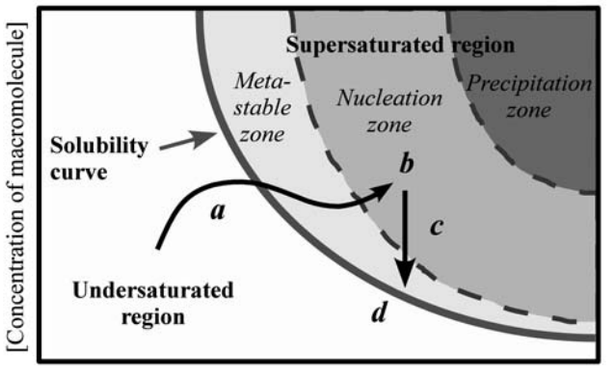

Biochemical parameters
Purity of the macromolecule
Ligands, inhibitors, effectors
Aggregation state of the macromolecule
Post-translational modifications
Source of macromolecule
Proteolysis or hydrolysis
Chemical modifications
Genetic variants
Inherent symmetry of the macromolecule
Stability of the macromolecule
Isoelectric point
History of the sample

Chemical parameters
pH
Crystallant type
Crystallant concentration
Ionic strength
Specific ion effects
Supersaturation
Reductive or oxidative environment
Concentration of the macromolecule
Metal ions
Crosslinkers or polyions
Detergents, surfactants or amphophiles
Non-macromolecular impurities

Physical parameters
Temperature variation
Contact surfaces
Methodology or approach to equilibrium
Gravity
Pressure
Time
Vibrations, sound or mechanical perturbations
Electrostatic or magnetic fields
Dielectric properties of the medium
Viscosity of the medium
Rate of equilibration
Homogeneous or heterogeneous nucleants

Figure 4.1.1.1
Crystallization is a multiparametric process under the control of a great variety of biochemical, chemical and physical parameters. The crystal grower can play with them to drive a macromolecule from the soluble towards the crystalline state. In the top left, a typical two-dimensional phase diagram illustrates how this can be achieved. (*a*) By modifying two parameters (concentrations of macromolecule and crystallant), the system will move from the undersaturated region where the macromolecule is soluble into the supersaturated region beyond the solubility curve where it will try to escape from the solution. The prenucleation trajectory essentially depends on the crystallization method (see Fig. 4.1.3.1). (*b*) As soon as they enter the supersaturation region, macromolecules will tend to aggregate. However, the system needs to cross an energy barrier to produce stable molecular assemblies, the nuclei, and this only happens in the so-called nucleation zone. (*c*) When stable nuclei are formed, they will capture more macromolecular entities from the mother liquor and produce three-dimensional crystals. (*d*) Crystals will grow until the system comes back to the solubility curve, crossing the metastable zone. Growth stops and crystals are in dynamic equilibrium with the mother liquor. When the system is driven to high supersaturation, macromolecules may rather produce amorphous or microcrystalline precipitates than useful monocrystals. Note that the limits between zones of the supersaturated zone (dashed curve) move with time.

nucleation occurs spontaneously and a metastable domain (at low supersaturation) where nucleation does not occur spontaneously but where crystals grow. This domain is favourable for seeding. The wisdom of the crystal grower will be to take advantage of an overall understanding of phase diagrams for designing crystallization strategies and selecting favourable solvent conditions (Sauter, Lorber *et al.*, 1999; Asherie, 2004).

4.1.2. Main parameters that affect crystallization of macromolecules

4.1.2.1. Crystallizing agents

Crystallizing agents (also referred to as crystallants or precipitants) are defined as those chemical species that are able to precipitate a soluble macromolecule and hence to bring it into a supersaturated state needed for its crystallization. They fall into four categories: (i) salts, (ii) organic solvents, (iii) long-chain polymers, and (iv) low-molecular-mass polymers and non-volatile organic compounds (McPherson, 1990). The first two classes are typified by ammonium sulfate and ethanol; higher polymers such as PEGs (polyethylene glycols) of molecular weight (M_r) > 1000, are characteristic of the third. In the fourth are placed compounds such as MPD (2-methyl-2,4-pentanediol) and low-molecular-mass PEGs.

