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Improving quality and harvest period of protein crystals for structure-based drug design: effects of a gel and a magnetic field on bovine adenosine deaminase crystals

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The overall crystal quality as well as the harvest period of bovine adenosine deaminase containing a zinc ion at the active centre has been compared in three different environments: crystallization as a control, crystallization with agarose gel and crystallization in a high magnetic field. In crystallization with agarose gel, the probability of obtaining high-quality crystals was somewhat increased, but the harvest period was elongated. On the other hand, in crystallization in a magnetic field, the probability of obtaining high-quality crystals was greatly increased. Furthermore, the harvest period for crystal growth in a magnetic field was much shorter than that with agarose gel.

1. Introduction

The quality of crystals is most important for determining the precise structure for structure-based drug design (SBDD), one of the most powerful processes for producing effective drugs. Accurate and reliable structures are available when X-rays are diffracted to high resolution in all directions. Therefore, a number of more or less general means of improving the quality of protein crystals during the course of crystal growth are being developed. At present, methods to increase the stability of the crystal-growth environment include growth in a high magnetic field (Ataka *et al.*, 1997; Sakurazawa *et al.*, 1999; Lin *et al.*, 2000; Sato *et al.*, 2000; Wang *et al.*, 2002), growth with gels (Cudney *et al.*, 1994; García-Ruiz & Moreno, 1994; Zhu *et al.*, 2001), growth in microgravity (Borgstahl *et al.*, 2001) and growth in the presence of cross-linking agents (Pakhomova *et al.*, 2000).

A supplementary requirement is that the harvest period for obtaining crystals must be sufficiently short for a smooth and efficient SBDD cycle. In our case, a complex with a new class of inhibitor must be analyzed within two weeks, since other investigations, except for X-ray analysis, can usually be finished within at least one month. To obtain effective drugs (not inhibitors), a number of SBDD cycles are necessary. Therefore, speedy acquisition of complex crystals can also be advantageous for successful SBDD.

We have already shown that crystals of bovine adenosine deaminase (ADA) containing a zinc ion at the active centre are easily grown in the presence of an inhibitor in two weeks (Kinoshita *et al.*, 1999); the structure of the complex has been solved to 2.5 Å resolution (Kinoshita *et al.*, 2003). However, most of the crystals were of relatively low quality with a large amount of sticky brown precipitation in the normal environment. Only 5% of all crystals grown could be used to collect diffraction data. For the other 95% of the crystals, data could not be measured because diffraction spots were streaked or split. Moreover, even a large crystal scarcely diffracted to over 2.5 Å resolution.

In order to supply precise information for SBDD for ADA on a suitable timescale and successively, methods to improve the quality of crystals must be developed in which the harvest period is maintained or reduced. In the present example with bovine ADA, the harvest period of two weeks was acceptably short; however, since the crystal quality was generally poor in most batches, we could not carry out SBDD effectively without some means of improving the crystal quality. We therefore tested two alternative environments. One was crystallization with agarose gel, which can be used easily and is a popular method. The other was crystallization in a magnetic field, which has recently been shown to improve the quality of certain protein crystals (Lin *et al.*, 2000). We compared crystals grown in the three environments in order to determine the optimum conditions.

2. Methods

Bovine ADA was purchased from Roche Diagonostics Inc. and used without further purification. Several lots of commercial ADA were characterized and were found to be almost identical in solution based upon SDS–PAGE and dynamic light scattering. Moreover, the activities of the lots used were 194–210 U mg^{-1}, approximately the same as expected (200 U mg^{-1}). All crystallization experiments using the normal method led to the same results. Purine riboside, a potent inhibitor, and agarose gel (type V: high gelling

temperature) were purchased from Sigma. ADA crystals were prepared by the sitting-drop vapour-diffusion method in all the studied environments. All operations were carried out using 24-chamber crystallization plates purchased from Charles Supper Co. Throughout all experiments, the temperature was maintained at 293 K as far as possible.

