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Crystallogenesis of Biological Macromolecules: Facts and Perspectives

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Abstract

This paper gives an overview of the science of crystals of biological macromolecules. The historical background of the field is outlined and the main achievements and open problems are discussed from both biological and physical–chemical viewpoints. Selected results, including data from the authors, illustrate this overview. The perspectives of crystallogenesis for structural biology, but also more general trends, are presented.

1. Introduction

Biological crystallogenesis, the multidisciplinary science that seeks to describe and understand the crystal growth of biological macromolecules, has emerged together with the modern biotechnologies and particularly macromolecular engineering. The reason is the need for structural knowledge of biological macromolecules (and their assemblies), not only for basic biological research but also for understanding biotechnological problems. Thus, an increasing number of molecular biologists became interested in growing crystals for X-ray crystallography despite the frequent difficulty of the task. From another point of view, understanding the crystal growth of molecules as complex as proteins represents a great challenge for the physicists and physical chemists of materials science who are attracted by the mysteries of macromolecules.

In the past, research in the field was seldom conducted in a cooperative way between biologists and physicists. The lack of interdisciplinary contacts was perceived about one decade ago by both communities. An attempt towards an interdisciplinary approach of macromolecular crystal growth came from the French communities of small-molecule crystal growers and structural biologists who organized, in 1984 at Marseille, a joint meeting (with a limited international attendance) on the specific topic of protein crystallization. Simultaneously, American scientists organized, in 1985 at Stanford, the first international conference on the crystal growth of macromolecules (Feigelson, 1986). The Stanford meeting was the real start of the discipline and brought the nucleation for interdisciplinary research combining structural biology, physical chemistry, materials science and engineering that is now carried out in many places. Microgravity programs helped greatly to overcome the energy barrier needed to nucleate this new science. This was true in the United States with NASA, in Europe with ESA, and in France with CNES. The development of the field became apparent in the next international conferences that attracted an increasing number of participants, first at Bischenberg in France (Giegé, Ducruix, Fontecilla-Camps, Feigelson, Kern & McPherson, 1988) and in the following conferences, 1989 in Washington (Ward, 1991); 1991 in Freiburg, Germany (Stezowsky & Littke, 1992); and 1993 in San Diego (these proceedings). Presently, biological crystallogenesis is becoming a major scientific discipline at the borders of biology and physics.

The interest of the scientific community in biological crystallogenesis also became apparent with the increasing number of published papers dedicated to understanding crystallization. Starting with 21 papers for the Stanford Conference (Feigelson, 1986), about three times more papers were published for the Freiburg conference (Stezowsky & Littke, 1992). The importance of crystallogenesis in structural biology is also convincingly reflected by the number of crystallization papers appearing in the biological literature. For instance, 74 such papers were published in 1993 in the first 13 issues of the *Journal of Molecular Biology*, mainly on protein crystallization, with an average of five to six papers per issue. With such a number of crystallized proteins, one can wonder whether crystallization remains a bottleneck in structural biology and, thus, one can question the need for crystallogenesis.

This review will show why crystallogenesis is essential. The first reason, already discussed in the

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closing lecture at the Bischenberg Conference (Drenth, 1988), is the will of scientists to understand a process they do not really control. Another reason, of a more practical nature, is that many macromolecules are relunctant to crystallize, especially hydrophobic membrane proteins, nucleic acids and multi-molecular complexes. Therefore, better control of crystal growth is still important for X-ray biocrystallographers. From another and more general point of view, biological crystallogenesis could have its interest for itself and develop as an independent field. Because of the large size of macromolecules, studying their crystallizability could bring breakthroughs in the physics of crystal growth and permit an experimental approach, from the molecular point of view, to some of the unsolved problems in the science of crystals, such as nucleation phenomena.

This report, starting with an historical perspective, gives a short overview of the field which is necessarily incomplete and likely to reflect our own scientific interests. It emphasizes the most significant breakthroughs, especially those occurring in the last decade since the conferences on the crystal growth of biological macromolecules have been organized. In conclusion, future trends in the field, which will emerge most likely from present day research, will be presented. Additional information may be found in books and reviews by McPherson (1982), Carter (1990), McPherson (1990), Weber (1991) and Ducruix & Giegé (1992).

2. History and selected advances

2.1. *Towards structural biology*

Crystallization is one of the oldest sciences (for a review, see Scheel, 1993), and for molecules of biolgical origin the field started more than a century ago in both the physical–chemical and biological directions. During the decade 1847–1857, Louis Pasteur established in Strasbourg the rules of stereochemistry when studying tartaric acid crystals (Pasteur, 1986). Independently, with a first success dating back to 1840, physiological chemists crystallized several proteins at a time where their macromolecular nature was unknown (reviewed by McPherson, 1991). At the beginning of this century several hundred species of hemoglobins had been crystallized (Reichert & Brown, 1909). In those early days, crystallization was a purification tool for protein characterization and shortly before the advent of X-ray crystallography, crystallization experiments allowed researchers to establish definitively that the biological catalysts, the enzymes, are proteins (reviewed by Dounce & Allen, 1988). At present we know that the first macromolecules crystallized by biochemists (*e.g.* storage proteins from plants, globins, proteases, nucleases and even symmetrical virus particles) were stable molecules with compact structures which, in retrospect, explains their rather easy crystallization.

