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Crystal engineering: deletion mutagenesis of the 24 kDa fragment of the DNA gyrase B subunit from *Staphylococcus aureus*

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The 24 kDa fragment of DNA gyrase B from *Staphylococcus aureus* was expressed in *Escherichia coli* and purified for crystallization. Crystals of the wild-type protein grew in the presence of cyclothialidine but proved difficult to reproduce. In order to improve the crystallization, the flexible regions of the protein were deleted by mutagenesis. The mutant proteins were analyzed by differential scanning calorimetry and the most stable mutants produced crystals. It was possible to reproducibly grow in the microbatch system single well defined crystals which belonged to the space group *C*2 and diffracted isotropically to approximately 2 Å resolution.

1. Introduction

The bacterial DNA topoisomerase II, DNA gyrase, introduces negative supercoils into covalently closed DNA using the free energy derived from ATP hydrolysis. The enzyme is composed of two subunits which form an A_2B_2 tetramer. The A subunit is responsible for DNA breakage and reunion, while the B subunit catalyzes ATP hydrolysis (Reece & Maxwell, 1991). As DNA gyrase is an essential enzyme in prokaryotes, it has been seen as an attractive target for the design of antibiotics. The coumarins (*e.g.* coumermycin and novobiocin) act by inhibiting ATP hydrolysis by the B subunit of DNA gyrase. The X-ray structure of the 43 kDa N-terminal fragment–AMPNP complex (NGyrB–AMPNP) and the 24 kDa N-terminal fragment–novobiocin complex of the gyrase B subunit from *Escherichia coli* have been described (Lewis *et al.*, 1996; Wigley *et al.*, 1991). The 24 kDa fragment of DNA gyrase has no inherent activity of its own; it has been shown to bind a novobiocin–Sepharose affinity column with similar avidity to GyrB (Gilbert & Maxwell, 1994). We are interested in designing inhibitory ligands binding to the ATPase site of the B subunit of DNA gyrase, based upon the X-ray structure. Although structures exist of the binary complex of the 24 kDa protein with novobiocin, the crystallization remains a challenge and to the best of our knowledge there are no reports to date regarding the crystallization of the apo-enzyme.

One of major obstacles for success in macromolecular crystallography studies is reproducibly obtaining X-ray quality crystals; in addition, there is also the problem of establishing a crystallization system for the study of protein–ligand complexes either for crystal soaking with apo-enzyme or co-crystallization trials. Analysis of proteins prior to crystallization using dynamic light scattering has proven to be an important criterion in judging the suitability of a protein for crystallization. Our studies have demonstrated that approximately 70% of proteins which have a monodisperse size distribution when analyzed by dynamic light scattering (DLS) will produce crystals in our initial screen (Zulauf & D'Arcy, 1992). The remaining 30% may require the addition of a specific ligand to the crystallization mixture or a protein modification before crystallization, such as limited proteolysis or deglycosylation.

In the past, this problem could often be solved by using a homologous enzyme from another species for crystallization. Generally, these enzymes differed by only a few amino-acid residues, which are often found on the surface of the protein, but the effect on crystallization could be dramatic. In this context, protein engineering has become a powerful tool for influencing a protein's crystallization properties. Introducing single amino-acid substitutions has proved effective in improving the crystallization properties of a protein. In many cases, this has proved critical to the successful completion of the project (Braig *et al.*, 1994; Chen *et al.*, 1996; Dyda *et al.*, 1994; Lawson *et al.*, 1991; McElroy *et al.*, 1992; Oubridge *et al.*, 1995; Schwede *et al.*, 1999; Zhang *et al.*, 1997). More recently, the successful use of deletion mutagenesis to improve the crystallization of proteins has been described (Armstrong *et al.*, 1998; Chen *et al.*, 1998; Yeh *et al.*, 1996). In our initial studies with the 24 kDa fragment of DNA gyrase from *E. coli*, we used single amino-acid substitutions to influence the crystallization process (D'Arcy *et al.*, 1999). Although we obtained better diffracting crystals of the novobiocin complex, we did not reach our goal of obtaining a crys-

Table 1
Data-collection statistics.

