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Crystal engineering: a case study using the 24 kDa fragment of the DNA gyrase B subunit from *Escherichia coli*

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Site-directed mutagenesis was used to determine the efficacy of changing surface residues to improve crystal quality. Nine mutants of the 24 kDa fragment of the *Escherichia coli* DNA gyrase B subunit were produced, changing residues on the protein's surface. The mutations changed either the charge or the polarity of the wild-type amino acid. It was found that single amino-acid changes on the surface could have a dramatic effect on the crystallization properties of the protein and generally resulted in an improvement in the number of crystal-screen hits as well as an improvement in crystal quality. It is concluded that crystal engineering is a valuable tool for protein crystallography.

1. Introduction

Knowledge-based lead identification and optimization has become an integral part of the drug-discovery process in the pharmaceutical industry. In many cases, X-ray structures can be solved in a very short period of time as a consequence of the advances made in data collection and crystallographic software. However, obtaining crystals of suitable quality remains a major stumbling block in this process.

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 (D'Arcy, 1994). The remaining 30% may require the addition of a specific ligand or modification of the protein such as limited proteolysis or deglycosylation prior to crystallization. In this context, protein engineering has become a powerful tool for influencing a protein's crystallization properties (Price & Nagai, 1995). In the past, when an enzyme from one source failed to crystallize, the homologous enzyme from another species was tried, even though these enzymes may differ by only a few amino-acid residues found on the surface of the protein. A very elegant study by Villafranca (McElroy *et al.*, 1992) demonstrated that even single amino-acid changes had a dramatic effect on the crystallization of thymidylate synthase (TS) and this work was the initial inspiration for our study.

For this study, the 24 kDa fragment of the *Escherichia coli* DNA gyrase B subunit (*Ec*GyrB) was chosen. DNA gyrase is an essential bacterial-specific type II DNA topoisomerase and has been seen as an attractive target for the design of antibiotics (Reece & Maxwell, 1991). The coumarins (*e.g.* novobiocin) are naturally occurring compounds which inhibit the ATPase activity of the B subunit. The X-ray structures of the 43 kDa N-terminal fragment–AMPNP complex and the 24 kDa N-terminal fragment–novobiocin complex of the gyrase B subunit from *E. coli* have been described (Wigley *et al.*, 1991; Lewis *et al.*, 1996). Although these structures exist, the crystallization remains a challenge and our attempts to obtain reproducible high-quality crystals of the 24 kDa fragment alone or in complex with novobiocin have proven only partially successful. In this study, we report the results of our attempts to improve the reproducibility and crystal quality by using selective site-directed mutagenesis to influence the crystallization process.

2. Materials and methods

2.1. Site-directed mutagenesis

Mutagenesis was performed directly on the plasmid expressing residues 1–220 of *Ec*GyrB, p*Ec*GyrB(1–220). The mutations were introduced using the primer extension, *Dpn*I digestion approach of Stratagenes quick-change site-directed mutagenesis kit. The oligonucleotides primer pairs used were, 5′–3′: K14E, CAGTATCAAAGTCCTGGAAGGCTGGATGCGG; K57R: CGCGGGTCACTGTAGAGAAATTATCGTACC; I82T, CCGACCGGTACTCACCCGGAAG; I82N, CCGACCGGTAATCACCCGGAAG; F104Y, CGCAGGCGGTAAATATGACGATAACTCC; L173N, GGCCCAGCAACGAAACCTTCACC and the respective complementary oligonu-

Table 1
Crystallization results of surface mutants.

