Interest of the normalized second virial coefficient and interaction potentials for crystallizing large macromolecules

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It has been shown for several years that the second virial coefficient, A_2, can be helpfully used to describe the thermodynamic behavior of biological macromolecules in solution prior to crystallization. The coefficient, which reflects either repulsive or attractive interactions between particles, can allow a rapid determination of crystallization conditions. Different biological systems, from 14 kDa to 4600 kDa, were studied by small angle X-ray scattering. With large macromolecules, the A_2 values were found at the low end of the crystallization slot described by George & Wilson [(1994) *Acta Cryst.* D50, 361-365]. This led us to investigate the physical meaning of the second virial coefficient and to propose the use of the dimensionless second virial coefficient independent of the molecular weight and the size of the particle, which only takes into account the interaction potential between macromolecules, to predict successful crystallization conditions for large macromolecules. With this normalized coefficient (a_2), the effect of salt on small proteins becomes equivalent to the effect of PEG on large macromolecules in terms of interaction potentials.

Keywords: interaction potentials, second virial coefficient, protein crystallization

1. Introduction

For the last two decades, the understanding of protein crystallization has progressed thanks to a better knowledge of the thermodynamic properties of protein solutions (Arakawa & Timasheff, 1985; Boistelle & Astier, 1988). Changes in the immediate environment of proteins in solution (temperature, pressure, concentration of salts, organic solvents, neutral polymers) can modify their solubility by altering the interactions protein-solvent and protein-protein and thus lead to crystallization (Riès-Kautt & Ducruix, 1997; Veesler & Boistelle, 1999). Characterizing protein interactions to predict phase diagrams became therefore of fundamental interest in crystallogenesis, the aim of these studies being a better understanding of the laws which govern crystallization for the physico-chemists and more rationalized trials for getting crystals for crystallographers.

Proteins in solution are under control of weak interactions forces (Israelachvili, 1992), which are mainly excluded volume (or hard sphere) forces, coulombic electrostatic repulsions and van der Waals attractions. These three interactions, which depend on pH, temperature and solvent composition, are taken into account in what is called the DLVO model (Derjaguin & Landau, 1941; Verwey & Overbeek, 1948). The addition of crystallizing agents like salts (George & Wilson, 1994; Muschol & Rosenberger, 1995; Boyer *et al.*, 1996; Ducruix *et al.*, 1996) or PEG (Budayova *et al.*, 1999; Finet & Tardieu, 2001) can also induce other additional attractive forces. The attractive interaction induced by the addition of polymer is the depletion attraction, which is function of the polymer mass and concentration (Lekkerkerker, 1997; Kulkarni *et al.*, 2000; Vliegenthart & Lekkerkerker, 2000). Proteins in solution can also interact through other non-specific forces like hydration or hydrophobic forces, which are still poorly characterized.

The resultant of macromolecular interactions between particles in solution is generally characterized by scattering techniques *via* the osmotic second virial coefficient (A_2 or B_{22}). It was shown that crystallization occurs within a narrow range of slightly negative second virial coefficients from about –1.0 to -8.0×10^{-4} mol.ml.g^{-2}, called the "crystallization slot" (George & Wilson, 1994). More recently, the correlation between solubility and second virial coefficient was shown (Bonneté *et al.*, 1999; Guo *et al.*, 1999; Haas *et al.*, 1999). Consequently, the second virial coefficient turned out to be the suitable parameter to predict the crystallization of biological macromolecules.

In order to determine the crystallization conditions of *Aspergillus flavus* urate oxidase, interactions in solution were investigated by small angle X-ray scattering (SAXS), in different physicochemical conditions of pH, temperature, addition of salts and of PEG (Bonneté *et al.*, 2001; Vivarès & Bonneté, 2002). In parallel, crystallization trials were performed in conditions corresponding to negative values of the second virial coefficient. Crystals of urate oxidase were obtained with PEGs of different sizes and at various concentrations, for A_2 values in a restricted range (about -0.4×10^{-4} to $+0.1 \times 10^{-4}$ mol.ml.g^{-2}) at the low end of the "crystallization slot".

