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Purification, crystallization and preliminary X-ray analysis of the HsdR subunit of the *EcoR124I* endonuclease from *Escherichia coli*

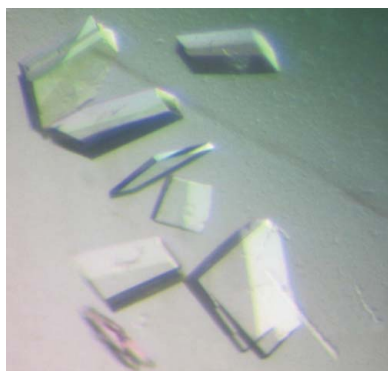
EcoR124I is a multicomplex enzyme belonging to the type I restriction-modification system from *Escherichia coli*. Although *EcoR124I* has been extensively characterized biochemically, there is no direct structural information available about particular subunits. HsdR is a motor subunit that is responsible for ATP hydrolysis, DNA translocation and cleavage of the DNA substrate recognized by the complex. Recombinant HsdR subunit was crystallized using the sitting-drop vapour-diffusion method. Crystals belong to the primitive monoclinic space group, with unit-cell parameters $a = 85.75$, $b = 124.71$, $c = 128.37$ Å, $\beta = 108.14^\circ$. Native data were collected to 2.6 Å resolution at the X12 beamline of EMBL Hamburg.

1. Introduction

Type I restriction-modification (R-M) systems are multifunctional molecular machines (Davies *et al.*, 1999) that are key players in defence against bacteriophage infection (McClelland *et al.*, 2005). DNA sequences that are predicted to encode type I R-M systems have been identified in more than 30 different bacterial species. Based on amino-acid conservation, enzymatic properties, cross-hybridization tests between genes and antibody cross-reactivity, type I restriction enzymes are grouped into the four families IA, IB, IC and ID (Murray, 2000; Bourniquel & Bickle, 2002; Dryden *et al.*, 2001).

EcoR124I is an endonuclease belonging to the type IB restriction-modification system; similar to other representatives of this type, it is composed of three different kinds of subunits: HsdS (where S stands for specificity and Hsd for host specificity for DNA), HsdM (methylation) and HsdR (restriction). HsdM and HsdS are sufficient to form a functional DNA methyltransferase (MTase; M₂S complex) which protects the host genome from cleavage by methylation of the adenine residues in the recognition sites in one or both strands (Murray, 2000). In association with HsdR, MTase forms a multi-subunit complex that possesses DNA-endonuclease and ATP-dependent DNA-translocase activities (Burckhardt *et al.*, 1981; Dryden *et al.*, 1993; Taylor *et al.*, 1992; Janscak & Bickle, 1998). The subunit stoichiometry of the functional endonuclease is R₂M₂S (Dryden *et al.*, 1997; Janscak *et al.*, 1996, 1998). This system recognises a specific bipartite nonpalindromic DNA sequence 5'-GAANN-NNNRNRTCG-3', but cleaves DNA at nonspecific sites distant from the recognition sequence (Janscak & Bickle, 2000). The HsdR subunit is supposed to make a second contact with nonspecific sequences near to the recognition site and pulls DNA through the complex in a reaction that is dependent on ATP hydrolysis (Janscak, MacWilliams *et al.*, 1999).

Current work is focused on characterization of the HsdR subunit of the *EcoR124I* enzyme (R.*EcoR124I*). The protein composition has been identified by limited proteolysis, mutational and sequence analysis (Janscak, Sandmeier *et al.*, 1999). At the N-terminus, there is a defined domain that is common to all endonucleases. A C-terminal domain is thought to be involved in the MTase core interface. The superfamily 2 (SF2) helicase motif (DEAD-box motif) is situated



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between the C-terminal and endonuclease domains (Gorbalenya & Koonin, 1991). This DEAD-box motif is responsible for ATP binding, ATP hydrolysis and DNA translocation. On the basis of this, *EcoR124I* and the whole family can be defined as helicase-like proteins (McClelland & Szczelkun, 2004), although no evidence of DNA unwinding by type I enzymes has been reported using classical oligoduplex helicase assays (Stanley *et al.*, 2006).

Although their mechanisms of activity have been extensively studied in recent years, further accurate investigations have been hampered by the absence of structural information on the enzymes of this system. In the present work, we report some progress in the structural study of the *EcoR124I* enzyme from *Escherichia coli*, describing the purification, crystallization and preliminary crystallographic analysis of its restriction subunit HsdR.