Salts. Salts exert their effects by dehydrating proteins through competition for water molecules (Green & Hughes, 1955). Their ability to do this is roughly proportional to the square of the valences of the ionic species composing the salt. Thus, multivalent ions, particularly anions, are the most efficient. One might think there would be little variation between different salts, so long as their ionic valences were the same, or between two different sulfates, such as lithium and ammonium sulfates. This, however, is often not the case. In addition to salting out, which is a dehydration effect, or lowering of the chemical activity of water, there are specific protein–ion interactions that have other consequences (Riès-Kautt & Ducruix, 1991; Ducruix & Giegé, 1999). This is true because of the polyvalent character of individual proteins, their structural complexity, and the dependence of their physical properties on environmental conditions and interacting molecules. Therefore, restricting the search to one or two salts is never sufficient when attempting to crystallize a protein. Changing the salt may produce crystals of varied quality, morphology and diffraction properties.

It is usually not possible to predict the molarity of a salt required to crystallize a particular protein without some prior knowledge of its behaviour. In general, the concentration is just a small percentage less than that yielding an amorphous precipitate. To determine the precipitation point with a given crystallant, a 10 µl droplet of a 5–15 mg ml^{-1} protein solution is placed in the well of a depression slide and observed under a microscope as increasing amounts of salt solution or organic solvent (in l–2 µl increments) are added. If the well is sealed between additions with a coverslip, the increases can be made over a period of many hours. Indeed, the droplet should equilibrate 10–30 min after each addition, and longer in the neighbourhood of the precipitation point.

Widely used and the most successful salt to grow protein crystals is ammonium sulfate. A variety of other salts included in crystallization screens yield success as well. Sodium malonate, not systematically present in screens, deserves particular attention, since it gave the highest success rate (better than ammonium sulfate) in a comparative study designed to identify the most effective salts for protein crystallization (McPherson, 2001). Following this example, other organic salts have been added to the repertoire (McPherson & Cudney, 2006).

Organic solvents. The most common organic solvents (volatile compounds) utilized are ethanol, methanol, isopropanol, acetone and dioxane. They have been frequently used for crystallizing nucleic acids, particularly tRNAs and duplex oligonucleotides

(Dock *et al.*, 1984). This, in part, stems from the greater tolerance of polynucleotides to organic solvents and their polyanionic character, which appears more sensitive to dielectric effects than proteins. Volatile organic solvents should be used at low temperature and should be added slowly and with good mixing.

Long-chain polymers. PEGs are polymers of various lengths that are useful in crystallogenesis (McPherson, 1976). The larger species with $M_r > 1000$ exist at room temperature as either waxy solids or powders. The M_r's specified by manufacturers are mean values, and the distribution around these means varies. In addition to volume exclusion properties, PEGs share characteristics with salts that compete for water and produce dehydration, and with organic solvents that reduce the dielectric properties of the medium. PEGs also have the advantage of being effective at minimal ionic strength and providing low-electron-density media. The first feature is important because it leads to stronger affinities for ligands than high-ionic-strength media. Thus, there is greater ease in obtaining heavy-atom derivatives and in forming protein–ligand complexes. The second characteristic, their low electron density, implies a lower noise level for structures derived by X-ray diffraction.

The most useful PEGs in crystallogenesis are those in the range 2000–8000. Sizes are generally not completely interchangeable for a given protein, and this parameter has to be optimized by empirical means. An advantage of PEG over other crystallants is that most proteins crystallize within a rather narrow range of PEG concentrations (∼4–18%). In addition, the PEG concentration at which crystals form is rather insensitive, and if one is within 2–3% of the optimal value, some success will be achieved. The advantage is that, when conducting initial trials, one can use a fairly coarse selection of concentrations. This means fewer trials with a corresponding reduction in the amount of material expended. Note that PEGs are often used in conjunction with salts.

