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A flash-annealing technique to improve diffraction limits and lower mosaicity in crystals of glycerol kinase

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Abstract

A flash-annealing method has been developed that increases the diffraction limits, and simultaneously decreases the mosaicity of glycerol kinase crystals. This technique utilizes brief thawing and rapid freezing cycles of the crystal in the cold nitrogen stream. The effective resolution limits increased almost by 0.8 Å, from 3.6 to 2.8 Å, and mosaicity values halved.

1. Introduction

X-ray diffraction data collection at cryogenic temperatures has recently become a standard technique as radiation damage is minimized or virtually eliminated and higher quality diffraction data are produced. However, this technique has the drawbacks of possibly increasing mosaicity and lowering the diffraction limit of data obtainable for some crystals (Garman & Schneider, 1997; Rodgers, 1994). A technique, crystal annealing, was recently introduced and shown to decrease mosaicity of certain protein crystals (Harp *et al.*, 1997). In this technique, a frozen crystal is removed from the cold stream and transferred to a solution containing cryoprotectant at room temperature. The crystal is equilibrated in the cryoprotectant solution or oil and allowed to remain at room temperature for at least 3 min and then refrozen. Here we present a technique, called flash annealing, which utilizes this principle of thawing and refreezing of the crystal but in rapid cycles and directly under the cold nitrogen stream while keeping the crystal on the goniometer head. The results from this technique when applied to crystals of the *Enterococcus casseliflavus* glycerol kinase, a dimeric protein with 55 kDa subunits (Charrier *et al.*, 1997), are described.

2. Materials and methods

2.1. *Crystallization*

Crystals of *E. casseliflavus* glycerol kinase were obtained by the hanging-drop technique, from a solution containing 35% polyethylene glycol 4000, 0.25 *M* ammonium acetate, 7.5% polyethylene glycol 200, and 0.1 *M* sodium citrate pH 6.4. They crystallized in space group *C*2 with cell parameters a = 101.2, b = 148.1, c = 73.4 Å. Crystal morphology ranged from rods to three-dimensional plates, with crystal dimensions of 0.2 × 0.2 × 0.1 to 0.4 × 0.2 × 0.1 mm

2.2. *Flash freezing*

Glycerol kinase crystals were transferred from the drops to a solution containing the artificial mother liquor and 20% ethylene glycol and then immediately transferred to a loop. The loop assembly was placed on the goniometer head after excess mother liquor was blotted away with a wick of filter paper. Following the blotting step, flash freezing of the crystal was achieved directly under the nitrogen stream at 103 K.

2.3. *Flash annealing*

Three frames at spindle settings of 0, 45 and 90° with 1° oscillations and exposure times of 30 s were collected at 103 K on beamline 7-1 at the Stanford Synchrotron Radiation Laboratory. These first images showed very high mosaicity (> 2° for some) and the diffraction pattern effectively fades at the inside edge of the diffuse ring, which is 3.7 Å (Fig. 1). To flash anneal, the cold stream was blocked for 1.5–2 s, three times, with intervals of 6 s between thawing steps. The film around the crystal visibly thawed and refroze during these thawing–freezing cycles, as observed on the video monitor.

3. Results

Following the flash-annealing procedure, a second set of images was taken and the resulting frames showed dramatic

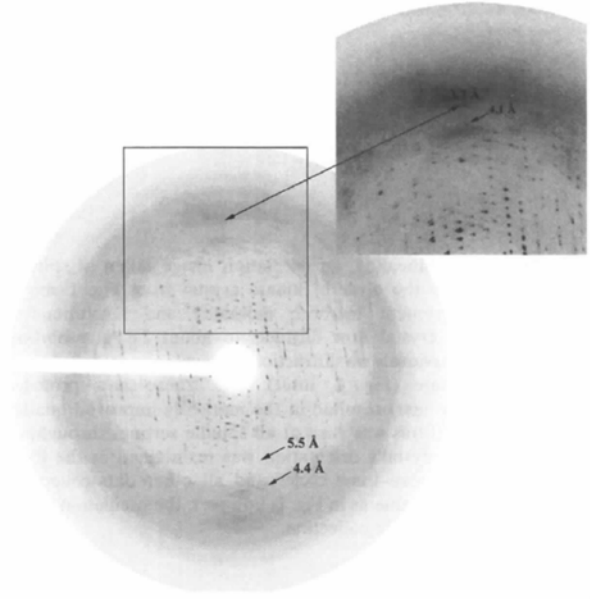

Fig. 1. Prior to flash annealing, a 1° oscillation image taken at spindle setting of 90° on a glycerol kinase crystal shows very high mosaicity and useful diffraction to only about 4 Å resolution. Even with significantly higher contrast, diffraction is not seen beyond about 3.7 Å resolution (inset). The image was recorded on a Mar 18 cm plate at a crystal-to-film distance of 185 mm and with an exposure time of 30 s at beamline 7-1 at SSRL.