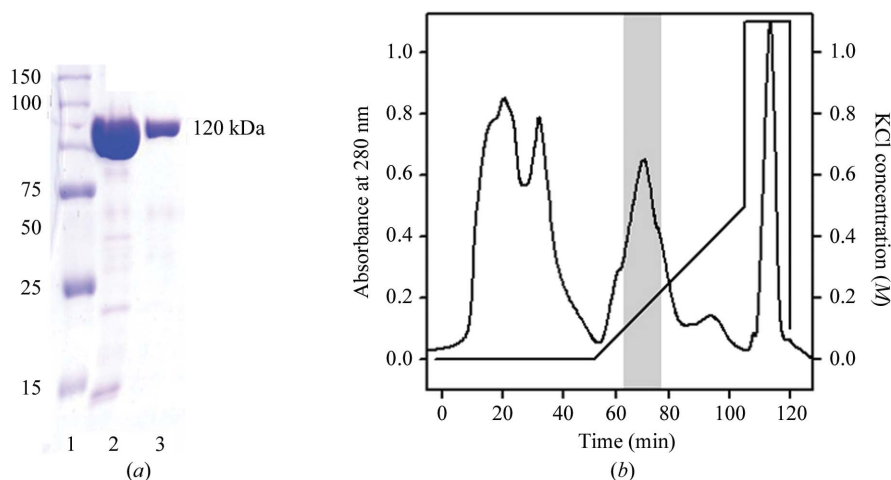


Figure 1 (a) SDS-PAGE (12%) showing the purification of *R.EcoR124I*. Lane 1, molecular-weight markers (kDa); lanes 2 and 3, purified *R.EcoR124I*. (b) Elution profile from purification of the HsdR subunit of the *EcoR124I* endonuclease on a 20 ml DEAE Sepharose Fast Flow column at 4 ml min⁻¹. The shaded area represents the peak fraction containing *R.EcoR124I*.

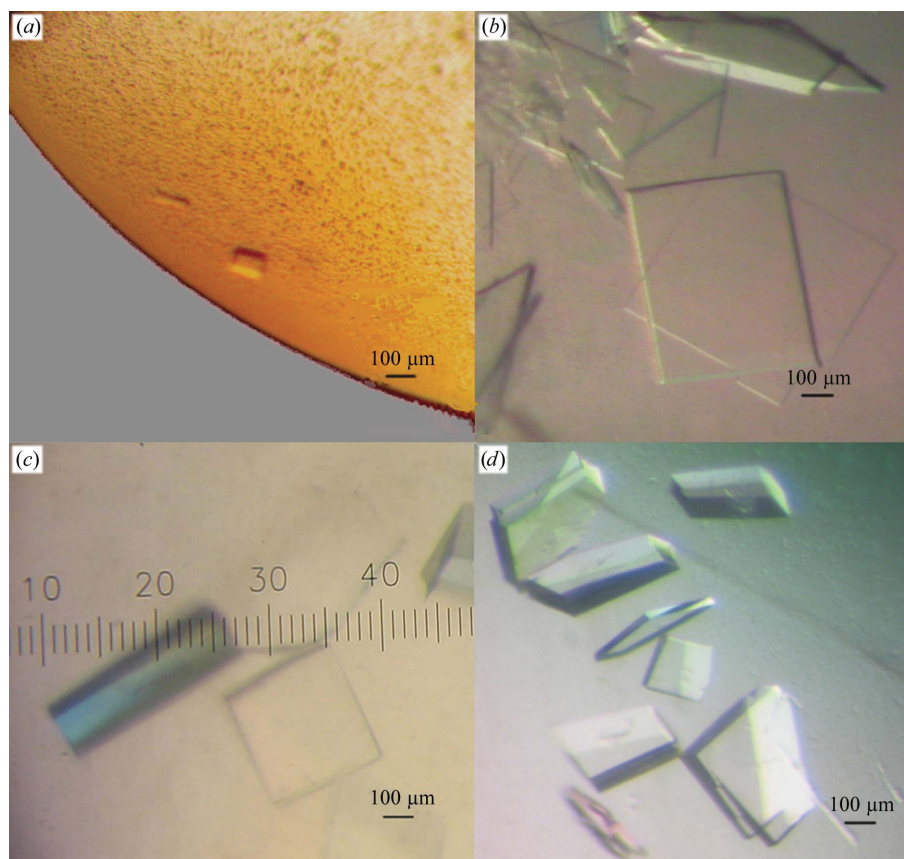


Figure 2 Various crystal forms of the *R.EcoR124I* protein appeared during screening of crystallization conditions. (a) A crystal of the apoprotein (protein without ATP) in 0.2 M KSCN, 8% PEG 20 000, 8% PEG 550 MME at 298 K. (b) Numerous plates growing within 30 min from the protein with 5 mM ATP. The precipitant consisted of 8% PEG 20 000, 8% PEG 550 MME, 0.2 mM Li₂SO₄ at 298 K. (c) Crystals of protein with ATP appearing in a single drop at 277 K. (d) Crystals of protein with ATP in the presence of 11 mM CoCl₂ at 277 K.

