

**Influence of Sulfolane on ESI-MS Measurements of Protein-Ligand Affinities**

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## Abstract

The results of an investigation into the influence of sulfolane, a commonly used supercharging agent, on electrospray ionization mass spectrometry (ESI-MS) measurements of protein-ligand affinities are described. Binding measurements carried out on four protein-carbohydrate complexes, lysozyme with  $\beta$ -D-GlcNAc-(1 $\rightarrow$ 4)- $\beta$ -D-GlcNAc-(1 $\rightarrow$ 4)- $\beta$ -D-GlcNAc-(1 $\rightarrow$ 4)-D-GlcNAc, a single chain variable fragment and  $\alpha$ -D-Gal-(1 $\rightarrow$ 2)-[ $\alpha$ -D-Abe-(1 $\rightarrow$ 3)]- $\alpha$ -D-Man-OCH<sub>3</sub>, cholera toxin B subunit homopentamer with  $\beta$ -D-Gal-(1 $\rightarrow$ 3)- $\beta$ -D-GalNAc-(1 $\rightarrow$ 4)[ $\alpha$ -D-Neu5Ac-(2 $\rightarrow$ 3)]- $\beta$ -D-Gal-(1 $\rightarrow$ 4)- $\beta$ -D-Glc and a fragment of galectin 3 and  $\alpha$ -L-Fuc-(1 $\rightarrow$ 2)- $\beta$ -D-Gal-(1 $\rightarrow$ 3)- $\beta$ -D-GlcNAc-(1 $\rightarrow$ 3)- $\beta$ -D-Gal-(1 $\rightarrow$ 4)- $\beta$ -D-Glc, revealed that sulfolane generally reduces the apparent (as measured by ESI-MS) protein-ligand affinities. To establish the origin of this effect, a detailed study was undertaken using the lysozyme-tetrasaccharide interaction as a model system. Measurements carried out using isothermal titration calorimetry (ITC), circular dichroism and nuclear magnetic resonance spectroscopies reveal that sulfolane reduces the binding affinity in solution but does not cause any significant change in the higher order structure of lysozyme or to the intermolecular interactions. These observations confirm that changes to the structure of lysozyme in bulk solution are not responsible for the supercharging effect induced by sulfolane. Moreover, the agreement between the ESI-MS and ITC-derived affinities indicates that there is no dissociation of the complex during ESI or in the gas phase (i.e. in-source dissociation). This finding suggests that supercharging of lysozyme by sulfolane is not related to protein unfolding during the ESI process. Binding measurements performed using liquid sample desorption ESI-MS revealed that protein supercharging with sulfolane can be achieved without a reduction in affinity.

## Introduction

The direct electrospray ionization mass spectrometry (ESI-MS) assay is a valuable tool for detecting and quantifying non-covalent protein-ligand interactions *in vitro* [1]. In addition to its speed, sensitivity and selectivity, the assay is free of any requirements of immobilization or labeling of either the protein or the ligand, making it a highly versatile binding assay. Moreover, the method is well suited for screening libraries of compounds to simultaneously identify and quantify the highest affinity ligands [2]. And when implemented using a catch-and-release (CaR) format (i.e., CaR-ESI-MS), the assay enables the screening of libraries against high molecular weight (MW) protein assemblies, such as viral capsid protein particles, for which the protein-ligand complexes are difficult to analyze directly by ESI-MS [3]. A semi-quantitative ESI-MS assay suitable for screening natural compound libraries at unknown concentrations, such as mixtures of human milk oligosaccharides isolated from breast milk, against target proteins to identify interactions and rank their affinities was also recently described [4].

While these ESI-MS assays allow for the detection and quantification of protein-ligand interactions *in vitro*, they do not, on their own, provide insight into the location or nature of the interactions. There is evidence that intermolecular interactions are largely preserved upon transfer of protein-ligand complexes from solution to the gas phase using ESI from studies of several protein-ligand complexes using the blackbody infrared radiative dissociation/functional group replacement strategy [5-10]. Consequently, one possible strategy for localizing ligand binding sites involves so-called “top-down” MS using electron-mediated activation methods, such as electron capture dissociation (ECD) and electron transfer dissociation (ETD). With this approach, the identification of peptide fragments retaining the ligand serves to localize the

binding site(s). To date, binding sites of several protein–ligand complexes [5-11], including  $\alpha$ -synuclein-spermine [9], adenylate kinase-ATP [10] and anterior gradient-2-PTTIYY hexapeptide interactions [11], were identified using this approach.

In order for such a top-down approach to be effective for ligand localization, there must be extensive fragmentation of the protein-ligand complex ions in the gas phase. One of the limitations of the electron-mediated dissociation methods is the low efficiency of the electron transfer/capture processes [6,11-16]. Because electron capture cross sections increase quadratically with charge state, the efficiency of the process can be improved by increasing the charge state of the gaseous complex [6]. As shown by a number of different laboratories, the charge states of proteins and protein complexes in ESI can be significantly increased through the use of so-called “supercharging reagents”, such as 3-nitrobenzoic acid (*m*-NBA) and sulfolane [17-21]. The mechanisms responsible for enhanced charging induced by supercharging agents have been extensively studied [17-23]. In a recent review, Loo and co-workers concluded that efficient positive ion supercharging reagents must be weak Brønsted bases, soluble in aqueous solution and be less (or similarly) volatile as the solvent [24]. They also advanced the hypothesis that high concentrations of the supercharging reagent in the ESI progeny droplets reduce the extent of acidic residue ionization, which effectively results in an increase in the protonation state of the gaseous protein ions [24].

While there have been many reported examples of protein supercharging in ESI-MS, there have been relatively few studies of the effects of supercharging agents on the structures and stabilities of multiprotein and protein-ligand complexes. Loo and coworkers reported that the supercharging of several protein-ligand and multiprotein complexes using *m*-NBA was

accompanied with little or no change their native structures and non-covalent interactions [18]. More recently, it was shown by the same group that the use of sulfolane in reactive liquid sample desorption ESI (DESI)-MS can produce supercharging without destabilizing protein–ligand complexes [25]. However, studies have also shown that the structures and interactions of other protein complexes are susceptible to supercharging agents. For example, heme loss from myoglobin was observed upon addition of *m*-NBA or sulfolane to solution [19-21].

The goal of the present study is to investigate the influence of sulfolane on ESI-MS measurements of protein-ligand affinities *in vitro*. With this in mind, ESI-MS binding measurements were carried out on four protein-carbohydrate complexes in the presence and absence of sulfolane. The interactions between lysozyme (Lyz) and the tetrasaccharide ligand  $\beta$ -D-GlcNAc-(1→4)- $\beta$ -D-GlcNAc-(1→4)- $\beta$ -D-GlcNAc-(1→4)-D-GlcNAc (**L1**), a single chain variable fragment (scFv) of the monoclonal antibody Se155-4 and the trisaccharide ligand  $\alpha$ -D-Gal-(1→2)-[ $\alpha$ -D-Abe-(1→3)]- $\alpha$ -D-Man-OCH<sub>3</sub> (**L2**), cholera toxin B subunit homopentamer and the GM1 pentasaccharide  $\beta$ -D-Gal-(1→3)- $\beta$ -D-GalNAc-(1→4)-[ $\alpha$ -D-Neu5Ac-(2→3)]- $\beta$ -D-Gal-(1→4)- $\beta$ -D-Glc (**L3**) and a fragment of galectin 3 and the tetrasaccharide ligand  $\alpha$ -L-Fuc-(1→2)- $\beta$ -D-Gal-(1→3)- $\beta$ -D-GlcNAc-(1→3)- $\beta$ -D-Gal-(1→4)- $\beta$ -D-Glc (**L4**) served as model systems for this study. Having found evidence that sulfolane generally reduces the apparent affinity, a detailed study of the origin of the reduced affinity was undertaken using the Lyz-**L1** interaction as a model. Measurements were carried out using isothermal titration calorimetry (ITC), circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy to establish how sulfolane affects the structure and stability of the protein-tetrasaccharide complex in solution. Finally, the effect of sulfolane on protein-ligand affinity measurements

carried out using liquid sample DESI-MS was explored.

## **Experimental**

### **Materials**

Ubiquitin (Ubq, MW 8565 Da), lysozyme from chicken egg white (Lyz, MW 14310 Da), cholera toxin B subunit homopentamer from *Vibrio cholera* (CTB<sub>5</sub>, MW 58 kDa) were purchased from Sigma-Aldrich Canada (Oakville, Canada). The C-terminal fragment (residues 107-250) of human galectin-3 (Gal-3C, MW 16340 Da) and single chain variable fragment (scFv, MW 26539 Da) of the monoclonal antibody Se155-4 were produced and purified as described previously [26, 27]. The tetrasaccharide **L1** (MW 830.3 Da) was purchased from Dextra Science and Technology Centre (Reading, UK); the trisaccharide **L2** (MW 486.5 Da) was a gift from Prof. D. Bundle (University of Alberta); the GM1 pentasaccharide **L3** (MW 998.3 Da) was purchased from HyTest Ltd. (Turku, Finland) and the tetrasaccharide **L4** (MW 854.6 Da) was purchased from IsoSep AB (Tullinge, Sweden). Stock solutions of each protein (in 200 mM ammonium acetate) and each oligosaccharide (in deionized water) were prepared and stored at -20 °C until needed.

### **Mass Spectrometry**

A Synapt G2 quadrupole-ion mobility separation-time-of-flight (Q-IMS-TOF) mass spectrometer (Waters UK Ltd., Manchester, UK) with an 8k quadrupole mass filter and a Synapt G2S Q-IMS-TOF mass spectrometer (Waters, Manchester, UK) with a 32k quadrupole mass filter were used for ESI-MS and liquid sample DESI-MS measurements. Both instruments were equipped with nanoflow ESI (nanoESI) sources, which were operated in positive ion mode. NanoESI tips (~5 µm i.d.) were produced from borosilicate capillaries (1.0 mm o.d., 0.68 mm i.d.), using a P-1000 micropipette puller (Sutter Instruments, Novato, CA, USA). A capillary

voltage of 1.0 - 1.3 kV was applied to a platinum wire to initiate the spray on the Synapt G2 instrument, where the platinum wire was inserted into the nanoESI tip, and a Capillary voltage of between 0.8 and 1.0 kV applied to the Synapt G2S instrument. In both instruments, the Cone voltage was maintained at 30 V and the source block temperature was 60 °C. The Trap and Transfer collision energies were 5 V and 2 V, respectively, and the argon pressures in the Trap and Transfer regions were  $8.56 \times 10^{-3}$  mbar and  $8.62 \times 10^{-3}$  mbar, respectively, for the Synapt G2S and  $9.17 \times 10^{-3}$  mbar and  $9.28 \times 10^{-3}$  mbar, respectively, for the Synapt G2. For the liquid sample DESI-MS measurements, a modified OMNI SPRAY Ion Sources 2-D OS-6205 (Prosolia Inc., Indianapolis, IN) was used. Details of the experimental setup can be found elsewhere [28]. Briefly, the liquid sample solution was delivered through a silica capillary (360 nm o.d., 100 nm i.d.) at a flow rate of  $15 \mu\text{L h}^{-1}$  using a syringe pump. The outlet of the sample capillary was located between ESI spray tip and the inlet to the mass spectrometer. The ESI solution flow rate was  $2\text{-}3 \mu\text{L min}^{-1}$ . Capillary and Cone voltages of 3.5 kV and 30 V, respectively, were used and the pressure of the  $\text{N}_2$  nebulizing gas was 65 psi. The source block temperature was the same as for the ESI-MS binding measurements. All data were processed using MassLynx software (v4.1).

