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Stability of human serum albumin structure upon toxin uptake explored by small angle neutron scattering

Shun Yu [a, b, c], Ben Kent [a], Charl J. Jafta [a], Albrecht Petzold [a], Aurel Radulescu [d], Mirjam Schuchardt [e], Markus Tölle [e], Markus van der Giet [e], Walter Zidek [e], Matthias Ballauff [a, b, c, *]

[a] Institute Soft Matter and Functional Materials Helmholtz-Zentrum Berlin, Hahn-Meitner Platz 1, 14109 Berlin, Germany
[b] Department of Physics, Humboldt-Universität zu Berlin, Newtonstr. 15, 12489 Berlin, Germany
[c] Helmholtz Virtual Institute "Multifunctional Biomaterials for Medicine", Kantstr. 55, 14513 Teltow, Germany
[d] Jülich Centre for Neutron Science (JCNS), Outstation at MLZ, Forschungszentrum Jülich GmbH, Lichtenbergstr. 1, 85748 Garching, Germany
[e] Charité - Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Department of Nephrology, Campus Benjamin Franklin, Berlin, Germany

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ABSTRACT

Possible denaturation or tertiary structural changes of the protein human serum albumin (HSA) upon adsorption of uremic toxin is investigated using small-angle neutron scattering (SANS). Calorimetric data in previous studies give proof of the binding between HSA and two classes of uremic toxins: i) small molecular weight and ii) middle molecular weight molecules. A representative polyelectrolyte of negative net charge is used as a model middle molecule and two molecules phenylacetic acid (PhAA) and indoxyl sulfate (IDS) represent the small molecular weight toxins. The present study find no proof of destabilization of the protein structure upon toxin uptake. Analyzing the structure factor of scattering intensities from high concentrated protein samples complexed with PhAA and IDS show that interaction between native and complexed HSA is also not altered. However, a small effect of the net charge of HSA is found in the case of urea modified proteins.

1. Introduction

The interaction between proteins and polyelectrolytes is involved in many processes of modern life science involving biomaterials and medical devices [1]. In particular the uptake of ligands by proteins such as metabolites is an extensively investigated subject involved in all biological organisms [2]. These interactions are often accompanied by unwanted destructive influences on the protein, where either secondary or tertiary structures are altered and the protein functions are disabled. Therefore determining structural changes to proteins during ligand uptake is necessary to provide insight into deleterious effects of polyelectrolyte interaction with proteins.

Human serum albumin (HSA), as the most abundant protein in the human blood, plays a central role in the transport of naturally occurring compounds such as fatty acids and hormones [3,4], as well as introduced compounds like pharmaceuticals [5]. Notably, these transport properties facilitate the removal of high concentrations of uremic toxins that accumulate in the blood of chronic kidney disease (CKD) patients [6]. The toxins otherwise interact detrimentally with plasma proteins and lead to a high risk of cardio-vascular diseases [7–9]. Despite these well-known effects, their effect on the HSA protein structure remains unknown [6,10,11].

In our previous study, we have shown that a considerable amount of toxins interact strongly with HSA [12]. The analysis done there rests entirely on binding events, the tertiary structure of the protein was assumed to be undisturbed when adsorbing a toxin. However, conformational changes of the protein, such as those that occur when HSA binds with certain drugs [13,14], can affect the calorimetric signal, which then cannot purely be associated to the binding process itself. Such concerns can be eliminated by a structural study as SANS.

SANS is well suited for the study of macromolecular structures

* Corresponding author. Institute Soft Matter and Functional Materials Helmholtz-Zentrum Berlin, Hahn-Meitner Platz 1, 14109 Berlin, Germany.
E-mail address: matthias.ballauff@helmholtz-berlin.de (M. Ballauff).

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in solution, such as the protein structure [15–18]. The high contrast of proteins in heavy water and it's non destructive nature has led to its wide use as a probe of spatial structures of protein polyelectrolyte complexes [15,19–21]. Alternative techniques, such as crystallography [22,23], NMR, Fourier-transformed infrared spectroscopy (FTIR) [24] and dynamic light scattering (DLS) [25] are restricted either in the necessity for crystallization or in the size of the studied system. FTIR can be performed under physiological condition, but is only sensitive to secondary structure. SANS has the advantage of the possibility of analyzing protein-ligand/protein-polyelectrolyte complexes under equilibrium conditions.

We focus the present study on the interaction of HSA with two protein-bound uremic toxins, indoxyl sulfate (IDS) and phenylacetic acid (PhAA), a short polyelectrolyte, polyacrylic acid (PAA), as well as the effects of urea modification on HSA. IDS and PhAA are representative protein-bound uremic toxins which are small hydrophobic molecules bearing an ionic moiety.

PAA is a short polyelectrolyte, a class of middle molecular weight toxin. Whereas the interactions between high molecular weight polyelectrolytes have been well-studied, showing the formation of large agglomerations and phase separation leading to complex coacervation, (a useful mechanism for protein purification in pharmaceutical industries [26]) short polyelectrolytes of middle molecular weight remain comparatively unknown. Of the available studies, the interaction between a short polyelectrolyte, polyacrylic acid (PAA) with HSA in terms of driving forces and binding mechanisms has been explored [12], however, the structure of such a polyelectrolyte-protein complex has not yet been investigated.