In the present work, we analyze the ranges of second virial coefficient values obtained for different biological systems of different molecular weight and size, studied in various physicochemical conditions (pH, salt and PEG), which led to crystallization: lysozyme (Bonneté *et al.*, 1999), gammaD crystallin (data kindly provided by S. Finet (1999)), urate oxidase (Vivarès & Bonneté, 2002), brome mosaic virus (Casselyn *et al.*, 2001) (Table 1). From data obtained for small proteins like lysozyme (14300 Da) or gammaD crystallin (20000 Da) and for large particles like urate oxidase (128000 Da) and BMV (4.6×10^6 Da), it turns out that all values of A_2 obtained for large macromolecules in crystallization conditions were found in a more restricted range close to zero. This led us to propose the use of a dimensionless second virial coefficient normalized to molecular weight and excluded volume of particles to predict and control the nucleation and crystal growth of large globular proteins.

2. Experiments and methods

All these macromolecules were prepared and studied at the Laboratoire de Minéralogie Cristallographie (LMCP). All experimental conditions were already described elsewhere (Bonneté *et al.*, 1999; Finet, 1999; Casselyn *et al.*, 2001; Vivarès & Bonneté, 2002). Interactions between particles were characterized by small angle X-ray scattering using the instrument D24 on the ring DCI at L.U.R.E (Orsay-France). The advantage of SAXS compared to the more common light scattering was already emphasized in our previous study. Small angle X-ray scattering allows us to get, in only one experiment, the extrapolated zero-angle scattered intensity necessary for the determination of A_2 (the extrapolation being valid in the attractive regime (Bonneté *et al.*, 1997)) and the form factor of the protein solution giving informations about the presence or not of oligomers (Hamiaux *et al.*, 2000) or of aggregates. On the other hand, the coupling of SAXS experiments (experimental structure factor) with numerical simulations (calculated structure factor) (Tardieu *et al.*, 1999) allows us to determine the different underlying interaction potentials acting between proteins in solution in the

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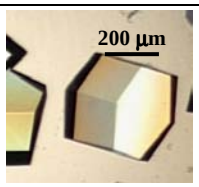
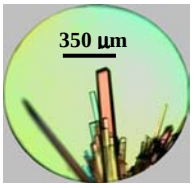
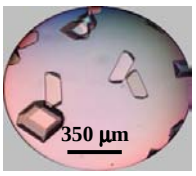
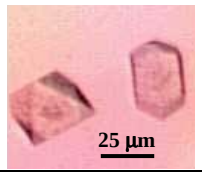
Table 1

Macromolecule parameters (M, molecular weight; σ, particle diameter; v_e, partial excluded volume), second virial coefficient A_2, normalized second virial coefficient a_2 and crystallization conditions for our four model systems (error bars on A_2 values were chosen such as MA_2 = +/-1 $cm^3.g^{-1}$).

Macromolecule : $M/\sigma/v_e$	Crystallization condition	Corresponding A_2 (10^{-4} mol.ml.g^{-2})	Corresponding a_2	Picture of crystals
Lysozyme (Bonneté *et al.*, 1999; Tardieu *et al.*, 1999) 14300 Da 32.4 Å 0.74 $cm^3.g^{-1}$	500 mM NaCl pH 4.5	**- 3.3 (+/- 0.7)**	- 6.4 (+/- 1.4)	
Gamma-D crystallin (Finet, 1999) 20000 Da 36 Å 0.74 $cm^3.g^{-1}$	500 mM NaCl pH 4.5	- 2.0 (+/- 0.5)	- 5.4 (+/- 1.4)	
Urate oxidase (Vivarès & Bonneté, 2002) 128000 Da 70 Å 0.84 $cm^3.g^{-1}$	4% PEG 20000 pH 8.5	- 0.26 (+/- 0.08)	- 4.0 (+/- 1.2)	
BMV (Casselyn *et al.*, 2001) 4.6 x 10^6 Da 268 Å 1.32 $cm^3.g^{-1}$	6% PEG 8000 pH 5.0	+ 0.008 (+/- 0.002)	+ 2.8 (+/- 0.8)	