### **Isothermal Titration Calorimetry**

The ITC affinity measurements performed on the Lyz-**L1** interaction were carried out using a VP-ITC (MicroCal, Inc., Northampton, MA). For each ITC experiment, the Lyz solution (0.2 mM) in the sample cell was titrated with a solution of **L1** (2 mM); both the Lyz and **L1** solutions were aqueous ammonium acetate (50 mM, pH 6.8, 25 °C) containing varying percentages of sulfolane (2%, 5% or 10%).

### **Circular Dichroism**

Circular dichroism (CD) spectra were recorded at 25 °C on an OLIS DSM CARY-17 spectrophotometer conversion and circular dichroism module (On-line Instrument Systems Inc.) using a 0.2 mm path length quartz cuvette. Protein solutions (56 μM) were prepared in phosphate buffer (20 mM, pH 7.0) and varying percentages of sulfolane (0 to 20%). Data were collected in scanning mode from 250 to 190 nm and the average value of five repetitions was reported. Data were analyzed with OLIS Spectral Works (v4.3) and converted into molar ellipticity units. For each solution, the CD spectrum of the solvent alone was subtracted from the sample spectrum.

### **NMR spectroscopy**

All NMR spectra were acquired at 27 °C on a 700 MHz Agilent/Varian spectrometer equipped with a cold probe (Agilent Technologies Inc., CA). 1D  $^1\text{H}$  and TROESY [29] NMR spectra were obtained for **L1** in  $\text{D}_2\text{O}$  and in 2% (v/v) sulfolane– $\text{D}_2\text{O}$ . The spectra were referenced to an external standard of acetone (2.22 ppm for  $^1\text{H}$ ), and the intensity of the residual HOD peak was decreased using a presaturation pulse sequence, irradiating at 4.76 ppm. The spectral window was 8389 Hz (from 10.76 to –1.22 ppm). The 1D  $^1\text{H}$  spectra were acquired in 16 transients, and no window functions were applied in the Fourier transformation. The TROESY spectra were acquired with 8 transients in F2, 256 increments in F1, and a mixing time of 0.4 s. Sine-bell functions were applied interactively to improve signal-to-noise in the TROESY, but no line-broadening was used.

Natural abundance  $^1\text{H}$ – $^{15}\text{N}$  gHSQC NMR spectra were obtained for Lyz and Lyz–**L1** complex in 10% (v/v)  $\text{D}_2\text{O}$ – $\text{H}_2\text{O}$  with varying concentrations of sulfolane – 0, 2, 5, and 10% (v/v). The Lyz concentration was 5 mM, and the **L1** concentration was 20 M. The spectra were acquired in a 5 mm  $\text{D}_2\text{O}$ -matched Shigemi tube at 27 °C on a 700 MHz Agilent/Varian

spectrometer equipped with a cold probe and referenced using the default parameters in the VNMR software. The spectral window for  $^1\text{H}$  was 8389 Hz (from 10.71 to  $-1.26$  ppm), and the spectral window for  $^{15}\text{N}$  was 3545 Hz (139.8 to 90.2 ppm). The spectra were acquired with 16 transients in F2, 64 increments in F1 – except for Lyz in 10%  $\text{D}_2\text{O}$ – $\text{H}_2\text{O}$ , which was acquired with 76 increments in F1. The proton signals were decoupled during acquisition, and a  $^1J_{\text{N,H}}$  value of 90 Hz was used. Sine-bell functions were applied interactively to improve signal-to-noise, but no line-broadening was used. An additional  $^1\text{H}$ – $^{15}\text{N}$  gHSQC NMR spectrum was acquired for denatured Lyz in 8M urea and 10% (v/v)  $\text{D}_2\text{O}$ – $\text{H}_2\text{O}$ . This spectrum was acquired in a 3mm NMR tube, with 128 transients in F2 and 64 increments in F1. The remaining parameters were the same as for the folded protein.

## Data Analysis

### Average Charge State Calculation

The average charge state (*ACS*) of the protein-ligand complexes ions was calculated from the ESI mass spectrum using eq 1:

$$ACS = \frac{\sum_n [Ab(\text{PL}^{n+}) \times n]}{\sum_n Ab(\text{PL}^{n+})} \quad (1)$$

where  $Ab(\text{PL}^{n+})$  is the abundance of the protein-ligand complex ions and  $n$  is the charge state.

The *ACS* of the protein ions was calculated in the same way.

### Association Constants from ESI Mass Spectra

The general procedure for determining association constants ( $K_a$ ) for protein-ligand interactions from ESI mass spectra has been described in detail elsewhere [28,30-32] and only a brief description is given for the case where the protein has single ligand binding site. The assay relies

on the detection and quantification of the gas-phase ions of the free and ligand-bound protein. Following correction of the mass spectrum for the occurrence of nonspecific ligand-protein binding during the ESI process using the reference protein method [31, 32], the ratio ( $R$ ) of the total abundance ( $Ab$ ) of ligand-bound protein (PL) to free protein (P) ions is taken to be equal to the concentration ratio of PL to P, eq 2:

$$R = \frac{\sum Ab(PL)}{\sum Ab(P)} = \frac{[PL]}{[P]} \quad (2)$$

and  $K_a$  calculated from eq 3:

$$K_a = \frac{R}{[L]_0 - \frac{R}{1+R}[P]_0} \quad (3)$$

where  $[P]_0$  and  $[L]_0$  are the initial protein and ligand concentrations, respectively. Details on the determination of  $K_a$  for the stepwise binding of **L3** to CTB<sub>5</sub> [33, 34] are given in Supplementary Data.

## Results and Discussion

### Influence of sulfolane on protein-carbohydrate affinities

*Direct ESI-MS binding measurements.* ESI-MS binding measurements were carried out on four protein-carbohydrate complexes to assess whether protein-ligand interactions are generally influenced by the presence of sulfolane in solution. Shown in Figures 1 and 2 are representative ESI mass spectra acquired in positive ion mode for aqueous ammonium acetate solutions (50 mM, pH 6.8 and 25 °C) containing each protein/carbohydrate ligand pair in the absence and presence of sulfolane (H<sub>2</sub>O:sulfolane 98:2 v/v). A reference protein ( $P_{ref}$ ) was added to each solution in order to identify the occurrence of nonspecific carbohydrate-protein binding during the ESI process [31, 32].

Inspection of the mass spectrum measured for a solution of Lyz (10  $\mu\text{M}$ ), **L1** (15  $\mu\text{M}$ ) and  $\text{P}_{\text{ref}}(\text{Ubq}, 5 \mu\text{M})$  reveals the presence of protonated  $\text{Lyz}^{n+}$  and  $(\text{Lyz} + \mathbf{L1})^{n+}$  ions, at  $n = 7$  and  $8$ , together with  $\text{Ubq}^{n+}$  ions at  $n = 5$  and  $6$  (Figure 1a). The absence of  $(\text{P}_{\text{ref}} + \mathbf{L1})^{n+}$  ions indicates that nonspecific binding was negligible under these experimental conditions. From the ratio of the total abundance of  $(\text{Lyz} + \mathbf{L1})^{n+}$  to  $\text{Lyz}^{n+}$  ions and the known initial concentrations, a  $K_a$  value of  $(7.6 \pm 0.2) \times 10^4 \text{ M}^{-1}$  was determined from the mass spectrum (eqs 2 and 3). This value is in reasonable agreement with previously reported values,  $0.8\text{-}1.1 \times 10^5 \text{ M}^{-1}$  [28, 35, 36]. Upon addition of 2% sulfolane to the solution, the charge states of the  $\text{Lyz}^{n+}$  and  $(\text{Lyz} + \mathbf{L1})^{n+}$  ions shifted to  $n = 8 - 12$  (Figure 1b). This shift corresponds to an increase of 35% in ACS for the  $(\text{Lyz} + \mathbf{L1})^{n+}$  ions; the  $\text{Lyz}^{n+}$  ions exhibited a similar increase in ACS (~40% increase). The  $\text{P}_{\text{ref}}^{n+}$  ions also shifted to higher charges states,  $n = 6$  to  $10$ , which corresponds to a 50% increase in ACS. Also evident in the mass spectrum are  $\text{Lyz}^{n+}$  and  $(\text{Lyz} + \mathbf{L1})^{n+}$  ions bound to one or more sulfolane molecules. Similar observations have been reported for other proteins analyzed in the presence of sulfolane by ESI-MS [37]. Notably, there is a significant reduction in the abundance of  $(\text{Lyz} + \mathbf{L1})^{n+}$  ions, relative to  $\text{Lyz}^{n+}$  ions; the measured  $K_a$  is  $(4.1 \pm 0.1) \times 10^4 \text{ M}^{-1}$ , which corresponds to a 46% decrease in affinity compared to the value measured for the same solution but in the absence of sulfolane.

Analogous measurements were carried out on solutions with up to 10% sulfolane. Plotted in Figure 3 are the ACS values determined for the  $(\text{Lyz} + \mathbf{L1})^{n+}$  ions and the corresponding  $K_a$  values measured for the solutions at different percentages of sulfolane. It can be seen that the dependence of ACS on sulfolane concentration is most pronounced up to ~2% sulfolane; at higher concentrations only a slight increase in ACS was observed, reaching a value of  $(10.93 \pm$

0.01) at 10% sulfolane. The dependence of  $K_a$  is also most pronounced at the lower concentrations of sulfolane, but the affinity continues to decrease with increasing sulfolane concentrations, dropping to  $(5.5 \pm 0.3) \times 10^3 \text{ M}^{-1}$  at 10% sulfolane. This change in  $K_a$  corresponds to a 93% decrease in affinity.

