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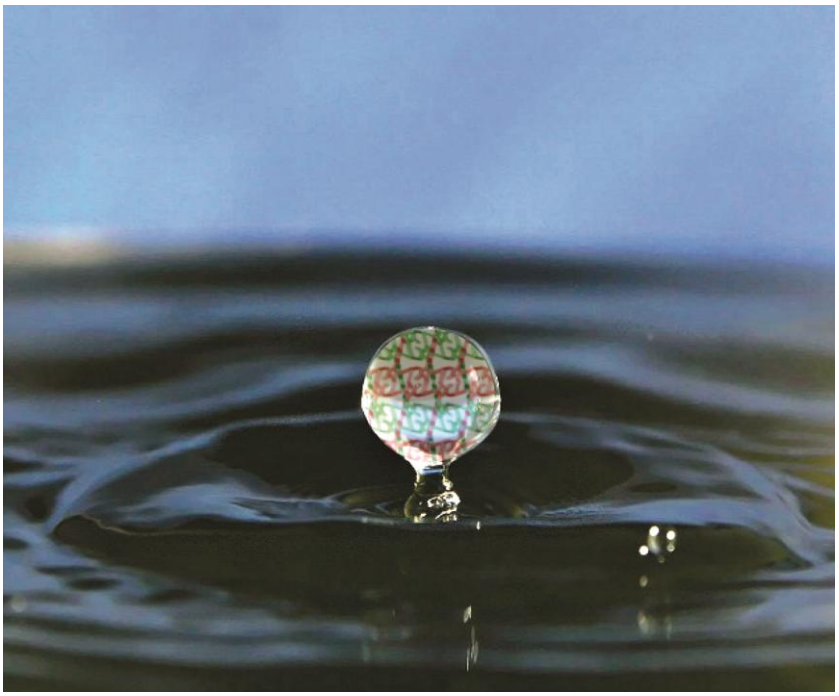

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PAPER

Hofmeister effects of ionic liquids in protein crystallization: Direct and water-mediated interactions†‡

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We have performed experiments on the crystallization of two low molecular weight, positively charged proteins, lysozyme and ribonuclease A, using ionic liquids as either crystallization additives or, in particular cases, as precipitating agents. The ionic liquids (ILs) have been ordered according to their salting-in/out ability and the relative position of these ionic liquids in this ranking has been rationalized by considering their hydration properties (positive–negative, hydrophobic–hydrophilic). The ability to screen the effective charge of cationic proteins and aid protein nucleation (salting-out) has been shown to be superior for large polarizable anions with low charge density, negatively hydrated-Cl^-, Br^-, $[SCN]^-$, methane-$[C_1SO_3]^-$ and ethanesulfonates $[C_2SO_3]^-$, than for anions with a relatively stable hydration shell, positively hydrated-lactate $[Lac]^-$, butylsulfonate $[C_4SO_3]^-$ and acetate $[Ac]^-$. Upon increasing the background salt concentration, where electrostatic interactions are already effectively screened, the ability of the IL ions to stabilize proteins in solution (salting-in) has been shown to increase as the ions are likely to migrate to the non-polar protein surface and lower protein–water interfacial tension. This tendency is enhanced as the focus moves from those ions with positively hydrated hydrophilic compartments (*e.g.* $[Ac]^-$) to those with negatively hydrated groups (*e.g.* $[C_1SO_3]^-$) and the prevailing hydrophobic hydration (*e.g.* $[C_4SO_3]^-$). The observed inversion in the relative effect of ILs on protein crystallization with increasing ionic strength of the aqueous media has been interpreted as the differing effects of ion adsorption: charge screening and interfacial tension modification. Moreover, this work can further help in our understanding of the influence of ionic liquids on conformational changes of biomacromolecules in solution. Identification of the specific incorporation sites for choline and acetate ions, localized in two lysozyme crystals grown in pure IL solutions without any buffer or inorganic precipitant, can give us some insight into the role of the ionic liquid ions in protein structure development.

Introduction

Obtaining a good quality protein crystal adequate for X-ray structure determination is still a bottleneck in proteomics. Many efforts have been made in order to understand both the mechanism of protein crystallization and the effect of particular additives on conformational changes of biomacromolecules. In recent years, there has been an increasing attention to the application of ionic liquids in different areas of the protein science[1] – from protein stability,[2–5] activity,[6–8] and extraction[9–12] to protein crystallization.[13–20] One of the reasons for this relevance is the tunable nature of ILs that allows tailoring ILs with specific chemical and physical properties.[21] In regard to protein crystallization, studies have mainly focused on model proteins in order to understand the origin of the ILs influence on biomacromolecular crystals formation. ILs have been shown to often exert an advantageous effect on crystallization. Nevertheless only a few cases of the successful application of ILs to overcoming difficulties in real case scenarios are reported.[14,22] Any systematic or consistent theory that could explain the mechanism of action of ILs on protein crystallization, and, therefore, that could serve as a guide to select (tailor) ILs presenting a specific, desirable effect, has not yet been presented.

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† Electronic Supplementary Information (ESI) available: Table with detailed collection statistics for protein crystals and crystal images. See DOI: 10.1039/c2ce25129a

‡ Data for RNase crystal grown in the presence of IL as well as coordinates and observed structure factor amplitudes for the choline- and acetate-bound lysozyme have been deposited in the Protein Data Bank in Europe (PDBe), www.ebi.ac.uk/pdbe (PDB ID codes: 4AO1 for RNase 4AGA for lysozyme).

The aim of the current study is to contribute to further understanding the molecular phenomena underlying the influence of ILs on crystallization of biomacromolecules. This work focuses on model proteins and tends to systematize and rationalize the observed trends. ILs used in our experiments have been chosen based on previous evidence about the impact of ILs with particular characteristics on inorganic crystal precipitation. That information is found in one of our previous studies.[23] Apparently, close similarities are found between the response of simple ionic systems and that of complex biomacromolecular ones to the presence of ILs. Ionic liquids themselves, while being composed of relatively simple organic ions but also possessing charges, hydrophobic and hydrophilic parts, have been shown to respond to the Hofmeister series of ions in a manner similar to that observed for proteins.[24–28] Some underlying mechanisms of interactions (direct binding, surface tension effects) have been proposed. On the other hand, ILs have been ordered according to their salting-in/out ability towards organic species.[29] These aforementioned studies performed on simpler systems together with existing theories in the field of protein crystallization have served as the starting point for the current rationalization presented in this work of the effect of ILs on model protein crystallization.

