Chapter 3

Preparing 3D protein nano-crystals for electron diffraction studies

Abstract

Single crystal X-ray diffraction is still the leading technique for structure determination in the solid state. However, one of the major bottlenecks in general and particularly in protein crystallography is the limited availability of well diffracting macro-crystals. Often only micro- or nano-sized protein crystals grow and there are no standard protocols for optimizing such crystals (if they can be optimized at all). Since electrons interact much more strongly with matter than X-rays we investigated whether electron diffraction can be applied as an alternative technique for studying proteins. 3D nano-crystals of potato serine protease inhibitor and lysozyme, grown as described in Chapter 2, were used for testing. The diffraction signal obtained from nano-crystals of both proteins indicated that the crystals are well ordered and can be used for diffraction studies.

In this chapter we describe how to optimally prepare 3D protein nano-crystals for electron diffraction studies.

3.1 Introduction

Despite substantial progress in crystallization techniques, growing protein crystals remains a process of trial and error. Development of protein nano-crystallization methods allows thousands of crystallization trials to be set up in a single experiment. However, even if crystals are obtained in nano-volumes there is no guarantee that the crystallization experiment can be reproduced in larger volumes and that macro-crystals can be further grown [1].


Heterogeneous crystallization techniques relax to a certain extent the requirement for high protein concentrations needed to induce nucleation, facilitating crystallization in this respect. However, even using heterogeneous nucleants, crystal growth is not easily controlled. Often showers of crystals are obtained and those crystals need to be further optimized for X-ray studies. If optimization is not possible, X-ray diffraction techniques cannot be used for structure determination (nano-crystals are far too small to be studied even with synchrotron X-ray sources).

The development of synchrotron X-ray powder techniques relaxes the requirement for macroscopic single crystals in special cases [2]. Powder diffraction usually yields accurate unit cell parameters and in some cases it may allow a full structure determination. Unfortunately, the collapse of three-dimensional reciprocal space into a one-dimensional powder diffraction pattern leads to a significant loss of information. There is not only the usual phase problem, but due to significant peak overlap it is frequently not even possible to determine the intensity of the individual Bragg reflections, but only their sum. This has limited ab initio structure determination from powder diffraction to smaller molecules, though in favorable conditions protein structures have been determined with this technique. Another practical requirement which is also a severe limitation in the case of proteins is that milligram quantities of crystalline powder need to be available for successful structural studies.

Protein crystals absorb energy during data collection, which reduces their crystallinity and the resolution in a predictable fashion. The X-ray dose (energy per unit mass) a cryo-cooled crystal can absorb before the diffraction pattern decays to half of its original intensity is defined as $2 \times 10^7$ J/kg (the “Henderson limit”) [3]. Since modern synchrotron X-ray sources have sufficient brilliance to very quickly reach the Henderson limit, beyond which further data collection is no longer useful, radiation damage has become the second main limiting factor in X-ray crystallography of proteins, especially when only micro-crystals are available.

However, the statistics are much more favorable in the case of electrons. The main advantage of electrons is that for each elastic scattering event, accompanying inelastic event they deposit at least three orders of magnitude less energy in the sample, compared to X-rays [4]. Hence even sub-micron crystals can still yield useful diffraction data when electrons are used rather than X-rays. This explains the success of electron diffraction of 2D crystals of (membrane) proteins, which are far too thin for diffraction analysis by X-rays. In view of the success of electrons in 2D protein crystallography, electron diffraction
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should be an attractive alternative for 3D protein nano-crystals as well, since the high scattering cross-section of electrons allows very small crystalline volumes (more than $10^6$ times smaller than those required for X-rays) to be studied. A comparison of the atomic cross-section for electrons, X-rays and neutrons as a function of wavelength is given in Figure 3.1. The ratio of inelastic to elastic scattering events is also more favorable in the case of electrons (see Table 3.1).