Since PEG is not volatile, this agent must be used like salt and equilibrated with the protein by dialysis, slow mixing or vapour diffusion. This latter approach is the most popular. When the reservoir concentration is in the range 5–12%, the protein solution to be equilibrated should initially be at about half that concentration. When the target PEG concentration is higher than 12%, it is advisable to initiate the equilibration at no more than 4–5% below the final value. This reduces lags in time during which the protein might denature. Crystallization of proteins with PEG has proved most successful when ionic strength is low, and more difficult when high. If crystallization proceeds too rapidly, addition of some neutral salt may be used to slow growth. PEG can be used over the entire pH range and a broad temperature range. It should be noted that solutions with PEG may serve as media for microbes, particularly moulds, and if crystallization is attempted at room temperature or over extended periods of time, then retardants, such as azide (∼0.1%), must be included in the protein solutions.

Low-molecular-mass polymers and non-volatile organic compounds. Low-molecular-mass PEG species with $M_r < 1000$, MPD and glycerol are widely used. These are oily liquids that increase the viscosity of the crystallization samples. Glycerol and other polyols have structure-ordering potential and might be useful for crystallizing flexible proteins (Sousa, 1995). MPD has properties midway between PEG and organic solvents, which makes it one of the most successful crystallants for promoting crystallization of biological macromolecules (Anand *et al.*, 2002). Besides efficacy with proteins, ∼50% of oligonucleotides were crystallized with MPD (Li *et al.*, 2007). These low-molecular-mass compounds are often used in combination with salts or long-chain PEGs.

Recent data show that room-temperature ionic liquids have the potential for macromolecular crystallogenesis (Pusey *et al.*, 2007) and can be added to the list of crystallants. They are organic salts that can solubilize proteins while preserving structural stability and enzymatic activity. With regard to crystallization applications, it is worth noticing that imidazolium-based ionic liquids enhance protein folding and suppress aggregation (Lange *et al.*, 2005). As a promising result, several ionic liquids used in the range 3–30%(*v*/*v*) yielded crystals of trypsin and lysozyme (Judge *et al.*, 2009).

4.1.2.2. Physical, physical–chemical and biochemical variables

Many physical, chemical and biological variables influence, to a greater or lesser extent, the crystallization of macromolecules (Fig. 4.1.1.1). The difficulty in assigning the relative importance for each factor is substantial for several reasons. First, each macromolecule has different surface properties and this even applies to molecules that differ by no more than one or a few residues. Therefore one can hardly predict in advance the specific values of a variable or sets of conditions that might be most profitably explored. Furthermore, crystallization parameters are not independent and their interrelations may be difficult to discern. Thus, it is not easy to elaborate firm guidelines related to physical or chemical factors that can increase the success in crystallizing a particular macromolecule. Among them, only temperature and pH have been studied carefully. For pressure or magnetic and electric fields, few investigations have been carried out and virtually nothing is known on the effects of sound, vibrations or viscosity on the growth or final quality of protein crystals (see Section 4.1.4.2).

Current physical–chemical variables. Temperature may be of great importance (Judge *et al.*, 1999; Astier & Veesler, 2008; Lin *et al.*, 2008). In general, it is wise to conduct parallel investigations at 4 and 20 °C. Even if no crystals are observed at either temperature, differences in the solubility behaviour of a protein with various crystallants and effector molecules may give some indication as to whether temperature is likely to play an important role (Christopher *et al.*, 1998). Generally, the solubility of a protein is more sensitive to temperature at low ionic strength than high. One must remember, however, that diffusion rates are less, and equilibration occurs more slowly at colder than higher temperature, so that the time required for crystal formation may be longer at colder temperatures. Although most crystallization trials are done at low (∼4° C) or medium (∼20 °C) temperatures, warmer temperatures in the range 35–40 °C should not be ignored, particularly with molecules that tend to aggregate and with nucleic acids (Dock-Bregeon *et al.*, 1988).

