

# Complementary approaches to obtaining thermodynamic parameters from protein ligand systems-challenges and opportunities

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**Abstract.** Protein ligand interactions play an important role in biology. Increasingly the aim is to understand and influence protein ligand binding. The binding process is heavily influenced by its thermodynamic parameters. In order to understand how the whole system thermodynamics work it is important to characterise the individual contribution of each of the systems components. While the change in conformational entropy of the protein can be determined using QENS complementary methods are necessary in order to characterise all components. This paper will describe the challenges that can occur when combining the different methods, as well as how they can be overcome.

## 1 Protein ligand interactions

Protein ligand interactions play an important role in biology [1]. A ligand is any particle that forms a complex with a biomolecule for a biological purpose. These particles can be vitamins, minerals, sugars, or other small particles [2]. Protein ligand interactions induce structure changes, facilitate ligand transport or ligand trapping, as well as signal transfer [3–5].

The strength of a protein ligand interaction is described by the equilibrium dissociation constant  $K_d = K_a^{-1} = k_{on}/k_{off}$ , here  $K_a$  is the association constant,  $k_{on}$ ,  $k_{off}$  are the binding and release rates respectively. This dissociation constant describes the concentration at which the ligand free and ligand bound state exist in solution. If a complex is diluted below this threshold the ligand will be continuously released [6–8].

Protein ligand interactions are governed by thermodynamic parameters. Any protein ligand binding that happens spontaneously needs to be accompanied by an advantageous change in Gibb's free energy  $\Delta G = \Delta H - T\Delta S < 0$ , which means that the reaction is exergonic. A reaction can be favourable even if  $\Delta H$  or  $\Delta S$  are not favourable due to entropy enthalpy compensation [7, 9]. In addition to the whole system contributions to these parameters, each parameter contains the individual

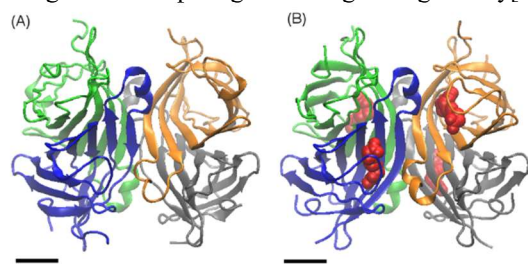
contributions of each component to the system. These components are not easily accessible and require a combination of different techniques to be measured. While it is tempting to assume that all individual contributions are either opposing or favourable for the reaction this is not the case, as has been described for the binding of biotin (B) to streptavidin (STV), where entropy-entropy compensation was observed [10, 11].

This paper will focus on discussing the different components of a protein ligand system and what their contributions are to the total change in entropy  $\Delta S$ . For this purpose, neutron scattering, as well as other complementary methods will be discussed, since the different components cannot be separated from the whole change in entropy  $\Delta S$  using only calorimetric techniques. Using different methods that were not originally designed to be used on the same systems poses several different challenges. However, some of these differences also provide unique opportunities and information that might not have been explored without these methods.

In order to illustrate this approach, the system of STV and B will be used as an example. STV is a homotetramer with a molecular weight of 13271.6 Da [10, 12]. Its ligand B, which has a molecular mass of 244.3 g mol<sup>-1</sup>, binds to it with a stoichiometry of 4:1, see Fig. 1 [13]. This binding is among the strongest bindings known in biology with a

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dissociation constant of  $K_d \approx 10^{-14}\text{M}$  [14, 15]. The biological function in this case is the trapping of the ligand biotin, which creates a slightly antibacterial environment, and gives the bacterium *Streptomyces avidinii* an advantage over competing bacteria growing nearby[16].



**Fig. 1.** This image shows the pdb structures of the ligand free STV tetramer (A), and of the complex STV+B (B), with one B per STV binding pocket [10]. Reprinted with permission from [10] M. Sarter et al., “Strong Adverse Contribution of Conformational Dynamics to Streptavidin-Biotin Binding,” *J. Phys. Chem. B*, vol. 124, no. 2, pp. 324–335, 2020 American Chemical Society.

While this biological cause for the binding is not of particular relevance for current applications, the strong binding affinity of STV and B has led to a specific application. In order to fix a molecule to a surface the surface has biotin attached to it, and the molecule has biotin attached as well, while STV is added. STV then binds to the surface and the molecule so that the molecule is affixed [14].

