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Crystallization of the archaeal transcription termination factor NusA: a significant decrease in twinning under microgravity conditions

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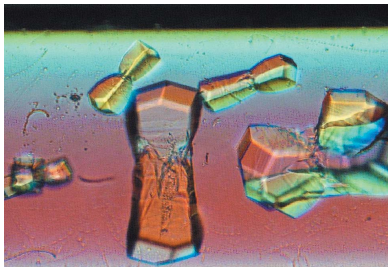


The transcription termination factor NusA from *Aeropyrum pernix* was crystallized using a counter-diffusion technique in both terrestrial and microgravity environments. Crystallization under microgravity conditions significantly reduced the twinning content (1.0%) compared with terrestrially grown crystals (18.3%) and improved the maximum resolution from 3.0 to 2.29 Å, with similar unit-cell parameters. Based on a comparison of the crystal parameters, the effect of microgravity on protein crystallization is discussed.

1. Introduction

Bacterial NusA is a multifunctional protein involved in transcriptional pausing, termination and antitermination processes (Nudler & Gottesman, 2002; Borukhov *et al.*, 2005). NusA is an elongated protein composed of several domains: an RNA polymerase-binding N-terminal domain (NTD), an RNA-binding S1 domain and two RNA-binding K-homology (KH) domains (Worbs *et al.*, 2001; Gopal *et al.*, 2001; Zhou *et al.*, 2002). NusA-related proteins are phylogenically conserved among eubacteria and archaea; however, the archaeal NusA protein is composed of only tandem KH domains, which are supposed to bind RNA. Although the G*XX*G motifs in the KH domains of bacterial NusA are functional in both RNA binding and antitermination of transcription (Zhou *et al.*, 2002), the role of archaeal NusA in RNA-mediated events has yet to be elucidated. Thus, we sought to crystallize the *Aeropyrum pernix* NusA protein in order to solve its tertiary structure.

Hemihedral twinning was observed in archaeal NusA crystals grown in the terrestrial environment using the hanging-drop vapour-diffusion technique and this caused severe difficulty in determining the crystal structure. Hence, we attempted to crystallize this protein under microgravity conditions, which may improve the crystal quality, as indexed by resolution or mosaicity (McPherson, 1999; Kundrot *et al.*, 2000; Yoon *et al.*, 2001).

We performed the crystallization of archaeal NusA in a microgravity experiment as a part of the JAXA–GCF project[1]. Here, we report a comparative study of microgravity-grown and terrestrially grown crystals of archaeal NusA and discuss the effects of microgravity on improving the quality of protein crystals.

2. Materials and methods

2.1. Protein expression and purification

The gene encoding full-length archaeal NusA (144 amino acids; calculated molecular weight 16 042 Da; accession code BAA80854) from *A. pernix* K1 was amplified by PCR with forward primer 5′-GGAATTCATATGAGTGGTGATTATAGGATAAC-3′ and

[1] The JAXA–GCF project is the 'High-Quality Protein Crystallization Project on The Protein Structure and Function Analysis for Application' conducted by Japan Aerospace Exploration Agency (JAXA) from 2002 to 2006, using the Granada Crystallization Facility (GCF). The GCF is a crystallization-device container developed by the European Space Agency (ESA) and the University of Granada.

reverse primer 5′-GGAATTGGATCCTTATTAACGTATAACCA-CCTTCTCGACGCC-3′ using *A. pernix* genomic DNA as the template. The amplified fragment was cleaved with *Nde*I and *Bam*HI, subcloned into pET11a (Novagen) and expressed in *Escherichia coli* Rosetta (DE3) (Novagen). Approximately 4.0 g of *E. coli* cells were harvested from the 2 l culture by centrifugation, resuspended in 20 m*M* Tris–HCl buffer pH 8.0 containing 200 m*M* NaCl, 5 m*M* $MgCl_2$, 2 m*M* DTT and 1 m*M* EDTA and lysed by sonication. The *E. coli* lysate was subjected to heat treatment at 343 K for 30 min. Denatured proteins and cell debris were removed by centrifugation at 16 000*g* for 20 min at 277 K. The resulting supernatant was dialyzed overnight against 20 m*M* Tris–HCl buffer pH 8.0 containing 2 m*M* DTT. The crude sample was applied onto a HiTrap Q column (5 ml; GE Healthcare Biosciences). The protein was eluted with a linear gradient of NaCl (0–1.0 *M*; the protein eluted at around 0.5 *M*).

