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Preliminary X-ray crystallographic analysis of the complex between the DNAase domain of colicin E9 and its cognate immunity protein

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We have crystallized and performed preliminary X-ray characterization of the complex between the DNAase domain of the E9 colicin and its cognate immunity protein Im9. The dissociation constant for this complex, $K_d = 1 \times 10^{-16}$ *M*, reveals it to be one of the highest affinity protein–protein interactions known. Single crystals of the 1:1 complex were grown from microseeding experiments using PEG 4K as precipitant. The space group is $P2_12_12_1$ with one molecule of complex in the asymmetric unit, and crystals contain approximately 43% solvent. These crystals are inherently non-isomorphous and so selenomethionine-derivatized protein has been prepared and crystals grown for MAD phasing experiments.

1. Introduction

The colicins are plasmid-encoded antibacterial proteins produced by the *Enterobacteriacae* which are classified on the basis of the cell-surface receptor to which they bind. The E-class colicins all bind to the product of the chromosomal *btuB* gene which forms an essential component of the high-affinity transport system for vitamin B_{12} in *Escherichia coli* (DiMasi *et al.*, 1973). This class has been further subdivided into nine immunity groups (ColE1–ColE9) (Males & Stocker, 1982; Cooper & James, 1984) which exhibit three modes of cytotoxic activity: a periplasmic membrane-depolarizing (or pore-forming) agent (ColE1) (Cramer *et al.*, 1990), RNAases (ColE3, ColE5 and ColE6) (Bowman *et al.*, 1971) and DNAases (ColE2, ColE7, ColE8 and ColE9) (Schaller & Nomura, 1976; Toba *et al.*, 1988; Eaton & James, 1989; Chak *et al.*, 1991). In this paper we are concerned with E colicins of the latter group and specifically with the colicin E9.

The DNAase E colicin plasmids code for the production of an approximately 61 kDa colicin toxin, a 9.5 kDa inhibitor protein (known as an immunity protein) which protects the producing cell against the cytotoxic activity of the toxin and a 5–6 kDa lysis protein which releases the resulting colicin complex from the bacterium. Synthesis of these proteins in *E. coli* K12 strains results from DNA damage which activates an inducible promoter (SOS promoter) located proximal to the E colicin structural gene (Pugsley & Schwartz, 1983). The expressed lysis protein causes partial cell lysis upon strong induction, which is thought to be responsible for the release of the 71 kDa heterodimeric colicin complex into the extracellular medium.

The bactericidal activity of these colicins towards sensitive cells is dependent on three steps (receptor binding, translocation and cytotoxicity), each of which is associated with a specific protein domain. The cytotoxic DNAase activity of E-type colicins is associated with the 15 kDa C-terminal domain (approximately 130 amino acids) and whilst these colicins show a high degree of sequence conservation in the regions responsible for receptor binding and translocation, they are only ~80% homologous in their DNAase domains. This domain can be isolated as a stable folded protein and it is to this domain that the immunity protein (84–86 amino acids) binds forming a stoichiometric complex that neutralizes its activity (Schaller & Nomura, 1976; Wallis *et al.*, 1994). However, whilst these colicin-producing cells are naturally immune to high concentrations of their own toxin, they are sensitive to the action of other DNAase colicins from the same family. Therefore, although significantly homologous (sharing ~50% sequence homology), the immunity proteins provide varying degrees of protection towards the action of a colicin. For example, for colicin E9, the order of protection afforded by immunity proteins is seen to be Im9 >> Im2 > Im8 (no protection is given by Im7). However, this cross-reactivity is only seen when the immunity proteins are over-expressed. Thus, non-cognate immunity proteins will bind to the colicin E9 DNAase but with weaker affinities (the K_d range is from 10^{-4}–10^{-8} *M*) (Wallis, Leung *et al.*, 1995). Dissociation constants for colicin–immunity protein complexes therefore cover 12 orders of magnitude and may provide a useful source of information relating to the structural basis of the stability and specificity of protein–protein interactions.

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Whilst the structures of two pore-forming colicins, colicin A (Parker *et al.*, 1989) and colicin Ia (Wiener *et al.*, 1997), have been reported, the structures of E colicins remain unknown. However, structural data is available for some of the E colicin immunity proteins. The solution structure of the colicin E9 immunity protein Im9 has been solved by multidimensional NMR (Osborne *et al.*, 1996) and the DNAase-domain binding site has been defined through ^{1}H–^{15}N two-dimensional and three-dimensional NMR experiments (Osborne *et al.*, 1997). The structure of Im7 has also been solved by X-ray crystallography (Chak *et al.*, 1996). The structure of an intact E colicin or of any of its individual domains has yet to be reported. However, the crystallization of the RNase colicin E3 (homologous to the DNAase colicins in its receptor binding and translocation domains) in complex with its immunity protein has appeared in the literature (Frolow & Shoham, 1990; Shoham & Djebli, 1992).

