Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray diffraction studies of the $\varepsilon\zeta$ addiction system encoded by *Streptococcus pyogenes* plasmid pSM19035

Anton Meinhart,[a]† Claudia Alings,[a]† Norbert Sträter,[a] Ana G. Camacho,[b] Juan C. Alonso[b] and Wolfram Saenger[a]*

[a]Institut für Kristallographie, Freie Universität Berlin, Takustrasse 6, D-14195 Berlin, Germany, and [b]Departamento de Biotechnología Microbiana, Centro Nacional de Biotecnología (CSIC), Campus Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain

† These authors contributed equally to this work.

Correspondence e-mail: saenger@chemie.fu-berlin.de

Received 1 February 2001
Accepted 7 March 2001

The proteins encoded by the *Streptococcus pyogenes* broad-host range and low copy-number plasmid pSM19035 form a toxin–antitoxin module that secures stable maintenance by causing the death of plasmid-free segregants. The $\varepsilon\zeta$ protein complex was crystallized in four different forms at pH 5.0 and pH 7.0 using the vapour-diffusion method with PEG 3350 and ethylene glycol as precipitants. Three of the crystal forms were obtained in the same droplet under identical conditions at pH 5.0. One form belongs to the enantiomorphic space groups $P4_32_12$ or $P4_12_12$. For the other two, the X-ray reflection conditions match those of space group $P2_12_12_1$, one representing a superlattice of the other. A crystal form growing at pH 7.0 also belongs to space group $P2_12_12_1$, but there is no indication of a structural relationship to the other orthorhombic forms. Initially, the crystals diffracted to 2.9 Å resolution and diffracted to 1.95 Å after soaking at pH 7.0. A preparation of selenomethionyl $\varepsilon\zeta$ protein complex yielded single crystals suitable for X-ray diffraction experiments using synchrotron sources.

1. Introduction

Genetic evidence suggests that the ω, ε and ζ gene products encoded by region SegB of the broad-host range and low copy-number plasmid pSM19035 from *S. pyogenes* actively prevent plasmid loss at cell division. Plasmid mutants lacking the $\omega\varepsilon\zeta$ operon show a loss of stabilizing capacity (>1000-fold) and certain impairments to cell growth (*e.g.* longer cell-doubling time than plasmid-free cells; Ceglowski *et al.*, 1993). The ω repressor controls its own synthesis and indirectly controls that of the ε and ζ gene products (de la Hoz *et al.*, 2000). The functional mechanism of the ζ toxin is unknown, but continuous synthesis of the ε antitoxin is required to counteract ζ toxicity (reviewed in Gerdes, 2000). Both the 90-residue protein ε and the 287-residue protein ζ show no significant sequence homology to any other structurally known protein using a BLAST search (Corpet *et al.*, 1998). The ε and ζ proteins appear to form a stable complex as they could not be separated by high salt concentration (2 *M* NaCl) or by certain chaotropic agents (Camacho *et al.*, 2001). In solutions containing 50 m*M* Tris–HCl adjusted to pH 7.5 and 150 m*M* NaCl, the ε and ζ proteins form a $\varepsilon_2\zeta_2$ heterotetramer; after extensive dialysis the heterotetramer partially dissociates to a $\varepsilon_1\zeta_2$ heterotrimer (Camacho *et al.*, 2001). As we could not distinguish between $\varepsilon_1\zeta_2$ and $\varepsilon_2\zeta_2$ owing to the small molecular mass of ε, the term $\varepsilon_x\zeta_y$ is used for the complex formed by ε and ζ, unless otherwise stated.

2. Experimental and results

2.1. Materials and methods

All buffer compounds were *pro analysi* grade. Tris was purchased from Roth, Karlsruhe and all other chemical compounds from Merck, Darmstadt. The buffers used for purification and protein storage are buffer *A*, 50 m*M* Tris–HCl pH 7.5; buffer *B*, 50 m*M* Tris–HCl pH 7.5 and 500 m*M* NaCl; buffer *C*, 100 m*M* Tris–HCl pH 7.5 and 1.3 *M* $(NH_4)_2SO_4$; buffer *D*, 100 m*M* Tris–HCl pH 7.5; buffer *E*, 50 m*M* Tris–HCl pH 8.0; buffer *F*, 50 m*M* Tris–HCl pH 8.0 and 500 m*M* NaCl; SDS–PAGE gels were prepared according to standard protocols (Laemmli, 1970). All protein concentrations were determined according to Bradford (1976).