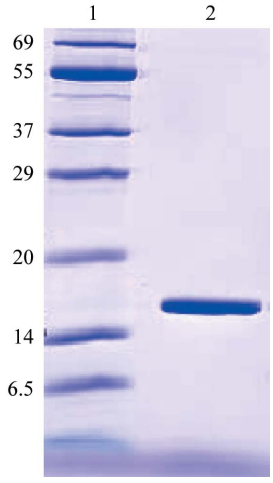


Figure 1
SDS–PAGE profile of the archaeal NusA protein. Lane 1, molecular-weight markers. The molecular weight of each protein band is shown in kDa on the left. Lane 2, purified archaeal NusA protein.

The NusA protein was identified as a band with a molecular weight of approximately 16 kDa on SDS–PAGE. The NusA-containing fractions were then loaded onto a HiTrap Butyl column (GE Healthcare Biosciences) and the protein was eluted with a linear gradient of ammonium sulfate (1.2–0 *M*; the protein eluted at around 0.3–0.4 *M*). The eluted fractions containing the protein were dialyzed overnight against 20 m*M* Tris–HCl buffer pH 8.0 containing 50 m*M* NaCl and 2 m*M* DTT. The dialysate was collected and loaded onto a Resource S (1 ml) column (GE Healthcare Biosciences), which was eluted with a linear gradient of NaCl (0–1.0 *M*; the protein eluted at around 0.3 *M*). The pooled fractions were then loaded onto a Mono S (1 ml) column (GE Healthcare Biosciences) and the protein was eluted with a linear gradient of NaCl (0–1.0 *M*; the protein eluted at around 0.3 *M*). The protein was finally fractionated by gel filtration on a Superdex 75 HR 10/30 column (GE Healthcare Biosciences). The NusA protein, purified to near-homogeneity (Fig. 1), was concentrated to 9.1 mg ml^{-1} in 20 m*M* Tris–HCl buffer pH 8.0 containing 150 m*M* NaCl and 2 m*M* DTT. The activity of the NusA protein was confirmed by an RNA-binding analysis (data not shown). The same protein preparation was used for both the microgravity and terrestrial crystallization experiments.

2.2. Crystallization

Initial crystals were obtained by the hanging-drop vapour-diffusion method using a solution containing 25% PEG 3350 and 150 m*M* sodium acetate pH 4.5 or a solution containing 400 m*M* monoammonium dihydrogen phosphate pH 4.1 as the precipitant, but the resulting crystals were not of sufficient quality to diffract X-rays. Therefore, crystallization screening by a counter-diffusion technique was attempted using polyethylene glycol (20% PEG 1500, 20% PEG 4000 or 20% PEG 8000; Tanaka *et al.*, 2004*a*). The optimized counter-diffusion condition, 20% PEG 8000 and 200 m*M* monoammonium dihydrogen phosphate pH 8.0, was then utilized for the microgravity experiment as well as for the terrestrial control experiment. The same preparation of NusA (buffered in 20 m*M* Tris–HCl pH 8.0, 150 m*M*

