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No Salting-In of Lysozyme Chloride Observed at Low Ionic Strength over a Large Range of pH

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ABSTRACT Solubility of lysozyme chloride was determined in the absence of added salt and in the presence of 0.05–1.2 M NaCl, starting from isoionic lysozyme, which was then brought to pH values from 9 to 3 by addition of HCl. The main observation is the absence of a salting-in region whatever the pH studied. This is explained by a predominant electrostatic screening of the positively charged protein and/or by adsorption of chloride ions by the protein. The solubility increases with the protein net charge at low ionic strength, but the reverse is observed at high ionic strength. The solubility of lysozyme chloride seems to become independent of ionic strength at pH ~9.5, which is interpreted as a shift of the isoionic pH (10.8) to an isoelectric pH due to chloride binding. The crystallization at very low ionic strength, where lysozyme crystallizes at supersaturation values as low as 1.1, amplifies the effect of pH on protein solubility. Understanding the effect of the net charge and of ionic strength on protein-protein interactions is valuable not only for protein crystal growth but more generally also for the formation of protein-protein or protein-ligand complexes.

INTRODUCTION

The association between proteins is clearly a very important process in biology (Briggs, 1997) when protein complexes are involved. In the particular case of protein crystal growth, identical protein molecules have to build contacts although weaker than in protein complexes (Riès-Kautt and Ducruix, 1997). This work aims at showing the importance of long-range electrostatic interactions to allow the protein molecules to approach each other, depending on the net charge and on the ionic strength, as it has been recently shown for the formation of the barnase-barstar complex (Schreiber and Fersht, 1996).

Protein solubility may be considered as a macroscopic property resulting from various interactions between protein-protein, protein-ion, ion-water, and water-protein molecules. Protein solubility is affected by the properties of the protein itself: its net charge, the ratio of charged, polar, and neutral amino acids, hydrophobicity or hydrophilicity, and overall stability versus temperature, pH, and solvent compounds. Protein solubility is also dependent on the crystallization parameters such as temperature, pH, ionic strength, and the presence of either stabilizing or denaturating agents in the solution. In addition, all these parameters may interact. Their effects can be experimentally quantified by measuring the solubility as a function of these parameters. Solubility then becomes a thermodynamic characteristic of the protein/solvent system as long as it corresponds to the concentration of soluble protein in equilibrium with the crystalline protein at a given temperature, pH, and solvent composition. Protein concentrations measured in the presence of precipitates usually exceed the solubility (Riès-Kautt and Ducruix, 1997; Guilloteau et al., 1992) and additionally depend on initial protein concentration in contrast to the values determined at equilibrium with crystals (Shih et al., 1992; Leavis and Rothstein, 1974).

Solubility data on proteins have been reported in the literature since the beginning of the century, especially over two periods, in the 1930s and in the last decade. In the first period, the most extensively studied was probably the solubility of hemoglobin, as a function of the nature of salt at high concentration (Green, 1931a) as well as versus pH (Green, 1931b) or at low ionic strength (Green, 1932). The work of Green gave the experimental basis of the rules that protein solubility is minimal at its pI and that it first increases (salting-in) and then decreases (salting-out) with ionic strength. Solubility versus ionic strength has been expressed empirically by the Cohn-Green formula (Green, 1932):

$$\log S = \log S_w + k_i \sqrt{I} - k_o I \quad (1)$$

where k_i and k_o are, respectively, the salting-in and salting-out constants and S and S_w are the protein solubility and the protein solubility in pure water, respectively; I is the ionic strength:

$$I = \tfrac{1}{2} \sum (m_i Z_i^2) \quad (2)$$

where m_i is the molality of salt and Z_i its charge valence. At high salt concentrations, the solubility expression can be simplified as a linear relationship:

$$\log S = \beta - K_s I \quad (3)$$

with S the solubility of the protein, β the intersect with the ordinate axis, K_s the salting-out constant, and I the ionic strength as defined above.

Based on these experimental data, theories have been developed to describe the ionic strength dependence of the solubility of proteins in terms of an initial increase, because

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of a dominant electrostatic effect (salting-in), and a subsequent decrease, because of a predominant hydrophobic effect (salting-out). A nice synthesis was provided by Melander and Horvath (1977) who expressed the free energy of a protein molecule in salt solution as resulting from the contributions of free energy for 1) formation of a cavity in the solvent, 2) electrostatic interactions between protein and solvent, 3) van der Waals interactions between protein and solvent, and 4) a term of free volume. They combined the Debye-Hückel theory, valid for simple ions at low ionic strength, and the Kirkwood model of dipolar ions, which they showed to be valid at high ionic strength, to the solubility of the hemoglobin. The electrostatic contribution between protein and ions was described using the first approximation of Debye-Hückel theory, which takes into account only the monopole-monopole interaction and neglects interactions of dipoles, quadrupoles, etc.

