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Crystallization of the 43 kDa ATPase domain of *Thermus thermophilus* gyrase B in complex with novobiocin

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The 43 kDa ATPase domain of *Thermus thermophilus* gyrase B was overproduced in *Escherichia coli* and a three-step purification protocol yielded large quantities of highly purified enzyme which remained stable for weeks. Crystals of the 43 kDa domain in complex with novobiocin, one of the most potent inhibitors of bacterial topoisomerases, were obtained. Crystals obtained in the presence of PEG 8000 do not diffract, but a different crystal form was obtained using sodium formate as a precipitating agent. The plate-shaped crystals, which were less than 10 μm in thickness, could be cryocooled directly from the mother liquor and a full diffraction data set was collected to 2.3 Å allowing the determination of the first structure of a gyrase B 43K domain in complex with a coumarin.

1. Introduction

DNA gyrase is involved in DNA replication, repair, recombination and transcription (Menzel & Gellert, 1994). This enzyme introduces negative supercoils into closed circular DNA using free energy derived from ATP hydrolysis (Kreuzer & Cozzarelli, 1980). The active enzyme is an A_2B_2 enzyme, where the A subunit is responsible for DNA breakage and reunion while the B subunit catalyzes ATP hydrolysis (Krueger *et al.*, 1990; Reece & Maxwell, 1991). As DNA gyrase is an essential enzyme in prokaryotes, it has attracted much interest in the design of antibiotics. The coumarins (*e.g.* novobiocin) are natural inhibitors of the enzyme's ATP-dependent activity (Gellert *et al.*, 1976; Ali *et al.*, 1993).

The gyrase B domain 1 (also called the 24 kDa domain) comprises *Escherichia coli* residues 1–220 and contains most of the residues implicated in the coumarin binding. The crystal structure of the *E. coli* 24 kDa domain–novobiocin complex has been described previously (Lewis *et al.*, 1996). Nevertheless, it was shown that the 24 kDa domain is unable to bind and hydrolyze ATP or to bind ADP or ADPNP (Gilbert & Maxwell, 1994). The crystal structure of the 43 kDa domain (comprising domains 1 and 2 from residues 1–392) in complex with ADPNP has been solved and shows that both domains 1 and 2 are implicated in nucleotide binding (Wigley *et al.*, 1991; Brino *et al.*, 2000).

No crystal structure of the 43 kDa domain in complex with novobiocin has yet been reported that would provide information on the consequences of the inhibitor binding on the whole ATPase domain. We present here the crystallization of the *T. thermophilus* gyrase B 43 kDa domain in complex with novobiocin.

2. Materials and methods

2.1. Overproduction and purification of recombinant *Thermus thermophilus* 43 kDa domain

The sequence coding for *T. thermophilus* gyrase B was inserted into the pJW282 vector (Hallett *et al.*, 1990). The details of the cloning will be published elsewhere (Hoermann *et al.*, in preparation). The resulting construct containing *T. thermophilus* gyrase B was digested by *Sac*I and *Hin*dIII in order to restrict the 43 kDa domain comprising the initial methionine to valine 392. Annealing of oligonucleotides TN155 (5′-TGTCCGCTA-GTGATAAA-3′) and TN156 (5′-AGCTTT-TATCACTAGCGGACAAGCT-3′) allowed the insertion of a stop codon in the C-terminal part of the protein. The plasmid was transformed in *E. coli* strain DH5α and the cells were grown to an OD_{600nm} of 0.5 prior to induction by 0.8 m*M* IPTG. Expression of the 43 kDa protein was induced overnight at 310 K. The pellet from a 6 l culture was disrupted by chemical lysis and centrifuged. The supernatant was precipitated using a series of saturated ammonium sulfate cuts between 30 and 45%. The protein contained in the 45% ammonium sulfate fraction was dialyzed and loaded on a DEAE Sephadex A-50 column (Pharmacia) at pH 9.0 and recovered in the flowthrough fraction. After concentration, the

protein was eluted on a gel-filtration column (Superdex S200 Pharmacia) in a buffer containing 10 m*M* Tris pH 7.2, 1 m*M* EDTA pH 8, 5 m*M* 2-mercaptoethanol, 200 m*M* NaCl before proceeding to the preliminary crystallization trials.