The results obtained for the scFv-**L2**, CTB<sub>5</sub>-**L3** and Gal3C-**L4** interactions are qualitatively similar to those described above for the Lyz-**L1** system. Shown in Figures 1c and 1d are the ESI mass spectra for a solution of scFv (10  $\mu\text{M}$ ), **L2** (10  $\mu\text{M}$ ) and P<sub>ref</sub>(Lyz, 5  $\mu\text{M}$ ), in the absence and presence of sulfolane, respectively. In the absence of sulfolane, protonated scFv<sup>n+</sup> and (scFv + **L2**)<sup>n+</sup> ions, at n = 9 to 12, together with P<sub>ref</sub><sup>n+</sup> ions at n = 8 to 11, were detected. The measured  $K_a$  value for the (scFv + **L2**) complex,  $(8.2 \pm 0.1) \times 10^4 \text{ M}^{-1}$ , is in good agreement with the reported values, which are in the  $0.66\text{-}1.2 \times 10^5 \text{ M}^{-1}$  range [28, 38]. Upon addition of sulfolane, the ACS of the scFv<sup>n+</sup> and (scFv + **L2**)<sup>n+</sup> ions increased by ~14% and the presence of sulfolane adducts was evident. The measured  $K_a$ ,  $(2.9 \pm 0.1) \times 10^3 \text{ M}^{-1}$ , is 96% smaller than the value  $((8.2 \pm 0.1) \times 10^4 \text{ M}^{-1})$  measured in the absence of sulfolane. Shown in Figures 2a and 2b are representative ESI mass spectra for a solution of CTB<sub>5</sub> (5  $\mu\text{M}$ ), **L3** (5  $\mu\text{M}$ ) and P<sub>ref</sub>(scFv, 3  $\mu\text{M}$ ) in the absence and presence of sulfolane, respectively. In the absence of sulfolane, protonated CTB<sub>5</sub><sup>n+</sup> and (CTB<sub>5</sub> + q**L3**)<sup>n+</sup> ions, where q = 1 – 4, at n = 15 to 18 are detected, along with P<sub>ref</sub><sup>n+</sup> ions at n = 10 to 13. The association constants for the stepwise binding (i.e.,  $K_{a,q}$ ) of three molecules of **L3** to CTB<sub>5</sub>  $((1.5 \pm 0.1) \times 10^7 \text{ M}^{-1}$  ( $K_{a,1}$ ),  $(8.1 \pm 0.1) \times 10^6 \text{ M}^{-1}$  ( $K_{a,2}$ ) and  $(5.4 \pm 0.1) \times 10^6 \text{ M}^{-1}$  ( $K_{a,3}$ )) are consistent with reported values ( $K_{a,1} = 1.6 \times 10^7 \text{ M}^{-1}$ ,  $K_{a,2} = 7.3 \times 10^6 \text{ M}^{-1}$  and  $K_{a,3} = 3.8 \times 10^6 \text{ M}^{-1}$ ) [34]. Upon addition of sulfolane, the ACS of the CTB<sub>5</sub><sup>n+</sup> and (CTB<sub>5</sub> + q**L3**)<sup>n+</sup> ions increased by ~ 6%, while the  $K_{a,q}$  values decreased by 98%  $((2.6 \pm 0.1) \times 10^5 \text{ M}^{-1}$

( $K_{a,1}$ ),  $(1.3 \pm 0.1) \times 10^5 \text{ M}^{-1}$  ( $K_{a,2}$ ) and  $(1.1 \pm 0.1) \times 10^5 \text{ M}^{-1}$  ( $K_{a,3}$ )). ESI mass spectra measured for a solution of Gal3C (10  $\mu\text{M}$ ), **L4** (10  $\mu\text{M}$ ) and  $P_{\text{ref}}$ (Ubq, 6  $\mu\text{M}$ ), in the absence and presence of sulfolane are shown in Figures 2c and 2d, respectively. In the absence of sulfolane, protonated Gal3C<sup>n+</sup> and (Gal3C + **L4**)<sup>n+</sup> ions, at n = 7 to 9, together with  $P_{\text{ref}}$ <sup>n+</sup> ions at n = 5 to 6, were detected. The measured  $K_a$ ,  $(1.45 \pm 0.02) \times 10^5 \text{ M}^{-1}$ , is similar to the reported value,  $2 \times 10^5 \text{ M}^{-1}$  [39]. The introduction of sulfolane to the solution resulted in an increase of 28% in the ACS of the Gal3C<sup>n+</sup> and (Gal3C + **L4**)<sup>n+</sup> ions; sulfolane adducts were also observed. The  $K_a$ ,  $(1.25 \pm 0.02) \times 10^5 \text{ M}^{-1}$ , is ~14% lower than the value measured in the absence of sulfolane ( $(1.45 \pm 0.02) \times 10^5 \text{ M}^{-1}$ ).

The present results suggest that protein-carbohydrate affinities measured by ESI-MS are generally sensitive to the presence of sulfolane, and that the magnitude of the reduction in affinity (at a given sulfolane concentration) is dependent on the nature of the protein-carbohydrate interactions. However, it is not clear from the ESI-MS data alone whether this effect occurs in bulk solution, during the ESI process or as a result of in-source (gas-phase) dissociation. With the goal of establishing the origin(s) of the reduced affinity (as measured by ESI-MS), ITC measurements, CD spectroscopy and NMR spectroscopy experiments were used to quantify the Lyz-**L1** interaction in the absence and presence of sulfolane and to establish whether the sulfolane influences the higher order structure of Lyz or the Lyz-**L1** complex in solution, or interacts strongly with **L1** or Lyz.

#### **Influence of sulfolane on the (Lyz + L1) complex in solution.**

Isothermal titration calorimetry was used to quantify the effect of sulfolane on the stability of the (Lyz + **L1**) complex in solution. Binding measurements were carried out for aqueous solutions of

Lyz (0.2 mM) and **L1** (2.0 mM) with 2%, 5%, and 10% sulfolane, respectively (Figures S1, S2 and S3, Supplementary Data). Plotted in Figure 3 are the corresponding  $K_a$  values, as well as the reported value measured in the absence of sulfolane [28], together with the values measured by ESI-MS. Notably, the ITC data reveal that sulfolane reduces the thermodynamic stability of the Lyz-**L1** interaction in solution. Moreover, the  $K_a$  values measured by ESI-MS are in good agreement with the values determined by ITC. This finding confirms that ESI-MS provides an accurate measure of the distribution of free and **L1**-bound Lyz in the sulfolane-containing solutions, which implies that the (Lyz + **L1**) complex does not dissociate during the ESI process or in the gas phase (i.e., in-source dissociation).

The decrease in  $K_a$  upon introduction of sulfolane to solution could be due to changes in higher-order structure of Lyz or due to general or specific solvent effects. CD spectroscopy was employed to assess whether the addition of sulfolane alters the secondary structure of Lyz, which contains five standard alpha helical regions, five  $\beta$ -pleated sheet regions and four disulfide bonds [40]. Shown in Figure S4 (Supplementary Data) are CD spectra measured for solutions of Lyz (56  $\mu$ M) and sulfolane (0-20%) in phosphate buffer (20 mM, pH 7, 25 °C). CD spectra of Lyz in solutions with different concentrations of sulfolane did not show significant change in the 190 – 250 nm region [41-43]. The results from CD experiments suggested that sulfolane does not significantly influence the secondary structure of Lyz in bulk solution. This is consistent with the findings of Alexander and co-workers, who previously reported that Lyz is resistant to significant structural changes in the presence of high concentrations (up to 60% (v/v)) of organic solvents (e.g., acetonitrile, tetrahydrofuran and 1-propanol) [41].

To gain further insight into the origin of the reduced affinity resulting from the addition to

sulfolane, solution NMR experiments were performed on Lyz, **L1** and the (Lyz + **L1**) complex, in the absence and presence of sulfolane (0-10%, v/v) to identify any structural changes in Lyz or the (Lyz + **L1**) complex. Natural abundance  $^1\text{H}$ - $^{15}\text{N}$  gHSQC NMR spectra for Lyz in 10% (v/v)  $\text{D}_2\text{O}$ - $\text{H}_2\text{O}$  and with varying concentrations of sulfolane (0, 2, 5, and 10% (v/v)) are shown in Figures S5 and S6 (Supplementary Data), respectively. Backbone NH signals for Lyz were assigned based on comparisons with literature values [44, 45]. The addition of sulfolane does not cause major changes in the  $^{15}\text{N}$  chemical shifts of the backbone NH peaks (Figure S6, Supplementary Data), and Lyz remains folded at sulfolane concentrations of up to 10% (Figures S6 and S7, Supplementary Data). Notably, the resonances with the largest  $^1\text{H}$  chemical shift changes – R5, A42, S50, C80, S81, S100, N106, W108, K116, A122, L124, R128 – are not part of the **L1** binding site [46-48].

Alternatively, the reduced affinity could be due to interactions between sulfolane and **L1** or the (Lyz + **L1**) complex.  $^1\text{H}$  NMR spectra of **L1** in  $\text{D}_2\text{O}$  and in 2% (v/v) sulfolane- $\text{D}_2\text{O}$  are shown in Figure S8 (Supplementary Data). None of the chemical shifts or coupling constants for the **L1** change with the addition of sulfolane, which indicates sulfolane does not form any strong interactions with **L1**. To determine whether the addition of sulfolane affects the conformation of **L1**, TROESY spectra [29] were acquired in both solvent systems. The signals for **L1** are unchanged in both spectra (Figure S9, Supplementary Data), and there are no cross peaks between **L1** and sulfolane, which means sulfolane does not change the conformation of **L1** in solution. Natural abundance  $^1\text{H}$ - $^{15}\text{N}$  gHSQC NMR spectra for the (Lyz + **L1**) complex in 10% (v/v)  $\text{D}_2\text{O}$ - $\text{H}_2\text{O}$  and with varying concentrations of sulfolane (0, 2, 5, and 10% (v/v)) are shown in Figures S10 and S11 (Supplementary Data), respectively. The spectra for Lyz with and without

**L1** are very similar (Figure S10, Supplementary Data), as expected because the structure of Lyz changes very little upon ligand binding [49]. Three new signals are observed for the amide groups in **L1** [50]. The addition of sulfolane to the (Lyz + **L1**) complex causes similar changes in the spectra to those observed for Lyz alone (compare Figures S6 and S11, Supplementary Data).

Taken together, the results of the ITC, CD and NMR spectroscopy measurements reveal that the reduction in the affinity of the (Lyz + **L1**) complex induced by sulfolane is not the result of significant change in protein conformation, nor from solvent effects related to sulfolane interactions with **L1** or Lyz residues located in the binding pocket. Consequently, it would seem that loss of affinity is related to a general solvent effect, whereby sulfolane increases the energetic penalty associated with solvent reorganization of the **L1** and Lyz required for binding. That the addition of sulfolane to aqueous solutions affects the hydrogen bonding network of water provides indirect support for this hypothesis [51].