Construct	Hits	Crystal morphology
Wild type	3	Needles
K14E	4	Needles
K57R	10	Needles
I82T	1	Needles
I82N	10	Needles/prisms
F104Y	14	Needles/plates/prisms
K14E/F104Y	4	Needles/rods/prisms
K57R/I82N	8	Needles
F104Y/L173N	14	Needles
I82 N/F104Y/ L173N	23	Needles

cleotides. DNA sequencing of both strands verified the mutations and the mutant proteins were expressed and purified. All DNA work employed standard techniques (Sambrook *et al.*, 1989)

2.2. Expression and purification of 24 kDa *Ec*GyrB and mutants

The 24 kDa *Ec*GyrB fragment and mutations were overexpressed in *E. coli* M15(pRep4) cells grown at 310 K. Protein expression was induced with isopropyl-β-D-thiogalactopyranoside and 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 Biochemicals, Mannheim, Germany). After cell disruption and centrifugation, the supernatant was separated from cell debris by centrifugation. After filtration through a 0.2 µm filter (Sterivex-GP, Millipore products), the supernatant was loaded onto a 50 ml novobiocin–Sepharose column equilibrated with buffer *A*. Proteins were eluted with buffer *A* with 8 *M* urea, 2 m*M* DTT. The protein was diluted to 150 µg ml^{-1} and extensively dialyzed against buffer *A* with 2 m*M* DTT and 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.

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 μRPC C2/C18 ST 4.6/100 column (Pharmacia).

2.4. Crystallization

A modification of the 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 screen based on the original described by Jancarik & Kim (1991) was used for all proteins. Typically, 1 µl of protein 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.

2.5. Data collection

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 a MAR Research image-plate area-detector system and evaluated with *XDS* (Kabsch, 1988) or *HKL* (Otwinowski & Minor, 1997).

3. Results and discussion

Five single, three double and one triple mutant of the *Ec*GyrB 24 kDa protein were produced by altering single amino acids at five positions on the protein surface by oligonucleotide site-directed mutagenesis. The mutations were chosen on the basis of the X-ray structure of the 43 kDa fragment, and the residues mutated were not conserved over the known sequences. Our strategy was to choose either surface-exposed hydrophobic residues and mutate them to hydrophilic residues (I82T, I82N, F104Y) or mutate surface-exposed lysine residues to residues found in homologous proteins (K14E, K57R). The mutants were all purified to homogeneity and tested for their ability to bind novobiocin as an assay for activity. Our initial aim was to establish whether the mutations had any effect on the crystallization; thus, the classification of crystal quality was subjective and did not necessarily correlate with diffraction quality. The number of hits were noted after a period of one month