2.2. Crystallization trials and preliminary crystallographic analysis

All trials were carried out in Linbro 24-well tissue-culture plates (Hampton Research) using the hanging-drop vapour-diffusion method. The protein was concentrated by centrifugation on a Centricon 10. Drops containing 2 µl 4.5 mg ml^{-1} 43 kDa domain with 0.3 m*M* novobiocin and 2 µl crystallization solution were equilibrated against a well containing 1 ml crystallization solution. Initial crystallization conditions were screened using Crystal Screen and Crystal Screen II from Hampton Research at 276 and 295 K (Jancarik & Kim, 1991). The first crystal form was obtained at 295 K after refinement of the first screening hits with a crystallization solution consisting of 100 m*M* sodium cacodylate pH 6.5, 75 m*M* sodium acetate and 14.3% PEG 8000. The crystal form which diffracts to 2.3 Å was obtained at 295 K using 100 m*M* Tris pH 8.5 and 3 *M* sodium formate as the crystallization solution; the protein was concentrated after dialysis against a buffer containing 10 m*M* sodium cacodylate pH 6.9 and 20 m*M* KCl.

The crystals were separated in the drop using a needle. Before freezing, the first crystal form was mounted in a cryoloop (10 µm, Hampton Research) and quickly soaked in a cryoprotectant solution containing the mother liquor with 30% glycerol. The sodium formate crystal form was directly frozen from the mother liquor by liquid ethane refrigerated in a liquid-nitrogen bath. Data were collected on beamline ID14-EH4 (ESRF, Grenoble) and processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1996).

3. Results and discussion

3.1. Overproduction and first crystallization trials

The recombinant *T. thermophilus* GyrB 43K was overproduced in *E. coli* in a soluble form (Fig. 1*a*) and the lysate was precipitated using ammonium sulfate prior to dialysis and recovery from a DEAE column (Fig. 1*b*). The protein was then submitted to size-exclusion chromatography, yielding 95% pure protein in a buffer suitable for concentration and the first crystallization trials. The protein has a molecular weight of 43 352.34 Da measured by mass spectrometry under denaturing conditions (data not shown). This value corresponds to the weight derived from the peptidic sequence of the enzyme (MW = 43 346.98 Da).

The protein was mixed at room temperature with 0.3 m*M* novobiocin prior to crystallization. Initial screening using Crystal Screen and Crystal Screen II (Jancarik & Kim, 1991) gave heavy precipitates in the drops. In this case, the corresponding reservoirs were immediately replaced with a solution diluted twice with water so as to re-equilibrate the drops. At 295 K, thin needles appeared within 3 d in conditions 9 and 28 of Crystal Screen, in which the precipitates had dissolved. Condition 9 contains PEG 4000 combined with ammonium acetate and sodium citrate pH 5.6, whereas condition 28 contains PEG 8000 with sodium acetate and sodium cacodylate pH 6.5. Despite refinement of the crystallization conditions and screening for additives, the crystals would not grow thicker than 10 µm, although they achieved a surface of up to 600 × 50 µm. The combination of 14.3% PEG 8000 and 75 m*M* sodium acetate at pH 6.5 was finally selected and reproducibly gave needles (Fig. 2*a*). The needles could be separated and cryocooled with 30% glycerol. These crystals show no diffraction either on a classical laboratory diffraction apparatus or using a synchrotron-radiation source. In our experience, neither refinement of the crystallization conditions nor other cryocooling tests could improve this crystal form.

