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Resolution improvement from '*in situ* annealing' of copper nitrite reductase crystals

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Significant improvement of the resolution of copper nitrite reductase crystals was achieved by using the *in situ* annealing technique. The effective resolution limits increased by 1.5 Å from 2.5 to 1.0 Å, the mosaicity value decreased from 1.5 to 0.3° and the spot shape changed from elliptical to circular.

1. Introduction

Cryocrystallography coupled with highly collimated high-flux protein crystallography stations using synchrotron radiation has allowed smaller crystals to be used than was previously possible with in-house laboratory systems. One of the disadvantages of cryocrystallography is an increase in crystal mosaicity and as a result there is an occasional loss in diffraction quality and resolution. Recently, some successful examples of overcoming this problem by crystal annealing have been reported. Different annealing methods have been developed since it was discovered that crystal annealing could decrease mosaicity (Harp *et al.*, 1998). Multiple flash-annealing of glycerol kinase crystals changed the diffraction limit from 3.6 to 2.8 Å and halved mosaicity (Yeh & Hol, 1998); flash-cooling of *Escherichia coli* Ppase (Samygina *et al.*, 2000) combined with an increase in salt concentration changed the diffraction limit from 1.8 to 1.2 Å and decreased mosaicity by a factor of 10. In the case of nitrite reductase (NiR), a blue copper protein (Dodd *et al.*, 1998), significant resolution improvement from 2.5 to 1.0 Å was seen with the annealing of crystals of both the native (NiRNat) protein and a mutant D92E. It is noted that these crystals diffract to ~1.8 Å at room temperature on the same experimental setup.

2. Crystallization and flash-cooling

Similar-looking hexagonal crystals of both native (NiRNat) protein and a mutant D92E were grown using the hanging-drop vapour-diffusion method at 287 K using a 500 µl reservoir comprising of 40–50% PEG–MME 550, 10 m*M* $CuSO_4$, 0.1 *M* MES pH 6.5. The drop consisted of 2 µl protein solution and 2 µl reservoir solution. The protein was at an initial concentration of 5.6 mg ml^{-1} in 10 m*M* Tris–HCl buffer pH 7.1; microcrystals of another NiR mutant were used as seeds. Crystals grew within six months to $0.1 \times 0.1 \times 0.4$ mm in size.

X-ray diffraction data were collected at SRS, Daresbury Laboratory station 9.5 using the 165 mm MAR CCD detector for D92E and station 14.2 using the ADSC Quantum 4 CCD detector for NiRNat. Extensive exploration of cryoprotectants was carried out including PEG 400, PEG 550, methylpentanediol (MPD), ethylene glycol and glycerol. A range of

concentrations from 10–40% were used and mixtures of protectants were also attempted. In all cases diffraction was poor or the crystal redissolved rapidly. In both cases crystals were frozen to cryogenic temperatures with the reservoir solution as cryoprotectant. The data were processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The first images show very high mosaicity. To anneal the crystal *in situ*, the cold stream was blocked for 10–15 s, during which procedure the crystal was thawed before being refrozen. For comparison, data were also collected from a similar crystal from the same drop at room temperature using an identical setup.

3. Results

The D92E crystal initially diffracted to a resolution of 2.5 Å; the crystal was fairly mosaic (1.5°) and was slightly twinned. The shape of the diffraction spots was also highly elliptical (Fig. 1*a*). After annealing, a second image was taken using the same settings as before. The improvements in both spot shape, diffraction limits and mosaic spread were clear. Also, the twinning seen in the previous image was lost. Diffraction was observed to the edge of the plate (Fig. 1*b*); the spots were circular in nature and the mosaic spread decreased to 0.3°. After changing the X-ray wavelength and the crystal-to-detector distance, the maximum resolution observed was 1.10 Å. Data were collected and processed to a resolution of 1.12 Å.

