Strong Isotope Effects on Effective Interactions and Phase Behavior in Protein Solutions in the Presence of Multivalent Ions

Michal K. Braun, Marcell Wolf, Olga Matsarskaia, Stefano Da Vela, Felix Roosen-Runge, Michael Sztucki, Roland Roth, Fajun Zhang, and Frank Schreiber

Institut für Angewandte Physik, Universität Tübingen, Auf der Morgenstelle 10, 72076 Tübingen, Germany
Institut Laue-Langevin, 71 Avenue des Martyrs, 38000 Grenoble, France
ESRF - The European Synchrotron, 71 Avenue des Martyrs, 38000 Grenoble, France
Institut für Theoretische Physik, Universität Tübingen, Auf der Morgenstelle 14, 72076 Tübingen, Germany

ABSTRACT: In this article, we have studied the influence of the isotopic composition of the solvent (H2O or D2O) on the effective interactions and the phase behavior of the globular protein bovine serum albumin in solution with two trivalent salts (LaCl3 and YCl3). Protein solutions with both salts exhibit a reentrant condensation phase behavior. The condensed regime (regime II) in between two salt concentration boundaries (c* < c < c**) is significantly broadened by replacing H2O with D2O. Within regime II, liquid–liquid phase separation (LLPS) occurs. The samples that undergo LLPS have a lower critical solution temperature (LCST). The value of LCST decreases significantly with increasing solvent fraction of D2O. The effective protein–protein interactions characterized by small-angle X-ray scattering demonstrate that although changing the solvent has negligible effects below c*, where the interactions are dominated by electrostatic repulsion, an enhanced effective attraction is observed in D2O above c*, consistent with the phase behavior observed. As the LCST–LLPS is an entropy-driven phase transition, the results of this study emphasize the role of entropy in solvent isotope effects.

INTRODUCTION

Quantitative description of the effective protein–protein interactions in aqueous solutions is one of the major challenges in the study of soft and biological matter. As the effective interactions control the exact phase behavior of protein solutions, including crystallization, a complete picture of protein interactions at different length scales with various control parameters could ease the search of optimal conditions for crystal growth. Protein phase behavior is also crucial for a better understanding of protein aggregation-related physiological diseases. In particular, the existence of a metastable liquid–liquid phase separation (LLPS) in protein and colloid systems, emphasizing the role of short-ranged attractions.

Among the various environmental parameters, the solvent isotope effect on the effective interactions between proteins is still far from clear. The impact of the isotopic composition of the solvent strongly depends on the circumstances and the observable of interest. The physicochemical properties of D2O are very similar to those of H2O. It is thus commonly believed that the substitution of H2O with D2O causes only a very small observable of interest. The physicochemical properties of D2O are very similar to those of H2O. It is thus commonly believed that the substitution of H2O with D2O causes only a very small perturbation of the structural preferences of a solute. In fact, in many biophysical studies using neutron scattering and infrared and NMR spectroscopy, D2O is widely used as the solvent to obtain a useful signal.
assumed that D$_2$O has a negligible effect on the structure and interactions of these biological systems. However, while the effect of D$_2$O is generally considered weak in biological systems, studies have shown significant changes in the interactions between proteins and their dynamics when H$_2$O is substituted with D$_2$O. For instance, it has been found that D$_2$O can stabilize proteins against thermal denaturation and influence the macromolecular dynamics in *Escherichia coli.*

Some studies have found that the effective protein-protein attraction can be enhanced when H$_2$O is replaced by D$_2$O. Gripon et al. investigated the effective interactions in lysozyme solutions in H$_2$O and D$_2$O to understand the lower solubility of lysozyme in D$_2$O. The solubility line is shifted to higher temperatures by about 7.2 °C in D$_2$O. SANS measurements and data analysis based on the second virial coefficient indicate that the repulsive potential due to the net surface charge does not change. The attraction between lysozyme molecules, however, is stronger in D$_2$O than in H$_2$O. It is assumed that this is due to hydrophobic hydration and salt-specific interactions.

A recent study by Bucciarelli et al. on γ$_s$-crystallin solutions has shown an even stronger solvent isotope effect. Replacing H$_2$O with D$_2$O results in an increase of the critical LLPS temperature of up to 16 °C. While this systematic study demonstrates that the variation of the critical temperature can be described using the extended law of corresponding states, the underlying physical mechanism of the solvent isotope effect is still not clear.