The adverse change in entropy observed during the STV+B complex formation is of particular interest, as it is such a strong binding and contains an adverse component [17, 18]. Therefore, we would like to pursue how the different components contribute.

## 2 Entropic components and corresponding experimental approach

Any spontaneously happening reaction requires an advantageous change in Gibbs’s free energy  $\Delta G$ . To understand this change the change in enthalpy  $\Delta H$  and in entropy  $\Delta S$  need to be considered. To understand the change in enthalpy knowledge of the different bonds and during the protein ligand interaction, as well as the structures is sufficient to perform calculations [19]. The situation is more complex and involves more parameters for the change in entropy  $\Delta S$ , any simulation will require full knowledge and understanding of the components involved. We have therefore chosen to focus on the components to the change in entropy  $\Delta S$  here.

Before discussing how to experimentally obtain the different components that make up the total change in entropy during protein ligand interactions it is important to define them.

Protein ligand systems are analysed in solution, so the influence of the solvent needs to be considered, in addition to that of the protein, its ligand, and any conformational changes upon complex formation.

$$\Delta S = \Delta S_{protein} + \Delta S_{ligand} + \Delta S_{hydr} \quad (1)$$

$$\Delta S_{protein} = \Delta S_{conf,protein} + \Delta S_{trans,rot,protein} \quad (2)$$

$$\Delta S_{hydr} = \Delta S_{displaced} + \Delta S_{surrounding} \quad (3)$$

$$\Delta S_{ligand} = \Delta S_{conf,ligand} + \Delta S_{trans,rot,ligand} \quad (4)$$

Equation 1 shows that there are three main contributions to consider for a protein ligand system, each of which has its own parts. The main contributions are any entropy change experienced by the protein  $\Delta S_{protein}$ , and its ligand  $\Delta S_{ligand}$ , as well as entropy changes to the solvent  $\Delta S_{hydr}$ . Table 1 provides an overview of the methods which are appropriate for each component, which will be discussed in detail below.

In addition to the individual contributions the whole system also needs to be measured. A method that is able to obtain all the relevant experimental contributions is isothermal titration calorimetry (ITC). In addition to the change in entropy  $\Delta S$ , it also provides the binding stoichiometry, binding affinity  $K_a$ , change in enthalpy  $\Delta H$ , and change in Gibbs free energy  $\Delta G$ [20]. If the aim is to only get  $K_a$  without the thermodynamic parameters a labelled competition experiment is a viable alternative to ITC [21].

The first part of  $\Delta S$  is the protein’s contribution  $\Delta S_{protein}$ , the components of which are given in Equation 2[20]. To analyse the change in conformational entropy  $\Delta S_{conf,protein}$  quasi elastic neutron scattering (QENS) experiments in a  $\text{D}_2\text{O}$  based buffer are used [22]. If the energy resolution for the experiment is chosen appropriately, diffusive contributions for  $\Delta S_{trans,rot,protein}$  can also be observed [23]. However, it might also be opportune to use dynamic light scattering (DLS) to determine the proteins’ diffusive behaviour [24]. All QENS experiments proposed above are based on the assumption that the ligand is small compared to the protein, and will not provide a relevant scattering contribution. If the ligand should have a high number of H atoms compared to the number in the protein deuteration of the ligand becomes necessary.

With all experiments carried out in solution the hydration layer surrounding the proteins provides a relevant contribution to the thermodynamics of the whole system [25]. Its change in entropy  $\Delta S_{hydr}$  and the contributing components are given in Equation 3. In order to determine the influence of  $\Delta S_{displaced}$  it is at first necessary to know which water molecules are hydrogen bonded to the binding site, and how many of these will be displaced upon binding. This can be achieved by comparing the free protein and protein ligand bound crystal structures, as well as by MD simulations. The entropic component is calculated from this information. Another contributing component is  $\Delta S_{surrounding}$ , which gives the entropy change of the hydration layer surrounding the protein. It can be experimentally accessed using thermal diffusion forced Rayleigh scattering (TDFRS) [26, 27].

