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Spectacular improvement of X-ray diffraction through fast desiccation of protein crystals

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Succeeding in getting a protein to crystallize is not always the final hurdle in the determination of its three-dimensional structure. A relatively frequent and particularly vexing situation is the production of macroscopically well formed crystals that exhibit no suitable diffraction pattern. In this paper, three independent cases (*i.e.* proteins and crystallization conditions) are reported of spectacular diffraction-pattern improvement through a simple crystal-handling procedure that was discovered serendipitously. The procedure basically consists of removing a non-diffracting frozen crystal from the X-ray beam, plunging it into a soaking solution made of the original crystallization solution supplemented with a traditional cryoprotectant and then letting it dry in the evaporating sitting drop for some time (15 min to several hours). The treated crystals are then remounted and exhibit a huge improvement in their diffraction intensity and resolution. In all three cases presented here, the crystal quality shifted from unusable to perfectly suitable for structure determination. In addition to being a 'last resort' procedure for experimentalists struggling with non-diffracting crystals, this puzzling effect constitutes one more challenging problem for theoretical protein crystallographers.

1. Introduction

The rationalization of protein crystallogenesis (*i.e.* the identification of suitable crystallization conditions from first principles and/or protein-sequence information) has been the Holy Grail of protein crystallographers for quite some time now. However, if protein crystallization is less of a bottleneck in today's laboratory than it was ten years ago, it is mainly owing to our progress in high-throughput screening of crystallization conditions (using commercial screens, robotics and/or optimized experimental design; Abergel *et al.*, 2003; Audic *et al.*, 1997) rather than theoretical understanding of when and why a given protein will produce suitable crystals. In consequence, a number of experimental situations not uncommonly encountered by structural biologists have yet to receive a convincing theoretical explanation. During the course of the structural genomics project running in our laboratory (Abergel *et al.*, 2003), we serendipitously encountered several cases of initially poorly diffracting crystals that exhibited a significant increase in resolution after a long period of time (*e.g.* the 'forgotten crystallization plate' scenario). In order to better establish such phenomena, we initially followed the evolution of experiments in which freshly grown crystals produced poor diffraction (12 Å) or no diffraction image at all. Crystals from the same crystallization plates were tested at regular intervals on synchrotron beamlines. Over time (several months), we observed a significant increase in diffraction