A similar effect was seen with crystals of native NiR. Initial images diffracted to 2.0 Å resolution, with a mosaic spread in excess of 1.5°. Again, the spots were elliptical in nature and slightly twinned. Annealing was carried out as previously discussed. The next image diffracted to the edge of the plate. The spot shape was improved, the mosaic spread decreased to 0.33° and the twinning was lost. The maximum resolution

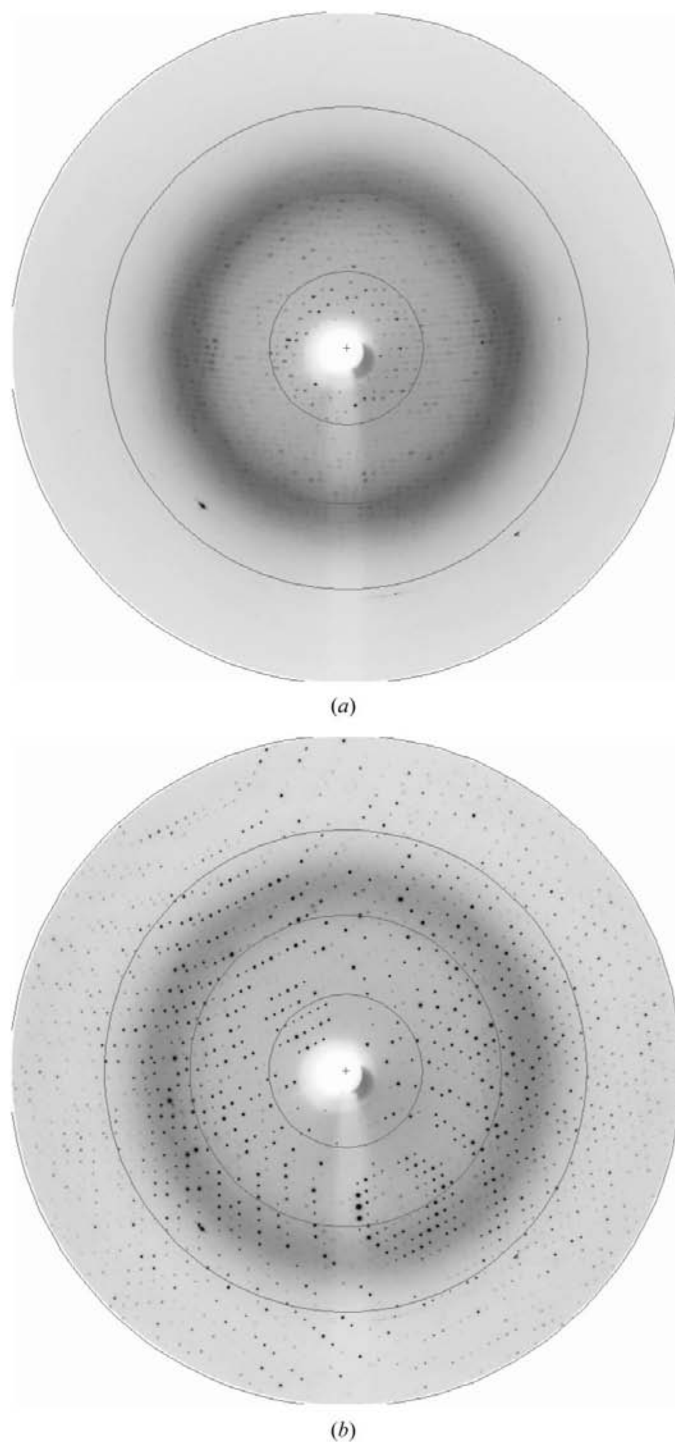

Figure 1
Diffraction from the D92E crystal (*a*) before annealing and (*b*) after annealing; the same settings were used in both cases. Resolution rings are 8.2, 4.1, 2.7 and 2.0 Å resolution.

Figure 2
The first diffraction image from the native NiR crystal at room temperature. Diffraction spots are seen to 1.8 Å in the intial images; however, the crystal rapidly decayed. The unit-cell parameters of the room-temperature data collection were slightly larger than the frozen crystals. Resolution rings are 6.0, 3.0, 2.0 and 1.5 Å.

observed for the crystals was better than 1.00 Å and data were collected and processed to 1.04 Å resolution. A room-temperature data collection carried out on a crystal of native NiR showed diffraction to 1.8 Å resolution (Fig. 2). Thus, it is clear that *in situ* annealing has provided a genuine improvement in crystal order and thus the resolution.

It can be seen from this that exceptional improvement in resolution has been made using a simple *in situ* technique. Annealing will not work for every case attempted; however, the technique should not be discounted. This technique may be of use in certain circumstances where limited resolution data have been collected previously or the level of mosaic spread is such that processing of the images is not possible.

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