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A single mutation of restriction endonuclease *Eco*RII led to a new crystal form that diffracts to 2.1 Å resolution

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R88A, a mutant of the type IIE restriction endonuclease *Eco*RII, has been crystallized in space group $P2_1$, with unit-cell parameters $a = 58.7$, $b = 92.4$, $c = 88.3$ Å, $\beta = 108.1°$. There are two monomers in the asymmetric unit and the solvent content is estimated to be 50% by volume. The crystals diffract to 2.1 Å resolution, which is much higher than that of the wild type, which diffracted to 2.8 Å resolution. The mutant crystals have been used in the identification of an excellent heavy-atom derivative.

1. Introduction

*Eco*RII (EC 3.1.21.4) is a type IIE restriction endonuclease (Krüger *et al.*, 1988). It recognizes the nucleotide sequence 5′-CCWGG (W = A or T) in double-stranded DNA and cleaves the phosphodiester bond preceding the first cytosine at both strands. Methylation at C5 of the second cytosine inhibits cleavage. The enzyme has the ability to search for the presence of two substrate sites before cleavage. Transmission electron microscopy provided evidence that *Eco*RII generates loops on linear DNA containing two 5′-CCWGG sites (Mücke *et al.*, 2000). The activation of the enzyme occurs when an *Eco*RII dimer interacts with two DNA-recognition sites (for reviews, see Krüger *et al.*, 1995; Pingoud & Jeltsch, 2001). *Eco*RII is a 45.6 kDa protein with 404 amino-acid residues whose sequence has been determined (Kossykh *et al.*, 1989; Bhagwat *et al.*, 1990). Biochemical studies suggest that *Eco*RII is a dimeric molecule with at least two domains per monomer (Reuter *et al.*, 1998, 1999). Limited proteolysis of *Eco*RII confirmed the existence of two stable domains that independently bind to specific DNA. It is speculated that the N-terminal domain (activator domain) binds to an activator DNA site and the C-terminal domain (endo domain) binds to a substrate DNA site (Mücke *et al.*, 2002). The structural basis of *Eco*RII–DNA interaction has not been elucidated. Crystal structure determination of *Eco*RII and its DNA complexes will help us elucidate its unique activation and cleavage mechanisms.

Wild-type *Eco*RII was first crystallized in the cubic space group $I23$ or $I2_13$ (Karpova *et al.*, 1999). The cubic crystal form only diffracted to 4 Å resolution at best. Recently, we reported a new crystal form of wild-type *Eco*RII that diffracted to much higher resolution (2.8 Å) than the cubic form (Zhou *et al.*, 2002). However, the wild-type crystals were difficult to reproduce. Here, we report the crystallization of a mutant *Eco*RII, R88A, which yielded a different crystal form (different unit-cell parameters) to those of the wild type and diffracted to even higher resolution (2.1 Å).

2. Materials and methods

2.1. Expression and purification of *Eco*RII mutant R88A

Site-directed mutagenesis was carried out according to a previously described protocol (Reuter *et al.*, 1999). The expression and purification protocols for mutant R88A were the same as those used in the wild-type preparation (Reuter *et al.*, 1999; Zhou *et al.*, 2002). In brief, *Escherichia coli* strain JM109 (pDK1) was transformed by a pQE-30 vector containing the *Eco*RII mutant R88A gene with a His_6 tag at the N-terminus. The correct colonies were selected on double resistance to kanamycin and ampicillin. Cells were grown in LB culture medium and expression was induced by isopropyl-β-D-thiogalactoside (IPTG). Protein was purified using an Ni–NTA affinity column followed by a HiTrap heparin HP column. The purity of the protein was checked by SDS–gel electrophoresis. We obtained ~32 mg of pure protein (concentrated to ~4.5 mg ml^{-1}) from a 1 l cell culture using the above protocol.

2.2. Crystallization

Purified *Eco*RII mutant protein was concentrated using CentriPrep YM-30 (Amicon Bio-Separations) to ~4.5 mg ml^{-1} and stored at 277 K. Crystallization was performed by the vapour-diffusion technique in hanging drops at room temperature. Crystals of *Eco*RII R88A were first grown under conditions similar to those used to grow the

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wild-type crystals. After optimization of these conditions, the best crystals were obtained when 1–2 µl of protein in 20 m*M* Tris–HCl buffer pH 7.5 and 5 m*M* β-mercaptoethanol was mixed with an equal volume of reservoir precipitant solution, which contained 100 m*M* cacodylate buffer pH 6.3–6.5, 3.5–4% methanol, 35–40 m*M* $MgCl_2$, and equilibrated against 1 ml of reservoir solution. Crystals usually appeared within one to three weeks (Fig. 1).