improvement when compared with the initial diffraction pattern. The resolution was extended by about 0.8 Å to 2.8 Å and the mosaicity was decreased from almost 2 to 0.8°, as determined through postrefinement in *DENZO* (Otwinowski & Minor, 1997) (Fig. 2). This improvement was uniformly seen for all settings and was reproducible when repeated on two additional crystals of glycerol kinase. In all of the crystals, resolution increased by 0.5 Å to 0.9 Å and mosaicity values decreased by 0.4° to more than 1° in the best case. Data were collected on the best crystal and after processing by *SCALEPACK* (Otwinowski & Minor, 1997), have an overall completeness of 89.8%, with a completeness in the last shell (2.93–2.8 Å) of 60.5%, and an overall R_{merge} of 9%.

The low volume of solution around the crystal was probably the key to allowing the same orientation to be obtained before and after annealing, where a larger volume of mother liquor in the loop might have promoted movement of the crystal when thawed. This facilitated assessment of the level of improvement at each spindle setting. The speed of refreezing was likely to have been an important factor in the improvement of the quality of diffraction and preventing excessive icing on the loop. To test whether increasing the annealing time and number of cycles further affected the diffraction, additional freeze–thaw cycles were performed. Increasing the thawing time in the freeze–thaw cycle or increasing the number of freeze–thaw cycles adversely affected the crystal and resulted in obliterating the diffraction. Even increasing the amount of time by only 2–3 s in the thawing step detrimentally affected both the mosaicity and diffraction. Thus, optimizing the timing and number of freeze–thaw cycles can apparently affect the results obtained and can be critical to obtaining a successful annealing protocol.

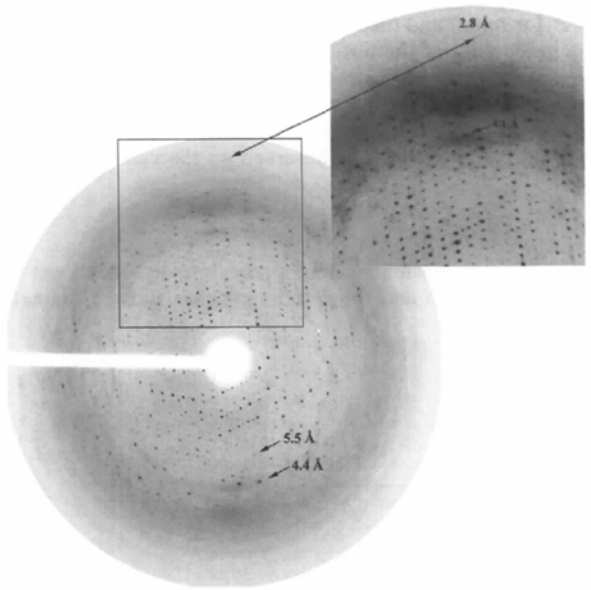

Fig. 2. After flash annealing, an oscillation image taken at spindle setting of 90° on the glycerol kinase crystal from Fig. 1 shows dramatic improvement in both mosaicity and resolution of diffraction. The crystal now diffracts to about 2.8 Å resolution (inset), where previously no diffraction spots were seen close to the edge of the plate (Fig. 1, inset). The freeze–thaw protocol (described in the text) resulted in the markedly improved quality of diffraction and this was seen at all spindle settings throughout the crystal. The crystal's orientation was maintained in the loop throughout the freeze–thaw cycles and all other data-collection parameters are the same as in Fig. 1; however, the oscillation angle here is 1.5°, set for data collection.

The sensitivity of these crystals to the exact length of time in the thawing step indicated that the crystal-annealing technique (Harp *et al.*, 1998) would not improve the quality of diffraction. This is because crystal annealing necessitates an increased amount of time at room temperature because of the equilibration and transfer steps. This technique was tested on another crystal of glycerol kinase that was flash frozen as described above. When the crystal was removed from the cold stream and equilibrated in 50 µl of the cryoprotectant solution at room temperature, then flash frozen under the cold nitrogen stream, the crystal no longer diffracted. Hence, the flash-annealing technique worked for the glycerol kinase crystals when crystal annealing did not.

It is likely that the flash-annealing procedure is generally applicable since crystals neither need to be physically removed from the cold stream nor need to be transferred to various solutions. This makes the procedure simple, reproducible and possibly less perturbing to the crystal. The dramatic improvements seen in the *E. casseliflavus* GK crystals indicates that it may be a worthwhile technique to try on other protein crystals.

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