Materials and methods

Ionic liquids

The following ionic liquids have been used: 1,3-dimethylimidazolium dimethylphosphate, [MMIM][DMP] (Io-li-tec, > 98%), 1-ethyl-3-methylimidazolium chloride, [EMIM]Cl (Io-li-tec, > 98%), bromide, [EMIM]Br (Fluka, ⩾ 97%), thiocyanate, [EMIM][SCN] (Io-li-tec, > 98%), acetate, [EMIM][Ac] (Io-li-tec, > 98%), ethanesulfonate, [EMIM][C_2SO_3] (synthesized in our lab), butylsulfonate, [EMIM][C_4SO_3] (synthesized in our lab), 1-butyl-3-methylimidazolium chloride, [BMIM]Cl (Io-li-tec, 99%) and acetate, [BMIM][Ac] (Aldrich, ⩾ 95%), 2-hydroxyethyl-trimethylammonium chloride, [Ch]Cl (Sigma, ⩾ 98%), acetate, [Ch][Ac] (Solchemar, 95%), methanesulfonate, [Ch][C_1SO_3] (synthesized in our lab) and lactate, [Ch][Lac] (synthesized in our lab). The structures of the cations and anions are presented in Fig. 1. The ILs were chosen based on their recognized effect on inorganic crystal precipitation from solution and depending on their hydration characteristics and tendency of the cation and anion to associate in solution.[23]

Lysozyme-ionic liquids as additives

Chicken egg white lysozyme (Fluka) has been dissolved in 50 mM sodium acetate buffer (pH = 4.57) and crystallized by the sitting drop diffusion method. Drops have been prepared by mixing 10 μl of the protein solution (40 mg ml^{-1}) with 10 μl of the reservoir solution (11% NaCl in 50 mM sodium acetate buffer of pH = 4.57) containing a particular concentration of the ionic liquid additive. Each 20 μl drop contained 20 mg ml^{-1} of lysozyme and 5.5% (w/v) of NaCl in 37.5 mM of Na[Ac] (pH = 4.57) and either no additive (control conditions) or 0.0625 M, 0.125 M, 0.25 M, 0.5 M, and 1 M, of a given IL. Each set of drops (with a specific concentration of the IL) has been equilibrated at 20 °C against 3.5 ml of the mutual reservoir solution. No IL was present in the reservoir.

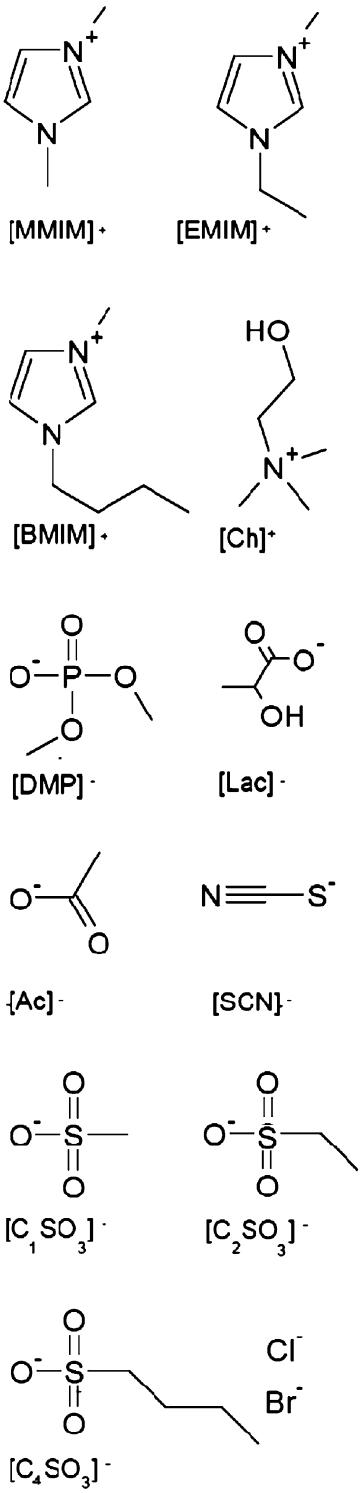

Fig. 1 Structures of the cations and anions composing the ionic liquids used in this study and corresponding abbreviations.

Lysozyme–ionic liquids as precipitating agents

Additional experiments have been performed using the batch crystallization method without the use of any inorganic

precipitant. Two different conditions have been applied: (a) drops containing 20 mg ml^{-1} of lysozyme and 1 M [Ch]Cl or 1 M [Ch][Ac] in 25 mM sodium acetate buffer of pH = 4.57, and b) drops containing 20 mg ml^{-1} of lysozyme and 1 M [Ch]Cl or 1 M [Ch][Ac] without buffer, in either a protein or IL solution. Drops have been prepared by mixing 10 μl of the protein solution (40 mg ml^{-1}) with (a) 10 μl of the solution containing 2 M IL dissolved in 50 mM sodium acetate buffer (pH = 4.57) or (b) 10 μl of the 2 M IL solution. 40 μl drops placed in sealed containers have been equilibrated at 4 °C.

RNase-ionic liquids as additives

Ribonuclease A from bovine pancreas (Sigma) has been dissolved in 50 mM sodium acetate buffer (pH = 4.57) and crystallized by the sitting drop diffusion method. For the preliminary scanning of crystallization conditions, 4 μl drops have been prepared by mixing 2 μl of the protein solution (10 mg ml^{-1}) with (a) 2 μl of the reservoir solution (3 M NaCl and 0.2 M $(NH_4)_2SO_4$ in 50 mM Na[Ac], pH = 4.57) or (b) 1 μl of the reservoir solution and either 1 μl of 2 M [Ch][C_1SO_3] or 1 μl of 2 M [Ch]Ac. Under these conditions the protein crystal has grown only in the IL-free sample, while drops containing ILs remained clear. Therefore considerably higher protein and/or inorganic precipitant concentrations had to be used in further experiments in order to induce protein crystallization in the presence of ILs. In all the following experiments reservoir solutions contained 3 M NaCl and 2 M $(NH_4)_2SO_4$ in 50 mM Na[Ac] (pH = 4.57) and 4 μl drops have been prepared by mixing 2 μl of the protein solution (10 mg ml^{-1} or 40 mg ml^{-1}) with 1 μl of the reservoir solution and 1 μl of the IL solution of a particular concentration or by mixing 3 μl of the protein solution (10 mg ml^{-1}) with 0.5 μl of the reservoir and 0.5 μl of the IL solution. Thus, four different crystallization conditions have been applied: (a) drops containing 0.25 M IL, 5 mg ml^{-1} RNase and 1/4 of the salts concentration with respect to their concentration in the reservoir solution; (b) 0.25 M IL, 7.5 mg ml^{-1} RNase and 1/8 of the reservoir solution; (c) 0.25 M IL, 20 mg ml^{-1} RNase and 1/4 of the reservoir solution; (d) 0.125 M IL, 20 mg ml^{-1} RNase and 1/4 of the reservoir solution. Each set of drops (with specific IL : RNase concentration ratio) has been equilibrated at 20 °C against 2 ml of mutual reservoir solution. Control experiments consisted of drops with RNase and precipitants but without IL additives.