The aim of this study is to define the structural basis for the recognition of immunity proteins by colicin DNAase domains. Our first target is the complex between the cytotoxic DNAase domain of colicin E9 and its cognate immunity protein Im9. The dissociation constant for this 25 kDa complex, $K_d = 1 \times 10^{-16}$ *M*, reveals it to be one of the highest affinity protein–protein interactions known (Wallis, Moore *et al.*, 1995). We have crystallized and performed preliminary X-ray analysis of the complex and have proceeded to prepare analogues of this protein modified by incorporation of selenomethionine in one or both of the protein subunits in preparation for structure solution by multiple-wavelength anomalous diffraction (MAD) phasing methods. In conjunction with future structural studies of non-cognate complexes, we hope to reveal the nature of interactions responsible for determining the affinity of protein–protein interactions in this system.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli strains JM105, BL21(DE3) and the methionine auxotroph BL21 derivative B834 (Novagen) were used. The pTrc99A (Pharmacia Biotech) derivatives pRJ352 and pRJ345 containing the genes encoding the colicin E9 DNAase–Im9 complex and Im9 alone, respectively, were as previously reported (Wallis *et al.*, 1994; Wallis, Reilly *et al.*, 1992). The pET21d derivative pRJ353 encoding the colicin E9 DNAase–Im9 complex with the Im9 protein modified by the addition of a $(His)_6$-tag at the C-terminus was used for the preparation of the uncomplexed DNAase domain (Garinot-Schneider *et al.*, 1996).

2.2. Bacterial expression

To produce native E9 DNAase–Im9 complex and free Im9 protein, *E. coli* JM105 (pRJ352) was grown in LB medium at 310 K to an optical density of 0.7–0.8 at 600 nm. Overexpression of protein was induced by addition of IPTG to a concentration of 1 m*M*. Bacterial growth was then continued for a further 5 h at 310 K before cell harvest. To produce free E9 DNAase domain the *E. coli* BL21(DE3) (pRJ353) was used. To produce selenomethionine-labelled proteins, the methionine auxotropic BL21 strain B834 that had been transformed with pRJ353 was grown in a minimal M9 medium containing 0.3 m*M* DL-selenomethionine, 1 mg ml^{-1} thiamine and supplemented with 40 mg l^{-1} of each of the common amino acids except cysteine and methionine. The control expression using DL-methionine was also carried out.

2.3. Protein purification

The purification protocols used to produce native E9 DNAase–Im9 complex (Wallis *et al.*, 1994) and the free uncomplexed components, E9 DNAase domain (Garinot-Schneider *et al.*, 1996) and Im9 (Wallis, Moore *et al.*, 1992) were as described in the respective references. Singly labelled selenomethionine complex proteins (*i.e.* with one subunit of the complex labelled but not the other) were prepared by mixing the appropriate selenomethionine-labelled free protein with its native partner in a 1:1 molar ratio and purifying the complex by gel filtration. To do this, the complexed protein was dialysed against water and concentrated by lyophilization. The freeze-dried protein was resuspended in 4 ml of 50 m*M* potassium phosphate buffer, filtered through a 0.45 µm filter and loaded onto a Sephadex G-75 (16/60) column mounted on a Pharmacia FPLC system equilibrated with the same buffer. The elution flow rate was 3 ml min^{-1}. The complex-containing fractions (as indicated by absorbance at 280 nm) were collected, dialysed against water and concentrated by lyophilization. From previous work it appears that lyophilization of the individual proteins has no effect upon the measured dissociation constant of the complex (Wallis, Moore *et al.*, 1995). The purified complex proteins were resuspended in water at a concentration of 55 mg ml^{-1} and filtered by passage through a 300 kDa NMWL filter (Ultrafree, Millipore) before crystallization experiments.

2.4. Electrospray mass spectrometry

The masses of all purified native and selenomethionine-labelled proteins were verified by mass spectrometry using a VG platform electrospray mass spectrometer calibrated with bovine heart myoglobin as previously described (Garinot-Schneider *et al.*, 1996).