As concerns more particularly the effect of low ionic strength on protein solubility, this has been extensively described (Tanford, 1961; Arakawa and Timasheff, 1985) in terms of the change of the chemical activity coefficient of the protein when changing the ionic strength of the solution, again based on Debye-Hückel theory. It does not take into account the electrostatic contribution between the two charged macromolecules. The screening of the protein, considered as a polyion, by ionic species in the solution decreases the chemical activity coefficient (related to the electrostatic free energy) of the protein and increases its solubility, according to:

$$\ln(S/S_w) = Z_p^2 \epsilon^2 \mathcal{N} \kappa / [2DRT(1 + \kappa a)] \quad (4)$$

where Z_p is the protein's net charge, ϵ is the electronic charge, $\mathcal{N}$ is Avogadro's number, D is the dielectric constant, R is the universal gas constant, T is the temperature (in degrees Kelvin), a is the sum of the radius of the protein and the average radius of the electrolyte ions of the solution, and κ is related to the square root of ionic strength I and to the temperature, according to:

$$\kappa = (8\pi \mathcal{N} \epsilon^2 / 1000 D k_B T)^{1/2} (I)^{1/2} \quad (5)$$

with k_B as Boltzmann's constant. It was specified that Eq. 4 is valid only at low ionic strength and for protein molecules surrounded by an excess of ions of charge opposite to its net charge (i.e., counterions) and provided that the chemical potential of the protein in the solid phase remains constant (Arakawa and Timasheff, 1985). This model was confirmed by more recent solubility studies with the β-lactoglobulin (Treece et al., 1964).

In the last decade, the goal of better understanding protein crystal growth has led to the reexamination of protein solubility, with lysozyme being very stable versus pH and temperature and available in large amounts, being the protein most often studied by many different techniques and becoming a well documented system. In the case of lysozyme in the presence of various salt types at pH 4.5 (Guilloteau et al., 1992; Riès-Kautt and Ducruix, 1989), the experimental data did not fit a linear representation of the logarithm of protein solubility over large ranges of salt concentrations. A steep increase of solubility, which was observed at low salt concentrations when decreasing the concentration of the crystallizing agent, was tentatively attributed to an increasing contribution of the acetate buffer, because sodium acetate was shown to be one of the salts in which lysozyme is very soluble. Alternatively, no salting-in may exist below 0.2 M NaCl in the lysozyme solubility diagram.

Concerning the effect of pH, protein solubility is expected to decrease with its net charge Z_p for a given (low) ionic strength and to become minimal at its isoelectric pH (pI), defined as the pH at which the protein has a net charge of zero. This expectation has effectively been experimentally confirmed for egg albumin by Sørensen and Høyrup (Green, 1931a), hemoglobin (Green, 1931a), β-lactoglobulin (Grönwall, 1942; Edsall, 1947), and insulin (Fredericq and Neurath, 1950). However, if a protein P binds z counterions X, it has to be considered as a protein salt, P^zX_z. The minimal solubility of such a protein salt is then observed when the protein polyion in solution bears a net charge equal to z and no longer when it equals zero (Tanford, 1961).

Most protein solubility data in the literature are given for a narrow pH range, because exploring a large range of pH requires the use of different types of buffers, which add another variable to the system, confounding the pH dependence. As previous work on lysozyme had shown large variations of solubility depending on the nature of the anions (Riès-Kautt and Ducruix, 1989) and indicated possible adsorption of anionic species by the protein (Riès-Kautt and Ducruix, 1991), we decided to start with completely desalted isoionic lysozyme solutions and study its solubility over a broad range of pH (3 to 9). All cations and anions are first exchanged (Riès-Kautt et al., 1994) for H^+ and OH^- to ensure accurate control of all chemical species present in the solution. To avoid the presence of buffers, isoionic lysozyme (pH = 10.8 ± 0.2) was then brought to the required pH by addition of HCl and crystallized in the presence of indicated NaCl concentrations by a batch method. Solubility measurements were undertaken at pH 9, 8, 6, 4, and 3 at constant temperature (18°C) at concentrations of NaCl between 0 and 1.2 M.

The observed experimental solubility data over a large range of pH, in the absence of buffer and from very low to high ionic strength, will be discussed based on the relevant theories. We believe that this and similar studies will be valuable to the understanding not only of crystallization but also of long-range interactions involving formation of stable protein-protein or protein-ligand complexes in essential biochemical processes (Schreiber and Fersht, 1996).

