Strong and specific effects of cations on lysozyme chloride solubility

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The influence of salt nature and concentration on tetragonal lysozyme chloride crystal solubility is presented for a set of mono-, di- and trivalent cations (Cs^+, Rb^+, Mn^{2+}, Co^{2+} and Yb^{3+}). The results show that cations have as strong an effect on protein solubility as anions and that they present their own particular effects as co-ions. Indeed, after decreasing at low ionic strength, lysozyme solubility increases with high concentration of polyvalent cations, probably due to co-ion binding and therefore the concomitant increase of the net charge of the protein-salt complex. These new results are discussed in order to progress in the understanding of the crystallisation process at the atomic level.

Keywords: solubility; cations; chloride salts; Hofmeister series; isoionic lysozyme.

1. Introduction

This work aims to address the effect of the nature of cations in protein crystallisation. Indeed the effect of ions is still unclear when using the approach of long-range interactions (Piazza, 2000; Tardieu *et al.*, 2002). We focus on the changes of the protein salt and the physical chemical properties that are perturbed when varying the nature and concentration of the salts during the protein crystallisation process.

Hofmeister was the first to rank salts according to their ability to precipitate a mixture of proteins, extracted from Hen Egg White (HEW) (Hofmeister, 1888). Later, Green (1931, 1932) analysed the behaviour of some salts in terms of salting-out and salting-in effects in solubility studies of carboxyhemoglobin. She concluded that anions of the Hofmeister series have a much stronger effect than cations, and that they could be ranked as follows:

$$SO_4^{2-} > HPO_4^{2-} > CH_3CO_2^- \sim \text{citrate}^{3-} > \text{tartrate}^{2-} > HCO_3^- > CrO_4^{2-} > Cl^- > NO_3^- > ClO_3^-$$

$$\text{and} \quad Li^+ > Na^+ \sim K^+ > NH_4^+ > Mg^{2+}.$$

However, the weaker effect of the cations was based on a very restricted sampling in comparison to the anions. A first aim of the present work is to investigate the effects of a much broader set of cations on the solubility of HEW lysozyme. The choice of this model protein is justified by the fact that it has been used for most studies of protein solubility in order to attempt a rationalisation of crystallisation conditions. Furthermore, it has been demonstrated that the hypothesis could be applied to other proteins in a more general rule (Riès-Kautt & Ducruix, 1997).

It has been shown that the solubility of proteins is affected by the nature of anions, depending on the net charge of the protein along with the relative anion-protein "affinity". Anions decrease the solubility of negatively charged proteins according to the Hofmeister series (Carbonnaux *et al.*, 1995), whereas the order of the series is reversed for positively charged ones (Riès-Kautt & Ducruix, 1989; Guilloteau *et al.*, 1992). This was attributed to a weak binding of the anions on the protein, which consequently affects the net charge of the resulting protein salt. Solubility is known to change is the same way as does the net charge, becoming minimal at the isoelectric pH. More generally, it was shown that the binding of anions to positively charged proteins, i.e. of opposite net charge, can be understood in terms of counter-ion binding, which lowers the solubility of the protein salt (Riès-Kautt & Ducruix, 1997). The effect of the anion series has since been confirmed by x-ray studies of several lysozyme anion salts (Vaney *et al.*, 2001) and is further discussed by Retailleau *et al.* (2002).

Conversely when anions bind to proteins bearing a negative net charge, there is a co-ion binding which would increase the net charge of the protein-salt, until saturation is reached.

As for cations, almost no experimental data is available in the literature in respect to protein solubility, except the increase of lysozyme solubility in the presence of $NiCl_2$ (Ataka & Katsura, 1992) or $MgBr_2$ (Broide *et al.*, 1996). Therefore, a second aim of the present work is to quantify the effect of cation binding to a positively charged protein (i.e. co-ions) in order to search for an increase of its solubility and estimate the order of magnitude of the required ionic strength for binding. A set of five mono-, di- and trivalent cations of different atomic numbers and hydration radii were therefore selected, namely Cs^+, Rb^+, Mn^{2+}, Co^{2+} and Yb^{3+}.

