Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray data investigation of the bacterial enterocin A immunity protein at 1.65 Å resolution

Bjørn Dalhus,[a]* Line Johnsen[b] and Jon Nissen-Meyer[b]

[a]Department of Chemistry, University of Oslo, PO Box 1033, Blindern, N-0315 Oslo, Norway, and [b]Department of Biochemistry, University of Oslo, PO Box 1041, Blindern, N-0316 Oslo, Norway

Correspondence e-mail: bjornda@kjemi.uio.no

Received 21 March 2003
Accepted 6 May 2003

Crystals of the bacterial enterocin A immunity protein have been prepared by the hanging-drop vapour-diffusion technique at 293 K. The crystals diffract to better than 1.7 Å resolution and X-ray diffraction data to 1.65 Å have been collected at 110 K using synchrotron radiation. The enterocin A immunity protein crystals belong to the monoclinic crystal system, with unit-cell parameters a = 116.32, b = 42.35, c = 66.17 Å, β = 111.3°. The symmetry and systematic absences in the diffraction pattern are consistent with space group $C2$. The presence of two molecules in the asymmetric unit with a molecular weight of ∼12.2 kDa gives a crystal volume per protein mass (V_M) of ∼3.1 Å^3 Da^{-1} and a solvent content of ∼60% by volume.

1. Introduction

Many Gram-positive bacteria produce ribosomally synthesized antimicrobial peptides (AMPs), often termed bacteriocins. A very dominant and important group of these AMPs consists of the pediocin-like AMPs produced by lactic acid bacteria (Nes *et al.*, 2002; Ennahar *et al.*, 2000; Nissen-Meyer *et al.*, 1997). These AMPs contain between 37 and 48 residues and have very similar primary structures: they are cationic, display anti-listeria activity and kill target cells by permeabilizing the cell membrane (Moll *et al.*, 1999; Chikindas *et al.*, 1993). At least 20 pediocin-like AMPs have been characterized (Nes *et al.*, 2002; Ennahar *et al.*, 2000; Nissen-Meyer *et al.*, 1997; Fimland *et al.*, 2002*a*) and the three-dimensional structures of some of these have been analyzed by NMR and mutagenesis (Fimland *et al.*, 2000, 2002*a*; Gallagher *et al.*, 1997; Wang *et al.*, 1999; Uteng *et al.*, submitted).

Genes encoding pediocin-like AMPs are usually co-transcribed with a gene encoding a cognate immunity protein which protects the AMP producer from their own AMP (Axelsson & Holck, 1995; Dayem *et al.*, 1996; Hüne *et al.*, 1996; Quadri *et al.*, 1995, 1997; Venema *et al.*, 1995). These immunity proteins consist of 88–115 amino-acid residues and display 5–85% sequence similarity (Eijsink *et al.*, 1998). The immunity proteins show a remarkably high degree of specificity with respect to the AMP they recognize, although some immunity proteins may recognize and render cells immune to a few other pediocin-like AMPs in addition to their cognate AMP (Fimland *et al.*, 2002*b*). The mode of action of the immunity proteins and the basis of their specificity are unknown and insight into their three-dimensional structure will be useful for elucidating these aspects. In this communication, we report the purification to homogeneity and crystallization of the enterocin A immunity protein (entA-im), a 103-residue cationic protein that confers immunity to the pediocin-like AMPs enterocin A, sakacin P and leucocin A (Fimland *et al.*, 2002*b*).

2. Experimental

2.1. Protein expression and purification

The protein-purification system IMPACT–CN (New England Biolabs) was used for expression and purification of the enterocin A immunity protein. The *Escherichia coli* DH5α strain was used for preparation of plasmids and cloning and *E. coli* 2566 (New England Biolabs) was used for expression of the fusion protein.

