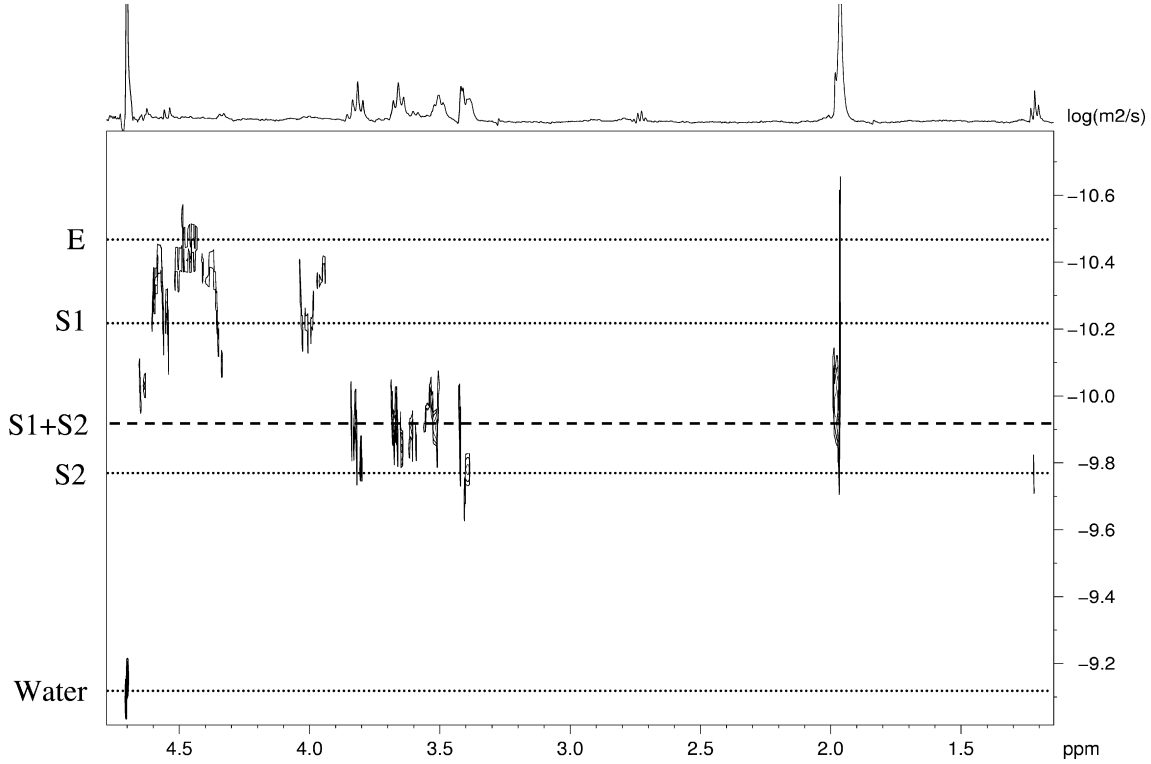

Fig. 3. STD-DOSY spectrum of a mixture of S1, S2 and WGA (50:50:1).

