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A new crystal form of restriction endonuclease *Eco*RII that diffracts to 2.8 Å resolution

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*Eco*RII, a type IIe restriction endonuclease, has been crystallized in space group $P2_1$, with unit-cell parameters a = 58.3, b = 127.8, c = 59.9 Å, β = 91.4°. There are two monomers in the asymmetric unit and the solvent content is estimated to be 49% by volume. The crystals diffract to 2.8 Å resolution, which is much higher than that of the previously reported cubic crystal form, which diffracted to 4 Å resolution.

1. Introduction

Restriction endonucleases (ENases) occur ubiquitously among prokaryotic organisms. Their principal function is to protect the host genome against invading foreign DNA, showing a great ability to recognize and cleave short specific DNA sequences hidden within the large background of DNAs. Type II ENases are remarkably recognition site-specific enzymes (for a review, see Pingoud & Jeltsch, 2001). More than 3000 type II ENases isolated from different sources have been described, representing more than 200 individual DNA-sequence specificities (Roberts & Macelis, 2001). They form one of the most comprehensive groups of functionally similar proteins with distinct DNA-binding specificities. There is a unique subgroup within type II ENases, termed subclass IIe and characterized by their essential interactions with two copies of the recognition site for DNA cleavage. *Eco*RII (EC 3.1.21.4) is the first ENase for which this special characteristic was described (Krüger *et al.*, 1988, 1995). Other type IIe ENases include *Nae*I (Conrad & Topal, 1989), *Nar*I, *Bsp*MI, *Hpa*II, *Sac*II (Oller *et al.*, 1991), *Atu*BI, *Cfr*9I, *Sau*BMKI, *Eco*571 and *Ksp*6321 (Reuter *et al.*, 1993), which represent a wide variety of bacterial species.

*Eco*RII recognizes the nucleotide sequence 5′-CC*W*GG (*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 a unique ability to search for the presence of two substrate sites before cleavage. The activation of enzyme occurs when an *Eco*RII dimer interacts with two DNA-recognition sites (Krüger *et al.*, 1988; Pein *et al.*, 1991; Gabbara & Bhagwat, 1992; Petrauskene *et al.*, 1994). *Eco*RII is a 45.6 kDa protein with 404 amino-acid residues whose primary sequence has been determined (Kossykh *et al.*, 1989; Bhagwat *et al.*, 1990). Biochemical studies suggest that *Eco*RII is a dimeric molecule with two domains per monomer (Reuter *et al.*, 1998, 1999; Mücke *et al.*, 2000, 2002). It is speculated that one domain (the endo domain) binds to a substrate DNA and another domain (the activator domain) binds to an activator DNA. The structural basis of *Eco*RII–DNA interaction has not been elucidated. Crystal structure determination of *Eco*RII and its DNA complexes will increase our knowledge of not only the catalytic mechanism of type II ENases in general, but also the unique activation mechanism of type IIe ENases in particular. This could lead to novel uses of type IIe ENases or their mutants to regulate sequence-specific DNA cleavage essential to many biological processes. Therefore, we have initiated a structure–function relationship study of *Eco*RII.

The first crystal structure of a type IIe ENase, *Nae*I, was solved only recently (Huai *et al.*, 2000), followed by the determination of its DNA complex (Huai *et al.*, 2001). The 2.5 Å crystal structure of *Nae*I in complex with a 17-base-pair DNA substrate reveals that two DNA molecules bind to two different domains in the *Nae*I dimer, a novel pattern in contrast to the one DNA molecule binding per dimer in ordinary type II endonucleases. The dramatic rearrangement of the *Nae*I dimeric structure upon DNA binding enables speculation about the allosteric cleavage of DNA by *Nae*I. It would be very interesting to see whether *Eco*RII also has the same or similar dramatic rearrangement of its dimeric structure upon DNA binding. Crystal structure determination of *Eco*RII and its DNA complexes will enable us to either confirm an allosteric activation mechanism similar to that of *Nae*I or to discover a new type of mechanism.