The first use of protein crystals for X-ray studies was made by Dorothy Hodgkin in 1934, when she obtained the first diffraction pattern of a pepsin crystal (Bernal & Crowfoot, 1934). She told the story of her first experiments in a stimulating lecture at the Bischenberg Conference. While X-ray methods were developed, the problem of crystallization was really not crucial and crystallization was by no means the bottleneck in X-ray crystallography. The situation changed as soon as the methods for solving structures became better established and when molecular biology gave access to more sophisticated molecules. More basic biological questions could then be addressed (*e.g.* molecular understanding of metabolic pathways, of genetic mechanisms, *etc.*) and a great need for crystals of selected macromolecules appeared.

2.2. *Crystallogenesis and protein synthesis*

The molecular biology of protein synthesis was a field that contributed most to the early stages of crystallogenesis. Several versions of the crystallization micromethods were developed by workers studying the translation machinery of genetic information. That was the case for vapor-phase crystallization micromethods, largely used for the crystallization of tRNAs, first in the sitting-drop version using the well known sandwich boxes (Hampel, Labanauskas, Conners, Kirkegard, RajBhandary, Sigler & Bock, 1968; McPherson, 1982). The microdialysis methods and the Cambridge buttons were first employed for the crystallization of aminoacyl-tRNA synthetases (Reid, Koch, Boulanger, Hartley & Blow, 1973). The discovery of the importance of spermine and other polyamines for crystallization of nucleic acids arose from studies on tRNAs (reviewed by Dock, Lorber, Moras, Pixa, Thierry & Giegé, 1984). From another point of view, the usefulness of thermophilic organisms for easier crystallization of proteins reluctant to crystallize, first documented with the work on tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* (Reid, Koch, Boulanger, Hartley & Blow, 1973), was an important breakthrough. At present, using proteins from the extreme thermophile *Thermus thermophilus*, as first advocated by the Russian school (Garber, Agalarov, Elisaikina, Sedelnikowa, Tishchenko, Shirokov, Yusupov, Reshetnikova, Trakhanov, Tukalo & Yaremchuk, 1991), led to an explosion of crystallizations of synthetases and their complexes with tRNAs (*e.g.* Poterszman, Plateau, Moras, Blanquet, Mazauric & Kern, 1993; Reshetnikova, Khodyreva, Lavrik, Ankilova, Frolow & Safro,

1993), of elongation factor (Reshetnikova, Reiser, Schirmer, Berchtold, Storm, Hilgenfeld & Sprinzl, 1991), and also of the ribosome, the most sophisticated structure so far studied by crystallographic methods (Yonath, 1992). It is likely that the easier crystallizations result from the higher stability of the thermophilic proteins [and also halophilic ones (Yonath, 1992)]. Finally, studying the association between synthetases and tRNAs allowed the unexpected discovery that ammonium sulfate stabilizes complex formation between these two types of macromolecules (Giegé, Lorber, Ebel, Moras & Thierry, 1980; Giegé, Lorber, Ebel, Moras, Thierry, Jacrot & Zaccaï, 1982). This property led to the crystallization of the complex between yeast aspartyl-tRNA synthetase and its cognate $tRNA^{Asp}$ (Giegé, Lorber, Ebel, Moras & Thierry, 1980; Lorber, Giegé, Ebel, Berthet, Thierry & Moras, 1983; Ruff, Cavarelli, Mikol, Lorber, Mitschler, Giegé, Thierry & Moras, 1988) and afterwards a series of other similar complexes (*e.g.* Perona, Swanson, Steitz & Söll, 1988; Eiler, Boeglin, Martin, Eriani, Gangloff, Thierry & Moras, 1992; Reshetnikova, Khodyreva, Lavrik, Ankilova, Frolow & Safro, 1993; Price, Cusack, Borel, Berthet-Colominas & Leberman, 1993). Although the effect of ammonium sulfate is not exactly understood, one may propose that it favors hydrophobic interactions as supported by biochemical studies on valyl-tRNA synthetase/tRNA or tRNA-like complexes (Florentz, Kern & Giegé, 1990).

2.3. *Crystallogenesis of hydrophobic proteins*

Crystallization of hydrophobic membrane proteins has already been discussed in the previous conferences (*e.g.* Garavito, Markovic-Housley & Jenkins, 1986; Gros, Groendijk, Drenth & Hol, 1988; Garavito & Picot, 1991) and was largely covered during the San Diego meeting (these proceedings). The breakthrough in the field arose when the first single crystals of bacteriorhodopsin (Michel & Oesterhelt, 1980) and porin from *E. coli* (Garavito & Rosenbusch, 1980) were obtained, which facilitated X-ray crystallography of integral membrane proteins [Deisenhofer, Epp, Miki, Huber & Michel (1984), reviewed in Michel (1991)]. These first successes were the consequence of better control of purification and solubilization of hydrophobic proteins thanks to the availability of new non-ionic detergents (*e.g.* Michel, 1983; Garavito & Rosenbusch, 1986; Garavito & Picot, 1990; Kuhlbrandt, 1988; Reiss-Husson, 1992). However, despite these advances, crystallizing membrane proteins still remains a difficult task which requires sophisticated biochemical and crystallization procedures and only a few of these proteins have been crystallized to date.