Several experiments have been performed at each experimental condition for lysozyme and RNase and the reported effects are based on average outcomes. Only reproducible results are reported.

Crystal cryo-preservation and X-ray data collection

Crystals of lysozyme and RNase with ionic liquids were flash frozen using paratone as a cryoprotectant. Diffraction data were collected at beam line Proxima-1 at SOLEIL (France) using a Pilatus detector.

3-D structure solution and refinement

Lysozyme

Data were processed using the XDS[30] software in the $P4_32_12$ space group ($a = b = 78.35$, $c = 36.90$ Å) with an overall completeness of 99.5%. The number of observed reflections was 132 855 (19 918) and, of those, 20 915 (3118) were unique reflections. The average $I/\sigma(I)$ was 5.06 (0.83). [Values in parentheses correspond to the last resolution shell (1.51–1.50 Å)]. Details of data collection statistics are listed in Table S1 in ESI†.

The structures were solved by rigid body refinement in Refmac 5.5[31] of the CCP4 package[32] using pdb entry 193L[33] as a search model. Final R_{work} and R_{free} converged to 16.75% and 21.09%, respectively (Table S1, ESI†). Data in the resolution range 39.17–1.50 Å were used for molecular replacement and refinement. Iterative model building with COOT[34] guided by $2mF_0$-DF_c and mF_0-DF_c maps, together with restrained refinement in Refmac, resulted in a good final model (as judged by the validation tools in COOT).

RNase

The CCP4 [32] programs POINTLESS and SCALA [35] were used to analyze the intensities against the possible Laue and point-group symmetries, followed by scaling. The structure solution and refinement were done using CCP4 programs. The structures were determined by the molecular replacement method using, as search model, the coordinates of bovine pancreatic ribonuclease (pdb code 3I67). Data collection statistics is given in Table S2 in ESI†.

Results

Ionic liquid additives

In control drops (containing no IL) lysozyme crystallized in the form of sea urchin aggregates and RNase formed a dense precipitate. We did not attempt to optimize control crystallization conditions as our aim was to establish if the addition of ILs (while keeping all other parameters constant) could result in any improvement of the crystallization outcome. Generally, with increasing concentration of a given IL in a drop we have observed reduction in the crystal nucleation density (Fig. 2 and 3a) accompanied by an advantageous effect on the crystal quality and Fig. 4 and S1†).

For instance, transitions from sea urchin-type of structures (or high density of less regular crystals) to more perfect lysozyme crystals—less in number and larger in size—have been witnessed. Fig. 4 shows crystallization differences induced by ILs with distinct cation's surface activity–surface active $[EMIM]^+$ *vs.* preferring-bulk-hydration $[Ch]^+$ (both anions, ethanesulfonate and lactate, exhibit a propensity to the hydrophobic interface). The origin of the relationship between the type of hydration (hydrophobic *vs.* hydrophilic) of the IL and the crystal's nucleation density is explained in the Discussion.

The presence of choline-based ionic liquids resulted in crystal quality improvement as compared to the IL-free conditions, but their effect on the nucleation density was different than that of imidazolium-based ILs. For [Ch]Cl, after the initial solubilization of the protein, the nucleation density was observed to increase with increasing IL concentration. For Ch-based ILs with surface active anions—[Ch][Ac], [Ch][C1SO3] and [Ch][Lac]—we observed a peak in the induced nucleation density at a given IL concentration (Fig. 5 and 6).

Ordering of ILs according to their ability to reduce nucleation density of protein crystals, resulting in the formation of larger

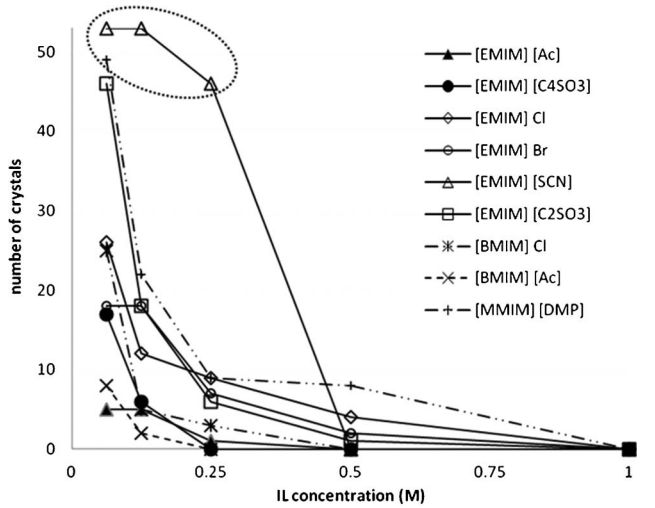

Fig. 2 Nucleation density (number of crystals/drop volume) of lysozyme upon the increase in the concentration of imidazolium-based ILs. Dashed ellipse indicates the region with the formation of sea-urchin type structures. Open (filled) symbols designate [EMIM]-based ILs combined with negatively (positively) hydrated anions. Positive hydration means ions that retard water mobility (due to strong ion-water affinity or as a result of hydrophobic cage formation). Negative hydration is related to ions that increase water mobility and break down its structure (due to the fact that the ions have a lower affinity for water than water to itself) and have, comparatively, a dynamic hydration shell.[23,36–39]

