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Purification, crystallization, X-ray diffraction analysis and phasing of an engineered single-chain *Pvu*II restriction endonuclease

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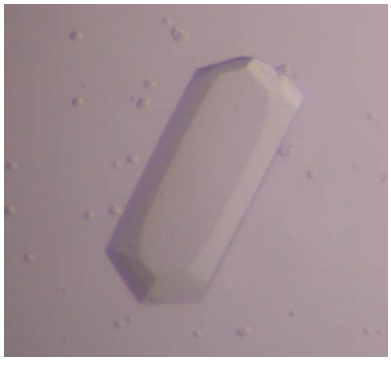
The restriction endonuclease *Pvu*II from *Proteus vulgaris* has been converted from its wild-type homodimeric form into the enzymatically active single-chain variant sc*Pvu*II by tandemly joining the two subunits through the peptide linker Gly-Ser-Gly-Gly. sc*Pvu*II, which is suitable for the development of programmed restriction endonucleases for highly specific DNA cleavage, was purified and crystallized. The crystals diffract to a resolution of 2.35 Å and belong to space group $P4_2$, with unit-cell parameters $a = b = 101.92$, $c = 100.28$ Å and two molecules per asymmetric unit. Phasing was successfully performed by molecular replacement.

1. Introduction

The *Pvu*II restriction endonuclease is the nuclease component of one of the *Proteus vulgaris* type II restriction-modification systems (Blumenthal *et al.*, 1985; Gingeras *et al.*, 1981). Like most type II endonucleases (Niv *et al.*, 2007; Pingoud & Jeltsch, 1997; Kovall & Matthews, 1999; Roberts & Halford, 1993; Roberts & Macelis, 2000), *Pvu*II is homodimeric and cleaves its double-stranded cognate DNA substrate in the presence of Mg^{2+} so that each subunit acts on one DNA strand in a concerted manner. The enzyme cleaves the palindromic 5′-CAGCTG-3′ sequence between the central GC residues and generates blunt ends with a 5′-phosphate group. With 157 amino-acid residues, *Pvu*II is one of the smallest type II restriction endonucleases (Athanasiadis *et al.*, 1990; Tao & Blumenthal, 1992). The crystal structure of the protein (Athanasiadis *et al.*, 1994; Cheng *et al.*, 1994) showed that the C-terminus of one subunit is in close proximity to the N-terminus of the other. This particular feature of the *Pvu*II restriction endonuclease raised the interesting possibility of engineering a single-chain (sc) enzyme by linking these termini with a short peptide linker. Simoncsits *et al.* (2001) converted *Pvu*II from its natural homodimeric form into a single polypeptide chain (sc*Pvu*II) by tandemly linking the two subunits of the wild-type enzyme (wt) through the peptide linker Gly-Ser-Gly-Gly. The protein (including the C-terminal histidine tag Gly-Ser-His_6) has a molecular weight of 36 912.3 Da and comprises 326 residues.

The results of *in vivo* and *in vitro* tests (Simoncsits *et al.*, 2001) showed that the cleavage specificity of sc*Pvu*II is indistinguishable from that of the wt enzyme; the engineered protein is a potent catalyst, although somewhat less efficient than wt *Pvu*II. Such a functional single-chain restriction endonuclease could prove to be an invaluable tool in protein-engineering studies, both in basic research and in practical applications. sc*Pvu*II has found applications in ongoing studies to develop programmed restriction endonucleases for highly specific DNA cleavage suitable for the analysis of genomic DNA or for targeting individual genes in complex genomes: sc*Pvu*II covalently coupled *via* a bifunctional cross-linker to a triple-helix-forming oligonucleotide (TFO) specifically cleaves high-molecular-weight DNA at *Pvu*II recognition sites if these are located at a distance of approximately one helical turn from a triple-helix-forming



site (TFS) which is complementary to the TFO (Eisenschmidt *et al.*, 2005); other *Pvu*II sites are left intact. Given the fact that TFO can be synthesized to form triple helices with any sequence that one would like to address (Rusling *et al.*, 2005) and that triple-helix formation can be used to target genes *in vivo* (Majumdar *et al.*, 2003), the sc*Pvu*II variant can be considered as a programmable restriction enzyme that offers a useful alternative to designed zinc-finger nucleases in highly efficient endogenous gene-correction applications (Durai *et al.*, 2005; Kandavelou *et al.*, 2005; Urnov *et al.*, 2005).