The solubility of lysozyme in the presence of these cations was determined by a scintillation method initially proposed by Rosenberger *et al.* (1993) and adapted by Gripon *et al.* (1997). This method gives not only access to the solubility data, but also to the crystallisation enthalpies of the crystal forms. As the measurement of one dissolution temperature for a given protein and salt concentration required almost one day, we choose to favour a screening of solubility changes for these salts in the broadest range of concentrations, rather than a more accurate data collection with many points for a single curve per salt.

2. Materials and methods

2.1. HEW lysozyme preparation

HEW lysozyme, crystallised three times, was purchased from Sigma (Cat. N° L6876, batch 73H7045). The purity of this batch was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by enhanced silver staining (Thomas *et al.*, 1996), which showed a single band.

In order to avoid artefacts of the solubility measurements due to the uncontrolled presence of impurities such as ligands or ions, the lysozyme was desalted to its isoionic state according to a published procedure (Riès-Kautt *et al.*, 1997). In this state, the only remaining ions in the solution are H^+ and OH^-. Then, the isoionic protein solution was acidified to pH 4.5 by addition of about ten molar equivalents of HCl. This acid was chosen for coherence with the solubility measurements in the presence of the chloride salts of the tested cations (Riès-Kautt *et al.* 1994; Retailleau *et al.* 1997). At this stage, the purity of lysozyme was confirmed by ESI mass spectrometry, which indicated a molecular weight of 14305 ± 3 Da.

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2.2. Water and reagents

Deionised, triply distilled water was used to prepare the solutions (Meram, France). All chloride salts were of ACS grade with purity greater than 99%. CsCl was purchased from Bethesda Research Laboratory, RbCl and $YbCl_3.6H_2O$ from Aldrich, $MnCl_2.4H_2O$ and $CoCl_2.6H_2O$ from Merck.

The refractive index was measured to check the salt concentrations of the stock solutions over time as well as of freshly prepared stock solutions. Linearity of the refractive index with concentration was confirmed for both the salts and the lysozyme solutions. It was also used to confirm the salt and protein concentrations when setting-up most samples, i.e. before crystallisation.

2.3. Sample preparation and crystallisation

Lysozyme (approx. 130 mg/ml) was dissolved and filtered through a 0.22 µm Millipore filter (Millex-GV4). The protein concentration was determined by UV absorption at 280 nm, using $\varepsilon^{0.1\%} = 2.66$ $l.g^{-1}.cm^{-1}$ and averaged from two to three measurements falling within 10% accuracy.

Sample solutions (300 µl) were prepared at room temperature by adding lysozyme to the salt solution, in siliconised glass tubes. The samples were then tightly sealed and stored to crystallise at 4°C. The crystal habit was verified by visual inspection using a light microscope. The macroscopic crystals were crushed into small crystallites by gentle magnetic stirring. To ensure equilibrium between solution and crystallites, the sample was stirred overnight at 4°C.

2.4. Sample preparation and crystallisation

The glass tube containing the crystallite sample to be measured was installed in the static light scattering device (red light laser 670 nm, 2 mW) designed by I. Rosenman (Groupe de Physique des Solides, Paris). A temperature sweep, as low as 0.6°C/hour was applied, while the crystallites were maintained in suspension by gently stirring (Gripon *et al.*, 1997). The intensity of the scattered light at 90° was recorded, a value of zero indicating full dissolution of the crystallites. At the temperature at which the crystallites completely dissolve, the solubility is equal to the protein concentration of the solution.

At the end of the experiment, the protein concentration was confirmed by 3 immediate UV measurements at 280 nm falling within 10% accuracy. The pH of the sample at 20°C was then verified.

3. Results

Crystals of HEW lysozyme were grown in the presence of Cs, Rb, Mn, Co and Yb chlorides in the protein or salt concentrations indicated in Figure 1. In parallel, large lysozyme monocrystals were grown under comparable conditions and were subjected to X-ray diffraction. The structure determination at high resolution is in progress, in order to identify the possible location of cations in the electron density maps. All crystals belong to the tetragonal space group $P4_32_12$, the standard known for low temperature HEW lysozyme chloride crystals. This shows that lysozyme chloride presents no polymorphism when changing the nature of the cation, contrary to what is observed when changing the nature of anions (Vaney *et al.*, 2001).

