Importance of the nature of anions in lysozyme crystallisation correlated with protein net charge variation

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Hofmeister anion series are studied to examine the coupled influence of pH and ionic strength on the solubility of previously desalted lysozyme. Solubility curves are measured at pH 4.3 and 8.3 and 18°C for nitrate, para-toluenesulfonate, citrate, sulphate, phosphate, and acetate. Extreme low ionic strength is explored, confirming the decrease of lysozyme solubility while increasing the protein net charge and the ionic strength. The classification of specific salt effects takes into account the valence of the anions with respect to the protein net charge.

Keywords: lysozyme; crystallisation; solubility; Hofmeister series; electrostatic effects; polyelectrolytes; anions.

1. Introduction

The interaction potentials that govern the distribution of biomacromolecules in solution are made up by numerous and weak forces that compete with those that structure water and solvate small electrolytes as well (Israelachvili, 1992). The potentials leading to crystal growth present a strong dependence for which electrostatic interactions play an important role at long distance (Neal *et al*, 1998; Elcock & McCammon, 2001). Therefore, pH and electrolytes are decisive solvent parameters that modulate the potentials through both non-specific and specific effects. A pH variation (in the extreme cases from about 2 to 12) modifies both the amplitude and sign of net charges of hydrosoluble proteins (for details see Riès-Kautt & Ducruix, 1997). Coulombic potentials between these macroions are affected by the concentrations of free electrolytes, which range from millimolar to molar. They decrease exponentially beyond the Debye screening length, λ_D, defined as

$$\lambda_D = (\varepsilon \varepsilon_0 k_B T / e^2 I)^{1/2} \quad (1)$$

where $I = 1/2 \sum_i c_i z_i^2$ is the ionic strength, c and z are the concentration and the valence of the electrolyte i respectively, and ε and ε_0, the dielectric constants of the solvent and the pure water respectively. Basically, 1M of a monovalent salt reduces the range of electrostatic interactions to λ_D = 3 Å (Israelachvili, 1992). As (1) depends on the square of the valence, multivalent ions should be particularly efficient to screen Coulombic interactions. Phosphate and citrate are therefore expected to present a higher screening ability at pH 8 than at 4, owing to their valence increase within pH.

Under conditions of strong electrostatic interactions (high protein net charge and low ionic strength) or dominant short-range interactions (high ionic strength), significant specific effects emerge, leading to failure in modelling protein systems as colloids (Piazza, 2000; Tardieu *et al*, 2002). Therefore the geometrical and chemical complexity of the protein surfaces, local protonation states and differential ionic affinity for protein binding sites have to be studied in more detail.

Ionic specificity is revealed by the effects of the so-called Hofmeister series that originally consisted in the ranking of ions as function of their effectiveness to precipitate a mixture of hen egg white proteins (Hofmeister, 1888) according to:

$$SO_4^{2-} > HPO_4^{2-} > CH_3CO^{2-} > HC_6H_5O_7^{2-} > C_4H_5O_6^{-} > HCO_3^{-} > CrO_3^{-} > Cl^{-} > NO_3^{-} > ClO_3^{-}$$

It was further observed that many properties of biomacromolecules in solution vary as a monotonic function of the Hofmeister series (Collins & Washabaugh, 1985; Parsegian, 1995; Cacace *et al*., 1997). This classification appears to be correlated with the sign and value of Jones-Dole viscosity B coefficients (Collins, 1997 and refs. therein) that merely reflect how these ions interact with the surrounding water molecules. Salts on the left side of the series are all divalent ions and therefore more hydrated than large monovalent ones (Israelachvili, 1992; Collins, 1997). They present a rather high charge density, structure the surrounding water molecules, increase the surface tension on a macroscopic level and are called lyotropic or cosmotropic. Salts on the right side of the series have a low charge density, increase the mobility of nearby water molecules, prefer to adsorb to non-polar surfaces and are named chaotropic.