2. Experimental procedure

2.1. Protein expression and purification

Recombinant *R.EcoR124I* was expressed in BL21(DE3) *E. coli* (Novagen) from the plasmid pTRC124 (Janscak & Bickle, 1998). The overnight cell culture was diluted 1:100 in 1 l fresh LB (Luria–Bertani) medium supplemented with ampicillin to a final concentration of 100 µg ml⁻¹ and grown with shaking at 310 K until the OD₆₀₀ (optical density at 600 nm) was about 0.4–0.5. Protein expression was induced by the addition of 0.8 mM isopropyl β-D-thiogalactopyranoside (IPTG) and the culture was incubated for an additional 3 h at 290 K. Cells were harvested (4500 rev min⁻¹ for 20 min) and resuspended in 60 ml buffer A (20 mM potassium phosphate pH 7.5) and broken by sonication on ice for 2 × 30 s bursts with 30 s cooling in between. The cell lysate was clarified by ultracentrifugation (25 000 rev min⁻¹ for 1 h at 277 K).

The supernatant was directly applied onto a 20 ml DEAE Hi-Trap Sepharose Fast Flow column (Amersham) pre-equilibrated in buffer A. Bound proteins were eluted with a linear gradient of KCl (0.01–0.5 M, 600 ml). Fractions were analyzed by 10% SDS–PAGE (Bio-Rad; Maniatis *et al.*, 1982), which showed that the purity of the peak fraction was more than 95% (Fig. 1*a*). Recombinant HsdR was eluted from the DEAE Sepharose column as a single peak, which was monitored using a dual-path UV monitor (Pharmacia; Fig. 1*b*). The chromatographic process was performed using FPLC (Pharmacia). Fractions containing recombinant protein were pooled and concentrated to 15 mg ml⁻¹ using 50 kDa cutoff spin concentrators (Millipore). The protein concentration was calculated from the absorbance at 280 nm using a molar extinction coefficient derived from the amino-acid sequence (98 225 M⁻¹ cm⁻¹; Gill & von Hippel, 1989) using the protein identification and analysis tools on the ExPASy server. Prior to crystallization, the enzyme solution was supplemented with ATP to a final concentration of 5 mM. The protein was stored at 250 K.

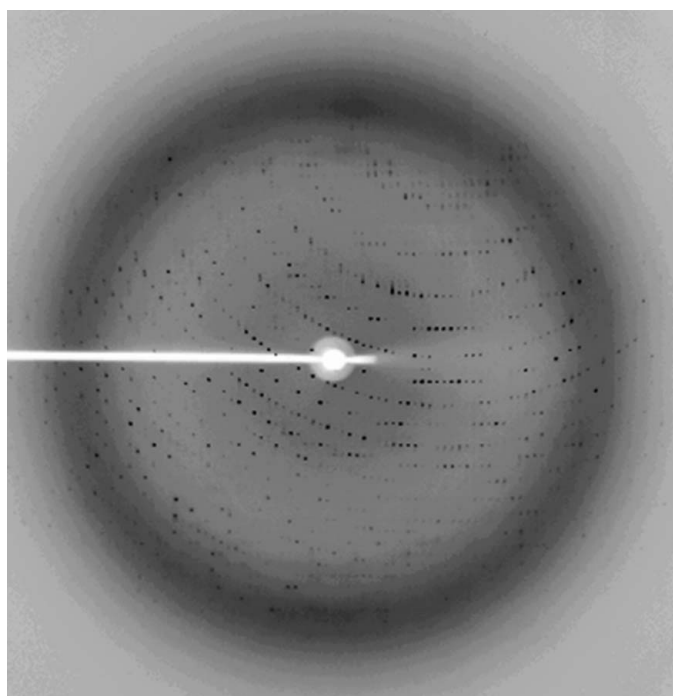


Figure 3
Diffraction image of the native *R.EcoR124I* enzyme. The crystal diffracted to a resolution of 2.6 Å.

Table 1

Data-collection statistics for the *R.EcoR124I* crystal.

Values in parentheses are for the highest resolution shell.

Beamline	X12
Wavelength (Å)	1.0
Unit-cell parameters (Å, °)	$a = 85.75, b = 124.71,$ $c = 128.37, \beta = 108.14$
Space group	$P2_1/P2_1$
Resolution range (Å)	20–2.6 (2.64–2.60)
Redundancy	7.3 (6.7)
Measured reflections	582265
Unique reflections	79374
Completeness (%)	99.3 (96.5)
R_{merge}^\dagger (%)	10.9 (55.0)
$I/\sigma(I)$	18.2 (2.9)
Mosaicity (°)	0.99

$^\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum |I_i - \langle I \rangle|}{\sum \langle I \rangle}$, where I_i is the intensity of the i th measurement of reflection hkl and $\langle I \rangle$ is the average intensity of a reflection.