We have shown previously that trivalent salts, such as YCl$_3$, can induce a reentrant condensation (RC) phase behavior in several acidic proteins. For a given protein concentration c$_p^*$ when the salt concentration c$_s$ is below a certain value c$, or above a value c$^**(*)$, protein solutions are clear. At salt concentrations between c$^*$ and c$^**$, protein condensation occurs, including aggregation or clustering, LLPS, and crystallization. In particular, the metastable LLPS has been demonstrated to be an entropy-driven process, as the solvent fractions of D$_2$O was determined by visual inspection. The state diagram of protein solutions with YCl$_3$ and different solvent fractions of D$_2$O was determined by visual inspection. To discriminate between LLPS and aggregation, the turbid samples were examined using a transmission microscope (AxioScope A1, Zeiss) equipped with an AxioCam ICcS charge-coupled device (CCD) camera.

**Determination of the LCST.** The dependence of the transition temperature on the solvent fraction of D$_2$O was determined using a UV–vis spectrometer equipped with a water bath for temperature control (Haake A10B and SC 150, Thermo Fisher Scientific Inc.). The sample was slowly heated from the bath temperature of 12 °C at a rate of 0.1 C/min. The temperature of the sample solution was calibrated using a thermocouple attached to the sample holder. At a heating rate of 0.1 °C/min, the sample temperature was given by $T = (0.092t + 12)$ °C, with time t (in minutes) after the start of the experiment. The absorbance spectra in the range of 500–800 nm were collected every 2 min, and the intensities were integrated and plotted against temperature. The LCST was determined from the main peak of the first derivative of the integrated absorbance as a function of temperature. Before the UV–vis measurement, the samples were centrifuged for 2 min with an RCF of 6860g to remove large aggregates.

**Small-Angle X-ray Scattering (SAXS).** Effective protein–protein interactions in the solutions were characterized by SAXS. SAXS experiments were conducted at beamline ID02 at the ESRF, Grenoble, France. The X-ray energy was 12 keV, which corresponds to a wavelength of 1.0 Å. For all measurements, the sample-to-detector distance was set to 2 m, covering a q-range of 0.005–0.5 Å$^{-1}$. The data were collected by a high-sensitivity fiber-optic-coupled CCD detector placed in an evacuated flight tube. The samples were prepared right before the measurement. The protein solution was loaded into a flow-through quartz capillary with a diameter of 2 mm and a wall thickness of 0.01 mm. The data sets were reduced by subtracting the scattering of a pure salt solution as a background and by normalizing to absolute intensity. Further details on q-resolution, calibration, and data reduction can be found in refs 56 and 57. Data fitting was performed using IGOR PRO with macros provided by NIST.

**RESULTS**

Phase Behavior and Effective Interactions of BSA–LaCl$_3$ in H$_2$O and D$_2$O. We first present the phase behavior of BSA with LaCl$_3$ in both pure H$_2$O and D$_2$O at room temperature. As shown in Figure 1, an RC is found in both cases. The solutions are clear below the salt concentration c$^*$(regime I). In between c$^*$ and c$^**(*)$, condensation takes place, which is the so-called regime II.
absorbance of the samples with 80 mg/mL BSA, 13 mM LaCl₃, and D₂O solvent fraction. The line is a linear fit to the first five data points.

Table 1. Determination of the LCST for a Series of Samples with 80 mg/mL BSA, 13 mM LaCl₃, and Different D₂O Solvent Fractions

<table>
<thead>
<tr>
<th>D₂O Solvent Fraction</th>
<th>cₚ (mg/mL)</th>
<th>LCST (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>80.1</td>
<td>31.5</td>
</tr>
<tr>
<td>0.05</td>
<td>80.7</td>
<td>28.8</td>
</tr>
<tr>
<td>0.10</td>
<td>80.8</td>
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<tr>
<td>0.15</td>
<td>80.1</td>
<td>23.6</td>
</tr>
<tr>
<td>0.20</td>
<td>80.4</td>
<td>22.2</td>
</tr>
</tbody>
</table>

*Underlined numbers indicate a decrease of protein concentration in the supernatant due to aggregation.*

investigate higher D₂O solvent fractions with our method. From a linear fit of the first five data points shown in Figure 2c, an LCST of −16.1 °C is estimated in pure D₂O. This would be 47.6 °C below the LCST in pure H₂O.