conditions studied. The intensity scattered by a solution of quasi-spherical particles is therefore the product of the form factor, I(0,s), which depends on the particle geometry, and the structure factor, S(c,s), which depend on the pair distribution function by:

$$S(c,s) = 1 + \rho \int 4\pi r^2 (g(r) - 1) \frac{\sin 2\pi rs}{2\pi rs} dr \qquad (1)$$

where $\rho = cN_a/M$ is the number of particles per unit of volume and c is the particle concentration (g.cm^{-3}).

The structure factor at the s-origin, S(c,0), is related to the osmotic pressure Π of the particle solution (Guinier & Fournet, 1955) by:

$$S(c,0) = k_B T \left(\frac{\partial \Pi}{\partial \rho}\right)^{-1} \text{ or } S(c,0) = \frac{RT}{M}\left(\frac{\partial \Pi}{\partial c}\right)^{-1} \qquad (2)$$

where k_B is Boltzmann's constant, T the absolute temperature and Π the osmotic pressure described by (Eisenberg, 1976):

$$\frac{\Pi}{\rho k_B T} = 1 + B_2 \rho + (\text{ terms of order } \rho^2) \text{ or}$$

$$\frac{\Pi}{cRT} = \frac{1}{M} + A_2 c + (\text{ termsof order } c^2) \qquad (3)$$

A_2 or B_2 depends on the interaction pair potential U(r) between particles in solution by the expression:

$$A_2 = \frac{B_2 N_a}{M^2} = \frac{2\pi N_a}{M^2} \int_0^\infty \left(1 - \exp\left(-\frac{U(r)}{k_B T}\right)\right) . r^2 dr \qquad (4)$$

with U(r) = + ∞ for r < σ

where r is the interparticle distance and σ the particle diameter.

A_2 or B_2 is positive for repulsive interactions and negative for attractive ones.

Since it is easier to measure particle concentrations than particle numbers, the experimental value of A_2 is generally obtained from the slope of the linear fit:

$$\frac{1}{S(c,0)} = 1 + 2.M.A_2.c \qquad (5)$$

3. Results and discussion

Values of second virial coefficient A_2 have been measured by SAXS experiments, in pre-crystallizing conditions, for the four macromolecular model systems: lysozyme, gammaD crystallin, urate oxidase and Brome Mosaic Virus (BMV). Crystallization trials were performed in parallel. Interactions in solution and crystallization trials of lysozyme (Bonneté *et al.*, 1999) and gammaD crystallin (Finet, 1999) were investigated with addition of salt. Urate oxidase (Vivarès & Bonneté, 2002) and BMV (Casselyn *et al.*, 2001), which do not present attractive interactions and do not crystallize with only (monovalent) salt, were studied with addition of PEG. A_2 values (expressed in mol.ml.g^{-2}) are plotted for each system as a function of salt or PEG concentrations in Fig. 1a. The zone where crystals were obtained for the different systems studied, is in grey squaring and some crystals are shown in Table 1.

The crystallization zone, - 6 < A_2 (10^{-4} mol.ml.g^{-2}) < 0 for the four model systems, corresponds approximately to the well-known "crystallization slot". However, one can notice that for large macromolecules - urate oxidase and BMV - the A_2 crystallization zone falls at the low-end of this slot. This was already observed with some other large macromolecules, halophilic malate dehydrogenase (Ebel *et al.*, 1999), OmpF porin (Hitscherich *et al.*, 2000) horse spleen apoferritin (Petsev *et al.*, 2000; Tanaka, 2002). It seems that such a restricted range of A_2 values for large macromolecules, which

gives rise to crystals, is in agreement with recent theoretical predictions (Haas & Drenth, 1998).