Parameter	(1–234)	(1–234; D105–127)
Space group	$P2_1$ (No. 4)	$C2$ (No. 5)
Generator	Enraf–Nonius FR591	Enraf–Nonius FR591
Detector	30 cm MAR IP	30 cm MAR IP
Data-collection temperature (K)	298	120
Data-processing program	*XDS*	*XDS*
Unit-cell dimensions		
a (Å)	73.7	143.6
b (Å)	69.1	55.9
c (Å)	91.85	51.6
β (°)	109.8	100.9
Resolution range (Å)	20.0–3.0	40.0–2.2
Measured reflections	61541	73168
Unique reflections	17228	20575
R_{sym},† overall/ outer shell‡ (%)	19.2/46.4	10.4/37.9
$I/\sigma(I)$, overall/outer shell‡	8.7/3.0	10.1/3.6
Completeness, overall/ outer shell‡ (%)	97.9/97.9	98.3/98.3

† $R_{sym} = \sum_h \sum_i |I_i(h) - \langle I(h)\rangle| / \sum_h \sum_i I(h)$, where $I_i(h)$ and $\langle I(h)\rangle$ are the ith and mean measurement of the intensity of reflection h. ‡ The outer shell is 3.2–3.0 Å for *Sa*GyrB(1–234) data and 2.3–2.2 Å for *Sa*GyrB(1–234; D105–127) data.

tallization system of the N-terminal 24 kDa fragment (p24) as an apo-enzyme. In this report, we describe the use of deletion mutagenesis to improve the crystallization of the 24 kDa fragment from the B subunit of DNA gyrase from *Staphylococcus aureus* [*Sa*GyrB(1–234)].

2. Materials and methods

2.1. Deletion mutagenesis of *Sa*GyrB(1–234)

Mutagenesis was performed directly on the plasmid expressing residues 1–234 of the *S. aureus* gyrB, p*Sa*GyrB(1–234) (Stieger *et al.*, 1996). The oligonucleotides used were: P1, 5′-CAGAATTCATTAAAGAGGAGA-AATTAACTATGGGTTTAGAAGCAGT-ACGTAAAAGACC-3′; P2, 5′-TCAT-CAGTTGTAAACGCATTGTC-3′; P3, 5′-AGTTAAAATAACTTCGACAGCTGG-3′, as well as the vector-specific primers F and O (Stuber *et al.*, 1990). The PCR fragment P1/Owas cloned into the *Eco*RI and *Hin*dIII sites of the expression plasmid pDS56/RBSII (Stuber *et al.*, 1990), generating the plasmid p*Sa*GyrB(23–234). The PCR products from P2/O and P3/F using p*Sa*GyrB(23–234) or p*Sa*GyrB(1–234) as template were blunt-end ligated and then cloned into the *Eco*RI and *Hin*dIII sites of the expression plasmid pDS56/RBSII, giving rise to the plasmids p*Sa*GyrB(23–234;Δ105–127) and p*Sa*GyrB(1–234;Δ105–127), respectively. DNA sequencing of both strands verified the deletion mutations and the mutant proteins were expressed and purified. All DNA manipulations were carried out using standard techniques (Sambrook *et al.*, 1989).

2.2. Expression and purification of *Sa*GyrB(1–234) and deletion mutants

The proteins *Sa*GyrB(1–234) and deletion mutants were overexpressed in *E. coli* M15(pRep4) cells grown at 310 K. Protein expression was induced with isopropyl-β-D-thiogalactopyranoside; cells were then harvested by centrifugation. Bacterial cell paste from 2 l of cell culture (10–20 g) was resuspended in 100 ml 50 m*M* Tris pH 8.0, 100 m*M* NaCl, 5% glycerol, 0.02% azide (buffer *A*) with one tablet of COMPLETE protease-inhibitor cocktail (Roche Molecular Biochemicals). Cells were disrupted using a pre-cooled cell homogenizer (Rainin) and the supernatant was separated from cell debris by centrifugation at 20000*g*. After filtration through a 0.2 µm filter (Sterivex-GP, Millipore Products), the supernatant was loaded onto a 50 ml novobiocin–Sepharose column 26/30 (Pharmacia) equilibrated with buffer *A*. Proteins were eluted with buffer *A* with 8 *M* urea. The protein was diluted to 150 µg ml^{-1} and extensively dialyzed against buffer *A* with 0.1% CHAPS {3-[{3-cholamidopropyl)dimethylammonio]-1-propanesulfonate}. The protein was concentrated and loaded onto a Superdex 75 XK26/100 column (Pharmacia) equilibrated with buffer *A*. The protein eluted at the volume predicted for a 20 kDa protein. Analytical size-exclusion chromatography was performed on a Superdex-75 column (PC 3.2/30) using the SMART system (Pharmacia), with 50 µg of purified protein and buffer *A* as the mobile phase. The calibration standards (Pharmacia) were ribonuclease, chymotrypsin, ovalbumin, albumin, aldolase, catalase and ferritin.

