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Influence of temperature during crystallization setup on precipitate formation and crystal shape of a metalloendopeptidase

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It is well known that protein crystallization is affected by several different parameters such as the composition of the reservoir solution, the protein concentration, the pH and the temperature. An effect of different temperatures during setup of crystallization experiments was observed for a metalloendopeptidase ($AsaP1_{E294A}$). Spontaneous protein precipitation was reduced and the crystal shape could be improved by decreasing the temperature during crystallization setup.

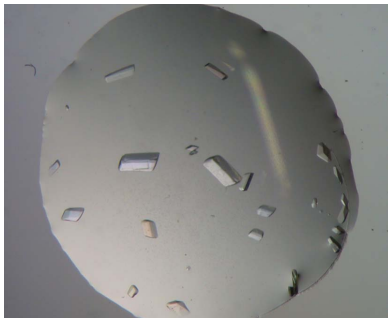
1. Introduction

X-ray crystallography enables us to visualize protein structures at an atomic level and to better understand the function of a protein. The first requirement for structure determination by X-ray crystallography is a well ordered single protein crystal. The nucleation and growth of protein crystals is dependent on several different factors such as the composition of the reservoir solution, the protein purity and concentration, the pH and the temperature. The parameters of successful crystallization trials are not predictable and it is only partly understood which effect is a consequence of a certain factor. The relevance of each parameter may differ considerably in importance for different proteins. For example, the crystallization of α-amylase and catalase is sensitive to temperature, while this is not the case for ovalbumin and ferritin (McPherson, 1990).

To investigate the influence of temperature, crystallization plates with identical crystallization conditions are often stored at different temperatures. Here, we report the effect of temperature during crystallization setup while mixing protein solution with reservoir solution. We observed that placing the crystallization plate on ice, cooling the reservoir solution (to about 277 K) and using pre-chilled pipette tips had a positive effect on the crystal shape and decreased precipitate formation. There was also a slight increase in diffraction quality.

The protein for which this effect was observed is an inactive mutant of the metalloendopeptidase AsaP1 ($AsaP1_{E294A}$), a major virulence factor of the fish-pathogenic bacterium *Aeromonas salmonicida* subsp. *achromogenes* (Gudmundsdóttir *et al.*, 1990; Arnadóttir *et al.*, 2008).

2. Materials and methods



Protein expression, purification and crystallization of $AsaP1_{E294A}$ have recently been described (Bogdanović *et al.*, 2009). For the crystallization experiments reported in this manuscript, a protein solution with a concentration of 16–25 mg ml^{-1} in 20 m*M* Tris–HCl buffer pH 7.6 and 200 m*M* NaCl was used. The protein solution was placed on ice or stored in a refrigerator (~277 K) at all times. Crystallization was carried out manually using hanging-drop vapour diffusion in 24-well crystallization plates (Greiner Bio-One, Item No. 662102). Each well contained 500 µl reservoir solution and the drop consisted of a mixture of 2 µl protein sample and 2 µl reservoir solution. Preparation of each plate took about 20 min.

The crystallization condition for $AsaP1_{E294A}$ (16–25 mg ml^{-1}) comprises 14%(*w*/*v*) PEG 4000, 0.1 *M* HEPES pH 7.5, 0.2 *M*

ammonium sulfate and 10%(*v*/*v*) 2-propanol. This condition was repeated 24 times per plate for three crystallization plates in each of the four different crystallization experiments as explained below.

The first crystallization experiment was set up with the crystallization plate on the bench at 294–296 K and with reservoir solutions at about 294–296 K. The crystallization plate was subsequently stored in a temperature-controlled room at 293 K. The second experiment was performed with the crystallization plate on the bench at 294–296 K and reservoir solutions that had been pre-chilled overnight in a refrigerator (∼277 K) and were kept on ice during the crystallization setup. The crystallization plates were stored at 293 K. In the third experiment the crystallization plate was placed on ice as were the pre-chilled (∼277 K) reservoir solutions. Additionally, pipette tips were cooled to 253 K overnight in a freezer and were placed on ice during crystallization setup. The crystallization plates were stored at 293 K.

In a fourth experiment, crystallization plates were placed on ice as well as the reservoir solutions, which were additionally pre-chilled to 277 K overnight in a refrigerator. These crystallization plates were stored in a temperature-controlled room at 277 K.

The X-ray diffraction resolution of the crystals was tested using a rotating-anode X-ray generator (MicroMax-007, Rigaku) with Osmic multiple layer optics (beam size 0.3 × 0.3 mm) and a CCD detector (SATURN92, Rigaku).