2.1.1. Construction of the expression plasmid. PCR fragments containing the enterocin A immunity gene were cloned into the vector pTYB11 (New England Biolabs). Specific primers for PCR amplification were synthesized. The forward primer was entFsap, GGTGGTTGCTCTTCCAACATGAAAAA-AAATGCTAAGC, and the reverse primer was entR, AGACTGCAGCATTAAAATT-GAGATTTATCTCCATAAT. The primers were constructed to contain the restriction sites *Sap*I and *Pst*I, respectively. After double digestion with *Sap*I and *Pst*I and purification from an agarose gel, the amplification product was ligated into the vector pTYB11 (New England Biolabs) cut by the same restriction enzymes. This construct allowed the fusion of the target protein N-terminus to the intein tag and the protein was obtained without any vector-derived residues.

2.1.2. Expression and purification of enterocin A immunity protein. The enterocin A immunity protein was overexpressed and purified by use of the IMPACT–CN system according to the protocol of New England Biolabs, with some modifications. *E. coli* strain ER2566 transformed with pTYB11/entA-im was grown in LB containing 100 µg ml^{-1} ampicillin at 310 K to an OD_{600} of 0.6–0.8. ITPG was added to a final concentration of 0.5 m*M* and induction was conducted at 289 K overnight. The cells were harvested by centrifugation (5000*g* for 10 min), resuspended in column buffer (20 m*M* Tris–HCl at pH 8, 500 m*M* NaCl, 1 m*M* EDTA) containing 0.1% Triton X-100 and broken by sonication. The clarified cell extract obtained by centrifugation was directly applied onto a chitin column pre-equilibrated with ten volumes of column buffer. The column was then washed with ten volumes of column buffer. Induction of on-column cleavage was conducted by quickly flushing the column with three volumes of column buffer containing 50 m*M* DTT. The column was left at 291 K for 40 h before elution of the target protein in three volumes of column buffer.

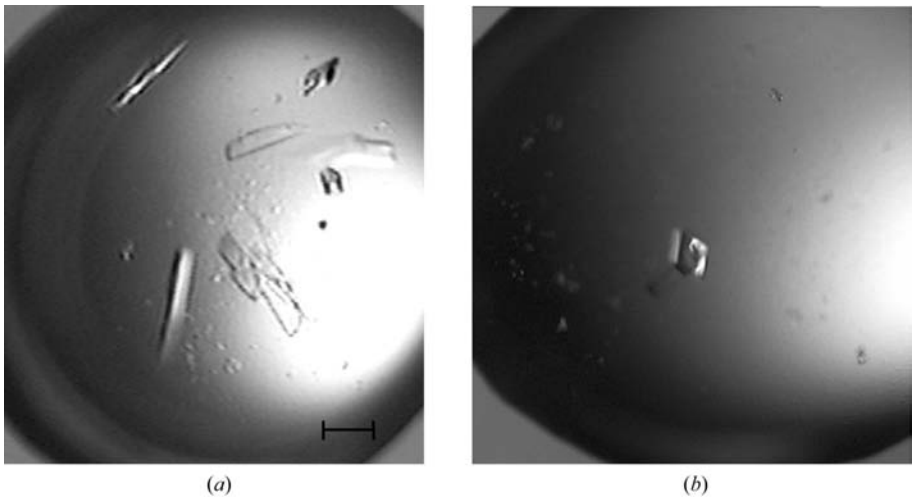
The protein was concentrated by using a Centricon centrifugal filter device (molecular-weight cutoff 3000 Da; Millipore) and further purified by chromatography on a μRPC SC 2.1/10 C_2C_{18} column (Amersham Biosciences) using the SMART chromatography system (Amersham Biosciences) and water/2-propanol containing 0.1% trifluoroacetic acid as the mobile phase. The primary structure was confirmed by mass spectrometry on a Voyager-DE RP matrix-assisted laser desorption time-of-flight mass spectrometer (Perseptive Biosystems); 3,5-dimethoxy-4-hydroxycinnamic acid was used as the matrix.

