Laser-Microdissection of Protein Crystals Down to Submicron Dimensions

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Abstract

We studied laser-microdissection of standard and Langmuir-Blodgett (LB) nanotemplate protein crystals in glycerol solution. The time required for microdissection was significantly longer for LB-crystals as compared to standard-crystals which also more rapidly dissolve. Microfragmentation of lysozyme crystals was observed after extended solvent exposure. Synchrotron radiation nanobeam mapping allowed localizing and aligning cryofrozen lysozyme microfragments. 3D data-sets obtained from two microfragments were refined to atomic resolution. The well-defined electron density maps showed no evidence for damage of radiation of sensitive side-groups. Our results suggest applications of laser-microdissection techniques in structural studies on crystals with a high mosaicity. They also provide a new window for the characterization of protein crystal organization down to the submicron scale, pointing to a new emerging biophysical technique.

Keywords: Langmuir-Blodgett; Synchrotron radiation; X-ray topography; Lysozyme

Introduction

While, a lot of efforts are being invested at Synchrotron Radiation (SR) sources on high-throughput protein-to-structure pipe-lines in the context of structural genomics [1-4], methods allowing manipulating and tailoring of protein micro-crystals in a laboratory or SR-beamline environment have been less explored. Such techniques could, however, be useful for separating and sorting crystals from a batch, for dissection of crystals, polymorphs or crystalline domains from an aggregate or a cellular environment. We also note the idealized model of a protein crystal composed of coherently scattering mosaic blocks [5] which can be experimentally studied by X-ray line-profile analysis or X-ray topography [6,7]. The properties and interactions of individual mosaic blocks are, however, rarely addressed. As an example we mention the observation of radiation damage induced accumulation of hydrogen at the mosaic-block boundaries of insulin crystals resulting in a reduction of block-size and increase of mosaicity [8,9]. A more detailed understanding of mosaic block interactions could contribute to more refined models of protein crystal mechanics and is of fundamental importance for defining the frontiers of nanomedicine [10,11]. Finally we note that raster-diffraction techniques allow identifying high-quality diffraction volume-elements in a batch of microcrystals or a larger crystal [12,13]. We are aware that the selection of mosaic blocks for X-ray nanodiffraction techniques would represent a methodological progress and could in particular improve the quality of structural refinements for high mosaicity crystals. For all of these reasons it is of interest exploring techniques allowing dissecting protein crystals, ultimately down to the level of individual mosaic blocks.

In view of the generally fragile nature of biological matter, laser-optical techniques appear to be well suited for protein crystal manipulation [9,14-16] and microdissection [17-25]. Our aim was exploring the ultimate size in coherently scattering crystal fragments obtainable by microdissection, principally for the model protein lysozyme. Preliminary results have been reported elsewhere in Pechkova and Nicolini [26]. In the following, we will use the term laser-microdissection for the cutting of a crystal by a laser beam while microfragmentation will be used for the separation of a microdissected crystal into smaller fragments due to effects such as cavitations at domain boundaries and solvent interpenetration. Given the rather small microfragments obtained in the present study, we used X-ray nanocrystallography for localizing individual microfragments and collecting data sets. We were also interested in the question whether crystals differing in perfection and X-ray radiation stability differ also in microdissection and microfragmentation behavior. This issue is of importance for the generation of very small crystals which might be more prone to laser-radiation damage than larger crystals. We will compare in this paper the microdissection behavior of Langmuir-Blodgett (LB) based crystals (called LB-crystals) and crystals obtained by standard (e.g. hanging drop) crystallization techniques (called standard-crystals) for lysozyme. Indeed, LB-crystals appear to have significantly higher radiation stability as compared to standard crystals for a wide range of proteins [27-32]. The LB nanotemplate crystallization method was first introduced by Pechkova and Nicolini [26]. According to this method, based on LB nanotechnology [34], a LB protein monolayer deposited on the glass slide by means of Langmuir-Blodgett trough previously proved to be highly ordered and thermostable [34] in the common hanging-drop method causes the acceleration of the nucleation and the crystal growth rate [33,35,36]. During the last decade, LB nanotemplate crystallization method was successfully used for crystallization of the protein not crystallizable by classical crystallization techniques [31,37,38], for producing significantly more radiation stable [26,28-32] and higher quality crystals [31,32] in comparison with classical ones for a wide range of proteins. The reason of these LB crystals qualities is mostly due to the more compact inner water molecules organization.