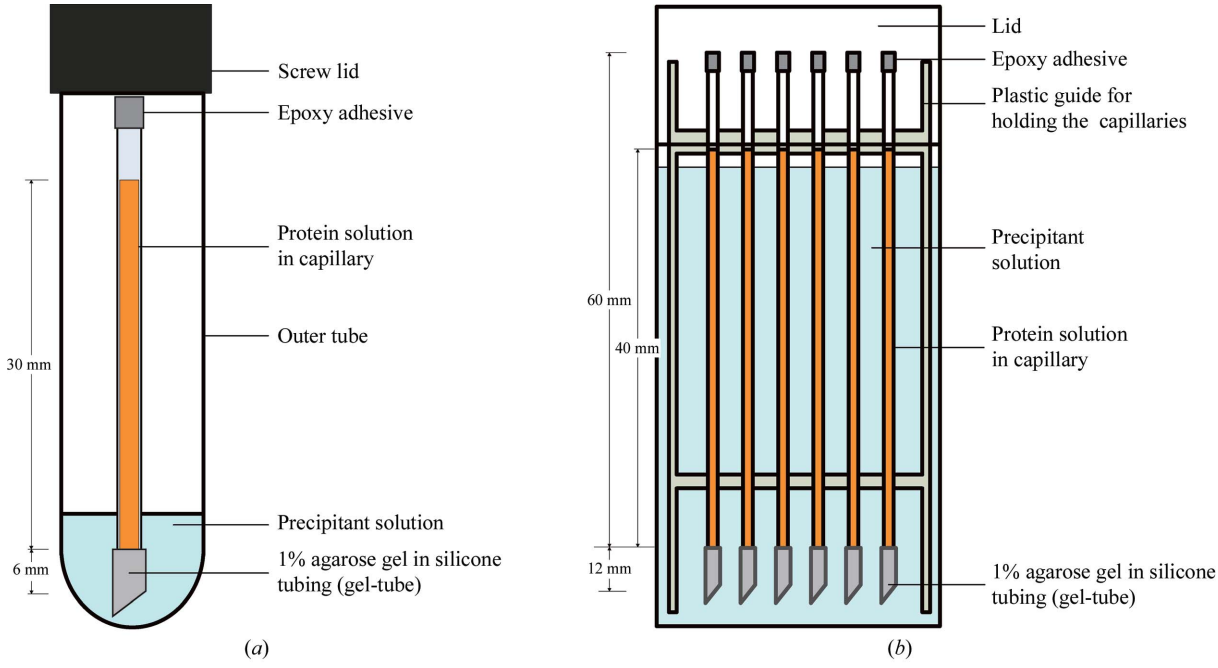
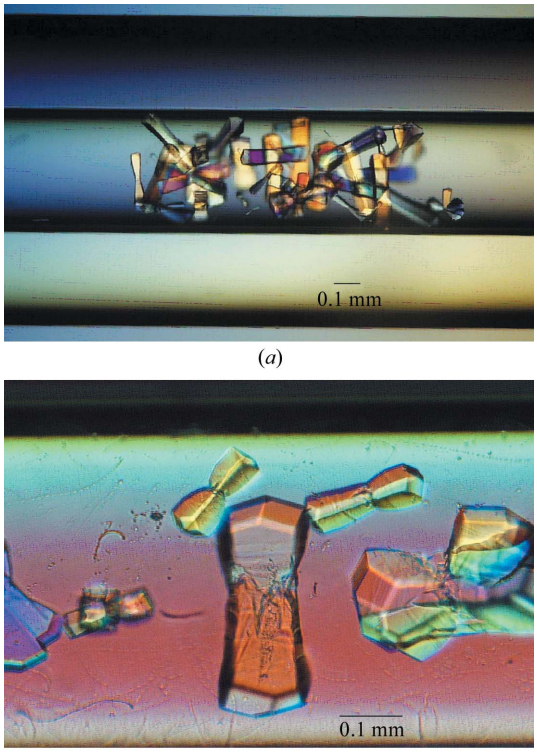


Figure 2
Crystallization devices used for the counter-diffusion experiments in this study. (*a*) Gel-tube method. (*b*) Gel-tube method in the Granada Crystallization Box.

sodium chloride and 2 m*M* DTT) and the same temperature (293 K) were used for both the terrestrial and microgravity experiments.

For the preliminary experiments of the JAXA–GCF project in the terrestrial environment, the gel-tube method (Tanaka *et al.*, 2004*b*) was used. This is a modification of the original counter-diffusion method of García-Ruiz & Moreno (1994). A capillary (0.5 mm diameter) was filled with protein solution at 9.1 mg ml^{-1} concentration to a height of 30 mm (6 µl). A 6 mm gel-tube consisting of 1% polymerized agarose inside a piece of silicone tubing was then attached to the end of the capillary, as shown in Fig. 2(*a*). 2 ml aliquots of the precipitant solutions (20% PEG 1500, 20% PEG 4000 or 20% PEG 8000 in monoammonium dihydrogen phosphate buffer pH 8.0) were poured into the outer tube. Single crystals appeared after 3 d in the capillaries.