An alternative way to reach the structure of membrane proteins is electron microscopy or cryo-microscopic crystallography of two-dimensional crystals (*e.g.* Henderson, Baldwin, Ceska, Zemlin, Besckmann & Downing, 1990; Havelka, Henderson, Heyman & Oesterhelt, 1993; Wang, Kuhlbrandt, Sarabia & Reithmeier, 1993). This field, which gave important results recently, will certainly expand in future.

From another point of view, methods developed for membrane-protein crystallization may become useful for soluble proteins. Considering the fact that all macromolecules possess a partial hydrophobic character should encourage use of non-ionic detergents as universal crystallization additives. The expectation was verified with a number of different proteins and tRNAs for which new crystal forms were obtained in the presence of octyl-glucoside (McPherson, Koszelak, Axelrod, Day, Williams, McGrath, Robinson & Cascio, 1986).

2.4. *Crystallogenesis and immunology*

Structural biology of immunoglobins is presently an active field (reviewed by Jones, 1993) and useful developments for protein crystallization arose from studies on antibodies and antibody–antigen complexes. This was first the case for two-dimensional crystallization techniques on lipid monolayers (Uzgiris & Kornberg, 1983). This is also the case for various seeding methods, such as micro- and macro-seeding techniques, streak-seeding, and cross-seeding between related macromolecular species (*e.g.* Stura & Wilson, 1990, 1992). These methods gave most promising results in many instances, such as for the crystallization of a Fab fragment that recognizes the principal determinant of the gp-120 glycoprotein of HIV-1 (Stura, Stanfield, Fieser, Silver, Roguska, Hincapie, Simmerman, Profy & Wilson, 1992) or that of a murine major histocompatibility complex with single peptides (Stura, Matsumura, Fremont, Saito, Peterson & Wilson, 1992).

2.5. *Other methodological advances*

Important methodological advances were brought about over the years by many workers studying various types of proteins. Mentioned here are the crystallization methods in capillaries (Zeppenzauer, 1971) and by interface diffusion (Salemme, 1972), which are widely used. Other methods which are less universally employed include, for instance, crystallization in gels, in electric fields, under pressure, under micro- or supergravity, under levitation, or methods controlling or varying parameters as a function of time, pH or temperature [see reviews in Ducruix & Giegé (1992) and in these proceedings]. It can be expected that some of them will become very

popular in future, as gel methodologies first applied to lysozyme and trypsin crystallization (Robert & Lefaucheux, 1988) and crystallizations triggered by temperature or pH variations (*e.g.* McPherson, 1985; Mikol & Giegé, 1989).

A final point worth mentioning is the methods using co-solvents either to yield homogeneous precipitant solutions that have special solvational properties or heterogeneous solutions with liquid–liquid phase separations. The first category includes co-solvents composed of glycerol or sucrose and salts that have stabilizing effects on protein structures and were used to crystallize T7 RNA polymerase (Sousa, Lafer & Wang, 1991). Phase separations are often observed during crystallization experiments but are not well understood and seldom studied explicitly. Interesting investigations on these lines are crystallizations of phosphoglucomutase in co-solvents of ammonium sulfate, polyethylene glycol 400 and water, where PEG would act as a nucleation catalyst (Ray & Bracker, 1986) and especially studies on detergent–salt or PEG co-solvents (*e.g.* Garavito & Rosenbusch, 1986; Lorber, DeLucas & Bishop, 1991).

3. Purity, biochemistry and crystallographic aspects

3.1. *The importance of purity*

Purity is certainly one, if not the major, parameter to control in crystallogenesis (Giegé, Dock, Kern, Lorber, Thierry & Moras, 1986), and lack of its control explains many non-reproducible results. Although crystallization can be used to purify molecules, lack of purity hampers growth of monocrystals, especially when the impurities share a structural resemblance with the molecules to be crystallized. So one has to care about the structural and conformational homogeneity of samples, to avoid degradation (by proteases or nucleases), and to control the post-translational (or post-transcriptional) modifications (reviewed by Lorber & Giegé, 1992).

Numerous examples have been reported in the literature where a lack of purity and sequence microheterogeneities prevent crystallization of proteins, and conversely where improved purity leads to better crystals (*e.g.* Bott, Navia & Smith, 1982; Anderson, Boodhoo & Mol, 1988; Van der Laan, Swarte, Groendijk, Hol & Drenth, 1989). Structural heterogeneities can be detected in samples, apparently pure according to conventional electrophoretic analysis, by using more powerful analytical tools like isoelectric focusing (reviewed by Lorber & Giegé, 1992). Batch variabilities in protein preparations are often encountered. They are due mostly to heterogeneities within samples generated during purification, often of proteolytic origin, and explain many non-reproducible crystallization experiments. Many of the problems related to purity and purification can be minimized when working with overexpressed recombinant proteins and actually the number of such proteins that are crystallized is increasing steadily.