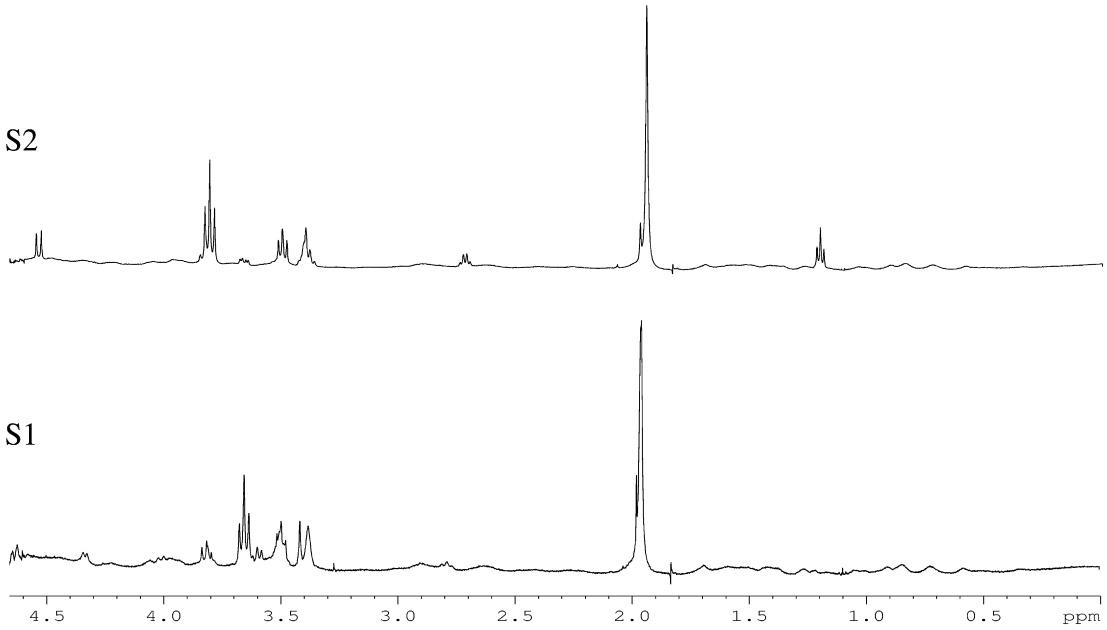

Fig. 4. Separate STD spectra of S1 and S2.

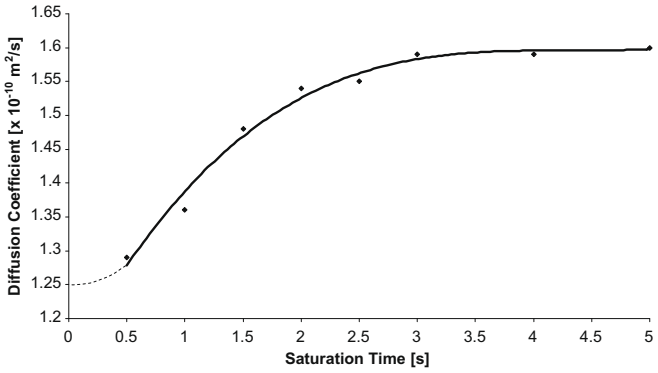

Fig. 5. Dependence of the calculated diffusion coefficient on the saturation time (the dotted course of the curve is assumed).

$$D_{obs} = x_1 D_{S1} + x_2 D_{S2} = x_1 D_{S1} + (1 - x_1) D_{S2} = (1 - x_2) D_{S1} + x_2 D_{S2} \quad (1)$$

(with D_{obs}, observable diffusion coefficient; x_1, x_2, integral fractions of the signals of S1 and S2; D_{S1}, D_{S2}, mean value of the diffusion coefficient of S1 and S2 as shown in Table 2) [9].

The diffusion coefficients of the pure components (D_{S1} and D_{S2}) were obtained from the standard DOSY experiment of the same mixture using the non-overlapping signals at around 4 and 4.4 ppm for substrate 1 and at 1.2 and 4.6 ppm for substrate 2 (arrows in the ^{1}H spectra of S1 and S2, Fig. 6).

They calculated to the values shown in Table 1.

With the aid of Eq. (1) we could calculate the ratio of saturation of the two substrates at every saturation time (Table 2).