MATERIALS AND METHODS

Reagents

The following reagents were purchased: lysozyme (three times crystallized, dialyzed, and lyophilized powder from chicken egg white reference

L-6876) from Sigma (Saint Louis, MO), 2) NaCl and HCl (0.1 N Titrisol) from Merck (Darmstadt, Germany), 3) HCl (1 N Normadose) from Prolabo (Paris, France), and 4) AG 50W-X8 (20–50 mesh, H^+ form, reference 142-1421), AG 1-X8 (20–50 mesh, OH^- form, reference 140-1422) from Bio Rad (Richmond, CA). The water used to prepare the solutions is a commercially available deionized and three times distilled water for injectable preparation (Meram, Melun, France).

Materials

The purity of the lysozyme batch was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis with enhanced silver staining by Thomas and Vekilov according to the procedure they have described (Thomas et al., 1996). The batch had a very high purity, which was otherwise confirmed by electro-spray ionization mass spectrometry. The protein was dialyzed against water with Spectrapor 7 (cutoff 6000–8000 Da) membranes. The protein concentration was measured by absorbance with a UV spectrophotometer (Beckman DU 640) at 280 nm and in Hellma cells (l = 1 cm). The absorbance of a 1 mg/ml lysozyme solution is assumed to be 2.66 at 280 nm. The experiments of 100 μl were prepared in polymerase chain reaction tubes (200 μl volume), those of 300 μl in glass microtubes (800 μl volume and 6.5 mm outer diameter). The crystallization tubes were stored at 18 ± 0.1°C in a thermal regulated incubator. Some samples (see text below) were kept at 4°C in a refrigerator. Shaking was performed by Rotomix (Bioblock) installed inside the thermoregulated chamber. The pH was verified with a Metrohm 632 pH meter equipped with a Tacussel MI-410 or a Mettler InLab 410 electrode.

Preparation of isoionic lysozyme

Commercial lysozyme contains salts the nature and amount of which depend on the commercial source and batch. Lysozyme of four different commercial sources was found to contain from 3.5 to 10.2% (w/w) chloride, representing 15 to 46 chloride ions per lysozyme molecule (Jolivalt et al., 1997). Desalting lysozyme by strong cation and anion exchange resins aims at exchanging cations with H^+ and anions with OH^-. X-ray fluorescence showed that this desalting procedure reduces the number of chloride ions to less than 0.3 per lysozyme molecule (Jolivalt et al., 1997). It is performed in three steps: dialysis, cation exchange, and anion exchange (Riès-Kautt et al., 1994). The isoionic lysozyme solution (pH ≤ 10.9 with only H^+ and OH^- as counterions) was rapidly deep frozen in test tubes in liquid nitrogen and freeze-dried. Due to the high basicity of isoionic lysozyme solutions, their contact with air had to be minimized to avoid absorption of air CO_2, which would lower the pH.

Preparation of lysozyme solutions at various pH values

The solutions (30–40 mg/ml) were prepared by acidifying 40–50 mg/ml isoionic lysozyme solutions in water to pH 9, 8, 6, 4, and 3 by adding respectively 4.5, 6, 8.5, 11, or 15 molar equivalents of H^+, as 0.1 N HCl, under pH meter control. Stock solutions of isoionic lysozyme in water at concentrations higher than 40–50 mg/ml were not achievable, although solutions of higher lysozyme concentrations were required for the crystallization at pH 8, 6, and 4. Therefore, these solutions were prepared by dissolving directly the required amount of isoionic lysozyme powder with dilute solutions of HCl at concentrations such that the pH was approximately one unit above the expected value, and then the solutions were adjusted accurately with a pH meter control.

Methods

Crystallization of lysozyme for the solubility determination was 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, three different initial protein concentrations were tested toward crystallization, two of them being duplicated to follow the equilibration kinetics. The duplicates were periodically checked for equilibration, i.e., for the protein concentration of the supernatant being constant with time. Table 1 indicates initial and final protein concentrations and the solubility values for lysozyme in the presence of increasing NaCl concentrations at different pH values. Indicated initial protein concentrations correspond to conditions for which crystals appear within a week at 18°C. Final concentrations are indicated to illustrate the differences of the protocols (see below).

To reduce the equilibration time of the crystallizing solutions, different approaches were tested and the results are presented in Table 1. Data in italic are given for comparison but were not included in the calculation of solubility values as these experiments had not yet reached equilibrium. In a first series, undisturbed assays did not reach equilibrium when measuring the final protein concentration after 2 months whereas the controls did (column 1). Equilibrium was more rapidly achieved in the control assays because the solutions were periodically mixed during the aliquot withdrawing and the observation under binocular microscope. The measurements of the undisturbed samples of the first series were repeated after 9 months (column 5) and were similar to the values of the controls measured after 2 months. A second series of assays were prepared and gently shaken once a week; after 2 months the protein concentrations (column 2) were similar to unshaken experiments of the first series after 9 months (column 5).