3.2. *Biochemical aspects and crystallization of nucleic acids*

One of the great challenges in structural biology remains the elucidation of nucleic acid structures, especially those of RNAs and of their complexes with proteins. For RNAs, the bottleneck lies obviously in their purification and crystallization. The first nucleic acids to be crystallized were tRNAs (reviewed by Dock, Lorber, Moras, Pixa, Thierry & Giegé, 1984). However, only a few of them gave crystals suitable for high-resolution X-ray studies, despite the fact that tRNAs are considered among the most compact nucleic acid structures. The reason for this lies in the intrinsic chemical fragility of these molecules which are easily cleaved by hydrolytic or metal-mediated processes. Further, purification of these molecules in large amounts is difficult. Crystallization of tRNAs complexed to synthetases, which for a long time was considered as a difficult task, appears at present much easier than crystallization of the free tRNA. This is due to the structural stabilization of the nucleic acid in its complexed form. Thus studying RNA/protein structures may represent a way for easier access to RNA structures.

An important breakthrough in nucleic acid crystallization came from the automated chemical synthesis of DNA fragments, which allowed the preparation of well defined double-stranded domains for crystallization purposes. Thus, structural biology and the underlying crystallogenesis of DNA [reviewed by Dock-Bregeon & Moras (1992), Joshua-Tor & Sussman (1993), and *e.g.* Wang & Teng (1988), Timsit & Moras (1991) and DiGabriele & Steitz (1993)] and of DNA/protein complexes [*e.g.* Joachimiak & Sigler (1991), for a review on crystallization methods] became easier. In particular, crystallography of complexes developed rapidly with an increasing number of structures crystallized and solved at high resolution [*e.g.* DNA operator/repressor complexes (Aggarwal, Rodgers, Drottar, Ptashne & Harrison, 1988; Otwinowski, Schevitz, Zhang, Lawson, Joachimiak, Marmorstein, Luisi & Sigler, 1988; Sommers & Phillips, 1992; Shimon & Harrison, 1993); a homeodomain/DNA complex (Wolberger, Vershon, Liu, Johnson & Pabo, 1991); TATA-box binding protein complexes with TATA elements (Kim, Geiger, Hahn & Sigler, 1993; Kim, Nikolov & Burley, 1993); the DNA polymerase I Klenow fragment bound to duplex DNA (Beese,

Derbyshire & Steitz, 1993); a complex between the human oncogen *GLI* containing five Zn fingers and a DNA duplex (Pavletich & Pabo, 1993); and the HIV reverse transcriptase complexed with double-stranded DNA (Jacobo-Molina, Ding, Nanni, Clark, Lu, Tantillo, Williams, Kamer, Ferris, Clark, Hizi, Hughes & Arnold, 1993)]. In all these examples, the choice of the correct length and sequence of the DNA fragments was the determinant for the success of crystallization. Further, overhanging bases in the DNA duplexes, which can make Watson–Crick hydrogen bonds between neighboring molecules for crystal lattice stabilization, can be prerequisites for crystal growth.

In the RNA field (reviewed by Dock-Bregeon & Moras, 1992), chemical synthesis is more difficult, due to the presence of free hydroxyl groups on the ribose moieties, and also to the frequent presence of post-transcriptional modified nucleotides. Enzymatic methods, based on *in vitro* transcription of synthetic genes under the control of phage polymerases may facilitate synthesis of large amounts of unmodified molecules (Milligan, Groebe, Witherell & Uhlenbeck, 1987). However, defining a globular and stable RNA domain amenable to crystallization within the often modular and large RNA structures remains difficult.

Several recent results have shown that RNA crystallization is becoming more easily accessible. A significant result concerns a double-stranded RNA fragment crystallized at high temperature (Dock-Bregeon, Chevrier, Podjarny, Moras, DeBear, Gough, Gilham & Johnson, 1988). Other examples are the crystallization of a chemically synthesized 5S RNA domain (Lorenz, Fürste, Bald, Zhang, Raderschall, Betzel, Dauter, Wilson & Erdmann, 1993) and especially that of ribozyme domains and other small RNA motifs (Doudna, Grosshans, Gooding & Kundrot, 1993). Interestingly, in the case of a 160-nucleotide domain of group I self-splicing intron from *Tetrahymena thermophilus*, the diffraction limit of the crystals extends to 2.8 Å (Doudna, Grosshans, Gooding & Kundrot, 1993).

Using *in vitro* synthesis of RNA by the transcriptional methods mentioned above, we prepared unmodified yeast $tRNA^{Asp}$ which could be crystallized in the complexed form with cognate aspartyl-tRNA synthetase. The complex with the unmodified tRNA exhibits solubility properties in ammonium sulfate solutions different from those found for the complex formed with the modified wild-type $tRNA^{Asp}$. As a consequence the two complexes crystallize under different conditions. While the native complex, with the modified tRNA, crystallizes in a high-resolution diffracting form (space group $P2_12_12_1$) at pH 6.0 in Tris–maleate buffer and 60% ammonium sulfate (Ruff, Cavarelli, Mikol, Lorber, Mitschler, Giegé, Thierry & Moras, 1988), the complex with the unmodified transcript yields, under the same solvent conditions, cubic crystals (Fig. 1) similar to the low-resolution diffracting form of the native complex (space group $I432$) obtained at pH 7.8 (or higher) in Tris–HCl buffer and 50% ammonium sulfate (Lorber, Giegé, Ebel, Berthet, Thierry & Moras, 1983).