3.2. Diffraction of the new crystal form

We noticed that the protein only nucleated in the presence of PEG (tiny needle urchins), but required carboxylate compounds to give crystals of reasonable size. Therefore, we tried carboxylates as major precipitating agents in the pH range 6.0–8.5. With all the carboxylates tested (acetate, formate and citrate), square plates (80 × 80 × 10 µm) appeared within 2 d at 295 K in conditions containing 3.0 *M* sodium formate at pH 8.5 (Fig. 2*b*). Under the same conditions, we also obtained similar plates at 278 K but within a week. In each case, we could easily separate the crystals from each other (Fig. 2*c*) and directly freeze them from the mother liquor.

We used the crystals grown at 295 K for the data collection. Despite their size and especially their thinness, these crystals diffracted to 2.3 Å on beamline ID14-EH4

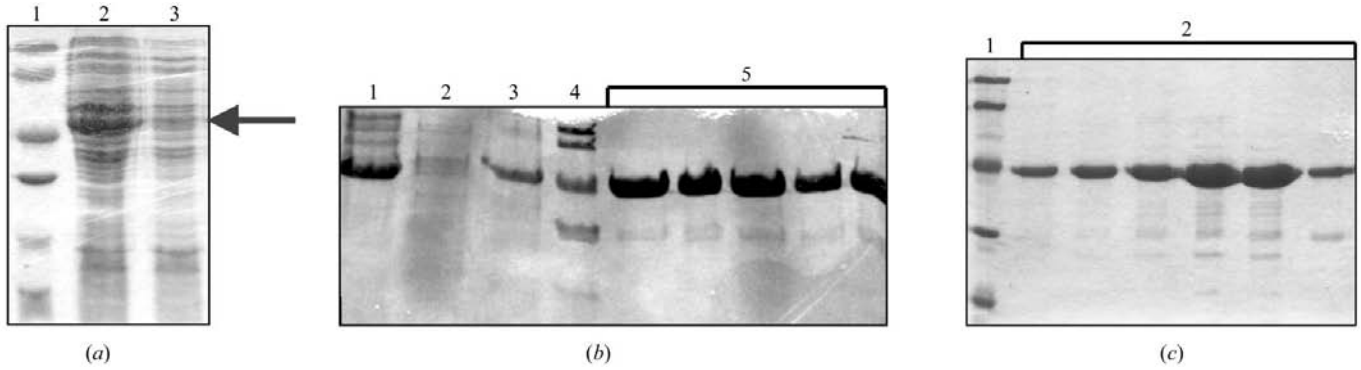
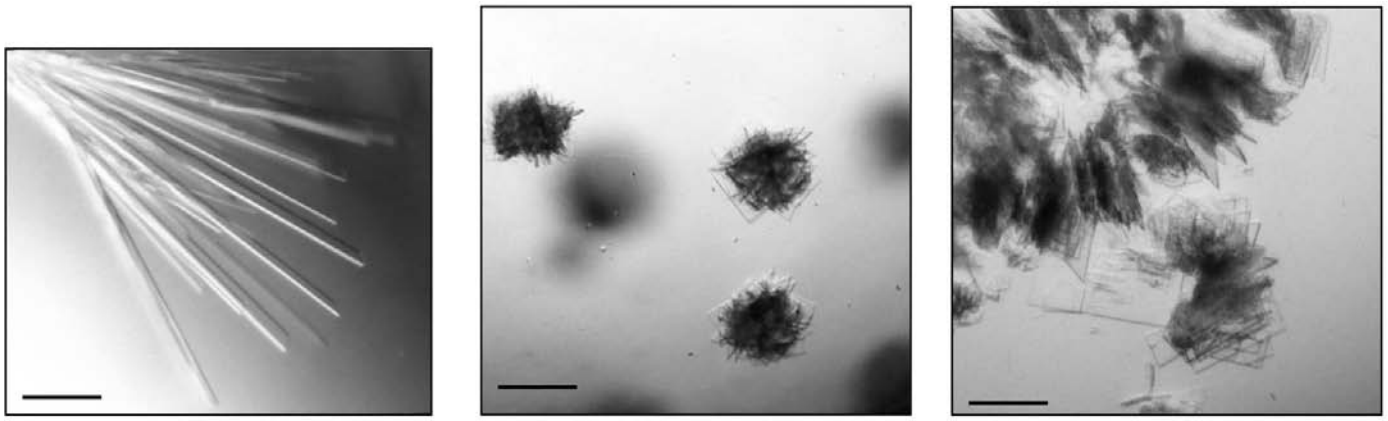


