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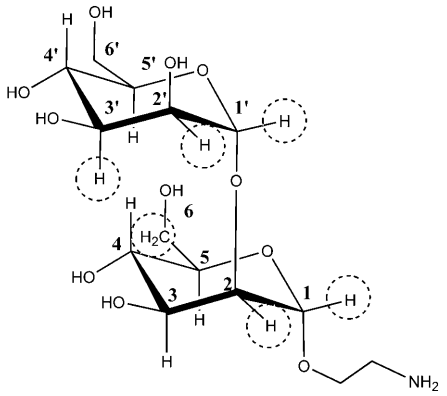
Saturation Transfer Difference (STD) NMR Spectroscopy Characterization of Dual Binding Mode of a Mannose Disaccharide to DC-SIGN

Jesús Angulo,[a] Irene Díaz,[a] José J. Reina,[a] Georges Tabarani,[b] Franck Fieschi,[b] Javier Rojo,[a] and Pedro M. Nieto*[a]

Saturation transfer difference NMR spectroscopy (STD-NMR), together with transfer NOE, is one of the most widespread NMR methods for the study of the interactions between small ligands and macromolecular receptors.[1] Originally proposed as a technique for the rapid screening of compound libraries, its scope has been extended to include mapping the interaction epitope by determining the ligand regions in contact with the receptor.[2] More recently, it has been applied to the study of receptor-bound ligand conformations by using quantitative STD theoretical calculations.[3–5] Herein, we investigate the effect of multiple ligand-binding modes on the quantitative analysis of STD experiments by analyzing the multimodal binding of a mannose disaccharide to DC-SIGN (Dendritic Cell-Specific ICAM-3 Grabbing Non-integrin).[6] Our results demonstrate that STD-NMR is sensitive to the existence of multiple binding modes. Furthermore, we propose an approach for the quantitative analysis of experimental NMR data and theoretical predictions in the case of multiple binding modes. This approach has allowed us to confirm the dual character of the multiple binding modes of the Manα(1→2)Man$OC_2H_4NH_2$ disaccharide (Scheme 1) to DC-SIGN in solution and to elucidate the ligand orientation in the less populated bound conformation, which was undefined in the crystallographic study.[6]

The interaction between Manα(1→2)Man and DC-SIGN was selected as a model system to verify the sensitivity of STD to multiple binding modes. DC-SIGN, also known as CD209, is a dendritic cell surface receptor. Specifically, DC-SIGN is a C-type lectin receptor with a carbohydrate recognition domain (CRD) at the C terminus able to interact with highly glycosylated proteins found on several pathogens.[7] DC-SIGN is considered an universal pathogen receptor and plays a key role in HIV *trans* infection.[8] The interaction of the CRD with different carbohydrates has been studied both the liquid and the solid state, and by STD-NMR.[6,9,10] Recent crystallographic structures demonstrate that DC-SIGN binds mannose oligosaccharides in a multimodal fashion within the same binding site.[6]



Scheme 1. Disaccharide Manα(1→2)Man$OC_2H_4NH_2$: Dashed circles indicate the protons whose NMR signals have been used for this study.

Multiple binding modes of a ligand in a single site can be considered a special receptor–ligand–competitive inhibitor system, where one complex is an inhibitor of the others. As the ligand and inhibitor(s) are the same species, the chemical shifts and equilibrium intensities of their signals are equivalent and undistinguishable. Therefore, the total saturation observed in a STD experiment would be the sum of the accumulated saturations corresponding to each binding mode.

$$L + P \rightleftharpoons LP_1 + LP_2 + \ldots + LP_i + \ldots + LP_n$$

CORCEMA-ST calculates the theoretical intensities of STD spectra via full relaxation matrix analysis based on the three-dimensional co-ordinates of the complex, concentrations, and association and dissociation rate constants.[3] The saturation due to each complex considered (derived from an alternative binding mode) can be calculated separately. Nevertheless, this approach is not directly applicable because the STD values can be affected by cross-rebinding, where a previously saturated ligand re-enters the binding pocket in an alternative mode, as the initial intensities of the signals are different from those at equilibrium. Equally, it is not possible to estimate the cross-rebinding contribution as CORCEMA-ST does not implement algorithms for multiple binding modes. When the saturation times are short enough, the potential effect of cross-rebinding becomes negligible. Unfortunately, under these conditions, the quality of the experimental data is very poor due to low magnetization transfer from the receptor. Our approach uses the STD initial growing rates (STD_0) to avoid possible rebinding contributions.