Another important variable is pH (Judge *et al.*, 2009). This follows since the charge character of a macromolecule and all of its consequences are intimately dependent on the ionization state of its components. Not only does its net charge change with pH (and the charge distribution), but also its dipole moment, conformation and often its aggregation state. Thus, an investigation of the behaviour of a specific macromolecule as a function of pH is an essential analysis that should be carried out in performing crystallization assays. Various buffer systems that are compatible with macromolecules are available for that purpose (Newman, 2004). As with temperature, the procedure is, first, to conduct trials at coarse intervals over a broad pH range and then to refine trials in the neighbourhood of those that showed

promise. In refining the pH for optimal growth, it should be recalled that the difference between amorphous precipitate, microcrystals and large single crystals might be due to a ΔpH of less than half a unit.

Other physical variables. Convection and diffusion are two correlated parameters that govern mass transport during the crystallization process, and studies on the physics of macromolecular crystal growth, in particular in relation to microgravity research (Giegé *et al.*, 1995; McPherson, 1996; Kundrot *et al.*, 2001; Helliwell & Chayen, 2007), have evaluated their importance and led to advanced methods of crystallization (see Section 4.1.4). On the other hand, mechanical or other types of physical perturbation of the crystallization media, *e.g.* by external electric or ultrasonic fields (Nanev & Penkova, 2001; Kakinouchi *et al.*, 2006), by continuous light irradiation (Veesler *et al.*, 2006) and by laser pulses (Lee *et al.*, 2008), might be used to induce nucleation. Local increase of protein concentration due to laser-induced cavitation could be a trigger of nucleation (Yoshikawa *et al.*, 2009).

Biochemical variables. It is now generally appreciated that, indeed, the protein is itself the most important and influential parameter in the crystallization endeavour (Dale *et al.*, 2003). When conventional approaches to crystallization have been exhausted there are two remaining options: further purifying the protein, or modifying the protein. The latter may be accomplished by genetic means using recombinant DNA techniques; that is, single or multiple point mutations may be introduced, or truncated forms of the polypeptide generated (see Section 4.1.2.4). Alternatively, traditional chemical reactions may be used to modify existing amino acids, or exposure to modifying enzymes may be employed, for example, the production of truncations by limited proteolysis. There are many examples of truncated proteins being successfully crystallized when the full-length polypeptide could not, *e.g.*, with many aminoacyl-tRNA synthetases (Giegé *et al.*, 2008). With the predictive capability of modern amino-acid sequence analysis and mass spectrometry to identify domains within proteins, designed constructs of predetermined lengths are becoming increasingly used. It has also been suggested that some surface amino acids, such as lysine and glutamic acid, inflict entropic costs when a protein crystallizes (Derewenda, 2004). Modification of such residues by reductive methylation appears to be a useful approach (Kim *et al.*, 2008) that in some cases enhances crystallizability. For instance, the addition of two methyl groups on the side chain of nine lysine residues of a nuclease introduced 44 cohesive CH intramolecular contacts and resulted in crystals diffracting at 1.2 Å resolution (Shaw *et al.*, 2007).

Histidine tags and proteins conjugated with a second protein, such as the maltose binding protein, are frequently produced as a basis for purification procedures for recombinant proteins. There is no reason why these 'tagged' or conjugated proteins should not crystallize and, indeed, many of them do. On the other hand, removal of the tag or conjugate and subsequent crystallization trials provide a further opportunity to obtain crystals of a protein, and should certainly be tried (Carson *et al.*, 2007).

Another possibility to gain access to the structure of proteins recalcitrant to crystallize is to try crystallization of their orthologues. Indeed, proteins are adapted to their cellular environments and orthologues of the same physiological function can exhibit structural and physical–chemical idiosyncrasies leading to modified crystallizability. Thus proteins and macromolecular assemblages from extremophile organisms often crystallize more readily than their cousins from mesophiles or from higher eukaryotes, as shown for instance with aminoacyl-tRNA synthetases (Giegé *et al.*, 2008).