Figure 1
Expression and purification of *T. thermophilus* gyrase B 43 kDa domain. (*a*) SDS–polyacrylamide gel of the 43 kDa domain overproduction stained by Coomassie Blue. Lane 1 shows molecular-weight markers: 97, 67, 43, 30 and 20.1 kDa. Lane 2 is an aliquot of the lysate; the 43 kDa protein is shown by an arrow. Lane 3 shows an aliquot of the cells before induction. (*b*) Ammonium sulfate precipitation and DEAE. An aliquot of the precipitate with 45% saturated ammonium sulfate was loaded onto an SDS–polyacrylamide gel (lane 1). Lane 2 shows an aliquot of the 45% ammonium sulfate soluble fraction, showing that all the protein was precipitated at 45% ammonium sulfate. Lane 3 shows the soluble fraction of the 30% ammonium sulfate precipitate, indicating that the protein was not fully precipitated at this concentration. Lane 4 shows the molecular-weight markers: 97, 67, 43, 30 and 20.1 kDa. After dialysis, the 43 kDa domain was retrieved in the flowthrough fractions of the DEAE column (lane 5). (*c*) SDS gel on fractions from the size-exclusion column. Aliquots of the elution fractions from a Superdex S200 (Pharmacia) were loaded onto an SDS–polyacrylamide gel (lane 2). After this stage, the protein was directly concentrated for the crystallization trials.



(*a*) (*b*) (*c*)

Figure 2
(*a*) PEG crystal form obtained with 14.3% PEG 8000 at pH 6.5. (*b*) Formate crystal form obtained with 3.0 *M* sodium formate at pH 8.5. Both crystal forms were obtained at 295 K. (*c*) Picture of the drop after separation of the plates with a thin needle. A single plate was mounted in a loop and frozen directly from the mother liquor. The black bar corresponds to 100 μm in each case.

Table 1
Diffraction statistics.

Values in parentheses refer to the highest resolution shell (2.38–2.30 Å).

Beamline	ID14-EH4
Wavelength (Å)	0.9397
Resolution range (Å)	15–2.3
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 44.9, b = 125.5, c = 79.8 β = 96.4
No. of reflections	96975
Unique reflections	37265
Completeness (%)	96.6 (90.4)
Redundancy	2.6
$I/\sigma(I)$	14 (5.5)
$R_{\rm merge}$ (%)	6.4 (15.5)

(ESRF, Grenoble) and a full data set could be collected from a single crystal. The crystal belongs to space group $P2_1$, with unit-cell parameters a = 44.9, b = 125.5, c = 79.8 Å, β = 96.4°. The Matthews volume (Matthews, 1968) suggests two molecules per asymmetric unit, with $V_{\rm M}$ = 2.6 Å^3 Da^{-1} (solvent content 51.5%). The data-processing statistics are summarized in Table 1.

4. Concluding remarks

The stability of the *T. thermophilus* GyrB 43 kDa domain allowed us to obtain large quantities of highly purified enzyme for the crystallization experiments. The protein could be stored for weeks either at 253 or 277 K without impairing its solubility or crystallizability. Both previous crystal structures of *E. coli* 43 kDa domain with ADPNP were crystallized with PEG as a precipitating agent. The first crystal form we obtained using PEG did not diffract, but the use of formate as a crystallization agent gave very thin crystals with a very good diffraction power. Combined with the powerful beamline ID14-EH4 at ESRF, we obtained a full data set which allowed the determination of the structure by molecular replacement (Lamour *et al.*, 2002). These data provide the first crystal structure of the complete ATPase domain of gyrase B complexed with novobiocin, one of the most potent inhibitors of bacterial topoisomerases.

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