2.3. X-ray data collection and analysis

Crystals were first characterized uaing an in-house X-ray source. A cryoprotectant solution was made by adding 30% (final concentration) glycerol to the reservoir solution. After transferring a crystal to the cryoprotectant solution, it was picked up in a fiber loop and flash-frozen in liquid nitrogen and then mounted on the goniometer head. Diffraction data were first collected at 93 K on a Rigaku R-AXIS IV image-plate detector using Cu $K\alpha$ radiation from a Rigaku RUH3R rotating-anode X-ray generator operating at 50 kV and 100 mA. The X-ray beam was focused using Osmic Blue Confocal Optics. The programs *DENZO* and *SCALEPACK* were used for data processing and analysis (Otwinowski & Minor, 1996). Synchrotron data from native R88A crystals were collected on the APS SBC-CAT beamline 19BM, Chicago, USA. Heavy-atom soaking experiments were carried out in-house and the most promising derivative data were collected on the APS SER-CAT beamline 22ID.

3. Results and discussion

Crystallization of a mutant protein instead of the difficult-to-crystallize wild-type counterpart has been used previously in protein-structure determination. An example is *E. coli* chaperonin GroEL, which gave the best crystals when subjected to two point mutations (Braig *et al.*, 1994). In the field of restriction endonucleases, the same trick has also been applied to *Mun*I, where a mutation in the active center was necessary to produce X-ray quality crystals (Deibert *et al.*, 1999). Crystals of wild-type *Eco*RII only diffracted to 3.3 Å resolution in-house and to 2.8 Å on a synchrotron source (Zhou *et al.*, 2002) and were difficult to reproduce, which hindered our in-house search for heavy-atom derivatives. Therefore, we decided to crystallize *Eco*RII mutants to see if they produce better crystals. Mutant R88A was the first mutant chosen because Arg88 is known to be located in DNA-binding site I of *Eco*RII (Reuter *et al.*, 1999) and in the absence of DNA its side chain may be quite flexible. Therefore, mutating arginine to alanine at position 88 might improve crystal order and thus resolution. Originally, R88A was constructed to prove the contribution of Arg88 to specific DNA binding of *Eco*RII. It was shown that R88A has only a slightly decreased DNA-binding affinity and an unaffected cleavage efficiency compared with the wild type and therefore is expected to have a similar structure to the wild type. In addition, it was also expressed well in large quantities. Our results seem to validate our selection. R88A crystals were easier to grow and diffracted to about 2.6 Å resolution with our conventional in-house rotating-anode X-ray source. Oscillation photos were used in autoindexing to determine the unit-cell parameters. The crystal had the symmetry of a monoclinic form. A data set to 2.6 Å resolution was collected in-house (Table 1). On the APS synchrotron beamline 19BM, these monoclinic crystals diffracted to 2.1 Å resolution. A complete data set to 2.1 Å resolution was collected with a total of 240° rotation at a crystal-to-detector distance of 180 mm (Table 1). It is interesting to note that both the wild-type and R88A crystals belong to the same space group $P2_1$, but have different unit-cell parameters. The unit-cell parameters for the wild-type crystals are a = 58.3, b = 127.8, c = 59.9 Å, β = 91.4°. This clearly hints that the packing arrangements in the two crystal forms differ from each other.

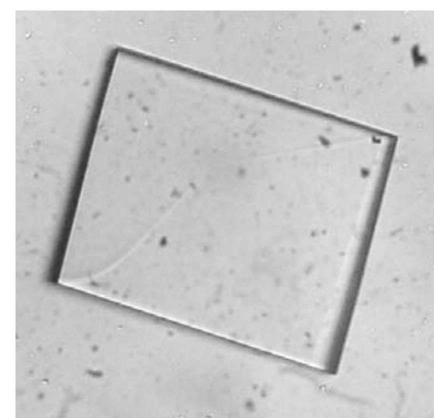
With a calculated molecular mass of about 46.6 kDa for the His_6-tagged *Eco*RII monomer and a crystal volume per asymmetric unit of 227 600 Å^3, assuming two monomers per asymmetric unit would yield a Matthews coefficient V_M of 2.4 Å^3 Da^{-1} (Matthews, 1968) with a solvent content of 50% (Westbrook, 1985) for the *Eco*RII mutant crystals. These values of V_M and solvent content are typical for protein crystals (Matthews, 1968) and strongly support the presence of two monomers per asymmetric unit.