2.2. CD spectroscopy

Circular dichroism (CD) spectra were recorded using a Jasco J-810 spectropolarimeter (Jasco International Co. Ltd, Tokyo, Japan) calibrated with ammonium D-camphor-10-sulfonate (Icatayama Chemicals, Tokyo, Japan). Measurements were performed at 296 K using a quartz cuvette (Starna, Essex, England) with a path length of 0.1 cm. All measurements were performed with a protein concentration of 0.1 mg l^{-1} in 10 m*M* potassium phosphate buffer pH 6.5. Samples were scanned five times at 20 nm min^{-1} with a band width of 1 nm and a response time of 1 s over the wavelength range 190–260 nm. The data were averaged and the spectrum of a protein-free control sample was subtracted. The spectra were smoothed (means movement, convolution width 5) and all measurements were conducted at least twice.

2.3. Crystallization and X-ray data collection

Crystallization was performed by the hanging-drop vapour-diffusion technique at 298 K using 24-well tissue-culture plates. The experiments were carried out by mixing 1 µl protein solution with an equal volume of reservoir solution and allowing the drop to equilibrate against 500 µl reservoir solution. The protein concentration was approximately 5 mg ml^{-1}. Initial crystallization conditions were explored using Crystal Screen from Hampton Research (Jancarik & Kim, 1991). However, no condition producing crystalline protein was detected at first, although several droplets with PEGs with molecular weight in the range 4000–8000 showed phase separation. Therefore, we turned to the PEG/Ion Screen from Hampton Research in order to explore the effect of various ionic additives. This strategy turned out to be successful: relatively large plate-shaped crystals with quite rough surfaces were obtained in a few instances, particularly when tartrate or citrate anions were present in the precipitant solution. Optimization of the most promising experiments resulted in conditions producing a few plate-shaped crystals per droplet. In general, these crystals were large in one dimension (in some instances more than 0.5 mm long) but generally less than 50 µm thick (Fig. 1*a*). In a few instances, block-shaped crystals, which were somewhat thicker than the plate-shaped crystals, were also obtained (Fig. 1*b*). These crystals were formed using 15–25% PEG 3350 as the main precipitant agent together with 200 m*M* diammonium hydrogen citrate. Diffraction data were collected under liquid-nitrogen cryoconditions at 110 K. To avoid damage on freezing, crystals were transferred and soaked for about 10 s in a cryoprotectant solution prepared by mixing three parts of the precipitant solution with one part pure PEG 400. Crystals were flash-cooled by rapidly moving them into the cold nitrogen stream. Native X-ray diffraction data were collected at station BM01A at the Swiss–Norwegian Beamline (SNBL) belonging to the ESRF synchrotron in Grenoble, France. The station is equipped with a MAR 345 image-plate detector. The sample temperature during data collection was maintained at 110 K. All data were indexed, integrated and scaled using the *DENZO*/*SCALEPACK* program package (Otwinowski & Minor, 1997).



(*a*) (*b*)

Figure 1
(*a*) Plate-shaped and (*b*) block-shaped crystals of the enterocin A immunity protein. The scale bar is approximately 0.2 mm long.

3. Results and discussion

The plate-shaped crystal selected for mounting on the MAR 345 diffracted to at least 2.0 Å (Fig. 2). The unit-cell parameters were determined to be a = 116.32, b = 42.35, c = 66.17 Å, β = 111.3°. Analysis of the data shows that the crystal belongs to the monoclinic crystal system and has a *C*-centered crystal lattice. The molecular mass of the enterocin A immunity protein is about 12.2 kDa, which gives a crystal volume per protein mass (Matthews coefficient; Matthews, 1968) V_M = 3.1 Å^3 Da^{-1} and a solvent content of about 60% by volume, assuming two molecules in the asymmetric unit. Scaling and merging of the obtained