![Figure 3.1 Comparison of the atomic cross-section for electrons, X-rays and neutrons as a function of wavelength. (Henderson, 1995)](image)

Table 3.1 shows that relative to the number of elastic scattering events, electrons cause less radiation damage compared to X-rays [4]. Because of the lack of sufficiently bright neutron sources the use of neutrons for studying biological samples is not feasible yet. For X-rays, the amount of damage per useful elastic scattering event is several hundred times greater than for electrons at all wavelengths and energies. Consequently, the
requirements on specimen volume are correspondingly higher. Therefore, electrons provide at present the most information for a given amount of radiation damage.

Table 3.1 Comparison between the energy deposited per elastic and inelastic scattering by electrons, X-rays and neutrons. (From Henderson, 1995)

<table>
<thead>
<tr>
<th></th>
<th>Electrons 80-500 keV</th>
<th>X-rays 1.5Å</th>
<th>Neutrons 1.8Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio (inelastic/elastic) scattering events</td>
<td>3</td>
<td>10</td>
<td>0.080</td>
</tr>
<tr>
<td>Energy deposited per inelastic event</td>
<td>20 eV</td>
<td>8 keV</td>
<td>2 keV</td>
</tr>
<tr>
<td>Energy deposited per elastic event</td>
<td>60 eV</td>
<td>80 keV</td>
<td>160 eV</td>
</tr>
<tr>
<td>Energy deposited relative to electrons (inelastic)</td>
<td>1</td>
<td>400</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1000</td>
<td>2.5</td>
</tr>
</tbody>
</table>

The development of cryo-electron microscopy was an important step, as it allowed at present thin biological specimens to be preserved in a frozen-hydrated state. While maintaining specimens at liquid nitrogen temperature or colder, the samples can be introduced into the high vacuum of the electron microscope column and observed at cryogenic conditions. The low temperature of cryo-electron microscopy provides also an additional protective factor against radiation damage [5].

Since protein crystals contain 50-70% water and need to remain intact, most of the cooling techniques, including high pressure or spray freezing, cannot be used. Because of the very small size of the crystals, the “fishing” techniques used in X-ray crystallography of selecting and mounting individual crystals for freezing, are anything but trivial. Immersion or plunge freezing therefore seems to be by far the best choice
for preservation of 3D nano-sized protein crystals. The higher thermal capacity of liquid ethane compared to liquid nitrogen, as well as the development of robotic systems for automated high-speed plunging of the specimen, can reproducibly lead to successful vitrification of three-dimensional biological samples.

A protocol for vitrification of thin 3D protein nano-crystals (in particular lysozyme) is presented in this chapter. Ice formation or secondary contamination of ice during the sample transfer into the microscope present additional problems and may impede successful cryo-electron experiments. An optimization of the cryo-transfer system is introduced and the advantages of the system over existing cryo-transfer systems are discussed. Initial diffraction experiments using selected area diffraction mode are also reported.

3.2 Experimental procedures

3.2.1 Materials

Commercial proteins used in this study were chicken egg-white lysozyme (Sigma, EC 3.2.1.17). Potato serine protease inhibitor 6.1 (the number representing the isoelectric point) was expressed and purified following the procedure given by Thomassen et al., [6]. All the chemicals used for the crystallization experiments were purchased from Merck and solutions were further filtered with Millipore filters (0.22 µm) directly before use.