As a control, we used crystals grown outside the magnetic field and without agarose gel. The previously described procedure for crystallization (Kinoshita *et al.*, 1999) was modified in order to eliminate experimental differences. Firstly, 4 µl drops of the sample buffer, 25 m*M* HEPES pH 7.5, were placed in the sitting basin in the centre of the wells. 4 µl drops of reservoir solution and 8 µl drops of protein solution were then mixed with the previously set-up sample buffer. Crystallization with agarose gel was performed using the same procedure as that for the control, except that agarose gel was added to the sample buffer. The concentration of agarose gel was optimized at 0.1%(*w*/*v*), a concentration at which the solution was not gelled but was somewhat viscous. The procedure for crystallization in a magnetic field was also performed in a similar manner as that for the control. Immediately after making up the crystallization drops, the wells were inserted into the bore of a liquid-helium-free superconducting magnet (JASTEC), which is not commercially available, but was specially manufactured for protein crystallization at National Institute of Advanced Industrial Science and Technology (Lin *et al.*, 2000). The bore was 100 mm in diameter and oriented vertically. Therefore, the plates were cut up into four-well sections owing to the bore size. The container pieces were placed at the position of maximum field (the centre of the magnet). Crystals were grown under a homogeneous and static magnetic field of 10 T. The container was placed in an incubator for a few days until data collection. The crystals did not grow further after removal from the magnetic field.

A single crystal from each condition was selected with the same size as far as possible; it was difficult to prepare a large number of similar-sized crystals from all environments. The crystals were dipped into a solution of NVH oil (Hampton Research Inc.) before data collection. The crystals were also placed in the X-ray stream with a similar orientation. Optical microscope observation and photographs were used to evaluate the size and orientation of crystals. For equal comparison, diffraction data sets were collected on the same equipment, an R-AXIS VI++ image plate on a Rigaku rotating-anode generator, operated at 50 kV and 100 mA. This source is rather weak and inferior but is a convenient source of a constant X-ray. For exact comparison, common experimental parameters were used. Each of the 1.0° oscillation images were exposed for 15 min. The crystal-to-image distance was 100 mm. The total oscillation ranges of the respective data sets were decided according to indications from the program *CrystalClear* (Rigaku) to give the same redundancies with 99% completeness. Data were also processed with *CrystalClear*. The statistics of the crystallization and data collection are shown in Table 1.

3. Results and discussion

Most crystals grown as controls had poor shape (Fig. 1*a*) and their diffraction spots were streaked or split (Fig. 2*a*). Only one batch of the 20 batches we prepared for structure determination contained a high-quality crystal with clear edges; this crystal diffracted to 2.5 Å resolution. The crystal used for data collection grew within two

Table 1
Crystallization and preliminary crystallographic data of ADA crystals in three environments.

Values in parentheses refer to the highest resolution shells: 2.65–2.50 Å for the control crystal, 2.33–2.20 Å for the agarose gel crystal and 2.08–2.00 Å for the magnetic field crystal.

	Control	Agarose gel	Magnetic field
Crystallization			
Optimum precipitant concentration (*M*)	2.0–2.1	2.1–2.2	2.3–2.4
Time	2 weeks	1 month	9 d
Average crystal size (mm)	0.20 × 0.15 × 0.15	0.30 × 0.20 × 0.20	0.30 × 0.20 × 0.20
No. of crystals per batch	5–10	1–5	1–3
No. of batches containing good-shaped crystals/ No. of batches tried	1/20	5/10	6/6
X-ray data collection at 100 K			
Total oscillation range (°)	105	98	90
Unit-cell parameters (space group $P4_32_12$)			
a (Å)	77.19	77.30	77.22
c (Å)	135.62	135.58	134.90
Diffraction limit (Å)	2.5	2.2	2.0
$I/\sigma(I)$	10.5 (3.4)	7.7 (3.1)	9.6 (3.3)
$R_{\rm merge}$ (%)	5.0 (18.7)	6.8 (26.4)	6.6 (21.6)
Redundancy	8.9	6.3	5.2
Completeness (%)	99.2 (99.9)	98.8 (99.1)	99.1 (98.1)