2. Materials and methods

2.1. Expression and purification

The *scpvuII* gene was cloned in the expression vector pRIZ′-scPvR-GSH6 (Simoncsits *et al.*, 2001) containing a C-terminal His tag and transformed into XL1 BlueMRF′ hosts (*Escherichia coli* strains) which contain the pLGM *Pvu*II methylase construct. A sufficient amount of soluble protein for structural studies was obtained after expression using the following conditions. Cells were grown in 1 l LB medium containing 100 µg ml^{-1} ampicillin, 25 µg ml^{-1} kanamycin and 5 µg ml^{-1} tetracycline at 310 K until an OD_{600} of 0.9 was reached. The culture was induced with 0.5 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4–6 h at 310 K and harvested by centrifugation at 6000 rev min^{-1} for 30 min at 277 K. The pelleted cells were resuspended in 50 ml lysis buffer containing 50 m*M* Na_2HPO_4/NaH_2PO_4 pH 8, 300 m*M* NaCl, 5 m*M* imidazole with 5 m*M* 2-mercaptoethanol. Subsequently, 1 m*M* PMSF, 20 µg ml^{-1} leupeptin and 150 µg ml^{-1} benzamidine were added and homogenized by sonication at 277 K. The cell debris was removed by centrifugation at 14 000 rev min^{-1} for 1 h at 277 K. Purification was effected *via* the His tag by affinity chromatography at 277 K on a 5 ml Ni–NTA agarose column (Qiagen) pre-equilibrated in lysis buffer. The column was washed with ten column volumes of lysis buffer containing 10 m*M* imidazole and ten column volumes of lysis buffer containing 20 m*M* imidazole, followed by a gradient from 50 to 300 m*M* imidazole in lysis buffer. sc*Pvu*II started to elute at 100 m*M* imidazole in lysis buffer. Fractions containing more than 95% homogeneous sc*Pvu*II, as visualized by SDS–PAGE gels, were dialyzed extensively against 20 m*M* Tris–HCl pH 7.5 buffer containing 20 m*M* NaCl. The protein solution was concentrated to 7 mg ml^{-1} using Amicon Centriprep (YM-10) filters and kept in 20 m*M* Tris–HCl pH 7.5 buffer for subsequent crystallization experiments. The protein concentration was determined using the Bradford assay with bovine serum albumin as a standard (Bradford, 1976). A total of 14 mg pure protein was the final yield from 3 g cell paste.

2.2. Crystallization

Crystallization trials were performed using the hanging-drop vapour-diffusion method and Linbro 24-well cell-culture plates. The drops consisted of 3 µl protein (at 5–9 mg ml^{-1}) plus 3 µl reservoir solution and were equilibrated against 1000 µl reservoir solution at 290 K. Well formed parallelepiped-shaped crystals of reasonable size for crystallographic studies were obtained within 3 d with 1.28–1.36 *M* ammonium sulfate, 4–5% MPD, 100 m*M* bis-Tris pH 6.5–7.4. The best results were reproducibly obtained using protein at a concentration of 7 mg ml^{-1} no later than 5 d after purification. The approximate dimensions of these crystals were 0.9 × 0.3 × 0.3 mm (Fig. 1). Crystals of comparable quality were also obtained when fresh protein was flash-frozen in liquid nitrogen, stored at 190 K and defrosted on ice prior to crystallization.

2.3. Data collection, processing and phasing

X-ray diffraction data were collected from a single crystal (Fig. 1) to a resolution of 2.35 Å (Fig. 2) using synchrotron radiation at the EMBL X11 beamline at the DORIS storage ring, DESY, Hamburg (wavelength 1.84 Å). The crystal was flash-cooled to 100 K in a nitrogen-gas cold stream using an Oxford Cryosystem device and a cryoprotection solution consisting of 1 *M* ammonium sulfate, 100 m*M* bis-Tris pH 7.2 and 25% glycerol. 360 images with an oscillation range of 0.5° were collected. The diffraction data were recorded on a MAR CCD165 detector with a diameter of 165 mm. X-ray diffraction data were indexed, integrated and scaled with *DENZO* and *SCALEPACK* from the *HKL* program suite (Otwinowski & Minor, 1997).