In nucleic acid crystallization, the large size of natural DNA and of many RNA molecules necessitates the design and preparation of adequate structural modules or domains amenable to crystal production. Tailoring an adequate module is often the prerequisite for its crystallization (Ducruix & Giegé, 1999; Holbrook *et al.*, 2001; Golden & Kundrot, 2003). Since RNA architecture is very modular, specific protein-binding domains can be introduced (typically small RNA hairpins) in the target RNA sequence to promote the association with a protein partner (*e.g.* the spliceosomal protein U1A) and hence to increase the possibilities of making packing interactions (Ferré-D'Amaré, 2010). Similarly, this engineering approach can be applied to proteins. A typical example is the production of crystals diffracting to high resolution of a G-protein-coupled receptor. In that case one flexible loop of this membrane protein was replaced by the sequence of T4 lysozyme and this globular exogenic domain created new contacts leading to three-dimensional packing (Cherezov *et al.*, 2007). Another example of engineering is the introduction of a leucine half-zipper into an aminoacyl-tRNA synthetase that has engendered multiple high-quality crystals (Guo *et al.*, 2010).

4.1.2.3. Additives

Certain chemical compounds or small molecules may have dramatic effects on the success of protein crystallization. Additives, as they are often called (McPherson, 1982, 1999; Sauter, Ng *et al.*, 1999), can be decisive in macromolecular crystallization. The most commonly used type of additives, and the only class for which we have any rational basis, are those which may, for physiological reasons, be bound by the protein with consequent favourable changes in its physical–chemical properties or conformation. These include coenzymes and prosthetic groups, inhibitors, enzymatic products, ions and other effector molecules. Often the liganded form is structurally defined and stable, while the unliganded form is not, which will lead to the crystallization of the former and not of the latter. Polyamines and metal ions are useful for nucleic acids (Dock *et al.*, 1984; Ducruix & Giegé, 1999). 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.

Numerous cases have, however, been reported where molecules were observed to make crucial interactions between macromolecules in the crystal that either helped guide or secure formation of the lattice. Such molecules sometimes had a physiological basis for their unexpected presence, but frequently not. They simply provided essential or at least helpful cross-links within the crystal. Additives that are used in protein crystallization or that might be appropriate for use in crystallization may be classified into nine categories.

Small physiological ligands. Physiologically or biochemically relevant small molecules such as coenzymes, substrate analogues, inhibitors, metal ions, prosthetic groups *etc*. are the first. These bind at the active sites of enzymes, or at specific sites elsewhere on protein molecules, and may promote more stable, homogeneous conformations, or they may induce conformational

changes into alternate states. In any case, the ultimate protein–ligand complex may exhibit a more monodisperse, less dynamic character. The pertinent molecules are, here, specific to the individual protein under study, and their selection for inclusion in mother liquors is amenable to rational analysis informed by the enzymology and biochemistry of the protein under study. That is, one considers all of the possible ligands of the protein and includes them in the screen of potential crystallization conditions.

Chemical protectants. These include reductants, such as 2-mercaptoethanol, gluthathione or dithiothreitol, which prevent oxidation, heavy metal ions, scavengers such as EDTA and EGTA (ethylenediaminetetraacetic acid and ethylene glycol tetraacetic acid), and compounds intended to prevent microbial infection such as sodium azide, pheno- or chlorobutanol. These compounds are generally included for well understood reasons, their effects are predictable and their impact on the crystallization process usually, but not always, of marginal significance. They prevent protein 'ageing', as do protease or nuclease inhibitors (Ducruix & Giegé, 1999), and are desirable when crystallization requires a long period of time to reach completion or when carried out at room temperature in PEG, or in low-ionic-strength solutions (*i.e.* conditions favourable for the growth of contaminating microorganisms).

