RESEARCH NEWS

A new model for promoting protein crystallization in solution

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Efficient crystallization of folded proteins from solutions is essential for three dimensional structure determination of the proteins. It is, however, not easy to grow large single crystals of proteins. In the absence of any microscopic understanding of protein crystallization, the growing of protein crystals has remained more of an art than science[1].

In an article entitled 'Crystallization of Macromolecules: General Principles', Alexander McPherson wrote: 'In principle, the crystallization of a protein, nucleic acid, or virus is little different than the crystallization of conventional small molecules. It requires the gradual creation of a supersaturated solution of the macromolecule and follows the spontaneous growth centres or nuclei'[2]. Several recent studies, however, have questioned this age-old wisdom that crystallization of proteins is essentially the same as of small molecules and instead suggested that the kinetics of crystallization of proteins and colloids can indeed be *very different* from the crystallization of small molecules[3,4].

What are the factors that inhibit growth of single crystals? First, of course, is the fact that proteins tend to aggregate and precipitate if the conditions are not correct. This has formed a vicious cycle because we need high concentration of proteins so that the critical nucleus required to start crystal growth can form. Second, there is always the possibility of the formation of poly-crystals which can happen if multiple nucleation sites are present in the solution.

The way to facilitate the growth of

crystals is to find the conditions ideal for the formation of a 'stable' crystal nucleus. In ordinary crystallization where the size of the molecules is small, the nucleation can be understood in terms of the competition between the surface tension of the liquid–crystal interface and the relative stability of the crystalline phase over the liquid phase. The basic science of this problem is well-understood. One finds the following relation for the free energy of activation of the nucleus and for the size of the critical nucleus

$$\Delta G^* = 16\pi\gamma^3/3(\Delta G_v)^2, \quad (1)$$

$$r_c = 2\gamma/\Delta G_v. \quad (2)$$

Here ΔG_v is the free energy difference per unit volume between the liquid and the solid, γ is the surface tension. This is the classical picture of nucleation. This picture seems to be valid when the range of the attractive interaction is comparable to that of the molecular size, that is, molecular diameter, as in the Lennard-Jones potential between two Argon atoms. In Figure 1 *a* we show both the potential and the phase diagram of such a simple system, showing the gas, liquid, solid boundaries. The above mechanism appears to provide a satisfactory description of nucleation in atomic and molecular systems.

What is the difference between crystallization in molecular systems and in proteins and colloids? This is the question recently addressed to by several workers[1,2]. According to ten Wolde and Frenkel[3], the main difference is the *range of the attractive interaction.* In molecular systems, this range is comparable to the size of the molecule itself. But in proteins and many colloids, this range is much smaller than the size of the molecule. A schematic description of such a potential is shown in Figure 1 *b*. Now, this much smaller range of potential can have a very interesting consequence. It is known that if the attractive potential is altogether absent, then the system cannot exist in the liquid phase and one considers only the gas–solid transition. When the range of the attractive interaction gradually decreases, one finds that the gas–liquid critical point gets depressed and eventually goes below the gas–solid coexistence. For some ranges of attractive potential, this critical point can be considered a *metastable critical point.* This phase diagram is shown in Figure 1 *b*. Now, what is really interesting is that the formation of a crystal nucleus can be greatly affected by the presence of this metastable critical point. The study of ten Wolde and Frenkel was motivated by the earlier studies of George and Wilson[5] and of Rosenbaum *et al.*[6] on the osmotic second virial coeffecient of protein solutions. These authors have observed that the conditions under which a large number of globular proteins can be made to crystallized map into a narrow range of the osmotic second virial coefficient value. In addition, earlier studies on the phase diagram of uncharged, suspended colloids[7,8] also suggested the scenario as noted by ten Wolde and Frenkel.

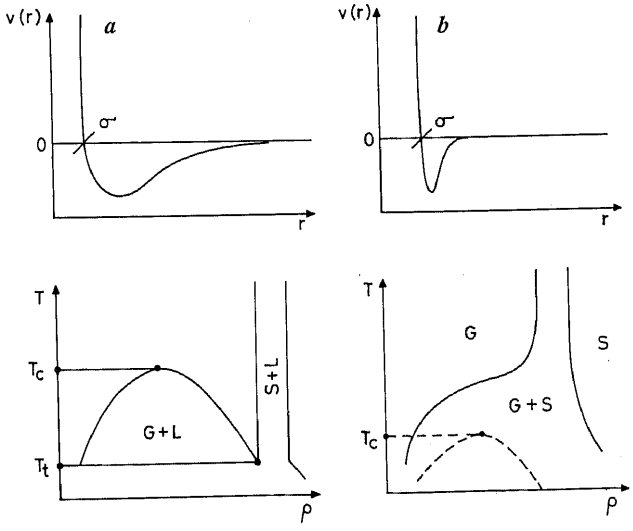

Figure 1. Relation between intermolecular potential $v(r)$ and the phase diagram. ***a*** shows the intermolecular potential and the phase diagram of a simple atomic or molecular system, such as argon or methane. ***b*** shows the same for a large colloidal system. In ***b*** the metastable critical point has been shown by a dashed line. T and ρ denote the temperature and the density of the system, respectively. G, L and S denote the gaseous, the liquid and the solid phases, respectively. See the text for discussion.