2.3. Activity assay

Activity was determined by the binding of novobiocin to the proteins. 50 µg of protein was brought to a fivefold molar excess of novobiocin and separated by gel filtration on a Superdex 75 PC 3.2/30; absorbance was measured at 280 and 310 nm. Peak fractions were collected and analyzed by HPLC on a reverse-phase mRPC C2/C18 ST 4.6/100 column (Pharmacia).

2.4. Differential scanning calorimetry

Protein samples for calorimetry were desalted using the SMART system (Pharmacia Biotech) on a PC 3.2/10 fast desalting column in 25 m*M* Tris pH 8.5, 100 m*M* NaCl and 0.02% azide. Calorimetry was performed at a protein concentration of 50 µ*M* at a scan rate of 1.0 K min^{-1} and the data were recorded every 1 s. The dependencies of temperature on the differential heat capacities for the proteins were recorded against the reference buffer using a differential scanning calorimeter (VP-DSC, Microcal Inc. Northampton, MA, USA). Subtraction of the instrument baseline and the buffer–buffer scanning curve from the raw data allowed analysis of the data using established procedures. Analysis of thermograms was performed with the help of *Microcal Origin* software (Microcal Inc., Northampton, MA, USA). The denaturation temperature (T_d) was determined as the temperature at the maximum relative specific heat capacity (ΔC_p^{max}).

2.5. Crystallization and data collection

A modified microbatch method (D'Arcy *et al.*, 1996) was used with an IMPAX robot (Douglas Instruments) to screen the proteins. All proteins were screened in the presence and absence of 1 m*M* novobiocin. A sparse-matrix crystallization screen based on the original described by Jancarik & Kim (1991) was used; typically, 1 µl of protein at 12–15 mg ml^{-1} was mixed with 1 µl of screen solution under a layer of 6 ml silicone and paraffin oil mixture (Hampton Research). Screens were stored at room temperature and observations were made one day after set up and at weekly periods thereafter. All X-ray data were measured using Cu $K\alpha$ radiation from a Nonius FR591 rotating-anode generator equipped with a Supper mirror system, recorded on MAR Research image-plate area-detector system and evaluated with *XDS* (Kabsch, 1988).

3. Results and discussion

The 24 kDa fragment of DNA gyrase from *S. aureus* was expressed in *E. coli* and purified. Crystallization trials with the purified enzyme have only been partially successful and it was only possible to grow crystals in the presence of the ligand cyclothialidine (Table 1). The structure was solved by molecular replacement and could be refined

to 3 Å resolution only after applying four-fold electron-density averaging techniques (D. Kostrewa, unpublished results). Analysis of the X-ray structure of the *Sa*GyrB(1–234)–cyclothialidine binary complex as well as comparison with the *E. coli* p24–novobiocin complex revealed two regions for which there was no electron density. In the *E. coli* complex, electron density is lacking for the N-terminal 13 amino acids and the loop comprising residues 99–118 is disordered (Lewis *et al.*, 1996). In the NgyrB–ADPNP complex, this glycine-rich loop forms a lid over the ATP-binding site, but in the p24–novobiocin complex it is not involved in ligand interactions. These regions correspond to residues 1–23 and 109–127 of the *S. aureus* enzyme (Brockbank & Barth, 1993). We hoped that deletion of the disordered and highly flexible regions would be of potential aid to structural studies; thus, we systematically removed these elements by deletion mutagenesis. Based on modelling studies, residues 105–127 were deleted from the loop region as we felt that this would be the shortest possible linkage between the elements of secondary structure. The proteins were expressed in *E. coli*, purified to homogeneity and subsequently tested for enzyme activity on the basis of their ability to bind novobiocin.

In the initial crystallization trials, only mutants with the deletion of the loop region corresponding to residues 105–127 crystallized readily. These conditions were optimized to reproducibly grow single crystals for X-ray analysis; the final conditions were 3 µl of protein at 12–15 mg ml^{-1} and 3 µl of 25% PEG 3350 (Hampton Research), 200 m*M* $MgCl_2$, 100 m*M* HEPES pH 7.5 in the microbatch system. The crystals belong to the space group *C*2 and diffract isotropically to approximately 2 Å resolution (Fig. 1). The crystal structure of the deletion mutant was determined and will be described in detail elsewhere (D. Kostrewa, manuscript in preparation). The N-terminal 23 amino-acids region had little effect on the crystallization properties of the protein, but seems to have a minor effect on protein stability (Fig. 2). Similar crystallization improvements to those described in our study have been reported previously. The ligand-binding domains of the ionotropic glutamate receptor (iGluR2) and the bacterial aspartate receptor have been studied in this context (Chen *et al.*, 1998; Yeh *et al.*, 1996). In the case of iGluR2, crystallization was only feasible after a process involving limited proteolysis followed by deletion mutagenesis, resulting in crystals diffracting beyond 1.5 Å resolution. For the bacterial aspartate receptor, an initial structure of the ligand-binding domain was used as a model for deletion mutagenesis. The authors removed the regions which were disordered in the initial electron-density map, which improved the resolution of the structure from 3.0 to 1.85 Å.