2.2. Purification

For purification of the wild-type $\varepsilon_x\zeta_y$ protein complex the protocol described by Camacho *et al.* (2001) was used with slight modifications. The paste of *Escherichia coli* BL21(DE3) cells harbouring pBT288-borne ε and ζ genes was resuspended in buffer *A* and processed using a French press. After centrifugation, the supernatant was loaded on a Heparin column equi-

librated with buffer *A* and the proteins were eluted as the $\varepsilon_x\zeta_y$ complex in a gradient of NaCl from 150 to 250 m*M* in buffer *B*. The selected fractions were adjusted to 1.3 *M* $(NH_4)_2SO_4$ and bound to a phenylether column equilibrated with buffer *C*. The $\varepsilon_x\zeta_y$ protein complex was eluted in a reverse gradient to buffer *D* at 500 m*M* $(NH_4)_2SO_4$. The pooled fractions were dialyzed against a reservoir solution containing buffer *E* and loaded onto a Q-Sepharose column equilibrated with the same buffer. The $\varepsilon_x\zeta_y$ protein complex eluted around 150 m*M* NaCl in buffer *F* using a gradient to 500 m*M* NaCl. The collected fractions appeared to be more than 99% pure according to Coomassie blue stained SDS–PAGE gels. They were dialyzed against buffer *A* and concentrated to 16 mg ml^{-1} protein. Because the $\varepsilon_2\zeta_2$ protein complex is in equilibrium with the $\varepsilon_1\zeta_2$ protein complex above 150 m*M* NaCl (Camacho *et al.*, 2001) and the difference between the molecular masses of the two complexes is small (10 721 Da), preparation of a stoichiometrally homogeneous solution proved not to be possible.

The purification procedure of selenomethionyl $\varepsilon_x\zeta_y$ protein complex was the same as for wild-type protein. The *E. coli* AM943(met$^-$) cells harbouring pBT288-borne ε and ζ genes were grown in New Minimal Medium containing selenomethionine (Budisa *et al.*, 1995). In order to prevent possible oxidation of selenomethionine, all steps were carried out in the presence of 5 m*M* DTT. 2 mg of $\varepsilon_x\zeta_y$ were obtained from 24 g bacteria paste and concentrated to 8 mg ml^{-1} protein. The storage buffer contained 50 m*M* Tris–HCl pH 7.5 and 5 m*M* DTT.

2.3. Crystallization

Crystallization experiments with the $\varepsilon_x\zeta_y$ protein complex were performed using the hanging-drop vapour-diffusion method at 291 K. In a typical screening experiment, 2 µl of the protein solution, containing about 16 mg ml^{-1} $\varepsilon_x\zeta_y$ in buffer *A*, was mixed with the same volume of reservoir solution and equilibrated against 1 ml of reservoir solution. For screening of crystallization conditions, the sparse-matrix protocol described by Jancarik & Kim (1991) was used in the form of the Crystal Screen kit (Hampton Research, Laguna Hills, CA, USA). With a reservoir solution containing 100 m*M* sodium citrate pH 5.6, 20%(*v*/*v*) 2-propanol and 20%(*w*/*v*) PEG 4000 crystallization was successful. Subsequent refinements provided better crystals when 100 m*M* sodium citrate pH 5.6 was replaced by 100 m*M* sodium acetate pH 4.6. Also, PEG between 1500 and 8000 Da could be used as precipitants and 2-methyl-2,4-pentanediol and ethylene glycol were possible additives. A solution containing equal volumes of the reservoir solution and buffer *A* showed the final pH value after mixing to be 5.0.

Since the molar ratio of ε and ζ ($\varepsilon_1\zeta_2$:$\varepsilon_2\zeta_2$) could not be controlled, possible fluctuations led to difficulties in crystallization experiments and consequently the crystallization conditions for each preparation were different. When using solutions containing 100 m*M* sodium acetate pH 4.6, 10%(*w*/*v*) PEG 6000 and 15%(*v*/*v*) 2-methyl-2,4-pentanediol for the reservoir, three different crystal forms appeared in one droplet. This occurred only for purified protein in one preparation and could not be reproduced. Nevertheless, enough single crystals with sufficient X-ray diffraction power were obtained. The final optimum conditions for the reservoir solution were 100 m*M* sodium acetate pH 4.6 and 16%(*w*/*v*) PEG 3350. Here, only crystal form 1 (see Table 1) appeared.

Problems with isomorphous heavy-atom derivatization suggested altering the pH value of the reservoir solutions. The refined crystallization conditions for the reservoir solution were 50 m*M* sodium acetate, 50 m*M* PIPES adjusted to pH 7.0, 10%(*w*/*v*) PEG 3350 and 15% ethylene glycol. The change of the pH value led to different packing of molecules and to the new crystal form 3 (see Table 1).

For the selenomethionyl $\varepsilon_x\zeta_y$ protein complex the same conditions as for the wild-type protein were chosen. Owing to the lower protein concentration (8 mg ml^{-1}) the concentration of precipitants had to be increased. After optimization of crystallization conditions, the best crystals were obtained when the reservoir solution contained 100 m*M* sodium acetate pH 4.5, 15%(*w*/*v*) PEG 1500 and 10%(*v*/*v*) 2-methyl-2,4-pentanediol.