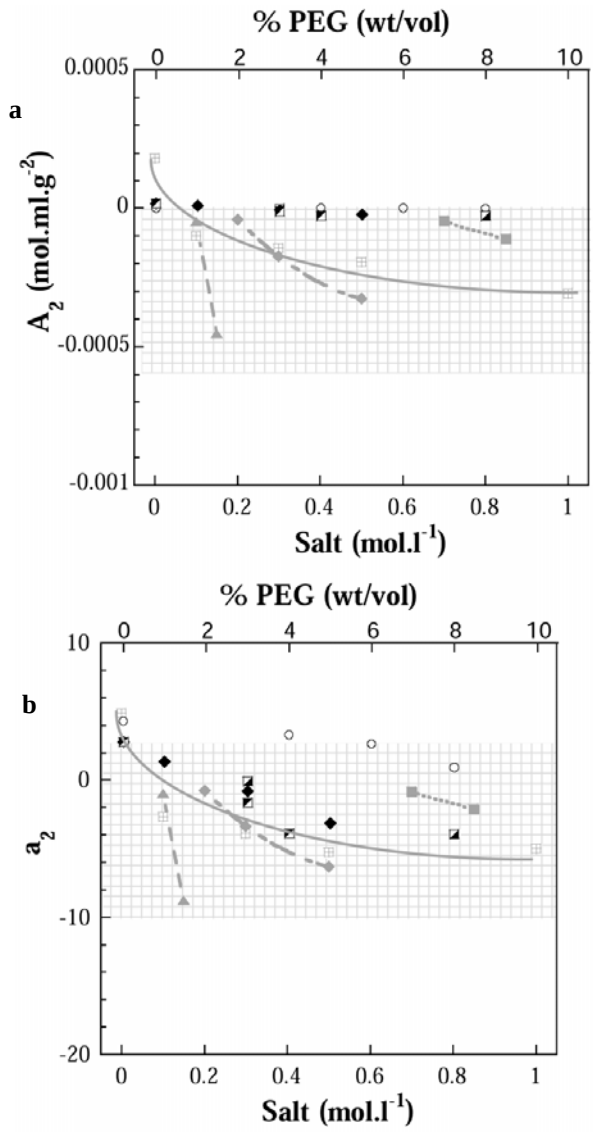


Figure 1
Second virial coefficients obtained from SAXS experiments for lysozyme (Bonneté *et al*., 1999), gammaD crystallin (Finet, 1999), urate oxidase (Vivarès & Bonneté, 2002) and BMV (Casselyn *et al*., 2001) and plotted in double x-axis. The different conditions are for each system: for lysozyme, at pH 4.5 $NaNO_3$ (▲), NaCl (●), NaOAc (■); for gammaD crystallin at pH 4.5 NaCl (⊞); for urate oxidase at pH 8.5 PEG 3350 Da (◪), PEG 8000 Da (◆), PEG 20000 Da (◩); and for BMV at pH 5.0 PEG 8000 Da (○).a) Second virial coefficient A_2 in mol.ml.g^{-2}; b) Dimensionless second virial coefficient a_2. (There is no correspondance between % of PEG and molarity of salt).

Experimentally, according to Eq. 5, it is more convenient to measure A_2 in mol.ml.g^{-2} but in that case, A_2 depends upon the radius and the mass of the particle (Eq. 4). In order to understand the origin of the different values of A_2 obtained with small and large macromolecules – difference predicted by Haas & Drenth (Haas & Drenth, 1998) - we considered the dimensionless second virial coefficient, which depends only upon the nature and the strength of the pair interaction potential between particles, already used by other authors (Rosenbaum *et al*., 1996; Petsev *et al*., 2000; Petsev & Vekilov, 2000; Piazza & Pierno, 2000; Poon *et al*., 2000; Vliegenthart & Lekkerkerker, 2000). Generally, this dimensionless second virial coefficient is found equal to the parameter B_2 normalized either to the second virial coefficient due to the only hard sphere pair potential $B_2^{HS} = \frac{2\pi\sigma^3}{3}$ (Rosenbaum *et al*., 1996; Piazza & Pierno, 2000; Poon *et al*., 2000) or to the spherical particle excluded volume $V_e = \frac{\pi\sigma^3}{6}$, where σ is the particle diameter (Petsev *et al*., 2000; Vliegenthart & Lekkerkerker, 2000).