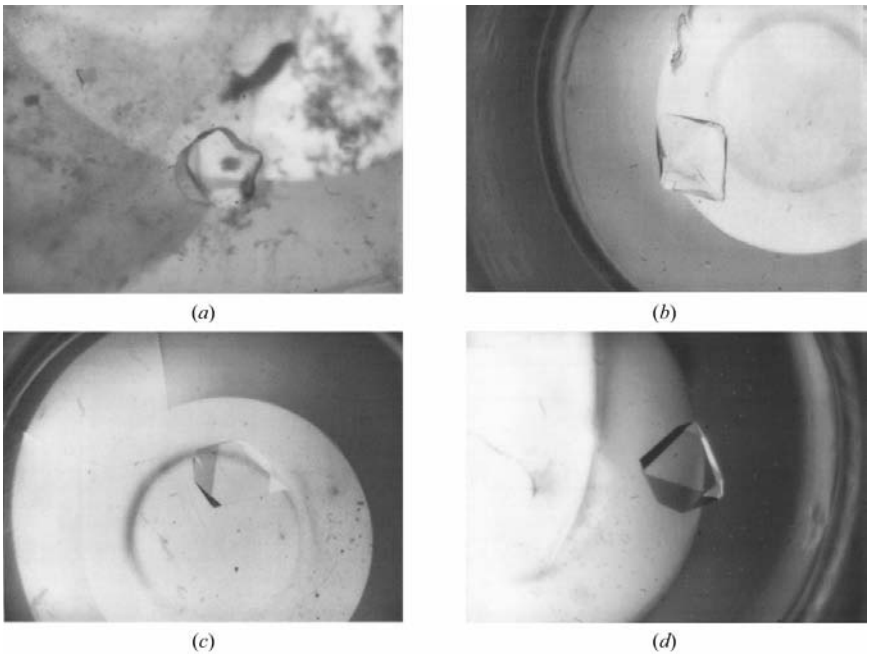

Figure 1
Photographs of ADA crystals prepared under several environments. (*a*) A typical crystal in the control environment without a gel or a magnetic field. Crystals with poor edges emerged with a large amount of additional brown precipitation. (*b*) A poor crystal grown in agarose gel. A crystal having poor edges emerged with a small amount of brown precipitation. (*c*) A good crystal from an environment with agarose gel. (*d*) A crystal grown in a magnetic field. In a 10 T magnetic field, all crystals in all batches had sharp edges. Furthermore, no precipitation occurred. The crystals diffracted to a resolution of 2.0 Å.

weeks and had dimensions of 0.20 × 0.15 × 0.15 mm. In almost all batches, 5–10 crystals per batch emerged with a large amount of additional brown precipitation. The optimum concentration of ammonium sulfate as a precipitant was 2.0–2.1 *M*.

In the crystallization with agarose gel, the probability of obtaining high-quality crystals was somewhat increased compared with the control. Furthermore, the brown precipitation was decreased. This indicates that the random aggregation of protein molecules may be suppressed. Generally, the situation of aggregation, as judged by a test such as dynamic light scattering, almost always leads to precipitation. All batches prepared contained 1–5 crystals. Five out of the ten batches prepared had good crystals. A poor-shaped crystal is shown in Fig. 1(*b*) and a well shaped one in Fig. 1(*c*). The well shaped crystals had clear edges, dimensions of 0.30 × 0.20 × 0.20 mm and diffracted to 2.2 Å resolution. However, the harvest period necessary to obtain a crystal such as that in Fig. 1(*c*) was extended to one month. We assume that this increase results from the role of the agarose gel in controlling the molecular motion in solution. In other words, agarose gel suppresses crystal nucleus formation as well as crystal growth. The slow growth leads to the suppression of random aggregation and also to improvement in crystal quality. The optimum concentration of ammonium sulfate as a precipitant was slightly increased to 2.1–2.2 *M*. In agreement with the previous work (Zhu *et al.*, 2001), we demonstrated the superiority of the gel environment, but also experienced disadvantages in terms of the speed of obtaining high-quality crystals.