The last contributions to the total change in entropy are those provided by the ligand  $\Delta S_{ligand}$ , as given in Equation 4. A change in the structure of the ligand  $\Delta S_{conf,ligand}$  can often be ruled out due to the ligands size and structure, if it is a small compact ligand such as most vitamins or minerals. If this is not the case, QENS experiments on the free ligand, as well as on the protein ligand complex with the protein deuterated are necessary to determine  $\Delta S_{conf,ligand}$ . A reduction in

$\Delta S_{trans,rot,ligand}$  will invariably take place, since the ligand will go from free movement in solution to being bound to the protein. Depending on the ligands size and the amount required it might be negligible or not. If QENS experiments are carried out on the ligand they can be used as discussed for the protein. Otherwise DLS experiments on the free ligand can determine its diffusion coefficient, which can be used to calculate how much the entropy is reduced if that ligand diffusion is slowed down upon ligand binding.

**Table 1** This table provides an overview about the methods which are appropriate for which entropy component.

	$\Delta S$	$\Delta S_{protein}$	$\Delta S_{hydration}$	$\Delta S_{ligand}$
ITC	Yes	No	No	No
QENS	No	Yes	No	Using deuteration
TDFRS	No		Yes	No

### 3 Challenges posed by the different methods

Before discussing the challenges of each method a brief description of how they are experimentally applied and how they work will be given.

ITC, used to determine the thermodynamic parameters of the whole system, is a calorimetric method. As such the protein solution is placed in one sample cell, while the buffer solution is placed in a reference cell. The cells are temperature controlled, with a highly concentrated solution of the ligand being titrated to the sample cell [11]. Due to the great sensitivity of the method it is important to determine the concentration of the protein solution and the ligand solution to a very high level of accuracy. Additionally, avoiding aggregation of the protein in solution is of utmost importance, so this has to be checked beforehand.

For data analysis it is first important to utilise the correct binding model during data analysis. While it can be 1:1, meaning that each ligand binds with the same affinity to the protein this is not necessarily the case for all systems [28]. A literature analysis and comparison of binding sites if a crystal structure is available are of great use.

Here the data analysis will be discussed for the simplest 1:1 model, which was used for the example data.  $\Delta H$  is determined from the difference in energy required to keep the reference and sample cell at the same temperature. The binding stoichiometry can be determined from the molar ratio of ligand and protein at the point where the binding reaction stops. To determine the change in Gibbs free energy  $\Delta G = -RT \ln(K_a)$  it is necessary to know the binding affinity  $K_a$  and the temperature at which the experiment is carried out [20]. Titrating the ligand to the protein solution provides a sigmoidal curve, with the change of energy plotted against the molar ratio of ligand to protein. By fitting the sigmoidal curve  $K_a$  can be determined as the turning point

of this curve. With this information the total change in entropy  $\Delta S = T^{-1}(\Delta G - \Delta H)$  can now be calculated [28].

Now that the method has been introduced it is time to talk about the associated challenges. Due to the high sensitivity of ITC it is important to keep the temperature in the lab where the ITC stands stable, otherwise a drift in the data will make the analysis more difficult. To ensure that no concentration effects exist for the observed system experiments should be performed at different concentrations and both data sets used simultaneously during data analysis. In order to obtain good results, it is helpful to repeat the experiments several times and perform a statistical analysis of the results, as the sensitivity of the method means that the slightest variation in cell filling or room temperature might affect the results. If a reaction is very strong, meaning it has a small  $K_d$ , or very weak, it is difficult to analyse as the reaction will not be slow enough to allow for thorough analysis [29][30]. This holds for the later discussed example of STV+B. In these cases a solution is to obtain  $K_d$  from additional experiments, such as labelled ligand displacement experiments, and use it as a known quantity to calculate  $\Delta G$  and thus  $\Delta S$ , or to perform ligand displacement ITC [21, 30].