Finally, HSA modification has been observed in pathophysiological conditions caused by chronic renal failure [27]. Under these conditions, a high urea concentration accompanies high levels of toxins, leading to protein modification [28]. Urea is known to induce carbamylation of HSA on multiple lysine- and arginine-containing chains. However, the links between this modification and the disease remains unclear [29]. At higher than pathophysiological urea concentrations, more extreme modifications of HSA have been observed, with denaturing HSA secondary structure occurring [30]. Thus the effect of urea modification on HSA structure and its possible impact on HSA-HSA interaction is of particular interest.

The uptake of a ligand by protein is analyzed by several authors using SAXS. Upon complex formation the radius of gyration R_g increased by 1 nm and an unexpected monomer-dimer equilibrium was found controlled by ionic strength and the protein concentration in the solution [31].

To elucidate the impact of toxin adsorption to HSA on the protein structure, we performed a series of SANS experiments of HSA under equilibrium conditions, to monitor the structural impact of toxins on the protein. By separating the particle form factor and the structure factor, we probed interactions with HSA concentrations as high as 40 g/L, which is also the physiological concentration in human blood plasma.

2. Experimental section

2.1. Materials

Phenylacetic acid (PhAA) and indoxyl sulfate potassium salt (IDS) and polyacrylic acid (PAA) with $M_W = 1800\ g\ mol^{-1}$ were purchased from Sigma-Aldrich. PhAA and IDS were used as received, while PAA was dialyzed several weeks to match buffer pH while avoiding a change of ionic strength in the solution. Human serum albumin (HSA) was also purchased from Sigma-Aldrich (lyophilized powder, fatty acid free, globulin free, 99%) with molecular weight calculated to be $M_W = 66{,}400\ g\ mol^{-1}$ and its purity verified by SDS-gelelectrophoresis. The buffer morpholin-N-oxide (MOPS) was purchased from Sigma-Aldrich and used as received.

2.2. Sample preparation

The ionic strengths of the solution were achieved by adding NaCl as a monovalent salt to a 10 mM MOPS buffer solution. To study the effect of divalend ions at an ionic strength of I = 20 mM, the solution were prepares as follows: 1 mM $MgCl_2$ + 2.5 mM $CaCl_2$ + 10 mM MOPS = 20 mM. The final pH of all samples studied was 7.2. All samples and buffers were prepared using 100% D_2O as the solvent, to provide increased contrast and minimal incoherent scattering background for SANS measurements.

For the HSA-PAA study, a concentration of 4.7 g/L of HSA was used for all samples, with 1 g/L and 0.25 g/L PAA added for the two different molar ratios, respectively. For the neutron scattering experiments of HSA-ligand complexes, three concentrations of HSA were prepared (30, 35 and 40 g/L) from a stock solution 40 g/L HSA in 10 mM buffer. Toxin concentrations were chosen to achieve full saturation of binding as determined by ITC. In the case of the HSA-urea system, 40 g/L HSA was mixed with 30 mM urea and incubated for 18 h at 37 °C in 10 mM MOPS buffer solution with pH 7.2. Two ionic strengths 10 mM and 150 mM were adjusted by adding NaCl to the buffer solution. After incubation the mixture was dialyzed against buffer using centrifugation dialysis several times. Final HSA concentrations were determined by characteristic UV-vis spectra to be approximately 25 g/L. These HSA-toxin samples were subsequently diluted by buffer solution to 22 g/L and 19 g/L.

2.3. Small angle neutron scattering (SANS)

SANS measurements were performed on the V4 SANS instrument at Helmholtz-Zentrum Berlin and the KWS-II SANS instrument at the MLZ in Munich. Samples were pipetted into Hellma 100 QS quartz cuvettes with a path length of 1 mm.

Measurements on the V4 instrument were made in a multi-sample holder maintained at 37 °C using a circulating water bath. Data were collected on a 1 m diameter area detector at sample to detector distances of 1 m, 4 m and 8 m, with a neutron wavelength of 6 Å and a wavelength resolution $\Delta\lambda/\lambda$ of 10%. Combined the total q range was 0.01 $Å^{-1}$ to 0.48 $Å^{-1}$. Additional high q measurements using a wavelength of 4.5 Å and a sample to detector distance of 1 m were made for the highest concentration samples of each sample type. These were combined with the data from the other instrument configurations to extend the maximum q for these samples to 0.75 $Å^{-1}$. Measurements on the KWS-II instrument were also maintained at 37 °C using a circulating water bath. KWS-II measurements were made at sample to detector distances of 1.12, 3.73, 5.73, and 19.73 m, with a wavelength of 5.27 Å.

Raw data from the V4 SANS measurements were reduced using the BerSANS software, while data from the KWS-II instrument were reduced using qtiKWS software. In both cases data were normalized to sample transmission, corrected for scattering from the empty cell, background detector noise, and variations in detector efficiency. Scattering arising from the sample buffer measurement and a flat incoherent scattering background, were both subtracted from the data. Conversion to an absolute intensity scale was achieved on V4 using a standard water sample measurement, while KWS-II used a Plexiglas secondary standard [32]. Finally, the data were radially averaged.

Differences in instrument configuration and wavelength ranges between the V4 and the KWS-II SANS instruments can result in differences of q resolution. This can lead to different representations of data features, however the difference in q resolution is insignificant in the context of the measured SANS curves for the