amorphous precipitate, microcrystals and large single crystals may be only a ΔpH of no more than 0.5.

4.1.3.3. *Additives*

Intriguing questions with regard to optimizing crystallization conditions concern which additional compounds should comprise the mother liquor in addition to solvent, macromolecule and crystallizing agent (Sauter, Ng *et al.*, 1999). Polyamines and metal ions are useful for nucleic acids. Some useful effectors for proteins are those that maintain their structure in a single, homogeneous and invariant state (Timasheff & Arakawa, 1988; Sousa *et al.*, 1991). Such effectors, sometimes named cosmotropes (Jerusalmi & Steitz, 1997), are polyhydric alcohols, like glycerol, sugars, amino acids or methylamino acids. Sulfobetaines also show remarkable properties (Vuillard *et al.*, 1994). Reducing agents, like glutathione or 2-mercaptoethanol, which prevent oxidation, may be important additives, as may chelating compounds, like EDTA, which protect proteins from heavy- or transition-metal ions. Inclusion of these compounds may be desirable when crystallization requires a long period of time to reach completion. When crystallization is carried out at room temperature in PEG or in low-ionic-strength solutions, the growth of microbes that may secrete enzymes that can alter the integrity of the macromolecule under study must be prevented (see below).

Substrates, coenzymes and inhibitors can fix a macromolecule in a more compact and stable form. Thus, a greater degree of structural homogeneity may be imparted to a population of macromolecules by complexing them with a natural ligand before attempting crystallization. In terms of crystallization, complexes have to be treated as almost entirely separate problems. This may permit a new opportunity for growing crystals if the native molecule is obstinate. Just as natural substrates or inhibitors are often useful, they can also have the opposite effect of obstructing crystal formation. In such cases, care must be taken to eliminate them from the mother liquor and from the purified protein before crystallization is attempted. Finally, it should be noted that the use of inhibitors or other ligands may sometimes be invoked to obtain a crystal form different from that grown from the native protein.

4.1.4. How to crystallize a new macromolecule

4.1.4.1. *Rules and general principles*

The first concern is to obtain a macromolecular sample of highest quality; second, to collate all biochemical and biophysical features characterizing the macromolecule in order to design the best crystallization strategy; and finally, to establish precise protocols that ensure the reproducibility of experiments. It is also important to clean and sterilize by filtration (over 0.22 μm porosity membranes) all solutions in contact with pure macromolecules to remove dust and other solid particles, and to avoid contamination by microbes. Inclusion of sodium azide in crystallizing solutions may discourage invasive bacteria and fungi. In vapour-diffusion assays, such contamination can be prevented by simply placing a small grain of thymol in the reservoir. Thymol, however, can occasionally have specific effects on crystal growth (Chayen *et al.*, 1989) and thus may serve as an additive in screenings as well.

Crystallization requires bringing the macromolecule to a supersaturated state that favours nucleation. Use of phase diagrams may be important for this purpose (Haas & Drenth, 1998; Sauter, Lorber *et al.*, 1999). If solubilities or phase diagrams are unavailable, it is nevertheless important to understand the correlation between solubility and the way supersaturation is reached in the different crystallization methods (see Fig. 4.1.2.1). In dialysis, the macromolecule concentration remains constant during equilibration. The initial concentration of the crystallizing agent in the exterior solution leaves the macromolecule in an undersaturated state. With increasing concentration of the agent in the exterior solution, a state of supersaturation can be attained, leading to crystallization or precipitation. In a vapour-diffusion experiment, where the concentration of crystallizing agent in the reservoir exceeds that in the drop, the macromolecule will begin to concentrate from an undersaturated to a supersaturated state, with both macromolecule and crystallizing-agent concentrations increasing. Crystals appear in the metastable region. For crystals that appear first, the trajectory of equilibration is complex and the remaining concentration of macromolecule in solution will converge towards a point located on the solubility curve. In batch crystallization using a closed vessel, three situations can occur: if the concentration of the macromolecule is undersaturated, crystallization never occurs (unless another parameter such as temperature is varied); if it belongs to the supersaturated region between solubility and precipitation curves, crystals can grow until the remaining concentration of the macromolecule in solution equals its solubility; if supersaturation is too high, the macromolecule precipitates immediately, although in some cases, crystals can grow from precipitates by Ostwald ripening (Ng *et al.*, 1996).

