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Solution stirring initiates nucleation and improves the quality of adenosine deaminase crystals

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Crystals of bovine adenosine deaminase (ADA) grown over a two-week period in the presence of an inhibitor (ADA complex) were found to be of low quality for X-ray diffraction analysis. Furthermore, ADA incubated in the absence of an inhibitor (ADA native) did not form any crystals using conventional crystallization methods. A solution-stirring technique was used to obtain high-quality ADA complex and ADA native crystals. The crystals obtained using this technique were found to be of high quality and were shown to have high structural resolution for X-ray diffraction analyses. The results reported here indicate that the solution-stirring technique promotes nucleation and improves the quality of protein crystals.

1. Introduction

Adenosine deaminase (ADA) is found in most mammalian tissues and is one of the enzymes involved in the purine pathway. Several studies have reported that elevated ADA levels are associated with a number of diseases. ADA inhibitors have been used as antimetabolic and antineoplastic agents and as modulators of neurological function because they strongly influence adenosine levels (Glazer, 1980; Centelles *et al.*, 1988). However, these drugs are seldom used because they are highly toxic. The development of toxin-free ADA drugs would have several practical applications for treating neurological diseases. Structure-based drug design is considered to be an effective method for discovering new and improved drugs. To achieve this, it is necessary to resolve the crystalline structure of ADA in the presence and absence of an inhibitor (ADA complex and ADA native, respectively).

The crystalline structure of ADA complex with a zinc ion at the active center has been solved at 2.5 Å (Kinoshita, Nishio *et al.*, 2003) and 2.0 Å (Kinoshita, Ataka *et al.*, 2003). ADA complex crystals were produced after an incubation period of two weeks (Kinoshita *et al.*, 1999). However, most of the crystals were of relatively low quality and large amounts of sticky brown precipitate formed in the crystallization solutions. Further investigation showed that 95% of the crystals grown using this method were not appropriate for collecting X-ray diffraction data because of streaking and splitting. Data could be measured in only 5% of the crystals. It has recently been reported that growth in the presence of a high-strength magnetic field improves the quality of ADA complex crystals (Kinoshita, Ataka *et al.*, 2003). It was also reported that the probability of obtaining high-quality crystals of ADA complex was increased and no brown precipitates were produced during the crystallization process. This experiment used a liquid-helium-free superconducting magnet (JASTEC; not commercially available; specially manufactured for protein crystallization at the National Institute of Advanced Industrial Science and Technology of Japan; Lin *et al.*, 2000).

In contrast, ADA produced in the absence of an inhibitor (ADA native) has not been reported to form crystals using conventional crystallization methods, even under the influence of a high-strength magnetic field. Therefore, the crystalline structure of ADA native has not been reported.

We previously developed a unique method for growing large high-quality protein crystals (Adachi *et al.*, 2002; Adachi, Takano, Yoshimura *et al.*, 2003; Adachi, Matsumura *et al.*, 2003; Adachi, Takano *et al.*, 2004; Adachi, Niino, Mutsumura, Takano, Inoue *et al.*, 2004; Yaoi

et al., 2004). This method involved the growth of protein crystals in a continuously stirred solution. The technique was shown to be very effective and similar in principle to the floating and stirring technique (FAST; Adachi *et al.*, 2002), the Micro-stirring technique (Adachi, Takano, Yoshimura *et al.*, 2003), and the Micro-FAST (Adachi, Matsumura *et al.*, 2003). Solution stirring prevents excess nucleation and accelerates the growth rate of protein crystals.

In this study, we tested the use of the Micro-stirring technique for the crystallization of ADA complex and ADA native, as well as the Micro-FAST for ADA native crystal development. All of these techniques are desirable as they are simple and easy to use. The Micro-stirring method combines traditional sitting-drop vapour-diffusion and stirring methods. Fig. 1(*a*) schematically illustrates the Micro-stirring technique using a rotary shaker. The principle of the method is very simple. A crystallization plate is rotated gently to shake the drops as in the sitting-drop vapour-diffusion technique. The Micro-FAST is used to stir the protein solution indirectly during the crystallization process (Fig. 1*b*). Application of the Micro-FAST enables the protein solution to be stirred mildly in combination with the vapour-diffusion technique using micro-scale samples. Protein crystals can be grown at the interface of the two liquids without contacting the vessel using the Micro-FAST, a characteristic that leads to the development of structurally perfect crystals and improved crystallinity. The Micro-FAST facilitates convenient extraction of the protein crystals after growth without leading to mechanical damage. In our experiments, we used Fluorinert (registered trademark of 3M) for the insoluble and very dense liquid. Fluorinert is a clear liquid with no observable effect on the growth of protein crystals. When the protein solution is added to this high-density liquid, it separates from the liquid and floats to the surface, forming an interface.