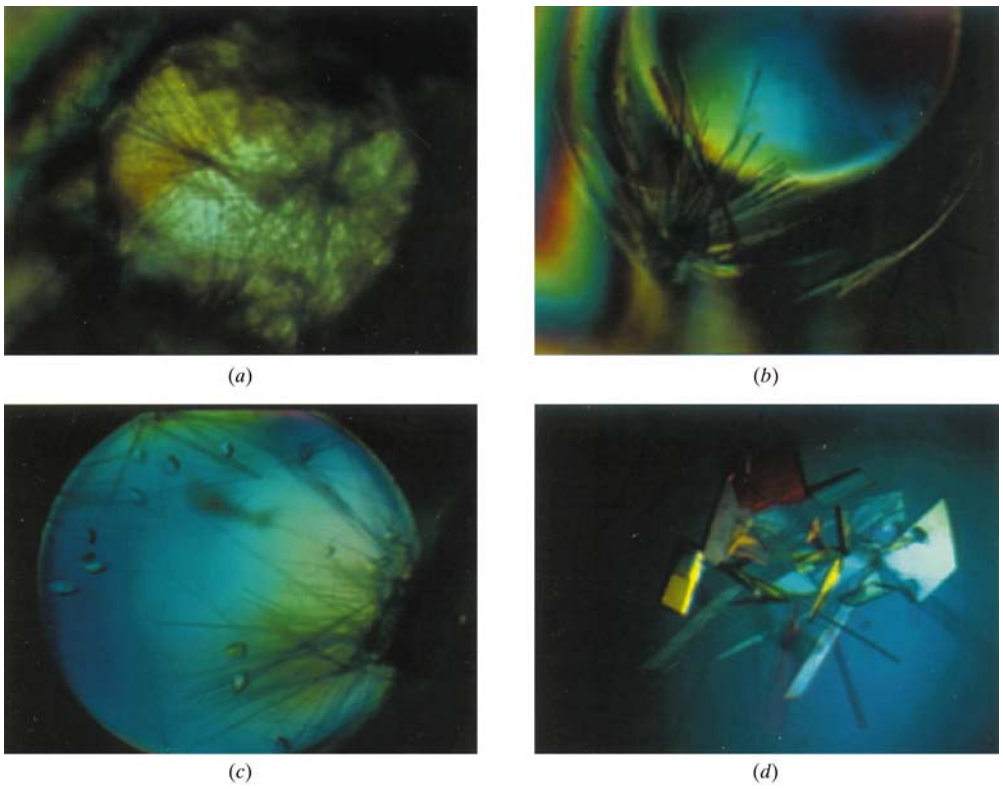

Figure 1
Crystal morphology. Crystals grown in the presence of novobiocin; (*a*)–(*c*) crystals from original microbatch, (*d*) optimized for X-ray analysis. (*a*) Wild type, (*b*) K14R/F104Y mutant, (*c*) K57R/I82N mutant, (*d*) F104Y mutant.

Table 2
Data-collection statistics.

	Wild-type data	F104Y mutant data
Space group	$P2_12_12_1$ (No. 19)	$P2_12_12_1$ (No. 19)
Data-collection temperature (K)	120	120
Data-processing program	*HKL*	*XDS*
Unit-cell dimensions: *a*, *b*, *c* (Å)	39.2, 47.3, 112.8	39.6, 47.6, 111.8
Resolution range (Å)	20.0–2.9	20.0–2.44
Number of observed reflections	20193	28631
Number of unique reflections	4625	7913
R_{sym}† overall/outer shell‡ (%)	15.8/39.8	3.5/11.2
$I/\sigma(I)$ overall/outer shell‡	8.3/2.4	36.4/15.9
Completeness overall/outer shell‡ (%)	92.1/73.6	94.8/93.0

† $R_{sym} = \sum_h \sum_i |I_i(h) - \langle I(h)\rangle| / \sum_h \sum_i I_i(h)$, where $I_i(h)$ and $\langle I(h)\rangle$ are the *i*th and mean measurement of the intensity of reflection *h*, respectively. ‡ The outer shell is 3.0–2.9 Å for wild-type data and 2.50–2.44 Å for F104Y mutant data.

for each mutant and observations made concerning the morphology of the crystals (needles, plates, rods, prisms); the results are summarized in Fig. 1. A list of the mutants and the number and type of crystals which they produced is given in Table 1. Several of the mutants gave significantly more crystals than the wild type and a number of the mutations gave crystals of better quality (Table 2). The mutant F104Y yielded five times more hits and, in addition to needles, also gave plates and prisms. Additionally, the K14E mutant produced crystals of the apo-enzyme, which to our knowledge is the first time that crystals of this protein have been obtained without an inhibitor bound. Crystals of the wild-type and mutant enzymes were predominantly obtained using polyetheylene glycol 3350 (Hampton Research) as a precipitating agent over a wide pH range and with a number of different salts. Crystals of the F104Y mutant used for X-ray analysis were grown in 25% PEG 3350, 200 m*M* NaCl, 100 m*M* Bis–Tris pH 6.5. It can be concluded from this study that single mutations can have a dramatic effect on the crystallization properties of proteins and generally result in an improvement in the number of hits as well as an improvement in crystal quality. An important indication from this study is that only a limited number of mutations are required to achieve an improvement in the yield or quality of crystals. However, the proteins have to be expressed, purified and characterized, and since the assay is crystallization, an efficient screening method is essential. Nevertheless, we believe that the results justify the time and effort involved.

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