This shows that equilibration time can be reduced by periodic mixing of the samples. However, for crystallization at low ionic strength (≤0.2 M NaCl), which requires very high initial protein concentration and, thus, solutions being very viscous, equilibration is always very slow. Shaking the tubes in the thermal regulated chamber did not significantly increase the kinetics, as 6 months were necessary to obtain protein values of the supernatant (column 4) remaining constant with time. In the last series, lysozyme was first allowed to crystallize at 18°C, then the tubes were stored for 1 month at 4°C, and finally the solutions were kept at 18°C to equilibrate for solubility being reached (column 3). Equilibration at low ionic strength was achieved this way within 2 months (column 3) instead of 6 months when the samples were simply shaken (column 4).

The protein concentrations were determined by diluting 2 to 5 μl of supernatant with water for UV absorbance measurement at 280 nm. Depending on the crystallization volume, these measurements were duplicated with a second aliquot. Solubility S, in mg/ml, is calculated as the mean of protein concentrations at equilibrium for a given salt concentration and for a given pH. Values of final protein concentration, solubility value S and standard deviation (SD) are reported in Table 1. pH values indicated in Table 1 are those measured when solubility was determined.

RESULTS

Lysozyme solubility versus NaCl concentrations at different pH values

The solubility curves of lysozyme plotted as a function of NaCl concentrations for various pH are shown in Fig. 1. The most striking result is the absence of salting-in, whatever the pH. Moreover, the slopes of the solubility curves are not only negative but they are steepest at low ionic strength.

Below 0.1 M NaCl, the solubility is very high. The inset of Fig. 1 shows the solubility at pH 4.3, plotted versus total ionic strength, i.e., due to added NaCl and to the counterions of the protein. This result shows that the contribution of the concentration of counterions can no longer be neglected below 0.2–0.3 M at pH 4.3 for lysozyme and serves as a reminder that an ionic strength equal to zero cannot be achieved in a not infinitely dilute solution of a protein bearing a net charge different from zero. Indeed, to bring

TABLE 1 Crystallization conditions, final protein concentrations, and solubility of lysozyme at indicated pH and NaCl concentrations

