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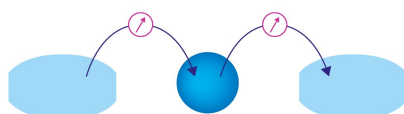
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Thermodiffusion as a Probe of Protein Hydration for Streptavidin and the Streptavidin-Biotin Complex

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Abstract. Molecular recognition via protein–ligand interactions is of fundamental importance to numerous processes in living organisms. Microscale thermophoresis (MST) uses the sensitivity of the thermophoretic response upon ligand binding to access information on the reaction kinetics. Additionally, thermophoresis is promising as a tool to gain information on the hydration layer, as the temperature dependence of the thermodiffusion behaviour is sensitive to solute-solvent interactions. To quantify the influence of structural fluctuations and conformational motion of the protein on the entropy change of its hydration layer upon ligand binding, we combine quasi-elastic incoherent neutron scattering (QENS) and isothermal titration calorimetry (ITC) data from literature. However, preliminary results show that replacing water with deuterated water leads to changes of the thermophoretic measurements, which are similar to the changes observed upon binding by biotin. In order to gain a better understanding of the hydration layer all measurements need to be performed in heavy water. This will open a route to develop a microscopic understanding of the correlation between the strength and number of hydrogen bonds and the thermophoretic behaviour.

INTRODUCTION

Thermodiffusion or thermophoresis describes the mass transport in a temperature gradient [1]. Nowadays one of the most important applications is the so-called microscale thermophoresis (MST), which monitors protein-ligand binding interactions and is especially used to determine equilibration constants of biochemical reactions [2]. As sketched in Fig.1 the tendency of a protein to accumulate in the cold regions often changes substantially once a protein binds to a small ligand molecule. The complex has typically only a slightly higher molecular mass compared to the free protein, but during the binding process the hydration layer changes. This can for instance be caused by a conformational change of the protein or due to a different hydrophilicity of the bound ligand molecule compared to the protein in the region of binding. The hypothesis is that changes in the hydration layer influence thermophoretic behaviour upon binding. In order to test this hypothesis on a well-known system and gain a better understanding of hydrogen bonding we investigated the thermophoresis of streptavidin (SA) and compare it with the streptavidin-biotin (SA-B) complex.

Thermodiffusion is characterized by the Soret coefficient S_T , which is equal to the ratio of the thermal diffusion coefficient D_T and the diffusion coefficient D . Interpretation and prediction of thermodiffusion behaviour is difficult due to its sensitivity to the properties of solute (mass, size, charge, moment of inertia) and solvent (ionic strength, chemical interactions). A striking difference between aqueous and unpolar solutions is the strong temperature dependence of the Soret coefficient in water. A sign change from thermophilic (negative S_T) to thermophobic (positive

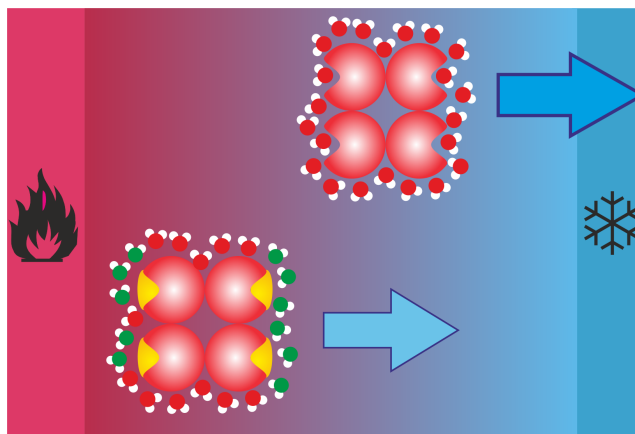


FIGURE 1. In this schematic illustration the free streptavidin tetramer has a stronger tendency to accumulate in the cold compared to the streptavidin-biotin complex. It is suspected that the change of the hydration upon binding (indicated by the water molecules with green oxygen atoms) influences the thermophoretic behaviour.