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Received September 24, 2013; Accepted October 29, 2013; Published October 31, 2013


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in the structure [39]. In the present work, the higher stability of LB crystal allows successful X-ray nanodiffraction analysis of their microfragments after the laser microdissection.

**Methods**

**Protein crystallization**

Lysozyme protein crystals were obtained by a state-of-the-art crystallization technique based on Langmuir-Blodgett (LB) nanotemplates [28,40-47]. LB-nanofilms were generated by the LB-technique and its variation, a modified Langmuir–Schaeffer (LS) technique [27,28,42]. Typically, protein was spread at the air-water interface of an in-house LB-trough and immediately compressed with 70 cm/min to a surface pressure of ~20 mN/m. The protein monolayers were then transferred from the water surface onto siliconized cover slips by touching the support in parallel to the surface according to the LS-technique at a pressure of ~20 mN/m. These LB-nanofilms were then used as crystallization templates [28,31,39]. Lysozyme was crystallized from 40 mg/ml lysozyme in 50 mM sodium acetate solution using the hanging drop vapor diffusion method. Crystallization conditions were the same for standard- and LB-crystals. The 4 µl droplet of protein solution was mixed with 4 µl droplet of precipitant (0.9 M NaCl) on the glass cover slide covered with two LB lysozyme monolayers and equilibrated over reservoir containing 1ml of 0.9 M NaCl. Further details can be found in Pechkova and Nicolini [33].

**Laser-microdissection**

Experiments were performed using a Zeiss PALM laser-microdissection system with a laser wavelength of λ=355nm and 2ns pulses of 90 µJ each at 100 Hz [48]. This corresponds for a ~4 um3 laser spot to a laser fluency of ~562 J/cm2 and a flux density of ~4×1010 photons s-1 mm-2. The laser beam was focused on the surface of the crystal avoiding thus shockwave induced crack formation. In order to allow in-situ observation and manipulation of crystal cuts, we performed microdissection operations for all four proteins being utilized (standard and LB) initially in an open aqueous solution drop rather than in a flash-frozen cryoloop [22]. The freely floating protein crystals and the convective flow induced by evaporation did, however, not allow clear cuts. In addition, evaporation resulted also in salt precipitation which limited the time available for sample manipulation. The movement of crystals and fragments could, however, be significantly reduced by electrostatically fixing them to a mica sheet and using a more viscous (~20%) glycerol solution. This also reduced the evaporation rate and allowed subsequent cryocooling of the sample. The microdissected protein crystals were transferred from the drop into a cryoloop or cryomesh for flash-freezing [28,49]. This method could, however, not be used for the much smaller microfragments which were instead generated directly from protein crystals in glycerol solution, kept in a cryoloop. The cryoloop was transferred to a micromanipulator and aligned in the laser-beam for microdissection. In order to avoid evaporation of the thin glycerol film, we first froze the loop containing one or more lysozyme crystals, then cut a selected crystal at room temperature (r.t.) and finally froze the loop again. Crystals which were not hit by the laser beam did not show any degradation during this procedure. Two single shots resulted only in several larger fragments while longer waiting times resulted in a microfragmentation into crystallites down to about 1 µm dimension (Figure 4). After microfragmentation the loops were flash-frozen to 100 K on the beamline microgoniometer.

**Synchrotron radiation experiments**

We used the ID13 undulator beamline at the ESRF-Grenoble [50]. A monochromatic SR-beam of λ=0.09611 wavelength was focused by crossed linear Fresnel lenses to a ~400 nm spot at the sample position [51]. The flux of ~7×1010 photons/s corresponded to a flux density of ~3×1014 ph/s/µm2. A scanning goniometer with integrated rotation axis was used to position and rotate the samples [52]. Samples were aligned normal to the beam by an on-axis Olympus microscope, which was calibrated to the beamline focal spot. Experiments were performed in transmission geometry at 100 K using an Oxford Cryoflow system. Diffraction data were collected by a MAR CCD 165 detector with 2K×2K pixels of 78.94×78.94 µm2 (binned to 1024×1024 pixels) at a sample-to-detector distance of 114.41 mm (refined) for a typical exposure time/pattern of 1s and a 1° of rotation. Raster-diffraction experiments were performed in step-scanning mode along two orthogonal axes, normal to the SR-beam. The raster-diffraction experiments were analyzed with the FIT2D program [53].