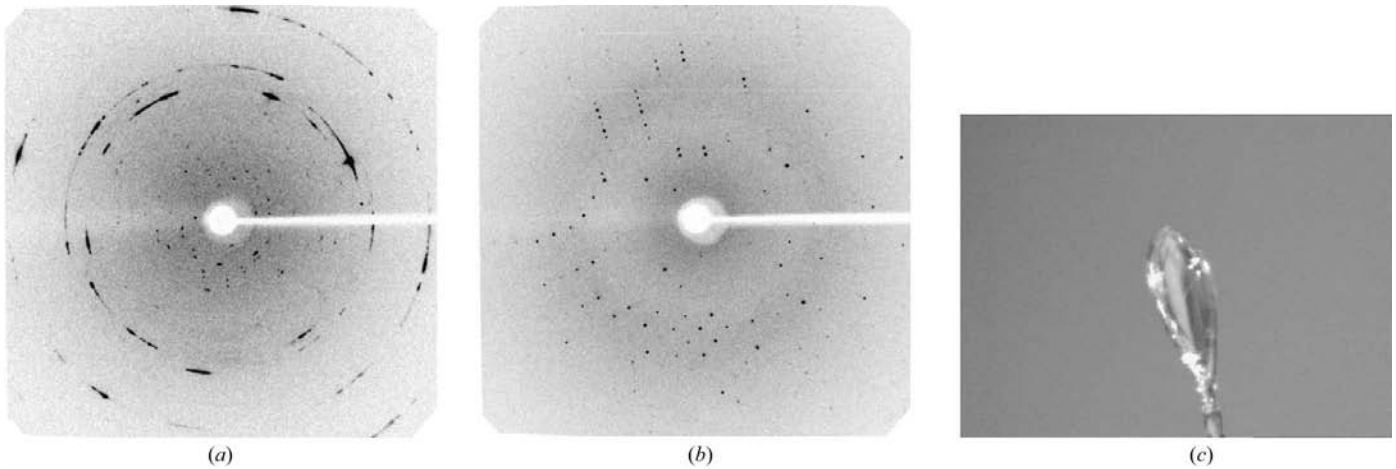

Figure 1
Before and after diffraction patterns for crystals of the *E. coli* YbgL protein ($\Delta\varphi = 0.5^\circ$, $d = 90$ mm, 30 s). (*a*) Diffraction pattern of the flash-frozen aggregated crystals, (*b*) diffraction pattern of a single crystal after a 2 h soaking time, (*c*) picture of the crystal after the procedure. In this figure and Figs. 2 and 3, the ice rings seen in the first images attest to the absence of cryoprotectant.

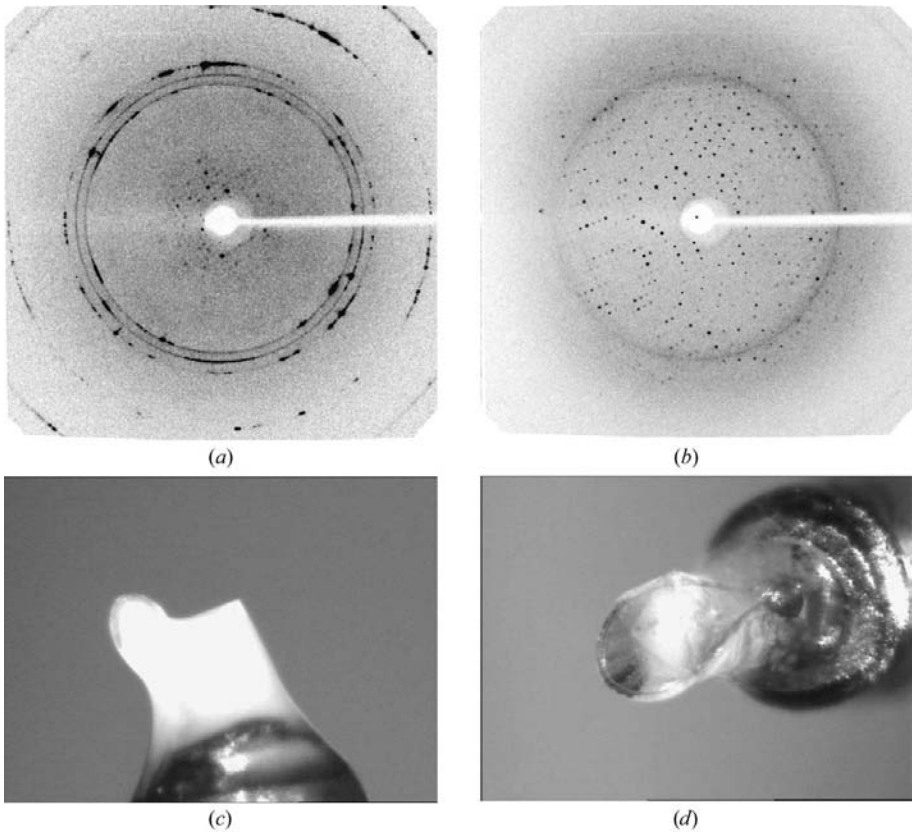

Figure 2
Before and after diffraction patterns for a crystal of the *E. coli* HAM1 protein ($\Delta\varphi = 0.5^\circ$, $d = 90$ mm, 30 s). (*a*) Diffraction pattern of the flash-frozen crystal, (*b*) diffraction pattern of the same crystal after a 30 min soak, (*c*) and (*d*) pictures of the corresponding crystal before and after the procedure.

resolution (8 Å), encouraging us to continue testing them. Over 2 y later, diffraction reached 2.3 Å resolution, while the wells in the crystal plate had almost evaporated. This prompted us to experiment further in order to test the simplistic hypothesis that the evaporation process was key to this crystal self-improvement phenomenon. This work finally converged toward defining a simpler and much faster protocol that could be systematically applied to poorly diffracting crystals. The procedures used in three independent cases are described in the following section.