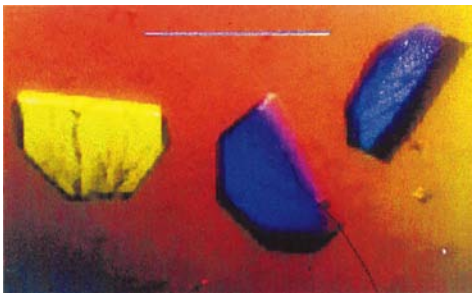

Figure 1
Crystal of *Sa*GyrB(1–234; D105–127). The bar is 500 µm long.

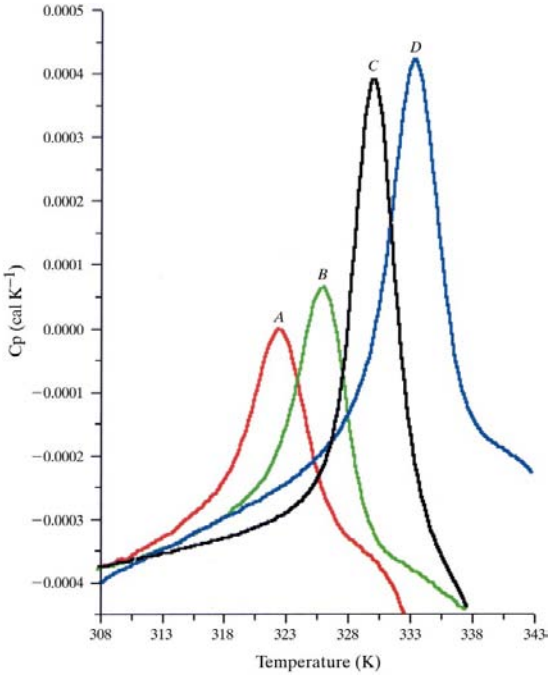

Figure 2
DSC profile of *Sa*GyrB(1–234) and deletion mutants. *A*, *Sa*GyrB(23–234); *B*, *Sa*GyrB(1–234); *C*, *Sa*GyrB(23–234; D105–127); *D*, *Sa*GyrB(1–234; D105–127). The thermal denaturation temperatures (T_d) for the proteins were 322.6, 325.9, 330.1 and 333.5 K for *A*, *B*, *C* and *D*, respectively.

Investigation by differential scanning calorimetry can be used to correlate the thermostability for the mutants with the crystallization properties. Under the experimental conditions used, thermal denaturation proved not to be reversible according to the reheating criterion; two successive scans were not superimposable. The values for T_d increased from 322.6 K to 333.5 K for the least stable [*Sa*GyrB(23–234)] to the most stable [*Sa*GyrB(1–234; D105–127)], respectively (Fig. 2). The mutant proteins which crystallized showed the highest thermostability. Deletion of the loop region encompassing residues 105–127 resulted in a 7.5 K increase in T_d, whereas the N-terminal truncation destabilized the proteins by 3.3 K (Fig. 2). Loops are typically the regions with the largest thermal factors in protein structures, indicating that they are likely to unfold first during thermal denaturation. In the four-helix bundle protein ROP, it has been shown that there is an inverse correlation between loop length and protein stability (Nagi & Regan, 1997). Moreover, it was demonstrated that several crystal properties of ROP loop mutants became systematically poorer with decreasing stability (Kokkinidis *et al.*, 1993). Thermozyme loops are often shorter than those found in their mesozyme counterparts, and it is believed that this increases the compactness of the protein and thus reduces the flexibility (Vielle & Zeikus, 1996). If such flexibility in a protein results in structural heterogeneity, this could be one reason why enzymes of thermophilic origin are found to crystallize more readily.

The conclusion that can be drawn from this and previous studies is that mutations can have a dramatic effect on the crystallization properties of proteins and that an improvement in crystal quality can generally be obtained. Deletion of flexible regions in proteins provides an additional method in crystal engineering. An important indication from the various studies is that only a limited number of alterations are required to achieve an improvement in the yield or quality of crystals.

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