		Protein concentration (mg/ml)						
			Final [protein]#					
	NaCl (M)	Initial [protein] (β)*	1	2	3	4	5	Solubility§ S (±SD)
pH 3.3 ± 0.3	0.20	127–168 (1.6–2.2)	*90/126*	73			70/75/82/83	77 ± 5
	0.30	97–130 (2.8–3.8)		32			35/36	34.3 ± 1.7
	0.40	55–84 (2.3–3.6)	22.6/23.8/26.7	22			19/25	23.2 ± 2.4
	0.60	18–40 (3.2–7.0)	5.3/*8.7/9.9/9.9*	5.1			5.8/6.2	5.6 ± 0.4
	0.80	7–22 (2.8–8.8)	2.6 *3.5/3.6/3.9*	2.9			2.7/2.5/2.4/2.2/2.7/2.7/ 2.0/2.5/2.6/2.3/2.2	2.5 ± 0.2
	1.20	9–15 (9–15)		0.7			0.95/1.01/0.99/0.96/ 1.15/1.08/1.0/1.03	0.99 ± 0.1
pH 4.3 ± 0.3	0.00	410–420 (1.12–1.15)				365/383/365/ 372/364/348		366 ± 10
	0.05	325–360 (1.3–1.4)			287/287	238/230/260/ 240/222/237		250 ± 23
	0.10	255–270 (1.4–1.5)			190/193/182/186	172/169/181/ 176/173		180 ± 8
	0.15	210–250 (1.8–2.1)			133/125/121/123	108/117/109/111		118 ± 8
	0.20	145–200 (2.2–3.0)			79/80	47/78/76/50	57	67 ± 14
	0.25	130–160 (3.0–3.6)			52/49	34/43/42/51/43/39		44 ± 6
	0.30	82–102 (3.1–3.9)		26.4				26.4
	0.40	45–58 (2.3–3.0)	19/19/20/23	19.3			14.8	19.2 ± 2.4
	0.60	22–40 (2.8–5.0)	5.6/8.3/8.7/9.9	8.4			7.5/7.9	8.0 ± 1.2
	0.80	10–20 (3.2–6.4)	3.1/3.8/*5.1/5.8*	2.8			2.6/3.2/3.1/3.3	3.1 ± 0.3
	1.20	5.5–11 (3.7–7.3)		1.5				1.5
pH 6.5 ± 0.2	0.00	370–375 (1.06–1.07)			350/345	325/330/370/ 365/365		350 ± 16
	0.05	300–330 (1.4–1.6)			207/205/213/200	216/225/210/206		210 ± 7
	0.10	260–280 (1.8–2.0)			127/127/118/115	165/150/180/155		142 ± 22
	0.15	220–240 (3.4–3.8)			59/60/59/57	68/74/70		64 ± 6
	0.20	170–190 (5.0–5.6)	*52*	*64*	30/30	34/38/37	33/35/37	34 ± 3
	0.25	120–140 (5.7–6.7)			17.3/17.7	22.5/25/22		21 ± 3
	0.30	68–86 (3.9–4.9)		*34*			17/18	17.5 ± 0.5
	0.40	46–60 (3.7–4.8)	13/13/*16/23*	*16.4*			10.6/12.7/12.6/ 11.5/14.1	12.5 ± 1.0
	0.60	44–58 (5.1–6.7)	9.2/9.2/9.5/*13.8*	6.8			8.2/8.8	8.6 ± 0.9
	0.80	23–41 (4–7)	5.5/5.5/6.1/6.3	6.1			5.0/5.2/5.3/6.3	5.7 ± 0.5
	1.20	16 (6.4)		2.1/2.8			2.6	2.5 ± 0.3
pH 8.4 ± 0.2	0.00	235–250 (1.3–1.4)			190/190	186/172/189/ 178/174		183 ± 7
	0.05	190–225 (2.5–2.9)			80/78/80/89	75/79/69/80		77 ± 4
	0.10	150–170 (5–6)			30/32/33/30	28/28/27/33/32		30 ± 2
	0.15	120–150 (10–12.5)			13/13/14/14	8.4/8.8/13.0/*27.0*		12 ± 2
	0.20	60–110 (6–10)	*24.2/36.1/18.0/28.6*		9/8/8/9	14/13.5/18.21		10.3 ± 2.5
	0.25	75–90 (7–9)				11.2/9.5/11.2/8.8		10.2 ± 1
	0.40	36–54 (4.5–6.8)	9.0/8.5/6.6/8.0					8.0 ± 0.9
	0.60	27–35 (3.8–4.9)	5.4/6.3/5.0				8.4/8.9/8.6/*11.5*	7.1 ± 1.6
	0.80	15–22 (2.7–4.0)	4.3/4.4/4.2/4.5				7.7/6.0/7.3	5.5 ± 1.4
	1.20	13.5 (2.2)					6.1/5.3/6.0/7.2	6.1 ± 0.7
pH 8.7 ± 0.2	0.20	66–78 (6.5–7.7)	8.0/7.6 *25.3/24*				10.5/10.0/10.3/ 11.9/11.6/11.3	10.1 ± 1.5
	0.30	40 (5)					8.4/7.3	7.9 ± 0.6
	0.40	29–52 (2.7–4.9)	10.0/9.6/9.4/7.7				8.1/10.3/12.2/ 13.2/14.9	10.6 ± 2.2
	0.60	20–29 (3.7–5.3)	5.9/4.8/5.0/4.5				6.2/5.8	5.4 ± 0.6
	0.80	16–29 (2.7–4.8)	4.2/3.7/3.7/4.2				10.2/8.8/7.3	6.0 ± 2.5

*Supersaturation, β, given in parentheses, was calculated a posteriori by dividing the initial protein concentrations by the corresponding solubility value.
#The values of final protein concentrations were measured after 2 months of crystallizations for columns 1–3, after 6 months for column 4, and 9 months for column 5. Volumes of assays are 100 μl except for column 3 (300 μl).
§Solubility value was calculated as the average of final protein concentration, except values in italic.