Solubilizing agents and detergents. These include quaternary ammonium salts (Mirzabekov *et al.*, 1972), sulfobetains (Goldberg *et al.*, 1996), chaotropes like urea (Bolen, 2004), and a range of surfactant and detergent molecules (Neugebauer, 1990; Zulauf, 1990; Wiener, 2004). Because of the interest in membrane proteins, this class of additives was extensively studied and has been broadly applied to many proteins. Remarkably, there is still no consensus on which are most useful, which should be included in screening conditions, or even how they function in the solubilization of macromolecules. Note that ionic liquids at low concentration can enhance protein solubility and were shown to improve crystallization (Chen *et al.*, 2010).

Poisons. Poisons, as they have traditionally been called (McPherson, 1982, 1999), were originally employed to reduce twinning. These are generally low concentrations, 1–5%(*w*/*v*), of common organic solvents. They include compounds such as ethanol, dimethyl sulfoxide, acetone, dioxane, butanol or MPD. Their role in the crystallization process, even after 50 years of use, remains obscure. They probably enhance the solubility of the proteins and slightly reduce the degree of supersaturation in the mother liquor, as well as lower the dielectric constant of the medium, but they may have other effects as well.

Osmolytes, co-solvents and cosmotropes. These are compounds that exert their effects at relatively high concentrations, 1 *M* or more, and include a wide range of molecules such as sucrose, trehalose and other sugars, proline, trimethyl amine *N*-oxide, glycine, betaine, taurine, sarcosine, and a host of others (Washabaugh & Collins, 1986; Jeruzalmi & Steitz, 1997; Bolen, 2004; Collins, 2004). The effect of their inclusion in the mother liquor is usually to stabilize the native conformation of the protein by altering the interaction of the protein's surface with water, or by altering the hydration layer and possibly the structured waters.

Reversible cross-linkers. It has been proposed that the conformations of proteins might be stabilized, and their dynamic character reduced, by providing the proteins with small molecules that could reversibly cross-link charged groups (carboxyl and amino groups) on the protein's surface, or form intramolecular hydrogen-bonding networks using surface polar groups (Maclean *et al.*, 2002). The molecules that have been explored are usually multivalent molecules such as diamino- or dicarboxylic acid-containing molecules, or aliphatic moieties of various lengths carrying some combination of charged groups. It is not known whether the stabilization of proteins by this means is significant enough to affect their crystallization or not. This potential mechanism of altering crystallization behaviour, however, may indeed be pertinent.

Compounds favouring lattice interactions. Classes of compounds useful for stabilizing proteins through non-covalent intramolecular bonds, as described above, may also help create and stabilize protein crystals by interposing themselves between protein molecules and forming intermolecular cross-links (McPherson, 1999; McPherson & Cudney, 2006; Larson *et al.*, 2007, 2008). These cross-bridges may involve purely electrostatic interactions, or they may rely on hydrogen-bonding arrangements as well. The compounds most favourable for forming such 'lattice interactions' are, again, likely to be multivalent charged compounds, but one might expect that their length, or 'reach', would need to be greater, since they would have to extend from one protein molecule to another.

Nucleation and solubility enhancers. A special class of additives would be those materials or compounds that somehow serve to enhance nucleation, including unique surfaces. These may include low concentrations of PEG (Ray & Bracker, 1986), or other polymeric substances such as jeffamine emulsified in solutions of high salt concentration (Kuznetsov *et al.*, 2000, 2001). The microdroplets of the polymeric phase serve to concentrate the protein locally and provide an interface for nucleation to occur. This category should probably also include things like the gel used in cubic lipidic phase crystallization (Nollert, 2004; Caffrey & Cherezov, 2009) and surfaces that promote epitaxy and heterogeneous nucleation (McPherson & Shlichta, 1988*b*; Chayen *et al.*, 2006; Thakur *et al.*, 2008; Saridakis & Chayen, 2009).

Nucleation enhancers when mixed with current crystallants can also enhance protein solubility (at solute/crystal equilibrium). This is the case, *e.g.*, for acetonitrile, dimethyl sulfoxide, glycerol, MPD and PEGs at relatively high concentrations. As shown with lysozyme and xylose isomerase crystallized, respectively, in the presence of glycerol and acetonitrile, the beneficial effects are nucleation at lower supersaturation and crystals of lower mosaicity (Gosavi *et al.*, 2009).

