Domain Organization and Properties of LB Lysozyme Crystals Down to Submicron Size

EUGENIA PECHKOVA and CLAUDIO NICOLINI

Nanoworld Institute, CIRSDNNOB University of Genova, 16132 Genoa, Italy and Fondazione ELBA, 00100 Rome, Italy

Abstract. New topographic details appeared evident in protein crystal buffered with glycerol solution native on mica by atomic force microscopy and after laser irradiation on glass by light microscopy. This observation indicates the existence of distinct domains in the 3D crystal organisation that are quite different in size and number between the lysozyme crystals grown by Langmuir–Blodgett (LB) nanotemplate with respect to traditional hanging-drop vapour diffusion. Nanodiffraction by highly focused synchrotron radiation of laser cut submicron crystals confirmed the atomic structure of all residues of LB lysozyme crystals as being the most resistant to radiation damage. Crystals grown by LB nanotemplate still diffracted at good resolution after several steps of X-ray ‘burning’, while the classical crystals decayed very quickly at the same exposure.

Synchrotron radiation facilities provide X-ray beams of well-tuned wavelength and size. Most macromolecular structures are now determined using synchrotron radiation, with X-ray crystallography being the most widely used method for structure determination. However successful structure determination and refinement needs higher X-ray doses and in the absence of such doses, structural information is often lost (1-10). Free radicals formed by photon absorption (primary effect) move throughout the crystal (secondary effect), leaving fingerprints on proteins (1-3). This problem in X-ray crystallography is termed radiation damage to protein crystal and appears highly reduced in the Langmuir–Blodgett (LB) nanotemplate protein crystal (4-7). Recently many techniques such as cryocooling, free radical scavengers and beam defocusing have been introduced to reduce radiation damage (1-3). However, radiation damage still persists and becomes highly problematic in microcrystallography (8-10), which represents the frontier of protein crystallography. Among the problems radiation damage causes are the reduction of diffractive power and resolution, the breaking of specific residues, the increase of mosaicity, overall B-factor and crystal unit cell volume (11). Highly ordered, well-diffracting and radiation stable crystals remain important for successful high-quality data collection. Crystal grown by LB-based methods have previously proven to be better in data quality and resolution (12, 13). Radiation stability in terms of reflection intensities have also been shown for lysozyme crystals based on LB-based methods (13). Recently, a detailed study of two types of numerous protein crystals grown by LB-based and classical-based methods proven indeed the effects of differential radiation damage before and after high X-ray dose (burning) at the beamlines of the Synchrotron Radiation Facility (ESRF) European, in Grenoble, being quite less pronounced in LB crystals (5, 14-21, 24). Extension of this study at the nanoscale is presented here to identify unexpected crystal topography probed by atomic force microscopy (AFM) (22) and laser microscopy (23).

Materials and Methods

The full technical details for the reader can be found in a recent review paper (25). Domain organisation was determined by a combination of AFM and Laser technologies, the first one using an Asylum Research (Santa Barbara, CA, USA) Atomic Force Microscope MFP-3D in the stand alone mode present at ESRF (Grenoble, France), with details of the laser technology to be found in references 25 and 26. The low noise head consisted of a sensed optical lever with diffraction limited optics and a low coherence light source virtually eliminated any interference artifacts. The MFP-3D XY Scanner warranted a precision and accuracy unlike any tube scanner. The MFP-3D Base warranted three configurations for illuminating and viewing a sample. The AFM instrument proved optimal for imaging protein crystals in a solution. The device was tested on crystals (LB) films of lysozyme proteins with quite different molecular weights. This approach enabled the periodicity and morphology of crystals to be studied in their mother liquid, thereby preserving the native periodic protein crystal structure and the domain structure, which is typically destroyed by drying.
The AFM instrument appeared to distinguish protein crystals from the types of preparation, either classical (Figure 1a) or LB (Figure 1b). When observed under an optical microscope, the protein crystals were quite similar in morphology, the difference between them often being revealed only during X-ray analysis. The LB technique and its variation, a modified Langmuir-Schaeffer (LS) method that is used in various scientific fields (24), were utilized. Proteins were brought to the air-water interface and then compressed to a surface pressure of 20 mN/m, using an LB trough. The protein monolayers were compressed immediately after spreading with a compression speed of 70 cm/min. Protein thin films were engineered on the solid substrate using the in house LB trough. The transfer of protein monolayers from water surface onto solid supports was performed by touching the support in parallel to the surface according to the LS technique at the pressure of 20 mN/m. Siliconised circle glass cover slides used for hanging-drop method were used as substrate for protein film deposition. These LB nanofilms over glass cover slides were used as template for crystallization (7). Crystallization conditions used for the LB-based and the classical-based methods used the similarly hanging-drop vapour diffusion method.