S_T) behaviour at a transition temperature T^* can often be observed [3]. For many biologically relevant systems the temperature dependence of S_T can be described by the following empirical equation

$$S_T(T) = S_T^\infty \left[1 - \exp\left(\frac{T^* - T}{T_0}\right) \right], \quad (1)$$

where S_T^∞ is the plateau value of S_T that is reached at high temperature [4]. The sensitivity to temperature is due to the contribution of solute-solvent interactions, a contribution that is close to zero in unpolar solvents and decreasing with rising temperature in water due to the breaking of hydrogen bonds at high temperature. While the contribution of these solute-solvent interactions is not strong enough to determine the absolute value of S_T , it dominates the temperature dependent part of the coefficient. The difference of S_T at two temperatures ΔS_T , which is proportional to the chemical contribution to S_T , was shown to correlate with $\log P$ [5].

The partition coefficient $\log P$ is a measure for hydrophilicity/hydrophobicity of a solute and can be used to model the transport of a compound in the environment or to screen for potential pharmaceutical compounds (Lipinski's 'Rule of Five' [6]). It describes the distribution of a solute A between aqueous and oil phase (most commonly 1-octanol) in equilibrium, with P being the ratio of solute concentration in oil and water $P = [A_{oil}]/[A_{water}]$. Due to the fact that measurement of $\log P$ can be quite costly, a number of algorithms have been developed that allow estimation of $\log P$ for any given compound, based on an experimental data base and incremental contributions by functional groups of the molecule. These methods give a reliable $\log P$ value only for room temperature, low concentrations and small (unfolded) molecules that have all contributing groups in contact with the surrounding water.

While the microscopic picture of solvation in water is not yet clear, the tendency of a solute A to accumulate in the oil or aqueous phase can be expressed as a difference in the solvation free energy upon transition between the two phases [7]

$$\Delta G_r(A) = -RT \ln([A_{oil}]/[A_{water}]) = -2.303RT \log P. \quad (2)$$

In an analogous view, the chemical contribution to thermodiffusion, that is the contribution that is due to solute-solvent interactions, can be understood as distribution of the solute between two 'phases' of different temperature, where the hotter water acts more oil-like due to a weakening of the hydrogen bond network.

This gives us only qualitative understanding, however. In order to quantify the entropic change of the hydration layer when biotin binds to SA, we used quasi-elastic incoherent neutron scattering (QENS) to access the conformational entropy of the protein structure, which does not include the entropic contribution of the hydration layer, and compared our results to ligand-displacement isothermal titration calorimetry (LDITC) measurements from the literature [8].

Quasi-elastic incoherent neutron scattering (QENS) observes inelastic scattering of neutrons, where the energy transfer is small compared to the energy of the incident neutrons. Therefore, a broadening of the elastic scattering peak is observed, this gives information about the dynamic properties of the samples [9, 10]. When applied to protein

dynamics QENS mainly observes the incoherent scattering of the hydrogen-atoms, while the deuterium atoms have a significantly smaller incoherent scattering cross section. Therefore, their contribution can be considered negligible when compared to the contribution from the hydrogen-atoms [9, 10]. By exchanging the interchangeable H-atoms in a protein for D-atoms and using a D₂O buffer the main scattering contribution of the sample then comes from the non-exchangeable H-atoms in the protein [11]. QENS observes the dynamics of these H-atoms, the movement of which can be related to a fluctuation of the residues to which they are bound. By measuring QENS from proteins we quantify the conformational fluctuations of the protein under different environmental conditions (i.e. with or without ligands) [12]. The loss of the elastic scattering peaks gives the information about the average amplitude of protein motions, which is given by the mean square displacement (MSD) calculated from the elastic incoherent structure factor EISF A_0 [12]

$$A_0(q) = \exp(-\text{MSD } q^2) (1 - p) + p, \quad (3)$$

where q is the scattering vector and p is the fraction of immobile H-atoms. The EISF is determined from the simplified scattering function

$$S(q, \omega) = A_0(q) \cdot L_G(q, \omega) + [1 - A_0(q)] \cdot L_{G+I}(q, \omega), \quad (4)$$

where $\hbar\omega$ is the energy transfer. The total theoretical scattering function plus linear background $B(q, \omega)$ was convoluted with the instrumental resolution function and fitted to the measured spectra. The HWHM (half width at half maximum) of the two Lorentzians

$$L_G(q, \omega) = \frac{1}{\pi} \times \frac{\Gamma_G(q)}{(\hbar\omega)^2 + \Gamma_G(q)^2} \quad (5)$$

and

$$L_{G+I}(q, \omega) = \frac{1}{\pi} \times \frac{\Gamma_G(q) + \Gamma_I(q)}{(\hbar\omega)^2 + [\Gamma_G(q) + \Gamma_I(q)]^2} \quad (6)$$

account for the global and internal protein diffusion, respectively. The change of the MSD between two states allows for the calculation of the change in the conformational entropy between these states [12].