2.4. Soaking experiments

A crystal (form 3) was soaked for 2 d in a solution containing 50 m*M* sodium acetate and 50 m*M* PIPES pH 7.0, 10%(*w*/*v*) PEG 3350, 15% ethylene glycol and 1 m*M*

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shell.

	Form 1				Form 2	Form 3	Form 4
	Native	MAD SeMet, high-energy remote	MAD SeMet, peak	MAD SeMet, inflection point	Native	$[PtCl_4]^{2-}$ derivative	Native
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P4_12_12$ or $P4_32_12$
Unit-cell parameters (Å)	a = 59.54	a = 59.54			a = 80.56	a = 50.47	a = 65.12
	b = 79.85	b = 79.67			b = 181.05	b = 143.18	b = 65.12
	c = 191.44	c = 195.21			c = 202.55	c = 153.74	c = 209.00
V_{asym} (Å^3)	113770	115749			369284	138871	55393
Beamline	X31 EMBL (Hamburg)	BM14 ESRF (Grenoble)			X11 EMBL (Hamburg)	X31 EMBL (Hamburg)	X11 EMBL (Hamburg)
Detector system	MAR Research IP	MAR Research CCD			MAR Research CCD	MAR Research IP	MAR Research CCD
Wavelength (Å)	1.01629	0.91841	0.97900	0.97931	0.9090	1.0688	1.0500
Resolution limits (Å)	20.00–1.95 (2.00–1.95)	20.0–3.1 (3.13–3.10)	20.0–3.1 (3.13–3.10)	20.0–3.1 (3.13–3.10)	55.0–3.1 (3.30–3.10)	20.0–3.0 (3.10–3.00)	30.0–3.3 (3.45–3.30)
Measured reflections	319120	55569	77845	55569	248291	202296	86962
Independent reflections	64215 (4067)	17130 (337)	17461 (406)	17457 (406)	51973 (8169)	22621 (1854)	7304 (859)
Completeness +|−† (%)	95.5 (91.8)	99.5 (99.6)	99.9 (100)	100 (100)	95.3 (91.6)	97.0 (81.0)	99.2 (97.2)
Mean (I/σ)	15.50 (5.14)	13.81 (4.60)	14.10 (5.30)	19.14 (6.83)	8.34 (1.34)	20.67 (13.10)	12.92 (1.64)
Mosaicity (°)	0.30	0.70	0.70	0.70	0.62	0.41	0.72
R_{lin}‡	0.051 (0.170)	0.070 (0.386)	0.063 (0.313)	0.074 (0.308)	0.087 (0.480)	0.080 (0.315)	0.075 (0.317)

† +|− for non-anomalous completeness. ‡ $R_{lin} = \sum(|I - \langle I\rangle I|)/\sum I$.

K_2PtCl_4. Although absorbance scans did not show a significant anomalous signal, the wavelength was adjusted to the absorption maximum of free $[PtCl_4]^{2-}$ ions in solution. After anomalous scaling using *SCALEPACK* (Otwinowski & Minor, 1997), the χ^2 values were significantly lower compared with non-anomalous scaling. This implies that a derivative may have been obtained. Whereas neither difference Patterson maps nor anomalous Patterson maps showed interpretable Harker peaks, an anomalous difference Fourier map based on model phases, which were obtained subsequently, confirmed binding of Pt to the protein. Cryoconditions could not be established for this crystal form.

When crystals grown at pH 5.0 were soaked for 45 min in cryosolutions containing 50 m*M* sodium acetate, 50 m*M* PIPES pH 7.0, 17%(*w*/*v*) PEG 1500 and 10%(*v*/*v*) 2-methyl-2,4-pentanediol, X-ray diffraction improved considerably. Compared with a resolution of 3.3 Å without soaking, the crystals now diffracted to 2.5 Å resolution or better.

2.5. X-ray diffraction studies

Crystals of form 1 were soaked as described above and crystals of forms 2 and 4 were soaked in cryobuffer containing 100 m*M* sodium acetate pH 4.5, 22%(*w*/*v*) PEG 3350 and 15%(*v*/*v*) ethylene glycol. They were flash-frozen in liquid nitrogen and exposed to X-rays in a nitrogen stream at 100 K. As no cryoconditions could be found for crystals of form 3, they were sealed in glass capillaries with small amounts of mother liquor and investigated at room temperature. X-ray diffraction data were collected on different beamlines (Table 1). Reflections were indexed and integrated using *DENZO* and scaled using *SCALEPACK* (Otwinowski & Minor, 1997). Crystal symmetry belongs either to the orthorhombic (forms 1, 2 and 3) or to the tetragonal (form 4) lattice system. The systematic extinctions of the diffraction data are compatible with space group $P2_12_12_1$ for forms 1, 2 and 3 and with $P4_12_12$ or the enantiomorphic space group $P4_32_12$ for form 4. Transformation of the lattice parameters of crystal form 2 using the matrix