Several crystals obtained in the first three crystallization experiments described above were tested using a cryoprotectant that consisted of the reservoir solution with 14%(*v*/*v*) PEG 400. Crystals were flash-cooled to 110 K in a stream of nitrogen (Oxford Cryosystems) and the diffraction images were analyzed using *CrystalClear* v.1.3.6 (Pflugrath, 1999). Reflections from the test images were determined using the *dtfind* option of the software with default settings of I/σ = 3.0 and a minimum pixel value of 20. These reflections were sorted by their resolution. On the best image recorded for each experiment, the 95th percentile of all reflections was considered as the resolution. Complete data sets were not collected.

3. Results

Recently, the crystallization of AsaP1$_{E294A}$ at a concentration of 7–10 mg ml^{-1} has been reported using crystallization conditions comprising 2.0 *M* ammonium sulfate and 0.1 *M* Tris–HCl pH 8.5, resulting in monoclinic crystals (space group *C*2), and 14%(*w*/*v*) PEG 4000, 0.1 *M* HEPES pH 7.5, 0.2 *M* ammonium sulfate and 10%(*v*/*v*) 2-propanol, resulting in orthorhombic crystals ($P2_12_12_1$) (Bogdanović *et al.*, 2009). In the crystallization experiments described here, we further characterized monoclinic crystals (*C*2) that were obtained using the same condition as used for the orthorhombic crystals but with a higher protein concentration (16–25 mg ml^{-1}; Fig. 1*a*). These crystals showed unit-cell parameters of a = 149.55, b = 48.23, c = 55.35 Å, β = 110.65°, which differed from the previously published monoclinic cell (Bogdanović *et al.*, 2009).

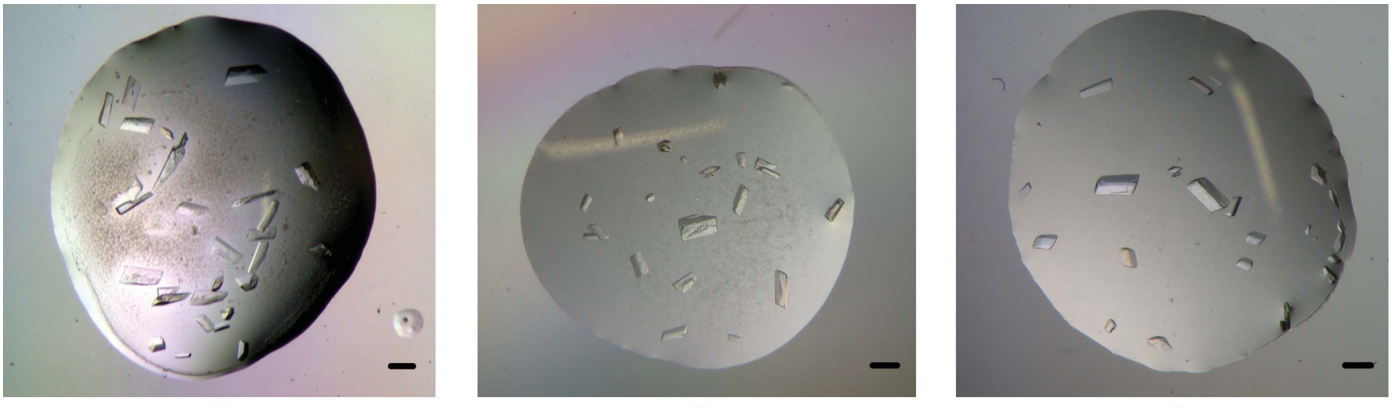
At a protein concentration in the range 16–25 mg ml^{-1}, the first setup of crystallization trials with the crystallization plate on the bench and with reservoir solutions that were not chilled (about 294–296 K) produced crystals within 2 d with rough edges and flaws and considerable amounts of precipitate within the drop (Fig. 1*a*). Protein aggregation and precipitate formation could be detected as a light turbidity immediately upon mixing the protein solution with the reservoir solution. After 2 d the growth of crystals as well as the observed Ostwald ripening (Fig. 1*a*) stopped and the amount of precipitate persisted. Crystals were stable for several weeks up to months and no further changes were detected within the drops.

Precipitate formation and the growth defects of the crystals could be reduced (Fig. 1*b*) by cooling the reservoir solutions to approximately 277 K before mixing them with the protein solution. By placing the crystallization plate on ice during crystallization setup and by additional cooling of the pipette tips (∼253 K) precipitate formation could be decreased even more and the shape of the crystals was also improved (Fig. 1*c*). In these experiments crystal growth again stopped after 2 d and crystals were stable for several weeks up to months.

The results were reproducible for different drops of the same crystallization experiment. All plates for these three experiments were stored at 293 K.