The sc*Pvu*II structure was solved by the molecular-replacement method using the program *AMoRe* (Navaza, 1994). The DNA-

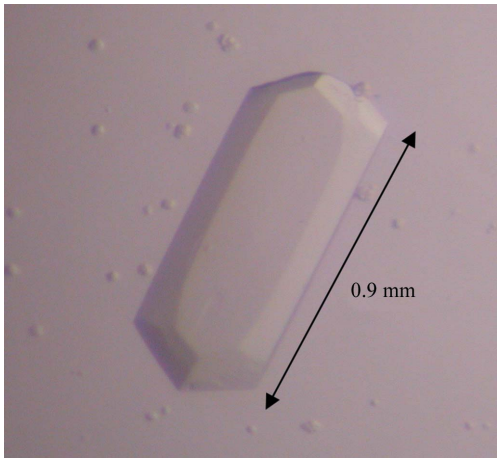


Figure 1
The crystal of sc*Pvu*II used for the collection of X-ray diffraction data. The approximate length of the crystal is 0.9 mm.

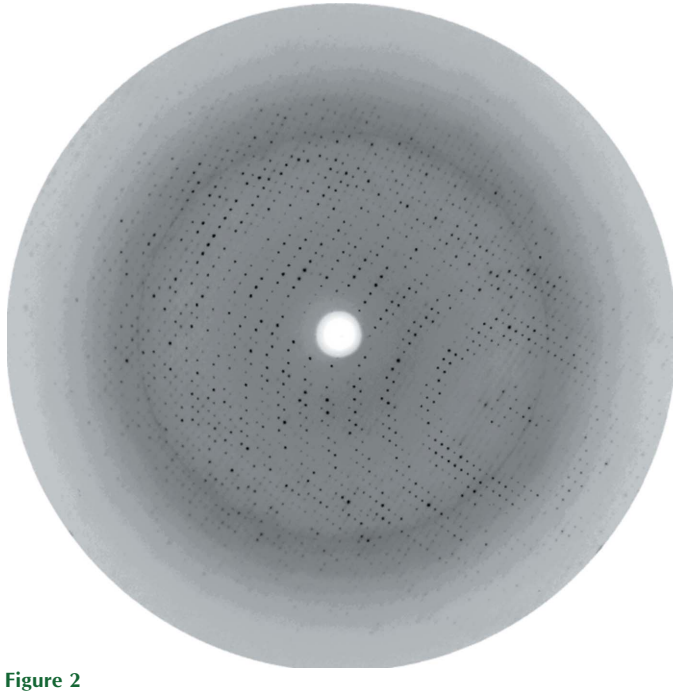


Figure 2
X-ray diffraction pattern from a sc*Pvu*II crystal. The detector edge corresponds to 2.3 Å resolution.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell (2.39–2.35 Å).

Space group	$P4_2$
Unit-cell parameters (Å)	$a = b = 101.92$, $c = 100.28$
Wavelength (Å)	1.84
Resolution (Å)	99–2.35
Observed reflections	1781833
Unique reflections	42448
Redundancy	4.0 (3.6)
Data completeness (%)	98.8 (99.2)
$R_{\rm merge}$† (%)	7.5 (58.0)
Average $I/\sigma(I)$	15.37 (2.70)
Mosaicity (°)	1.57
Matthews coefficient (Å^3 Da^{-1})	3.6
Solvent content (%)	65
sc*Pvu*II molecules per asymmetric unit	2

† $R_{\rm merge} = \sum_h \sum_i |I(h)_i - \langle I(h)\rangle| / \sum_h \sum_i I(h)_i$, where $I(h)$ is the intensity of reflection h, $\sum_h$ is the sum over all reflections and $\sum_i$ is the sum over I measurements of reflection h.

binding subdomain (residues 36–157) of the *Pvu*II restriction endonuclease (PDB code 1pvu; Athanasiadis *et al.*, 1994) provided the search model. Data in the resolution range 20.0–4.0 Å were used. A unique solution was only obtained on assuming space group $P4_2$. A final correlation coefficient of 0.47 and an R factor of 0.41 were obtained. There are two molecules of sc*Pvu*II in the asymmetric unit. Graphical interpretation of the crystal packing with the program *Xfit* from the *XtalView* program suite (McRee, 1999) confirmed the correctness of the solution. Subsequent refinement allowed the positioning of residues 1–35 and of the peptide Gly-Ser-Gly-Gly in the electron-density maps.

