In situ data collection and structure refinement from microcapillary protein crystallization

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In situ X-ray data collection has the potential to eliminate the challenging task of mounting and cryocooling often fragile protein crystals, reducing a major bottleneck in the structure determination process. An apparatus used to grow protein crystals in capillaries and to compare the background X-ray scattering of the components, including thin-walled glass capillaries against Teflon, and various fluorocarbon oils against each other, is described. Using thaumatin as a test case at 1.8 Å resolution, this study demonstrates that high-resolution electron density maps and refined models can be obtained from in situ diffraction of crystals grown in microcapillaries.

1. Introduction

Automating and miniaturizing the experimental aspects of macromolecular structure determination is greatly aiding investigators working on the elucidation of all types of biomolecules and their complexes (Weselak et al., 2003; Chayen, 2004; Pusey et al., 2005). The in situ collection of X-ray diffraction data from macromolecular crystals could significantly decrease the time and effort with which structures are obtained. Furthermore, by eliminating loop handling, the gene-to-structure pathway is streamlined, and crystals are less prone to damage caused by mechanical and environmental shock. While crystals from microcapillaries can be easily extracted for cryocooling (Zheng et al., 2004), the in situ method is not amenable to flash-cryocooling in general, making radiation damage a significant disadvantage. However, data sets from multiple isomorphous crystals can be used to overcome this drawback. With the advent of improved data processing software and faster CCD detectors (Yagi et al., 2004), this approach is now more feasible.

The advantages of in situ data collection have been demonstrated before (McPherson, 2000; López-Jaramillo et al., 2001; Ng et al., 2003; Luft et al., 1999), and there are undoubtedly much older unpublished accounts, when most crystal diffraction data were collected in capillaries. Commercial products (Watanabe, 2005) are now offered that can be used for plate-based X-ray exposures, for predicting resolution limit and space group. These products are impractical for complete data collection because of crystal dehydration and obstruction of the X-ray beam by the crystal container. In this paper, in situ collection is used in conjunction with a modern crystallization screening technique, where thin glass capillaries are filled with approximately 100 (replicate or unique) 20 nl microbatch trials (naming such capillaries as ‘microcapillaries’) via poly(dimethylsiloxane) (PDMS) stamp-based microfluidics (Chen et al., 2005; Song et al., 2003; Tice et al., 2003). The aqueous ‘plugs’, containing protein and precipitant, are separated by immiscible fluorocarbon oil at both ends (Fig. 1). This system has been used previously to obtain space-group and unit-cell information from crystal diffraction at room temperature (Zheng et al., 2004). In the present study, microcapillaries containing plugs which have crystals are mounted directly in a coldstream set to 277 K, and multiple frames of diffraction data are collected using synchrotron radiation. Despite the lack of cryocooling, here we demonstrate that sub-2.0 Å data sets and refined models are achievable, and that the collection of data from multiple crystals is convenient, given the large number of microbatch trials in a single capillary.

Although microfluidics-based free-interface diffusion chips (Hansen et al., 2002) have reached the market, our demonstration of microcapillary-based microbatch screening offers an alternative method to increase the efficiency of small volumes, and has the advantage of convenient in situ data collection and a potentially significant reduced cost of production.

2. Experimental methods

The apparatus used to fill microcapillaries with oil-spaced aqueous plugs was constructed as previously described (Chen et al., 2005; Song et al., 2003; Tice et al., 2003; Fig. 1) using PHD
2000 syringe pumps (Harvard Apparatus) and 10 and 50 μl Hamilton Gastight syringes (1700 series, TLL). Microchannels were fabricated using rapid prototyping in PDMS (Dow Corning Sylgard Brand 184 Silicone Elastomer). Devices were sealed using a Plasma Prep II (SPI Suppliers).

Thaumatin (Sigma Chemicals) was chosen as our initial model protein to demonstrate that the overall approach from crystal growth to data collection was feasible. Crystals were successfully grown (in each capillary material) using microfluidics by filling a single capillary with 100 replicate plugs that contained a 1:1 mixture, by volume, of 2.0 M sodium potassium tartrate and a thaumatin concentration of 25.0 mg ml⁻¹.

After generating the plugs containing tartrate and thaumatin, the first single crystals (25–50 μm) could be seen within hours. Pictures of microcapillaries were taken using a SPOT Insight color camera mounted on a Leica MZ 12 5 stereoscope.