3.1. Solubility as a function of temperature

Figs. 1 (a) - (e) show the solubility values as a function of temperature measured in the presence of the indicated concentrations of the five cations, respectively.

First, we observed that the solubility of lysozyme, in the presence of each of the cation type, increases with temperature, confirming the so-called direct solubility dependence with temperature. Indeed, no retrograde behaviour of solubility with temperature, sometimes observed for other protein salts, has so far been mentioned for lysozyme.

Second, the change of solubility with the ionic strength depends on the valency of the investigated cation.

(i) In the presence of the monovalent cations Cs^+ and Rb^+ (Figs. 1a & 1b), the lysozyme solubility decreases as the salt concentration increases, consistent with what is known for NaCl and KCl (Legrand *et al.*, 2001). It is worthwhile emphasising that protein

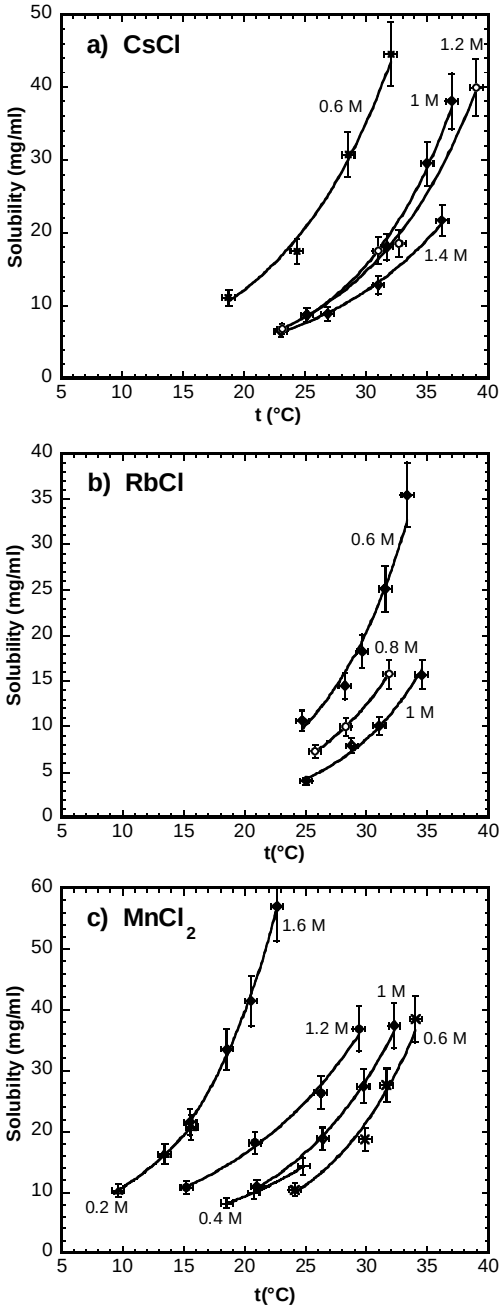

Figure 1

Solubility of lysozyme chloride as a function of temperature (pH 4.5) in the presence of (a) CsCl, (b) RbCl, (c) $MnCl_2$, (d) $CoCl_2$ and (e) $YbCl_3$.

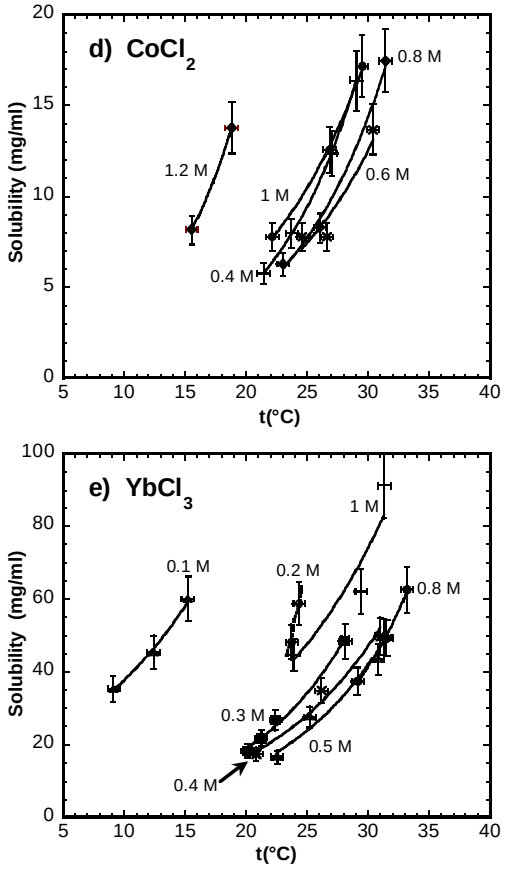

Figure 1 (continued)