**Data processing and refinement**

The lysozyme data-sets were indexed and integrated with XDS [54,55], scaled with XDSCSCALE and converted with XDSCONV. Lysozyme spacegroup P4321 was recognized by POINTLESS [56] and the data sets were processed in this spacegroup. FreeRerflag from the CCP4 [57] software package was used for the calculation of Free R factors (Rfree) [58]. According to the Matthews coefficient results [59,60], molecular replacement was performed with one molecule in the asymmetric unit assuming a protein mass of 14.3 KDa for lysozyme. Automated molecular replacement was performed using MOLREP using PDB templates with ID: 2AUB [61,62]. Both LB crystal fragments are solved by molecular replacement using the same template. 2AUB is the structure with the highest homology scoring of the Lysozyme LB structures. The PDB [63] file was then refined using REFMAC5 [64,65]. Before the final refinement step, electron density maps were inspected by COOT [66] at the same contour level. Dataset scaling was performed using both SCALA and FreeRerflag from the CCP4 software package [58]. In order to obtain significant statistics, data were processed at a resolution of 2.3 Å for the LB1 data set and 2.5 Å for the LB2 data set. The PDB file [63] was then refined for both datasets. Manipulation of PDB files was performed using RASMOL [67]. Visual inspection of protein structures was performed using PyMol [68].

**Results and Discussions**

**Laser-microdissection**

Microcrystals of lysozyme could be readily produced by laser-microdissection from LB-crystals while standard-crystals, obtained under the same conditions and of the same dimensions as LB crystals, disintegrated rapidly under the same conditions suggesting a lower stability. Indeed, cutting of lysozyme crystals along a user-defined line on the crystal required a continuous exposure of ~90 s to pulsing laser beam for LB-crystals and respectively ~20 s for standard-crystals. The cutting procedure is illustrated in Figures 1A and 1B. The crystal splits along the cutting surfaces immediately after being hit by a laser pulse. Within the first minutes after the onset of the cutting process, the characteristic fracture along lines could not be observed. Indeed, Figure 1A shows the crystal before cutting along the green line with the number 107 and Figure 1B after. The dissolution of a microdissected LB-crystal into microfragments after a waiting time is shown in Figures 1C and Figure 1D. The cutting of a standard-crystal into two parts is shown in Figures 2A and 2B. The surrounding solution developed a few larger black bubbles after a laser hit which did, however, not obstruct crystal observation. (Figure 2B) The cut surfaces of the two parts of the standard-crystal are not well defined and start breaking-up into smaller
fragments (from 1 to 3 µm) during the cutting-process. In contrast, the LB-crystal splits into two parts with clean cutting surfaces without immediate formation of microfragments. (Figures 2C and Figure 2D) This implies that microfragmentation of standard-crystals proceeds at a higher rate than for LB-crystals. In larger crystals of ~50 µm, one single laser impulse can -when hit in the right place- induce the crystals to split along certain lines producing fragments with ~15 µm minimum size.

Microfragmentation into smaller anisotropic crystallites was observed for lysozyme after some time depending on the type of the crystal. (Figures 3A- Figure 3C) The crystal shapes started dissolving into rows of microfragments which took up to 30 minutes for a standard-crystal and more than 45 minutes for a LB-crystal. These differences were reproducible and suggest a higher stability of the LB-crystals. By hitting at lines between the microfragments it was easy separating them. (Figures 3A-Figure 3C) Indeed, Figure 3A shows a standard- crystal, 20 minutes after only two laser pulses focused on one point. The morphology revealed displays large differences between standard- and LB-crystals [26,43,44]. Images of microdissected and microfragmented lysozyme LB-crystals in a cryoloop are shown in Figures 4A-4C. We tentatively attribute the microfragmentation of the cut crystals to a fast stress increase and cavitations at the domain boundaries induced by nanosecond laser flashes [69-71]. The shock waves have been shown to propagate hundreds of microns into biological matter such as cornea [70]. Slow interdiffusion of glycerol molecules is assumed to dissolve the hydrogen-bonded water network at the mosaic block boundaries resulting in their separation [72].