Before data collection at the synchrotron, we tested the background X-ray scattering/diffraction of capillaries made from thin glass (Hampton) and Teflon in combination with a set of three fluorocarbon oils, FC-(40, 70, 3283) (3M) (subset of data shown in Fig. 2). Capillaries were attached to pins using HoldFast epoxy aquarium sealant (Marineland) and mounted on the goniometer. Diffraction data collection was performed on a Rigaku MSC R-AXIS IV with 10 min exposures. For each combination of oil and capillary, an exposure was taken both at an aqueous plug and at an oil spacer centered in the beam in order to gain a practical understanding of the background intensity, given a typical 300 μm effective radius of a home-source X-ray beam.

Subsequently, thaumatin crystals grown in thin glass capillaries underwent diffraction data collection at the GM/CAT beamline at the Advanced Photon Source (APS) at 12 000 eV (1.03 Å). Capillaries were attached to pins as previously described and mounted on the goniometer under a coldstream (CryoJet XL, Oxford Diffraction) set to 277 K. Two data sets were collected (Table 1) from two crystals in adjacent plugs with 1.0° oscillation and 1.2 s exposure per frame, with the unfocused beam attenuated by 50%. Data were integrated and scaled in HKL2000 (Otwinowski & Minor, 1997) and the merged data set was produced using XPREP (Bruker AXS). Molecular replacement (using PDB ID 1thw as a model) was carried out using MOLREP, refinement using REFMACS (Murshudov et al., 1997) and solvent building using ARP/wARP (Perrakis et al., 1997) of the CCP4 (Collaborative Computational Project, Number 4, 1994) suite of programs.

3. Analysis of data

Previous reports of in situ data collection without cryocooling suggest the method is only suitable for weaker ‘home’ X-ray sources (Lopez-Jaramillo et al., 2001), but examples of data collections at synchrotrons exist (Lacy et al., 1998; Reinisch et al., 2000). We demonstrate that single crystals can provide high-resolution (Fig. 3) refinable data sets under the intensity of synchrotron radiation. In the case of acute decay or
Table 1
Diffraction data statistics for two thaumatin crystals in the same capillary.

<table>
<thead>
<tr>
<th></th>
<th>Data set 1</th>
<th>Data set 2</th>
<th>Merged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution range (Å)</td>
<td>27.94–1.86</td>
<td>36.34–1.90</td>
<td>36.34–1.86</td>
</tr>
<tr>
<td>Number of reflections</td>
<td>20 717</td>
<td>18 350</td>
<td>21 731</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>94.1</td>
<td>99.4</td>
<td>98.7</td>
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<tr>
<td>Completeness, outer (%)</td>
<td>94</td>
<td>99.2</td>
<td>94</td>
</tr>
<tr>
<td>Redundancy</td>
<td>7.9</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>Initial frame Iσ(I)</td>
<td>9.11</td>
<td>7.94</td>
<td></td>
</tr>
<tr>
<td>Final frame Iσ(I)</td>
<td>6.48</td>
<td>5.89</td>
<td></td>
</tr>
<tr>
<td>Initial frame Iσ_{out}(I)</td>
<td>2.96</td>
<td>2.64</td>
<td></td>
</tr>
<tr>
<td>Final frame Iσ_{out}(I)</td>
<td>1.38</td>
<td>1.28</td>
<td></td>
</tr>
<tr>
<td>Iσ(I)</td>
<td>24.07</td>
<td>22.0</td>
<td>30.56</td>
</tr>
<tr>
<td>Iσ_{out}(I)</td>
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<tr>
<td>R_{sym}</td>
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<td>R</td>
<td>0.145</td>
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<tr>
<td>R_{merge}</td>
<td>0.187</td>
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<td>0.188</td>
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<td>Initial frame outer resolution shell (Å)</td>
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<td>1.75–1.61</td>
<td></td>
</tr>
<tr>
<td>Final frame outer resolution shell (Å)</td>
<td>2.10–2.0</td>
<td>1.80–1.69</td>
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<tr>
<td>Number of frames</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3
Initial in situ diffraction image from data set 1 of a thaumatin crystal in a thin glass capillary. The frame parameters were 1.0° oscillation, 1.2 s exposure and detector distance of 150 mm.

Figures 2c and 2d show a comparison of the diffraction images for data set 1 and 2, respectively. The images obtained were suitable for indexing, suggesting that Teflon might be suited to screening-type diffraction studies, as opposed to those for structure determination (where blind spots could reduce data quality).

Unlike home sources, match the beam radius to the crystal (via focusing and slitting down). Therefore, we expect that, in a typical situation, the beam could be focused on single crystals in a microcapillary so that the radiation flux through the oil spacer would be minimal.