### **Supercharging and liquid sample DESI-MS**

Recently, it was shown that liquid sample and reactive liquid sample DESI-MS can be used to quantify protein-ligand affinities in solution [25, 28]. An attractive feature of the liquid sample DESI-MS assay is that it is significantly more tolerant to non-volatile buffers, such as PBS, than ESI-MS [25, 28]. With this feature in mind, the application of liquid sample DESI-MS for protein-ligand affinity measurements carried out in the presence of supercharging reagents was investigated. In these experiments, the “sample solution” contained Lyz, **L1** and  $P_{ref}$ , in 50 mM aqueous ammonium acetate, while the “ESI solution” contained either ACN:H<sub>2</sub>O (50:50, v/v) or ACN:H<sub>2</sub>O:sulfolane (49.75:49.75:0.5, v/v). Shown in Figures 4a and 4b are representative liquid sample DESI mass spectra for a sample solution of Lyz (10 μM), **L1** (15 μM), and  $P_{ref}$  (Ubq, 5

$\mu\text{M}$ ), measured in the absence and presence of sulfolane, respectively. It can be seen that the ACS for  $\text{Lyz}^{n+}$  and  $(\text{Lyz} + \mathbf{L1})^{n+}$  ions increased by  $\sim 20\%$  in the presence of sulfolane, which is similar to  $\sim 24\%$  increase of ESI-MS measurements acquired using 0.5% sulfolane. Overall, however, the ACS of Lyz and  $(\text{Lyz} + \mathbf{L1})$  complex in the presence and absence of sulfolane on liquid sample DESI spectra were lower than the ACS observed on their corresponding ESI spectra when the same solutions were sprayed. The lower ACS observed in the DESI spectra, which is consistent with the results of previous studies [51-53], may be due to the presence of ACN vapour in the source region. Acetonitrile has a higher gas phase basicity ( $178.8 \text{ kcal mol}^{-1}$ ) than  $\text{H}_2\text{O}$  ( $157.9 \text{ kcal mol}^{-1}$ ) [54], and would more efficiently deprotonate the Lyz ions in the source compared to  $\text{H}_2\text{O}$  vapour.

Notably, and in contrast to what was observed with ESI-MS, there is no measurable change in the  $K_a$  value for this interaction upon addition of sulfolane ( $(1.1 \pm 0.1) \times 10^5 \text{ M}^{-1}$  (0% sulfolane) and  $(1.4 \pm 0.1) \times 10^5 \text{ M}^{-1}$  (0.5% sulfolane). This finding, which is consistent with observations made by Loo and coworkers [25], is significant in that it reveals that protein supercharging and the reduction in the  $K_a$  of the  $(\text{Lyz} + \mathbf{L1})$  complex caused by sulfolane are independent processes. The absence of a change in the distribution of bound and unbound Lyz in liquid sample DESI-MS can reasonably be explained on the basis of the limited time available for re-equilibration of the  $\text{Lyz-L1}$  interaction upon introduction of sulfolane into the droplet. In fact, it was reported recently that, using liquid sample DESI-MS implemented with  $\mathbf{L1}$  in the ESI spray solution and Lyz in the sample solution, the lifetime of the resulting droplets (containing sample) was too short for the  $\text{Lyz-L1}$  interaction to reach equilibrium [28]. That supercharging is observed in the liquid sample DESI-MS measurements carried out with sulfolane demonstrates

that the process involved in supercharging occur on a much shorter timescale than the relaxation kinetics for the Lyz-**L1** interaction.

## **Conclusions**

The results of quantitative ESI-MS binding measurements performed on four protein-carbohydrate interactions (Lyz-**L1**, scFv-**L2**, CTB<sub>5</sub>-**L3** and Gal3C-**L4**) revealed that using sulfolane to supercharge protein ions is generally accompanied by a reduction in ligand affinity, although the magnitude of the effect (at a given sulfolane concentration) is dependent on the nature of the interaction. The affinities measured by ESI-MS for the Lyz-**L1** interaction in the presence of sulfolane agree with values measured by ITC. This finding indicates that there is no dissociation of the complex during the ESI process (i.e., in the droplets) or in the gas phase (i.e. in-source dissociation). Moreover, the results of CD and NMR spectroscopy measurements reveal that sulfolane does not cause any significant change in the higher order structure of Lyz, nor does it interact strongly with **L1** or the Lyz residues that make up the binding pocket in solution. It is proposed that sulfolane weakens the protein-ligand interactions through a general solvent effect. Taken together, the present findings suggest that supercharging of the (Lyz + **L1**) complex by sulfolane is not related to changes to protein structure in bulk solution or during the ESI process. It must be stressed, though, that the possibility of sulfolane-induced protein unfolding in the ESI droplets can't be definitively ruled out based on the current results. However, if protein unfolding does take place during the ESI process, the unfolding kinetics must be significantly faster than the dissociation kinetics. Finally, binding measurements performed on the Lyz-**L1** interaction using liquid sample DESI-MS revealed that the introduction of sulfolane into the ESI solution results in protein supercharging; however, this

effect is not accompanied by any loss in affinity. This finding, in addition to having mechanistic implications, suggests an inherent advantage of liquid sample DESI-MS over direct ESI-MS for protein-ligand affinity measurements in cases where supercharging is desirable.

### **Acknowledgement**

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## Figure captions

- Figure 1.** Representative ESI mass spectra acquired in positive ion mode for aqueous ammonium acetate (50 mM) solutions of Lyz (10  $\mu$ M), **L1** (15  $\mu$ M) and P<sub>ref</sub> (Ubq, 5  $\mu$ M) with (a) 0% sulfolane and (b) 2% sulfolane, or scFv (10  $\mu$ M), **L2** (15  $\mu$ M) and P<sub>ref</sub> (Lyz, 5  $\mu$ M), with (c) 0% sulfolane and (d) 2% sulfolane. Nonspecific sulfolane adducts are labelled as “S”.
- Figure 2.** Representative ESI mass spectra acquired in positive ion mode for aqueous ammonium acetate (50 mM) solutions of CTB<sub>5</sub> (5  $\mu$ M), **L3** (5  $\mu$ M) and P<sub>ref</sub> (scFv, 3  $\mu$ M) with (a) 0% sulfolane and (b) 2% sulfolane, or Gal3-C (10  $\mu$ M), **L4** (10  $\mu$ M) and P<sub>ref</sub> (Ubq, 5  $\mu$ M) with (c) 0% sulfolane and (d) 2% sulfolane. Nonspecific sulfolane adducts are labelled as “S”.
- Figure 3.** Plot of average charge state (ACS) of the (Lyz + **L1**) ions observed from ESI mass spectra acquired for aqueous ammonium acetate (50 mM) solutions of Lyz (10  $\mu$ M), **L1** (15  $\mu$ M) and Ubq (5  $\mu$ M) with 0 – 10% sulfolane. Also shown are the association constants ( $K_a$ ) for the (Lyz + **L1**) interaction measured by ESI-MS and isothermal titration calorimetry (ITC).
- Figure 4.** Liquid sample DESI mass spectra acquired in positive ion mode for aqueous ammonium acetate (20 mM) solutions of Lyz (10  $\mu$ M), **L1** (15  $\mu$ M) and Ubq (5  $\mu$ M) using an ESI spray solvent (ACN:H<sub>2</sub>O=50:50, v/v) containing (a) 0% sulfolane and (b) 0.5% sulfolane.