Protein solubility determination is a conventional means to integrate all the forces that maintain a fraction of protein molecules in solution in a thermodynamic equilibrium with those that assemble into an ordered three-dimensional array. Its theoretical interest also remains vivid because of difficulties in equating the experimental results on a physical basis. It must be reminded that liquid-liquid boundaries or precipitation curves are not relevant values for thermodynamics, because the phases of these systems are not at equilibrium, but they give a good qualitative approximation. Solubility values have been compared with liquid-liquid boundaries, and acknowledged discrepancies in response to pH and ionic strength (Taratuta *et al.*, 1990; Broide *et al.*, 1996; Grigsby *et al.*, 2001).

Analytic efforts were devoted to the data obtained by Green (1931a; 1931b; 1932) for carboxyhemoglobin in order to correlate salt-depending equation parameters to the changes in protein solvation or ion binding (Cohn, 1925; Jenkins, 1998). Other protein models (HEW lysozyme, Bovine Pancreatic Trypsin Inhibitor (BPTI) or *Hl* collagenase for instance) were investigated to revisit the original experiment by Hofmeister specifically. These studies (Riès-Kautt & Ducruix, 1989; Carbonnaux *et al.*, 1995) allowed to link the salt specificity to the protein net charge: the Hofmeister anion ranking holds for negatively charged protein (e.g. collagenase) while it is reversed with a positive net charge (e.g. lysozyme or BPTI). In the latter case, neutralisation of the protein net charge by anions has been proposed to be a major contribution of the decreasing lysozyme solubility (Riès-Kautt & Ducruix, 1991).

The description of the overall solubility variation *versus* the ionic strength should account for the non-specific effects, owing to the presence of electrolytes in the solvent. The solubility curves of carboxyhemoglobin as function of this parameter (Green, 1931 b; 1932) brought the experimental basis to suggest two non-specific salt effects, namely salting-in and salting-out. The moderate solubility increase, i.e. salting-in, would be due to the formation of pairs between the protein molecule and small ions, rather than between charged residue side chains. This can be equated by the Debye-Hückel theory over a 0.1-0.3 M range (Arakawa &

Timasheff, 1985). Above this range, the preferential hydration of the salts impacts the hydration of the protein as well. But proteins usually display hydrophobic patches on their surface and the system will tend to reduce the solvent-exposed surface by favouring the association of macromolecules (Melander & Horvath, 1977), hence the salting out.

Here we present a study of the solubility behaviour at very low ionic strength and as function of the net charge of lysozyme, in order to address the salt specificity question. The very basic isoionic point (pI 10.8) nevertheless restricted the studied range of pH values below the pI and consequently the net charge to positive values. The protocol of lysozyme deionisation (Riès-Kautt *et al.*, 1994) permitted to cover an extended pH domain and the accurate control of all electrolytes, avoiding the use of additional chemical buffers and exploiting the buffering capacity of the concentrated protein.

A previous study of the solubility of lysozyme bearing a net charge from + 3.5 to + 16 over a large range of ionic strength was carried out in the presence of NaCl (Retailleau *et al.*, 1997). This salt, being in the middle of the Hofmeister series, could thus be chosen as a "reference salt". The results showed that lysozyme solubility depends on the protein net charge even when electrostatic interactions are screened. Indeed the solubility increases with the net charge at low ionic strength (< 0.4 M), while it slightly decreases at high ionic strength (> 0.8 M). In addition, the absence of salting-in was evidenced at extremely low ionic strength (from 0 to 0.2 M of added NaCl) for lysozyme at pH values lower than the pI. The semi-log representation finally helped to delineate the zone of the Coulombic prevalence (steep linear decrease) and that of dominating short-range interactions apart from 0.6 M NaCl. This value was interpreted as an estimate for the affinity of chloride binding to lysozyme.

The present work extends the former study to other anions of the Hofmeister series, in order to assess their specificity. Solubility measurements at constant temperature (18°C) are undertaken at two pH values, 4.3 and 8.3, the protein net charge being expected to vary by a factor of two. Ionic strength is monitored by the concentration of these anions used either as sodium or ammonium salts. Indeed, the effect of these two cations is known to have a negligible effect on lysozyme solubility (Riès-Kautt & Ducruix, 1989). At acidic pH, experiments were performed with nitrate, para-toluenesulfonate (pTS), citrate, phosphate and acetate anions, additionally to chloride, which was previously reported (Retailleau *et al.*, 1997). At pH 8.3 sulphate was added to the previous list, since this anion is unfavourable for crystal growth of lysozyme at pH 4.3 whatever the monovalent cation chosen (Riès-Kautt *et al.*, 1994).