2. Materials and methods

The three proteins used in this study were cloned, expressed, purified and crystallized as described in Abergel *et al.* (2003). All crystals were grown by vapour diffusion using the hanging-drop method. X-ray diffraction images were recorded on the Xcalibur PX-ULTRA diffractometer (Oxford Diffraction) using a 90 mm distance between the crystal and the Onyx CCD detector.

2.1. The *Escherichia coli* YbgL protein

This protein (Swiss-Prot Accession No. P75746) is a 26 kDa molecular-weight protein belonging to the lamB family. Crystals were grown by mixing 1 µl of a reservoir solution consisting of 0.1 *M* Tris buffer pH 9.15, 0.8 *M* sodium citrate with 1 µl YbgL protein at a concentration of 80 mg ml^{-1} in 10 m*M* Tris buffer pH 9. Each recorded image corresponds to a 0.5° φ oscillation and 30 s exposure time. The first image corresponds to a crystal directly flash-frozen on the goniometer head (Fig. 1*a*), while the second image corresponds to the same crystal thawed and soaked for 2 h in a sitting drop consisting of 1 µl ethylene glycol and 9 µl reservoir solution (Fig. 1*b*). A picture of the crystal after soaking is shown in Fig. 1(*c*). The crystals belong to the monoclinic space group *C*2, with unit-cell parameters $a = 70.33$, $b = 36.73$, $c = 79.67$ Å,

$\beta = 93.51^\circ$. There is one molecule per asymmetric unit, with a solvent content of 56.6%.

2.2. The *E. coli* YggV (HAM1) protein

This protein is a NTPase of 21 kDa molecular weight (Swiss-Prot accession No. P52061). Crystals were obtained by mixing an equal volume (0.5 µl) of reservoir solution consisting of 0.1 *M* bicine buffer pH 9, 35% $(NH_4)_2SO_4$, 1 m*M* EDTA with 0.5 µl HAM1 protein at a concentration of 27.5 mg ml^{-1} in 10 m*M* Tris buffer pH 8, 0.1 *M* NaCl. Each recorded image corresponds to a 0.5° φ oscillation and 30 s exposure time. The first image (Fig. 2*a*) corresponds to a crystal directly flash-frozen on the goniometer head, while the second image (Fig. 2*b*) corresponds to the same crystal thawed and soaked for 30 min in a sitting drop consisting of 1 µl glycerol and 9 µl 0.1 *M* bicine buffer pH 9, 37.5% $(NH_4)_2SO_4$. The flash-frozen crystal is shown in Fig. 2(*c*). Fig. 2(*d*) shows the same crystal soaked for 30 min. The crystals belong to the tetragonal space group $P4_32_12$, with unit-cell parameters $a = b = 79.97$, $c = 79.93$ Å. There is one molecule per asymmetric unit, with a solvent content of 37.9%.

2.3. The *C. albicans* 3-dehydroquinate dehydratase protein

This protein is a homododecameric enzyme of 16 kDa molecular weight per subunit. Crystals were obtained by mixing 0.5 µl reservoir solution consisting of 0.1 *M* HEPES buffer pH 7, 11% PEG 8000, 0.2 *M* $LiSO_4$ with 1 µl protein at a concentration of 23.1 mg ml^{-1} in 50 m*M* Tris buffer pH 7.5, 1 m*M* EDTA and 1 m*M* β-mercaptoethanol. The first diffraction image (Fig. 3*a*) corresponds to the crystal directly flash-frozen on the goniometer head (Fig. 3*c*) using a 0.5° φ oscillation and 60 s exposure time. The second image (Fig. 3*b*) corresponds to the same crystal thawed and soaked for 15 min in a sitting drop made of 1 µl pure glycerol and 9 µl 0.1 *M* HEPES buffer pH 7, 11% PEG 8000, 0.2 *M* $LiSO_4$ (Fig. 3*d*) using the same φ oscillation and a 120 s exposure time. The crystal seems to belong to the monoclinic space group $P2$ or $P2_1$, with unit-cell parameters $a = 166$, $b = 82$, $1c = 286$ Å, $\beta = 97.8^\circ$, with a solvent content of 87.8% for 12 molecules in the asymmetric unit.