$$\Delta S_{\text{conformational}} = 3R \ln \left(\sqrt{\frac{\text{MSD}_{\text{complex}}}{\text{MSD}_{\text{free}}}} \right) \quad (7)$$

EXPERIMENTAL SECTION

Sample preparation

Streptavidin (SA) used in these experiments is Streptavidin Streptomyces Avidinii Recombinant produced in *E. coli* (Prospec, 7670308 Rehovot, Israel). The molecular weight of the tetramer is given by the manufacturer as 52 kDa and the amino acid sequence is MAEAGITGTWYNQLGSTFIVTAGADGALTGTYESAVGNAESRYVLTGRYDSAPAT DSGGTALGWTVAWKNNYRNAHSATTWSGQYVGGAERINTQWLLTSGTTEANAWKSTLVGHDTFTKVKP SAAS. SA was cleaned with PD-10 columns, lyophilized and then kept at -20°C. The biotin was purchased as lyophilized powder with $\leq 99\%$ purity (Sigma-Aldrich, 89555 Steinheim, Germany). The buffer stock solution has the following composition: 250 mM TrisHCl (Tris[hydroxymethyl]aminomethane, $\leq 99.9\%$, Sigma-Aldrich, 89555 Steinheim, Germany; Hydrochlorid acid 37%, Merck, 64271 Darmstadt, Germany), 1.2 M NaCl ($\leq 99.5\%$, Merck, 64271 Darmstadt, Germany), 50 mM KCl ($\leq 99.5\%$, Merck, 64271 Darmstadt, Germany), 30 mM MgCl₂ ($\leq 99.0\%$, Merck, 64271 Darmstadt, Germany).

For IR-TDFRS experiments the buffer stock solution was diluted with Milipore water 1:9. The concentrations of SA in buffer solution was 50 mg/mL (SA weight fraction 0.048 ± 0.001) for both samples. To the second sample we added biotin at a stoichiometry of SA:biotin = 1:4.

For the neutron scattering experiments the lyophilized SA powder was incubated in D₂O for 24h in order to exchange the interchangeable hydrogen atoms by deuterium. Afterwards the SA was again lyophilized and stored at -20°C. The D₂O based buffer has the following composition: 25 mM TrisDCL, 120 mM NaCl, 5 mM KCl, 3 mM MgCl₂, pH=7.4.

Thermal diffusion forced Rayleigh scattering

The thermal diffusion coefficients were measured in an optical quartz cell (Hellma) with optical path length of 0.2 mm by Infra-Red Thermal Diffusion Forced Rayleigh Scattering (IR-TDFRS), a laser-induced transient grating technique, which has been described in detail before [13, 14]. IR-TDFRS was measured in a temperature range from 10 to 50 °C, with steps of 5 °C. At least two measurements for each sample concentration were done. The error bars represent the standard deviation of the mean. All measurements have been performed in the buffer solution. Additional measurements of water/deuterated water mixtures with 10, 25 and 50 wt% of deuterated water were performed at 50°C, the temperature with the strongest signal.

Quasi-elastic neutron scattering

QENS experiments were performed on the backscattering spectrometer SPHERES [15, 16] operated by JCNS at the Heinz-Maier-Leibnitz Zentrum (MLZ) in Garching, Germany. The wavelength of the spectrometer is 6.27 Å, the resolution is approximately 0.65 µeV for the HWHM and the timescale observed is nanoseconds. The data were analyzed for the q -vectors between 0.5 and 1.6 Å⁻¹ and the measurements were performed for 25°C. A sample of SA and of SA-B with a concentration of 65 mg/mL were measured. For the SA-B complex biotin was added to streptavidin in a molar ratio of 4 to 1.

