Preparation of protein single crystals for electron diffraction

Nastasja Viktorovna Samochvalova[1,2]

[1]Department of Solid State Physics, Faculty of Nuclear Sciences and Physical Engineering, Czech Technical University in Prague
[2]Laboratory of Structure and Function of Biomolecules, Institute of Biotechnology of Czech Academy of Sciences

Abstract

The use of electron crystallography in structural biology is gradually increasing due to the properties of electrons such as strong interaction with matter and negative charge. Using these properties, it is then easier to determine the position of ions than in X-ray structural analysis or to study crystals of very small dimensions. Procedures for the preparation of protein crystal samples for electron diffraction as well as the subsequent processing of diffraction data are gradually being standardized. This work deals with the preparation of microcrystals of two selected proteins, thaumatin and a bilirubin oxidase mutant, by changing already known crystallization conditions and the subsequent detection of these microcrystals using SONICC technology.

Keywords: Protein crystallography, electron structural analysis, crystal growth.

Introduction

Proteins are organic substances consisting of amino acids linked by a peptide bond. Knowledge of the structure of these biopolymers is very important and often crucial for understanding various biological processes, drug development and much more. Methods such as nuclear magnetic resonance, diffraction-based methods or the now emerging cryo-electron microscopy are used to study proteins. So far, X-ray diffraction analysis remains the most widely used diffraction method for protein structure determination.
In general, for the phenomenon of diffraction to occur, an object with a periodic arrangement, such as a crystal, is needed. Although there are exceptions, most proteins are not crystalline in nature. Finding the right conditions for protein crystal growth is often a time-consuming task. In addition, for X-ray diffraction, crystals need to be large enough, which some proteins simply do not form. Therefore, electron crystallography, which uses crystals with dimensions of several tens of micrometers, has also been developed in the last few years.
In addition, electron diffraction makes it easier to identify light atoms such as hydrogen and shows a higher sensitivity to charged groups. These properties are due to the strong Coulombic interaction of individual electrons with matter.
The first protein structure determined by Micro-ED method was the tetragonal chicken egg white lysozyme in 2013, followed by several other structures successfully determined previously by X-ray diffraction. More recently, Micro-ED was used to determine the structure of the metalloenzyme SaR2lox, which was previously unknown [2]. In this experiment, SaR2lox crystals were diffracted at a resolution of 3.0 Å. Although the Micro-ED method was originally developed for the structural study of biological substances, it could have a major impact on study methods in various fields of chemistry and drug development. It can provide high atomic resolution structures of complex and small molecules without much preparation of the sample itself.

Theory

0.1 X-ray and electron diffraction

X-rays are most commonly used for diffraction experiments, as their wavelength is comparable to the interatomic distance in a crystal. X-rays interact only with the electrons in the electron shell. For this reason, it is difficult to determine the exact position of the hydrogens from this method since they have only 1 electron. The depth of penetration of X-rays is determined by the intensity of the source and the material under investigation and can be up to several millimetres. The relationship between the intensity obtained by X-ray diffraction and the volume of the crystal is given by Darwin's relation:

$$I_{hkl} = I_0 r_e^2 \frac{V_{crystal}}{V} \frac{\lambda^3 L}{\omega V} P A |\mathbf{F}_{hkl}|^2, \tag{1}$$

where I_{hkl} is the measured intensity, I_0 is the intensity of the incident beam, r_e is the classical radius of the electron, $V_{crystal}$ is the volume of the whole crystal, and V is the volume of the ground cell, λ is the wavelength of the X-ray beam, L is the Lorentz factor, ω is the angular frequency, P is the polarization factor, A is the absorption factor, and $\mathbf{F}_{hkl}$ is the structure factor.

As can also be seen from the relation (1), the problem of studying single crystals with X-rays arises when studying crystals with small volume. Electron diffraction therefore appears to be a potential alternative in the study of protein microcrystals.

Electrons interact strongly with matter because the interaction is not only with the electrons in the electron shell, but also with the nucleus [3]. This leads to a larger matter scattering cross section for electrons than for X-rays. High-energy electrons have shorter wavelengths (0.0251–0.0197 Å for 200–300 keV photons) than X-rays (1.0332 Å for 12 keV photons) [4].

Electrons also cause several orders of magnitude less damage than X-rays per elastic scattering, which is very important in the study of biological macromolecules.