For the space experiment of the JAXA–GCF project, a Granada Crystallization Box (GCB; García-Ruiz *et al.*, 2002) was used as the outer box instead of the test tube shown in Fig. 2(*a*). Six capillaries (60 mm in length, 0.5 mm in diameter) were filled with protein solution to a height of 40 mm and a gel-tube (12 mm in length) containing 1% polymerized agarose inside a piece of silicone tubing was attached to the end of the each capillary. The top of the capillary was sealed with epoxy adhesive. The capillaries were then placed into the GCB as shown in Fig. 2(*b*). The crystallization experiments were performed over a period of nine weeks at 293 K inside the Russian TBU incubator in the Russian Service Module of the International Space Station (ISS), developed by the Russian Federal Space Agency. Owing to the lack of an observation system in the ISS, it was not possible to know when the crystals began to grow during this nine-week period, but the crystallization experiments were set up to start after the samples had been placed in the microgravity environment. The terrestrial counter-diffusion crystallization experiments were set up at the same time in the same configuration using the GCB and a sample from the same preparation and for the same period of time as those on the ISS. The crystals grew to maximum dimensions of 0.31 × 0.06 × 0.06 mm under terrestrial conditions (Fig. 3*a*) and 0.35 × 0.13 × 0.13 mm under microgravity conditions (Fig. 3*b*).



(*a*)

(*b*)

Figure 3
Crystals of archaeal NusA. (*a*) Crystals from the counter-diffusion method grown in the terrestrial environment. (*b*) Crystals from the counter-diffusion method grown under microgravity conditions. The capillary diameters were 0.5 mm.

Table 1
Data-collection and processing statistics for archaeal NusA crystals.

Values in parentheses are for the last shell.

	Terrestrially grown crystal	Microgravity-grown crystal
Crystallization method	Counter-diffusion	Counter-diffusion
Precipitant	20% PEG 8000, 200 m*M* monoammonium dihydrogen phosphate pH 8.0	20% PEG 8000, 200 m*M* monoammonium dihydrogen phosphate pH 8.0
Space group	$I4_1$	$I4_1$
Unit-cell parameters (Å)		
$a = b$	99.96	99.48
c	45.46	45.72
Resolution (Å)	3.0 (3.11–3.0)	2.29 (2.37–2.29)
Mosaicity	2.369	0.591
$R_{\rm merge}$ (%)	0.042 (0.118)	0.042 (0.215)
Completeness (%)	0.878 (0.572)	0.965 (0.861)
Twinning fraction (%)	18.3	1.0
PDB code	Not deposited	2cy1

During the course of the microgravity experiment, we continued to optimize the terrestrial hanging-drop vapour-diffusion crystallization conditions. The optimized vapour-diffusion crystals were obtained by mixing 1.0 µl of the same NusA preparation (9.1 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0, 150 m*M* sodium chloride and 2 m*M* DTT) and 1.0 µl reservoir solution containing 22.5% PEG 3350 and 150 m*M* sodium acetate pH 4.8 and then equilibrating the droplet against 400 µl reservoir solution. Rod-like crystals (approximately 0.20 × 0.04 × 0.04 mm) grew at 293 K over the course of two months.

2.3. Data collection and analysis

After the return to earth of the space experiment, the crystals grown by the counter-diffusion method in both the terrestrial and microgravity experiments were harvested in mother liquor and soaked for less than 10 s in mother liquor containing 10% glycerol as a cryoprotectant. To harvest a crystal, the capillary was cut with a cutting stone at a position a few millimetres away from the target crystal and the crystal was washed out by micropipetting a gentle stream of mother liquor through the capillary. The crystals were then flash-cooled in a nitrogen-gas stream at 100 K. Diffraction data were collected from a single crystal on beamline BL12B2 at SPring-8, Harima, Japan using a CCD camera system (ADSC Quantum 4R). A wavelength of 1.0 Å and a crystal-to-detector distance of 200 mm were used. The oscillation range was 1.0°, with an exposure time of 120 s. All data were indexed, integrated and scaled with *DENZO* and *SCALEPACK* as implemented in the *HKL*-2000 program package (Otwinowski & Minor, 1997). The crystal parameters and data-processing statistics are shown in Table 1. The twinning fraction (Yeates, 1997) was calculated using *CNS* (Brünger *et al.*, 1998). The terrestrially grown crystal obtained by the counter-diffusion method exhibited an 18.3% hemihedral twinning fraction. On the other hand, the microgravity-grown crystal by the counter-diffusion showed a 1.0% twinning fraction.