RESULTS

Thermophoretic measurements

IR-TDFRS measurements were conducted for the two samples described above, and for buffer solution and biotin-buffer solution without SA. Although the buffered solutions are multi-component systems, the signal of SA and the SA-B complex can be well separated from those of the buffer salts and free biotin due to the large differences in diffusion speed. The signal of the buffer is very small compared to that of the protein and, as expected due to the strong binding between SA and biotin, we could not detect a separate biotin-signal in the mixture, so that all mixtures could be treated as 2-component systems in the evaluation. The diffusion coefficient of SA was measured as $D(20^\circ\text{C}) = (6.7 \pm 0.5) \cdot 10^{-7} \text{ cm}^2\text{s}^{-1}$, which agrees reasonably well with the literature value at room temperature of $D = 6.2 \cdot 10^{-7} \text{ cm}^2\text{s}^{-1}$ [17].

The Soret coefficient S_T is shown in Fig. 2 as a function of temperature. Both systems show the typical behaviour of aqueous protein solution with a temperature dependence according to Eq. 1. While the thermodiffusion of the SA-B complex shows no deviation from the free SA at 10°C, at higher temperatures S_T is significantly altered. This is due to

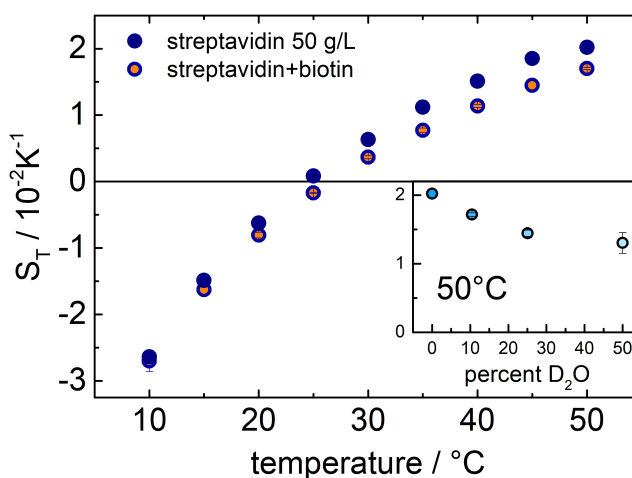


FIGURE 2. Soret coefficient of the unbound SA (blue) and the complex (orange filling) as function of temperature. The inset shows the variation of S_T of the free SA at 50°C with increasing D₂O content.

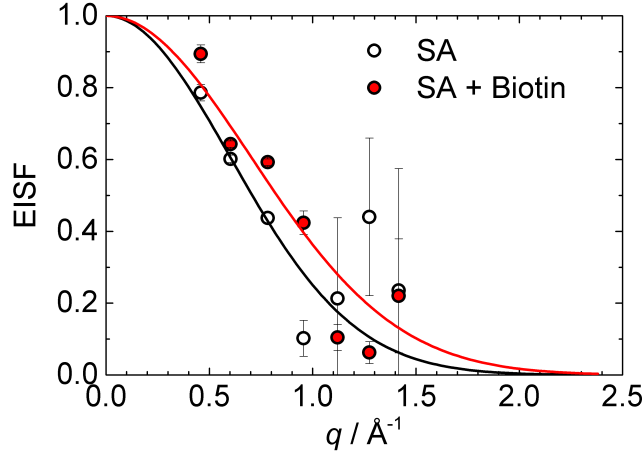


FIGURE 3. Results of QENS measurements performed on SPHERES. Elastic incoherent structure factor of streptavidin (black) and the streptavidin-biotin complex (red) as function of the scattering vector q .

a change in the thermodiffusion coefficient D_T , the diffusion coefficient D is not changed. The temperature sensitivity of S_T is reduced for the complex compared to the free protein. This indicates that the complex is less hydrophilic than the free SA, so that it is likely that the complex forms fewer hydrogen bonds with the surrounding water. Consequently, we expect a higher entropy of the water molecules in the hydration shell.

Due to the absence of an absorption band in D_2O we cannot perform IR-TDFRS measurements in pure heavy water. The inset of Fig. 2 shows that S_T decreases with increasing D_2O content and reaches a plateau at 50wt%. Additional temperature dependent measurements of the free protein and the complex are needed in order to investigate how the temperature sensitivity of S_T is altered.

Neutron scattering experiments

In Fig. 3 the EISF for SA and SA-B complex is shown, the EISF of the SA shows a steeper decline than that of the SA-B complex, this indicates that the MSD of SA-B is reduced when compared to the MSD of SA. This indicates that the SA-B complex is less flexible in its motions than the free SA at the nanosecond range. Calculating the change in conformational entropy for the different MSD yields $\Delta S^{QENS} = -2.0 \pm 0.2 \text{ kJ mol}^{-1} \text{ K}^{-1}$. This indicates a decrease in the conformational order of the protein upon ligand binding. It is expected that the more rigid structure of the complex is stabilised by an increase in the mobility of the water in the hydration layer, thus also compensating the decrease in conformational entropy by increasing in the hydration layer [18].