2.5. Crystallization and crystal characterization

Concentrated samples of purified native colicin E9 DNAase domain–Im9 complex were crystallized by vapour diffusion at 277 K following inoculation by microseeding. For initial microseeding trials a degreased Persian cat whisker was used to streak seed from crystalline deposits (first-stage crystal) grown in hanging-drop vapour-diffusion experiments into sitting drops containing a pre-equilibrated mixture of 4 µl of protein solution and 12 µl of a solution containing 24%(*w*/*v*) PEG 4K and 0.1 *M* sodium acetate pH 5.3 (crystals, however, could be grown in solutions containing from 22–26% PEG 4K). Subsequent crystallizations were performed by preparing seed stocks by homogenization of the first-stage crystal growth in 25%(*w*/*v*) PEG 4K and 0.1 *M* sodium acetate pH 5.3, followed by a 10^{-7}-fold dilution in the same solution. Seeding was then performed by direct inoculation of pre-equilibrated sitting drops with 1 µl of the diluted seed solution. Crystals of singly or doubly selenomethionine-labelled colicin E9 complex were grown by cross-streak seeding using direct inoculation of pre-equilibrated sitting drops with native crystal seed stock.

Soaking crystals prepared from either native or selenomethionine-labelled protein in a solution containing 15%(*v*/*v*) ethylene glycol, 30%(*w*/*v*) PEG 4K and 0.1 *M* sodium acetate pH 5.3 allowed them to be flash frozen by rapid immersion in a nitrogen-gas stream at 100 K. Diffraction data were collected with a laboratory-based Rigaku R-AXIS IV area detector and Oxford Cryosystems Cryostream cryocooler on an RU200HB rotating-anode generator with focusing mirror optics run at 50 kV, 100 mA. Synchrotron data collection was performed on station 9.5 at the Daresbury Laboratory (Warrington, UK) using a 30 cm MAR imaging-plate system and on station X31 of the DESY synchrotron (Hamburg, Germany) using an 18 cm MAR imaging-

Table 1
Colicin E9 DNAase–Im9 complex X-ray diffraction data-collection statistics.

Figures in parentheses refer to data in the highest resolution bin.

	E9 DNAase–Im9†	Se-Met-E9–Se-Met-Im9‡	Se-Met-E9–Im9§
Wavelength (Å)	0.92	0.979	0.979
Temperature (K)	100	100	100
Collected at	Daresbury 9.5	DESY X31	DESY X31
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Cell parameters (Å, °)	a = 43.3, b = 52.3, c = 87.4; $\alpha = \beta = \gamma = 90$	a = 43.3, b = 52.4, c = 87.0; $\alpha = \beta = \gamma = 90$	a = 43.9, b = 52.0, c = 87.6; $\alpha = \beta = \gamma = 90$
Resolution (Å)	2.15	2.6	2.05
Completeness (%)	90.3 (92.0)	98.5 (97.9)	99.6 (99.9)
$R_{\rm merge}$ (%)	5.6 (19.6)	7.2 (22.2)	6.1 (26.3)
$\langle I\rangle/\langle\sigma I\rangle$	17.8 (6.2)	14.3 (5.2)	22.8 (6.7)
Total observations	59535	37163	105376
Independent reflections	10647	6385	13096

† Native colicin E9 DNAase domain–native Im9. ‡ Selenomethionyl colicin E9 DNAase domain–selenomethionyl Im9. § Selenomethionyl colicin E9 DNAase domain–native Im9.

plate system. Indexing of images and data processing was performed using the *HKL* program suite (Otwinowski & Minor, 1996) and the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). Heavy-atom positions were found using direct methods with the program *SHELX* (Sheldrick, 1990).

3. Results and discussion

We have overexpressed and purified the E9 DNAase domain–Im9 complex in both the native and selenomethionine derivitized forms. Bacterial overexpression of the free uncomplexed immunity protein and the E9 DNAase domain with both in either native or selenomethionine-labelled forms has allowed us to prepare samples of complex bearing the selenium label in either of the subunits of the complex. ESI–MS characterization of all purified complexed proteins revealed the expected molecular masses of the subunits and verified effectively 100% incorporation of selenomethionine into the labelled proteins (data not shown).

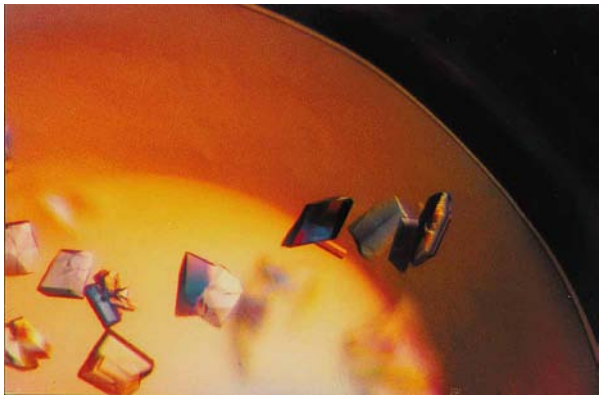

Figure 1
Crystals of the native colicin E9 DNAase–immunity protein complex.