Using the latter expression, this dimensionless second virial coefficient is defined by:

$$a_2 = \frac{12}{\sigma^3}\int_0^\infty \left(1-\exp\left(-\frac{U(r)}{k_B T}\right)\right).r^2 dr \qquad (6)$$

The excluded volume of the macromolecule (equal to $\frac{M.v_e}{N_a}$ with v_e the partial excluded volume in cm^3.g^{-1} and not constant for all proteins), which is higher than the dry volume (Véretout *et al*., 1989), allows to take into account the degree of compactness of the protein, due to the water content in the particle. It can be calculated from the ratio of v_e and $\bar{v}$, the partial specific volume generally found equal to 0.74 cm^3.g^{-1} for all proteins. For compact proteins, this ratio is equal to 1. The difference between the excluded volume of a macromolecule $V_e = \frac{M.v_e}{N_a}$ and the dry volume $\bar{V} = \frac{M.\bar{v}}{N_a}$ is schematically illustrated in Fig. 2. The excluded volume is determined from the numerical simulations (Véretout *et al*., 1989; Malfois *et al*., 1996).

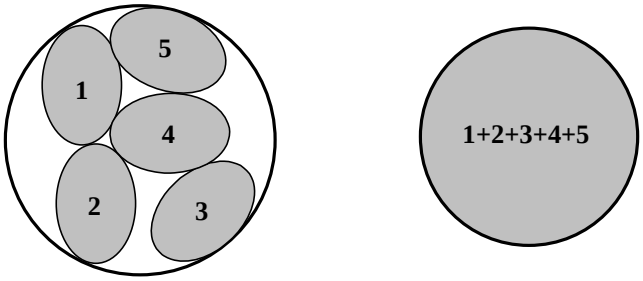


Figure 2
Illustration of the difference between the excluded volume V_e □ and the dry volume $\bar{V}$ ▭ supposing a quasi-spherical particle.

With this expression of a_2, for adhesive hard sphere, the parameter is equal to $a_2^{HS} = 4$ and as soon as $a_2 < 4$ or negative, it indicates an attraction between particles which can lead to crystallization.

Using accurate values of partial excluded volume (Table 1), a_2 was calculated from the corresponding A_2, $a_2 = \frac{M.A_2}{v_e}$, and plotted as a function of salt for small proteins and of PEG percentages for large particles (Fig. 1b). Whereas the crystallization ranges for small and large particles, expressed in mol.ml.g^{-2}, appeared in different decades, the a_2 crystallization zone becomes now similar for particles of different sizes and approximately corresponds to $-10 < a_2 < 3$, in only one decade. In the high limit of this range, phase separation – liquid-liquid or precipitation – was observed for urate

oxidase for $a_2 < -6$ ($A_2 < -0.4 \times 10^{-4}$ mol.ml.g^{-2}) at concentration of protein of 20-25 mg.ml^{-1}.

One can then wonder, what the characteristics (depth and range) of the attractive pair potential are for two macromolecules with different sizes but with the same a_2.

By coupling SAXS experiments and numerical simulations, we have calculated, as an example, the resulting pair potential in crystallizing conditions for a constant a_2 value of about -3.5 for lysozyme (Tardieu *et al.*, 1999) and urate oxidase (Vivarès *et al*, submitted). The experimental conditions determined for this a_2 value from Fig. 1b, corresponds to 280 mM NaCl pH 4.5 for lysozyme and 5% PEG 8000 Da pH 8.5 for urate oxidase. The resulting pair potentials were plotted as a function of $(r - \sigma)$ (Fig. 3a) and of $(r - \sigma)/\sigma$ (Fig. 3b). Whatever the representation, the attractive potential depth is almost constant for both proteins, which is consistent with the fact that the interaction contact energy between two particles shows little variations from one macromolecule to another (Haas & Drenth, 1998) in crystallization conditions. In Fig. 3a, the potential range is shown to be longer for urate oxidase than for lysozyme when represented as a function of $(r - \sigma)$. On the other hand, by normalizing the potential range of particle interactions to the particle diameter, i.e. by plotting the potential as a function of $(r- \sigma)/\sigma$ (Fig. 3b), the ranges for the two proteins become approximately identical. In other words, the range of the attraction leading to crystallization depends on the particle diameter.