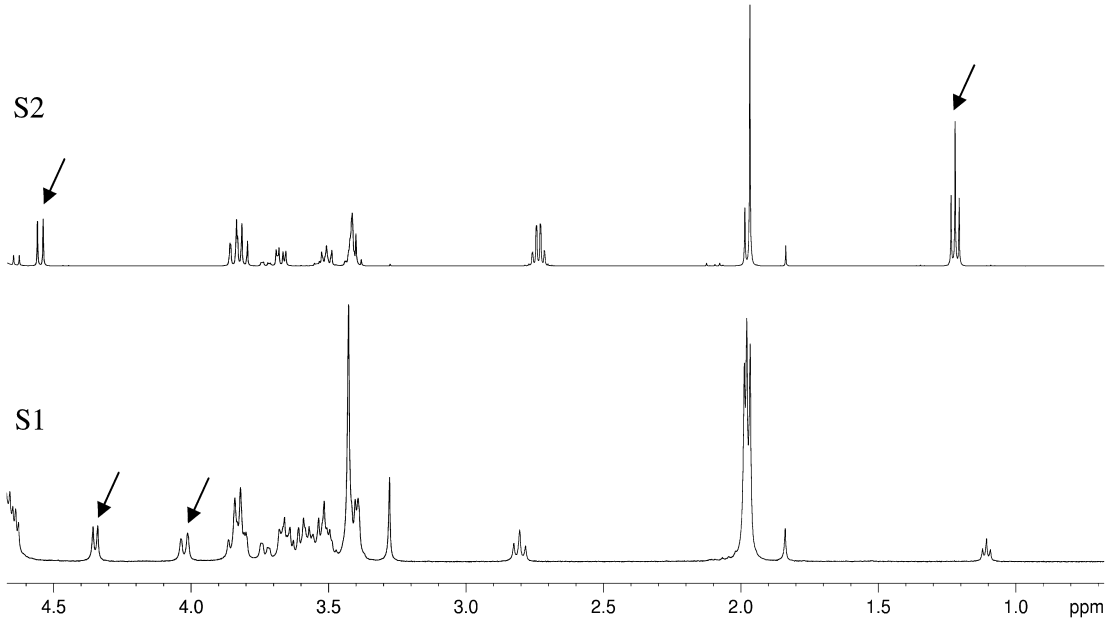

Fig. 6. ^{1}H spectra of S1 and S2 (the signals used to calculate D_{S1} and D_{S2} are marked by arrows).

Table 1
Diffusion coefficients of the pure components and its mean values.

Substrate	Chemical shift (ppm)	Diffusion coefficient (m^2/s)	Mean value (m^2/s)
S1	4	1.21×10^{-10}	1.25×10^{-10}
	4.4	1.29×10^{-10}	
S2	1.2	1.98×10^{-10}	1.93×10^{-10}
	4.6	1.87×10^{-10}	

Table 2
Calculated integral fractions and ratios of saturation.

Saturation time (s)	Diffusion coefficient (m^2/s)	x_1	x_2	S1:S2
0.5	1.29×10^{-10}	0.94	0.06	1:0.06
1	1.36×10^{-10}	0.85	0.15	1:0.18
1.5	1.48×10^{-10}	0.66	0.34	1:0.51
2	1.54×10^{-10}	0.56	0.44	1:0.79
2.5	1.55×10^{-10}	0.51	0.49	1:0.96
3	1.59×10^{-10}	0.5	0.5	1:1
4	1.59×10^{-10}	0.5	0.5	1:1
5	1.60×10^{-10}	0.49	0.51	1:1

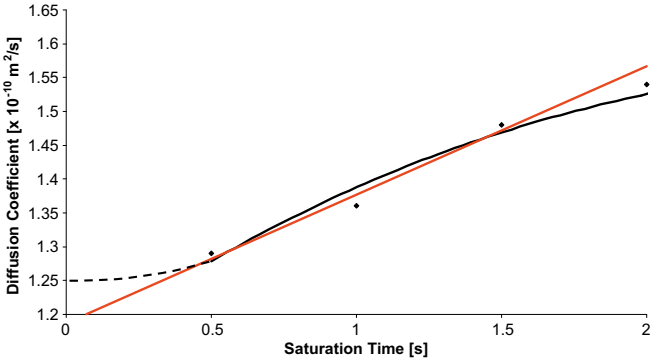

Fig. 7. Zoomed part of Fig. 5 used for the calculation of R_S.

For that case that both substrates have the same number of saturated protons for the signal investigated (as is for S1 and S2), the limit of the possible diffusion coefficient calculates to $D = ½(D_{S1} + D_{S2})$, for all substrate molecules being completely saturated. In our case this will result in 1.59×10^{-10} m^2/s by employing the mean values shown in Table 1 for D_{S1} and D_{S2}. This correlates well with the limit reached at high saturation times. These results lead to the conclusion, that substrate S1 seems to show a stronger affinity to WGA, because at smaller saturation times more of S1 is saturated. The saturation rate (R_S) for S2 in presence of S1 was estimated with the aid of Fig. 5. For that purpose we assumed that at lower saturation times between 0.5 and 1.5 s (the almost linear course of the curve in Fig. 5) the chance of the enzyme binding to a saturated S2 molecule is low and only becomes important with increasing saturation times. This simplification is valid working with large substrate excesses. Therefore, we applied the initial part of the curve in Fig. 5 and calculated the slope of the approximately straight line between 0.5 and 1.5 s (Fig. 7). At 0.5 s 6% and at 1.5 s 51% of the S2 molecules (0.02 mmol total concentration) are saturated (Table 2) leading to