**Effective Protein–Protein Interactions Characte**

ized by SAXS. For a quantitative understanding of the solvent isotope effects, we characterize the effective protein–protein interactions using SAXS. Representative SAXS profiles are shown in Figure 3 for BSA-LaCl₃ solutions in both H₂O and D₂O as a function of salt concentration. In both cases, SAXS data show similar trends. At low salt concentration, the effective protein–protein interactions are dominated by the net negative charge. A strong correlation peak is visible. With increasing salt concentration, the low q intensity increases, indicating the reduction of repulsion. In this region of the phase diagram (regime I), the solutions are clear.

With further increasing salt concentration, the systems become more and more dominated by attractive interactions, with the attraction reaching its maximum at 12 mM (H₂O) and 20 mM LaCl₃ (D₂O). In H₂O, starting from 15 mM, the strength of the attraction starts to decrease. In D₂O, the strength of the attraction starts to decrease at 25 mM. In D₂O, the decrease starts at a higher salt concentration, which corresponds very well to the finding that regime II also extends to higher salt concentrations (see also Figure 1). The decrease is found to start very close to the upper boundaries (c⁺⁺) of the second regime. In H₂O, c⁺⁺ (80 mg/mL) is located at 16 ± 2 mM. In D₂O, c⁺⁺ (87 mg/mL) is located at 26 ± 2 mM. The
The SHS model was introduced by Baxter for data analysis, see the Supporting Information. In H$_2$O, the scattering intensity decreases in (c) and (d). In the legend, SC indicates that the screened Coulombic potential was used for data fitting. The other data were fitted using the SHS potential. For further information on the SAXS data analysis, see the Supporting Information.

Figure 3. (a, c) SAXS data with model fits for samples in H$_2$O containing 85 mg/mL BSA. (b, d) SAXS data with model fits for samples in D$_2$O containing 87 mg/mL BSA. The scattering intensity at low q increases with increasing salt concentration in (a) and (b) and decreases in (c) and (d). In the legend, SC indicates that the screened Coulombic potential was used for data fitting. The other data were fitted using the SHS potential. For further information on the SAXS data analysis, see the Supporting Information.

To quantify the effective interactions, the SAXS data were fitted using models with an ellipsoid form factor and different interaction potentials. Figure 3 presents the SAXS data with model fitting for BSA–LaCl$_3$ in H$_2$O and D$_2$O, respectively. The fits are superimposed on the data as solid black lines. The corresponding structure factors are shown in Figure S3. In both cases, below c*, the interactions are dominated by electrostatics due to the surface charges. The two scattering curves for samples with very low salt concentrations (0 and 2 mM LaCl$_3$) were fitted using a screened Coulombic potential in both H$_2$O and D$_2$O. In H$_2$O, the fitted charges are 18.1 and 10.3 e and the ionic strengths are 7.4 and 10 mM for 0 and 2 mM, respectively. In D$_2$O, the charges are 16.3 and 8.71 e and the ionic strengths are 6.2 and 8.6 mM for 0 and 2 mM, respectively. The values in H$_2$O are thus very similar to those in D$_2$O. Therefore, the repulsive part of the potential shows only a weak dependence on the solvent (H$_2$O or D$_2$O).

In regimes II and III, where the effective interactions are attractive, a strong isotope effect is observed. This interesting finding will be further discussed and explained in the Discussion section.

To quantitatively describe the attractive potential, the stickiness parameter obtained for samples in regimes II and III. For these samples is below c*.