Initial crystallization experiments using purified native colicin E9 DNAase–Im9 1:1 complex gave crystalline deposits after 3 months in hanging-drop vapour-diffusion experiments using 32%(*w*/*v*) PEG 4K as precipitant and 0.1 *M* sodium acetate, pH 5.5. Optimization of these conditions by finer screening and by different seeding methods gave single crystals. The optimal method involved seeding into sitting drops preequilibrated for 24 h at 277 K to give single crystals of typical dimensions 200 × 150 × 100 µm after 5 d (Fig. 1). Alternatively, pre-equilibrated drops could be microseeded using crushed and diluted crystal material obtained from the first-stage crystal growth. These crystals can be harvested immediately but when left for prolonged periods at 277 K grew in size to reach maximum dimensions of 490 × 200 × 150 µm after several months. Electrospray mass-spectrometric analysis of washed and dissolved crystals showed component proteins of the expected molecular masses (data not shown). The complex thus appears stable to modification during crystallization.

Diffraction trials of these crystals mounted in glass capillaries sealed with wax were carried out at room temperature. Crystals diffract to 2.7 Å or beyond at room temperature on our laboratory X-ray source. Autoindexing and consideration of systematically absent reflections reveal that the crystals belong to space group $P2_12_12_1$ with typical unit-cell dimensions a = 45.5, b = 52.4, c = 88.4 Å, $\alpha = \beta = \gamma = 90°$. With the assumption of one molecule of complex in the asymmetric unit a value of the Matthews parameter of 2.15 Å^3 Da^{-1} is obtained with a corresponding solvent content of approximately 43%. However, these native crystals displayed an inherent non-isomorphism resulting in cell parameters that vary by up to 2% in the a and c cell edges. On cooling to 100 K, the diffraction pattern extends to 2.15 Å and the cell parameters change to typical values of a = 43.3, b = 52.3, c = 87.4 Å for the $P2_12_12_1$ cell. However, the non-isomorphism observed at room temperature persists on cryocooling. We attempted to stabilize the cell parameters by increasing the concentration of precipitant in the growth conditions to a maximum PEG 4K concentration of 35%(*w*/*v*) but this proved unsuccessful. This lack of isomorphism makes it impossible to consider solving the structure of the complex by classical isomorphous replacement methods. Therefore, the introduction of an Se atom into the complex as an anomalously scattering element was attempted to allow phasing by multi-wavelength anomalous diffraction (MAD).

The colicin E9 DNAase domain and immunity protein Im9 both contain an N-terminal methionine and an additional internal methionine residue in their sequences. Correspondingly, we prepared both proteins with selenomethionine substituted for methionine. We were interested to see how the substitution of selenium for sulfur affected the growth of crystals of the complex. Crystals of the doubly labelled complex, that is the complex with selenomethionine in both subunits (Se-Met-E9–Se-Met-Im9), grew from cross-microseeding experiments and were generally comparable in dimensions to their native protein counterpart crystals. At room temperature these crystals diffracted to 3.0 Å on station 9.5 at the Daresbury Laboratory. Crystals of the singly labelled complex, that is the complex with selenomethionine in the colicin E9 DNAase domain (Se-Met-E9 DNAase–Im9), also grew from cross-microseeding experiments and diffracted at room temperature to at least 2.7 Å during trials on station 9.5 at the Daresbury Laboratory.

Using cryoprotectant conditions established for native crystals, it was attempted to flash-freeze crystals of the doubly selenomethionine-labelled complex. Whilst their native counterparts could be frozen with an almost 100% success rate, these crystals behaved very differently, with only one in ten or so of the crystals freezing to give an useable diffraction pattern. For successfully frozen crystals, using synchrotron radiation X-rays of wavelength 0.979 Å (at the sele-

nium *K* edge for these crystals), a diffraction resolution limit of about 2.6 Å could be achieved depending on crystal size. The cryoprotection of the singly labelled complex crystals was successful in that the one crystal that was tested diffracted to a resolution of 2.05 Å at the same X-ray wavelength. Statistics for the data sets collected on the various forms of the complex are presented in Table 1.

Anomalous difference Patterson maps calculated from data sets collected from both singly and doubly selenomethionine-labelled crystals were noisy. However, it was possible to identify a consistent selenium site using direct methods in the program *SHELX* (Sheldrick, 1990). We assume that this site corresponds to the non-N-terminal selenomethionine in the colicin E9 DNAase domain. As a selenium site can be identified, we will now proceed to collect multi-wavelength data using the doubly labelled crystals and attempt to calculate phases using the MAD method.

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