Classical nucleation occurs from high density and at low temperature when the crystalline phase is thermodynamically much more stable than the liquid phase. Solid differs from liquid on two counts. First is the order, second is the density. Thus, one can describe crystallization in terms of two-order parameters, ρ for density and m for the crystalline order. Theoretical studies indicate in the case of ordinary crystallization, micro-crystalline embryo is characterized more by the crystalline order than by the enhanced density – the latter remains essentially the same as in the liquid. Only after the growth has occurred to an appreciable extent does the density build up occur.

It was noted by ten Wolde and Frenkel that the situation changes drastically for proteins and colloids when the nucleation occurs at conditions near the metastable critical point. Because of the presence of large density fluctuations which can occur here without involving large activation energy, nucleation rate was found to be enhanced by several orders of magnitude. This was offered as the reason for anomalous enhancement in the protein crystallization rate observed in some cases.

Talanquer and Oxtoby analysed the reason for this result by using a theoretical formalism of statistical mechanics called the density functional theory. Conclusions from this study are essentially the same as those from simulations and can be summarized as follows. In the presence of a metastable critical point, the nucleation scenario can be totally different from what is observed for small molecules. The free energy of activation undergoes a sharp decrease near the metastable critical point. Here, the formation of the critical nucleus may be an aggregation process. The periodic order may appear later and play a much smaller role in the whole nucleation process. As already mentioned, for small molecules, exactly the opposite is expected.

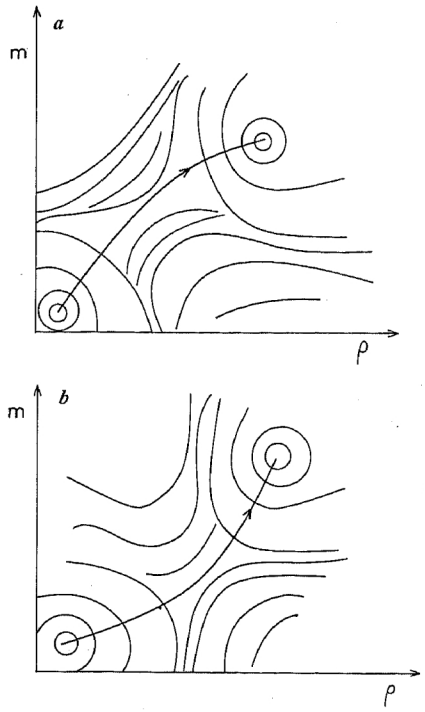

Figure 2. Free energy contour diagrams showing the fluid and the solid minima for the fluid (the circle at the left corner) and the crystalline (circle at the right corner) phases for simple molecules (*a*) and for colloids (*b*). Here ρ denotes the density and m the order of the system. The arrow shows the preferred pathway. For simple systems, the pathway is such that incipient order forms before the density build up occurs while for colloids (and presumably also for proteins), the density build up occurs before the order formation.

Thus, these new studies could at least partly explain the great sensitivity of protein crystallization to the experimental conditions. In the narrow temperature-density range near the critical point, large scale density fluctuations make the association of the proteins relatively easy.

This model of protein crystallization has certain similarities with Dill's model of protein folding where also a critical point was assumed to help the collapse of the coiled protein to the globular state – the proper connections required for the native state takes place later[9]. The same kind of model also appears in the analysis of Bryngelson and Wolynes[10]. All these models are separate applications of a general two-order parameter model. The underlying free energy surface determines the reaction pathway. The possible free energy surfaces are shown in Figure 2. Both in the Dill's model of protein folding and in the ten Wolde–Frenkel model of protein crystallization, the minimum energy pathway lies along the collapse or association direction, leading to first an increase of density which is then followed by the build up of order. This is shown by an arrow in Figure 2 *a*. For simple liquids, the situation seems to be reverse, as shown by the arrow in Figure 2 *b*.

The computer simulation and the density functional theory studies raise as many questions as they answer. Because of the favourable interactions among the hydrophobic patches between different proteins, one expects the association to be highly directional, that is like a polymerization or gelation process. Protein association is also expected to be highly dependent on electrical interactions between proteins. While it certainly helps to think in terms of the free energy surface, it is not clear how important these specific effects in protein crystallization are. In particular, while electrostatic interactions are long-ranged, the hydrophobic interactions are again rather short-ranged.

The analyses of ten Wolde and Frenkel and of Talanquer and Oxtoby are mainly for colloid systems. To what extent they can be extended to understand protein crystallization is not clear yet. The general picture suggested may still be true but for different reasons. As protein association may very well be guided by the hydrophobic patches and as the association itself may very well be the rate-determining stage, it is not surprising that the crystallization of proteins can be different from that of small molecules. In fact, earlier studies[11] had already discussed protein crystallization (such as nucleation and growth of orthorhombic form of hen egg-white lysozyme) as a self-assembly. The work of ten Wolde and Frenkel, however, seems to provide a thermodynamic explanation of the anomalous enhancement of protein crystallization observed in some systems. It may be useful to develop a kinetic version of this model and compare the results with the self-assembly models.

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ACKNOWLEDGEMENTS. This work was supported in part by grants from CSIR and DST, India.

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