$$\begin{pmatrix} 0 & 1/3 & 0 \\ 1 & 0 & 0 \\ 0 & 0 & 1 \end{pmatrix}$$

leads to converted unit-cell parameters similar to form 1. Comparison of the reciprocal plane (*hk*0) of form 1 and the transformed plane (*kh*0) of form 2 reveals that the latter possesses weak interlattice layers of multiplicity 3. This suggests that the reciprocal space of form 1 represents a superlattice of the reciprocal space of form 2 and, therefore, form 2 might be a superstructure of form 1. For forms 3 and 4, no such relationship could be found, although the lattice parameters *c* of forms 1, 2 and 4 are within the variance (±8 Å) observed for different crystals of each individual form.

As heavy-atom derivatization experiments were not successful, MAD data were collected on selenomethionylated crystals (see Table 1). Because the anomalous signal vanished at higher than 4 Å resolution, for structure solution a cutoff at 4 Å was applied to the data. A total of 13 selenium sites per asymmetric unit could be identified using *SOLVE* (Terwilliger & Berendzen, 1999) which delivered sufficient phasing information for interpretable electron-density maps. As in both proteins the N-terminal methionine is cleaved off (Camacho *et al.*, 2001), the ζ protein comprises six selenomethionines and the ε protein one selenomethionine. Closer inspection of the selenium sites suggested the presence of a twofold noncrystallographic axis in the asymmetric unit. A fourteenth selenium site could be identified in an anomalous difference Fourier map. This indicated the stochiometry $\varepsilon_2\zeta_2$ in form 1.

For determination of the protein and solvent content per asymmetric unit, the uncertainty in the exact stoichiometry of the crystallized $\varepsilon_x\zeta_y$ protein complex is the major obstacle. In form 4, the volume of the asymmetric unit suggests that the crystallographic twofold axis coincides with a symmetry axis of the complex. Consequently, the stoichiometry must be $\varepsilon_1\zeta_1$ per asymmetric unit and the solvent content 47% (1.28 Å^3 Da^{-1}). In form 1, the solvent content is 48% (1.32 Å^3 Da^{-1}) for one $\varepsilon_2\zeta_2$ molecule per asymmetric unit and in form 3 it is 57% (1.61 Å^3 Da^{-1}) ($\varepsilon_2\zeta_2$). For form 2 a decision on the stoichiometry could not be made as the solvent content would be 52% (1.43 Å^3 Da^{-1}) for $\varepsilon_6\zeta_6$ or 58% (1.64 Å^3 Da^{-1}) for $\varepsilon_3\zeta_6$. To illuminate all these features, structure refinement of forms 1 and 3 and structure determination of forms 2 and 4 is in progress.

We thank Paul Tucker at the EMBL Outstation at DESY (Hamburg), Vivian Stojanoff at the ESRF (Grenoble) and Peter Orth for help in data collection. These studies were partially supported by the Deutsche Forschungsgemeinschaft (Sa196/38-1) and Fonds der Chemischen Industrie to WS and by the Ministerio de Ciencia y Tecnología (BMC2000-0548) to JCA.

References

Bradford, M. (1976). *Anal. Biochem.* **72**, 248–254.
Budisa, N., Steipe, B., Demange, P., Eckerskorn, C., Kellermann, J. & Huber, R. (1995). *Eur. J. Biochem.* **230**, 788–796.
Camacho, A. G., Misselwitz, R., Ayora, S., Welfle, K., Meinhart, A., Lara, B., Lurz, R., Saenger, W., Welfle, H. & Alonso, J. C. (2001). Submitted.
Ceglowski, P., Boitsov, A., Chai, S. & Alonso, J. C. (1993). *Gene*, **136**, 1–12.
Corpet, F., Gouzy, J. & Kahn, D. (1998). *Nucleic Acids Res.* **26**, 323–326.
Gerdes, K. (2000). *J. Bacteriol.* **182**, 561–572.
Hoz, A. B. de la, Ayora, S., Sitkiewicz, I., Fernández, S., Pankiewicz, R., Alonso, J. C. & Ceglowski, P. (2000). *Proc. Natl Acad. Sci. USA*, **97**, 728–733.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–410.
Laemmli, U. K. (1970). *Nature (London)*, **227**, 680–685.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Terwilliger, T. C. & Berendzen, J. (1999). *Acta Cryst.* D**55**, 849–861.