2.2. Crystallization

15 mg ml⁻¹ recombinant HsdR in 20 mM phosphate buffer pH 7.5, 0.1 M KCl and 5 mM ATP was used for the crystallization experiments. Crystallization trials were performed in Hampton Research Linbro plates (Hampton Research, California, USA) at both room temperature and at 277 K using the sitting-drop vapour-diffusion method. Commercial crystal screening kits from Hampton Research (CA, USA) and Molecular Dimensions Ltd (Suffolk, England) were used for the initial screening of crystallization conditions. Crystals of the recombinant HsdR protein were grown from a mixture of polyethylene glycol solutions, yielding different crystal forms (Fig. 2). The best crystals were obtained from precipitant (500 µl reservoir solution) containing 8% PEG 20 000, 8% PEG 550 MME and 0.2 M Li₂SO₄ at 277 K. The crystallization drops consisted of 2 µl protein solution (protein in 20 mM potassium phosphate pH 7.5, 100 mM KCl, 5 mM ATP in water), 2 µl precipitant and 0.5 µl 0.1 M CoCl₂ as an additive. Crystals appeared within a few days and grew to maximum dimensions of about 0.5 × 0.4 × 0.1 mm (Fig. 2*d*).

2.3. Data collection and processing

A crystal mounted in a nylon loop (Hampton Research) was transferred for a few seconds to 10 µl of a cryosolution containing 25% glycerol (in water). The crystals were then flash-frozen in a cold nitrogen stream at 100 K for the diffraction experiment. Native diffraction data (Fig. 3) were collected to a resolution of 2.6 Å using synchrotron radiation of wavelength 1.0 Å at beamline X12 of the EMBL Hamburg Outstation. A total of 720 images were recorded with an oscillation angle of 0.5° and an exposure time of 60 s per image using a 225 mm MAR Mosaic CCD detector. The crystal-to-detector distance was set to 150 mm. The intensity data were processed and scaled using the *HKL* package (Otwinowski & Minor, 1997). Statistics of the crystallographic data are summarized in Table 1.

3. Results and discussion

Apoprotein (protein without ATP) was also successfully crystallized using condition No. 23 (0.2 M KSCN, 8% PEG 20 000, 8% PEG 550 MME) of Molecular Dimensions Clear Strategy Screen 1 (CSS-1, MD1-14, Molecular Dimensions Ltd, Suffolk, England). The initial crystals with dimensions of about 0.06 × 0.02 × 0.02 mm (Fig. 2*a*) were not suitable for diffraction experiments. Further improvement of the crystallization conditions did not lead to satisfactory results until the ATP complex was used for experiments. Crystallization of

the protein complex with ATP led to a rapid improvement of the crystal size and shape. Crystals grew from precipitant consisting of 8% PEG 20 000, 8% PEG 550 MME and 0.2 M Li₂SO₄ (condition No. 20, CSS-I, MD1-14 from Molecular Dimensions Ltd) at 298 K (Fig. 2*b*) and 277 K (Fig. 2*c*). Plate crystals appeared together with numerous intergrown crystals in a single drop. Crystals diffracted to a maximum resolution of 2.8 Å, but owing to high mosaicity and blurred spots it was not possible to process the diffraction data. The Hampton Research Additive Screen kit was used to screen various ions to attempt to improve the quality of crystals (Additive Screen HT, HR2-138, Hampton Research, California, USA). Crystals grew in the presence of various ions, but improvement of diffraction data quality was only achieved when 0.1 M CoCl₂ (Additive Screen HT, HR2-138, condition No. 4) was added to the drop to a final concentration of 11 mM. Crystals grew in 4–6 d at 277 K (Fig. 2*d*) and belong to the monoclinic space group, with unit-cell parameters $a = 85.75$, $b = 124.71$, $c = 128.37$ Å, $\beta = 108.14^\circ$. Assuming the presence of two molecules per asymmetric unit, the Matthews coefficient is estimated to be $2.7 \text{ \AA}^3 \text{ Da}^{-1}$, corresponding to a solvent content of 55% (Matthews, 1968), which is within the general range for protein crystals.

A protein-sequence search against the PDB did not reveal any structural homology; therefore, the molecular-replacement method was not attempted.

Heavy-atom-derivatized or selenomethionine-derivatized protein will be produced in order to solve the structure using single/multiple anomalous diffraction (SAD/MAD) experiments. A search for heavy-atom derivatives is presently in progress.

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to the X12 beamline at the DORIS storage ring of DESY in Hamburg.

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