The second method mentioned as a mayor experimental approach is QENS [31]. These experiments require a high protein concentration of around 50 to 100 mg/ml in order to achieve sufficient signal intensity while at the same time avoiding multiple scattering contributions to the recorded spectra. In addition, QENS requires a sample volume of around 1 to 2 ml that is large when compared to several other biological methods. All samples are prepared in a  $D_2O$  based buffer, so that the protein's and ligand's hydrogen provide the main scattering contribution [23, 32]. The buffer is still measured separately from the sample and subtracted as background. Experimental data is typically fitted with one or more Lorentzians to describe the protein's internal dynamics, as well as the global diffusion if an additional Lorentzian is fitted. Which dynamics can be observed depends on the timescale analysed. In addition to the peak broadening of the Lorentzian to determine the dynamics the system's mean-square displacement (MSD) is also analysed. This value can be used to calculate

$$\Delta S_{conf,protein} = Res \cdot 3R \ln \left( \sqrt{\frac{MSD_{protein+ligand}}{MSD_{protein}}} \right)$$

, here Res is the number of residues of the protein[33]. DSC and QENS experiments yielded the same values for  $\Delta S_{conf,protein}$  for the thermal unfolding of myoglobin, which are in quantitative agreement within the errors and hence, corroborate the QENS approach described above [34–36].

Having briefly summarised the experimental and data analysis approach for QENS the focus will now be on the challenges this method poses. As mentioned above, for larger ligands it can become necessary to deuterate both the protein, as well as the ligand. In this case access to a deuteration lab, and the knowledge of how to express the protein in a deuterated state will be necessary. The same will have to be achieved for the ligand. Another related



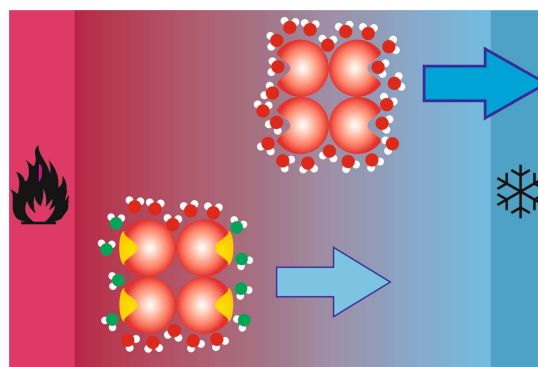
issue is sample availability, due to the comparatively large amount of sample required (100 to 200 mg protein per sample) it can be difficult to produce the required amount of protein. Even with the high sample concentration and volumes the time necessary to complete one data set is still around 12 h for a QENS experiment [10, 26]. If a protein ligand system has a very high binding affinity this is not a problem, as the complex will not dissolve during the measurement. Therefore, the ligand will not need to be present at oversaturation levels to ensure complex formation. For lower binding affinities over saturation with the ligand might be required to ensure that complex formation occurs. In this case the ligand free buffer needs to be measured, as well as the buffer at oversaturation levels. The ligand free buffer spectrum is then subtracted from the protein spectrum, while the buffer with the ligand at oversaturation levels is subtracted from the protein ligand complex. In the case of protein ligand systems with even lower binding affinities the complex will not be the only population present in the sample, instead a certain percentage of free protein will always be a part of the solution. For these samples it is important to calculate the percentage of ligand free protein that is part of the sample. After that calculation is performed during data treatment the spectrum of the free protein and the buffer spectrum are subtracted each with the appropriate factor. To do this the buffer data subtracted protein dataset will be used for subtraction, with the correct percentage applied as intensity scaling. Another challenge is that the measurements are performed in a D<sub>2</sub>O based buffer, while for many proteins this does not have any effects there have been reports about proteins being affected by the change in solvent from H<sub>2</sub>O to D<sub>2</sub>O. Therefore, it is necessary to check that the protein's, as well as the protein ligand complexes, properties are not affected by the change in solvent. For this purpose, small-angle scattering (SAS) experiments to analyse structural changes, ITC experiments in both buffers, TDFRS experiments in both buffers, as well as circular dichroism (CD) to check for structural changes, or DLS to check on the diffusive behaviour can be used. An appropriate selection of these methods should be made depending on the system. To confirm that the high concentration required does not affect the results it is necessary to perform dilution experiments, such as using DLS to ensure that the concentration has no negative influence and causes no aggregation. In addition, SAS, ITC, and TDFRS experiments at different concentrations can be used.