3. Results and discussion

sc*Pvu*II was successfully crystallized and a native data set was collected to 2.35 Å resolution using synchrotron radiation. The crystals belong to the tetragonal space group $P4_2$, with unit-cell parameters $a = b = 101.92$, $c = 100.28$ Å. Data-collection and processing statistics are given in Table 1. Scaling and merging of the crystallographic data resulted in an overall $R_{\rm merge}$ of 7.5%. The completeness of the data set was 98.8% to 2.35 Å resolution. Assuming the presence of two molecules in the asymmetric unit, the Matthews coefficient (Matthews, 1968) is 3.6 Å^3 Da^{-1} and the solvent content is 65%. Both the space group ($P4_2$) and the content of the asymmetric unit (two molecules of sc*Pvu*II) were confirmed by structure solution using molecular replacement. Model rebuilding and structure refinement are in progress.

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References

Athanasiadis, A., Gregoriu, M., Thanos, D., Kokkinidis, M. & Papamatheakis, J. (1990). *Nucleic Acids Res.* **18**, 6434.
Athanasiadis, A., Vlassi, M., Kotsifaki, D., Tucker, P. A., Wilson, K. S. & Kokkinidis, M. (1994). *Nature Struct. Biol.* **1**, 469–475.
Blumenthal, R. M., Gregory, S. A. & Cooperider, J. S. (1985). *J. Bacteriol.* **164**, 501–509.
Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.
Cheng, X., Balendiran, K., Schildkraut, I. & Anderson, J. E. (1994). *EMBO J.* **13**, 3927–3935.
Durai, S., Mani, M., Kandavelou, K., Wu, J., Porteus, M. H. & Chandrasegaran, S. (2005). *Nucleic Acids Res.* **33**, 5978–5990.
Eisenschmidt, K., Lanio, T., Simoncsits, A., Jeltsch, A., Pingoud, V., Wende, W. & Pingoud, A. (2005). *Nucleic Acids Res.* **33**, 7039–7047.
Gingeras, T. R., Greenough, L., Schildkraut, I. & Roberts, R. J. (1981). *Nucleic Acids Res.* **9**, 4525–4536.
Kandavelou, K., Mani, M., Durai, S. & Chandrasegaran, S. (2005). *Nature Biotechnol.* **23**, 686–687.
Kovall, R. A. & Matthews, B. W. (1999). *Curr. Opin. Chem. Biol.* **3**, 578–583.
McRee, D. E. (1999). *J. Struct. Biol.* **125**, 156–165.
Majumdar, A., Puri, N., Cuenoud, B., Natt, F., Martin, P., Khorlin, A., Dyatkina, N., George, A. J., Miller, P. S. & Seidman, M. M. (2003). *J. Biol. Chem.* **278**, 11072–11077.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Navaza, J. (1994). *Acta Cryst.* A**50**, 157–163.
Niv, M. Y., Ripoll, D. R., Vila, J. A., Liwo, A., Vanamee, E. S., Aggarwal, A. K., Weinstein, H. & Scheraga, H. A. (2007). *Nucleic Acids Res.* **35**, 2227–2237.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Pingoud, A. & Jeltsch, A. (1997). *Eur. J. Biochem.* **246**, 1–22.
Roberts, R. J. & Halford, S. E. (1993). *Nucleases*, edited by S. M. Linn, R. S. Lloyd & R. J. Roberts, pp. 35–88. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.
Roberts, R. J. & Macelis, D. (2000). *Nucleic Acids Res.* **28**, 306–307.
Rusling, D. A., Powers, V. E., Ranasinghe, R. T., Wang, Y., Osborne, S. D., Brown, T. & Fox, K. R. (2005). *Nucleic Acids Res.* **33**, 3025–3032.
Simoncsits, A., Tjörnhammar, M. L., Raskó, T., Kiss, A. & Pongor, S. (2001). *J. Mol. Biol.* **309**, 89–97.
Tao, T. & Blumenthal, R. M. (1992). *J. Bacteriol.* **174**, 3395–3398.
Urnov, F. D., Miller, L. C., Lee, Y. L., Beausejour, C. M., Rock, J. M., Augustus, S., Jamieson, A. C., Porteus, M. H., Gregory, P. D. & Holmes, M. C. (2005). *Nature (London)*, **435**, 646–651.