3.2.2 Crystallization experiments

Crystallization trials were carried out at 20°C using the sitting drop vapour diffusion technique in Q-crystallization plates (Hampton Research). Portions of 1 µL protein were added to 1 µL of reservoir solution in a sitting drop containing 7.5 mg/ml lysozyme, 0.1 M acetate buffer (pH 4.5), 1.6 M NaCl. Potato serine protease inhibitor was crystallized in 0.1 M HEPES (pH 7.5), 5% PEG8000 and 4% ethylene glycol complemented with 0.1 M glycine (Hampton Additive screen 2). The protein concentration was 7 mg/ml. Strands of hair were introduced in the crystallization droplets of both proteins, inducing nucleation and the growth of nano-crystals on its surface.
3.2.3 Sample preparation and preliminary electron diffraction of the nano-sized protein crystals

Chemical fixation

A hair fibre covered with tiny crystals of lysozyme or potato protease inhibitor was placed on a negatively charged 300 mesh continuous carbon grid. Next, a few microliter of the crystallization solution with the same composition as used for the crystal growth was pipetted onto the grid, resulting in detachment of most of the crystals. After that, the hair fibre was removed. The excess of buffer was blotted away with a filter paper and the crystals were negatively stained with 2% uranyl acetate.

Plunge freezing

Thin lysozyme crystals were grown as described in Chapter 2. For the freezing of the crystals three different cryo-solutions were prepared - one containing 1.6 \( M \) NaCl and 0.1 \( M \) Acetic buffer (pH 4.5) as in the mother liquor and the other two with the same composition, but additionally including 10% and 15% glycerol as cryo-protectant. The crystals were transferred on an electron microscopy grid as described in [7] and a few \( \mu \)L of a cryo-solution were pipetted onto the grid. Using the Leiden Vitrification System the grid was blotted between filter papers to remove the excess of the cryo-solution and plunge-frozen in liquid ethane. Two different kinds of 300-mesh electron-microscopy grids were used, with continuous and holey carbon film respectively.

Preliminary electron diffraction studies

Electron diffraction measurements were performed on a Philips CM30T LaB6 and CM200 FEG working at 300kV and 200kV respectively. A Gatan liquid nitrogen specimen holder was used for the data collection. The data were collected at -160º C, using selected area diffraction in low dose mode and recorded using a CCD camera.
3.3 Results and discussion

Small protein crystals are generally considered to be unsuitable for crystallographic studies. Whereas this assumption is valid for X-ray single crystal diffraction, it is not true for electron diffraction. In fact, for electron diffraction experiments the crystals have to be very thin because electrons undergo also coulombic interactions with matter which are $10^8$ stronger than electromagnetic interaction.

For electron diffraction experiments we used nano-sized lysozyme and potato protease inhibitor crystals grown on strands of hair. The advantage of using heterogeneous crystallization was that all the small crystals could be transferred easily onto an electron microscopy grid by transferring the fibre. No additional “fishing” techniques were needed.

Lysozyme was chosen as a test model since the protein has been studied extensively by X-ray crystallography techniques. Therefore it was possible to compare structural information obtained from X-ray and electron diffraction studies. Potato protease inhibitor (6.1) was selected as an example of a protein from which mostly small crystals are obtained and the optimization of the crystals for X-ray diffraction studies is rather difficult. Moreover, the crystals tend to intergrow which makes fishing out a single crystal for X-ray analysis is more difficult. Potato protease inhibitor was observed to crystallize readily on the hair fibres, as shown in Figure 2.4 in Chapter 2.

Transferring of the crystals on an electron microscopy grid did not present additional technical difficulties. It was also possible to image individual crystals, showing that they are suitable for electron crystallography studies (see Figure 3.2).

Optimizing the specimen preparation is an important step towards the acquisition of high quality diffraction data. Substantial progress in rapid freezing techniques allows biological structures to be studied in their hydrated state. However, formation of crystalline ice often occurs when the excess of water can not be removed completely. Ice crystals yield strong diffraction signals which disturb the diffraction produced by the protein crystals. Most importantly the formation of ice causes structural damage and may destroy the crystallinity of proteins.

There are no generally applicable rules for specimen preparation. Optimizing the thickness of the ice film is largely a process of trial and error However, from crystallization theory it is known that on increasing the viscosity of liquid water all rearrangements including the formation of nuclei occur more slowly.
Therefore compounds with high viscosity such as PEG, glycerol etc. are generally used as cryo-protectants for sample preparation in X-ray crystallography studies.