Nanodiffraction of LB-crystal microfragments

The optical image quality of the cryofrozen lysozyme LB-microfragments was not sufficient to clearly observe them at the beamline. (Figure 4D) We performed therefore raster-diffraction with
The laser-microdissection of protein crystals down to submicron dimensions offers unique advantages for structural biology.

**Figure 4**: LB-crystal in nylon cryoloop at r.t. prior to cutting; B: LB-crystal immediately after cutting with 2 single short laser pulses; C: LB-crystal after disintegrating into smaller fragments; D: Optical image of cryofrozen LB-crystal fragments in nylon cryoloop. Angular diffraction data were collected for a single domain at the red point (LB5). A ± 30 μm raster-diffraction scan with 1 μm step-increments was performed around the blue point.

**Conclusions**

Laser-microdissection of standard protein crystals poses problems as the cut surfaces of the two parts of the crystals are not well defined and start breaking-up into smaller fragments during the cutting-process. Clean cut surfaces can, however, be obtained for LB-crystals. Extended exposure of lysozyme LB-crystals to aqueous glycol solution allows separating the crystals into microfragments. The anisotropy of the microfragments appears to reflect anisotropic lysozyme mechanical properties [75]. Structural data from microfragments by SR microcrystallography [49,73].
Figure 6: Diffraction pattern of lysozyme with spot shapes for reflections at different resolutions of about 2.4 Å (a), 5.1 Å (b) and 12.1 Å (c) shown as histograms, analogue to [74].

<table>
<thead>
<tr>
<th>Parameters</th>
<th>4GG0</th>
<th>4GFZ</th>
</tr>
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<tbody>
<tr>
<td>Resolution (Å)</td>
<td>2.3</td>
<td>2.5</td>
</tr>
<tr>
<td>Unit cell a, c (Å) (where a=b) α= β= γ= 90°</td>
<td>78.8, 37.1</td>
<td>79.7, 37.2</td>
</tr>
<tr>
<td>Spacegroup</td>
<td>P 4,2,2</td>
<td>P 4,2,2</td>
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<tr>
<td>Mosaicity (°)</td>
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<td>~0.2</td>
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<tr>
<td>R_{wp}</td>
<td>0.21</td>
<td>0.16</td>
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<tr>
<td>R-factor</td>
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<td>0.20</td>
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<tr>
<td>R_{free}</td>
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<td>0.32</td>
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<tr>
<td>I/σ (values in brackets correspond to the outer resolution shell Å)</td>
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<td>9.1 (4.9)</td>
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<td>5.1</td>
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<tr>
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<tr>
<td>Mean B-value for side chains</td>
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<td>R.m.s. in bond angle (Å)</td>
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<tr>
<td>N° of water molecules</td>
<td>78</td>
<td>47</td>
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Table 1: Lysozyme LB-crystal structural parameters obtained by nanodiffraction for 4GG0 (LB1) and 4GFZ (LB2).
nanocrystallography correspond to a quality obtained by standard protein crystallography. Electron density maps reveal that radiation damage effects do not play a key role in agreement with other studies. The combination of LB technology for protein crystallography with laser cutting and nanobeam data acquisition opens therefore new crystallographic possibilities in particular for crystals which are notorious for their high mosaicity such as membrane protein crystals. We also noticed that microfragmentation of protein crystals in a microfluidic environment could provide a source of nanocrystals for serial crystallography experiments at ultrahigh brilliance X-ray sources [76].