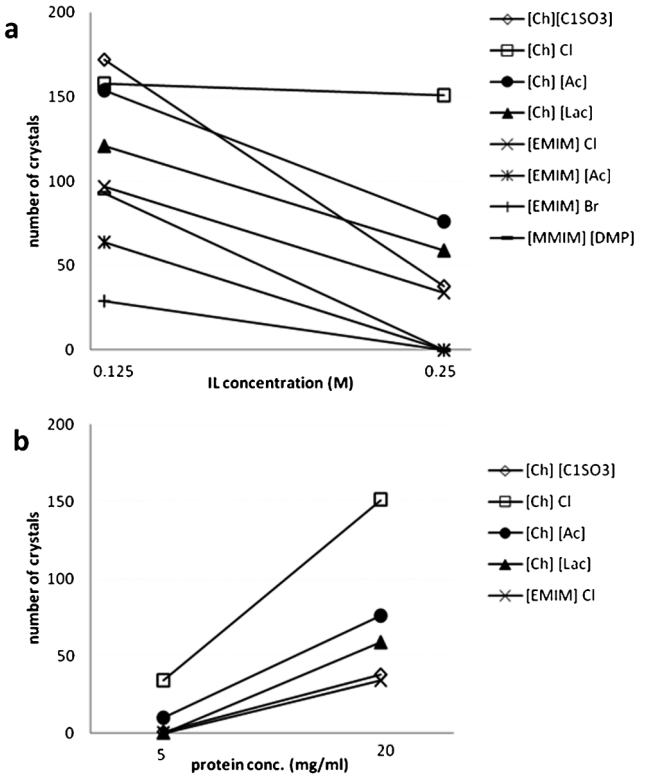

Fig. 3 Nucleation density (number of crystals/drop volume) of RNase (a) upon increasing concentration of ILs (at fixed protein: inorganic precipitants ratio, here 20 mg ml^{-1} RNase and 1/4 of the reservoir solution) and (b) upon increasing protein concentration (at constant concentration of the IL = 0.25 M and 1/4 of the reservoir solution). Open (filled) symbols designate [Ch]-based ILs combined with negatively (positively) hydrated anions.

and more perfect crystals (and further referred to as salting-in), increases from [Ch]-based ILs to imidazolium-based ILs. Here, in general the trend is commensurate with the increase in the chain length of the cation, *i.e.*, $[MMIM]^+ < [EMIM]^+ < [BMIM]^+$ (Fig. S2†). The relative salting-in effect of ILs with common cation and different counterions changes with increasing IL concentration. The ability of [EMIM]-based ILs to support salting-in of lysozyme at low IL concentration increases in the following order: [EMIM][SCN] < $[EMIM][C_2SO_3]$ < [EMIM]Cl < [EMIM]Br < $[EMIM][C_4SO_3]$ < [EMIM][Ac]. With increasing IL concentration, the relative positions change to $[EMIM]Cl^-$ < $[EMIM]Br^-$ < $[EMIM][C_2SO_3]^-$ < $[EMIM]\ [SCN]^-$ < $[EMIM]\ [Ac]^-$ < $[EMIM][C_4SO_3]^-$ (Fig. 2). With increasing concentration of [Ch]-based ILs, the relative salting-in effect towards lysozyme changes from $[Ch][C_1SO_3] \leqslant [Ch][Cl] \ll [Ch][Lac] < [Ch][Ac]$ to $[Ch][Cl] \ll [Ch][C_1SO_3] < [Ch][Ac] < [Ch][Lac]$ (Fig. 5).

For RNase the salting-in effect increases in the following order: $[Ch][C_1SO_3] < [Ch][Cl] < [Ch][Ac] < [Ch]][Lac] <$ [EMIM]Cl < [MMIM][DMP] < [EMIM][Ac] < [EMIM]Br (Fig. 3 and S3†). With increased IL concentration, the relative position of the $[Ch][C_1SO_3]$ changes in the ranking and its salting-in ability becomes superior to that of [Ch][Lac] and no crystallization is further induced in the drops containing [MMIM][DMP], [EMIM][Ac] and [EMIM]Br. In the presence of [EMIM]-based ILs with larger anions ($[SCN]^-$, $[C_2SO_3]^-$ and $[C_4SO_3]^-$) or imidazolium-based ILs with longer alkyl chain in the cation ([BMIM]Cl and [BMIM][Ac]) the RNase remains solubilized (clear drops are observed) at any of the conditions applied.

At higher initial concentrations of some particular ILs, we have observed that originally well-developed regular lysozyme crystals with smooth faces started to dissolve and become

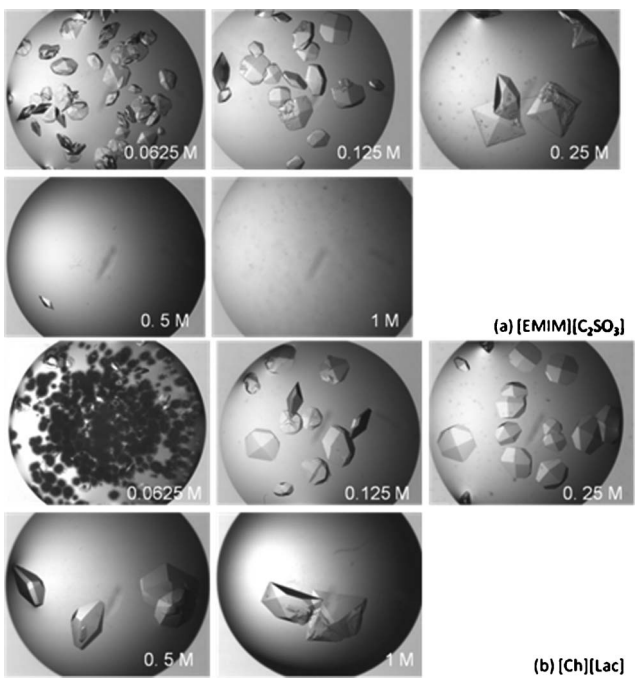

Fig. 4 Lyzosyme crystallization: Effect of (a) $[EMIM][C_2SO_3]$ and (b) [Ch][Lac] ionic liquids at increasing concentrations.

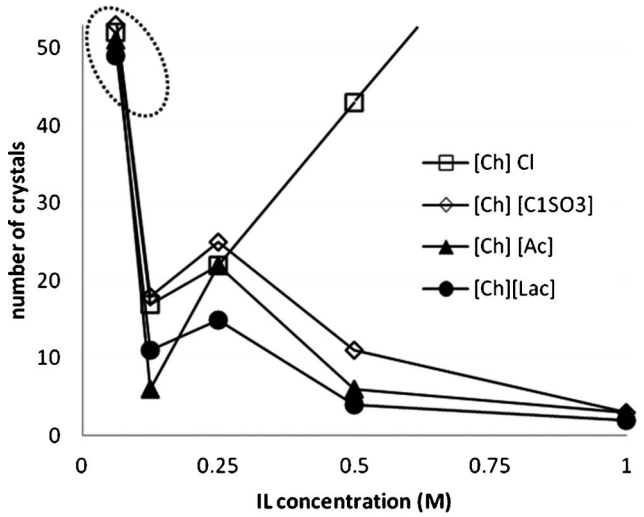

Fig. 5 Nucleation density (number of crystals/drop volume) of lysozyme crystals with the increasing concentration of choline-based ILs. Dashed ellipse indicates a region with the formation of precipitate or sea-urchin type structures. Open (filled) symbols designate ILs combined with negatively (positively) hydrated anions.