For the vitrification of the lysozyme nano-crystals we combined aspects of freezing techniques from X-ray crystallography and cryo-electron microscopy. The nano-crystals were plunge-frozen in liquid ethane. Three different cryo-solutions were used – containing 0%, 10% and 15% glycerol, respectively. Compared to X-ray crystallography, where the use of cryo-protectants is essential for the preservation of macro-crystals and their selection is often the most difficult step in the freezing procedure, the protein nano-crystals could be vitrified successfully in liquid ethane without any additives or cryo-protectants. This relaxes sample preparation requirements compared to X-ray crystallography where cryo-protectants are essential for the preservation of macro-crystals and the optimal cryo-protecting conditions need to be identified for each case individually. However, the formation of hexagonal ice was substantially minimized when the nano-crystals were frozen in cryo-solutions containing 10% glycerol. Vitrification of the crystals with 15% or even higher percentages of glycerol appeared to be less successful as this increased the background in the diffraction patterns. A schematic representation of plunge freezing and an electron micrograph of vitrified lysozyme nano-crystals are given in Figure 3.3.

![Figure 3.2](image)

**Figure 3.2** Electron micrograph of a negatively stained crystal of potato protease inhibitor (left image); Inset of diffraction pattern acquired from a negatively stained crystal of potato protease inhibitor (right image).
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Figure 3.3 Schematic representation of the vitrification of lysozyme nano-crystals by using immersion (plunge) freezing techniques. The bar is 2 µm.

Optimizing the cryo-transfer system is a continuing problem in electron microscopy. From the user's point of view the specimen-exchange procedure is one of the most important and technically most difficult parts of cryo-microscopy. Therefore the transfer system needs to provide a fast, smooth and relatively easy mechanism of exchange. Most of the commercially available Gatan cryo-holders require the use of threaded rings which need to be screwed on top of the grid. The system is difficult to manipulate in a fast and accurate way and especially the screwing on the clamping ring liquid nitrogen causes problems. This often limits the speed and efficiency of specimen exchange. The design of the home modified transfer device we used for our experiments is given in Figure 3.4.

This system uses a sliding mechanism that is easier to manipulate. The sliding devices are also relatively big and therefore easier to handle under liquid nitrogen. This facilitates specimen exchange shortening the time for loading and reducing the chance for specimen warming and the formation of cubic ice.
The diffraction signal itself is determined by the ordering of the atoms in the crystal. It cannot be controlled but the signal-to-noise ratio which co-determines the quality of the diffraction information may be influenced by external factors. Some of these, such as the increasing background noise caused by the radiolysis products formed during exposure, are not easily controlled. Other factors determined by the diffraction conditions, filtering out inelastically scattered electrons, the state of preservation of the crystals and the choice of support or grids as well as the choice of detector can be controlled to some extent. We have investigated whether the background diffraction of the carbon support or the vitrified water can be reduced by freezing the crystals in the thinnest possible water layer on a holey carbon support. Vitrification of the crystals in the holes was possible, but it was also observed that a lot of crystals shrunk or became slightly bent. Although the signal-to-noise ratio of crystals frozen in holey carbon should theoretically be better, bending of the crystalline specimen was of particular concern as it may affect the quality of the intensity data. The crystals vitrified on the carbon layer of the holey carbon film were better preserved and in the majority of the cases showed better diffraction than crystals frozen within the holes.

By using selected area diffraction, data of resolution close to 3.3Å was obtained from vitrified protein nano-crystals (see Figure 3.5). Insertion of a diffraction (selected area) aperture allows quasi-parallel illumination and therefore formation of diffraction spots instead of diffraction discs. Spots are easier to analyze, especially when the diffraction signal is relatively weak as is the case for protein crystals.