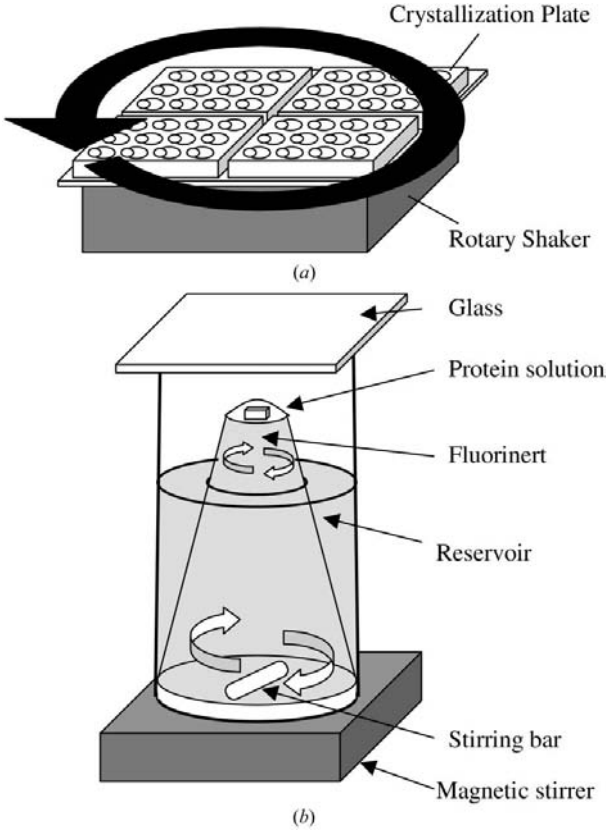

Figure 1
Schematic illustration of (*a*) the Micro-stirring technique and (*b*) the Micro-FAST, both of which combine vapour-diffusion and stirring methods (Adachi, Takano, Morikawa *et al.*, 2003; Adachi, Matsumura *et al.*, 2003).

Table 1
Crystallization data of ADA complex.

	With shaking	Without shaking
Crystallization time (d)	22–30	5–7
Batches tried	40	11
Batches containing crystals	26	11
Batches containing well shaped crystals	18	0

Table 2
Crystallographic parameters of ADA crystals grown by the Micro-stirring technique.

Values in parentheses are for the highest resolution shell.

	ADA complex	ADA native
Space group	$P4_32_12$	$P4_32_12$
Unit-cell parameters (Å)		
a	76.9	73.7
c	134.5	134.0
Diffraction limit (Å)	2.0	1.8
Average $I/\sigma(I)$	9.0	10.3
$R_{\rm merge}$ (%)	7.4 (33.2)	5.9 (32.2)
Completeness (%)	93.9 (84.2)	97.5 (97.2)

It has been demonstrated that stirring the protein solution prevents excess nucleation and encourages the growth of larger crystals. In our study, we also found that stirring improves the quality of the protein crystals and is a useful method of producing crystals of substances that do easily not crystallize using conventional techniques. In this paper, we summarize the results obtained for the crystallization of ADA using a solution-stirring technique.

2. Experimental procedures

2.1. Materials

Bovine ADA was purchased from Roche Diagnostics Inc. and used without further purification. Purine riboside (an inhibitor) was purchased from Sigma–Aldrich. All other chemicals were reagent grade. The sitting-drop and floating-drop (Adachi, Takano, Morikawa *et al.*, 2003) vapour-diffusion method was used in combination with the Micro-stirring and the Micro-FAST, respectively. The crystallization plate used for the Micro-stirring technique and the sealing tape were purchased from Emerald BioStructures. A rotary shaker was used to agitate the crystallization plates. A special crystallization vessel designed for the Micro-FAST was used in the experiments. This vessel was fabricated by laser stereolithography (Cybox). For application of the Micro-FAST, we used a small stirring bar (8 mm long and 1.5 mm in diameter) in combination with a magnetic stirrer.