Attempts to crystallize *Eco*RII protein have met with a lot of difficulty and frustration.

*Eco*RII was first crystallized in the cubic space group $I23$ or $I2_13$ (Karpova *et al.*, 1999). However, the cubic crystal form diffracted only to 4 Å resolution at best. Here, we report a new crystal form of *Eco*RII that diffracts to a much higher resolution than that of the cubic form.

2. Materials and methods

2.1. Expression and purification of *Eco*RII

A reliable expression and purification system has been established to produce pure N-terminally His_6-tagged *Eco*RII protein in large quantities (Reuter *et al.*, 1998, 1999). *Escherichia coli* strain JM109 (pDK1) was transformed by pQE30 vector containing the *Eco*RII 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 with isopropyl-β-D-thiogalactoside (IPTG). Protein was purified on 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 ~28 mg of pure protein (concentrated to ~5 mg ml^{-1}) from a 1 l cell culture using the above protocol.

2.2. Crystallization

We have successfully grown crystals of the N-terminally His_6-tagged *Eco*RII protein. Purified His_6-*Eco*RII protein was concentrated by CentriPrep YM-30 (Amicon Bio-Separations) to ~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 His_6-*Eco*RII were obtained after intensive screening and optimization of crystallization 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 consisting of 50 m*M* cacodylate buffer pH 7.0, 4–6% methanol, 80–100 m*M* $MgCl_2$ and equilibrated against 1 ml of reservoir solution. Crystals usually appeared within 1–3 d (Fig. 1).

Figure 1
Crystals of *Eco*RII protein (typical dimensions 0.15 × 0.10 × 0.05 mm).

2.3. X-ray data collection and analysis

Crystals were first characterized with an in-house X-ray source. Crystals were flash-frozen in liquid nitrogen. A variety of cryoprotection solutions and procedures were tried. So far, the best cryoprotectant solution was found by adding 20–30% (final concentration) glycerol to the reservoir solution. After transferring a crystal to the cryoprotectant solution, it was picked up by a fiber loop and flash-frozen in liquid nitrogen, 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 RU-H3R 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 were collected on APS beamline 19BM, Chicago, USA.

3. Results and discussion

It has been very difficult to grow *Eco*RII crystals. The cubic form crystals were obtained after a long period of extensive screening and optimization (Karpova *et al.*, 1999). However, these crystals diffracted poorly to less than 4 Å resolution on a rotating-anode X-ray source. A trip to the APS synchrotron beamline 19ID did not result in a significant improvement in the diffraction resolution of the cubic crystals. Because the protein sample used in crystallization was prepared from an expression clone that carries no tag, we decided to try an expression system with a His_6 tag at the N-terminus of *Eco*RII, hoping that a different expression and purification protocol might improve crystal quality. The N-terminally His_6-tagged *Eco*RII protein (His_6-*Eco*RII) was expressed and purified in a high yield that enabled us to screen many crystallization conditions. The His_6-*Eco*RII protein did not crystallize under the conditions used to grow the cubic form crystals. Massive screening and refining of crystallization conditions led to a different crystal form (Fig. 1).

Under optimal conditions, these crystals diffracted to about 3.3 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 and its space group was determined to be $P2_1$. A data set to 3.3 Å resolution was collected in-house (Table 1). On the APS synchrotron beamline 19BM, these monoclinic crystals diffracted to 2.8 Å resolution. A complete data set to 2.8 Å resolution was collected with a total of 240° rotation at a crystal-to-detector distance of 230 mm (Table 1). Several more data sets were also collected with similar statistics.

Table 1
*Eco*RII crystal data and data-collection statistics.

Values in parentheses are for the last resolution shell.