In order to determine the behaviour of  $\Delta S_{hydr}$  TDFRS can be used [27]. The method is based on thermophoresis, which is the transport of matter caused by a temperature gradient and shows a high sensitivity to the number and strength of hydrogen bonds formed between solute and water [37]. This transport is analysed in solution using the Soret coefficient  $S_T = D_T/D$ , with  $D_T$  as the thermal diffusion coefficient, and  $D$  the mutual diffusion coefficient. In order to induce a temperature gradient infrared TDFRS (IR-TDFRS) is applied. Interference of two infrared laser beams with a wavelength of  $\lambda = 980 \text{ nm}$  is used to create a thermal grating, which triggers the thermophoresis [26, 38, 39]. This wavelength

corresponds to an absorption band in H<sub>2</sub>O, but not in D<sub>2</sub>O. Experiments are carried out at different temperatures, to analyse the temperature sensitivity of  $S_T$ .  $S_T$  is determined from the amplitude of the concentration signal  $A$  as follows,  $A = \left(\frac{\partial n}{\partial c}\right)_{p,T} \left(\frac{\partial n}{\partial T}\right)_{p,c}^{-1} S_T c(1-c)$ . Here  $c$  is the concentration,  $n$  the refractive index increment with concentration,  $\left(\frac{\partial n}{\partial c}\right)_{p,T}$  was obtained from a refractometer and the refractive index increment with temperature  $\left(\frac{\partial n}{\partial T}\right)_{p,c}$  from interferometry. Further details can be found elsewhere [40].

In systematic experiments a strong correlation between thermodiffusion and hydrophilicity of the solute was found [37]. The more hydrophilic solutes have a stronger tendency to accumulate in the cold. This is illustrated in Figure 2 The free STV forms more hydrogen bonds with the surrounding water and shows a stronger tendency to accumulate in the cold, while biotin blocks some of the hydrogen bonding sites leading to a less hydrophilic complex and higher entropy  $\Delta S_{hydr}$  of the water molecules in the hydration shell. The lower hydrophilicity is also reflected by a larger temperature sensitivity of  $S_T$  often described by  $\Delta S_T$  the difference between  $S_T$  at two temperatures.

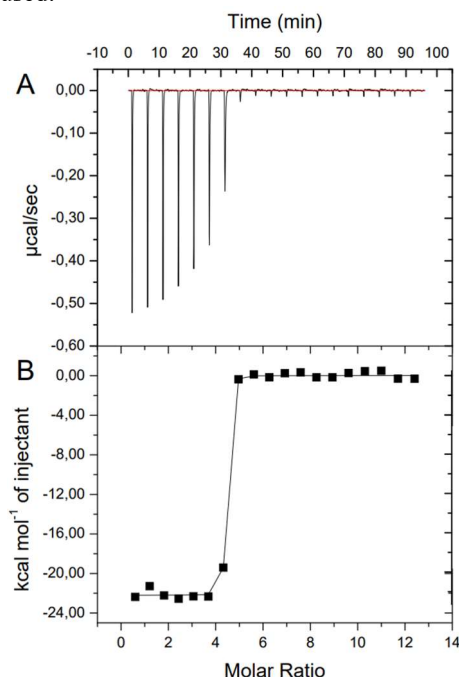
For the TDFRS experiments the protein needs to be measured in solution with and without the ligand, as well as the ligand in solution. If oversaturation is required for complex formation then the oversaturated buffer also needs precise analysis. Different temperatures and protein concentrations are used for the experiments. To ensure comparability with QENS the buffers need to be D<sub>2</sub>O based, which leads straight to the challenges faced with this method [26]. Due to the problems measuring in D<sub>2</sub>O mentioned above a pure D<sub>2</sub>O based buffer is not possible, instead the D<sub>2</sub>O concentration has to be steadily increased. An increase of up to 50% was found to be possible. If no D<sub>2</sub>O concentration dependent change of  $T$  dependent  $S_T$  behaviour can be observed it is safe to assume that no solvent dependence exists. However, data acquisition gets more difficult with increasing D<sub>2</sub>O concentration due to a lower refractive index contrast [38].



**Fig. 2.** Here it can be seen that the ligand free STV is more likely to accumulate in colder areas than the ligand bound STV+B complex. This is thought to be related to the higher hydrophilicity of free STV [26].

## 4 Experimental approaches discussed for the example of STV+B

STV+B will now be used as an example to explore the challenges faced for each method, the results obtained for this system will also be presented. The solutions chosen to face them, as well as reflections on which additional approaches might have been helpful in different circumstances will be presented. All experiments were performed in a 25 mM TRIS-HCl, 120 mM NaCl, 5 mM KCl, 3 mM MgCl, pH 7.4 buffer, either H<sub>2</sub>O, or 99.9% atom D based, or buffer mixtures depending on the method used.