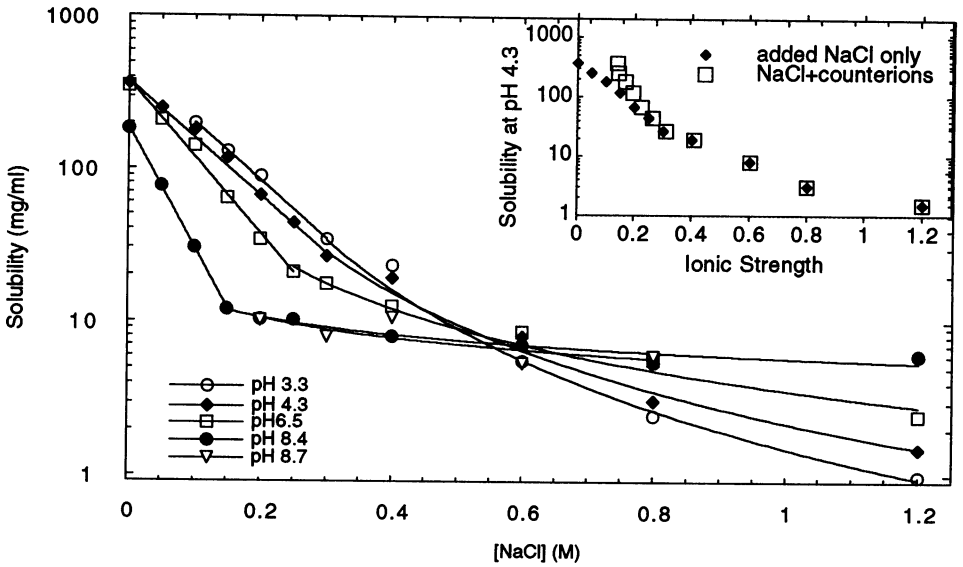

FIGURE 1 Solubility curves of lysozyme chloride versus the concentration of NaCl for indicated pH values at 18°C. In the inset, the solubility at pH 4.3 versus the concentration of NaCl is compared with the plot versus total ionic strength, i.e., due to added NaCl and to the counterions of the protein.

isoionic lysozyme to pH 4 by addition of HCl, 11 protons per lysozyme molecule are required, and thus 11 molar equivalents of chloride ions are present in the solution. At this pH and in the absence of NaCl, the solubility is 366 mg/ml (25.6 mM); consequently, 281 mM chloride ions are already present although no NaCl had been added. Similar calculations for solubility at 50, 100, 150, 200, 250, 300, and 400 mM NaCl at pH 4.3 give respective concentrations of counterions of 192, 138, 91, 52, 33, 20, and 15 mM. For the sake of clarity, solubility versus total ionic strength has not been represented for other pH values in the inset of Fig. 1, but a similar shift of the curves is observed; of course this shift is largest at low pH where the protein's net charge is highest.

The curves show a bimodal variation of lysozyme solubility: first a steep decrease up to 0.2–0.3 M NaCl and then a more moderate decrease at higher ionic strength. It is worth emphasizing that this change of slope is shifted toward lower NaCl concentration when the pH increases (approximately at 0.6, 0.3, 0.2, and 0.15 M NaCl for pH 3.3, 4.3, 6.5, and 8.3 respectively). These data confirm that the effect of pH on protein solubility is amplified at low ionic strength (Mikol and Giegé, 1989) as is the effect of temperature (Guilloteau et al., 1992; Ataka and Asai, 1988; Howard et al., 1988; Cacioppo et al., 1991).

Around 0.6 M NaCl, lysozyme solubility is nearly insensitive to pH variation. It suggests that the screening of the net charge has reached a saturation. The polyion behaves as if it bore a net charge of zero. This observation may be related to the observation of chloride adsorption by lysozyme carried out with a fluorescent quencher (Sibille and Pusey, 1994); the number of bound Cl^- ions increased between 0 and 0.35 M NaCl, remained constant up to 0.6 M NaCl, and then decreased rapidly at 0.8 M NaCl for a protein concentration of 20 mg/ml. The decrease of bound Cl^- at 0.8 M NaCl coincided with the occurrence of crystallization.

Lysozyme solubility versus pH at different NaCl concentrations

The solubility data are plotted as a function of pH for different NaCl concentrations in Fig. 2. Lysozyme solubility