Finally, in crystallization in a high magnetic field, the probability of obtaining high-quality crystals was greatly increased and brown precipitation was completely absent in all batches. It appears that random aggregation was strongly suppressed in this experiment. All batches contained only good crystals. The crystals had clear edges (Fig. 1*d*), diffracted to 2.0 Å resolution and had dimensions of 0.30 × 0.20 × 0.20 mm. Furthermore, the harvest period of the crystals was reduced to 9 d, much shorter than in the case of using gel. 1–3 crystals per batch emerged in every experiment. With the precipitant concentration used for the control and gel experiments (2.0–2.2 *M* ammonium sulfate), no crystals were obtained in a magnetic field. The optimum concentration of ammonium sulfate was found to be significantly higher (2.3–2.4 *M*). Using this concentration in the control and gel environments produced many tiny crystals, inappropriate for structure determination, within one week. Based upon our suggestion that the magnetic field strongly suppresses both the formation of crystal nuclei and precipitation, it is thought that the higher concentration of ammonium sulfate may effectively promote crystal growth within a short period.

4. Conclusions

It was shown that both agarose gel and a magnetic field improved the quality of ADA crystals. Both environments were favourable for ADA crystal growth. From a practical viewpoint, crystallization in a magnetic field gave a higher yield of high-quality crystals without brown precipitate. Furthermore, crystals grown in a magnetic field diffracted to a much higher resolution than the control crystals and to considerably higher than the gel-grown crystals. A crystal grown in a magnetic field diffracted to 2.0 Å resolution and crystal grown in gel to 2.2 Å when the same-sized crystals were used. On the basis of these experiments, the magnetic field most effectively improved the crystal quality. Additionally, crystals grown in a magnetic field could be obtained in the shortest time period of the three environments. Generally, it may be expected that the agarose gel extends the harvest period because of the suppression of crystal-nuclei formation and molecular motion in the gelled environment. In fact, the gel-grown crystal grew in one month or longer. Crystal growth in a magnetic field has also been reported to be slower than the control (Yin *et al.*, 2001). In our case, the magnetic field also may have slowed crystal nucleation; however, increasing and optimizing the precipitant concentration compensated for this and our crystals grew within 9 d without any detrimental side effects.

We therefore conclude that we could achieve our goal of sufficiently rapid growth of high-quality crystals of ADA by employing crystal growth in a magnetic field on the basis of these experiments. In order to verify this result statistically or confidently, further experiments are necessary.

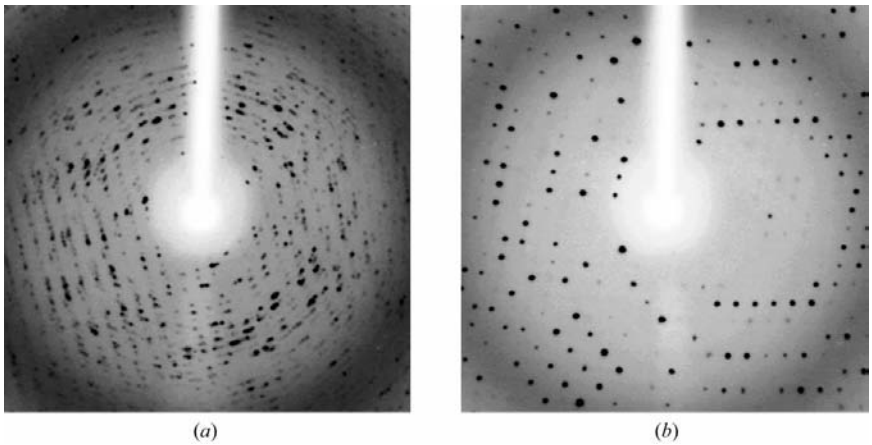

Figure 2
Diffraction images from ADA crystals. (*a*) An image from a typical crystal in the control environment (Fig. 1*a*). The diffraction spots were streaked. (*b*) An image from a good crystal (Fig. 1*d*). The diffraction spots were sharp and clear.

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