3. Results

We report here three cases of crystals grown using salts as precipitating agents in the presence and absence of cryoprotectant. These crystals were flash-frozen at 100 K and produced no or low-resolution diffraction (8–12 Å) after 30–120 s exposure time.

3.1. Initial observations: the *E. coli* YbgL protein

The initial observations were made on YbgL protein crystals obtained as aggregated thin plates. In order to separate these plates, we recovered the flash-frozen crystals from the goniometer head and soaked them in a solution corresponding to the reservoir solution containing 10% ethylene glycol. The soaking step succeeded in separating the plate-shaped crystals and one of them was immediately tested for diffraction. The image produced was typical of a single-crystal diffraction pattern; however, the resolution was still low. This crystal was therefore thawed again and placed back into the soaking solution which meanwhile had been evaporating slowly. Another crystal from the same drop was then tested and we observed a significant improvement in the resolution of the diffraction image. The resolution kept improving for several freeze/thaw rounds to an optimal state beyond which we started to observe a detrimental increase in crystal mosaicity. Following this initial observation, the procedure was optimized to preserve the best crystals from too many repetitions of the thawing/freezing rounds. For this, we worked in parallel using several crystals from the same crystallization droplet. On one hand, some crystals were used to follow the kinetics of the

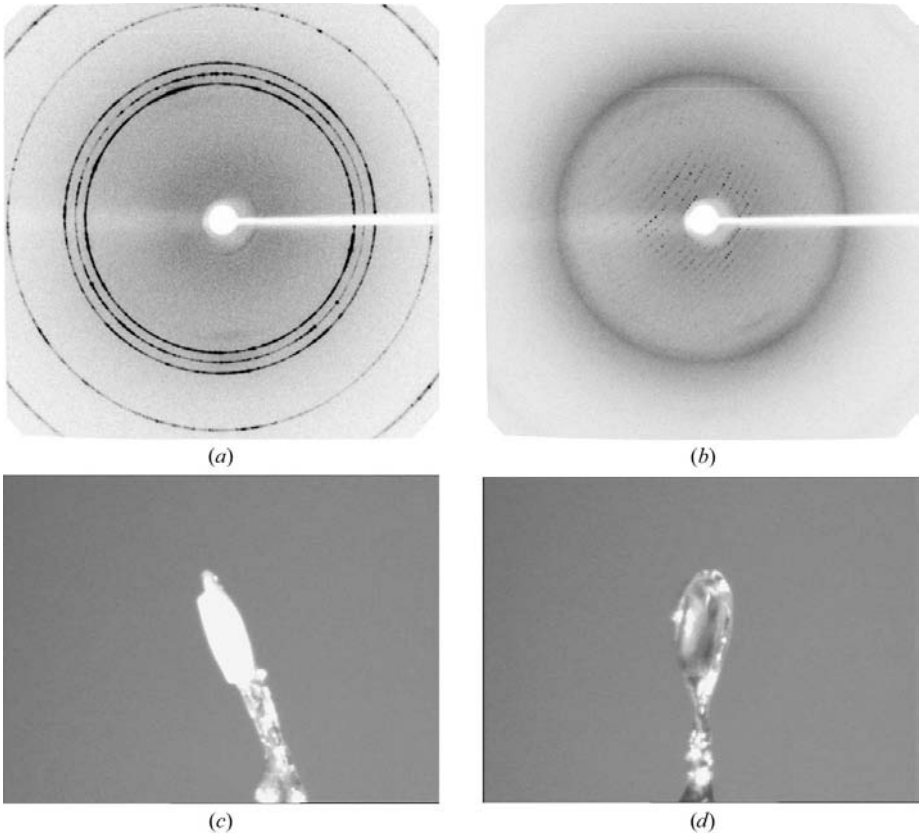

Figure 3
Before and after diffraction patterns for a crystal of the *C. albicans* 3-dehydroquinate dehydratase protein ($\Delta\varphi = 0.5^\circ$, $d = 90$ mm). (*a*) Diffraction pattern of the flash-frozen crystal, 60 s exposure time, (*b*) diffraction pattern after 15 min soak and 120 s exposure time, (*c*) and (*d*) picture of the mounted corresponding crystal before and after the procedure.