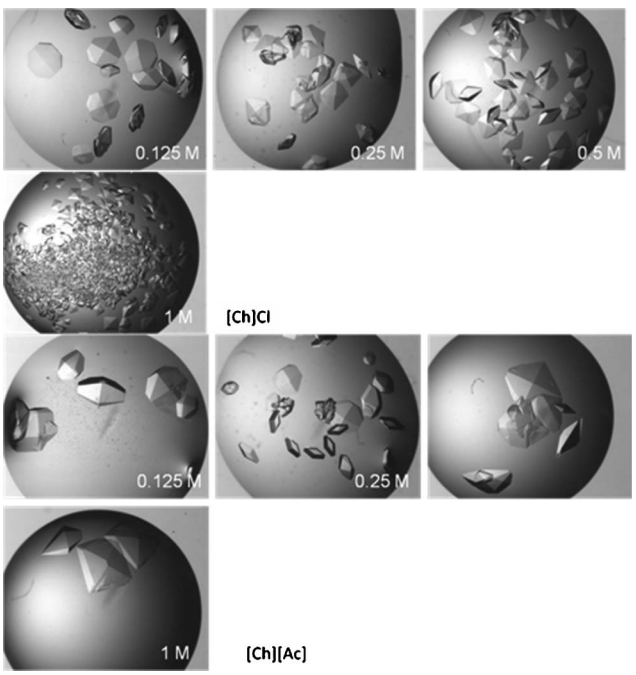

Fig. 6 Effect of [Ch]Cl and [Ch][Ac] on lysozyme crystallization with increasing ionic liquid concentration. Note the respective increase in number (and decrease in size) of the crystals grown in the presence of [Ch]Cl in comparison to the effect of [Ch][Ac] for which the highest number (and the smallest size) of the crystals corresponds to the intermediate (0.25 M) concentration of the IL.

significantly etched with time (Fig. S4†). The drops were not submitted to any change in the crystallization conditions and only the concentration of the inorganic precipitant (that does not cause solubilization effect) and that of IL were expected to increase when the system proceeds towards equilibrium.

[Ch]Cl and [Ch][Ac] as precipitating agents

We have observed induction of lysozyme crystallization at 4 °C in drops containing 1 M [Ch]Cl or 1 M [Ch][Ac] without the presence of inorganic precipitant, NaCl (Fig. S5†). The nucleation density has been significantly higher in the presence of [Ch]Cl. Crystals that have been formed solely in 1 M IL solutions without the presence of an acetate buffer (in either protein or IL solutions) and with no control of pH conditions were unstable when placed at 20 °C. Crystals formed in IL solutions buffered with Na[Ac] started to partially dissolve at 20 °C when [Ch]Cl was used as an additive (Fig. S5(e)†) and remained stable at room temperature in the presence of [Ch][Ac].

IL in the crystal structure

Careful analysis of the diffraction datasets collected from crystals of RNase† and lysozyme crystallized in the presence of ionic liquids reveals the existence of choline and acetate molecules in two of the crystal structures of lysozyme. The final model contains amino acid residues 1–129, one choline, one acetate, and one Na^+ and one Cl^- ions (Fig. 7). Na^+ and Cl^- ions come from the salt stabilized lyophilized protein sample that has been used for crystallization. The coordinates and structure factor amplitudes of the highest resolution dataset have been deposited in the PDB with accession number 4AGA.†

All lysozyme crystals were of the same tetragonal space group as the native one (193L). Choline and acetate molecules are found in a cavity close to residues 48–61 and 100–110, respectively. Acetate forms an intermolecular hydrogen bond with one of the methylene hydrogen's of choline. Superposition of this structure with the native lysozyme structure (193L)

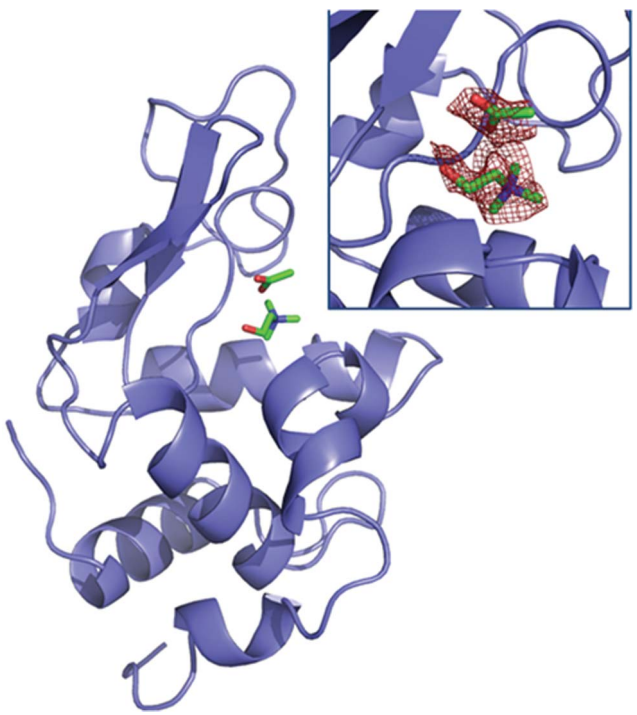

Fig. 7 Crystal structure of lysozyme with choline and acetate molecules bound. (Inset) Close-up view showing the calculated omit map of choline and acetate molecules contoured at 1.5σ.

(r.m.s.d. of 0.18 Å) indicates that the presence of choline and acetate does not affect the secondary structure of lysozyme with respect to the native form (193L). However, there is a significant difference in the unit cell volume of these two structures. The volume of the unit cell of this structure is $\sim$6470 Å^3 smaller than the native one. This contraction of unit cell upon binding of choline and acetate may occur as a result of desiccation/dehydration.

Discussion

Based on our crystallization experiments we have ranked the studied ionic liquids according to their salting-in/out effect exerted on two positively charged proteins, lysozyme and ribonuclease A. In the following sections we discuss the mechanism responsible for the observed behavior.

It has been proposed that the relative salting-in/out ability of ions can reverse upon increasing ionic strength conditions (IS) due to different factors determining the protein solubility in solution at low and high background ion concentrations.[40] The transition from the inverse to the direct Hofmeister series has been shown for lysozyme in the presence of inorganic sodium salts.[40] Here we rationalize the relative changes in the order of the ionic liquid ions according to their effect on the nucleation density of lysozyme and RNase crystals, with increasing IL concentration.