2. Materials and methods

The method used to measure the solubility curves is identical to the experiments carried out previously with NaCl (Retailleau *et al.*, 1997). The same overall strategy detailed therein was strictly replicated: the same batch of lysozyme (Sigma n°51H7150), deionisation according to the same protocol, the same batch method for crystal growth and incubating the samples at (18 ± 0.1)°C. Only additional chemicals and adjustments of the protocol for the current purpose are presented hereafter.

2.1. Reagents

The following chemical reagents were purchased from 1) Sigma (ACS grade): $(NH_4)_2HC_6H_5O_7$; 2) Merck: $NaNO_3$, NaH_2PO_4, Na_2HPO_4, Na_2SO_4, $(NH_4)_2SO_4$, $Na_3C_6H_5O_7$. $2H_2O$, *p*-toluenesulfonic acid, Na *p*-toluenesulfonate (pro analysis), sodium acetate and HNO_3 (1N), H_2SO_4 (0.1N-1N), H_3PO_4 (0.1N), acetic acid (1N) (Titrisol); 3) Prolabo: $C_6H_8O_7.H_2O$ (99 % Rectapur).

2.2. Lysozyme acidification

The acidification of the isoionic lysozyme for the preparation of our concentrated samples was assumed to be both protein concentration- and anion-independent, which is not strictly correct according to Tanford & Roxby (1972) and Kuehner *et al.* (1999) but remains a good approximation. Five protons are required to bring an initially isoionic lysozyme solution to a pH of about 8 (eleven for pH around 4) and these amounts are found approximately independent of the chemical nature of the acid.

Acidification brings not only the protons to the solution, but also eleven monovalent anions per lysozyme molecule at pH 4.3, and five at pH 8.3. If they are divalent their number of molar equivalents is reduced by a factor of two (5.5 divalent ions at pH 4.3 and 2.5 at pH 8.3). For trivalent citrate at pH 8.3 it becomes 5/3 molar equivalents.

Therefore it is necessary to add these ions for the calculation of the total ionic strength of the solution. At 25°C, pK_1 = 2.15, pK_2 = 7.20, pK_3 = 12.33 for phosphoric acid, and pK_1 = 3.13, pK_2 = 4.76, pK_3 = 6.40 for citric acid (Perrin & Dempsey, 1974). Hence phosphate is monovalent at pH 4.3 and divalent at pH 8.3, whereas citrate is mono- and divalent (in a 1:1 ratio) at pH 4.7 and completely trivalent at pH 8.2.

Although the initial pH of the two stock solutions of lysozyme was fixed to 4.3 and 8.3 respectively, the final pH values (given in Table 1) are those at the end of the experiments when measuring the solubility.

2.3. Crystallisation

All experiments were performed by the batch method by mixing salt solutions in water with the appropriate protein solution at the desired pH value. The tubes were stored tightly closed at 18°C. For a given ionic strength, at least two different initial protein concentrations were induced to crystallise and were duplicated to increase the statistic of the mean final value of the supernatant concentration. The equilibration kinetics was followed for each sample periodically until the protein concentration of the supernatant is constant with time. The experimental conditions are given in Table 1, all details being explained in the legend.

3. Results and discussion

The present study of lysozyme solubility aims at exploring the Hofmeister anionic series and pH effects along with ionic strength down to the lowest values experimentally reachable. The two pH values are chosen in order to compare the crystallisation of the lysozyme molecule bearing a net charge of either +11 or +5.

3.1. Crystal form

At pH 4.3 and very low ionic strength, crystallisation of lysozyme in phosphate or acetate is limited to salt concentrations above 0.2 M. Indeed extremely high protein concentrations (> 400 mg/ml) would be required below 0.2 M and the system would become too viscous for accurate measurements. No solubility curve for lysozyme in the presence of sulphate ions can be measured at pH 4.3 as a consequence of gel formation (Riès-Kautt *et al.*, 1994).