Ionic liquids. These crystallants (see Section 4.1.2.1) can also be considered as additives. Used in the concentration range 0.05–0.15 *M*, several of them (*e.g.* 1-ethyl-3-methylimidazolium tetrafluoroborate) improved the crystallizability of lysozyme, trypsin and an Fab complex, probably by subtle changes in solution conditions since no ionic liquid ions were observed in the crystal structures (Judge *et al.*, 2009; Chen *et al.*, 2010).

4.1.2.4. Purity and homogeneity

The concept of purity is of utmost importance in macromolecular crystallogenesis (Giegé *et al.*, 1986; Rosenberger *et al.*, 1996), even though some macromolecules may crystallize readily from impure solutions (Judge *et al.*, 1998). In practice, 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 can have deleterious effects when they interact with the macromolecule or compete for sites on growing crystals and consequently generate growth disorders (Vekilov & Rosenberger, 1996). Thus, part-per-million amounts of foreign mole-

cules can induce the formation of non-specific aggregates, alter macromolecular solubility, or interfere with nucleation and crystal growth (McPherson *et al.*, 1996, 2004; Moreno *et al.*, 2005; Thomas *et al.*, 1998). These effects are reduced in gel media (Hirschler *et al.*, 1995; Provost & Robert, 1995).

On the other hand, macromolecules that are apparently pure may be microheterogeneous in sequence and/or conformation. Their causes are multiple and can be revealed by analytical tools, such as polyacrylamide gel electrophoresis, isoelectric focusing, nuclear magnetic resonance and mass spectroscopy, but are often overlooked. The most common causes are partial hydrolysis and post-synthetic modifications. Proteolysis represents a major difficulty that must be overcome during protein isolation. In RNAs, hydrolytic cleavages induced by nucleases, metal ions or alkaline pH are common causes of microheterogeneity. These processes can be inhibited by addition of protease or nuclease inhibitors and metal chelators during purification (Ducruix & Giegé, 1999). Heterogeneity in post-synthetic modification patterns in proteins or RNAs can be the result of functional necessity but can also occur when cloned macromolecules are overproduced. Conformational heterogeneity may also 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 (see Section 4.1.3). This can also be done *in situ* by addition of trace amounts of protease to crystallization assays (Wernimont & Edwards, 2009). The many reasons that can account for such subtle degradations or modifications explain why altered versions of a macromolecule can be the worst contaminants for its crystallization. Accordingly, the macromolecule itself must be considered as an essential parameter in crystallization (Dale *et al.*, 2003). Control of these phenomena is of crucial importance for the crystallization of macromolecular assemblages, such as the ribosome (Auerbach-Nevo *et al.*, 2005).

Many crystal growers have found a correlation between the outcome of crystallization assays (*i.e.* number of crystals, crystal habit, volume and best diffraction properties) and the quality of macromolecular samples. For this reason, one should never spoil a 'pure' batch by mixing it with another 'pure' one, that may differ as far as microheterogeneities or minute contaminants are concerned. Altogether, purity, good solubility, structural homogeneity and absence of aggregates are good criteria for protein crystallizability (D'Arcy, 1994; Ferré-D'Amaré & Burley, 1997). Dynamic light scattering (DLS) is the appropriate analytical method to verify sample homogeneity, detect aggregates and find solvent conditions that prevent aggregation (Mikol, Hirsch & Giegé, 1990; Borgstahl, 2007; Niesen *et al.*, 2008).

4.1.3. Crystallization arrangements and classical methodologies

Many methods can be used to crystallize macromolecules (Ducruix & Giegé, 1999; McPherson, 1982, 1999). They all aim to bring the macromolecules to an appropriate state of supersaturation (Fig. 4.1.3.1). Although vapour-phase equilibrium and dialysis techniques are favoured, batch and free interface diffusion methods are often used. Besides the physical and chemical variables that affect crystallization (Fig. 4.1.1.1), macromolecular crystal growth is influenced by the crystallization method itself and the geometry of the setup. 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, however, can be achieved in special arrangements based on liquid circulation cells (Vekilov & Rosenberger, 1998).