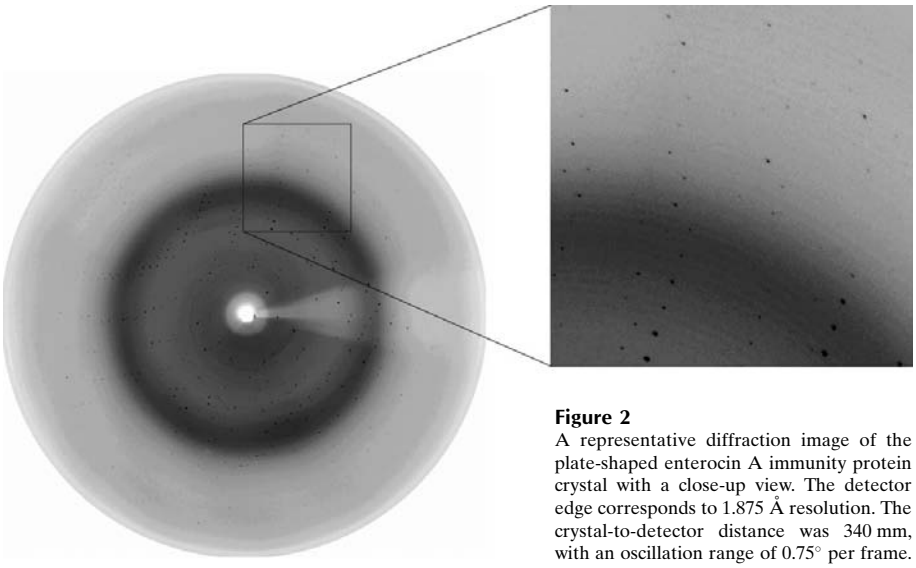


Figure 2
A representative diffraction image of the plate-shaped enterocin A immunity protein crystal with a close-up view. The detector edge corresponds to 1.875 Å resolution. The crystal-to-detector distance was 340 mm, with an oscillation range of 0.75° per frame.

Table 1
Data-collection and processing statistics for enterocin A immunity protein.

Values in parentheses refer to the outermost resolution shell (1.71–1.65 Å).

Data collection	
Beamline	BM01A, Swiss–Norwegian Beamline, ESRF
Detector	MAR 345
Wavelength (Å)	0.8727
No. of frames†	264/218
Oscillation per frame† (°)	0.75/0.50
Crystal-to-detector distance† (mm)	340/275
Temperature (K)	110
Crystal data	
Approximate crystal size (mm)	0.05 × 0.1 × 0.3 (plate-shaped crystal)
Unit-cell parameters (Å, °)	a = 116.32, b = 42.35, c = 66.17, β = 111.3
Space group	$C2$
Matthews coefficient‡ (Å^3 Da^{-1})	3.1
Solvent content‡ (%)	60
Processing statistics	
Resolution (Å)	1.65
Total No. of reflections	143745
Unique reflections	35815
Redundancy	4.0
Average $I/\sigma(I)$	18.7 (2.2)
$R_{\rm merge}$§ (Laue group 2/m)	0.086 (0.203)
Data completeness (%)	97.9 (87.6)

† Data for the plate-shaped and the block-shaped crystal, respectively. ‡ Assuming two monomers in the asymmetric unit, each with MW ≃ 12.2 kDa. § $R_{\rm merge} = \sum|I_i - \langle I\rangle|/\sum I_i$, where I_i is the intensity of the ith reflection.

data in space group $C2$ resulted in an overall $R_{\rm merge}$ of 0.051 and an $R_{\rm merge}$ of 0.387 for the highest resolution shell (1.97–1.90 Å). The block-shaped crystal (Fig. 1*b*) was also mounted on the beamline and this particular crystal diffracted to better than 1.7 Å resolution. Unfortunately, numerous Bragg peaks had irregular profiles or were split into a few separate components, especially at low resolution (>∼3.5Å). Thus, the 1.90 Å data set from the plate-shaped crystal was scaled and merged together with all data between 3.0 and 1.65 Å from the block-shaped crystal, making up a full data set for the range 25–1.65 Å. This data set is of good quality: $R_{\rm merge}$ is 0.086 and 0.203 for the full data range and the outer resolution shell (1.71–1.65 Å), respectively. Complete data-collection and crystallographic statistics are summarized in Table 1.

The relative content of different secondary-structure elements has been determined using CD spectroscopy. The best fit between the experimental CD data and the model is obtained when an α-helical content of about 50% is assumed. The phasing of the present X-ray data is not possible by molecular replacement using other structures as a search model, as there is probably no other known structure that resembles the folding in the enterocin A immunity protein. Hence, work is in progress to produce selenomethionine-labelled enterocin A immunity protein in order to solve the phase problem by the multiple-wavelength anomalous dispersion technique.