Most of the crystals grown under the investigated conditions display prismatic shapes, characteristic of the standard tetragonal $P4_32_12$ form, except for nitrate that favours monoclinic crystal packing. pH was expected to induce polymorphism from tetragonal to orthorhombic as often reported with NaCl > 0.3 M and at a pH near the isoelectric point (Alderton *et al.*, 1945; Artymiuk *et al.*, 1982; Tanaka *et al.*, 1996 ; Velev *et al.*, 1998).

Table 1 Experimental data of lysozyme concentrations at indicated pH values and salt concentrations.

[a] Salt conditions		[b] Initial			[c] Solubility (mg/ml)
	C (M)	Protein Conc.	I total (M)	N	S (± SD)
$NaNO_3$	0.00	165-207	0.043	5	111 ± 3
pH 4.4 ± 0.1	0.05	96-110	0.060	3	27 ± 2
monoclinic	0.10	55-83	0.102	5	4.2 ± 0.8
	0.15	30-41	0.150	5	1.3 ±0.1
$NaNO_3$	0.00	115-140	0.014	6	85 ± 4
pH 8.3 ± 0.2	0.05	40-92.5	0.051	8	8.1 ± 1.2
monoclinic	0.10	10-55	0.100	9	2.2 ± 0.3
	0.15	8-19	0.150	8	1.47 ±0.23
Na pTS	0.00	68-100	0.028	3	72 ± 5
pH 4.4 ± 0.2	0.01	90	0.034	1	62
tetragonal	0.05	60-92	0.068	6	46 ± 5
	0.10	40-82	0.111	4	29 ± 3
	0.20	30-50	0.205	3	12.2 ± 1.8
	0.40	5-20	0.401	2	1.7 ± 0.1
Na pTS	0.00	95-150	0.014	4	82 ± 9
pH 8.2 ± 0.2	0.01	86-90	0.020	1	60
tetragonal	0.05	74-89	0.057	6	41.2 ± 2.0
	0.10	63-82	0.103	5	15.8 ± 1.7
	0.20	10-50	0.201	7	4.3 ± 1.1
	0.40	2.9-20	0.400	6	1.36 ±0.18
Amm. citrate	0.00	216-255	0.014	3	112 ± 5
pH 4.7 ± 0.1	0.05	180-230	0.137	4	30.5 ± 1.9
tetragonal	0.10	155-180	0.270	3	23.3 ± 0.3
	0.40	77-84	1.069	4	20.0 ± 0.6
	0.60	37-40	1.602	4	13.8 ± 1.2
Na citrate	0.00	63-102	0.009	8	18 ± 6
pH 8.2 ± 0.1	0.01	38-87	0.062	6	3.5 ± 0.6
tetragonal	0.03	35-47	0.180	6	4.3 ± 0.3
	0.05	35-47	0.300	4	5.3 ± 0.3
Na_2SO_4	0.00	205-240	0.058	6	156 ± 12
pH 8.3 ± 0.3	0.025	174-198	0.096	6	59.5 ± 3
tetragonal	0.05	163-174	0.160	7	36.4 ± 2.4
	0.10	119-148	0.310	10	28.5 ± 2.7
	0.20	97-119	0.610	6	29.1 ± 2.8
orthorhombic	*0.20*	*107-128*	*0.610*	*3*	*12.4 ±1.8*
	0.40	*83-107*	*1.20*	*10*	*6.7 ±1*
$(NH_4)_2SO_4$	0.05	130-180	0.170	8	52 ± 6
pH 7.8 ± 0.5	0.20	94-128	0.620	7	47 ± 4
orthorhombic	0.40	78-107	1.200	6	50 ± 6
NaH_2PO_4	0.00	375	-	soluble	*> 375*
pH 4.3 ± 0.3	0.10	320	-	soluble	*> 320*
tetragonal	0.20	280-315	0.305	3	274 ± 13
	0.60	163-183	0.643	8	112 ± 10
	1.00	100-120	1.017	3	43 ± 8
Na_2HPO_4	0.00	255-290	0.048	4	136.5 ± 6
pH 8.3 ± 0.3	0.05	230	0.166	6	44.5 ± 5.5
tetragonal	0.10	205-230	0.309	4	26.2 ± 0.9
	0.20	155-175	0.605	4	15.2 ± 1.1
NaAcO	0.00	450-	-	soluble	*>> 450*
pH 4.5 ± 0.3	0.20	315-320	0.307	3	279 ± 17
tetragonal	0.60	195-250	0.614	5	36 ± 3
	1.00	80-107	1.003	4	7.4 ± 1.6
NaAcO	0.00	307-317	0.043	2	244 ± 9
pH 8.3 ± 0.3	0.15	284	0.178	1	158
tetragonal	0.30	70-176	0.310	5	57 ± 6

