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José Luis R. Arrondo
Alicia Alonso (Eds.)

Advanced Techniques in Biophysics

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With 93 Figures, 17 in Color and 2 Tables

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Preface

The present book of the Springer Series in Biophysics deals with some techniques that are being implemented nowadays. One of the motors that have driven the biosciences, like daily life, has been the technological boost produced by the advancement of microprocessor technology. A whole array of possibilities have been opened to develop the classical techniques that were used some years ago.

Abrahams and coworkers contribute with a chapter on protein nanocrystallography which deals with obtaining protein crystals in small, confined volumes, trying to overcome one of the setbacks in crystallography, the amount of material needed to obtain good samples for diffraction. This chapter is followed by one by Ibarra-Molero and Sanchez-Ruiz reviewing the recent advances of differential scanning calorimetry in the field of protein energetics and also in the energetic analysis of other biological systems. The following two chapters look at recent advances of IR spectroscopy. IR reflection--absorption spectroscopy (IRRAS) looks at the air--water interface of membranes and in the chapter by Mendelsohn and coworkers the general basis as well as the application to lipids and peptides or proteins are reviewed. Arrondo and coworkers address the analysis of IR spectra by a new approach called two-dimensional generalized spectroscopy, where information on protein changes after a perturbation is analysed by synchronous or asynchronous maps. This approach, essentially different from that of 2D-NMR spectroscopy, uses correlation analysis of the dynamic fluctuations caused by an external perturbation to enhance spectral resolution.

Three chapters are devoted to different technical developments of NMR. Szypersky deals with the principles of ultrafast NMR spectroscopy through the use of G-matrix Fourier transform (GFT) NMR as a technique for rapid sampling of multidimensional NMR data. Freeman and Kup e approach the problem of fast multidimensional NMR by outlining two radical new approaches, one using spatially encoded single-scan multidimensional NMR and the other using projection--reconstruction of multidimensional spectra. Size is one of the problems that NMR has to face in the study of proteins, Fernández and Wider analyse the use of transverse relaxation-optimized spectroscopy (TROSY) in combination with isotope-labelling techniques to extend applications of NMR spectroscopy in solution to much larger molecules, such as integral membrane proteins in detergent micelles, large proteins in monomeric form and in macromolecular complexes, and intermolecular interactions in large complexes.

Carrión-Vázquez and coworkers have addressed protein nanomechanics, a new multidisciplinary area of research to directly measure mechanical forces in single molecules, by applying atomic force microscopy (AFM). Large unilamellar

vesicles are the subject of the chapter by Bagatolli, who reviews the use of two-photon fluorescence microscopy in studying the lateral structure of compositionally simple vesicles and more complicated membranes. San Martín and Valle look at the three-dimensional organization and structural features of macromolecular assemblies, knowledge of which is indispensable for understanding their functions, by using three-dimensional electron microscopy.

This book constitutes a privileged observatory for reviewing novel applications of biophysical techniques that can help the reader utilize the efforts of the scientists contributing to the volume to enter an area where the technology is progressing quickly and where a comprehensive explanation is not always to be found.

Bilbao, June 2006

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Protein Nanocrystallization

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1.1 Introduction

There is no theory that allows us to predict when or where proteins will crystallize. However, for several reasons the problem is a very pertinent one, especially when we consider crystallization of proteins that are physically confined within a very small volume.

There is also a practical reason for studying protein crystallization in small, confined volumes: crystals are required for determining three-dimensional protein structures by X-ray crystallography. As crystallization conditions can only be found through trial and error, current practice requires simultaneous testing of many different conditions. The obvious idea that minimizing the volume of single tests maximizes the number of different conditions that can be screened with a given quantity of protein prompted the development of high-throughput nanocrystallization systems (Stevens 2000; Rupp 2003a, b; Bard et al. 2004).

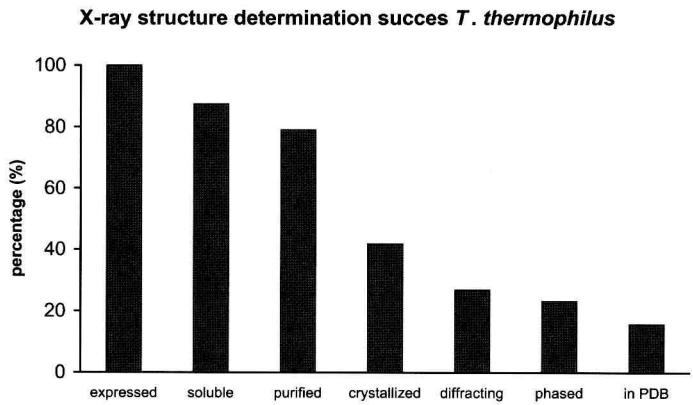

Fig. 1.1. The success rate of high-throughput crystallization. The overall success of the different stages in the high-throughput approach used by the RIKEN consortium is shown. The numerical data were presented at the ICCBM10 conference in Beijing by S. Yokoyama and represent the throughput obtained using expression in *Thermus thermophilus*. The high overall success rate in this example is not typical and expression in higher organisms shows a lower success rate

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Although nanocrystallization is quickly becoming a mainstream method, the crystallization step remains the major bottleneck in the structure production process (Blundell and Patel 2004). This is illustrated by recent data from a large structural genomics initiative, indicating that the least successful step in going from sequence to structure is the one from purified protein to crystal. Note that the overall trend illustrated in Fig. 1.1 is not very different from a report predating the widespread use of nanocrystallization (Chayen and Saridakis 2002; Chayen 2004). Probably microheterogeneity of the proteins is the prime cause of this bottleneck.