**Fig. 3.** (A) shows the raw data obtained from titrating biotin to STV plotted against time. The change in energy per second can be observed. It is also visible that between each titration sufficient time has passed that the reaction is finished and the solution equilibrated. (B) shows the energy change per mol of injectant, here B, plotted against the molar ratio of B and STV.

First the whole system was analysed with ITC. Two different concentrations of STV solution, as well as two different concentrations of B solution were used for the ITC experiments, with  $c_{STV} = 65$  mg/ml, and  $c_{STV} = 109$  mg/ml, all ITC experiments were carried out in a H<sub>2</sub>O based buffer. The B solutions were titrated to STV solutions, as shown Fig. 3. In Fig. 3 (A) it can be observed that the end of binding reactions occurring due to saturation of STV with B is very distinct. To observe the binding stoichiometry Fig. 3 (B) can be used. The change in enthalpy  $\Delta H$ , is easily determined from these results. In order to calculate  $\Delta S$  it is necessary to determine the turning point in Fig. 3 (B) to get  $K_a$ . It is obvious from Fig. 3 (B) that the turning point cannot be easily determined. As no literature value of  $K_d$  or  $K_a$  is known for this specific strain of STV it was decided to proceed with a literature value of wild strain STV for  $\Delta S_{STV} = -348$  J/(molK), instead of using its  $K_a$  and the ITC experiments. From the different experimental runs taking

the statistical fluctuations into account the following result for  $\Delta H = -427.1 \pm 47.2$  kJ/mol was calculated.

In this case the ITC specific challenges were solved by compensating with literature values. If a protein ligand system has not been previously characterised it would be necessary to experimentally obtain  $K_a$ . By performing the experiments at different STV and B concentrations it was possible to confirm that no concentration effects to the binding reaction or stoichiometry could be observed. The experiments were not repeated in D<sub>2</sub>O due to time constraints and the inaccessibility of  $K_a$ .

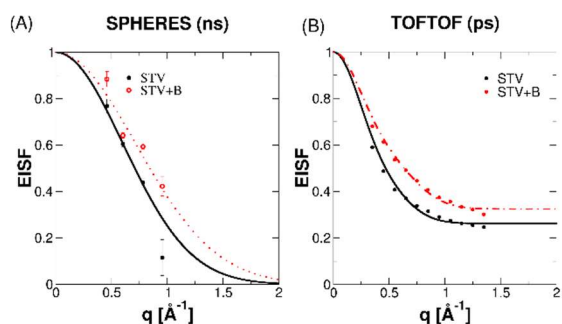
QENS experiments were performed in D<sub>2</sub>O based buffers. Due to the small and rigid structure of B, any conformational change on it was ruled out and  $\Delta S_{conf,ligand}$  was treated as negligible in this case. For  $\Delta S_{trans,rot,ligand}$  literature confirms that the change in entropy caused by desolvation is similar to the entropy contribution from its diffusion, so that any changes even out. Therefore, a deuteration of STV, or B apart from an exchange of free H to D was not necessary. The experiments were carried out on the ps-ns timescale for concentrations in the range of  $c_{STV} = 55$  mg/ml -  $c_{STV} = 65$  mg/ml. Due to the high binding affinity of this system no oversaturation of STV with B was necessary to ensure complex formation. Therefore, the amount of B necessary to saturate STV was calculated by weight and added to the STV solution. In addition to avoiding the need for an additional measurement of the buffer this system is also sufficiently stable in complex form over time that only the complex was present in solution during the experiments.

Neither DLS nor QENS experiments found a difference in the diffusion between STV and STV+B. In addition the different crystal structures for STV and STV+B did not produce a difference in diffusion coefficient using HYDROPRO [11, 41, 42]. Therefore, it was concluded that  $\Delta S_{trans,rot,protein}$  is negligible for this system. The value for  $\Delta S_{conf,protein}$  was in good agreement within the error on the different timescales and determined to be  $\Delta S_{conf,STV} = -2200 \pm 300$  J/(molK). The elastic incoherent structure factor (EISF) curves which provided the MSDs for this calculation can be seen in Fig. 4 This shows that the change in conformational entropy is more strongly opposed to the binding than the whole entropy change and illustrates the need for using several complementary methods to obtain each individual component of the total change in entropy.