[a] Salt conditions are given together with the final pH of the samples (measured when determining the solubility values), and the crystal form verified by X-ray diffraction or supposed (in italic) according to the crystal habit).
[b] Initial protein concentrations are given in mg/ml.
[c] Solubility value (mg/ml) is calculated as the average of final protein concentrations over N experiments, at a total ionic strength (salt plus counter-ions). SD is the standard deviation.

On the other hand a change in the lysozyme net charge was shown neither to modify the lysozyme iodide crystal form, nor the 3-D structure (Steinrauf, 1998). Under the conditions of our study, a pH variation from 4 to 8 does not modify the crystalline systems at 18°C and no polymorphism was apparent. A change of crystal habit and form is only suspected in the presence of sodium sulphate at high concentration and at pH 8.3 (see Table 1). Indeed, a discontinuity in the solubility curve is associated with a possible transition at pH 8.3 in the presence of 0.2 M Na_2SO_4: when starting with 97 - 119 mg/ml lysozyme, typical tetragonal crystals are observed, whereas a needle-like crystal form occurs with initial protein concentrations of 107 - 128 mg/ml. It suggests new crystal packing and therefore relevant solubility data must be kept apart (data in italic in Table 1).

The crystallisation conditions were such that nucleation occurs within one week. At basic pH, heterogeneous nucleation had to be initiated by streak seeding to overcome retarded nucleation in the case of chloride, phosphate and acetate. Tetragonal crystal seeds obtained at higher ionic strength or at acidic pH were deposited into the supersaturated samples. Consequently, clusters of needles rapidly filled the bulk of the crystallising solutions. However, the present needles are assumed to belong to the tetragonal system, because no discontinuity of solubility is observed and X-ray analysis is impossible. Similarly lysozyme crystals grown in MPD at pH 8 (Weiss *et al.*, 2000) exemplify that needle-like habit does not imply a change in crystalline system.

3.2. Effect of ionic strength on positively charged lysozyme

Figures 1 & 2 display the solubility curves of lysozyme as a function of the total ionic strength at the two investigated pH values. It must be emphasised that the curves do not start at the origin of the ionic strength abscissa, even when no salt is added (C = 0 M in Table 1). This initial ionic strength is due to the anions introduced to the solution during the acidification of isoionic lysozyme (see §2.2). Taking the example of lysozyme/NO_3 in the absence of any added sodium nitrate (first line in Table 1): C = 0 but I = 0.043. This comes from the solubility value of lysozyme, which is 111 mg/ml (7.76 mM) and therefore 85 mM nitrate ions are present due to the 11 molar equivalents of HCl used for acidification. The calculation supposes that the counterions are equally distributed in the bulk solution and the crystal, although the statement cannot yet been proved.

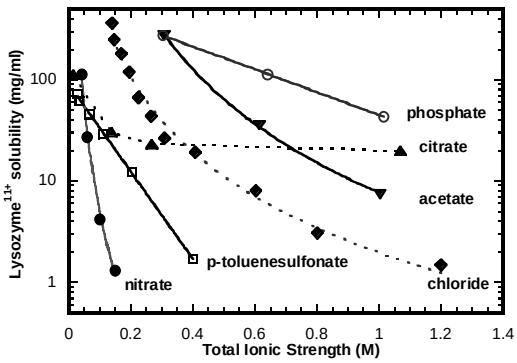

Figure 1

Semi-logarithmic representation of the lysozyme(+11) solubility at pH 4.3 and 18°C, in the presence of different anions. Data for NaCl are given for comparison and comes from Retailleau *et al.* (1997).