$$R_S = (0.51 \cdot 0.02\ \text{mmol} - 0.06 \cdot 0.02\ \text{mmol})/(1.5\ \text{s} - 0.5\ \text{s}) = 0.009\ \text{mmol/s}.$$

3. Conclusions and outlook

We presented a new method to separate different STD data sets of multiple components by their diffusion coefficients combining the STD and the DOSY NMR experiment. Unfortunately, the overlapping signals of the ring protons of both substrates could not be separated by either the *Exponential* or *CONTIN* method. Furthermore, we successfully used this experiment to calculate the ratio of saturation of two substrates S1 and S2 employing its diffusion coefficients instead of signal areas and we showed, that an estimation of the saturation rate for the weak-binding substrate could be performed. The advantage of the described method is that *overlapping* signals can be used for these calculations as shown for the NHAc groups of substances binding to WGA. This method is valid for all mixtures of two substrates binding to the same enzyme and possessing protons which produce signals with similar chemical shifts and experiencing an equal saturation transfer. If this is not the case one could calculate a correction factor by determining the integral ratios of the observed signals for S1 and S2 from the separately performed STD experiment with a high saturation time.

In addition, the possibility to determine the binding epitope of protein-bound substrates from an STD-DOSY spectrum and the validation of the experiment for two completely different binding substrates should be investigated.

4. Experimental

4.1. Sample preparation

0.02 mmol of S1 (8.26 mg), 0.02 mmol of S2 (5.3 mg) and 0.0004 mmol (6.8 mg) of WGA were dissolved in D_2O (lower concentrations are also possible, but for a better signal to noise ratio at lower measurement times, we used a 10-fold higher concentration as conventional for the standard STD experiment). The mixture was allowed to stand for about 10 min in an ultrasonic bath before carried over to an NMR sample tube.

4.2. NMR spectroscopy

4.2.1. Data acquisition

All measurements were performed on a Bruker Avance 500 MHz spectrometer operating at 500.17 MHz for ^1H, using a BBFO probe head equipped with a *z*-gradient. Gradient calibration was done using the method described in [10]. The diffusion measurements were carried out using a modified bipolar gradient pulse pair-stimulated echo sequence incorporating a longitudinal eddy current delay (BPP-LED) as shown in Fig. 1. The pulse sequence consists of a trim pulse (a), a selective saturation pulse (b), the BPP-LED sequence (c) and the acquisition part (d). For the selective saturation pulse an irradiation frequency list was generated including an on-resonance frequency of 10.02 ppm for WGA protons and an off-resonance frequency of 40 ppm. The gradient pulse length (δ) and the diffusion time (Δ) were kept at fixed values while gradually increasing the gradient strength. Typical values for δ and Δ are 3 and 100 ms, respectively. A longitudinal eddy current delay (T_e) of 5 ms was used. Sine-shaped gradients were linearly varied between 1 and 49 G cm^{-1} in 16 steps and at each step 512 scans with two FIDs per scan were acquired (for the measurement of the self diffusion coefficients 8 steps with 512 scans per step were used). Two experiments with identical transmitter phases (φ_1–φ_5) and opposite receiver phase (φ_6, φ_7) were performed before advancing to the next step in the phase cycle. The sample temperature was kept constant at 298 ± 0.1 K using a Bruker Variable Temperature Unit.

4.2.2. Data processing

The pseudo 2D data were processed, using exponential multiplication with a line broadening factor of 3.0 and zero filling to twice the size of the time domain in F2 direction. The DOSY spectra

were obtained by using the DOSY module of Bruker's Topspin 2.1 and processing method "exponential". The diffusion coefficients were obtained with the help of the T_1/T_2 relaxation module of Topspin as described in the diffusion manual of this software, whereas the fitting type "area" was used.

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