ESRF	**Experiment title:** Multivalent Cation Binding and Distribution around Protein in the Re-entrant Regime	**Experiment number**: SC-3004
Beamline: ID2	**Date of experiment**: from: 22th Oct. 2010 to: 26th Oct. 2010	**Date of report**: 1st Feb. 2011
Shifts: 12	**Local contact(s):** Michael Sztucki	*Received at ESRF:*

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Report:

Charge-induced protein-protein interactions attract much attention due to their direct relation to protein crystallization and protein association related diseases. For the interaction of proteins mediated by counter- and co-ions, the distribution of counterions is crucial [1]. However, little is known experimentally about the counterion distribution around charged proteins. So far, Anomalous Small Angle X-Ray Scattering (ASAXS) has provided the best way to study this issue by selecting the energies away and near the absorption edge of the target ions [2-5]. This work is devoted to the characterization of the counterion distribution around globular proteins in aqueous solution using ASAXS. The central question in this project is the spatial distribution of multivalent cations around the protein and the binding number of the cations.

We recently observed a reentrant condensation phase behavior for globular proteins in the presence of multivalent cations [1], as shown in Figure 1. Negatively charged globular protein in solution undergoes a phase-separation upon adding trivalent salt (YCl_3) up to a critical concentration C*. Further increasing the salt concentration above a second critical value, C**, the precipitates dissolve and the system turns back to a homogeneous solution. The reentrant phase behavior ensures the stability of the studied systems at high salt concentrations, which is important for ASAXS measurements. During this beamtime, we studied two proteins (BSA and BLG) in solution with YCl_3. Our goal is to get reliable ASAXS signals for the counterions by optimizing the samples and measurement parameters, such as protein/salt concentration, measuring time and number of energies, etc. Furthermore, we aim to establish the data analysis and fitting protocol for a direct description of the ion distribution around proteins in solution and the binding number on each protein. Further, this method will be extended to different proteins and salts of different valency.

Anomalous small-angle X-ray scattering (ASAXS) measurements were carried out at station ID2, ESRF, Grenoble. The experimental absorption K-edge of yttrium in solution was determined using a concentrated salt solution (1M) by an energy scan. The absorption edge is 17055 eV, with ΔE= 17 eV compared to the theoretical value (17038 eV). 12 energies were selected for following ASAXS measurements: with ΔE = -1000, -300, -100, -36, -12, -6, -4, -2, 0, 2, 6 and 36 eV.

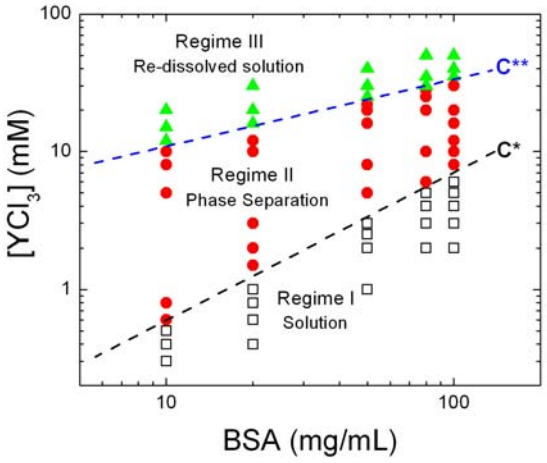

Figure 1 Phase diagram of protein solution in the presence of multivalent salt [1].

Protein solutions were filled into 2 mm quartz capillaries. The same salt solution without protein was measured as the background, in exactly the same way as the protein solutions and was subtracted from the sample scattering. All measurements were carried out at room temperature. The raw data were corrected for transmission, fluctuation of primary beam intensity, exposure time, and the response of the detector. Bovine serum albumin (BSA) and β-lactoglobulin (BLG) with protein concentration of 5, 20 and 50 mg/mL and salt concentration (YCl_3) from 1 to 50 mM were measured with a q-range from 0.06 to 4.5 nm^{-1}.

It has been estimated that in order to get a clear ASAXS signal, at least 0.1% of electrons participating is necessary. Indeed, we found that when using salt concentration below 5 mM, i.e. in the regime I of the phase diagram, no systematic shift of the scattering intensity could be observed in a full ASAXS run (data not shown). ASAXS signals become visible at higher salt concentrations in the reentrant regime III. Figure 2 presents the ASAXS data of BLG 50 mg/mL with 50 mM YCl_3, a clear ASAXS effect can be observed. The inset shows the scattering contribution of the Y^{3+} ions change strongly with the energy near the K-absorption edge, while the scattering contribution of the protein remains nearly constant. Systematic shifts in the scattered intensity can be observed at other concentrations samples after the background subtraction and fluorescence correction.

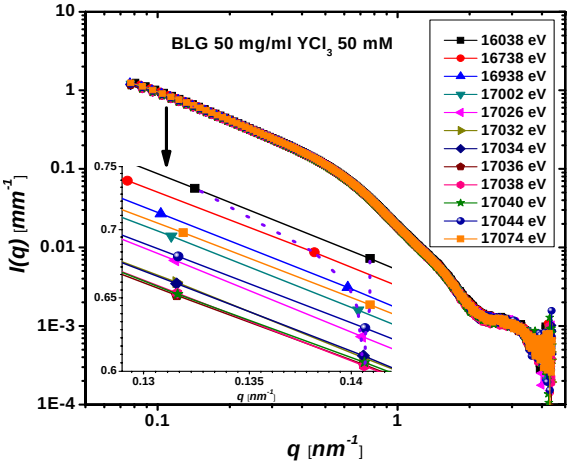

Figure 2 Typical ASAXS curves measured at 12 different energies .