The data are plotted against the normalized salt concentration, c$_s$/c$_o$. Results for two sets of samples with 150 and 85 mg/mL BSA in H$_2$O and one set of samples with 87 mg/mL BSA in D$_2$O are shown. Dashed lines are added as guides to the eye. Remarkably, the two series measured for the BSA–LaCl$_3$ system in H$_2$O fall onto one common master curve. In H$_2$O, $B_2/B_1^{HS}$ only touches −1.5 in its minimum. Clearly, the curve in D$_2$O is well below the one in H$_2$O. Thus, the strength of attraction is significantly enhanced in D$_2$O. The samples that showed macroscopic LLPS are marked by filled squares. $B_2/B_1^{HS}$ for these samples is below −1.5, which is in agreement with the findings reported by Wolf et al. Furthermore, $B_2/B_1^{HS}$ first decreases quickly below c*. After reaching the minimum, it starts to increase again but much more slowly.
nonsymmetric change is most likely attributed to the screening effect of the co-ion, Cl\(^-\). The increasing amount of co-ions screens the effective surface charge of the proteins. Fujihara and Akiyama studied the attractive interaction between macro-ions mediated by divalent cations and observed a similar trend of effective interaction potential as a function of cation concentration.\(^{52,54}\) This quantitative interpretation and analysis of the SAXS data nicely fits to the qualitative macroscopic phase behavior in the different systems described above (Figure 1). The qualitative observations as well as the quantitative results show that the attraction is enhanced when H\(_2\)O is replaced by D\(_2\)O.

**Effect of D\(_2\)O Solvent Fraction on the Phase Behavior and the Effective Interactions.** The general phase behavior, including LCST for BSA with YCl\(_3\) in H\(_2\)O, has been described in our previous work.\(^{41,46,50}\) Here, we focus on the effect of the solvent fraction of D\(_2\)O on the phase behavior. We first determined a state diagram for protein solutions with a fixed protein concentration (91.7 mg/mL BSA) as a function of the YCl\(_3\) concentration and the solvent fraction of D\(_2\)O. The results are summarized in Figure 5. The shaded area indicates regime II. This state diagram shows that above a D\(_2\)O solvent fraction of 0.8 there is no LLPS anymore but only amorphous aggregation. For example, samples with 12 mM YCl\(_3\) are all of this type.

Figure 5. Experimental state diagram for BSA with YCl\(_3\) at different solvent fractions of D\(_2\)O. The BSA concentration was fixed at 91.7 mg/mL. The shaded area corresponds to regime II. Sample series with selected salt concentrations for SAXS measurements were marked by green lines.

In regime III with 100 mM YCl\(_3\), where the samples are clear liquids, the correlation peak vanishes. An increase in the D\(_2\)O solvent fraction slightly shifts the scattering intensity at low \(q\) to higher values, indicating a slight increase in the attractive interactions.

In regime II with 12 mM YCl\(_3\), where macroscopic phase separation occurs as shown in Figure 5, the samples were centrifuged, and only the supernatant was used for SAXS measurements. The vertical shift of the SAXS profiles with increasing D\(_2\)O solvent fraction stays more or less constant.

To further investigate the solvent isotope effect on the effective interactions, four series of samples with salt concentrations well below \(c^*\), close to \(c^*\), in regime II (between \(c^*\) and \(c^{**}\)), and slightly above \(c^{**}\) were measured by SAXS as a function of the D\(_2\)O solvent fraction. The salt concentrations that were chosen for the SAXS experiments are marked by green lines in Figure 5. The measured SAXS profiles are shown in Figure 6.

![SAXS profiles for samples in different regimes with BSA 91.7 mg/mL and varying solvent fractions of D\(_2\)O. The YCl\(_3\) concentrations are given in each subplot (see also green dashed lines in Figure 5).](image)

Well below \(c^*_s\), at \(c_s = 0.5\) mM YCl\(_3\), the scattering curves exhibit a correlation peak. This is the result of the long-range Coulombic repulsion due to the net negative charge of the proteins. Varying the D\(_2\)O solvent fraction does not affect the scattering profiles and thus the effective interactions. This is in good agreement with the results for BSA with LaCl\(_3\) at low salt concentrations, which are presented in Figure 3.

Slightly below \(c^*_s\) with \(c_s = 5\) mM YCl\(_3\), the correlation peak in the SAXS profiles vanishes. An increase in the D\(_2\)O solvent fraction slightly shifts the scattering intensity at low \(q\) to higher values, indicating a slight increase in the attractive interactions.

In regime II with 12 mM YCl\(_3\), where macroscopic phase separation occurs as shown in Figure 5, the samples were centrifuged, and only the supernatant was used for SAXS measurements. The vertical shift of the SAXS profiles corresponds to the variation of the protein concentration in the supernatant. The downward shift of the SAXS profiles with increasing D\(_2\)O solvent fraction is consistent with an increase of attraction.