DISCUSSION

In the neutron scattering experiment only the entropic contribution of the protein is probed, while the isothermal titration probes the protein and the hydration shell. Assuming that the contributions of biotin are small compared to those of SA, the entropic contribution of the hydration shell $\Delta S^{hydration}$ can be calculated from the difference of the two.

$$\begin{aligned} \Delta S^{QENS} &= S_{SA-B}^{protein} - S_{SA}^{protein} \\ \Delta S^{ITC} &= S_{SA-B} - S_{SA} = (S_{SA-B}^{protein} + S_{SA-B}^{hydration}) - (S_{SA}^{protein} + S_{SA}^{hydration}) \\ \Delta S^{ITC} - \Delta S^{QENS} &= S_{SA-B}^{hydration} - S_{SA}^{hydration} = \Delta S^{hydration} \end{aligned} \quad (8)$$

Ligand-displacement ITC (LDITC) measurements of the binding of SA with biotin in the *same* buffer solution used in this work were carried out by Kuo *et al.* [8], who calculated the entropic contribution at 25°C as $\Delta S = -52.48 \text{ cal mol}^{-1} \text{ K}^{-1}$ with an error of 15% and the biotin/SA ratio of $n = 4.0$. Note that Kuo *et al.* used water instead

of heavy water. The entropic contribution of the whole tetramer is then $\Delta S^{\text{ITC}} = n \cdot \Delta S$.

$$\begin{aligned}\Delta S^{\text{QENS}} &= -2.0 \pm 0.2 \text{ kJ mol}^{-1} \text{ K}^{-1} \\ \Delta S^{\text{ITC}} &= -0.88 \pm 0.13 \text{ kJ mol}^{-1} \text{ K}^{-1} \\ \Delta S^{\text{hydration}} &= -0.88 \pm 0.13 \text{ kJ mol}^{-1} \text{ K}^{-1} + 2.0 \pm 0.2 \text{ kJ mol}^{-1} \text{ K}^{-1} = 1.12 \pm 0.33 \text{ kJ mol}^{-1} \text{ K}^{-1}\end{aligned}\tag{9}$$

These calculations show that the entropy of the hydration shell increases upon ligand binding, compensating in large parts the entropic loss of the complex. To connect these results with a microscopic picture, a comparison can be made to the work of Liese *et al.* [18], who investigated hydration effects on stretched polyethylene glycol (PEG) chains. They, too, found that hydration has a significant contribution towards the free energy of the observed system: the loss in conformational entropy of the PEG chain due to the increase in stretching energy is compensated by an increase of entropy in the hydration shell. Their molecular dynamics simulations showed that the entropy gain was due to the replacement of double by single hydrogen-bonded hydration water.

Applying these observations to our system, the increasing entropy of the hydration shell upon ligand binding could indicate a reduction of hydrogen bonds between the complex and the hydration water compared to the number of hydrogen bonds between free SA and hydration water. Reasons for this behaviour are not clear, but might include the displacement of hydration water by biotin, the increased rigidity of the protein or a reduction of surface area of the protein due to conformational changes. The finding that the number of hydrogen bonds between SA and the surrounding water is reduced upon ligand binding is in agreement with the IR-TDFRS results indicating less hydrophilic behaviour for the complex.

It has to be noted, however, that the neutron scattering experiments are performed in D₂O, while Kuo *et al.* used normal water for their LDITC experiments. As can be seen in Fig. 2 the thermophoretic behaviour is strongly influenced by the addition of D₂O. Similar results are expected for the SA-B complex. The change of S_T is of the same order of magnitude as the change upon binding with biotin. Since it is known [19, 20, 5] that the thermophoretic measurements are very sensitive to changes of the hydrogen binding, also the entropic changes of the ITC measurements will be influenced, if water is replaced by D₂O. Therefore, it is necessary to perform the ITC measurements in a buffer with deuterated water, in order to determine the correct $\Delta S^{\text{hydration}}$ by comparing ΔS^{ITC} with ΔS^{QENS} determined by the QENS measurements.

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