It has been recognized that, at low salt concentrations when electrostatic forces are dominant, the effect of ions depends on their ability to screen effective charges on the protein surface and reduce the repulsive interactions between equal-sign biomacromolecules. At higher salt concentrations, when charges have already been effectively screened, the influence of ions has been explained by considering their effect on protein–water interfacial tension. The same ions, which at low ionic strength (IS) conditions bind to the protein surface and screen the charges (promote salting-out), at high IS reduce protein–water interfacial tension by remaining hydrated while attached to the surface (induce salting-in).[40]

Charge screening and ion specific binding: Strength of hydration

Ions can bind to both, non-polar and charged compartments of the protein molecule, by means of hydrophobic and electrostatic interactions, respectively.[41,42] For cationic proteins studied, the charge screening will be determined by anions. Binding to the charged residues requires that the dehydration costs are lower than the energy gain on binding.[43] In accordance with this constraint, we have observed that at lower background salts concentration the salting-out effect (nucleation induction) exerted by ILs with common cation generally increases on going from ILs with anions having a stable hydration shell and relatively high dehydration costs (positively hydrated $[Ac]^-$, $[Lac]^-$, and $[C_4SO_3]^-$), to ILs with (negatively[23,36–39] hydrated Cl^-, Br^-, $[SCN]^-$, $[C_1SO_3]^-$ and $[C_2SO_3]^-$) anions that interact weakly with water and for which dehydration is energetically more favorable (Fig. 5 and 6).

When electrostatic forces determine protein–protein interactions in solution (low IS), the binding of the cations has the opposite effect of that of binding anions. While positive charges at the protein surface are screened by anions (which supports salting-out), the binding of the cations to the non-polar residues counteracts this effect. Cation binding also reduces the protein–water interfacial tension thereby aiding protein solubility. Therefore, the potential of ILs to solubilize protein increases along a range with increasing cation hydrophobicity (increasing surface affinity) starting with [Ch]-based ILs (due to the known relatively strong hydrophilic hydration of $[Ch]^+$ and its weak propensity to the protein surface)[44] and continuing on to [MMIM]—to [EMIM]—and finally to [BMIM]-based ILs (according to increasing alkyl chain length) (Fig. 2, 6 and S1†).

Protein–water interfacial tension and hydrophobic attraction

For lysozyme, the general trend in the salting-out ability, increasing as the focus moves from positively to negatively hydrated anions, is preserved with increasing ionic strength. This indicates the importance of the electrostatic interactions in the system. The relative inversion in the salting-in/out effect of ILs is observed within each particular group of ILs with anions of specific hydration characteristics (positive or negative). At high IS, the salting-in ability of the ILs towards lysozyme increases (at low IS decreases (with an inversion between Cl^- and Br^- positions) in the order: $[EMIM]^+$ ($Cl^- < Br^- < [C_2SO_3]^- < [SCN]^-$) and $[Ch]^+$($Cl^- < [C_1SO_3]^-$) for negatively hydrated anions and $[EMIM]^+$ ($[Ac]^- < [C_4SO_3]^-$) and $[Ch]^+$($[Ac]^- < [Lac]^-$) for positively hydrated anions. This inversion can be rationalized by considering the previously discussed differences in the effect of ion adsorption on increasing salt concentration—the transition from salting-out by charge screening to salting-in by surface tension modification. For RNase, the switchover between electrostatic and hydration forces is expressed as the change of the relative effect of $[Ch][C_1SO_3]$. The negatively hydrated anion,[23] $[C_1SO_3]^-$ which is supposed to preferentially bind to charged residues, is also expected to have a high affinity to the non-polar compartments (large polarizable ions with low charge density exert propensity to hydrophobic surfaces). Nevertheless, a high concentration of inorganic precipitants, used to induce RNase crystallization, hinders electrostatic interactions and the relative effect of the IL ions is generally determined by their affinity to the hydrophobic compartments and induced changes of the protein–water interfacial tension. According with the proposed mechanism of interaction on increasing IS in the system, the salting-in capacity of ILs with common cation was observed to increase with the predicted rise in the surface activity of the anion. Therefore, Cl^- as a counter ion exhibited the weakest salting-in ability. For [Ch]-based ILs this ability increases across a range starting with $[Ac]^-$, having hydrophobic methyl group and hydrophilic carboxylic group, continuing to $[C_1SO_3]$, with a methyl group and negatively hydrated[45,46] $-SO_3^-$ and finally ending with $[Lac]^-$ (which has been shown to have significant surface activity at higher concentration).[47] For [EMIM]-based ILs acting on lysozyme the salting in power of negatively hydrated anions is the most expressed for $[SCN]^-$, due to its large size and correspondingly low charge density, making it behave as a hydrophobic solute.[48] It is followed by $[C_2SO_3]^-$, with a short hydrophobic alkyl chain and a negatively hydrated $-SO_3^-$ group, and then by less negatively hydrated small halides ($Cl^- < Br^-$). Among [EMIM]-based ILs with positively hydrated anions, $[C_4SO_3]^-$ exerts

higher salting-in ability than $[Ac]^-$ due to the longer hydrophobic chain of the former. For RNase the salting-in ability has been observed to be stronger for $[EMIM]^+$ combined with negatively hydrated Br^- than for [EMIM][Ac] with a hydrophilic compartment in the anion.

At this point it is interesting to note that for simple anions their affinity to the protein hydrophobic surface is inversely dependent on the strength of hydration[40] (often expressed by the viscosity B-coefficient used to rationalize the effect of respective ions on protein stability[49]). Our results suggest that, in the case of more complex ions, it is better to state that the affinity of particular ions to the protein surface and, correspondingly, their salting-in ability increases with the rise in ion hydrophobicity. In this context, it is important to distinguish between the hydrophilic hydration resulting in the retardation of water mobility (and viscosity enhancement) due to the strong ion-water affinity (as in the case of the $[Ac]^-$ carboxylic group) and the hydrophobic hydration that restricts water mobility due to cage formation around the respective solute (*e.g.* butyl chain of $[C_4SO_3]^-$).[50] Apart from representing greater hydration strength, as expressed in higher viscosity,[23] it is expected that, due to its greater hydrophobicity, the latter anion will exert a higher propensity to non-polar surfaces. Interestingly, with regard to inorganic ionic crystal (barium sulfate) precipitation, the structuring of the aqueous solvent by hydrophobic IL ions (as well as other hydrophobic solutes) merely results in a salting-out effect as there are no specific interactions.[23,51]

Protein–water interfacial tension *vs.* the energy of cavity formation. Choline affinity to the protein and water surface

