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).

references