Equation 1 shows that the normalized intensity measured at a minimum of three energies can be decomposed into three parts: energy-independent normal SAXS term (F_0^2), a cross-term (F_0*ν) involving the amplitudes of normal SAXS and the resonant scattering of the counterions, and the spatial distribution of resonant scattering term (ν^2) due to the ions.

$$I(q,E) = F_0^2(q) + 2f'F_0(q)\nu(q) + (f'(E)^2 + f''(E)^2)\nu^2(q) \qquad (1)$$

These m equations can be solved by the matrix method (Eq 2) [6] if m ≥ 3 were measured:

$$\begin{bmatrix} I(q_i,E_1) \\ \vdots \\ I(q_i,E_m) \end{bmatrix} = \begin{bmatrix} 1 & 2f'(E_1) & f'^2(E_1)+f''^2(E_1) \\ \vdots & \vdots & \vdots \\ 1 & 2f'(E_m) & f'^2(E_m)+f''^2(E_m) \end{bmatrix} \times \begin{bmatrix} F_0^2(q_i) \\ F_0(q_i)\nu(q_i) \\ \nu^2(q_i) \end{bmatrix} \qquad (2)$$

Figure 3 shows the results of the decomposition for typical samples as derived by Eq 2. The data analysis was done with the software package, *SAXSutilities*, developed by Sztucki [6] for X-ray scattering research. The pure resonant term can be successfully deduced using 12 energies, which is about 0.1% of the

total SAXS signal. The successful separation of pure resonant signal for the multivalent ion makes it possible for further understanding of the distribution of counterion around charged protein molecules.

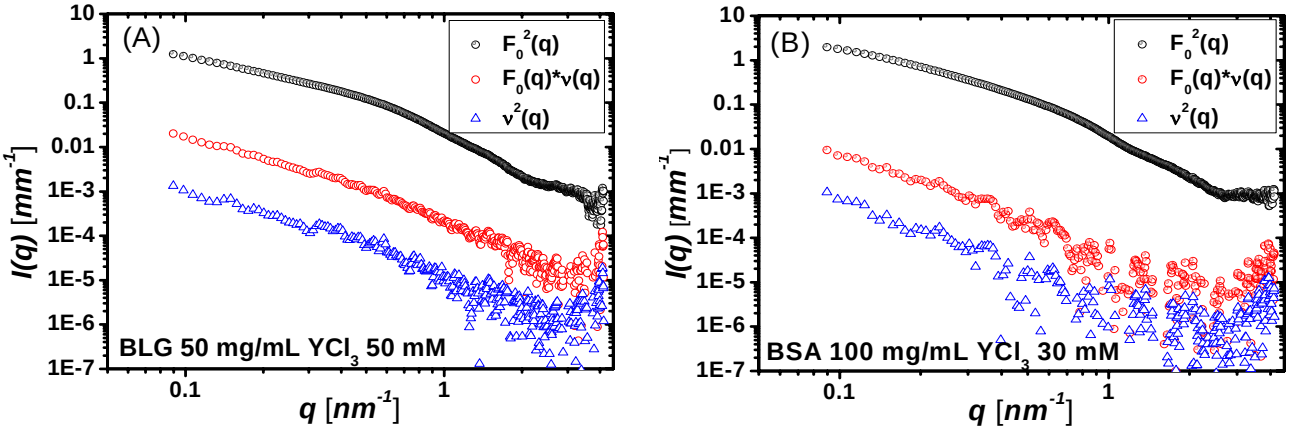

Figure 3 Typical separated ASAXS curves from 12 energies for two examples. Normal SAXS term (F_0^2), cross-term (F_0*ν), and the pure resonant scattering term (ν^2) .

According to the method introduced by Lois Pollack et al.[7], one can calculate the number of ions around the protein. For measurements carried out below the absorption edge, the *f''* term in Eq 1 is negligible. Using f_p for the effective number of electrons from a protein and N_{ions} the number of excess ions in the ion atmosphere around the protein, Eq 1 can be rewritten as:

$$I(q,E) = af'(E)^2 + bf'(E) + c \quad (3)$$

$$\text{where } a(q) = N_{ions}{}^2 \nu^2(q) \quad (4)$$

$$b(q) = N_{ions}(2f_p F_0(q)\nu(q) + 2f_0 N_{ions}\nu^2(q)) \quad (5)$$

$$c(q) = (f_p F_0(q))^2 + 2f_p f_0 N_{ions} F_0(q)\nu(q) + f_0{}^2 N_{ions}{}^2 \nu^2(q) \quad (6)$$

The q-dependent functions $a(q)$, $b(q)$, and $c(q)$ are extracted from measurements of $I(q)$ at several different energies. The number of ions around the protein calculated by Eq. 7-9 using two energies: one is far away and another is near but below the absorption edge. The values of $b(0)$ and $c(0)$ at q=0 are determined using the program GNOM [8]. Theoretical values are used for the scattering factor f' at different energies. For example, the numbers of trapped ions around a single protein for Figure 3A and 3B are N_{ions}=10.8±0.5 and 4.8±0.5, respectively.

$$N_{ions} = \frac{b(0)}{2\sqrt{c(0)}} \quad (7) \qquad b(q) = \frac{I(E_1) - I(E_2)}{f'(E_1) - f'(E_2)} \quad (8) \qquad c(q) = \frac{I(E_1)f'(E_2) - I(E_2)f'(E_1)}{f'(E_2) - f'(E_1)} \quad (9)$$

In summary, we have demonstrated that the ASAXS measurements on the counterion distribution around charged protein molecules are feasible and that information on the ionic cloud can be obtained. The present results encourage us to continue our research on the biological implications of such counterion atmosphere in protein solutions.

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