During the course of the nine-week microgravity experiment, we determined the structure of archaeal NusA using the MAD data sets obtained from the terrestrially grown crystals prepared using the hanging-drop vapour-diffusion method. The data-collection statistics and the structure of the crystals will be reported elsewhere (Shibata *et*

al., submitted). The crystals diffracted to well beyond 2.0 Å resolution; however, they exhibited a 21.8% hemihedral twinning fraction. For this model refinement, the data were processed using the program *CNS* (Brünger *et al.*, 1998) with the hemihedral twinning method (α = 0.218; k, h, $-l$; Shibata *et al.*, submitted). Using this archaeal NusA structure (PDB code 2cxc) as a model, we solved the structure of the microgravity-grown archaeal NusA crystals by molecular replacement (PDB code 2cy1).

3. Results and discussion

The NusA protein from *A. pernix* was purified to near-homogeneity (Fig. 1) and was crystallized by a counter-diffusion technique in terrestrial and microgravity environments after initial terrestrial experiments using vapour diffusion failed to yield satisfactory results. The shapes of the NusA crystals grown under terrestrial (Fig. 3*a*) and microgravity (Fig. 3*b*) counter-diffusion conditions were essentially identical to each other and the crystals belonged to the tetragonal space group $I4_1$ (Table 1). However, the microgravity-grown crystals were larger than the terrestrially grown crystals (Fig. 3). Near-complete data sets were collected using a single crystal from the terrestrial and microgravity experiments. It is interesting that the twinning fraction of the microgravity-grown crystals (1.0%) was reduced compared with that of the terrestrially grown crystals (18.3%). In addition to the decreased twinning, the resolution and mosaicity of the microgravity-grown crystals were also improved remarkably (Table 1). Crystallization in the microgravity environment improved the maximum resolution from 3.0 to 2.29 Å and the mosaicity was improved from 2.369 (terrestrial) to 0.591 (microgravity).

Protein molecules in solution are incorporated into the surface of the growing crystal. Therefore, a so-called depletion zone forms around a growing crystal in which the protein concentration is lower. Under microgravity conditions, density-driven fluid motion is suppressed in the depletion zone around the growing crystal and this is thought to lead to the protein molecules having more ordered orientations in the crystal. This seems to be one of the significant reasons for the improvement of the quality of crystals grown in the microgravity environment (McPherson, 1999). The fluid motion can be avoided in terrestrial conditions by the inclusion of a low-concentration [0.12%(*v*/*v*)] agarose solution in the protein solution (García-Ruiz *et al.*, 2001). However, the inclusion of agarose in the crystallization solution occasionally spoiled successful crystallization in our preliminary counter-diffusion experiment (data not shown). Another method to avoid or prevent density-driven fluid motion is to place the protein solution inside a capillary with a very small diameter (*i.e.* 0.1–0.2 mm diameter) that is subsequently positioned horizontally (García-Ruiz *et al.*, 2001). However, in our experience, better X-ray diffraction data collection was obtained from larger crystals, which thus required that they be grown in a capillary of at least 0.3–0.5 mm diameter.

The solution concentration of the protein in the vicinity of the surface of the growing crystal is determined by the ratio of protein molecules in solution around the crystal to their uptake into the crystal. The rate of protein-molecule diffusion can be represented by the diffusion coefficient of the protein molecules D. The rate of protein-molecule uptake into the crystal can be represented by the kinetic coefficient for the trapping of protein molecules into the crystal β. Therefore, the protein concentration on the surface of the crystal can be expressed as D/β (Chernov, 1998; Tanaka *et al.*, 2004*a*). If this D/β value is small enough, then the crystal can grow from relatively low levels of protein, even though the resulting crystal will be small. To decrease D/β, D can be reduced by the addition of viscous reagents to the crystallization solution, but it is difficult to change β experimentally. In this study, we used PEG 8000, because of its high viscosity, to reduce D. A 20% PEG 8000 solution behaves like a non-Newtonian fluid and the actual effect of the viscosity change caused by PEG does not decrease D by very much. Nonetheless, the viscosity coefficient of a solution containing 20% PEG 8000 is about 20 times higher than that of a pure aqueous solution (Tanaka *et al.*, 2006). In this study, PEG 8000 was a suitable viscous reagent because it had no influence on the crystallization. The choice of a suitable viscous reagent depends on the characteristics of the protein itself.