Traditionally in electron crystallography (when inorganic crystals are studied), three dimensional diffraction data are collected through the combination of rotation and additional tilt (beta tilt) which allows orienting the crystal and recording a set of diffraction patterns at various chosen crystallographic zones. Protein crystals absorb energy during data collection, which reduces their crystallinity and consequently the resolution obtained. Therefore orienting the crystals in the beam and acquiring only well oriented diffraction patterns is largely limited by the high beam sensitivity of the proteins and often even not possible. A different approach is used for collecting 3D diffraction data in protein X-ray crystallography where the beam sensitivity of the crystals is also a pertinent problem. In this case the protein crystal is rotated during exposure and (usually) for every degree a diffraction pattern is taken. Applying this method allows maximizing the diffraction information that can be obtained before the crystal is exhausted as no diffraction is wasted on orienting the crystal. The diffraction patterns are subsequently integrated, scaled and merged.
Figure 3.4 Illustration of the home modified cryo-transfer system. In figure 3.4 (a) is shown the tip in which the EM grid is placed (indicated with a black arrow). There are two removable parts as given in figure 3.4 (a) and marked with 1 and 2. After the grid is loaded, the part indicated with 1 is positioned on the top of part 2 as shown in figure 3.4 (e) and used to prevent possible instability or moving of part 1 in the holder. The cryo-holder itself was also modified such that it can accommodate the removable tips as shown in figure 3.4 (b). After the grid is placed in the tip, the latter is sliced in the cryo-holder as illustrated on figure 3.4 (c) and 3.4 (d). The final set-up before inserting the holder in the microscope, is given on figure 3.4 (e).
In the case of protein nano-crystals it is possible to obtain multiple diffraction patterns from a single crystal using low dose diffraction mode (see Figure 3.6). However, since the crystals used for electron-microscopy are $10^6$ smaller than those used for X-ray diffraction studies (even though electrons are three orders of magnitude less damaging than X-rays) the number of diffraction patterns that can be acquired from a protein nano-crystal is much less compared to the number of patterns that can be collected from a macro-crystal. The diffractograms in Figure 3.6 show that after each exposure the protein diffraction signal deteriorates significantly. If a full 3D dataset of sufficient quality can be obtained from a protein macro-crystal, diffraction data-sets from different nano-crystals need to be collected and merged.

### 3.4 Conclusions

Sample preparation requirements for electron microscopy studies are significantly relaxed compared to X-ray single crystal and powder diffraction. In the case of protein crystals the development of plunge freezing techniques allows vitrification of nano-crystals without additional cryo-protectants
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Figure 3.6 Diffraction patterns acquired from the same area of a vitrified lysozyme nano-crystal. The rotation angle is 1°, exposure time 1s. After every exposure and tilt the diffraction signal deteriorates significantly. It is hardly possible to distinguish the weak diffraction spots on the last diffractogram.

For preservation of macro-crystals in X-ray crystallography, the use of cryo-protectants is essential and the optimal cryo-protecting conditions need to be identified for each case individually.

The strong scattering cross-section of electrons allows diffraction information to be acquired from thin protein nano-crystals. The small electron wavelength permits collection of a complete diffraction zonal pattern at a single crystal setting.

Due to the high beam sensitivity and the small size of the protein nano-crystals it is not possible to apply existing strategies for data collection from X-ray protein crystallography or electron crystallography of small molecules. Since diffraction patterns from different crystals need to be collected in order to reconstruct the 3D reciprocal space, exhausting a single crystal in order to collect as many as possible diffraction patterns at the expense of poor quality and low resolution is highly unlikely to provide a successful structure solution. An alternative is to collect patterns with high quality diffraction information (high signal-to-noise ratio, high resolution, less dynamical perturbations) preferably from undamaged areas in a wide range of crystals settings. In this case the angular relationship between the diffraction patterns is lost. This makes data analysis definitely more difficult. However, the more diffraction information is present on a single pattern and the more kinematical the data are, the easier will be to identify the crystal phase and index the reflections.
References