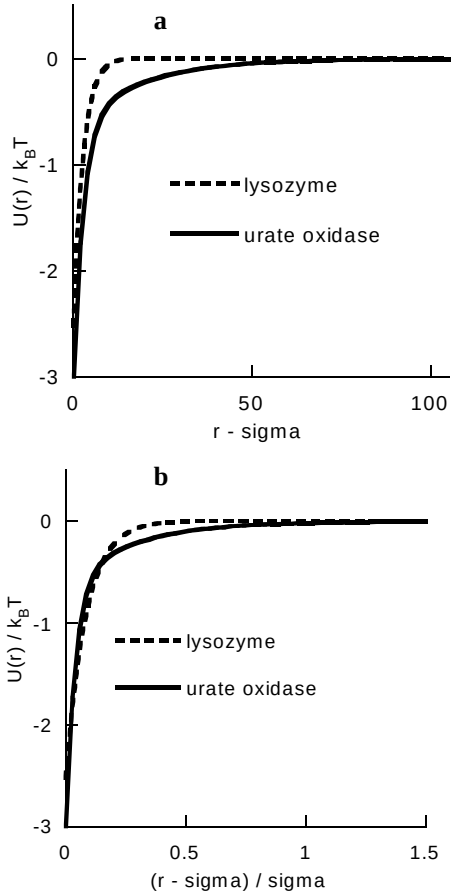


Figure 3

Resulting pair potentials in crystallizing conditions for a constant a_2 value ($a_2 \approx -3.5$) for lysozyme (with salt) and urate oxidase (with PEG): a) as a function of $r - \sigma$; b) as a function of $(r - \sigma)/\sigma$.

The examples of lysozyme and urate oxidase show that salts on small proteins and PEG on large proteins play quite equivalent roles. Even if salts induce a small-range attractive potential, which seems not sufficient for large particles, and PEG a medium-range one, they both induce an attractive potential whose range is the same diameter fraction of the proteins considered. The importance of the interaction potential range, function of the particle diameter, on the phase diagram of colloidal particles has been previously shown in the literature (Hagen & Frenkel, 1994; Ilett *et al.*, 1995; Asherie *et al.*, 1996; Malfois *et al.*, 1996; ten Wolde & Frenkel, 1997; Rosenbaum *et al.*, 1999).

4. Conclusion

For some years, interactions of macromolecules in solution have been studied by small angle X-ray scattering in order to better understand and control crystallization and nucleation. Different biological objects presenting different characteristics (size, oligomeric state, pI) were intensively studied as a function of various physicochemical conditions (pH, temperature, ionic strength, nature of salt, addition of polymer of different sizes). For each system studied (lysozyme, gammaD crystallin, urate oxidase, brome mosaic virus) interactions were characterized, via the second virial coefficient, which helped us to determine crystallization conditions.

In this paper we have compiled A_2 data for the four macromolecular systems studied in the laboratory and compared to the well-known "crystallization" slot of George & Wilson (1994). For small macromolecules, the second virial coefficient was found to correspond to the slot. As soon as the macromolecule size increases, and as it was also observed with other large biological systems, the range of A_2 shifts in a restricted part at the low end of the "slot". One could have thought that, with such low negative or even positive values at usual concentrations, large biological macromolecules would not crystallize. But by using a dimensionless second virial coefficient a_2, which only takes into account the interaction potential between the biological macromolecules and not the particle size and molecular weight, one enlarges the slot for large particles while keeping that of small particles almost unchanged. Thus since the solubility and the second virial coefficient are well correlated, one can control the crystallization and the nucleation rate of macromolecules whatever their size. In terms of interaction potential, the efficiency of salt with small proteins was found comparable to that of polyethylene glycol with large ones to induce to crystallization.

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