2.2. Crystallization

The ADA complex was crystallized at a protein concentration of 20 mg ml^{-1} by vapour diffusion in 2.2 *M* $(NH_4)_2SO_4$, 2% 2-methyl-2,4-pentanediol (MPD), 0.1 *M* 2-*N*-morpholino-ethanesulfonic acid (MES) buffer pH 6 at 293 K (Kinoshita, Ataka *et al.*, 2003; Adachi, Matsumura *et al.*, 2004). For ADA native crystallization, a commercially available sparse-matrix screening kit (96 conditions) obtained from Hampton Research was used (Adachi, Niino, Matsumura, Takano, Kinoshita *et al.*, 2004). The concentration of this solution was 64 mg ml^{-1}.

The crystallization trials for the vapour-diffusion technique were conducted under shaken (Micro-stirring and Micro-FAST) and

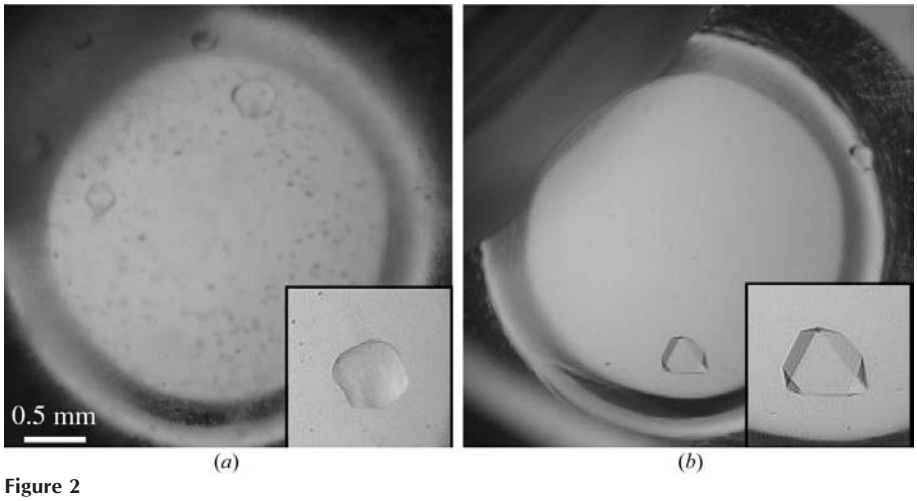

Figure 2
Typical results obtained for the crystallization of ADA complex, achieved at 293 K from the sitting-drop vapour-diffusion experiments: (*a*) without shaking and (*b*) with shaking.

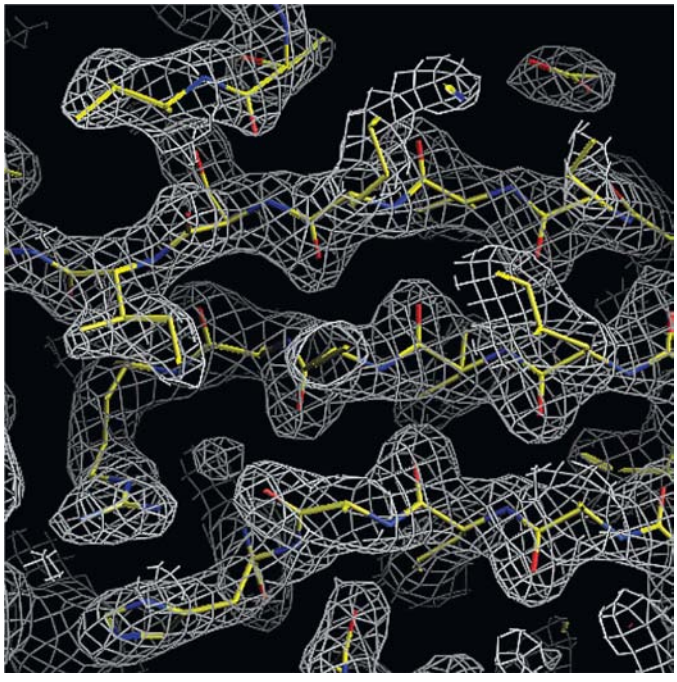

Figure 3
Electron-density map of ADA complex around the N-terminal domain. The $2F_o - F_c$ map is contoured at 1.2σ.

unshaken conditions. We used rotation speeds of 50 and 100 rev min^{-1} to stir the protein solution for the Micro-stirring and Micro-FAST techniques, respectively. The trials were carried out at 293 K.