0.2 Protein crystallization

Protein crystals are macromolecular crystals, therefore the search for crystallization conditions for protein crystal growth is an empirical and complex process. There is no theory that leads flawlessly to the desired results. Because of the complicated structure of protein molecules and the relatively weak interaction between individual protein molecules in solution per unit molecular surface area, the crystallization process is very sensitive to the physical and chemical conditions of the system [5]. In experiments using X-rays, the aim is to produce small numbers of large single crystals. For this purpose, it is important that the system has a small nucleation rate and gradually reaches the metastable zone. In the case of experiments using electrons, microcrystals are used and therefore the nucleation rate must be very high. For this purpose, additives can be added to the crystallization solutions to expand the nucleation zone of the protein in question [6].
Traditional crystallization methods such as the batch method, microcrystallization under oil or diffusion-based methods such as the sitting and hanging drop method can be modified to grow microcrystals. Moreover, due to the requirement for small crystals, non-standard methods such as in-cell crystallization or crystallization of proteins using liquid-liquid phase separation, which is typical only for some proteins, can also be used.

Results and Discussion

The proteins thaumatin and bilirubin oxidase were selected for experiments to optimize crystallization methods for targeted growth of protein microcrystals. Several crystallization methods such as the sitting and hanging drop method and the microcrystallization under oil were used to produce thaumatin crystals. To modify these conditions for the growth of micro- and nanocrystals, two approaches were used, namely changing in the concentrations of crystallization and protein solution or changing the ratio of the volumes of crystallization solution to protein solution. In the case of thaumatin, the hanging drop method was found to be the most suitable method for the preparation of microcrystals, where the ratio of the volumes of protein solution to crystallization solution was 8:10. In this experiment, the same initial crystallization condition was used in all drops: $20\,\%\frac{w}{v}$ Potassium sodium tartrate tetrahydrate (NaKTart), $15\,\%\frac{v}{v}$ ethylene glycol and 0.1 M BIS-TRIS at pH 6.6, and the same protein solution containing $50\,\mathrm{mg}\cdot\mathrm{ml}^{-1}$ thaumatin and 275 mM Sodium acetate (NaAc). As can be seen in Fig.1 and Fig.2, microcrystals grew in the droplet at the given ratio. The formation of these microcrystals under these conditions could be monitored using the patented technology SONICC (Formulatrix).

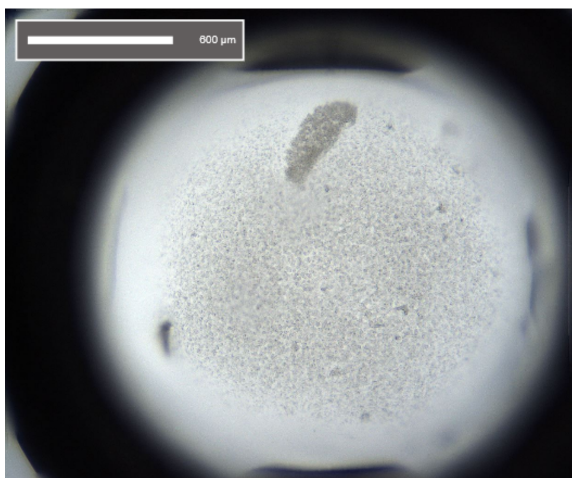

Figure 1: Thaumatin microcrystals grown by the hanging drop method. Ratio of protein solution to crystallization condition 8:10. With a crystallization condition of $20\,\%\frac{w}{v}$ NaKTart, $15\,\%\frac{v}{v}$ ethylene glycol, 0.1 M BIS-TRIS at pH 6.6 and a protein solution of $50\,\mathrm{mg}\cdot\mathrm{ml}^{-1}$ thaumatin and 275 mM NaAc. The line corresponds to 600 µm. (RI 1000)

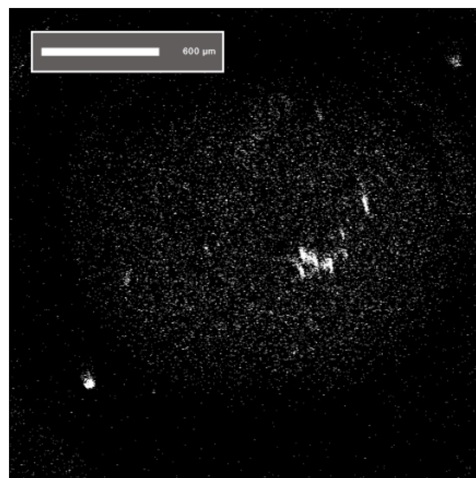

Figure 2: Image of the same droplet taken with SONICC technology in SHG mode, where visible particles correspond to protein microcrystals. The line segment corresponds to 600 µm. (RI 1000)

In the case of bilirubin oxidase, several functional variants were prepared for the purpose of the analysis of the role of the Trp396-His398 adduct. In the experiments described in this paper, one of these variants, more precisely MvBOxW396A, was worked with when Trp396 was mutated to alanine. This protein was prepared and described in detail in the work of Kovale et al. [7].