[a] *Dr. J. Angulo, I. Díaz, J. J. Reina, Dr. J. Rojo, Dr. P. M. Nieto*
Grupo de Carbohidratos, Instituto de Investigaciones Químicas
CSIC-Universidad de Sevilla
Américo Vespucio 49, 41092 Seville (Spain)
Fax: (+34) 954-46-05-65
E-mail: pedro.nieto@iiq.csic.es

[b] *G. Tabarani, Prof. F. Fieschi*
Laboratoire des Protéines Membranaires
Université Joseph Fourier/CEA
DSV/CNRS, UMR 5075, Institut de Biologie Structurale Jean-Pierre Ebel
41, rue Jules Horowitz, 38027 Grenoble Cedex 1 (France)

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$$\mathrm{STD}_0(\text{multiple-binding}) = \frac{I_0 - I_{sat}^{exp}}{I_0}\Big|_{t_{sat}\to 0} = \frac{\sum_i (I_0 - I_{sat}^i)}{I_0}\Big|_{t_{sat}\to 0} = \sum_i \mathrm{STD}_0^i \quad (1)$$

The experimental STD_0 were estimated from the initial slope of STD verses saturation time build-up curves ($STD = STD_{max}[1-\exp[-k_{saturation}\ t_{saturation}]$), as previously described.[11] The theoretical STD_0 values correspond to the sum of the initial growing rates calculated independently for each binding mode by CORCEMA-ST according to Equation (1).

$$\mathrm{NOE}(R\text{-factor}) = \sqrt{\frac{\sum W_k(\mathrm{STD}_{0,k}^{exp} - \mathrm{STD}_{0,k}^{calcd})^2}{\sum W_k(\mathrm{STD}_{0,k}^{exp})^2}} \quad (2)$$

We have determined the initial slopes from STD-NMR build-up experiments (0.5, 0.75, 1.0, 1.5, 2.0 and 3.0 s saturation times) on a sample containing the Manα(1→2)Man$OC_2H_4NH_2$ disaccharide (Scheme 1) in the presence of the soluble extracellular domain (ECD) of DC-SIGN. The experimental results have been compared with the theoretical initial slopes predicted using CORCEMA-ST. The agreement between the calculated and the experimental NMR data was evaluated using the NOE R factor given by Equation (2), where STD_0 is the initial slope and W is a weighting factor proportional to $1/STD_0^{exp\ or\ calcd}$.[4] For the sake of accuracy in the present study we used only well resolved and isolated NMR resonances of the ligand, avoiding uncertain contributions in the case of overlapped signals. Thus, seven protons have been included in the NOE R factor analysis (H1′, H2′, H3′, of the nonreducing sugar ring, and H1, H2, and the two H6 protons of the reducing sugar ring, Scheme 1).

The three-dimensional structures used for the full relaxation matrix calculations were based on the known crystal structure of the CRD of DC-SIGN with Manα(1→2)Man (PDB ID: 2IT6).[6] Two different ligand binding modes in complexation with the CRD were identified in a ratio 3:1 (major/minor). The major (**M**) complex was given the coordinates described in the crystal data,[6] however, the structure of the minor (**m**) complex is ambiguous as some ligand atoms were not defined. Two possible complex structures (**m1** and **m2**), with differing ligand orientations, are compatible with the X-ray data (Figure 1, details are given in the Supporting Information).

The magnitude of STD is strongly dependent on the dissociation off-rate constant, which is propagated to the equilibrium constant (K_D), assuming that the association process (on-rate) is controlled by diffusion.[1b] Since only relative affinities from competition experiments for oligomannosides binding to DC-SIGN are reported in the literature, a series of dissociation constants in the millimolar range were considered in the CORCEMA calculations. In the case of the bimodal systems, for each K_D value corresponding to the major orientation binding, the corresponding value for the minor orientation was calculated for a 3:1 ratio of binding modes.[6] The concentrations of the

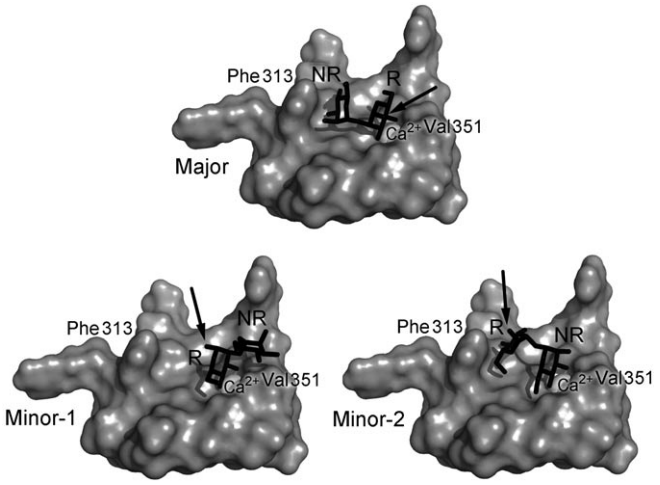

Figure 1. Structures of the DC-SIGN CRD–Manα(1→2)Man complexes used for the calculation of the theoretical STD values showing the orientation of the disaccharide relative to the Ca^{2+}. Only residues within 8 Å from a ligand atom are represented. Figure were prepared with PyMOL.[12] (R, reducing; NR, nonreducing).

complexes were calculated for monomodal or bimodal binding using equations for competitive binding.