solubility seems more sensitive to the temperature variation at low than at high ionic strength as previously reported (Guilloteau *et al.*, 1992). For example, the lysozyme solubility in the presence of CsCl (Fig. 1a) between 25 and 30°C varies by about 14 mg/ml at 0.6 M, but only 4 mg/ml at 1.2 M CsCl.

(ii) In the presence of the divalent Mn^{2+} and Co^{2+} (Figs. 1c & 1d), the solubility of lysozyme decreases until 0.6 M salt (ionic strength of 1.8 M), but then increases with the ionic strength. In the case of $MnCl_2$ (Fig. 1c), the relative order of the solubility curves is the following:

$$0.2 \gg 0.4 > 0.6 < 1.0 < 1.2 \ll 1.6\ \text{M}.$$

The solubility curves at 0.2 M and 1.6 M $MnCl_2$ seem to be a continuity of each other, likewise those at 0.4 and 1.0 M. Here, the high sensitivity of lysozyme solubility as a function of temperature can no longer be explained in terms of low versus high ionic strength. A more simple explanation may be provided from a derivation of equation [2]:

$$dS/dt = -(\Delta H_{cryst}/R\,T_0^2)\,S(t) \qquad [1]$$

where t is the temperature expressed in degrees Celsius, T_0 is 273 K. Equation [1] shows that the higher the solubility S, the higher dS/dt, if one considers that ΔH_{cryst} is independent of the salt concentration in a first approximation.

The relative order of the lysozyme solubility curves in the presence of $CoCl_2$ is

$$0.4 > 0.6 < 0.8 < 1 \ll 1.2\ \text{M}$$

The solubility increases once a minimum is reached at 0.6 M and the solubility curves at 0.4 and 1.0 M salt are similar, within the accuracy of the measurements, as for $MnCl_2$.

(iii) In the presence of the trivalent $YbCl_3$, the solubility variation is even more pronounced than with the divalent cations, the relative order is:

$$0.1 \gg 0.2 > 0.3 > 0.4 > 0.5 \sim 0.8 \ll 1.0\ \text{M}$$

Here the solubility also decreases monotonically until the salt concentration reaches 0.5 M $YbCl_3$, and then increases.

3.2. Crystallisation enthalpies

Considering the equality of the protein chemical potentials at the equilibrium between crystal and solution, its solubility S (e.g. in mg/ml) can be expressed as

$$\ln(S) = \Delta H_{cryst}/RT + E_1 \qquad [2]$$

where R is the molar gas constant, ΔH_{cryst} the crystallisation enthalpy and T the temperature in Kelvin. E_1 includes the entropic terms. The slope of ln (S) as a function of 1000/T, S being expressed in mg/ml, directly gives the crystallisation enthalpy of the crystal form.

Figs. 2 give the Arrhenius plots corresponding to the solubility of the lysozyme salts shown in Figs.1. Although the solid lines are the best linear fit, it must be stressed that the accuracy of a given plot depends not only on the accuracy of S (± 10%) but also on the number of points per curve.