It is difficult to determine the actual D/β value, because β is not easily measured. However, in the case of NusA crystallization in microgravity, the improvement of the maximum resolution and the mosaicity of the crystals grown in microgravity suggested that D/β may have been small enough to reduce the concentration of protein in solution at the surface of the crystal.

In summary, this paper is the first case to report the reduction of hemihedral twinning under microgravity conditions. The reason for the detwinning in the microgravity environment is not clear; however, it is likely that the slower growth of the protein crystal in the low-saturation solution under microgravity conditions makes the protein molecules adopt well ordered orientations at the surface of the crystal. This may reduce the number of molecules in the twinning orientation, if the positional potentials are different between the main fraction and the twinning fraction and if the slow crystal growth allows the transition from a quasi-stable orientation to a more stable one. Further examples of the detwinning of protein crystals under microgravity conditions will be necessary to examine the phenomenon in more detail.

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References

Borukhov, S., Lee, J. & Laptenko, O. (2005). *Mol. Microbiol.* **55**, 1315–1324.
Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J.-S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T. & Warren, G. L. (1998). *Acta Cryst.* D**54**, 905–921.
Chernov, A. A. (1998). *Acta Cryst.* A**54**, 859–872.
García-Ruiz, J. M., Gonzalez-Ramirez, L. A., Gavira, J. A. & Otálora, F. (2002). *Acta Cryst.* D**58**, 1638–1642.
García-Ruiz, J. M. & Moreno, A. (1994). *Acta Cryst.* D**50**, 484–490.
García-Ruiz, J. M., Novella, M. S., Moreno, R. & Gavira, J. A. (2001). *J. Cryst. Growth*, **232**, 165–172.

Gopal, B., Haire, L. F., Gamblin, S. J., Dodson, E. J., Lane, A. N., Papavinasasundaram, K. G., Colston, M. J. & Dodson, G. (2001). *J. Mol. Biol.* **314**, 1087–1095.
Kundrot, C. E., Judge, R. A., Pusey, M. L. & Snell, E. H. (2000). *Cryst. Growth Des.* **1**, 87–99.
McPherson A. (1999). *Crystallization of Biological Macromolecules.* Cold Spring Harbor, New York, USA: Cold Spring Harbor Laboratory Press.
Nudler, E. & Gottesman, M. (2002). *Genes Cells*, **7**, 755–768.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Tanaka, H., Inaka, K., Sugiyama, S., Takahashi, S., Sano, S., Sato, M. & Yoshitomi, S. (2004*a*). *Ann. NY Acad. Sci.* **1027**, 10–19.
Tanaka, H., Inaka, K., Sugiyama, S., Takahashi, S., Sano, S., Sato, M. & Yoshitomi, S. (2004*b*). *J. Synchrotron Rad.* **11**, 45–48.
Tanaka, H., Yoshizaki, I., Takahashi, S., Yamanaka, M., Fukuyama, S., Sato, M., Sano, S., Motohara, M., Kobayashi, T., Yoshitomi, S. & Tanaka, T. (2006). *Microgravity Sci. Technol.* **18**, 91–94.
Worbs, M., Bourenkov, G. P., Bartunik, H. D., Huber, R. & Wahl, M. C. (2001). *Mol. Cell*, **7**, 1177–1189.
Yeates, T. O. (1997). *Methods Enzymol.* **276**, 344–358.
Yoon, T.-S., Tetreault, S., Bosshard, H. E., Sweet, R. M. & Sygusch, J. (2001). *J. Cryst. Growth*, **232**, 520–535.
Zhou, Y., Mah, T. F., Greenblatt, J. & Friedman, D. I. (2002). *J. Mol. Biol.* **318**, 1175–1188.