In regime III with 100 mM YCl\(_3\), where the samples are clear again, the scattering intensity at low \(q\) and hence the attraction increase significantly with increasing D\(_2\)O solvent fraction. This is also consistent with the macroscopic observation that the samples are closer to \(c^{**}\) with increasing D\(_2\)O solvent fraction (Figure 5).
We have studied the solvent isotope effect (H2O vs D2O) on the phase behavior of BSA solutions in the presence of two trivalent salts, LaCl3 and YCl3. While the RC phase behavior is found under all experimental conditions (salt or solvent), LCST—LLPS occurs only under certain conditions. The phase behaviors found in different systems are summarized in Table 2.

In the BSA–YCl3 system, LLPS occurs in pure H2O. At room temperature, the region of LLPS shrinks with increasing D2O solvent fraction until it vanishes in pure D2O. In the BSA–LaCl3 system, however, LLPS occurs in pure D2O at room temperature, whereas in pure H2O, LLPS occurs only at temperatures above 30 °C (Figure 2). At room temperature, only mesoscopic protein clusters exist, and there is no macroscopic phase separation.

The effective protein–protein interactions characterized by SAXS demonstrate that the attraction increases by replacing H2O with D2O (Figure 4). This explains the phase behavior that is summarized in Table 2. In BSA–LaCl3 in H2O, there is no LLPS at room temperature because the attraction is too weak. In BSA–YCl3 in D2O, there is no LLPS because the attraction is too strong and only amorphous aggregates are formed.

The significant decrease of the LCST with increasing D2O solvent fraction should be compared with the effects of the solvent isotope on the solubility of lysozyme. A difference of about 7.2 °C in D2O versus H2O was reported.29,38 In another system, namely, in solutions of γ-R-crystallin, the (upper) critical temperature of LLPS increases by 16 °C in D2O versus H2O.30 As the 7.2 °C difference in protein solubility of lysozyme is consistent with the temperature difference of the maximum density of light and heavy water, the larger difference observed in γ-R-crystallin solutions and in the work presented here indicates that other contributions may play a crucial role.

The existence of an LCST phase behavior in our systems demonstrates that the LLPS is driven by entropy. Thus, the observed significant response of the effective interactions (mainly the attractive part) to the solvent composition in protein solutions must be due to the solvent-isotope-dependent entropy contribution. Before discussing further the possible entropy contribution, we emphasize that the protein condensation observed is not caused by a change of the protein structure induced by YCl3 or LaCl3. We have studied the stability of the protein secondary structure in the presence of multivalent salts in H2O and D2O using circular dichroism (CD) and Fourier transform infrared.42,57,85 The consistency of the results of the different techniques suggests that replacing H2O with D2O has no significant effect on the secondary structure of proteins. Moreover, the successful growth of high-quality crystals and structural analysis confirm that the proteins are still in their native state.47,63,64

We have recently discussed the mechanism of the LCST phase behavior in our system using the patchy colloid model. We propose that the key entropy contribution is due to the release of hydration water molecules upon ion bridging.60 Upon heating, both the carboxy groups and the trivalent ions are partially dehydrated, resulting in a high entropy gain. Substitution of H2O with D2O certainly influences the hydration and dehydration of both proteins and cations. Unfortunately, studies on the hydrogen bonds in H2O or D2O for systems involving different types of functional groups turn out to be a great challenge.31,63–68 Nevertheless, we find that these isotope effects lead to a higher entropy contribution for our system in D2O, which results in an enhanced effective attraction between proteins and a lower LCST. The entropy contribution to the solvent isotope effects may also shed light on the unusual strong effects observed in other protein systems.29,30,38

It is worth noting that although similar entropy-driven LCST phase behavior is common in some polymer solutions, the isotope effects of the solvents are different. In some polymer solutions (e.g., PNIPAM), replacing H2O with D2O increases the LCST by 1 or 2 °C69,70 which is in contrast to our system, where we observe a significant decrease in the LCST. This opposite trend of the solvent isotope effect may be due to the different types of entropy contributions involved. In aqueous solutions of polymers, the entropy contribution comes from the dehydration of the hydrophobic part of the molecules.70,71 Therefore, the stability of this hydration shell is enhanced when H2O is replaced by D2O, leading to a higher LCST. In our system, the entropy contribution comes from the reduced translational and rotational entropy of the hydration waters of the hydrophilic carboxyl groups on the protein surface and the metal cations. The different significance of the solvent isotope effect between the polymer and protein systems might be due to the different levels of cooperation of the hydrogen bond or the different amounts of hydration water involved.