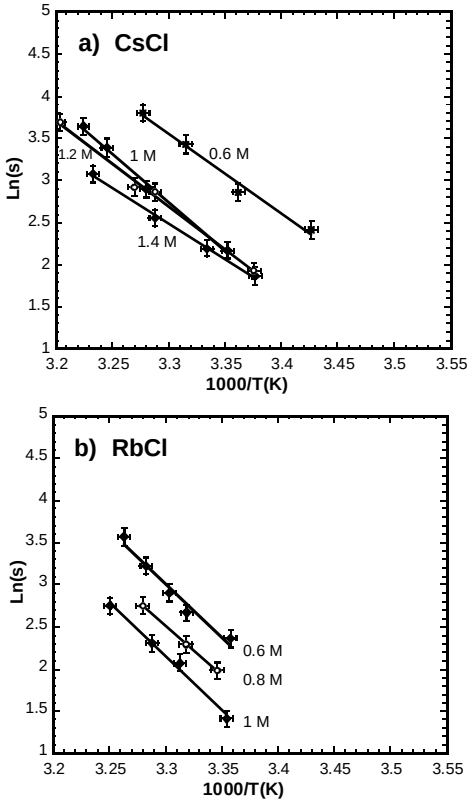

Figure 2

Arrhenius plots of lysozyme chloride solubility as a function of 1000/T (T in Kelvin) in the presence of (a) CsCl, (b) RbCl, (c) $MnCl_2$, (d) $CoCl_2$ and (e) $YbCl_3$.

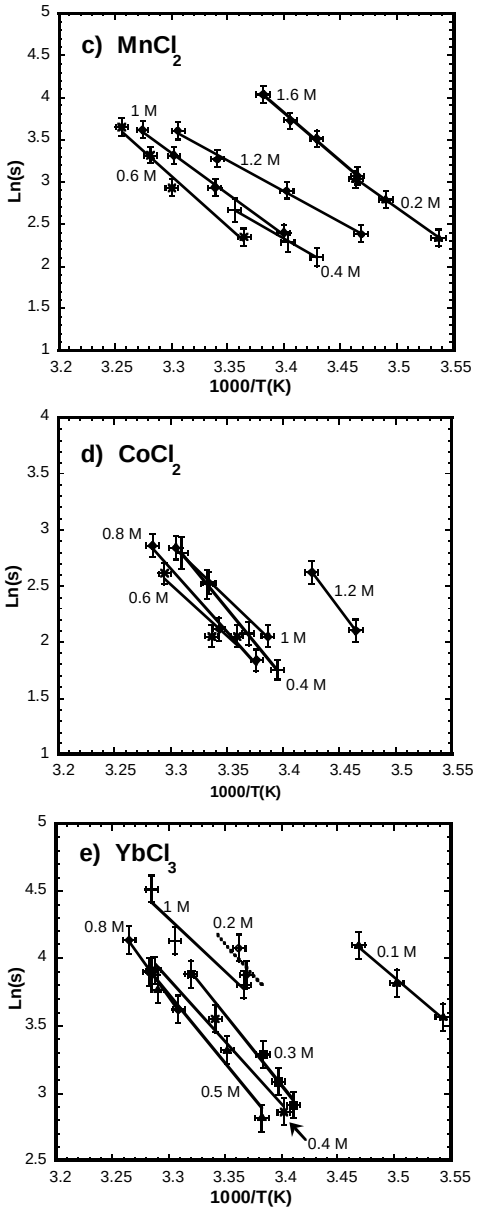

Figure 2 (continued)

As expected from the formation of the crystallites by decreasing the temperature, all solubility curves have a negative slope for a given salt concentration, meaning that this crystallisation is an exothermic process.

The crystallisation enthalpies determined from these slopes are reported in Table 1. With an accuracy of about 15 %, the average value of crystallisation enthalpies ΔH_{cryst} is -85 kJ.mol^{-1}. These values agree with the values so far reported in the literature for the tetragonal lysozyme crystals (Table 1). The previously reported values are only for crystals grown in the presence of either NaCl or KCl. Interestingly the new results show that the nature of the cation strongly affects the solubility of the protein, but not significantly the crystallisation enthalpies. This implies that strong effects on solubility can arise from slight differences inside the crystals: packing interactions and/or interactions between the lysozyme molecules and other solute components or the solvent.

Table 1

Crystallisation enthalpies of tetragonal lysozyme grown in the presence of different chloride salts. The values from this work are deduced from the best fit of the Arrhenius plots shown in Figs.2.