Nucleation density increasing on increasing [Ch]Cl concentration and a maximum in the salting-out effect exerted by other [Ch]-based ILs with surface active anions ($[Ac]^-$, $[C_1SO_3]^-$ and $[Lac]^-$) can be explained by considering the effect of ILs on solvent properties. Apart from the protein–water (hydration modification) and protein-additive (electrostatic and hydrophobic adsorption) interactions, protein stability in solution is affected by the easiness to create a cavity in the surrounding solvent structure in order to accommodate a protein molecule.[52] The relative energy of cavity formation can be inferred from the changes in the surface tension of water exerted by the respective solutes.[52] Among the ILs studied, only the [Ch]-based ones increase the surface tension of water[53] therefore augmenting the energy of cavity formation. The surface tension of the [Ch]Cl solution increases with concentration due to the fact that $[Ch]^+$ is repelled from the water surface and prefers bulk hydration [44] (this is reflected in the increasing salting-out behavior of these ILs with concentration). The contrary occurs with imidazolium cations which are surface active species. In the case of $[Ch]^+$ combined with surface active anions, we can expect a local maximum in surface tension resulting from the influence of $[Ch]^+$, followed by a decrease in the surface tension induced by anions with a propensity to the air/water interface (as observed *e.g.* for $[Ac]^-$ salt of strongly hydrated Mg^{2+} in[54] contrast to the $[Ac]^-$ salt of weakly hydrated Na^+, which only exhibited a monotonic decrease in surface tension[55]). The suggested maximum in the surface tension is reflected in the maximum salting-out effect exerted by [Ch][Ac], [Ch][C1SO3] and [Ch][Lac] at specific concentrations. Due to the increased energy of creating a cavity in water, the [Ch]Cl and [Ch][Ac] at appropriate concentrations can be used as precipitating agents for lysozyme without the addition of any inorganic salt or other precipitant (Fig. S5†). Nevertheless, all [Ch]-based ILs can be concluded to solubilize protein to a greater extent than the IL-free solution (as expressed in the formation of well developed crystals in the former as compared to sea urchin aggregates in the latter). This suggests that direct interaction with the protein surface is also taking place. In fact, $[Ch]^+$ has been shown to represent weak unspecific binding to the protein surface.[56] Adding hydroxyls to the short aliphatic chains of the IL cation has been previously found to increase protein stability (native folded conformation) in imidazolium-based ILs.[57] This property has been ascribed to the increased H-bond capacity of ILs with oxygen-containing functional groups. The stabilizing effect of H-bonding ILs can result from both, the ability of respective ILs to provide intra- or intermolecular H-bond bridges, and from their tendency to form H-bonded networks with water (resulting in hydrophilic character and increased surface tension).

Salt specific effects: Hydration and association of IL ions

The specific ordering of the ILs according to their salting-in/out ability at low ionic strength conditions (here considered for lysozyme) cannot be explained by taking only direct (electrostatic and hydrophobic) interactions into account. The relative ability of ILs with anions of particular hydration characteristics (positive or negative) to assist on the protein nucleation correlates with the effect of those ILs on the hydration of other charged species present in solution.[23] The salting-out of lysozyme is increasing from [EMIM][Ac] to $[EMIM][C_4SO_3]$ for ILs with positively hydrated anions, and from [EMIM]Br < [EMIM]Cl < $[EMIM][C_2SO_3]$ to [EMIM][SCN] and from [Ch]Cl to $[Ch]C_1SO_3$ for ILs with negatively hydrated anions. This increase in the salting-out is in absolute agreement with the effect of those ILs on the precipitation (salting-out) of an inorganic crystal-barium sulfate and correlates with the expected decrease in stability of the hydration shells of the charged species (here compartments of biomacromolecules) immersed in the respective IL solutions.[23] This IL-induced modification of hydration has been correlated (i) with the tendency of the IL cation and anion to associate in solution and (ii) with their hydration properties.[23] (i) The potential energy of water molecules aligned in hydration shells of the charged species immersed in IL solution is reduced due to attractive interaction between the respective water dipoles and the IL ions.[58,59] This mechanism enhances hydration [43] stabilizing hydrated species in solution (corresponding to salting-in) and is determined by the distribution of IL ions (or other background salts) in solution.[60–62] The more associated the background electrolyte ions are, the more screened are their charges and less expressed is their hydrating (salting-in) ability towards other hydrated species. (ii) ILs can also compete (for water of hydration) with hydrated species in solution (when positively hydrated) or facilitate the breaking of the water structure and its alignment in an electric field of the other charged species (when negatively hydrated), corresponding to salting-out/in, respectively. The interplay of these two factors (i) and (ii) ranks ILs according to their relative hydrating/

dehydrating ability.[23] The reported IL-specific effects exerted on lysozyme (salting-out increasing on the easiness of the dehydration of the charged compartments) can be explained by the fact that protein folding is crucially determined by dehydration of charged residues forming salt bridges and other interactions within the native protein.[63] The influence of the addition of salt on hydration and the consequent modification of attractive interactions between like-charged solutes in water has been shown by Kinoshita and Harano.[43] We have herein demonstrated its relevance for conformational transitions of biomacromolecules.

Hydrophobic adsorption *vs.* hydration modification

The proposed water-mediated mechanism of interaction depends strongly on the hydration of ILs (ii). As a result, its effect on the conformational changes of lysozyme in solution corresponds, to a great extent, to the usually considered effect of charge screening induced by (iii) ion adsorption to the non-polar regions at the protein surface (Fig. 8). This is because the affinity of the ions to the hydrophobic compartments is determined by their hydration characteristics, so that the more hydrophobic or more negatively hydrated hydrophilic ions (large polarizable ions with low charge density), the higher their propensity to the non-polar surface. The ranking position of [EMIM]Cl and [EMIM]Br, in regard to the relative effect of the Cl^- and Br^- anions usually reported for lysozyme at low IS, is nevertheless observed to reverse. This fact can be explained by considering that the $[EMIM]^+$ and the Cl^- have a higher mutual tendency to associate in aqueous solution than the cation and the anion composing [EMIM]Br.[23,64,65] Therefore, apart from the lower energy of hydration of Br^- in comparison to that of Cl^- (and easier binding of the former to both the charged and the non polar residues at the protein surface (iii), that promotes salting-out at low IS), the less associated [EMIM]Br(I) additionally stabilizes the hydration of charged compartments of the biomacromolecule (supports salting-in by water-mediated mechanism). The need to take into account the mutual affinity of the ions and their counter-ions as salting-out/in agents was stressed by Mason and coworkers [56] who concluded that ions can only be properly ranked in respect to their salting-in/out behavior if their counter-ion partners are taken into due consideration.