Constructing genetic variants and developing more advanced means of protein production and purification might increase the success rate. Nevertheless, advances in nanocrystallization should also accompany this, as nanocrystallization favors throughput whilst substantially reducing demands on large-scale production and purification platforms.

Here we focus on miniaturization aimed at increasing the probability of finding crystallization conditions when the amount of protein available is limited. First we will review current understanding of nucleation and crystallization of proteins, and focus mainly on those aspects affected by the volume of the mother liquor. Subsequently we will review in detail the major practical obstacles typical of protein nanocrystallization. Problems typically associated with nanovolumes (500 nL or less) concern their dispensing, evaporation and mixing[1]. We also discuss the limits imposed by the design of substrates suitable for storing liquid arrays, the robotic accuracy of dispensing strategies, and strategies for scoring nanocrystallization trials.

1.2 Nucleation and Crystallization in Nanovolumes

Naively, one might think that the protein concentration determines the level of supersaturation regardless of the volume. However, this may not be the case, considering that in tiny droplets the surface tension forces become relevant and below a certain volume even predominant. Inside a small nanodroplet the pressure can be substantially higher than the ambient pressure and can be calculated using the Young–Laplace equation (for a review see de Gennes 1985; Blokhuis 2004). However, these effects are less likely to influence protein crystallization in the microliter range. The pressure difference between the inside of a water droplet of 100-μm radius and the gas phase for a surface tension of 72 mN/m is only equal to 1.44 kPa (kN/m^2). Giegé and coworkers studied the influence of external hydrostatic pressure on the nucleation and growth of lysozyme crystals and reported that increasing the pressure from 0.1 MPa (atmospheric pressure) to 250 MPa leads to reduction of the size and number of lysozyme crystals. Moreover a transition to urchinlike particles made of crystalline needles progressively occurs (Lorber et al. 1996; Kadri et al. 2003).

These considerations are obviously irrelevant when the protein is confined within a lipid membrane and thus do not apply for proteins dissolved in the cytoplasm

[1] Classical numerical rounding separates the nanoliter from the microliter range: less than 0.5 is rounded to zero, if one wants to define the nanoliter regime its upper boundary is 500 nL.

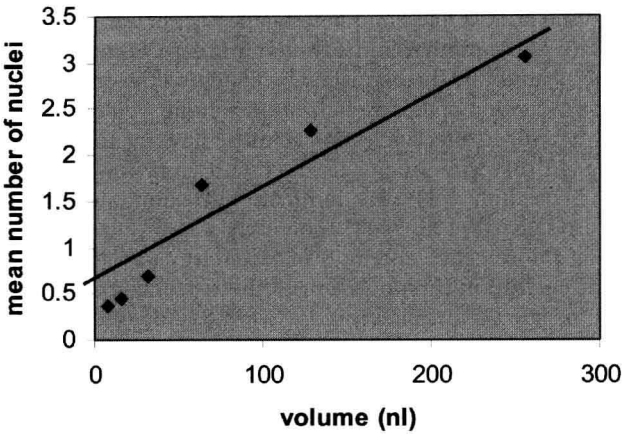

Fig. 1.2. Heterogeneous nucleation in submicroliter volumes. The average number of tetragonal crystals per droplet detected 24 h after mixing as a function of the volume of the droplet. Each data point is the count obtained from 16 droplets. In the smaller droplets needlelike crystals showed a higher relative abundance. (From Bodenstaff et al. 2002)

of living cells. The pressure inside a living cell is well regulated and partially determined by the presence of surrounding tissue. In plant cells the turgor or intracellular pressure can reach several atmospheres at most (Tomos and Leigh 1999).

For practical purposes it is more important that the homologous nucleation rate in protein crystallization is theoretically determined by the level of supersaturation, and it is independent of the volume of the mother liquor. If at a certain level of supersaturation it takes on average a full day to form a stable nucleus that grows into a macroscopic protein crystal in say 1 μL, then it would take 50 days on average for a similar event to occur in a volume of 20 nL. If the nucleation rate per unit volume is constant, reduction of the crystallization volume therefore results in a reduced chance of finding crystals. In other words, one has to increase the level of supersaturation in nanoliter crystallization trials in order to observe rare nucleation events. The relation between the crystallization volume in submicroliter volumes and the observed number of crystals is shown in Fig. 1.2 and indicates that there is a dependence on the droplet volume (Bodenstaff et al. 2002). The relation appears to be linear, but does not go through the origin, indicating that a basic assumption of the homogeneous nucleation theory is not satisfied. This suggests that heterogeneous nucleation plays an important role in low volumes. Vekilov et al. report that despite precautions, heterogeneous nucleation is always observed in their experiments and led to a nonzero intercept of the linear dependence of N (mean number of observed crystals) as a function of the induction time, Δt, in a volume of 700 nL (Galkin and Vekilov 1999; Chernov 2003; Vekilov and Galkin 2003).

Note that although the probability of finding a crystal is very low, a nucleus can always be formed owing to a spontaneous (homogeneous) nucleation event because of density fluctuations (ten Wolde and Frenkel 1997) At this point two types of heterogeneous nucleation should be distinguished: heterogeneous nucleation that de-