The first observation is that solubility always decreases as soon salt is added and no salting-in occurs as already observed in the presence of sodium chloride (Retailleau *et al.*, 1997). Hence this observation is extended to all salts presently tested, emphasising a non-specific effect. One difference with the first experimental data showing salting-in is that the solubility of carboxyhemoglobin was measured close to its pI (Green, 1931a; 1931b;1932). Furthermore,

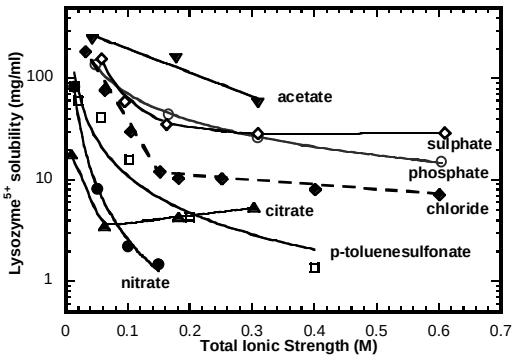

Figure 2

Semi-logarithmic representation of the lysozyme(+5) solubility at pH 8.3 and 18°C, in the presence of different anions. Data for NaCl are given for comparison and comes from Retailleau *et al.* (1997).

the 14.3 kDa lysozyme has a larger charge density than does tetrameric carboxyhemoglobin (64.5 kDa), which exhibits wide hydrophobic patches. It thus makes sense that lysozyme is more sensitive to charge effects in solution and that the correlation between positive net charge and surrounding anions determines the trend for its solubility variation.

Lysozyme solubility is seen to monotonically decrease over the studied ranges of ionic strength for each salt, except for Na_3Citrate at pH 8.3 where the decrease is limited between 0 - 0.060 M. The decrease of lysozyme solubility is less steep in most of the salts above 0.4 M, when the solubility becomes nearly insensitive to any further salt addition as already observed in studies carried out at higher ionic strength (Riès-Kautt & Ducruix, 1989).

If salting-in does not apply to lysozyme at low ionic strength under the present conditions, salting-out *via* preferential protein hydration does not seem to be affected by the divalent anions either. Indeed it is striking to see that 0.2 to 0.8 M sulphate, known for its strong salting-out effect, leaves the lysozyme solubility almost invariant at 50 mg/ml (Table 1), and 30 mg/ml (Table 1 and Fig.2) when using ammonium or sodium sulphate respectively. The weak efficiency of the divalent sulphates or phosphates stresses that non-specific effects, appear albeit taking place in the bulk solution.

Finally a particular behaviour is observed for the curve of lysozyme/citrate at pH 8.3, showing that the solubility increases after an initial decrease. This is not predicted by models known for colloids. Furthermore a gel formation occurs with lysozyme in the only presence of the citrate counter-ions (no citrate added). Once crystals nucleate and grow, the gel disappears as already observed for phosphate (Riès-Kautt & Ducruix, 1989). Binding of hydrated ions may be invoked as observed for lysozyme in the presence of $MnCl_2$, $CoCl_2$ and $YbCl_3$ (Bénas *et al*, 2002), or 1M $MgBr_2$ (Broide *et al.*, 1996). When the interactions between slightly negatively charged apoferritin molecules (450 kDa) have been investigated (Petsev & Vekilov, 2000), hydrated sodium ions (> 0.2 M) were assumed to bind preferentially to the protein surface, increasing the repulsive hydration forces between the protein surfaces. Binding of co-ions may also explain why carboxyhemoglobin solubility still increases above 3 M NaCl, well beyond the validity limit of the Debye-Hückel theory. The increase of solubility observed for our Data occurs at about 20 mM citrate (ionic strength ~ 0.1 M). Repulsive forces are probably related to the trivalent citrate anion rather than the sodium counter-ion (60 mM).

3.3. Effect of pH variation and evidence for ion binding

A change of pH directly affects the protein ionisation state *via* the protonation of its charged groups (aspartic and glutamic acids, tyrosine, free cysteines, arginines, lysines, histidines and C- and N-terminal residues). This modifies the physical chemical properties of the protein molecule, namely its solubility or the second virial coefficient, which quantifies a deviation of the protein solution from ideal behaviour (Tardieu *et al*, 1999; Elcock & McCammon, 2001). A pH variation also affects weak acids and bases of low molecular weight used as buffers and/or precipitating agents, e.g. acetate, citrate, phosphate. Carbonate can also be accidentally present after dissolution of CO_2 in solutions at high pH.