4.1.4.2. *Purity and homogeneity*

The concept of purity assumes a particular importance in crystallogenesis (Giegé *et al.*, 1986; Rosenberger *et al.*, 1996), even though some macromolecules may crystallize readily from impure solutions (Judge *et al.*, 1998). In general, macromolecular samples should be cleared of undesired macromolecules and small molecules and, in addition, should be pure in terms of sequence integrity and conformation. Contaminants may compete for sites on growing crystals and generate growth disorders (Vekilov & Rosenberger, 1996), and it has been shown that only p.p.m. amounts of foreign molecules can induce formation of non-specific aggregates, alter macromolecular solubility, or interfere with nucleation and crystal growth (McPherson *et al.*, 1996; Skouri *et al.*, 1995). These effects are reported to be reduced in gel media (Hirschler *et al.*, 1995; Provost & Robert, 1995).

Microheterogeneities in purified samples can be revealed by analytical methods, such as SDS–PAGE, isoelectric focusing, NMR and mass spectroscopy. Although their causes are multiple, the most common ones are uncontrolled fragmentation and post-synthetic modifications. Proteolysis represents a major difficulty that must be overcome during protein isolation. Likewise, nucleases are a common cause of heterogeneity in nucleic acids, especially in RNAs that are also sensitive to hydrolytic cleavage at alkaline pH and metal-induced fragmentation. Fragmentation can be inhibited by addition of protease or nuclease inhibitors during purification (Lorber & Giegé, 1999). Conformational heterogeneity may originate from ligand binding, intrinsic flexibility of the macromolecule backbones, oxidation of cysteine residues, or partial denaturation. Structural homogeneity may be improved by truncation of the flexible parts of the macromolecule under study (Price & Nagai, 1995; Berne *et al.*, 1999).

4.1.4.3. *Sample preparation*

Preparation of solutions for crystallization experiments should follow some common rules. Stocks should be prepared with chemicals of the purest grade dissolved in double-distilled water and filtered through 0.22 μm membranes. The chemical nature of the buffer is an important parameter, and the pH of buffers, which must be strictly controlled, is often temperature-dependent, especially that of Tris buffers. Commercial PEG contains contaminants, ionic (Jurnak, 1986) or derived from peroxidation, and thus repurification is recommended (Ray & Puvathingal, 1985).

Mother liquors are defined as the solutions that contain all compounds (buffer, crystallizing agent, *etc.*) at the final concentration for crystallization except the macromolecule. Samples of macromolecules often contain quantities of salt of unknown composition, and it is therefore wise to dialyse new batches against well characterized buffers. Whatever the crystallization method used, it almost always requires a high concentration of macromolecule. This may imply concentration steps using devices operating under nitrogen pressure, by centrifugation, or by lyophilization (notice that lyophylization may denature proteins and that non-volatile salts also lyophilize and will accumulate). Dialysis against high-molecular-weight PEG may also be used. During concentration, pH and ionic strength may vary and, if not kept at the appropriate values, denaturation of samples may occur.

4.1.4.4. *Strategic concerns: a summary*

Homogeneity: Perhaps the most important property of a system to be crystallized is its purity. Crystallization presupposes that identical units are available for incorporation into a periodic lattice. If crystallization fails, reconsidering purification protocols often helps achieve success.

Stability: No homogeneous molecular population can remain so if its members alter their form, folding, or association state. Hence, it is crucial that macromolecules in solution are not allowed to denature, aggregate, or undergo conformational changes.

Solubility: Before a molecule can be crystallized, it must be solubilized. This means creation of monodisperse solutions free from aggregates and molecular clusters. Solubility and crystallizability strongly depend on substances (organic solvents and PEGs) that reduce the ionic strength of the solution (Papanikolau & Kokkinidis, 1997).

Supersaturation: Crystals grow from systems displaced from equilibrium so that restoration requires formation of the solid state. Thus, the first task is to find ways to alter the properties of the crystallizing solutions, such as by pH or temperature change, to create supersaturated states.

Association: In forming crystals, molecules organize themselves through self-association to produce periodically repeating three-dimensional arrays. Thus, it is necessary to facilitate positive molecular interactions while avoiding the formation of precipitate or unspecific aggregates, or phase separation.