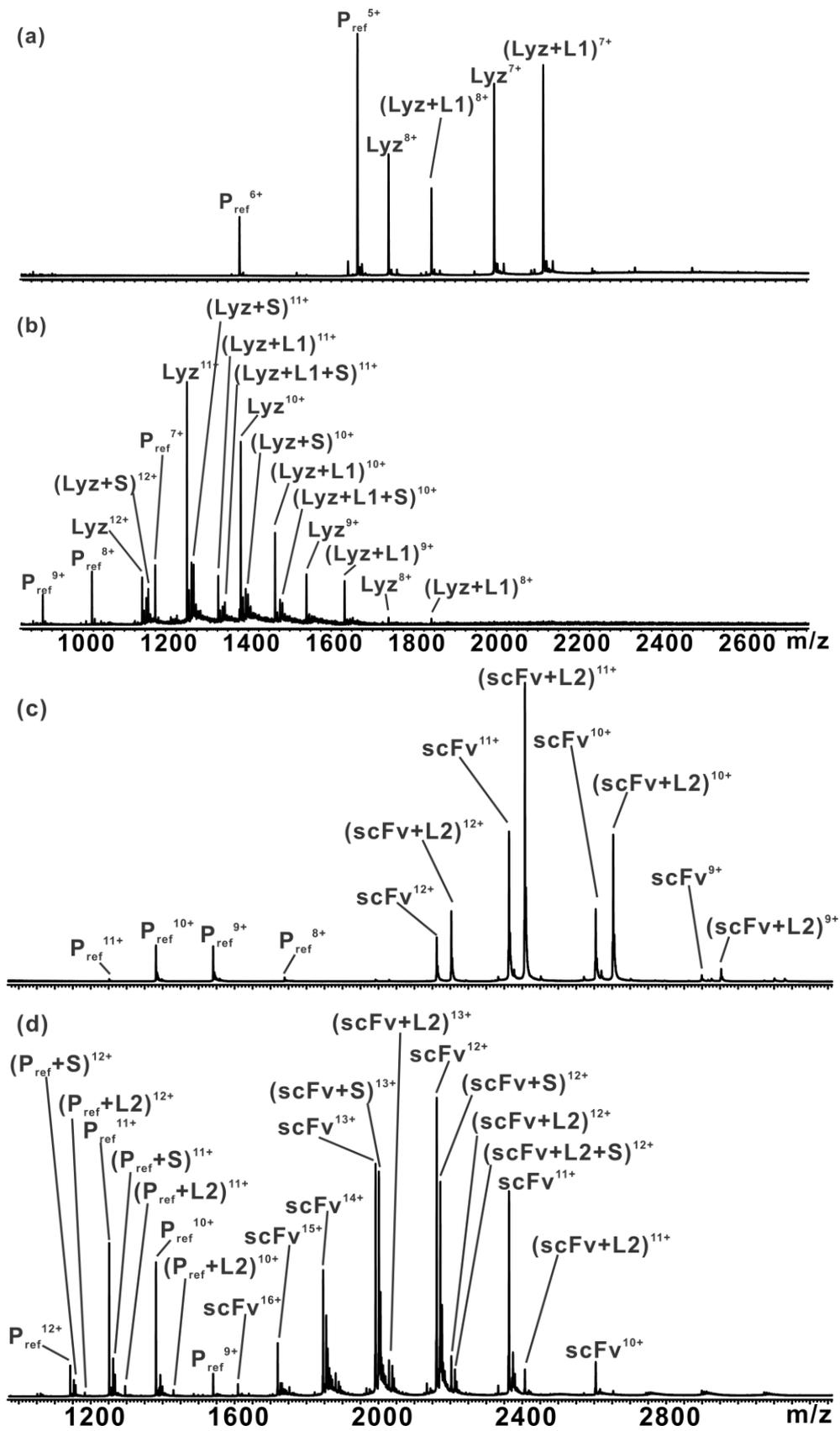


Figure 1

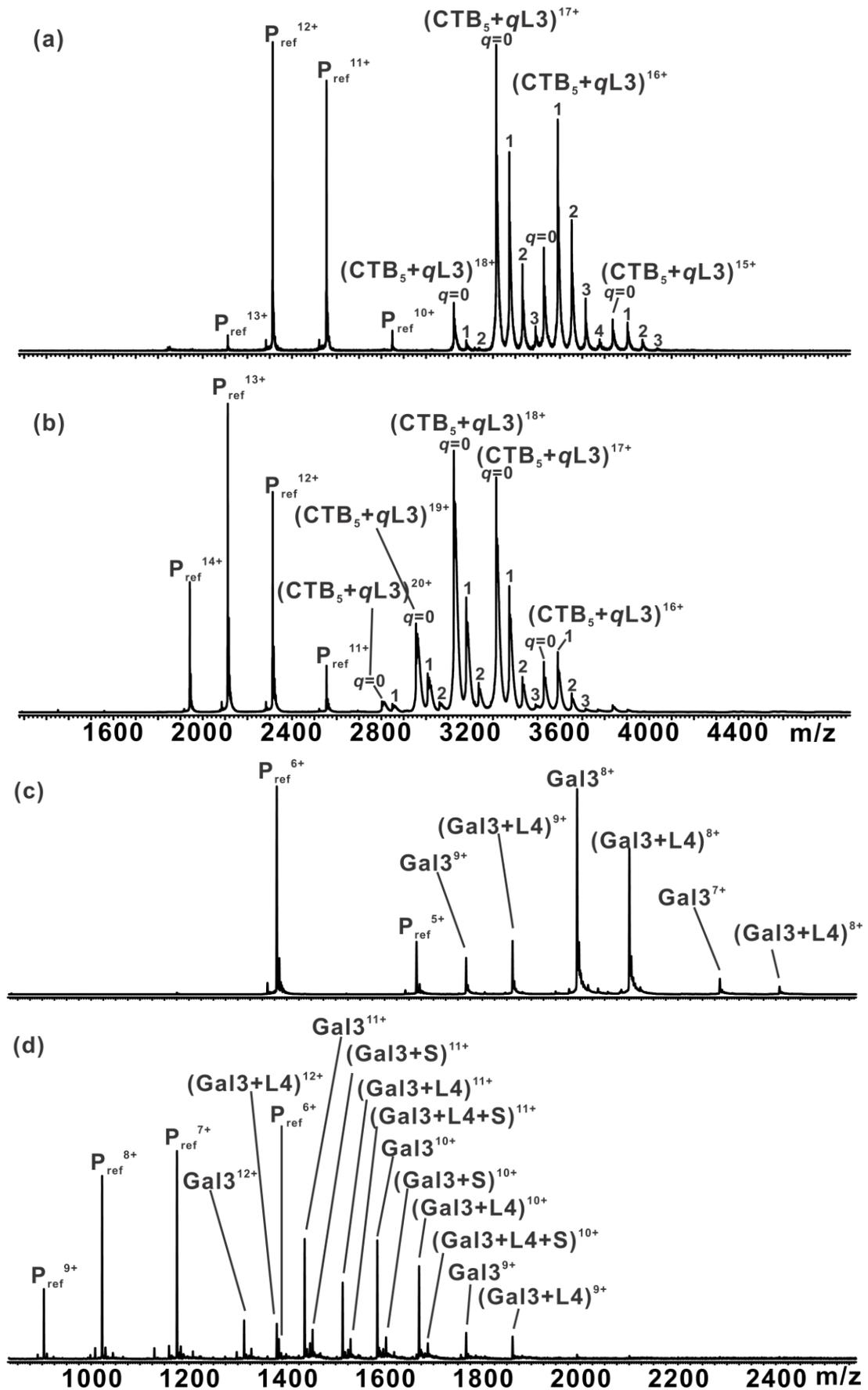


Figure 2

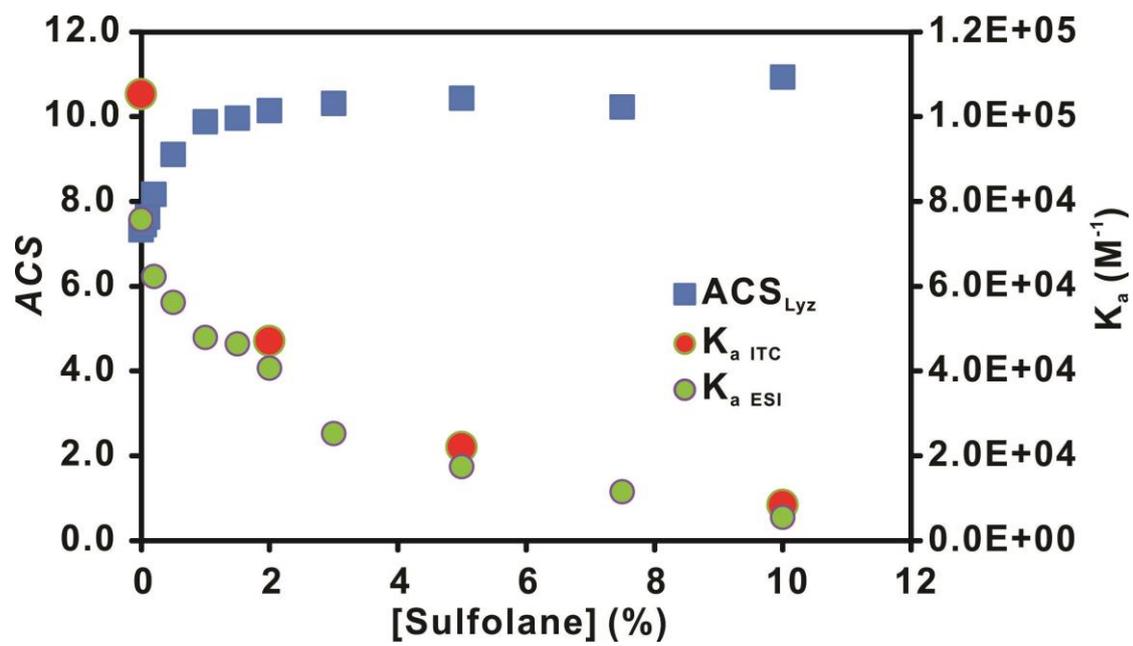


Figure 3

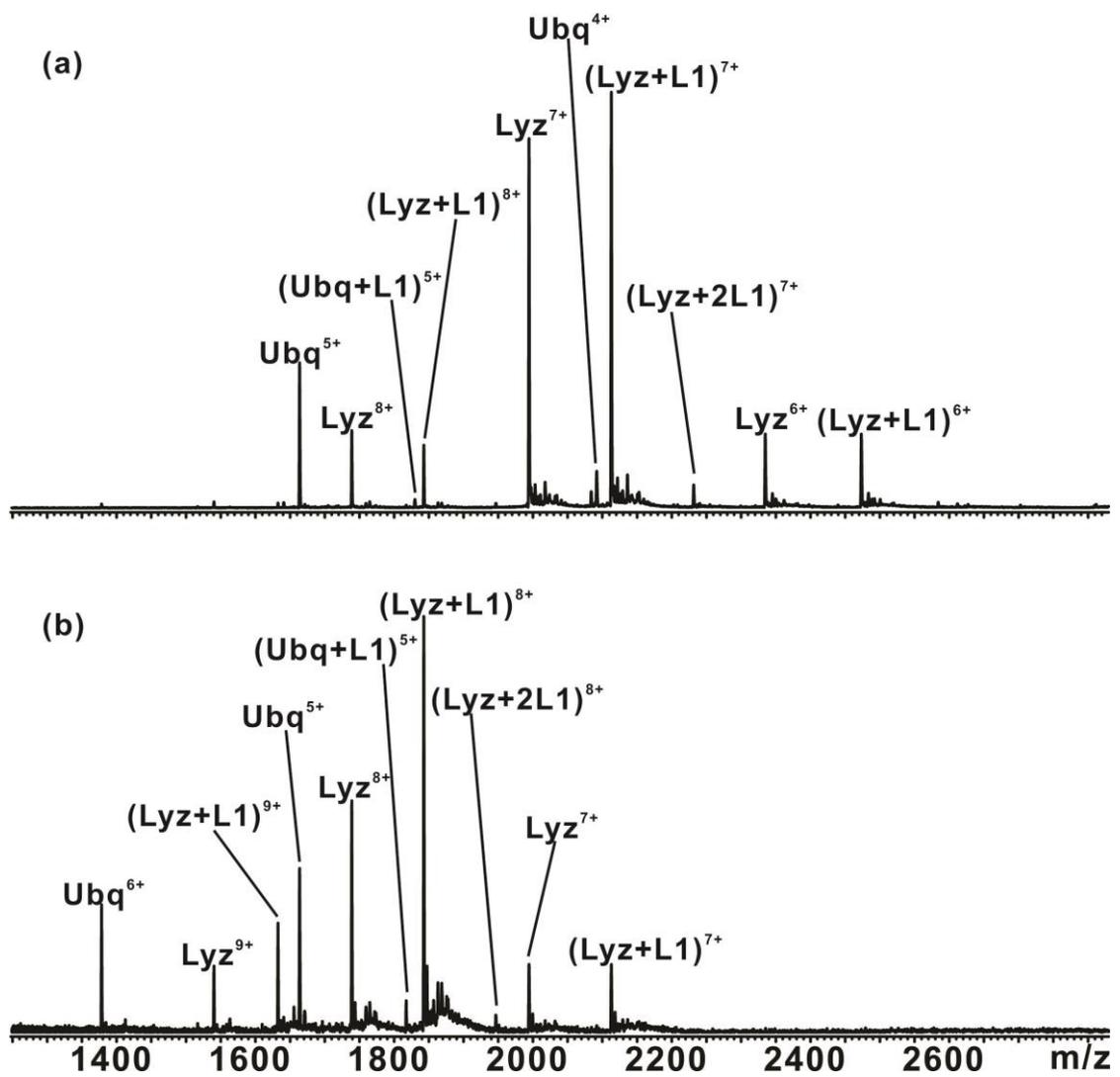


Figure 4

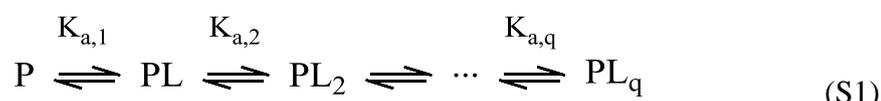
## Supplementary Data for:

### Influence of Sulfolane on ESI-MS Measurements of Protein-Ligand Affinities

Yuyu Yao, Michele R. Richards, Elena N. Kitova and John S. Klassen

#### Determination of association constants for stepwise binding of L3 to CTB<sub>5</sub>

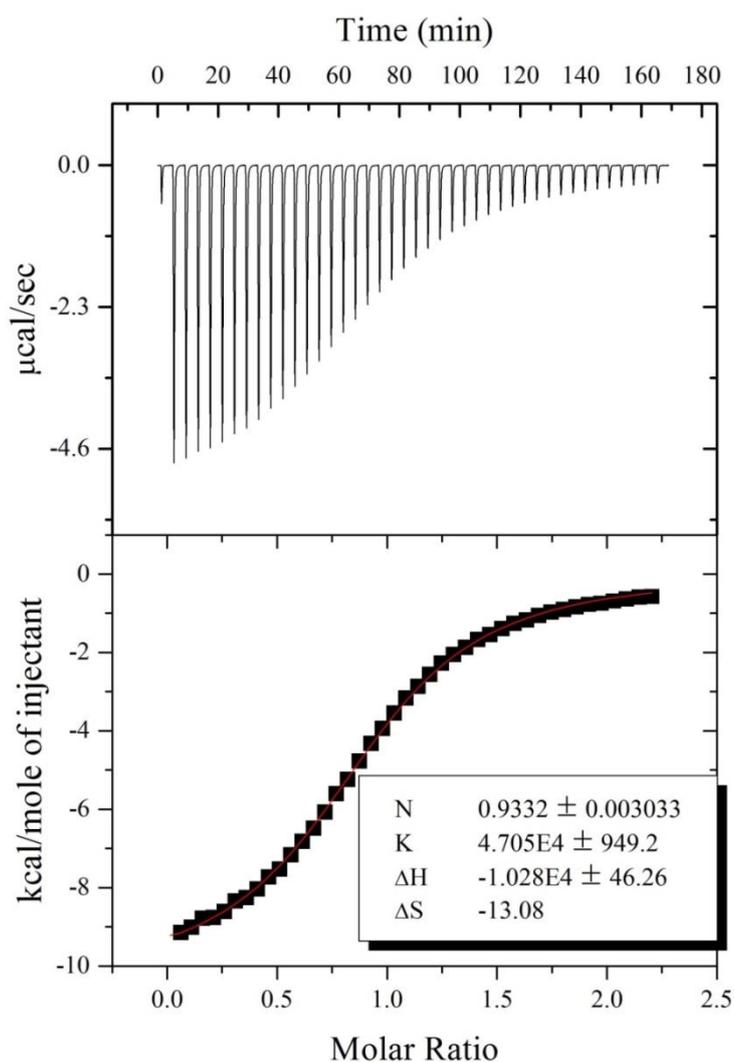
A general expression for the association constants ( $K_{a,q}$ ) for the stepwise binding of ligand (L) to protein (P) (eq S1) is given by eq S2 [1]:



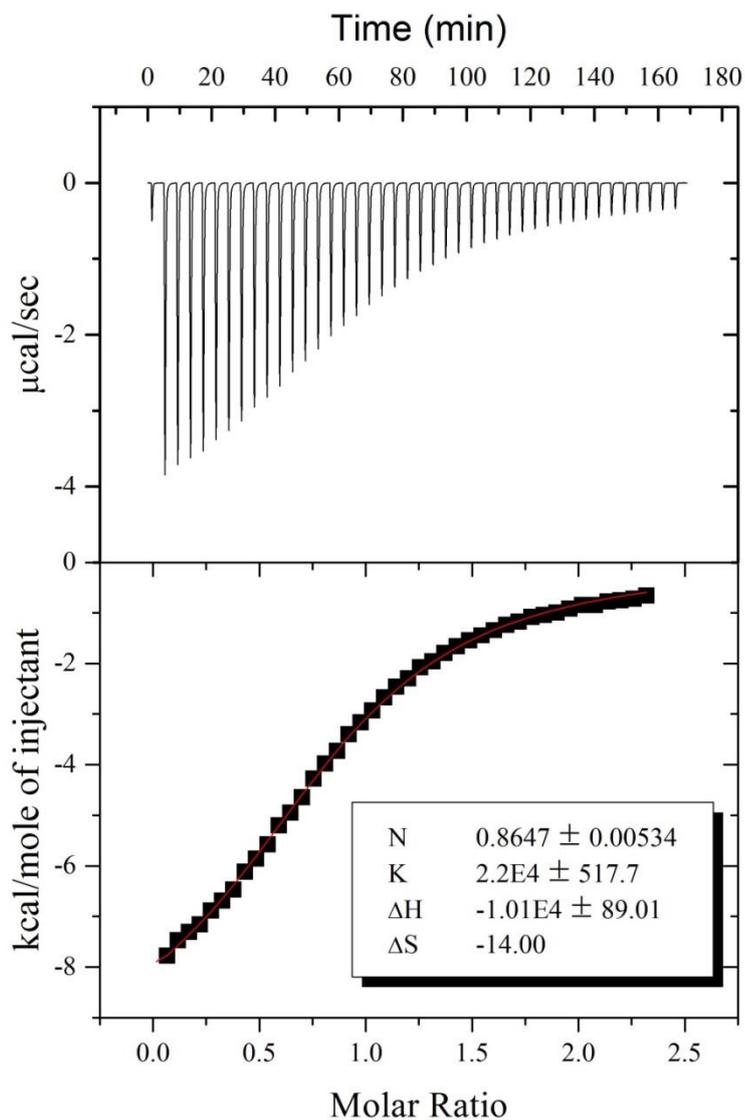
$$K_{a,q} = \frac{R_q}{R_{q-1}([L]_0 - \frac{(R_1 + 2R_2 + \dots + qR_q)[P]_0}{1 + R_1 + R_2 + \dots + R_q})} \quad (S2)$$

where  $[L]_0$  and  $[P]_0$  are the initial concentrations of L and P, respectively.  $R_q$  is the concentration ratio of ligand-bound (to q molecules of L) to free P, which is taken to be equal to the total ion abundance ratio of the corresponding gas-phase ions as determined from the ESI mass spectrum, eq S3:

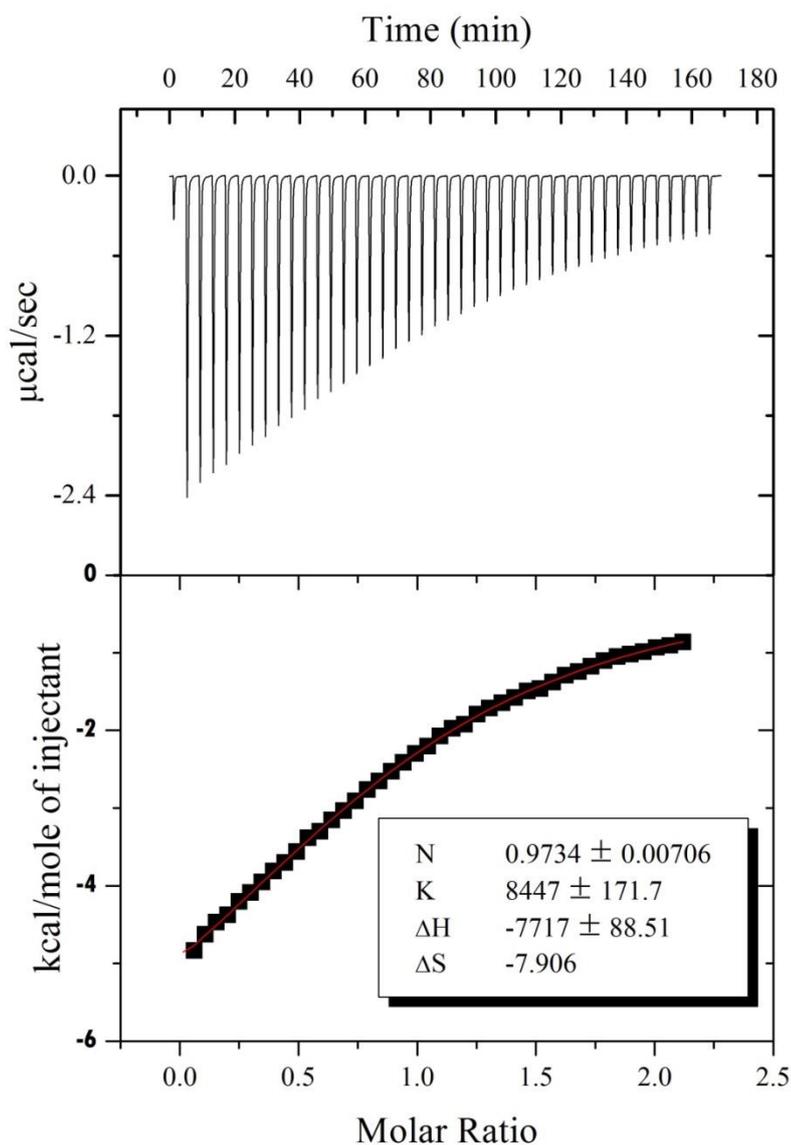
$$R_q = \frac{\sum Ab(PL_q)}{\sum Ab(P)} = \frac{[PL_q]_{eq}}{[P]_{eq}} \quad (S3)$$



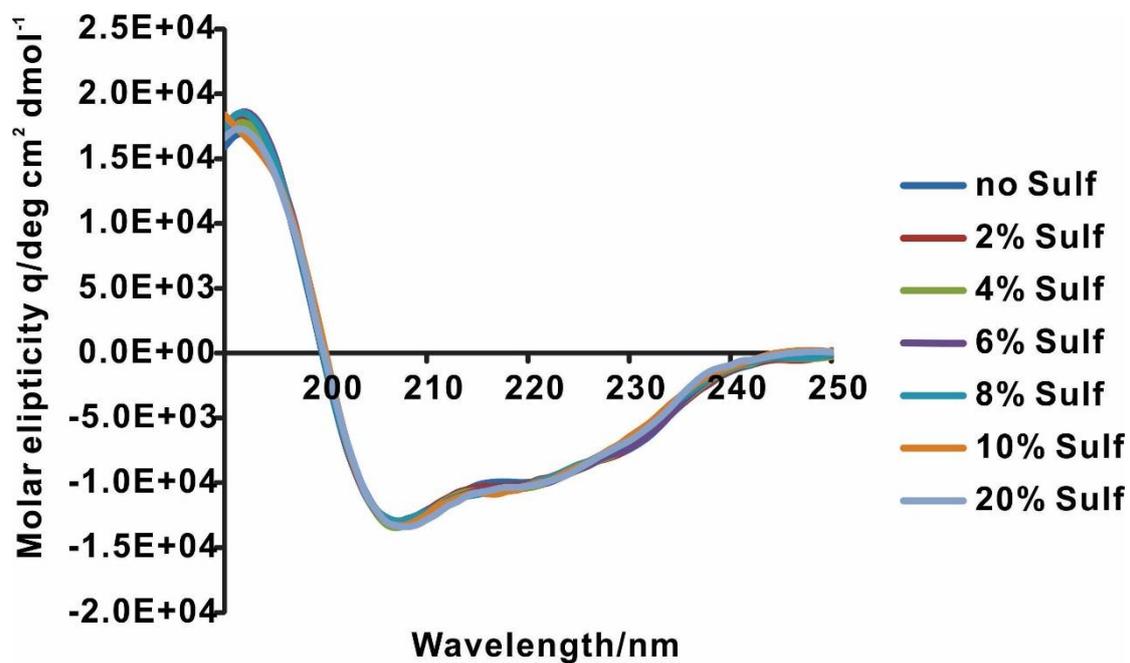
**Figure S1.** ITC profiles obtained for the binding of Lyz (0.2 mM) to **L1** (2.0 mM) in aqueous ammonium acetate (50 mM, pH 6.8 and 25 °C) solution containing 2% (v/v) sulfolane. Raw data are shown in the top panel, in the lower panel integrated data (black squares) and fitting curve (solid red line) obtained using Origin 8.0 and a single site binding model, are presented. The inset in the lower panel shows best fit parameters: stoichiometry (N), association constant ( $K \equiv K_a$ ) and enthalpy of association ( $\Delta H$ ). The entropy of association ( $\Delta S$ ) was calculated from the free energy ( $\Delta G = -RT \ln K$ ) and enthalpy values ( $\Delta G = \Delta H - T\Delta S$ ).