The high diffraction quality of these mutant crystals has permitted us to easily carry out an extensive search of heavy-atom derivatives using an in-house X-ray source. After some initial failures, we have identified an excellent gold derivative using $KAu(CN)_2$. The heavy-atom soak was performed in crystallization buffer with 5 m*M* $KAu(CN)_2$ for 2 d. Data collection from this derivative on the APS synchrotron beamline 22ID provided both isomorphous and anomalous phase information to 2.1 Å resolution. Both isomorphous and anomalous difference Patterson maps clearly

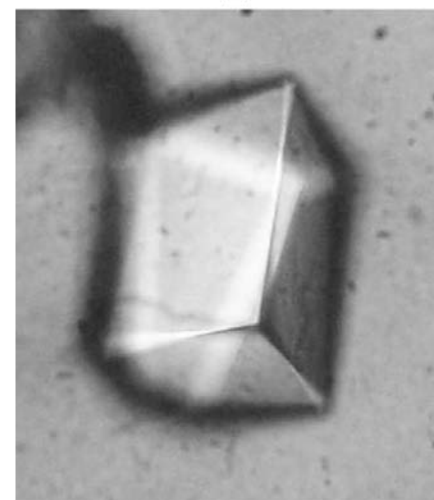
Table 1
Crystal data and data-collection statistics of *Eco*RII mutant R88A.

Values in parentheses are for the highest resolution shell.

	In-house	Synchrotron
Space group	$P2_1$	$P2_1$
Unit-cell parameters (Å,°)	a = 58.8, b = 92.4, c = 88.4, β = 108.2	a = 58.7, b = 92.4, c = 88.3, β = 108.1
Temperature (K)	93	93
Wavelength (Å)	1.5418	1.0088
Oscillation range per frame (°)	1.0	1.0
Exposure time (s)	900	15
Resolution (Å)	2.6	2.1
Total No. of reflections (unique/measured)	27738/119135	52027/259113
Completeness (%)	100 (100)	99.6 (100)
R_{merge}	0.057 (0.421)	0.053 (0.387)
Average $I/\sigma(I)$	19.5 (4.0)	11.5 (3.8)



(*a*)



(*b*)

Figure 1
Crystals of *Eco*RII mutant R88A protein (typical dimensions 0.31 × 0.27 × 0.08 mm).

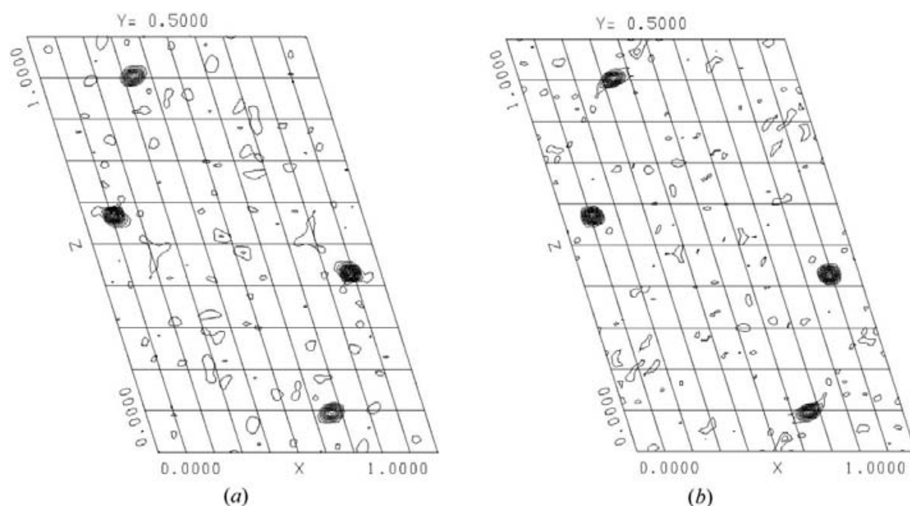


Figure 2
Harker sections of the isomorphous (*a*) and anomalous (*b*) difference Patterson maps of the gold derivative.

showed the presence of two gold sites (Fig. 2). We have solved and refined the *Eco*RII R88A structure using phases from the gold derivative. The coordinates have been deposited in the Protein Data Bank with code 1na6. A full description of the structure will be published elsewhere. Co-crystallization of both wild-type and mutant R88A *Eco*RII with its DNA substrates is in progress.

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