Nucleation: The number, size and quality of crystals depend on the mechanisms and rates of nuclei formation. In crystallization for diffraction work, one must seek to induce limited nucleation by adjustment of the physical and chemical properties of the system.

Variety: Macromolecules may crystallize under a wide spectrum of conditions and form many polymorphs. Thus, one should explore as many opportunities for crystallization as possible and explore the widest spectrum of biochemical, chemical and physical parameters.

Control: The ultimate value of any crystal is dependent on its perfection. Perturbations of the mother liquor are, in general, deleterious. Thus, crystallizing systems have to be maintained at an optimal state, without fluctuations or shock, until the crystals have matured.

Impurities: Impurities can contribute to a failure to nucleate or grow quality crystals. Thus, one must discourage their presence in the mother liquor and their incorporation into the lattice.

Perfection: Crystallization conditions should be such as to favour crystal perfection, to minimize defects and high mosaicity of the growing crystals, and to minimize internal stress and the incorporation of impurities. Predictions from crystal-growth theories may help to define such conditions (Chernov, 1997*b*, 1999).

Preservation: Macromolecular crystals may degrade and lose diffraction quality upon ageing. Thus, once grown, crystals may be stabilized by temperature change, addition of more crystallizing agent, or by some other suitable alteration in the mother liquor.

4.1.5. Techniques for physical characterization of crystallization

Crystallization comprises four stages. These are prenucleation, nucleation, growth and cessation of growth. It proceeds from macromolecules in a solution phase that then 'aggregate' upon entering a supersaturated state and which eventually undergo a phase transition. This leads to nuclei formation and ultimately to crystals that grow by different mechanisms. Each of these stages can be monitored by specific physical techniques. Although systematic characterization of crystallization is usually not carried out in practice, characterization of individual steps and measurement of the physical properties of crystals obtained under various conditions may help in the design of appropriate experimental conditions to obtain crystals of a desired quality (*e.g.* of larger size, improved morphology, increased resolution or greater perfection) reproducibly.

4.1.5.1. *Techniques for studying prenucleation and nucleation*

Dynamic light scattering (DLS) relies on the scattering of monochromatic light by aggregates or particles moving in solution. Because the diffusivity of the particles is a function of their size, measurement of diffusion coefficients can be translated into hydrodynamic radii using the Stokes–Einstein equation. By making measurements as a function of scattering angle, information regarding aggregate shape can also be obtained. For single-component systems, the method is straightforward for determining the size of macromolecules, viruses and larger particles up to a few μm. For polydisperse and concentrated systems, the problem is more complex, but with the use of autocorrelation functions and advances in signal detection (Peters *et al.*, 1998), DLS provides good estimates of aggregate-size distribution.

In bio-crystallogenesis, investigations based on light scattering have been informative in delineating events prior to the appearance of crystals subsequently observable under the light microscope, that is, the understanding of prenucleation and nucleation processes. Many studies have been carried out with lysozyme as the model (Kam *et al.*, 1978; Durbin & Feher, 1996), though not exclusively, and they have developed with two objectives. One is to analyse the kinetics and the distribution of molecular-aggregate sizes as a function of supersaturation. The aim is to understand the nature of the prenuclear clusters that form in solution and how they transform into crystal nuclei (Kam *et al.*, 1978; Georgalis *et al.*, 1993; Malkin & McPherson, 1993, 1994; Malkin *et al.*, 1993). Such a quantitative approach has sought to define the underlying kinetic and thermodynamic parameters that govern the nucleation process. The second objective is to use light-scattering methods to predict which combinations of precipitants, additives and physical parameters are most likely to lead to the nucleation and growth of crystals (Baldwin *et al.*, 1986; Mikol, Hirsch & Giegé, 1990; Thibault *et al.*, 1992; Ferré D'Amaré & Burley, 1997). A major goal here is to reduce the number of empirical trials. The analyses depend on the likelihood that precipitates are usually linear, branched and extended in shape, since they represent a kind of random polymerization process (Kam *et al.*, 1978). Aggregates leading to nuclei, on the other hand, tend to be more globular and three-dimensional in form. Thus, a mother liquor that indicates a nascent precipitate can be identified as a failure, while those that have the character of globular aggregates hold promise for further exploration and refinement. Other analyses have been based on discrimination between polydisperse and monodisperse protein

references