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Full-length nisin immunity protein NisI from *Lactococcus lactis* in a lipid-free form: crystallization and X-ray analysis

Jin Hee Jeong and Sung Chul Ha*

Beamline Department, Pohang Accelerator Laboratory, Pohang University of Science and Technology, 80 Jigok-ro 127 beon-gil, Pohang, Gyeongbuk 37673, Republic of Korea. *Correspondence e-mail: scha2@postech.ac.kr

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NisI is a lantibiotic-binding lipoprotein that is specific for nisin. Nisin-producing microorganisms use NisI as an immunity protein for self-protection against nisin. Here, the purification, crystallization and preliminary X-ray diffraction of full-length NisI from *Lactobacillus lactis* in a lipid-free form ($NisI_{22\text{-}C}$) are reported. Importantly, reductive methylation of the lysine residues in $NisI_{22\text{-}C}$ was essential for initial crystallization. Only methylated $NisI_{22\text{-}C}$ crystallized. The optimized crystals of methylated $NisI_{22\text{-}C}$ were grown in 30–40 m*M* ammonium sulfate, 0.1 *M* sodium acetate pH 4.6, 16–18% PEG 4000 at 295 K and diffracted to 1.9 Å resolution. The crystal belonged to space group $P2_12_12_1$, with unit-cell parameters a = 45.99, b = 76.67, c = 76.39 Å, $\alpha = \beta = \gamma = 90.0^\circ$. Assuming the presence of one molecule in the asymmetric unit, the estimated Matthews coefficient (V_M) is 2.58 Å^3 Da^{-1} and the estimated solvent content is 52.3%.

1. Introduction

Lantibiotics are lanthionine- or methyllanthionine-containing polypeptides that are produced by Gram-positive bacteria. Lanthionine and methyllanthionine are formed from an inactive precursor polypeptide by the dehydration of serine and threonine, respectively, followed by cyclization with adjacent cysteine residues, and this modification requires specific enzymes. When only the dehydration reaction occurs for serine or threonine without the cyclization step, serine and threonine remain as dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively. The fully modified lantibiotics are secreted after transportation to the extracellular side of the cytoplasmic membrane and cleavage of the leader sequence, resulting in the active forms of the lantibiotics (Willey & van der Donk, 2007). Because of the antimicrobial activity of lanthionine- and methyllanthionine-containing polypeptides, these polypeptides are named 'lantibiotics' (Schnell *et al.*, 1988).

Nisin, the first lantibiotic to be identified, is produced by Gram-positive bacteria such as *Lactococcus lactis* and *Streptococcus uberis* (Rogers & Whittier, 1928; Wirawan *et al.*, 2006). Nisin is produced by the following sequential steps: post-translational modification of an inactive NisA precursor (Kaletta & Entian, 1989), transportation to the extracellular side of the cytoplasmic membrane by NisT (Qiao & Saris, 1996) and cleavage of a leader sequence containing 21 amino acids by the protease NisP (van der Meer *et al.*, 1993). For the formation of lanthionine or methyllanthionine, NisB and NisC are involved in the enzymatic dehydration and cyclization reactions (Karakas Sen *et al.*, 1999; Koponen *et al.*, 2002). The secreted active nisin has five lanthionine- or methyllanthionine-based rings, which are designated rings A, B, C, D and E. Nisin

exhibits antimicrobial activity by forming lipid II-mediated pores (Ruhr & Sahl, 1985; Brötz *et al.*, 1998). In addition, nisin inhibits or kills bacteria by impairing the continuous synthesis of peptidoglycan by complex formation with lipid II (Hasper *et al.*, 2006).

The immune system for nisin, which protects nisin-producing bacteria from nisin, has been identified. In nisin-producing *L. lactis*, NisFEG, the integral ABC transporter, and NisI, the nisin-binding lipoprotein on the extracellular side of the cytoplasmic membrane, are involved (Kuipers *et al.*, 1993; Engelke *et al.*, 1994; Siegers & Entian, 1995). It has been shown that NisFEG can eliminate nisin from the cytoplasmic membrane by pumping nisin out from the membrane, and that NisI can remove nisin by forming a complex (Stein *et al.*, 2003). NisI is a lipoprotein and, as a preprotein, consists of 245 amino acids. NisI is attached to the extracellular side of the cytoplasmic membrane by the modification of cysteine residues in a lipobox (16-GLSGCY-21) with a diacylglycerol moiety (Kuipers *et al.*, 1993; Qiao *et al.*, 1995). NisI also exists as a lipid-free form (LFNisI) in the medium (Koponen *et al.*, 2004). Although the mechanism of NisI immunity remains unknown, it is believed that NisI functions as an immunity protein by removing nisin molecules through the formation of a complex (Stein *et al.*, 2003; Takala *et al.*, 2004; Hacker *et al.*, 2015). It has been shown that the C-terminal fragment consisting of 21 amino acids is very important for the immunity characteristics of NisI (Takala & Saris, 2006). Recently, NMR structures of the isolated N- and C-terminal domains of NisI showed that NisI consists of two structurally similar domains that are similar to SpaI, a LanI immunity protein for subtilin in *Bacillus subtilis* (Hacker *et al.*, 2015). However, the structure of full-length NisI is not available. Therefore, how the two structurally similar domains are organized into one polypeptide is unknown. Accordingly, in order to gain insight into the organization of the N-terminal and C-terminal SpaI folds and to understand the underlying molecular mechanism of the immunity function of NisI against nisin, the structure of full-length NisI is necessary. In this study, we have expressed, purified and crystallized full-length NisI in a lipid-free form.