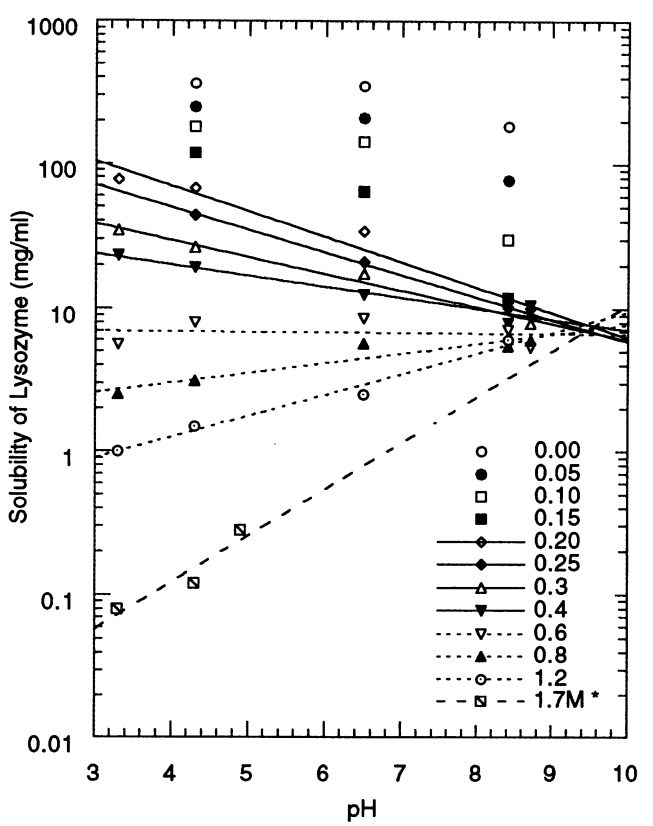

FIGURE 2 Solubility curves of lysozyme chloride versus pH for indicated NaCl concentrations (M) at 18°C. *Data for 1.7 M NaCl are taken from Cole et al. (1969).

effectively increases with the protein net charge at low ionic strength, but curiously, the reverse is observed at high ionic strength. It must be noted that the semi-logarithmic scale may be misleading; in fact, the difference of lysozyme solubility is 183 mg/ml in the absence of added NaCl and only 5 mg/ml at 1.2 M NaCl when comparing the data at pH 4.3 and 8.4. Such a slight increase of solubility when approaching the pI has earlier been reported by Cole et al. (1969) and Cacioppo and Pusey (1991).

The curves seem to intersect for a pH value around 9.5 and a solubility of approximately 10 mg/ml. The equivalence of this intersection with the isoelectric pH of lysozyme chloride will be discussed in the next section.

Supersaturation and crystallization kinetics

The supersaturation values were calculated a posteriori as the ratio of initial protein concentration over protein solubility (Table 1). The supersaturation required for nucleation appears to be very small (1.1 to 1.5) when lysozyme is crystallized at low ionic strength, i.e., at high protein concentrations (180–366 mg/ml). This may be interpretable in terms of the high volume fraction of protein in solution, which reaches 10–33%. For such values of volume fractions, the intermolecular distance becomes comparable or less than the diameter of the protein (Chernov and Komatsu, 1995). For comparison, the volume fraction of protein inside the crystals is approximately 70%.

As concerns the crystallization kinetics, periodic mixing of the solutions has been shown to reduce the equilibration time from 9 months to 2 months for solutions containing initially less than 100 mg/ml but was not effective at higher protein concentrations. For solutions containing initially 100–400 mg/ml, the equilibration time can be reduced from 6 months to 2 months by cooling them to 4°C for 1 month after nucleation has started at 18°C and before allowing the equilibration at 18°C.

DISCUSSION

Predominance of electrostatic protein-protein interactions for charged proteins at low ionic strength

As mentioned in the introduction, it is generally claimed that salting-in is expected at low ionic strength. However, most examples of salting-in reported in the literature concern protein solubility data near the pI of the protein, as in the case of carboxyhemoglobin and β-lactoglobulins. In this work on lysozyme, we study a protein that is a positively charged polyion and extend the knowledge of solubility behavior to charged proteins. The fact that at low ionic strength the solubility of charged protein molecules is decreased by increasing ionic strength may be due to a predominant effect on protein-protein interactions, rather than on the protein activity coefficient, and/or to a decrease of the protein's net charge due to anion adsorption to the positively charged protein (Riès-Kautt and Ducruix, 1997). It should be added that our observations corroborate those in the field of colloids, where the solubility of charged particles is known to decrease with ionic strength (Rosenbaum et al., 1996).

Equation 4 has been introduced to describe protein solubility via the chemical activity coefficient of the protein when its environment undergoes an increase of ionic strength. The question may alternatively be addressed by considering protein-protein interactions. A relevant theory has been applied to the field of colloids; DLVO (Derjaguin-Landau-Verwey-Overbeck) theory (Israelachvili, 1992) is used to describe interactions between nonpenetrable, spherical, and uniformly charged particles. The net interaction between two particles is expressed as resulting from a repulsive electrostatic contribution and an attractive van der Waals contribution. The repulsive electrostatic interactions occurring between two charged particles, e.g., protein molecules, are decreased by increasing ionic strength. Small-angle x-ray scattering measurements have effectively shown that the net interaction between lysozyme molecules (100 mg/ml at pH 4.5 in acetate buffer) is repulsive but becomes attractive when increasing the ionic strength to approximately 0.25 M NaCl (Ducruix et al., 1996). In a more general context, it was suggested that macromolecular association proceeds through the formation of a weakly specific complex, which is dominated by long-range electrostatic interactions, followed by precise docking to form the high-affinity complex (Schreiber and Fersht, 1996). A similar process may be involved for protein crystallization, where the first step can be predicted by DLVO theory (Veretout et al., 1988; Malfois et al., 1996), whereas DLVO theory does not take into account the docking step, which involves opposite charge-charge interactions, H bonding, etc.