Laser-microdissection method was here proved able to obtain pieces of crystals with dimensions so small to be considered quite-domain. This method can be used before X-ray micro and nanodiffraction experiments in order to overcome the very common problem of twinned, defect, aggregated and mosaic crystals. Indeed, the mosaicity in microdissected fragments was reduced in respect to standard structures. Therefore, we envision that this method could be useful also for the crystals with high mosaicity such membrane protein crystals. Laser-microdissection was here proved able to obtain pieces of crystals with dimensions so small to be considered quite-domain. This method can be used before X-ray micro and nanodiffraction experiments in order to overcome the very common problem of twinned, defect, aggregated and mosaic crystals. Indeed, the mosaicity in microdissected fragments was reduced in respect to standard structures. Therefore, we envision that this method could be useful also for the crystals with high mosaicity such membrane protein crystals. Laser-microdissection was here proved able to obtain pieces of crystals with dimensions so small to be considered quite-domain. This method can be used before X-ray micro and nanodiffraction experiments in order to overcome the very common problem of twinned, defect, aggregated and mosaic crystals. Indeed, the mosaicity in microdissected fragments was reduced in respect to standard structures. Therefore, we envision that this method could be useful also for the crystals with high mosaicity such membrane protein crystals. Laser-microdissection was here proved able to obtain pieces of crystals with dimensions so small to be considered quite-domain. This method can be used before X-ray micro and nanodiffraction experiments in order to overcome the very common problem of twinned, defect, aggregated and mosaic crystals. Indeed, the mosaicity in microdissected fragments was reduced in respect to standard structures. Therefore, we envision that this method could be useful also for the crystals with high mosaicity such membrane protein crystals. Laser-microdissection was here proved able to obtain pieces of crystals with dimensions so small to be considered quite-domain. This method can be used before X-ray micro and nanodiffraction experiments in order to overcome the very common problem of twinned, defect, aggregated and mosaic crystals. Indeed, the mosaicity in microdissected fragments was reduced in respect to standard structures. Therefore, we envision that this method could be useful also for the crystals with high mosaicity such membrane protein crystals. Laser-microdissection was here proved able to obtain pieces of crystals with dimensions so small to be considered quite-domain. This method can be used before X-ray micro and nanodiffraction experiments in order to overcome the very common problem of twinned, defect, aggregated and mosaic crystals. Indeed, the mosaicity in microdissected fragments was reduced in respect to standard structures. Therefore, we envision that this method could be useful also for the crystals with high mosaicity such membrane protein crystals. Laser-microdissection was here proved able to obtain pieces of crystals with dimensions so small to be considered quite-domain. This method can be used before X-ray micro and nanodiffraction experiments in order to overcome the very common problem of twinned, defect, aggregated and mosaic crystals. Indeed, the mosaicity in microdissected fragments was reduced in respect to standard structures. Therefore, we envision that this method could be useful also for the crystals with high mosaicity such membrane protein crystals. Laser-microdissection was here proved able to obtain pieces of crystals with dimensions so small to be considered quite-domain. This method can be used before X-ray micro and nanodiffraction experiments in order to overcome the very common problem of twinned, defect, aggregated and mosaic crystals. Indeed, the mosaicity in microdissected fragments was reduced in respect to standard structures. Therefore, we envision that this method could be useful also for the crystals with high mosaicity such membrane protein crystals. Laser-microdissection was here proved able to obtain pieces of crystals with dimensions so small to be considered quite-domain. This method can be used before X-ray micro and nanodiffraction experiments in order to overcome the very common problem of twinned, defect, aggregated and mosaic crystals. Indeed, the mosaicity in microdissected fragments was reduced in respect to standard structures. Therefore, we envision that this method could be useful also for the crystals with high mosaicity such membrane protein crystals.

### Supporting Material

The atomic coordinates and structural factors have been deposited in the Protein Data Bank (www.pdb.org). LB1: PDB ID code 4GG0, RCSB ID code is RCSB074134; LB2: PDB ID code 4GFZ, RCSB ID code is RCSB074135.

### Acknowledgement

We thank Dr. I Snigireva of the ESRF microimaging laboratory for help with the use of the PALM MicroBeam microdissection system. We thank Dr. M. Burghammer for instrumental support at the ID13 beamline. This work was supported by FIRB–MIUR grants to Claudio Nicolini of the University of Genoa for Functional Proteomics (RBNE01X3IC) and for Nanosensors (RPBPR05JH2P) and by a MIUR grant to the Fondazione El.B.A. for Funzionamento (DM48527).

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