Electrostatics and hydration forces at low IS

Focusing on the interplay of the IL-protein binding (electrostatic and hydrophobic) and the modification of the hydration of charged residues by the IL ions allows us to understand the observed peculiarities in the ranking of ILs according to their salting-in/out effects towards lysozyme at low IS conditions. For a given IL cation, the charge screening by specific anion binding to the charged residues on the protein surface seems to predominate over hydrophobic interactions and hydration changes in determining the salting-out ability of IL additives. Such a conclusion results from the observation that the salting-out power generally increases as the focus moves from positively to negatively hydrated ions (according to the decreasing dehydration costs). In agreement with this statement, $[EMIM][C_4SO_3]$, regardless of the more hydrophobic character of the anion (higher propensity to the non polar residues (iii)) and the predicted greater ability to dehydrate charged compartments (i) + (ii),[23] exerts a lesser salting-out effect than $[EMIM][C_2SO_3]$ due to the stronger net hydration

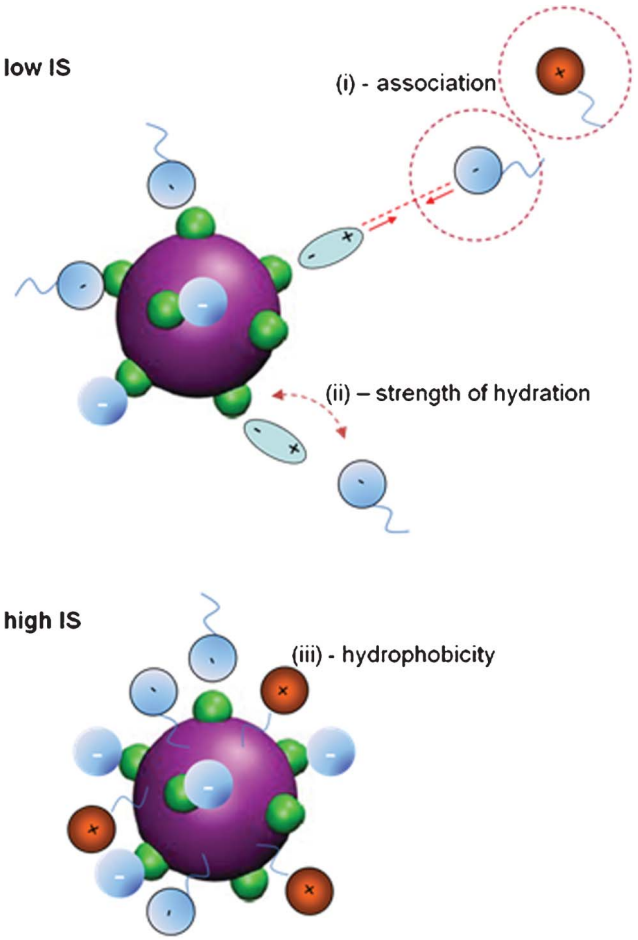

Fig. 8 Schematic representation of interactions of a macromolecule containing hydrophobic (purple) and cationic (green) surface groups [adapted from Lund *et al.*, 2008[41]] with its surroundings (IL ions, water, and inorganic ions). **Low IS**-specific binding of weakly hydrated anions to the cationic residues (charge screening → salting out) and water mediated interactions: electrostatic stabilization of hydration (salting in) and exchange of water molecules hydrating the protein with positively (salting-out) and negatively (salting-in) hydrated ions. **High IS**-non specific adsorption of weakly hydrated ions to the hydrophobic surface (surface tension reduction → salting-in). Symbols: spheres – charged groups, tails (hydrophobic chains), dotted circles – electric fields, light blue ellipses – water dipoles.

(expressed in the effect of the ion on the adjacent water mobility [23]) of the $[C_4SO_3]^-$ anion.

For ions of the same net hydration characteristics, the type of ion–water interaction (hydrophobic or hydrophilic) defining non-specific adsorption at the hydrophobic surface (iii) and the effect on the hydration of charged residues (determined by the hydration strength of IL (ii) ions and their tendency to associate (i)) will determine the relative salting-in/out ability.

ILs in the task of crystallization: Reaction environment *vs.* IL–protein interaction

There has been some debate about whether the improvement of crystallization outcomes caused by the presence of ILs is in fact determined by their binding to the protein surface. It has been suggested that the IL advantage may rather stem, from some

discrete charges in the crystallization conditions in a drop, such as slower equilibration due to the significantly reduced evaporation rates in the presence of the ILs.[14] An X-ray analysis of our crystals has shown that ILs can indeed be incorporated into the structure of lysozyme. This finding agrees with the results of a recent study where IL fragments were identified in lysozyme crystals.[66] ILs are not persistent in the structure, but can be sporadically localized. This is a relatively common phenomenon for the small molecule compounds used as crystallization additives. Small molecules have been recognized as often being crucial for protein crystallization, but they have seldom been identified within the crystal structure.[67,68] There are also experimental results showing that long alkyl chain imidazolium-based IL ([C_{14}MIM]Br) in fact binds to the surface of a globular protein bovine serum aluminum (BSA).[69] In agreement with the mechanisms of IL–protein interactions discussed in our work, it has been found that, at lower concentration, [C_{14}MIM]Br binds to the BSA surface due to electrostatic attraction while at higher IL concentration the mechanism is that of hydrophobic interaction.[69]

Conclusions

We have shown the systematic effect of ILs of particular characteristics on the crystallization of two positively charged low molecular weight proteins, lysozyme and ribonuclease A. The effect of complex IL ions on protein crystallization has been systematized and rationalized for the first time based on the intrinsic properties of IL solutions (type of hydration, association) stemming from their structures in contrast to the ordering of ions and the extraction of their properties (*e.g.* kosmotropic and chaotropic hydration) from the response of the protein.

It has been shown that the preferential binding of the IL ions to the protein surface (enhanced with increasing the negative or hydrophobic hydration) on the one hand facilitates protein nucleation by electrostatic charge screening. On the other hand, it stabilizes the protein in solution by lowering the protein–water interfacial tension. As a result, the anions that exert the greatest salting-out effect at low ionic strength turn out to have the most prominent salting-in ability when hydration forces start to dominate. Apart from the direct protein–water interactions, the effect of ILs on the hydration of charged residues and on solvent properties had to be considered in order to understand the influence of ILs on protein crystallization. To the best of our knowledge, this work reports for the first time hydration modification (caused by induction, *via* the presence of an additive) of protein charged compartments, with this mechanism being partially responsible for the success of crystallization. The combined effect of the IL binding to the protein surface (initial solubilization) and increasing the water surface tension (subsequent salting-out) as recognized for [Ch]-based ILs suggests that the application of IL ions with specific characteristics can be beneficial, for example in those systems where protein crystallization is restricted due to its limited solubility.

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