While thin-walled glass and quartz have long been used for data collection from macromolecular crystals, we considered the use of Teflon-based capillaries as a flexible and cheaper replacement. While about three distinct ‘rings’ are visible in resolution shells under 10 Å (Figs. 2c and 2d), we found that the images obtained were suitable for indexing, suggesting that Teflon might be suited to screening-type diffraction studies, as opposed to those for structure determination (where blind spots could reduce data quality).

Unexpectedly, crystals in microcapillaries appeared visibly stationary under φ axis rotation, as confirmed by the observation of minimal variation in the orientation matrix during data reduction. We suggest that this steadfastness may be a consequence of the small 20 nl volumes and small dimensions of the plugs. The inertial effects for fluids become less significant at lower values of the Reynolds number. The Reynolds number decreases as the dimensions of the system decrease, as viscocity increases and as velocity of fluid flow decreases. The confinement of crystals at the water/fluorocarbon or the water/glass interface may provide additional stability. Furthermore, data collection on home sources necessitates the use of much larger crystals, which often dislodge owing to gravity. The intense synchrotron beam allows much smaller samples to be used, which are less likely to be dislodged.

Radiation damage induced by a lack of cryocooling deserves special attention (Blake & Phillips, 1962), as it could limit the applicability of the overall approach we are proposing. As an initial proof of concept, we have biased our experiments towards large crystals of thaumatin (100 μm scale), offsetting primary radiation damage effects which are independent of temperature. For crystals of this size, the total radiation exposures required for minimally complete data sets are well below the Henderson (1990) limit. Secondary damage from the diffusion of reactive radiolytic products is likely to be a much larger effect in the case of non-frozen microcapillaries than it is in flash-cryocooled experiments. We tried to minimize secondary damage and local heating effects by setting the cold stream to 277 K. Despite these measures we can still, as expected, observe weakening of diffraction spots much more rapidly than is typically seen under cryoconditions. This can be observed in both data sets by the decreased overall intensities and/or an expected increased sensitivity to vibrations from low mosaicity (initial mosaicities are 0.056 and 0.049°).

Mosaicity
has a constant upward drift as a function of frame number (Fig. 5), but variations for other integration parameters are small. The final difference in unit-cell lengths for both data sets is only about 0.1%. There is no clear inflection point or threshold that would indicate a reasonable frame at which to cut off the integration. The two data sets were merged with a threshold that would introduce errors from subtle non-isomorphisms. Virus crystallographers seem to be apprehensive about introducing errors from subtle non-isomorphisms. Protein crystallographers routinely use multiple crystals [see Grimes et al. (1998) for an example of 1000 crystals] in merging complete data sets.

It is difficult to estimate exactly how the decay process affects the quality of the data set, given the low R factors for the refined model. We note that disulfide bonds, known to be susceptible to radiation-induced reduction, have faithful electron density in the refined thaumatin maps (Fig. 6). Some glutamate side chains in each structure demonstrate carboxylate electron density that is less precise than other parts of the model, but it is difficult to determine if this difference is due to decarboxylation.

In order to evaluate the general applicability of the microcapillary approach, we attempted to grow crystals of a protein target from the SARS genome. A specific example for a conserved domain of SARS-CoV protein nsp3 (Saikatendu et al., 2005) is shown in Fig. 7. We were successful in growing single crystals in most cases, which suggests a surprising equivalence between vapor diffusion and microbatch type screening. The crystals grown in the microcapillary apparatus appear to be identical to those grown using traditional methods, under X-ray diffraction. Current efforts are focused...
on increasing the rate of successful crystal growth via microfluidics-based seeding and on data collection strategies for crystals that suffer acute decay under X-ray exposure.

4. Results and discussion

We have demonstrated an integrated in situ approach to crystallization screening and X-ray data collection for macromolecules. Plug-based microfluidics using PDMS stamp lithography to load nanovolume crystallization experiments into glass capillaries offers an inexpensive platform to increase volume efficiencies by tenfold over conventional techniques. This method is amenable to automated sparse matrix screening (Zheng & Ismagilov, 2005) and gradient fine screening (Zheng et al., 2003). Microcapillaries provide convenient handling of multiple crystals for data collection, and this technique can be used to offset the shortened lifetime of exposed crystals without the aid of cryocooling. We have validated this approach by using thaumatin as a model system to the point of providing sub-2.0 Å structures. The overall approach is well suited towards the automation of macromolecular structure determination.

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References