3.3. *Crystal packing*

Mechanical stability of protein crystals, and probably also their internal order and diffraction power, rely on the nature and strength of packing contacts within crystalline lattices (Salemme, Genieser, Finzel, Hilmer & Wendlowski, 1988). Packing contacts involve molecular recognition of complementary regions of protein by hydrogen bonds, van der Waals contacts and salt bridges, which is reminiscent of the interactions occurring at subunit interfaces in oligomeric proteins. Changing solvent conditions can affect the nature and number of these contacts and yield other crystal forms as observed and rationalized for pancreatic RNase crystals (Crosio, Janin & Jullien, 1992). Contacts are often located along lattice symmetry axes, as in lysozyme and cytochrome crystals (Salemme, Genieser, Finzel, Hilmer & Wendlowski, 1988), thus implying an anisotropy of intermolecular contacts within crystals. Consequently, as accounted for by the 'Periodic Bond Chain' theory of Hartman & Perdock applied to protein crystallization (Frey, Genovesio-Taverne & Fontecilla-Camps, 1988), growth kinetics of the different faces of a crystal should be correlated with the structural anisotropy of the contacts. Following these lines, it becomes understandable then that mutation

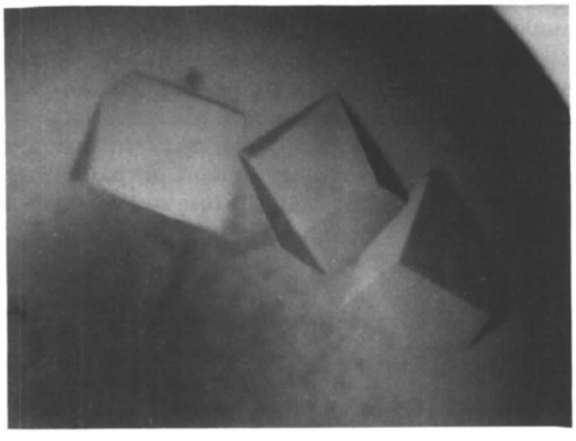

Fig. 1. Crystals of the complex between *in vitro* synthesized yeast $tRNA^{Asp}$ and its cognate yeast aspartyl-tRNA synthetase. Crystallization was performed at 277 K using the vapor-diffusion technique in hanging drops as indicated in the text. The largest crystals measure 0.5 mm.

of surface residues can affect crystallizability of proteins (Brennan, Wozniak, Faber & Matthews, 1988; McElroy, Sisson, Schoettlin, Aust & Villafranca, 1992; Mittl, Berry, Scrutton, Perham & Schulz, 1994) by packing contact modifications. This opens the possibility of crystal-contact tailoring in order to improve crystal quality. Such a rationale was applied for improving the quality of human ferritin crystals, by introducing a mutation at a position homologous to a critical contact in a related ferritin. This engineering ultimately allowed solution of the structure of the protein (Lawson, Artymiuk, Yewdall, Smith, Livingstone, Treffry, Luzzago, Levi, Arosio, Cesarini, Thomas, Shaw & Harrison, 1991).

Nucleic acid crystals are also stabilized by specific interactions between neighboring molecules, such as hydrogen bonds (*e.g.* Watson–Crick pairings as found in $tRNA^{Asp}$ crystals), base-stacking interactions (Moras & Bergdoll, 1988), Watson–Crick bonding between overhanging bases in head-to-tail stacked DNA duplexes (*e.g.* Pavletich & Pabo, 1993) or backbone/groove contacts (Timsit & Moras, 1991). But RNA crystals can become fragile by the progressive appearance of nicks in the ribose–phosphate chains, due to hydrolytic cleavages in flexible regions, as found in the D-loop of crystalline yeast $tRNA^{Asp}$ (Moras, Dock, Dumas, Westhof, Romby, Ebel & Giegé, 1986). The propensity of RNA molecules to show fragility points at particular sequences that generate structural microheterogeneities, explains failures in crystallization experiments, especially when the heterogeneities concern positions involved in packing contacts. This is the case with $tRNA^{Asp}$ which presents a fragile anticodon loop structure: if the level of cleavage in its anticodon is too severe, no crystallization is possible since anticodons make packing contacts by GUC/GUC Watson–Crick pairing (Moras, Dock, Dumas, Westhof, Romby, Ebel & Giegé, 1986). Returning to the crystallogenesis of modified and unmodified $tRNA^{Asp}$/synthetase complexes, it is likely that their differential crystallization properties are due to the structural differences introduced in the tRNA at potential packing contacts, in particular in their T-loop where the T and ψ residues are replaced by U residues in the transcripts.