Salt	Concentration (M)	ΔH_{cryst} (kJ.mol^{-1}) 4.2 < pH < 4.7	References
NaCl	0.2 - 0.6	– (100 ± 5)	(Gripon et al., 1997)
"	0.5	– (105 ± 21)	(Takizawa & Hayashi, 1976)
"	0.35	– 90	(Cacioppo & Pusey, 1991)
"	0.5 - 0.85	– 72	(Ataka & Asai, 1988)
KCl	0.3 - 0.6	– (85 ± 5)	(Legrand et al., 2001)
CsCl	0.6 - 1.4	– (81 ± 11)	This work
RbCl	0.6 - 1.0	– (103 ± 5)	"
$MnCl_2$	0.2 - 1.6	– (80 ± 15)	"
$CoCl_2$	0.4 - 1.2	– (92 ± 14)	"
$YbCl_3$	0.1 - 1.0	– (78 ± 14)	"

3.3. Extended Hofmeister series for cations

Taking the solubility values for lysozyme chloride in the presence of the five salts at 25°C (Figs. 1), the solubility curves can be drawn as a function of the ionic strength in order to determine the cation specificity (Fig. 3).

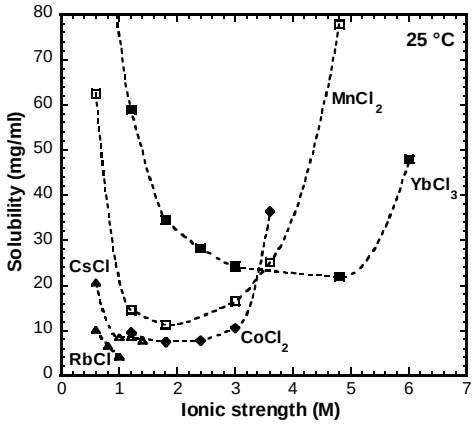

Figure 3

Solubility of lysozyme chloride as a function of ionic strength (pH 4.5 and 25°C).

For the first time we show that cations can modulate the solubility as strongly as do anions. Indeed, the solubility of lysozyme chloride at 25°C can be monitored from 4 to 80 mg/ml at 1 M ionic strength, depending on the nature of the cation. The highest value of the solubility in 0.1 M $YbCl_3$ (75 mg/ml, extrapolated at 18°C from Fig.1e) is close to the value obtained in the presence of $H_2PO_4^-$, shown to be the less efficient of the anionic series at the same temperature, pH and ionic strength (Riès-Kautt & Ducruix, 1989). The solubility values of lysozyme are around 20 mg/ml in $NiCl_2$ (Ataka & Katsura, 1992) and 10 mg/ml in $MgBr_2$ (Broide *et al.*, 1996). The lowest value of 4 mg/ml which is reached here for the action series with RbCl, was about 1 mg/ml in the presence of SCN^- in the anion series.

For an ionic strength ranging from 0.5 to 2 M, the efficiency of cation chlorides to decrease the lysozyme solubility can be extended as follows:

$$RbCl > CsCl \sim CoCl_2 > MnCl_2 >> YbCl_3$$

More striking is a specific effect of the studied di- and trivalent cation salts that increase lysozyme solubility at concentrations higher

than 0.6 M. This had been observed for $NiCl_2$ above 0.5 M (Ataka & Katsura, 1992) and for 0.3 M $MgBr_2$ (Broide *et al.*, 1996), but the reversal of a Hofmeister series has never been observed for a solid-liquid phase transition. Indeed, the polyvalent ionic (cation) series is reversed above an ionic strength of about 3.5 M:

$$CoCl_2 < MnCl_2 < YbCl_3$$

The solubility minimum occurs at a lower ionic strength (around 2 M) in the presence of the divalent Co and Mn than in the presence of the trivalent Yb (around 4 M).

4. Discussion and conclusions

The solubility results presented here bring not only new insights in the field of protein crystal growth, but also provide experimental evidence to support previous hypothesis of an important effect of ions, namely their direct interaction with the protein molecules during the process of crystallisation. Cations can affect the solubility of proteins as well as anions by at least one order of magnitude at a same ionic strength. An explanation may be provided by two complementary view points: i) a change of the net charge by ion adsorption on the protein when increasing the salt concentration with an ion-protein affinity depending on the charge density (polarisability) of the ions in each series, and ii) the hydration changes of the bound ions as well as of the protein when forming the protein salt.