Crystallization conditions with a lower protein concentration (down to 14 mg ml^{-1}) or a reduced precipitant concentration [down to 10%(*w*/*v*) PEG 4000] showed the same effect of protein precipitation and similarly shaped but fewer crystals.

Only phase separation and no crystals could be detected for the fourth crystallization experiment, in which the crystallization plate was placed on ice and chilled reservoir solutions were used. These crystallization plates were stored at 277 K for several months.



(*a*) (*b*) (*c*)

Figure 1
Influence of the temperature during crystallization setup on precipitate formation and crystal shape. Monoclinic crystals of AsaP1$_{E294A}$ from three different crystallization setups are shown. Typical examples of 72 trials after 2–4 d when crystal growth has already stopped are presented. All plates were stored at 293 K for several weeks, but the crystals and precipitate remained unchanged. (*a*) Crystallization at about 294–296 K, (*b*) crystallization at about 294–296 K with pre-chilled reservoir solutions (∼277 K) and (*c*) crystallization plate placed on ice during crystallization setup with pre-chilled reservoir solutions (∼277 K) and pre-chilled pipette tips (253 K). The drop volume is 4 µl and the solid bar at the bottom right represents 200 µm.

For several typical crystals from each crystallization experiment diffraction images were recorded to test the resolution. For crystals obtained in the first, second and third crystallization experiments the resolution of the 95th percentile of all reflections from the best image was 3.83, 3.64 and 3.27 Å, respectively.

4. Discussion

The probability of crystal nucleation and crystal growth is higher the farther the system is pushed into the supersaturation region. On the other hand, the probability of excess nucleation and protein aggregation also increases (McPherson, 1990). Additionally, the crystal-growth velocity is high, which leads to growth defects and flaws in the crystals. Temperature can be one parameter that pushes the system into the supersaturation region. Fig. 1(*a*) shows a condition with protein aggregation and numerous crystals with growth defects and flaws as an example of a system that has been pushed far into the supersaturation region owing to a high protein concentration and probably owing to an elevated temperature. By decreasing the temperature while mixing the protein solution with the reservoir solution (Figs. 1*b* and 1*c*), disordered aggregation of the protein molecules (precipitate) can be reduced and the protein molecules tend to arrange in better ordered single crystals. This is reflected in the slightly better resolution of the corresponding crystals.

In the case of $AsaP1_{E294A}$ the protein could not be concentrated to more than 25 mg ml^{-1} without precipitating; thus, the solubility limit of the protein solution was reached. The solubility as a function of temperature was not investigated. The effect of temperature on protein solubility varies for different proteins as well as for diversified precipitant conditions and should be determined for the particular protein. Proteins in salt solutions seem to be more soluble at lower temperatures, whereas proteins in solutions of polyethylene glycol, methylpentanediol or other organic solvents are less soluble in the cold (McPherson, 1990). The crystallization condition for $AsaP1_{E294A}$ contained polyethylene glycol as a precipitant, but protein precipitation was reduced in the cold. Taking into account the fact that the $AsaP1_{E294A}$ protein is already at the solubility limit owing to the high protein concentration, the observed effect of temperature on protein precipitation could suggest retrograde solubility. Thus, the improvement in crystal quality that was noted could have been because pre-chilling the various components (tips, solutions and trays) of the crystallization setup led to a lower level of supersaturation in the drops.

Blow *et al.* (1994) showed that a separation of nucleation and crystal growth can be achieved by a temperature shift from 283 to 291 K for lysozyme. They also showed that the higher the lysozyme concentration and the longer the incubation time at 283 K the more stable the nuclei that were observed. In our experiments, the number of crystals is the same but their quality has improved. This would suggest that the experiment still reaches the same level of supersaturation. However, seeding experiments (Bergfors, 2003) have shown that crystal quality can be dependent on the quality of the seeds that serve as nuclei. It may be that this is what explains the improvement that we noticed here when we pre-chilled the crystallization setup. The initial nuclei were more ordered owing to the slight and brief decrease in temperature.

However, the temperature response of the protein seems to be complicated because there was no crystallization or protein precipitation in experiment 4. It is not clear if nucleation occurs under these conditions and only crystal growth is not supported at 277 K. The energy barrier for the formation of stable nuclei might be overcome by random fluctuations (Blow *et al.*, 1994) that will increase with temperature.

It may be that nucleation in the different experiments occurs while the crystallization plates that are set up in the cold are stored at 293 K and the temperature slowly increases. At lower temperature the protein solution will pass through the nucleation region and the metastable phase more slowly because of retarded diffusion. This could result in crystallization nuclei with better quality and, owing to a decreased growth velocity, to fewer imperfections within the crystals.

This approach might be useful for proteins that precipitate immediately when added to the precipitant in the drops.

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