4. Physical chemistry and physics

4.1. *General features*

Crystal growth of macromolecules obeys the same laws as those governing the growth of small molecules (*e.g.* Boistelle & Astier, 1988; Feigelson, 1988; Chernov, 1993). This seemingly obvious statement was not really recognized or considered seriously by most of the crystal growers of macromolecules until recently. The first rule to remember is that the physical–chemical conditions need to reach nucleation are not the same as those needed for the growth of the crystals. Nucleation requires a higher supersaturation than growth. Knowing the hierarchy of parameters affecting supersaturation it then becomes possible to uncouple nucleation and growth. This often happens in an uncontrolled way in the laboratory as a consequence of pH and/or temperature fluctuations. Today it can be carried out in a programmed way, as first advocated by physicists of crystal growth (*e.g.* Rosenberger & Meehan, 1988; Rosenberger, Howard, Sowers & Nyce, 1993).

Only recently attempts were undertaken to study the growth mechanisms and to monitor growth kinetics (*e.g.* Ataka & Asai, 1990; Monaco & Rosenberger, 1993; Vekilov, Ataka & Katsura, 1993), in particular by electron microscopy (Durbin & Feher, 1990) and atomic force microscopy (Durbin & Carlson, 1992; Littke, Haber & Güntherodt, 1992). Interesting conclusions arose from the electron microscopy investigations on lysozyme crystallization: growth occurs by a lattice-defect mechanism at low supersaturation and by two-dimensional nucleation at high supersaturation (Durbin & Feher, 1990).

4.2. *Solubilities and phase diagrams*

Uncoupling nucleation and growth requires knowledge of solubilities and phase diagrams. Systematic studies on solubilities have been undertaken for several proteins such as lysozyme (Howard, Twigg, Baird & Meehan, 1988; Cacioppo, Munson & Pusey, 1991), concanavalin A (Mikol & Giegé, 1989) or canavalin (DeMattei & Feigelson, 1991). Emphasis was placed on understanding the specific influence of ions on these solubilities (Riès-Kautt & Ducruix, 1989, 1991). Micromethods to establish phase diagrams have been worked out in several laboratories (*e.g.* Chayen, Akins, Campbell-Smith & Blow, 1988; Mikol & Giegé, 1989; Cacioppo, Munson & Pusey, 1991). Some of these diagrams have illustrated the possibility of changing the solubility of proteins by pH or temperature changes, but have also shown how complex and unpredictable solubilities can be when varying conditions. In current phase diagrams, only temperature and pH are usually varied in addition tc the precipitating agent and protein concentrations. But other physical conditions that could affect solubility (and/or crystallization) may also be considered, such as pressure (Gross & Jaenicke, 1991) or microgravity. In that latter case, despite the increasing number of experiments and of promising data (*e.g.* Littke & John, 1984; DeLucas, Smith, Carter, Snyder, McPherson, Koszelak & Bugg, 1991; Strong,

Stoddard, Arrott & Farber, 1992), no definitive answer as to a positive effect of weightlessness can be given today.

Because of the complexity of phase diagrams, choosing the right conditions for crystallization is the challenge for crystal growers, especially when one considers the quasi-infinite parameter space to be screened. Thus different approaches have been proposed and used, like statistical methods first explored by C. Carter and now widely used in various versions (*e.g.* Carter & Carter, 1979; Betts, Frick, Wolfenden & Carter, 1989; Weber, 1990; Abergel, Moulard, Moreau, Loret, Cambillau & Fontecilla-Camps, 1991; Carter, 1992), and those using sparse matrices of precipitating agents or related strategies (*e.g.* Jancarik & Kim, 1991; Stura, Nemerow & Wilson, 1992). Defining the matrices may be facilitated by using macromolecule data bases (*e.g.* Gilliland & Bickham, 1990).

4.3. *Using light-scattering methods*

The first use of light-scattering methods in crystallogenesis was made in the pioneering work of G. Feher and coworkers on lysozyme crystallization, when they proposed different interaction schemes of this protein when it crystallizes or precipitates (Kam, Shore & Feher, 1978). Using dynamic light scattering (DLS), several years ago we addressed the very simple question concerning the aggregation state of proteins in under- and supersaturated solutions, with the aim of finding a qualitative correlation between crystallizability and solvent conditions (Mikol, Hirsch & Giegé, 1990). Studies were conducted on lysozyme and concanavalin A. The conclusion was that these two proteins remain essentially monodisperse in undersaturated conditions in solvents leading to crystal growth and show a strong tendency to aggregate much before their precipitation in solvents that do not allow crystallization. We and others could extend this conclusion to several other proteins. Thus DLS can be used as a tool for crystallization solvent diagnostics; it can also be used for protein homogeneity assays, as carried out systematically in Basel (D'Arcy, Banner, Janes, Winkler, Loetscher, Schönfeld, Zulauf, Gentz & Lesslauer, 1993).