The authors are grateful to Dimitris Mantzilas, Department of Biochemistry (UiO) for collecting the CD spectrum of the enterocin A immunity protein. We would also like to thank Dr Jon Are Beukes at BM01A (Swiss-Norwegian Beamline) at the ESRF for assistance during data collection. This work was supported by the Norwegian Research Council (project No. 138493/410 to BD and project No. 138404/432 to LJ as well as travel grants from the SYGOR Project).

References

Axelsson, L. & Holck, A. (1995). *J. Bacteriol.* **177**, 2125–2137.
Chikindas, M. L., Garcia-Garcera, M. J., Driessen, A. J. M., Ledeboer, A. M., Nissen-Meyer, J., Nes, I. F., Abee, T., Konings, W. N. & Venema, G. (1993). *Appl. Environ. Microbiol.* **59**, 3577–3584.
Dayem, M. A., Fleury, Y., Devilliers, G., Chaboisseau, E., Girard, R., Nicolas, P. & Delfor, A. (1996). *FEMS Microbiol. Lett.* **138**, 251–259.
Eijsink, V. G. H., Skeie, M., Middelhoven, H., Brurberg, M. B. & Nes, I. F. (1998). *Appl. Environ. Microbiol.* **64**, 3275–3281.
Ennahar, S., Sashihara, T., Sonomoto, K. & Ishizaki, A. (2000). *FEMS Microbiol. Rev.* **24**, 85–106.
Fimland, G., Eijsink, V. G. H. & Nissen-Meyer, J. (2002*a*). *Biochemistry*, **41**, 9508–9515.
Fimland, G., Eijsink, V. G. H. & Nissen-Meyer, J. (2002*b*). *Microbiology*, **148**, 3661–3670.
Fimland, G., Johnsen, L., Axelsson, L., Brurberg, M. B., Nes, I. F., Eijsink, V. G. H. & Nissen-Meyer, J. (2000). *J. Bacteriol.* **182**, 2643–2648.
Gallagher, N. L. F., Sailer, M., Niemczura, W. P., Nakashima, T. T., Stiles, M. E. & Vederas, J. C. (1997). *Biochemistry*, **36**, 15062–15072.
Hüne, K., Axelsson, L., Holck, A. & Kröckel, L. (1996). *Microbiology*, **142**, 1437–1448.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Moll, G. N., Konings, W. N. & Driessen, A. J. M. (1999). *Antonie van Leeuwenhoek*, **76**, 185–198.
Nes, I. F., Holo, H., Fimland, G., Hauge, H. H. & Nissen-Meyer, J. (2002). In *Peptide Antibiotics: Discovery, Modes of Action and Application*, edited by C. J. Dutton, M. A. Haxell, H. A. I. McArthur & R. G. Wax. New York: Marcel Dekker.
Nissen-Meyer, J., Hauge, H. H., Fimland, G., Eijsink, V. G. H. & Nes, I. F. (1997). *Recent. Res. Devel. Microbiol.* **1**, 141–154.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Quadri, L. E. N., Kleerebezem, M., Kuipers, O. P., de Vos, W. M., Roy, K. L., Vederas, J. C. & Stiles, M. E. (1997). *J. Bacteriol.* **179**, 6163–6171.
Quadri, L. E. N., Sailer, M., Roy, K. L., Vederas, J. C. & Stiles, M. E. (1995). *J. Bacteriol.* **177**, 1144–1151.
Venema, K., Kok, J., Marugg, J. D., Toonen, M. Y., Lederboer, A. M., Venema, G. & Chikindas, M. L. (1995). *Mol. Microbiol.* **17**, 515–522.
Wang, Y., Henz, M. E., Gallagher, N. L. F., Chai, S., Gibbs, A. C., Liang, Z. Y., Stiels, M. E., Wishart, D. S. & Vederas, J. C. (1999). *Biochemistry*, **38**, 15438–15447.