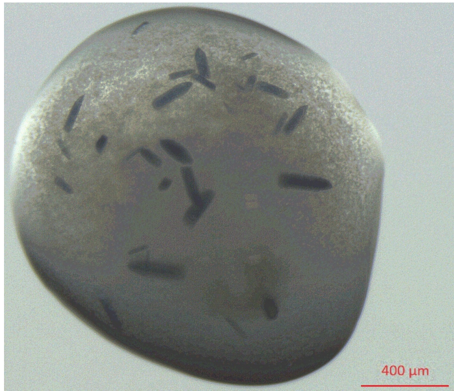

Figure 3: Crystals of MvBOxW396A, growth by the hanging drop method. Crystallization condition 0.09 M succinic acid, 14 % $\frac{w}{v}$ PEG 3350 and protein solution 25 mM Tris/HCl, pH 7.5 with 250 mM NaCl and 25 mg · ml^{-1} MvBOxW396A. The line corresponds to 400 µm. (Olympus Stereomicroscope)

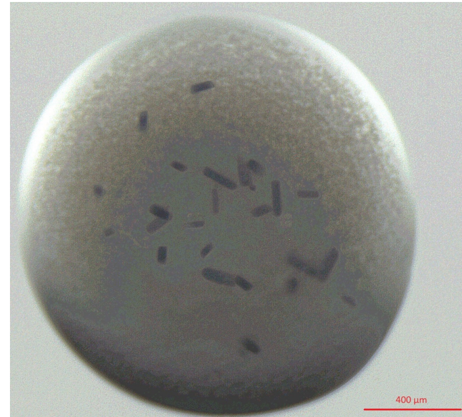

Figure 4: Crystals of MvBOxW396A, growth by the hanging drop method. Crystallization condition 0.1 M succinic acid, 16 % $\frac{w}{v}$ PEG 3350 and protein solution 25 mM Tris/HCl, pH 7.5 with 250 mM NaCl and 25 mg · ml^{-1} MvBOxW396A. The line corresponds to 400 µm. (Olympus Stereomicroscope)

For crystallization were used known crystallization conditions from [7]. Under these conditions, characteristic blue crystals of bilirubin oxidase grew, suitable in size for X-ray diffraction. The crystallization condition contained 0.09 M succinic acid, 14 % $\frac{w}{v}$ PEG 3350 (see Fig.3) or 0.1 M succinic acid and 16 % $\frac{w}{v}$ PEG 3350 (see Fig.4).

The optimization of crystallization conditions for the formation of microcrystals was performed by changing the ratio of crystallization solution to protein solution. In these experiments, bilirubin oxidase crystals were of different sizes, but neither microcrystals nor nanocrystals were detected. Thus, microcrystals of the bilirubin oxidase mutant either did not form or there may have been a problem in the actual detection of possible micro- or nanocrystals, due to the fact that bilirubin oxidase crystals grow most often from the precipitate, which is still partially present after crystal growth and may noticeably attenuate the SHG or UV-TPEF signal when detected by SONICC technology. Thus, it can be said that the application of the techniques used in the case of this target molecule is more complicated and requires further development of procedures to achieve unambiguously detectable growth of micro- and nanocrystals even with a small amount of sample.

Conclusions

The aim of this work was the modification of the crystallisation conditions for the formation of detectable micro-crystals. For selected proteins thaumatin and bilirubin oxidase, crystallization conditions were successfully found and crystals were grown. For thaumatin, the conditions for microcrystal formation were also successfully found by varying the ratio of the volumes of crystallization solution to protein solution and by varying the concentrations of the two solutions. The best crystallization method with emphasis on microcrystal formation in the case of thaumatin was the hanging drop method, where the ratio of protein solution to crystallization solution was 8:10. In the case of bilirubin oxidase, crystallization conditions suitable for X-ray diffraction were found.

The droplets were observed using the patented SONICC technology and in some cases a signal was detected in the SHG mode. In the case of bilirubin oxidase, only crystals suitable for X-ray diffraction but too large for electron diffraction could be detected. For the time being, SONICC technology is an inseparable and very important part of the microcrystal preparation process, as modes such as SHG and UV-TPEF can be used to detect very small crystals that would not be visible with other techniques.

The diffraction properties of the obtained microcrystals were also verified in an electron microscope. Diffraction was not observed probably due to damage of the crystals during transfer to the grid.

It appears that sample preparation for this structural technique with small amounts of rare material is not yet fully resolved and requires further development and optimization. This may include the introduction of new procedures aimed at targeted formation of microcrystals and nanocrystals with the least possible sample consumption.

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