At present, DLS methods are very popular among crystal growers (these proceedings), and attempts to use them for approaching nucleation and the early stages of crystal growth have already been undertaken (*e.g.* Mikol, Hirsch & Giegé, 1989; Skouri, Munch, Lorber, Giegé & Candau, 1992; Georgalis, Zouni, Eberstein & Saenger, 1993; Malkin & McPherson, 1993). Other scattering methods, such as X-ray (Guilloteau, 1991) and neutron (Boué, Lefaucheux, Robert & Rosenman, 1993) scattering, were also employed and brought useful complementary information. Of interest are the fluorescence techniques that can monitor small aggregates (Crosio & Jullien, 1992). Interpretation of results, however, may be delicate (see below), since rare events (appearance of nuclei) have to be extracted from averaged distributions of scattering data arising from the concentrated protein solutions.

4.4. *Impurities and crystal growth*

Minute amounts of contaminants may interfere with basic phenomena of macromolecular crystal growth. Observations made with lysozyme illustrate this statement. Batch-dependent variations in DLS measurements where first observed when searching for large aggregates that could occur during nucleation of this protein. Later it was shown that different lysozyme batches can exhibit different solubility properties and other crystal growth behaviour leading to different crystal morphologies (Fig. 2). These variabilities are due to protein contaminants not exceeding a few percent (Lorber, Skouri, Munch & Giegé, 1993). Using preparations devoid of detectable impurities, in which 1%(*w*/*w*) of foreign proteins were added on purpose, allowed mimicry of the properties described above. The largest monocrystals are formed with the purest and most homogeneous lysozyme preparations. Badly shaped crystals are formed when contaminants are present (in the present case they were the natural ovalbumin and conalbumin contaminants of lysozyme). These structurally unrelated contaminants would favor

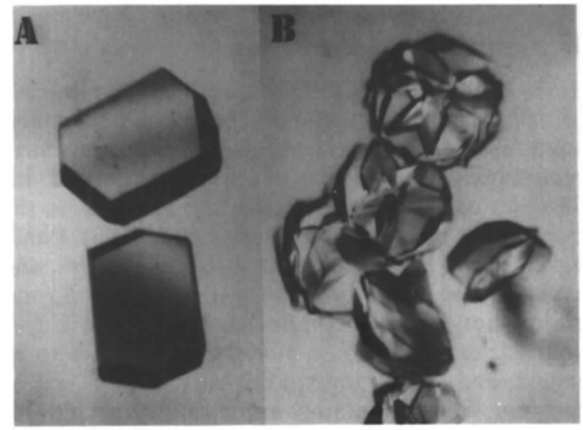

Fig. 2. Crystals of hen egg-white lysozyme obtained with two different batches. (*A*) Tetragonal crystals obtained with the purest batch. (*B*) Altered crystal habits obtained with lysozyme contaminated by traces of other proteins of egg white. Crystallization was carried out at 293 K under identical conditions using the batch technique in small cylindrical light scattering cells. Solutions (80 µl) contained 30 mg ml^{-1} lysozyme in 40 m*M* sodium acetate pH 4.5 and 5%(*w*/*v*) NaCl. The largest crystal size is 0.5 mm.

non-ordered associations of lysozyme molecules that become starting points for heterogeneous nucleations. The heterogeneous large aggregates that are formed in supersaturated conditions can be removed by filtration on microporous membranes, and the resulting lysozyme solution then exhibits properties similar to those of highly purified samples (Skouri *et al.*, to be published).

In conclusion, the importance of purity to obtain reproducible results in crystal growth experiments has to be emphasized again [see also the papers by Carter (1988), Abergel, Nesa & Fontecilla-Camps (1991) describing the effects of protein contaminants]. It is recalled that small organic molecules or minerals can also act as impurities affecting crystal growth. They will lead to unreproducible results as long as their nature is unknown as was observed for PEG contaminants (Jurnak, 1985). They could have positive effects if added on purpose and in a controlled way in crystallization trials as was done, for instance, by addition of minerals (McPherson & Shlichta, 1988), thymol (Chayen, Lloyd, Collyer & Blow, 1989) or glycerol (Sousa, Lafer & Wang, 1991). In these cases the 'impurities' have to be considered as additives.

5. Perspectives

The imagination of scientists working on the crystallogenesis of biological macromolecules will certainly find ways to circumvent many of the difficulties encountered presently. In what follows some of the unsolved problems and the likely new perspectives are outlined.

5.1. *Physics and physical chemistry*

It is expected that studying very large particles, like spherical viruses, could help to understand nucleation better. Investigations on these lines have already been initiated by A. McPherson and his colleagues using satellite tobacco mosaic virus as the model virus (*e.g.* Malkin & McPherson, 1993). Phase diagrams and the transitions between under- and supersaturated media are not well understood, and certainly not from a theoretical point of view. Therefore, basic experiments on model proteins have to be pursued, and continuing studies on lysozyme will be important, even if one may argue that lysozyme is a special protein because it crystallizes in sodium chloride and not in ammonium sulfate as most proteins do. In fact studies should be pursued to understand the solubility of proteins better (Timasheff & Arakawa, 1988).

