4. CRYSTALLIZATION

4.1. General methods

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4.1.1. Introduction

Crystallization of biological macromolecules has often been considered unpredictable, but presently we know that it follows the same principles as the crystallization of small molecules (Giegé *et al.*, 1995; McPherson *et al.*, 1995; Rosenberger, 1996; Chernov, 1997*a*). It is, similarly, a multiparametric process. The differences from conventional crystal growth arise from the biochemical properties of proteins or nucleic acids compared to quantitative aspects of the growth process and to the unique features of macromolecular crystals. Crystallization methods must reconcile these considerations. The methods described below apply for most proteins, large RNAs, multimacromolecular complexes and viruses. For small DNA or RNA oligonucleotides, crystallization by dialysis is not appropriate, and for hydrophobic membrane proteins special techniques are required.

Macromolecular crystals are, indeed, unique. They are composed of ~50% solvent on average, though this may vary from 25 to 90%, depending on the particular macromolecule (Matthews, 1985). The protein or nucleic acid occupies the remaining volume so that the entire crystal is, in many ways, an ordered gel with extensive interstitial spaces through which solvent and other small molecules may freely diffuse. In proportion to molecular mass, the number of bonds that a conventional molecule forms with its neighbours in a crystal far exceeds the few exhibited by crystalline macromolecules. Since these contacts provide lattice interactions responsible for the integrity of the crystals, this largely explains the difference in properties between crystals of small molecules and macromolecules. Because macromolecules are labile and readily lose their native structures, the only conditions that can support crystal growth are those that cause little or no perturbation of their molecular properties. Thus, crystals must be grown from solutions to which they are tolerant, within a narrow range of pH, temperature and ionic strength. Because complete hydration is essential for the maintenance of the structure, crystals of macromolecules are always, even during data collection, bathed in the mother liquor (except in cryocrystallography).

Although morphologically indistinguishable, there are important differences between crystals of low-molecular-mass compounds and crystals of macromolecules. Crystals of small molecules exhibit firm lattice forces, are highly ordered, are generally physically hard and brittle, are easy to manipulate, can usually be exposed to air, have strong optical properties and diffract X-rays intensely. Crystals of macromolecules are, by comparison, generally smaller in size, are soft and crush easily, disintegrate if allowed to dehydrate, exhibit weak optical properties and diffract X-rays poorly. They are temperature-sensitive and undergo extensive damage after prolonged exposure to radiation. The liquid channels and solvent cavities that characterize these crystals are primarily responsible for their often poor diffraction behaviour. Because of the relatively large spaces between adjacent molecules and the consequently weak lattice forces, every molecule in the crystal may not occupy exactly equivalent orientations and positions. Furthermore, because of their structural complexity and their potential for conformational dynamics, macromolecules in a particular crystal may exhibit slight variations in their folding patterns or in the dispositions of side groups.

Although the dominant role of the solvent is a major contributor to the poor quality of many protein crystals, it is also responsible for their value to biochemists. Because of the high solvent content, the individual macromolecules in crystals are surrounded by hydration layers that maintain their structure virtually unchanged from that found in bulk solvent. As a consequence, ligand binding, enzymatic and spectroscopic characteristics, and other biochemical features are essentially the same as for the native molecule in solution. In addition, the sizes of the solvent channels are such that conventional chemical compounds, such as ions, substrates or other ligands, may be freely diffused into and out of the crystals. Thus, many crystalline enzymes, though immobilized, are completely accessible for experimentation through alteration of the surrounding mother liquor (Rossi, 1992).

Unlike most conventional crystals (McPherson, 1982), protein crystals are, in general, not initiated from seeds, but are nucleated *ab initio* at high levels of supersaturation, usually reaching 200 to 1000%. It is this high degree of supersaturation that, to a large part, distinguishes protein-crystal formation from that of conventional crystals. That is, once a stable nucleus has formed, it subsequently grows under very unfavourable conditions of excessive supersaturation. Distant from the metastable zone, where ordered growth could occur, crystals rapidly accumulate nutrient molecules, as well as impurities; they also concomitantly accumulate statistical disorder and a high frequency of defects that exceeds those observed for most conventional crystals.

4.1.2. Crystallization arrangements and methodologies

4.1.2.1. *General considerations*

Many methods can be used to crystallize macromolecules (McPherson, 1982, 1998; Ducruix & Giegé, 1999), the objectives of which bring the macromolecules to an appropriate state of supersaturation. Although vapour-phase equilibrium and dialysis techniques are favoured, batch and free interface diffusion methods are often used (Fig. 4.1.2.1). Besides the current physical and chemical parameters that affect crystallization (Table 4.1.2.1), macromolecular crystal growth is affected by the crystallization method itself and the geometry of the arrangements used. Generally, in current methods, growth is promoted by the non-equilibrium nature of the crystallization process, which seldom occurs at constant protein concentration. This introduces changes in supersaturation and hence may lead to changes in growth mechanism. Crystallization at constant protein concentration can, however, be achieved in special arrangements based on liquid circulation cells (Vekilov & Rosenberger, 1998).

4.1.2.2. *Batch crystallizations*

Batch methods are the simplest techniques used to produce crystals of macromolecules. They require no more than just mixing the macromolecular solution with crystallizing agents (usually called precipitants) until supersaturation is reached (Fig. 4.1.2.1*a*). Batch crystallization has been used to grow crystals from samples of a millilitre and more (McPherson, 1982), to microdroplets of a few µl (Bott *et al.*, 1982), to even smaller samples in the µl range in

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