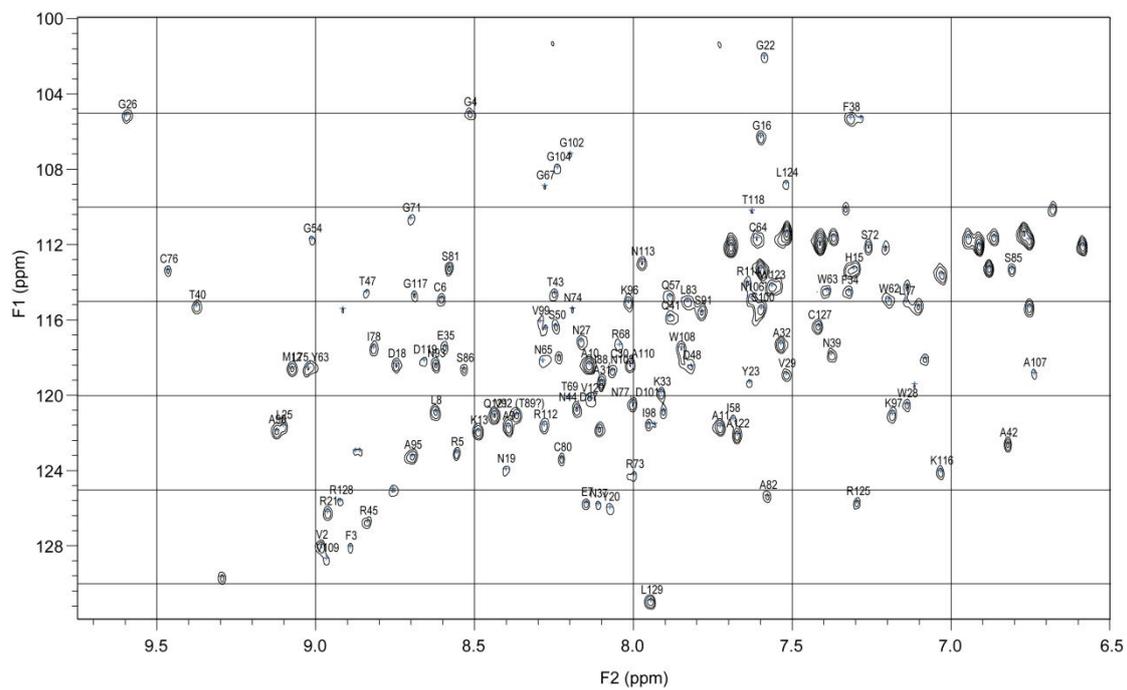
**Figure S2.** ITC profiles obtained for the binding of Lyz (0.2 mM) to L1 (2.0 mM) in aqueous ammonium acetate (50 mM, pH 6.8 and 25 °C) solution containing 5% (v/v) sulfolane. Raw data are shown in the top panel, in the lower panel integrated data (black squares) and fitting curve (solid red line) obtained using Origin 8.0 and a single site binding model, are presented. The inset in the lower panel shows best fit parameters as described in **Figure S1**.



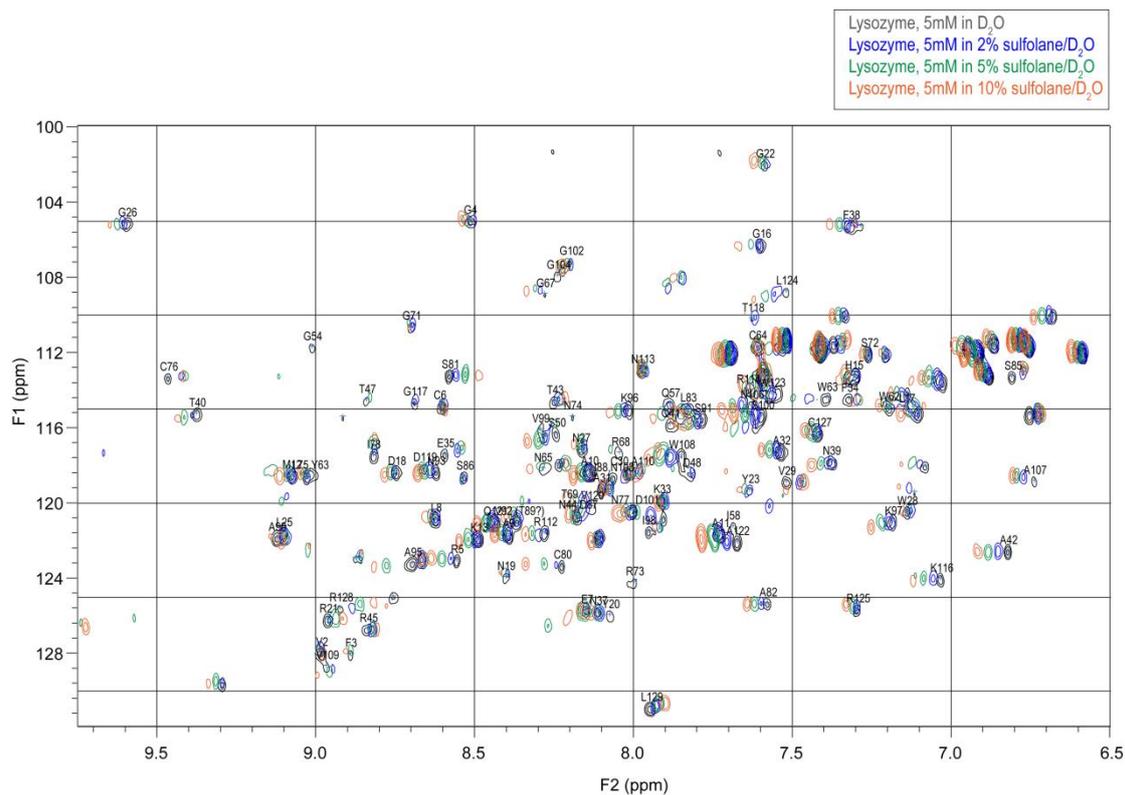
**Figure S3.** ITC profiles obtained for the binding of Lyz (0.2 mM) to **L1** (2.0 mM) in aqueous ammonium acetate (50 mM, pH 6.8 and 25 °C) solution containing 10% (v/v) sulfolane. Raw data are shown in the top panel, in the lower panel integrated data (black squares) and fitting curve (solid red line) obtained using Origin 8.0 and a single site binding model, are presented. The inset in the lower panel shows best fit parameters as described in **Figure S1**.



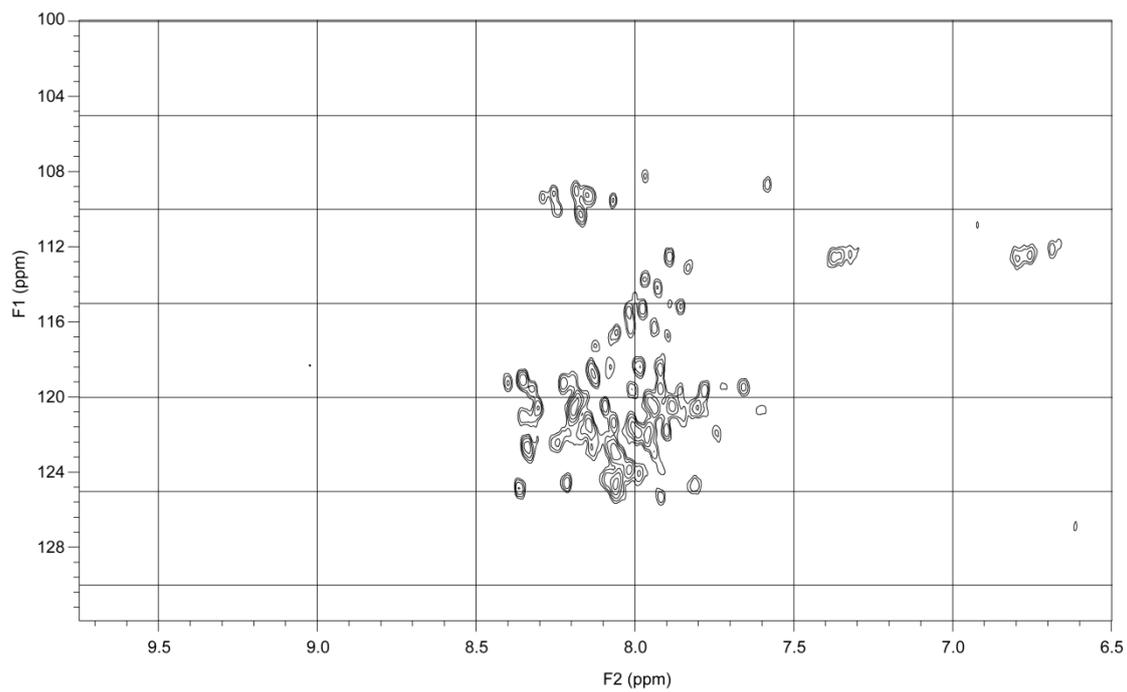
**Figure S4.** CD spectra acquired for an aqueous phosphate buffer (20 mM, pH 7.0) solutions of Lyz (56  $\mu\text{M}$ ) and sulfolane (0-20%).