Practically nothing is known on the physical chemistry of nucleic acid crystallization (*e.g.* Malinina, Tereshko, Ivanova & Borovik, 1991) or of nucleoprotein complexes. Easier preparation of DNA and RNA molecules by synthetic and macromolecular engineering methods will facilitate future studies. The physics of hydrophobic membrane protein crystallization is also in its infancy. Here new routes may be explored also. Two-dimensional crystallization methods will certainly develop and facilitate electron microscopy crystallography, not only for membrane proteins, but for macromolecules in general (*e.g.* Uzgiris & Kornberg, 1983; Schultz, Célia, Riva, Sentenac & Oudet, 1993). Theoretical approaches (Higo, Endo & Nagayama, 1992) and the search for new materials [*e.g.* as used for polymer crystal growth by Wittmann, Thierry & Lotz (1988)] that would orient proteins could bring interesting advances, as illustrated by the first data on epitaxial growth in the protein field (McPherson & Shlichta, 1988). From another point of view, growth of protein or nucleic acid crystals may be controlled or modified by additives able to specifically interact with the macromolecules. Here, advantage can be taken of the concepts explored experimentally in the small-molecule field (Weissbuch, Addadi, Lahav & Leiserowitz, 1991). Finally, active control of nucleation and growth parameters should permit tailoring of crystal shape and morphology. This should lead to the production of large single crystals of proteins, that could be used for special applications (*e.g.* neutron diffraction, crystal physics).

5.2. *Biological trends*

5.2.1. *Crystallization of selenium-containing proteins and use of anomalous scattering.* The anomalous-scattering properties of selenium make this atom an attractive marker of protein crystals for phase determination (Hendrickson, Horton & LeMaster, 1990), the other bottleneck in X-ray crystallography. Selenium can be incorporated *in vivo* into proteins as seleno-methionine at methionine positions under conditions where exogeneous seleno-methionine competes for methionine during protein synthesis. The resulting proteins keep their properties essentially unchanged, in particular their crystallizability [for practical details related to crystal growth see Doublié & Carter (1992)]. The first structure determination that took advantage of this possibility was that of RNase H (Yang, Hendrickson, Crouch & Satow, 1990). In future, engineering selenium-containing proteins may become more versatile. Indeed, under normal physiological conditions, selenium is incorporated into proteins as seleno-cysteine residues at particular stop-codon positions. The underlying metabolic pathways are very complex (Böck, Forchhammer, Heider & Baron, 1991) and require special mRNA structures (Heider, Baron & Böck, 1992). Thus, the possibility exists of

engineering mRNA sequences in such a way that seleno-cysteine would be incorporated at any position desired in a protein.

5.2.2. *Crystallization of mutants, structural domains, and of artificial macromolecules.* Crystallization of protein variants will certainly develop in future, but not necessarily aimed at studying crystal growth. However, in cases where large numbers of mutants are produced, their systematic crystallization may contribute to finding the structural rules underlying macromolecular crystal growth. This is the case for T4 lysozyme which is presently a good model to correlate structural alterations in a protein with its crystallizability (*e.g.* Brennan, Wozniak, Faber & Matthews, 1988; Eriksson, Baase & Matthews, 1993).

Synthetic methods have already been used to prepare and crystallize designed peptides (*e.g.* Eisenberg, Wilcox, Eshita, Pryciak, Ho & DeGrado, 1986) and in fact protein domains were employed long ago for easier crystallization experiments. For instance, a trypsic fragment of methionyl-tRNA synthetase crystallizes as a monomer (Waller, Risler, Monteilhet & Zelwer, 1971) while the dimeric native enzyme could never be crystallized. Thanks to macromolecular engineering, design of chimeric and even completely artificial proteins will certainly develop in future. Promising attempts have been made in those directions concerning crystallization of alloproteins containing non-natural amino acids (Judice, Gamble, Murphy, de Vos & Schultz, 1993).

Crystallization of domains is the only possible way to reach the fine structural knowledge of large RNA molecules (introns, catalytic RNAs, regulatory structures in mRNAs, *etc.*). Approaching this goal requires the design of compact structures corresponding to active and biologically significant RNA cores. Computer modeling based on chemical probing of RNA, and, if possible, on phylogenetic sequence comparisons, is the method of choice for the design of such structures. The method has already been employed to define autocatalytic RNA cores (*e.g.* Michel & Westhof, 1990) and viral rRNA-like domains (*e.g.* Felden, Florentz, Giegé & Westhof, 1994). It is expected that engineering of such domains will lead in the near future to the preparation and crystallization of biologically significant structures.

5.2.3. *Crystallization of antigen–antibody complexes.* In cases where macromolecules fail to crystallize or yield crystals of poor quality, an attractive method could be their crystallization as antigen–antibody complexes (Laver, 1990). This strategy has already been used to solve protein structures (Bentley, Boulot, Riottot & Poljak, 1990) and could become the method of choice for crystallizing proteins that are too flexible in their free state.

5.2.4. *Biomimetism.* A last example of novel trends is the research aimed at designing self-assembling macromolecular architectures. Attempts to construct crystal-like scaffoldings of nucleic acids were undertaken by Seeman (1991), and a cube-like molecule could already be prepared. To do this, appropriate DNA sequences were synthesized that could be hybridized and ligated so as to form double-stranded structures with branched junctions that assemble in the desired geometry.

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