2.3. X-ray diffraction (XRD) experiments

The crystal was mounted in a cryoloop. Data were collected at 100 K on a Rigaku R-AXIS IV^{++} imaging plate. Cu $K\alpha$ radiation was produced using a Rigaku ULTRaX18 rotating-anode generator operated at 50 kV and 100 mA. The crystal-to-detector distance was set between 100 and 140 mm and the crystal oscillation angle per image was set at 1 and 0.6° for the ADA complex and native ADA, respectively.

3. Results and discussion

3.1. ADA complex

ADA complex crystals were obtained using the Micro-stirring technique (shaken) and the traditional sitting-drop vapour-diffusion technique (unshaken). Fig. 2 depicts the crystals produced using these two methods. Table 1 summarizes observations taken during the crystallization process. The results show differences in the shape of nucleated crystals produced on the shaken and unshaken plates. The results demonstrated that all crystals grown without shaking were poorly shaped and the probability of obtaining well shaped crystals was greatly increased by shaking the solution. The amount of brown precipitate produced in the solution also decreased with shaking. Crystals obtained using the rotary shaker had clear edges and appeared similar to those produced under the influence of a high-strength magnetic field (Kinoshita, Ataka *et al.*, 2003). Clearly, stirring caused the homogenization of the protein solution and assisted in the control of solution flow toward the surface of the developing grown crystals (Kadowaki *et al.*, 2004). Nucleation time was found to be significantly longer under the influence of shaking. This observation indicates that homogenizing the protein solution by stirring decreases the spontaneous nucleation of crystals. The results of this experiment demonstrate that stirring is a more effective method of producing high-quality protein crystals than conventional techniques.

XRD data were obtained for the ADA complex crystals, to evaluate crystallinity. The crystals grown using the Micro-stirring technique diffracted at a resolution of more than 2.0 Å. In contrast, the diffraction spots of the crystals grown without shaking were streaked or split. Therefore, no XRD data were collected from their crystals. Table 2 summarizes the crystallographic parameters of the crystals grown using the Micro-stirring technique. They were similar to those grown in a high-strength magnetic field (Kinoshita, Ataka *et al.*, 2003). Structural analysis of the ADA crystals grown with the stirring treatment indicated a resolution of 2.0 Å. The resulting electron-density map for this crystal was of very high quality (Fig. 3). This result demonstrates that the Micro-stirring technique is effective for crystallizing protein–drug complexes with high diffraction quality.

3.2. ADA native

Crystallization screening of the ADA native was investigated with experiments carried out using the Micro-stirring technique. The crystals of ADA native were grown in a reservoir containing 20% 2-propanol and 20% PEG 4000 in 0.1 *M* citrate buffer pH 5.6. This solution differed from that used for the growth of the ADA complex. After two months of growth, crystals with dimensions of 0.3 × 0.3 × 0.2 mm were obtained for the first time (Fig. 4*a*). Crystals did not develop in the unshaken samples. Crystal development was extremely slow in the shaking condition (two months) indicating that stirring of the protein solution decreases spontaneous nucleation but is nevertheless a useful method for nucleating proteins that do not crystallize using conventional techniques. It is reasonable to suppose that stirring increases the interactions between the protein molecules, which might lead to the development of large clusters. These clusters might form the stable nuclei of critical radii required for spontaneous nucleation to occur. Although the crystallization conditions were

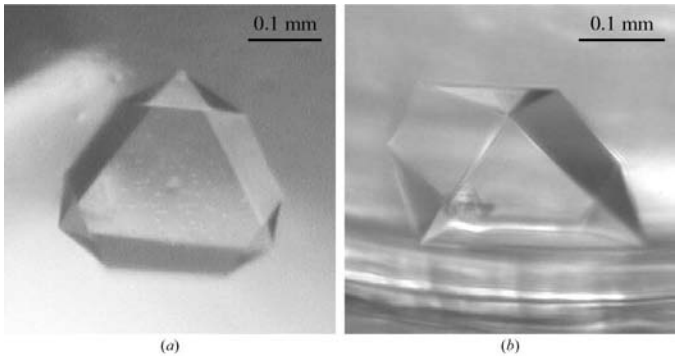

Figure 4
Typical crystallization results of ADA native grown using (*a*) the Micro-stirring technique and (*b*) the Micro-FAST.

clearly different between the ADA complex and ADA native solutions, the shapes of the crystals appeared similar. The similarity in crystal shape suggests that the ADA native crystals were also of high quality for XRD analyses. We obtained similar results using the Micro-FAST (Fig. 4*b*), confirming the efficacy of stirring on the production of ADA native crystals.