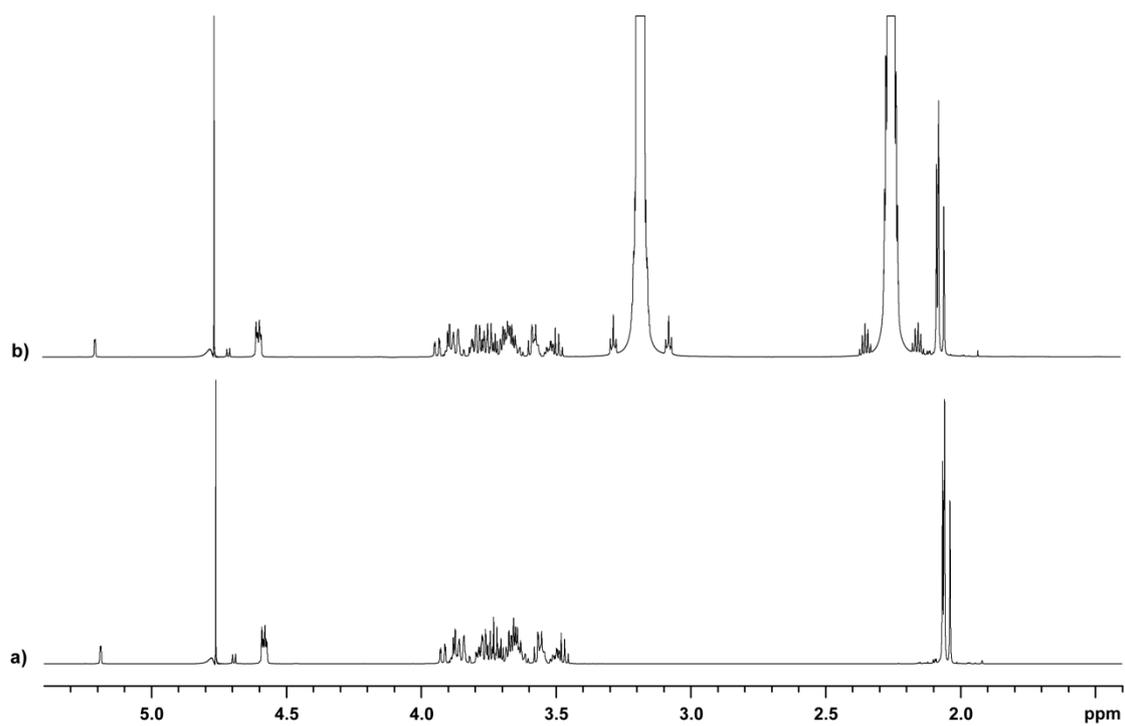
**Figure S5.** Natural abundance  $^1\text{H}$ - $^{15}\text{N}$  gHSQC NMR spectrum for Lyz in 10% (v/v)  $\text{D}_2\text{O}$ - $\text{H}_2\text{O}$ . Backbone NH signals were assigned based on comparisons with literature values [2,3].



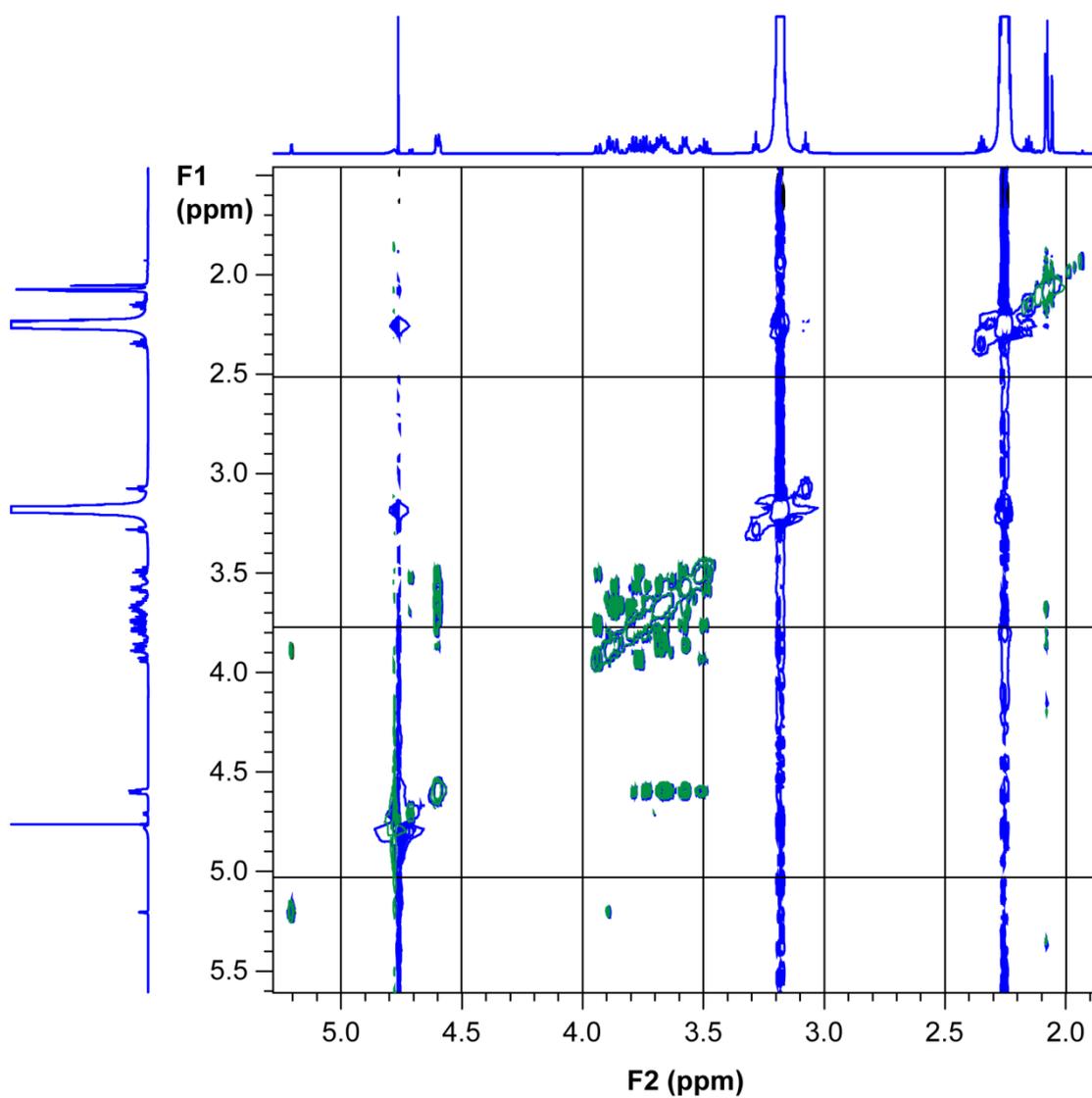
**Figure S6.** Natural abundance  $^1\text{H}$ - $^{15}\text{N}$  gHSQC NMR spectra were obtained for lysozyme in 10% (v/v)  $\text{D}_2\text{O}$ - $\text{H}_2\text{O}$  with varying concentrations of sulfolane – 0, 2, 5, and 10% (v/v). Backbone NH signals for lysozyme in 10% (v/v)  $\text{D}_2\text{O}$ - $\text{H}_2\text{O}$  were assigned based on comparisons with literature values [2, 3].



**Figure S7.** Natural abundance  $^1\text{H}$ - $^{15}\text{N}$  gHSQC NMR spectrum for denatured lysozyme in 8M urea and 10% (v/v)  $\text{D}_2\text{O}$ - $\text{H}_2\text{O}$ .

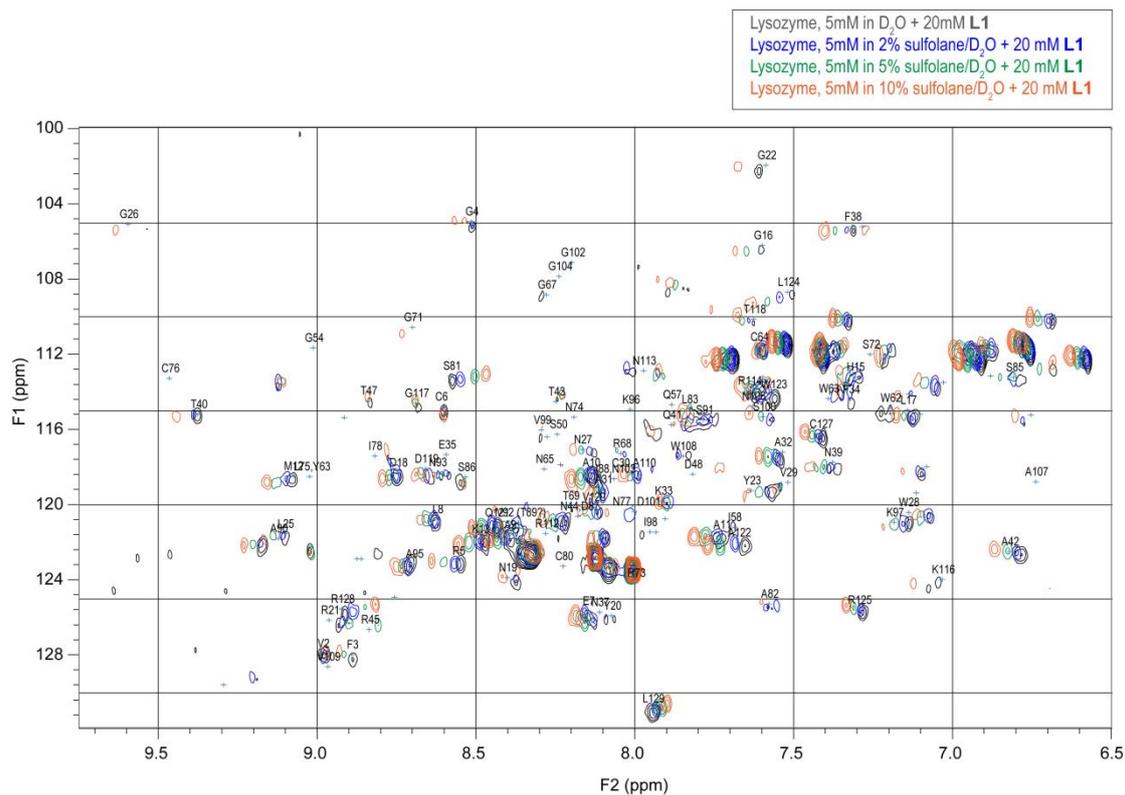


**Figure S8.** <sup>1</sup>H NMR spectrum of **L1** in D<sub>2</sub>O. b) <sup>1</sup>H NMR spectrum of **L1** in 2% (v/v) sulfolane–D<sub>2</sub>O.



**Figure S9.** TROESY spectra of **L1** in D<sub>2</sub>O (green) and **L1** in 2 % (v/v) sulfolane-D<sub>2</sub>O (blue).





**Figure S11.** Natural abundance  $^1\text{H}$ - $^{15}\text{N}$  gHSQC NMR spectra were obtained for Lyz with L1 in 10% (v/v)  $\text{D}_2\text{O}$ - $\text{H}_2\text{O}$  with varying concentrations of sulfolane – 0, 2, 5, and 10% (v/v). Backbone NH signals for Lyz in 10% (v/v)  $\text{D}_2\text{O}$ - $\text{H}_2\text{O}$  were assigned based on comparisons with literature values [2, 3].

## References

1. Lin, H., Kitova, E.N., Klassen, J.S.: Measuring Positive Cooperativity Using the Direct ESI-MS Assay. Cholera Toxin B Subunit Homopentamer Binding to GM1 Pentasaccharide. *J Am Soc Mass Spectrom* **25**, 104-110 (2014)
2. Redfield, C., Dobson, C.M.: Sequential <sup>1</sup>H NMR Assignments and Secondary Structure of Hen Egg White Lysozyme in Solution. *Biochemistry* **27**, 122-136 (1988)
3. Buck, M., Boyd, J., Redfield, C., MacKenzie, D.A., Jeenes, D.J., Archer, D.B., Dobson, C.M.: Structural Determinants of Protein Dynamics: Analysis of <sup>15</sup>N NMR Relaxation Measurements for Main-Chain and Side-Chain Nuclei of Hen Egg White Lysozyme. *Biochemistry* **34**, 4041–4055 (1995)