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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

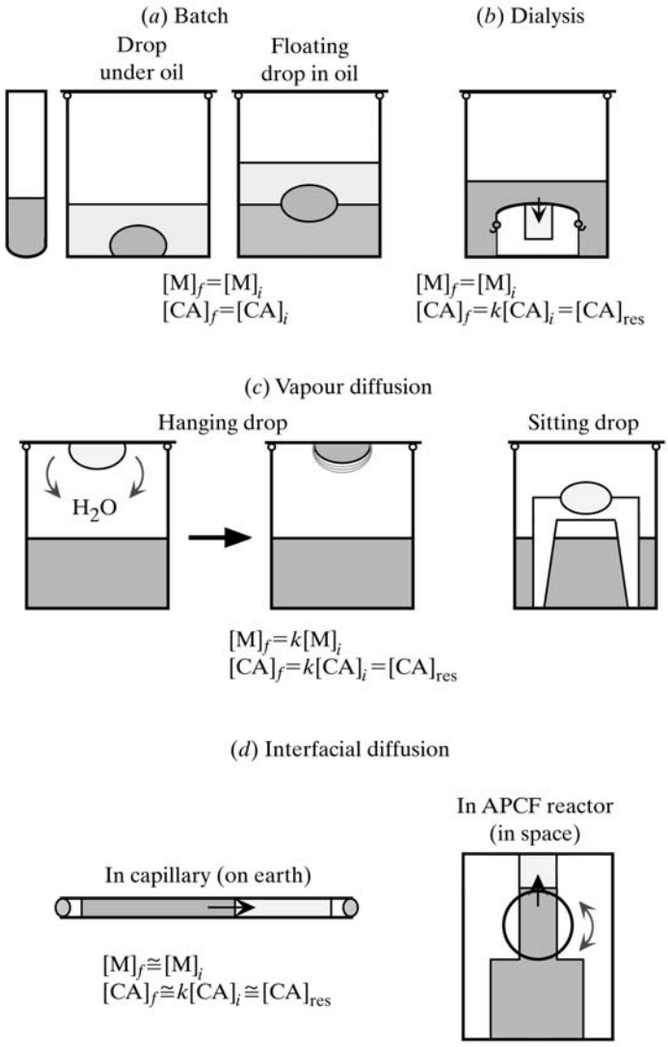

Fig. 4.1.2.1. Principles of the major methods currently used to crystallize biological macromolecules. (*a*) Batch crystallization in three versions. (*b*) Dialysis method with Cambridge button. (*c*) Vapour diffusion crystallization with hanging and sitting drops. (*d*) Interface crystallization in a capillary and in an arrangement for assays in microgravity. The evolution of the concentration of macromolecule, [M], and crystallizing agent (precipitant), [CA], in the different methods is indicated (initial and final concentrations in the crystallization solutions are $[M]_i$, $[M]_f$, $[CA]_i$ and $[CA]_f$, respectively; $[CA]_{res}$ is the concentration of the crystallizing agent in the reservoir solution, and k is a dilution factor specified by the ratio of the initial concentrations of crystallizing agent in the drop and the reservoir). In practice, glass vessels in contact with macromolecules should be silicone treated to obtain hydrophobic surfaces.

capillaries (Luft *et al.*, 1999*a*). Because one begins at high supersaturation, nucleation is often excessive. Large crystals, however, can be obtained when the degree of supersaturation is near the metastable region of the crystal–solution phase diagram.

An automated system for microbatch crystallization and screening permits one to investigate samples of less than 2 µl (Chayen *et al.*, 1990). Reproducibility is guaranteed because samples are dispensed and incubated under oil, thus preventing evaporation and uncontrolled concentration changes of the components in the microdroplets. The method was subsequently adapted for crystallizing proteins in drops suspended between two oil layers (Chayen, 1996; Lorber & Giegé, 1996). Large drops (up to 100 µl) can be deployed, and direct observation of the crystallization process is possible (Lorber & Giegé, 1996). The absence of contacts between the mother liquor and any solid surfaces yields a reduced number of nucleation sites and larger crystals. Batch crystallization can also be conducted under high pressure (Lorber *et al.*, 1996) and has also been adapted for crystallizations on thermal gradients with samples of ~7 µl accommodated in micropipettes (Luft *et al.*, 1999*b*). This latter method allows rapid screening to delineate optimal temperatures for crystallization and also frequently yields crystals of sufficient quality for diffraction analysis.

Batch methods are well suited for crystallizations based on thermonucleation. This can be done readily by transferring crystallization vessels from one thermostated cabinet to another maintained at a higher or lower temperature, depending on whether the protein has normal or retrograde solubility. In more elaborate methods, the temperature of individual crystallization cells is regulated by Peltier devices (Lorber & Giegé, 1992). Local temperature changes can also be created by thermonucleators (DeMattei & Feigelson, 1992) or in temperature-gradient cells (DeMattei & Feigelson, 1993). A variation of classical batch crystallization is the sequential extraction procedure (Jakoby, 1971), based on the property that the solubility of many proteins in highly concentrated salt solutions exhibits significant (but shallow) temperature dependence.

4.1.2.3. *Dialysis methods*

Dialysis also permits ready variation of many parameters that influence the crystallization of macromolecules. Different types of systems can be used, but all follow the same general principle. The macromolecule is separated from a large volume of solvent by a semipermeable membrane that allows the passage of small molecules but prevents that of the macromolecules (Fig. 4.1.2.1*b*). Equilibration kinetics depend on the membrane molecular-weight exclusion size, the ratio of the concentrations of precipitant inside and outside the macromolecule chamber, the temperature and the geometry of the dialysis cell. The simplest technique uses a dialysis bag (*e.g.* of inner diameter ~2 mm), but this usually requires at least 100 µl of macromolecule solution per trial.

Crystallization by dialysis has been adapted to small volumes (10 µl or less per assay) in microdialysis cells made from capillary tubes closed by dialysis membranes or polyacrylamide gel plugs (Zeppenzauer, 1971). Microdialysis devices exist in a variety of forms, some derived from the original Zeppenzauer system (Weber & Goodkin, 1970); another is known as the Cambridge button. With this device, protein solutions are deposited in 10–50 µl depressions in Plexiglas microdialysis buttons, which are then sealed by dialysis membranes fixed by rubber O-rings and subsequently immersed in an exterior solution contained in the wells of Linbro plates (or other vessels). The wells are sealed with glass cover slips and vacuum grease. Another dialysis system using microcapillaries was useful, for example, in the crystallization of an enterotoxin from *Escherichia coli* (Pronk *et al.*, 1985). In the double dialysis procedure, the equilibration rate is stringently reduced, thereby improving the method as a means of optimizing crystallization conditions (Thomas *et al.*, 1989). Equilibration rates can be manipulated by choosing appropriate membrane molecular-weight exclusion limits, distances between dialysis membranes, or relative volumes.

4.1.2.4. *Vapour diffusion methods*

Crystallization by vapour diffusion was introduced to structural biology for the preparation of tRNA crystals (Hampel *et al.*, 1968). It is well suited for small volumes (as little as 2 µl or less) and has became the favoured method of most experimenters. It is practiced in a variety of forms and is the method of choice for robotics applications. In all of its versions, a drop containing the

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