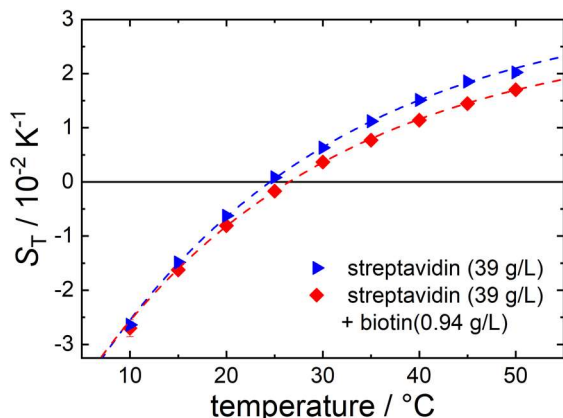
Due to the experiments being performed in D<sub>2</sub>O it was necessary to check that free STV, as well as the STV+B complex were not affected by this change in solvent. This was done by comparing the diffusion in DLS, structure with both SAXS and SANS, as well as CD spectroscopy and later during TDRFS experiments where the ratio of D<sub>2</sub>O to H<sub>2</sub>O in the buffer was altered. No buffer dependent changes could be observed. In addition, DLS dilution experiments were carried out to check for any changes in diffusive behaviour due to concentration and none were observed.

To gain information about the last entropic component TDRFS experiments were carried out. In accordance with the challenges previously mentioned the buffer used contained up to 50% D<sub>2</sub>O, while initial experiments were

carried out in H<sub>2</sub>O based buffers, the protein's concentration was  $c_{STV} = 39 \frac{\text{mg}}{\text{ml}}$ .



**Fig. 4.** EISF curves for STV and STV+B on the ns (A), and ps (B) timescale. It can be observed that free STV is more flexible than the STV+B complex [10]. Reprinted with permission from [10] M. Sarter et al., “Strong Adverse Contribution of Conformational Dynamics to Streptavidin-Biotin Binding,” *J. Phys. Chem. B*, vol. 124, no. 2, pp. 324–335, 2020 American Chemical Society.



**Fig. 5.** The Soret coefficient  $S_T$  shows that the free STV is more hydrophilic than the STV+B complex. This indicates that the entropy of the hydration layer increases upon complex formation.

From these experiments no changes in diffusion were observed upon ligand binding, while  $S_T$  of STV+B is reduced compared to free STV **Fig. 5**. This difference decreases with decreasing  $T$  and is almost gone below 298 K. These results indicate that STV is slightly more hydrophilic than STV+B, which agrees with observations made from the structural data and shows that the  $T$  dependent change of  $\Delta S_{hydr}$  relates as follows.  $\Delta S_{hydr}^{STV} < \Delta S_{hydr}^{STV+B}$ . From this it can be concluded that an advantageous change in  $\Delta S_{surround}$ . Increases of D<sub>2</sub>O to H<sub>2</sub>O ratio did not lead to a change in  $T$  sensitivity of  $S_T$ .

Regarding  $\Delta S_{displaced}$  this could be calculated, as crystal structures show that 5 H are contained in each binding pocket,  $\Delta S_{displaced} = 586 \text{ J}/(\text{molK})$ .

Using the system of STV+B as an example the advantage of using different complementary methods in addition to QENS to approach the thermodynamic parameters was shown. Several of the potential challenges which come from combining these methods were encountered and the solutions chosen for this system were

illustrated. By combining the different methods, it could be confirmed that entropy-entropy compensation occurs upon STV+B complex formation and that this is the reason that the reaction is still thermodynamically favourable, although the conformational contributions would oppose the binding.

## 5 Conclusion

In order to understand protein ligand systems, it is important to know what the thermodynamics of the ligand binding process are. This proceeding has provided an overview over complementary methods that are available to analyse the entropic component in depths. While there are several challenges in combining different complementary methods, these can be overcome by careful planning and characterisation of the system.

Using the example of the STV+B system it was shown that analysing the different components offers valuable insights about which part of the system influences the binding in what way. This is of special importance in the case of biopharmaceutical applications, where adjusting the binding affinity is often used to improve medications. A better understanding of each components' contribution will allow for more targeted approaches.

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