Comparison of our data with those previously reported seems to indicate that salting-in would be observed when the protein net charge is around zero, but as soon as the protein bears a high net charge, the screening effect of ionic strength on protein-protein interactions becomes predominant. To confirm this hypothesis, it should be checked whether salting-in occurs when the solubility of lysozyme is studied near or above the pI. This could not yet be achieved for experimental reasons, because of the difficulty of preventing denaturation of the protein and of absorption of CO_2 by the solution when working at such very alkaline pH values.

Adsorption of chloride anions by the protein

Interestingly, we observed in Fig. 2 that the solubility curves for different ionic strengths intersect at a pH value of approximately 9.5, which corresponds to a net charge of approximately +4. Similarly, it is known that titration curves become independent of ionic strength at their isoelectric point (Tanford and Wagner, 1954). We mentioned

that the solubility of a protein is reached for a net charge of zero, unless it crystallizes as a salt, P^zX_z, the minimal solubility of which is observed at a net charge equal to z. Indeed, solubility versus pH should become independent of the ionic strength, except in the case of ion binding, which induces a shift of pI (Leavis and Rothstein, 1974). In other words, the isoionic pH of the protein is shifted to an isoelectric pH in the presence of ions that interact with the protein. Therefore, the intersection of our solubility curves at a net charge of approximately +4 suggests that we deal no longer with lysozyme but with lysozyme chloride (lysozyme^{n+}, n Cl^-, with $n \approx 4$). More accurate values have to be determined by additional measurements under an atmosphere of nitrogen to prevent absorption of CO_2 by alkaline solutions. However, the evidence of four chloride ions being bound to the protein molecule in the crystal provides independent corroboration. Recently, one chloride ion has been identified by Vaney et al. (1996) by x-ray crystallography in the electronic density map of lysozyme at 1.33 Å resolution and crystallized with NaCl at 18°C and at pH 4.5. Three other putative bound chloride ions have been identified by Pusey (personal communication) after soaking the crystals in solutions containing bromide ions. In addition, the measurement of the concentration of free chloride ions in crystallization solutions of lysozyme at pH 4 showed that the number of chloride ions taken up in the crystal were 10 ± 6 at 5°C and 4 ± 3 at 35°C (Elgersma et al., 1992). More recently, Vekilov et al. (1996) estimated that 3.1–4.7 chloride ions per protein molecule were incorporated in the crystal of purified lysozyme.

The occurrence of anion binding to lysozyme, and formation of a protein salt, would decrease its positive net charge and consequently its solubility (Riès-Kautt and Ducruix, 1997). To confirm this hypothesis, it should be checked whether salting-in occurs when cations are adsorbed on lysozyme, which would increase its net charge and its solubility (Riès-Kautt and Ducruix, 1997). It is worth indicating that a similar work has been performed with fibrinogen (Leavis and Rothstein, 1974), although the study was carried out in the presence of precipitate.

Predominance of attractive interactions at high ionic strength

Electrostatic interactions and formation of a protein salt apparently predominate at low ionic strength. At high ionic strength, the increase of solubility when approaching the pI cannot yet be explained, but it shows that attractive interactions become predominant. The shielding of the protein's net charge by ions at high ionic strength allows the protein molecules to come closer to each other until they become able to form specific intermolecular contacts to build the crystal. New attractive interactions (Israelachvili and Wennerström, 1996), which are not included in the DLVO theory, then favor the crystal contacts (Salemme et al., 1988) via polar, nonpolar, or charged interactions. In the case of pancreatic ribonuclease, it has been shown that the nonpolar and the charged interface is higher when the crystals are grown in the presence of salt than in the presence of alcohols or polyethylene glycol, whereas the polar interface is higher in the latter case (Crosio et al., 1992).

Concluding remarks

In addition, as we observed in previous results (Riès-Kautt and Ducruix, 1989) that lysozyme solubility at pH 4.5 was strongly dependent on the nature of anions, solubility measurements at various pH values and low ionic strength with anions different from chloride are now under study. Preliminary results show important differences of solubility depending on the nature of the anion, which will be analyzed in terms of ion binding. Consequently, the very complex physicochemical system of protein-water-ions in solution and precrystalline state becomes progressively better understood. Furthermore, complementary thermodynamic and structural approaches are in progress to develop more general models to explain protein solubility and, more generally, protein-protein association.

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