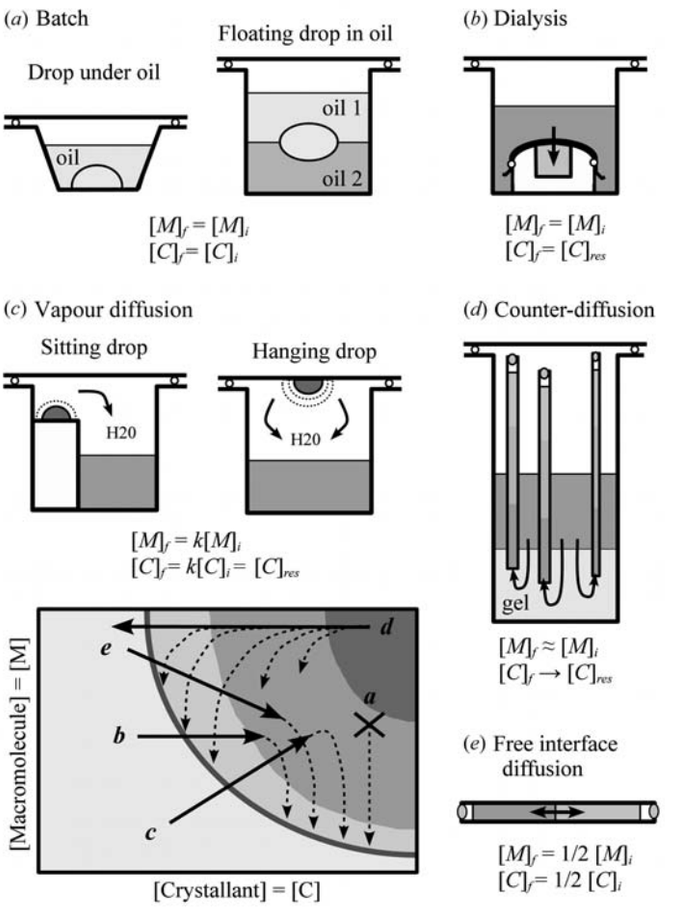

Figure 4.1.3.1
Principles of major methods used to crystallize biological macromolecules. (*a*) Two versions of batch crystallization. (*b*) Dialysis method with Cambridge button. (*c*) Vapour-diffusion crystallization with sitting and hanging drops. (*d*) Counter-diffusion setup in X-ray capillary tubes pinched in a gel layer. (*e*) Crystallization by free-interface diffusion in a capillary where two solutions of equivalent volume are brought into contact. The evolution of the macromolecule concentration, $[M]$, and crystallant concentration, $[C]$, in the different methods is indicated (initial and final concentrations in the crystallization solutions are $[M]_i$, $[M]_f$ and $[C]_i$, $[C]_f$, respectively; $[C]_{res}$ is the concentration of the crystallant in the reservoir, and k is a dilution factor specified by the ratio of the initial concentrations of crystallant in the drop and reservoir. In practice, glass vessels in contact with macromolecules should be silicone-treated in a way to obtain hydrophobic surfaces. Typical equilibration trajectories are illustrated in the phase diagram by black arrows, and the evolution of drops after nucleation and during growth are indicated by dashed arrows.

4.1.3.1. Historical development of methods

Protein crystallization is an old field that started more than 100 years ago (McPherson, 1991). Early methods included protein extractions with salts or organic solvents, or dialysis of salt solutions against water, and they were carried out on the gramme scale. Batch crystallization was the method of choice at that time. A first breakthrough that paralleled the development of X-ray methods occurred in the 1960s with the development of micromethods such as dialysis and vapour diffusion (with protein at the 1–100 mg scale and crystallization assays in the 10–50 µl range). However, it became rapidly apparent that screening of the entire