The structure of ADA native was investigated using XRD. The crystal grown using the Micro-stirring technique diffracted with a resolution of more than 1.8 Å (Table 2). Analysis of the ADA native crystals grown using the stirring technique indicated that the structure had a resolution of 1.8 Å. The electron-density map was found to be of very high quality. In addition, similar XRD results were obtained from the crystals grown using Micro-FAST. Both techniques enable one to stir the protein solution mildly based on indirect stirring, leading to logical results in production of the crystals. The structural details of ADA complex and native including the water molecules will be reported elsewhere (Kinoshita *et al.*, submitted work).

4. Conclusion

In this paper, we demonstrated that stirring a protein solution improves crystallinity. Stirring a protein solution was also found to promote nucleation of high-quality ADA native crystals. The results of this study indicate that the stirring technique is a useful method for obtaining crystals from proteins that do not readily crystallize using other more conventional methods.

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References

Adachi, H., Matsumura, H., Niino, A., Takano, K., Kinoshita, T., Warizaya, M., Inoue, T., Mori, Y. & Sasaki, T. (2004). *Jpn J. Appl. Phys.* **43**, L522–L525.
Adachi, H., Matsumura, H., Takano, K., Niino, A., Inoue, T., Yoshimura, M., Mori, Y. & Sasaki, T. (2003). *Jpn J. Appl. Phys.* **42**, L1161–L1163.
Adachi, H., Niino, A., Matsumura, H., Takano, K., Inoue, T., Mori, Y. & Sasaki, T. (2004). *Jpn J. Appl. Phys.* **43**, L243–L246.
Adachi, H., Niino, A., Matsumura, H., Takano, K., Kinoshita, T., Warizaya, M., Inoue, T., Mori, Y. & Sasaki, T. (2004). *Jpn J. Appl. Phys.* **43**, L762–L764.
Adachi, H., Takano, K., Matsumura, H., Inoue, T., Mori, Y. & Sasaki, T. (2004). *J. Synchrotron Rad.* **11**, 121–124.
Adachi, H., Takano, K., Morikawa, M., Kanaya, S., Yoshimura, M., Mori, Y. & Sasaki, T. (2003). *Acta Cryst.* D**59**, 194–196.
Adachi, H., Takano, K., Yoshimura, M., Mori, Y. & Sasaki, T. (2002). *Jpn J. Appl. Phys.* **41**, L1025–L1027.
Adachi, H., Takano, K., Yoshimura, M., Mori, Y. & Sasaki, T. (2003). *Jpn J. Appl. Phys.* **42**, L314–L315.
Centelles, J. J., Franco, R. & Bosal, J. (1988). *J. Neurosci. Res.* **19**, 258–267.
Glazer, R. (1980). *Rev. Drug Metabol. Drug Interact.* **3**, 105–128.
Kadowaki, A., Yoshizaki, I., Rong, L., Komatsu, H., Odawara, O. & Yoda, A. (2004). *J. Synchrotron Rad.* **11**, 38–40.
Kinoshita, T., Ataka, M., Warizawa, M., Neya, M. & Fujii, T. (2003). *Acta Cryst.* D**59**, 1333–1335.
Kinoshita, T., Nishio, N., Nakanishi, I., Sato, A. & Fujii, T. (2003). *Acta Cryst.* D**59**, 299–303.
Kinoshita, T., Nishio, N., Sato, A. & Murata, M. (1999). *Acta Cryst.* D**55**, 2031–2032.
Lin, S.-X., Zhou, M., Azzi, A., Xu, G.-I., Wakamiya, N. I. & Ataka, M. (2000). *Biochem. Biophys. Res. Commun.* **275**, 275–278.
Yaoi, M., Adachi, H., Takano, K., Matsumura, H., Inoue, T., Mori, Y. & Sasaki, T. (2004). *Jpn J. Appl. Phys.* **43**, L686–L688.