It has been established that protein solubility varies with the protein net charge and becomes minimal at the pI (Green, 1931a). The data for lysozyme chloride with a net charge varying from + 3.5 to + 16 agreed with this general rule at low ionic strength, where electrostatics dominates (Retailleau *et al.*, 1997). As expected, lysozyme solubility also decreases with decreasing net charge in the presence of the salts tested here.

The change of solubility between lysozyme(+11) and lysozyme(+5) depends strongly on the nature of the anion. The maximal decrease in the solubility is approximately 20 mg/ml with *p*-toluenesulfonate (pTS), 50 with nitrate, 100 with citrate, 200 with chloride and 300 with acetate or phosphate. The relative low amplitudes with pTS or nitrate bring strong evidence to the hypothesis of ion pairing in the decrease of solubility (Riès-Kautt & Ducruix, 1991). Indeed it may be understood by stronger association constants of these anions to bind to the protein, therefore a more efficient electrostatic screening or chemical decrease of the protein net charge at lower salt concentrations. The solubility decrease in the presence of phosphate and citrate is biased by the fact that the anions themselves change their valence at the same time. In addition the ionic strength takes only into account the ionic species like acetate at pH 4.5 omitting completely the presence of a nearly equivalent amount of non-charged acetic acid.

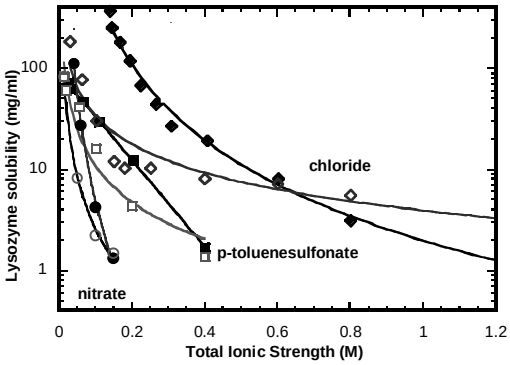

Figure 3

Superposition of lysozyme solubility curves at pH 4 (filled symbols) and pH 8 (open symbols) in the presence of three monovalent anions at 18°C. Data for NaCl are given for comparison and comes from Retailleau *et al.* (1997).

Therefore data at pH 4.3 and 8.3 are only strictly comparable for nitrate, pTS and chloride and is plotted in Fig.3. It clearly shows a pH-related curve intersection that coincides with the limits of the electrostatics prevalence. These intersections occur at about 0.15 M for nitrate, 0.4 M pTS and 0.6 M for chloride and provide an estimate for salt binding affinity together with another measure for the salt efficiency in the lysozyme solubility decrease. Indeed the intersection means an identical solubility at a given ionic strength, whatever the protein net charge. This can be understood as a saturation of the charged protein sites by counter-ions and the protein behaves as if its net charge would be screened.

The results of lysozyme/chloride (Retailleau *et al.*, 1997) also showed an inversion of lysozyme solubility with the net charge if the ionic strength becomes higher than the intersection point. This is likely to happen with the salts of the present study. An explanation of such an inversion may come from emerging hydration forces at higher ionic strength.

3.4. Effect of the chemical nature of the anion

For both sets of solubility curves shown in Fig 1 & 2, the different efficiency of the anions over the investigated range of ionic strength the salt specificity. The Hofmeister ranking established for lysozyme in the acetate buffer conditions (Riès-Kautt & Ducruix, 1989) is confirmed in the region of low ionic strength at both pH values for the following anions:

at pH 4.3 : NO_3^- > pTs^- > Cl^- > $CH_3CO_2^-$ ≥ $H_2PO_4^-$
at pH 8.3 : NO_3^- > pTs^- > Cl^- > SO_4^{2-} ~ HPO_4^{2-} > $CH_3CO_2^-$

All these salts (except citrate) are monovalent at pH 4.3 and display contrasted effects. Nitrate occupies the left side of the series, being the most chaotropic ion, because it has the strongest effect in decreasing lysozyme solubility at such a low ionic strength. In contrast, acetate is a lyotropic representative and is on the right side (solubility > 300 mg/ml around 0.3 M). The steep linear section of the solubility curves at low ionic strength can assess the respective ability in Coulombic pairing (Riès-Kautt & Ducruix, 1991): steepest for chaotropic anions, much less so steep for lyotropic ones, with an intermediate feature provided by the "neutral" chloride. The differential propensity of chaotropic anions to approach the protein surface intuitively explains the way it reduces the distances of electrostatic interactions. In contrast, lyotropic acetate or phosphate at pH 4.3, which are preferentially in the bulk solution, imply that other interactions are required for salting-out. Here short-distance hydrophobic contributions may be forced by the high concentration of electrolytes (protein and salt).