	In-house	Synchrotron
Space group	$P2_1$	$P2_1$
Unit-cell parameters (Å, °)	a = 59.1, b = 128.2, c = 60.6, β = 90.04	a = 58.3, b = 127.8, c = 59.9, β = 91.4
Temperature (K)	93	93
Wavelength (Å)	1.5418	1.0332
Oscillation range per frame (°)	1.0	1.0
Exposure time (s)	1800	15
Resolution (Å)	3.3	2.8
No. of unique reflections	11487	22008
Total No. of reflections	20799	103818
Completeness (%)	83.2 (82.1)	98.5 (89.8)
R_{merge}	0.078 (0.699)	0.087 (0.421)
$I/\sigma(I)$ (average)	10.8 (1.7)	6.3 (1.5)

With the known molecular weight of about 45.6 kDa for an *Eco*RII monomer and a crystal volume per asymmetric unit of 223 100 Å^3, assuming two monomers per asymmetric unit would yield a Matthews coefficient V_M of 2.4 Å^3 Da^{-1} (Matthews, 1968) and a solvent content of 49% (Westbrook, 1985) for the monoclinic form of *Eco*RII 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. In order to determine the relationship between the two crystallographically distinct monomers, we calculated the self-rotation function of the data with the program *MERLOT* (Fitzgerald, 1988). In the $\kappa = 180°$ section (Fig. 2), the strongest peak with the maximum peak height of 100 at $\varphi = 0$ and $\psi = 0°$ represents the only crystallographic twofold axis of the crystal. The other two peaks with the second highest peak height of about 75 at (i) $\varphi = 30$ and $\psi = 90°$ and (ii) $\varphi = 120$ and $\psi = 90°$ are non-crystallographic twofold axes relating the two monomers in the asymmetric unit. The positions of the two pseudo-twofold axes in relation to the crystallographic twofold axis in the rotation map imply that the crystal may possess pseudo-222 symmetry, while the relative peak heights indicate that the crystal

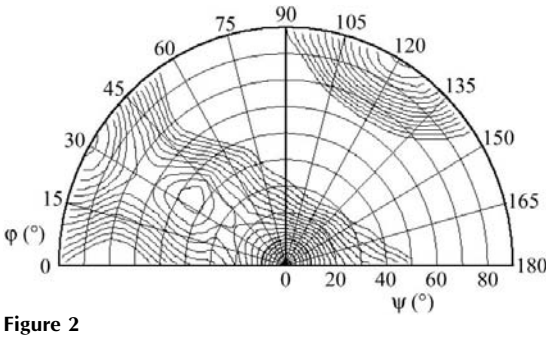

Figure 2
A plot of the self-rotation function ($\kappa = 180^\circ$) calculated using the program *MERLOT* (Fitzgerald, 1988) for the monoclinic *Eco*RII crystal

does not have true 222 symmetry. Careful reindexing and re-examination of the raw diffraction data reconfirmed the crystal Laue symmetry as 2/*m* but not *mmm*. The fact that an asymmetric unit in the crystal consists of a dimer is consistent with the finding that in solution *Eco*RII exists as a dimer.

It is very interesting to note that the cubic form *Eco*RII crystals were grown in the absence of divalent cations in the crystallization solution, while the monoclinic form crystals grew in the presence of Mg^{2+}. The cubic form crystals could not be grown in the presence of Mg^{2+} (Karpova *et al.*, 1999). They diffracted poorly to 4 Å resolution at best, while the monoclinic form crystals diffracted to much higher resolution (2.8 Å) on a synchrotron source. However, it is not clear whether the presence of Mg^{2+} dramatically improved the diffraction resolution of the monoclinic crystal form. Another factor might be the His_6 tag present at the N-terminus of *Eco*RII protein used to grow the monoclinic crystals. The *Eco*RII protein sample used to grow the cubic form crystals did not carry any tag. This is in contrast to the conventional wisdom that a His_6 tag would hinder protein crystallization.

We are currently crystallizing SeMet-*Eco*RII protein. We plan to use the Se-MAD or other phasing methods to solve the *Eco*RII structure. Co-crystallization of *Eco*RII with its DNA substrates is also in progress.

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