**DISCUSSION**

We have studied the solvent isotope effect (H2O vs D2O) on the phase behavior of BSA solutions in the presence of two trivalent salts, LaCl3 and YCl3. While the RC phase behavior is found under all experimental conditions (salt or solvent), LCST—LLPS occurs only under certain conditions. The phase behaviors found in different systems are summarized in Table 2.

<table>
<thead>
<tr>
<th>no.</th>
<th>solvent</th>
<th>salt</th>
<th>macroscopic phase behavior in regime II</th>
<th>attraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H2O</td>
<td>LaCl3</td>
<td>mesoscopic clusters</td>
<td>too weak for LLPS</td>
</tr>
<tr>
<td>2</td>
<td>D2O</td>
<td>LaCl3</td>
<td>LCST—LLPS</td>
<td>suitable for LLPS</td>
</tr>
<tr>
<td>3</td>
<td>H2O</td>
<td>YCl3</td>
<td>LCST—LLPS</td>
<td>suitable for LLPS</td>
</tr>
<tr>
<td>4</td>
<td>D2O</td>
<td>YCl3</td>
<td>amorphous aggregates</td>
<td>too strong for LLPS</td>
</tr>
</tbody>
</table>

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**CONCLUSIONS**

In this work, we have studied the effective protein–protein interactions and phase behavior in solutions with two trivalent salts (LaCl3 and YCl3). In particular, we focused on the solvent isotope effect when replacing H2O with D2O. For both systems in both solvents, an RC phase behavior is observed. Within regime II, LCST—LLPS occurs under certain specific conditions. This rich phase behavior is highly sensitive to the D2O solvent fraction. While c* is weakly affected by replacing H2O with D2O, c** shifts to higher salt concentrations, resulting in a broadening of regime II. The LCST—LLPS phase behavior for both salts shows strong solvent isotope effects, as summarized in Table 2. The LCST decreases significantly with increasing D2O solvent fraction. The effective protein–protein interactions characterized by SAXS are consistent with the phase behavior observed. At low salt concentrations below c*, interactions are dominated by electrostatic repulsion, which is not sensitive to the D2O solvent fraction. Above c*, the effective interactions become attractive and strongly depend on the D2O solvent fraction. The interaction potential can be well described using a SHS model, indicating the short-range nature of the attraction. The reduced second virial coefficients, B2/D2O, decrease steeply first and increase slowly after reaching a minimum with increasing salt concentration. Similar trends are observed in both H2O and D2O, but the values become more negative in D2O, indicating an enhanced attraction. The
entropy-driven LCST phase behavior suggests that the origin of the short-ranged attraction is closely related to entropy, which is most likely due to the release of hydration water from both metal ions and protein surfaces upon ion binding. The entropy contribution to the solvent isotope effects may also shed light on the unusual strong effects observed in other protein systems.29,30,38

■ ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpcb.6b12814.

Details on SAXS data fitting, determination of the LCST using UV−vis transmission, data for 150 mg/mL BSA, structure factors obtained from data fitting, series of microscope images for samples with varying D2O solvent fraction (PDF)

■ AUTHOR INFORMATION

Corresponding Author
*E-mail: fajun.zhang@uni-tuebingen.de.

ORCID
Michal K. Braun: 0000-0002-9642-7492
Fajun Zhang: 0000-0001-7639-8594

Present Addresses
1 Austrian SAXS Beaml ine, ELETTRA Sincrotrone, Outstation of the Institut für Anorganische Chemie, Technische Universität Graz, Steyrmgargasse 9/V, 8010 Graz, Trieste, Italy (M.W.).
2 Division of Physical Chemistry, Department of Chemistry, Lund University, Naturvetarvägen 16, 22400 Lund, Sweden (F.R.-R.).

Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank R. Akiyama (Kyushu University, Japan) and G. Lotze (ESRF) for valuable discussions and help for data analysis. This work was supported by the Deutsche Forschungsgemeinschaft (DFG). Furthermore, they thank the ESRF for allocation of beamtime on ID02. Olga Matsarskaia acknowledges a studentship by the Studienstiftung des Deutschen Volkes.

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