Investigation at basic pH allows to extend the study to divalent or trivalent anions and to evidence a significant impact on the solubility of lysozyme. While the solubility of lysozyme(+11) in the presence of the monovalent $H_2PO_4^-$ is at the upper position in the phase diagram, the divalent HPO_4^{2-} becomes more efficient than acetate and even almost as efficient as chloride with respect to the lysozyme(+5).

As already mentioned, multivalence confers anions lyotropic properties that place them at the right end of the Hofmeister series. Surprisingly, divalent anions present a "median" efficiency in decreasing the solubility of lysozyme(+5) and trivalent citrate is even among the most efficient. The particular high capacity of sulphate, relative to chloride, to enhance lysozyme-lysozyme attraction at pH 4.5 was already reported (Ducruix *et al.*, 1996). Positive lysozyme net charge readily attracts divalent negative charges in dilute conditions as evidence by the coupled lysozyme-sulphate diffusion (Retailleau *et al.*, 1999). Therefore Coulombic screening must have some impact on the solubility, as shown by the steep curve decrease at low ionic strength. In the meantime, preferential hydration maintains the anions in the bulk, away from the protein surface, moderating the screening efficiency, hence the intermediate positions of the corresponding solubility curves in the phase diagram.

This duality appears reinforced with citrate bearing a triple negative valence. The fully trivalent citrate is the most efficient anion by far with respect to lysozyme(+5) in the absence of any added salt: ~15 mg/ml *vs* ~85 mg/ml for the chaotropic anions). A quick decrease over >0-0.1M testifies that three negative charges at the time are very efficient in screening the +5 net charge. Then lyotropic properties and steric and/or electrostatic hindrance at proximity of the protein surface (because of the rather large tridentate shape of the citrate) tend to repel the counter-anions and consequently to limit the Coulombic screening. At the acidic pH, the corresponding curve presents similarities transposed to a larger scale of solubility values and ionic strength range. Citrate is almost as efficient as the chaotropic nitrate or pTS. The decrease is then brief (0.1 - 0.2 M) followed by a stable evolution rather than a small increase as observed for pH 8.3. This may mean that the contribution of divalent species in the solution is significant; curve determinations at pH 3 and 5.2 should therefore clarify the relative role of monovalent and divalent forms relatively to the intrinsic chemical ability to decrease the solubility. In the present study this leads lysozyme/citrate curves to cross the phase diagram horizontally. This results in a position of citrate in the Hofmeister series varying with the ionic strength. At low ionic strength I < 0.1 M

at pH 4.3 NO_3^- > pTs^- > $Citrate^{-/2-}$ >> Cl^-
at pH 8.3 $Citrate^{3-}$ > NO_3^- ~ pTs^- >> Cl^-

As more salt is added, the position of citrate is gliding to the level of chloride around 0.4 M.

4. Conclusion

Complementary data on the solubility of lysozyme as poly-electrolyte are provided by varying the protein net charge and studying protein-ion relationship in equimolar ratios.

Usefulness of solid-liquid phase diagrams is verified by the rationale for screening designs in crystal growth of hydrosoluble proteins using salt properties (Leavis & Rothstein, 1974; Riès-Kautt & Ducruix, 1997).

Investigation at basic pH throws light on the effects of the anion multivalence correlated with the ionic strength. While divalent (sulphate, phosphate) or trivalent (citrate) anions are considered proficient for protein salting-out, the present study stresses the duality of the balance between preferential protein charge screening and preferential hydration leading to a moderate action on solubility positively charged proteins.

Detection of anions by X-ray crystallography confirms the penetration of the first hydration layer by counter-ions (Vaney *et al.*, 2001). It remains to fully understand the microscopic interactions that account for the macroscopic properties.

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