The agreement between experimental and theoretical results obtained by full relaxation calculations for each system was assessed using the NOE R factor (Figure 2). The results for

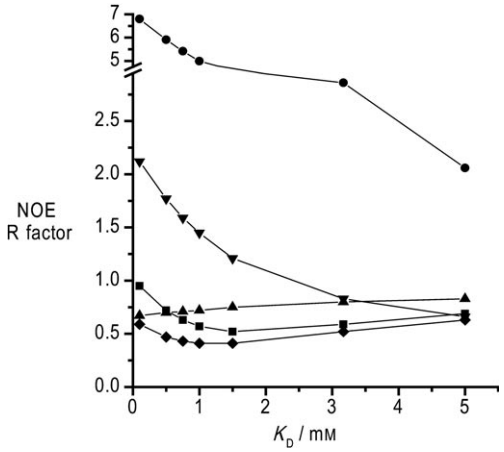

Figure 2. Deviations between the experimental and theoretical STD_0 values as a function of the dissociation constant used in the prediction. Monomodal complexes: **M** (■), **m1** (●), and **m2** (▲). Bimodal complexes: **M+m1** (▼), and **M+m2** (◆).

the monomodal binding of the ligand orientation **m1** did not correlate to the experimental results observed for the K_D values considered. The data for a hypothetical monomodal binding of the ligand orientation **m2** showed improved R factors with a growing tendency for increasing K_D values (Figure 2). Conversely, the major binding mode (**M**) had the smallest deviations relative to the experimental data for K_D values above 0.5 mM (Figure 2). This observation confirms the previous crystallographic analysis of the CRD of DC-SIGN with

Manα(1→2)Man as this mode corresponds to the largest contribution to the experimental data.[6]

Considering the bimodal systems, the combination of the modes **M** and **m1** led to very high NOE R factors, showing poorer values than the major component alone. In combination with the **m1** monomodal results, these results imply that there is a negligible, if any, contribution to the multiple binding equilibrium of the ligand orientation **m1** (reducing ring bound to Ca^{2+} site). In contrast, the contribution of the ligand orientation **m2**, together with the major mode (**M**), to the multiple binding mode equilibrium was readily inferred from the analysis of NOE R factor data (**M**+**m2**). The inclusion of **m2** in the calculations led to significant reductions in the NOE R factor values for the full range of K_D values considered when compared to both individual orientations of the ligand in the bound state: the major (**M**), and the minor-2 (**m2**). Moreover, the lowest NOE R factor was observed in the low millimolar range, in agreement with the K_D estimated from competition experiments.[9a,b]

These results refine the previous crystallographic studies by determining the precise structure and orientation of the minor binding mode of the disaccharide within the DC-SIGN binding site. In this binding mode the disaccharide interacts at the same sub-site as the major complex but the orientation of the reducing and nonreducing sugars, imposed by the coordination of the mannose with the Ca^{2+} atom, is reversed (Figure 1). This observation is particularly useful for further studies into the interactions of carbohydrates with these receptors, and the design of new ligands for DC-SIGN.

In conclusion, we have shown that STD experiments are sensitive to multiple binding modes of a ligand to a single binding site, provided that binding events fall within the fast exchange regime. Moreover, in favorable cases, the experimental STD initial rate can be calculated as the sum of the theoretical individual initial rates corresponding to the binding modes with known three-dimensional coordinates. This approach has been used to solve the ambiguities on the binding mode of the minor component present in crystallographic structures of a dimannoside ligand to DC-SIGN, defining its orientation relative to the binding site. Considering the wide use of STD as a method for solving the structure of bound ligands in combination with relaxation matrix analysis, our observation should be a useful tool in further studies.

Experimental Section

The synthesis of disaccharide Manα(1→2)Man$OC_2H_4NH_2$ was previously described.[,10c] The ECD (corresponding to amino acids 66–404) of DC-SIGN was overproduced as described previously.[13] NMR experiments were performed on an AVANCE Bruker instrument operating at 500 MHz. All NMR samples were prepared using carbohydrate (2 mM) and lectin DC-SIGN ECD (40 μM; binding site molarity) in D_2O (150 mM NaCl, 4 mM $CaCl_2$, 25 mM d-TRIS, pH 8.1). STD experiments were performed at 278 K using watergate solvent suppression (0.5, 0.75, 1.0, 1.5, 2.0 and 3.0 s saturation times) and a train of Gaussian shaped pulses (49 ms and 100–60 Hz power spaced by 1.0 ms delays). On-resonance irradiation was performed at 0.9 ppm and off-resonance at 40.0 ppm, appropriate blank experiments were carried out to assure the absence of direct irradiation on the ligand. Theoretical calculations of STD were done with the CORCEMA-ST protocol. A complete description of the proposed approach and further experimental details are given in the Supporting Information.

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