A study of chloride adsorption by lysozyme carried out with a fluorescent quencher (Sibille & Pusey, 1994) showed that the saturation of adsorption was reached around 0.6 M NaCl and 20 mg/ml lysozyme. The solubility was found to be nearly insensitive to pH variation around 0.6 M NaCl, suggesting that the protein behaves as if it had a net charge of zero (Retailleau *et al.*, 1997). Similarly, the solubility becomes independent of ionic strength at a pH of about 9, instead of the known lysozyme pI of 10.8. This was interpreted as an additional confirmation of chloride binding, equivalent to the neutralisation of about four positive charges of the protein. In the present study we observe that the solubility increases above a concentration of 0.6 - 0.8 M divalent cations or of $YbCl_3$ (Fig. 3), corresponding to 2 M and 4 M ionic strength respectively. We can therefore conclude that the adsorption of chloride onto this protein predominates on the cation adsorption in the presence of NaCl, CsCl, RbCl, $MnCl_2$, $CoCl_2$ and $YbCl_3$.

The adsorption of cations is also likely to occur, given the impressive increase of solubility in the case of the polyvalent species. However, this occurs apparently at higher salt concentrations than for chloride. Different chemical and physico-chemical reasons can be involved: i) the positively charged protein is surrounded by a double layer enriched in anions rather than cations, compared to the bulk concentration of the salt, ii) cation binding must fulfil the coordination constraints, iii) competition between anion and cation binding during salt concentration increase, and iv) hydration of all these species present in the solution as discussed hereafter. Further investigations at atomic level are in progress in order to have a more accurate view of the solution and to moreover understand predominating factors in the processes leading to crystallisation.

Finally hydration forces must be addressed in even more subtle solubility studies in the future. Although the solubility behaviour of the proteins seems to be predominated by the modification of the charge density at the surface of the protein, hydration of the ions as well as of the protein have to be taken into account. So far, very little is known on how ions perturb the hydration of the protein surface at an atomic scale.

Correlations have been found between the Hofmeister series and several physical properties of aqueous solutions of ions: the Jones-Dole viscosity B coefficient (Collins, 1997), the equivalent osmotic pressure (Li *et al.*, 1998), as well as the relative degree of hydration of the ions (Zavitsas, 2001). In fact, all these three classifications are sustained by a simpler physical quantity: the surface charge density of the ions (Collins & Washabaugh, 1985) and therefore their ability to modify the water structure. This property can be quantified by the ion entropy of hydration (from dissolving the ion in water).

The hydration of anions is known to be very different from the one of the cations (Collins, 1995; 1997). In the Hofmeister series, cations are ranked according to their decreasing hydration entropy. Small singly charged cations, such as Na^+, are strongly hydrated, whereas large singly charged cations, such as NH_4^+, are weakly hydrated. The former possess a higher surface charge density and therefore more negative entropy of hydration than the latter. Divalent and trivalent cations are even more hydrated than small monovalent cations, bearing a higher surface charge density and therefore leading to a more negative entropy of hydration.

The binding of hydrated cations on a protein surface can produce an energy barrier, known as hydration force. As shown by Pashley (1981; Pashley & Israelachvili 1984), such repulsive hydration forces exist between negatively charged mica surfaces at high electrolyte concentrations. Strongly hydrated cations, such as di- and tri-valent cations, would give rise to higher repulsive energy barriers than low hydrated ones, agreeing with the cationic Hofmeister series. While revising this manuscript, we were informed of a contribution that supports our viewpoint (Kiriukhin & Collins, 2002).

PB was supported by the European Biocrystallogenesis initiative (contract N°:BIO4-CT98-0086(DG12-SSMI)), and thanks ICCBM9 and the 'Comité National de Biochimie' for travel grants that allowed his attendance at the meeting. PB is indebted to Prof. C.M. Lawrence for the opportunity to take time to write this paper. We thank the Centre National d'Etudes Spatiales (CNES) and the Centre National de la Recherche Scientifique (CNRS) for financial support of this project. The authors are indebted to Prof. A. Ducruix for helpful scientific discussions and comments on the manuscript. We acknowledge Dr. Izio Rosenman for helpful information on the experimental set-up. We thank Drs. M. Rogers and G. Gauss for careful reading of this manuscript.

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 Received 6 May 2002 • Accepted 7 August 2002