resolution improvement, while on the other hand the best crystal was reserved for data collection at the optimal time (about 2 h, no annealing). At this time, we noticed salt crystals starting to appear in the soaking drop. In this specific case, the diffraction quality increased from about 12 to 2.6 Å resolution (as measured locally; Fig. 1). Subsequently, a complete data set was recorded using the ESRF synchrotron BM30A beamline to 1.85 Å resolution. To assess the potential role of the cryoprotectant in this phenomenon, the same protein was co-crystallized with 10% ethylene glycol in the same crystallization conditions (*i.e.* the soaking solution). The crystals produced again exhibited the same initial poor diffraction. However, applying the above protocol (up to 2 h) did not improve the diffraction quality, thus excluding the sole effect of the presence of cryoprotectant within the crystal.

3.2. Crystals of the *E.coli* HAM1 protein

The same procedure was applied to poorly diffracting crystals of the *E. coli* HAM1 protein. A diffraction image was first recorded on the flash-frozen crystal, which was then thawed in the reservoir solution using 10% glycerol as cryoprotectant. At various times, the crystal was flash-frozen again and the diffraction images recorded. A spectacular improvement in diffraction quality (from 12 to 2.6 Å resolution, as measured locally) was again observed (Figs. 2*a* and 2*b*). Subsequently, a complete data set was recorded from this crystal to 2 Å resolution using the ESRF synchrotron BM30A beamline. As in the previous case, a crystal from the same drop was reserved for data collection at the optimal time, thus confirming that the annealing procedure was not essential to the success of the procedure.

3.3. Crystals of the *Candida albicans* 3-dehydroquinate dehydratase

In this last case, our procedure resulted in a diffraction improvement from no diffraction to 3 to 4 Å resolution diffraction (Figs. 3*a* and 3*b*). As in the first case, co-crystallization in presence of cryoprotectant did not improve crystal diffraction and suppressed the effect of the freeze/thaw desiccation procedure (15 min desiccation procedure). The same lack of effect was observed on another (poorly diffracting) crystal form of *C. albicans* 3-dehydroquinate dehydratase grown from MPD solutions.

4. Discussion

J. D. Bernal's historic discovery that usable protein crystals could only be produced within a highly hydrated environment allowed the birth of protein crystallography 70 y ago (Bernal & Crowfoot, 1934; Surridge, 1999). It thus seems ironic that desiccating protein crystals could now be reported as a way to largely improve their diffraction quality. Yet the effect is reproducible and has been observed in three independent cases (out of three tested), involving non-homologous proteins, different crystallization conditions and various solvent contents. The results described in this article are reminiscent of previous reports of incremental improvement in the diffraction of protein crystals following rapid temperature changes (annealing procedure; Harp *et al.*, 1998; Yeh & Hol, 1998) or controlled dehydration in concentrated soaking solutions taking place over months (Esnouf *et al.*, 1998; Fu *et al.*, 1999). However, these procedures achieved 'moderate' improvements in diffraction resolution (from 3 to 2 Å). Our method, perhaps by combining effects from various causes, results in diffraction improvement of an unprecedented magnitude in a matter of hours. If this method is to become of general use within the structural biology community as a systematic procedure to 'rescue' hopeless protein crystals, it seems important to give it some rational basis. Even a crude understanding of the physical phenomena at work could help to optimize its use and extend its application. We believe it is unlikely that the mere evaporation of the solvent is by itself responsible for the large improvement of the diffraction pattern. Rather, the procedure described here probably triggers a crystal-wide reordering of the protein molecules, induced by the stabilization of a preferential conformational state (Esnouf *et al.*, 1998; James & Tawfik, 2003). We would therefore like to propose this puzzling effect to the scrutiny of the protein crystallographic community.

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