The data were acquired after a sequence of laser irradiation described in (26). Laser irradiation parameters were: wavelength 0.1 nm, pulse duration 1 s, resulting in $7 \times 10^{10}$ photons/s, pulse beam diameter 500 nm resulting in a beam spot area $3.14 \times 0.25$ μm$^2 = 8 \times 10^{-07}$ mm$^2$ and approximately $0.9 \times 10^{15}$ photons/s/mm$^2$. The solution in which the proteins are stored has an important effect on their behavior. In a water-based solution, they tend to float around and therefore cannot be handled very precisely. Additionally, the water evaporates quickly, so there is a limited time in which the protein crystals can be manipulated, due to the increasing concentration of salt in the solution (around 15 minutes). In contrast, when stored in a viscous solution, the crystals cannot be separated after the cutting, and evaporation is not a problem. The solution utilised in the present study fitted the required needs (such as manipulation time and kinetics) the best (as shown in references 25 and 26). The laser cutting experiment (the laser was from PALM Microlaser technologies, as shown in reference 26) was carried out with two different types of samples: classical protein crystals and LB proteins. Both were stored in a glycerol solution and were electrostatically fixed to the mica and therefore did not move as fast as they did during the first experiments. This made a rather precise cut possible.

Results and Discussion

The following observations were made during the experiment. The proteins could be cut rather precisely, during their low mobility (Figure 2). During this cutting (within the first minutes after the beginning), the break that is typically seen along domain lines was not observed. After 20 to 40 minutes (depending on the type of the crystal), the proteins started to dissolve in doing so, showed their domain structure (Figure 2). When hit at one of these domain lines, they easily split. Images show another crystal broken along several domain areas. On the same images, the damage induced by the laser energy on the upper half of the crystal can be seen. Material was removed there, instead of the clean split along the domains. This dissolution took approximately 30 minutes for the ordinary crystals (Figure 2B) and more than 45 minutes for the LB crystals (Figure 2A). Striking and reproducible differences in domains were apparently visible in crystal produced by classical hanging-drop vapour diffusion (Figure 2B) with respect to LB crystal produced by nanotemplate hanging drop vapour diffusion (Figure 2A). Images were taken with the crystals maintained under the same buffer conditions but at different times, due to the higher stability of LB. In larger crystals (~50 μm), one single laser impulse was able to cause, when hit in the right place, the crystals to split along certain lines, like domains. These cuts revealed a very clean cutting surface. Clearly, there were only a small number of these domain lines per crystal, which meant that there was a minimum size which could be created by this effect (~5 μm). Additionally, it was possible to damage the proteins locally by the laser energy, like other materials analysed, which resulted in a rather rough surface.

For the first time, microcrystallography of submicron laser, cut LB lysozyme crystal was performed with a focused synchrotron-radiation beam of less than 1 micron. The crystal structure of lysozyme from the diffraction pattern (Figure 3) was determined with a flux density of about $3 \times 10^{10}$
photons/s/mm² at the sample. Sets of diffraction images collected from different sized crystals were shown to comprise data of good quality, which allowed a 2.0 Å resolution lysozyme structure to be obtained. The main conclusion of this experiment is that a high-resolution diffraction pattern can be obtained from a less than 1 micron cube crystal volume, corresponding to about 2×10⁷ unit cells. Despite the high irradiation dose in this case, it was possible to obtain an excellent high-resolution map and it could be concluded from the individual atomic B-factor patterns that there was no evidence of significant radiation damage in this ultrasmall LB crystal. The photoelectron escape from a narrow diffraction channel is a possible reason for reduced radiation damage as indicated by Monte Carlo simulations (6, 9, 10, 26). These results open many new opportunities in scanning protein microcrystallography and make random data collection from microcrystals a real possibility, therefore enabling structures to be solved from much smaller crystals than previously anticipated as long as the crystallites are LB prepared. Datasets from several LB and classical crystals were collected to choose the best diffracting crystals for complete analysis. Typical classical crystal dimension were 100×200×150 and LB crystal dimension were 100×300×150 microns. Beam size
used for analysis was 100x100 microns. The total dose absorbed by the two crystals during burning and normal data collection corresponded to 9.5 MGy. Data collection statistics for before and after burning of LB and classical crystals are presented elsewhere (5). In order to have a detailed study of radiation damage, electron density maps were compared for first and last data sets for classical- and LB-based crystals. The most characteristic specific structure effect of irradiation is electron density loss between disulfide bonds and carboxylic acid groups of aspartic and glutamic acid residues (1-3). Thus electron densities for some of these residues were compared before and after the burning process. The average B-factor indicates the positional spread of each atom in protein after burning; the increase for each residue was more significant for the classical crystal compared to the LB-based crystal, indicating more damage in the classical crystal (Figure 4).

Overall analysis indicated more damage suffered by classical crystal compared to LB-based crystal. Loss of electron density at the same contour levels and increase in average B-factors clearly indicated that radiation damage is more significant in the case of classical-based crystals compared to LB-based crystals.

Acknowledgements

This work was supported by the Functional Proteomics and Cell Cycle FIRB Grant from MIUR (Italy) to the Nanoworld Institute-CIRNNOB of the University of Genova and by the Funzionamento Grant 2008-2009 from MIUR (Italy) to Fondazione El.B.A. (Rome).

References

11 Ravelli RBG, Leiros HKS, Pan BC, Caffrey M and McSweeney S: Specific radiation damage can be used to solve macromolecular crystal structures. Structure 11: 217-224, 2003.