2. Materials and methods

2.1. Macromolecule production

The *nisI* gene encoding $NisI_{22\text{-}C}$ (residues 22–245) without the N-terminal signal peptide containing the leader sequence was amplified by a general PCR protocol with *Pfu* DNA polymerase and genomic DNA from *L. lactis* subsp. *lactis* using 5′-CGATACCATATGCAAACAAGTCATAAAAAG-GTGAGG-3′ as the forward primer and 5′-GGAATTCACT-CGAGGTTTCCTACCTTCGTTGCAAGC-3′ as the reverse primer. The resulting PCR product was cloned into a modified pET-28a (pSKB3) vector with a His_6 tag at the N-terminus and a TEV cleavage site using NdeI and EcoRI restriction-enzyme sites. The protein was expressed in *Escherichia coli* BL21 (DE3) Star cells (Invitrogen). When the OD_{600} of cells grown at 310 K in LB medium reached approximately 1.0, the cultures were induced with a final concentration of 0.2 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG) at 293 K for 15 h and harvested by centrifugation at 5000*g* at 277 K. The cell pellet was resuspended in buffer *A* (20 m*M* Tris–HCl pH 8.0, 0.1 m*M* EDTA, 2 m*M* *β*-mercaptoethanol) and lysed by sonication. The lysate was adjusted to 0.1 *M* NaCl and 30 m*M* imidazole and centrifuged at 15 000*g* for 30 min. The supernatant was loaded onto a 5 ml HisTrap FF Crude column (GE Healthcare) equilibrated with buffer *B* (20 m*M* Tris–HCl pH 8.0, 0.2 *M* NaCl, 30 m*M* imidazole). The bound protein was eluted using a linear gradient of 30–500 m*M* imidazole. The eluate was dialyzed with buffer *C* (10 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl, 2 m*M* *β*-mercaptoethanol) overnight. The N-terminal His_6 tag was removed by overnight treatment with TEV protease at 277 K. The target protein and TEV protease were used in a molar ratio of 50:1. The sample was then applied onto a 5 ml HisTrap FF Crude column (GE Healthcare) equilibrated with buffer *B* and the flowthrough fractions were collected. Finally, the protein was further purified using a Superdex 200 pg 26/600 column (GE Healthcare) equilibrated with 10 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl, 2 m*M* *β*-mercaptoethanol. The purity of the protein was checked by SDS–PAGE and the protein concentration was measured spectroscopically using an extinction coefficient of 28 880 M^{-1} cm^{-1} at 280 nm, which was calculated using *ProtParam* on the ExPASy website (http://web.expasy.org/protparam). Macromolecule-production information is summarized in Table 1. Reductive methylation of $NisI_{22\text{-}C}$, which contains 22 lysine residues, at a concentration of approximately 1.8 mg ml^{-1} in a buffer consisting of 20 m*M* HEPES pH 7.4, 0.1 *M* NaCl was performed using a protocol described elsewhere (Walter *et al.*, 2006). Briefly, 20 µl 1 *M* dimethylamine–borane complex (ABC) and 40 µl 1 *M* formaldehyde were added per millilitre of protein solution and incubated at 277 K for 2 h. The same amounts of 1 *M* ABC and 1 *M* formaldehyde were then added. After 2 h of incubation at 277 K, 10 µl 1 *M* ABC per millilitre of protein solution was added and incubated at 277 K overnight. To quench the methylation reaction, 1 *M* Tris–HCl pH 8.0 was added to give a concentration of 100 m*M*. Finally, the sample was further purified using a Superdex 200 pg 26/600 column (GE Healthcare) equilibrated with 10 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl, 2 m*M* *β*-mercaptoethanol.

Table 1
Macromolecule-production information.

Source organism	*L. lactis* subsp. *lactis* ATCC 11454
DNA source	Genomic DNA
Forward primer†	5′-CGATACCATATGCAAACAAGTCATAAAAAGG-TGAGG-3′
Reverse primer†	5′-GGAATTCACTCGAGGTTTCCTACCTTCGTTG-CAAGC-3′
Cloning vector	pSKB3 (modified pET-28a)
Expression vector	pSKB3 (modified pET-28a)
Expression host	*E. coli* BL21 (DE3) Star
Complete amino-acid sequence of the construct produced‡	MGSSHHHHHHDYDIPTTENLYFQGHMQTSHKKVR-FDEGSYTNFIYDNKSYFVTDKEIPQENVNNSK-VKFYKLLIVDMKSEKLLSSSNKNSVTLVLNNI-YEASDKSLCMGINDRYYKILPESDKGAVKALR-LQNFDVTSDISDDNFVIDKNDSRKIDYMGNIY-SISDTTVSDEELGEYQDVLAEVRVFDSVSGKS-IPRSEWGRIDKDGSNSKQSRTEWDYGEIHSIR-GKSLTEAFAVEINDDFKLATKVGNLE

Table 2
Crystallization.

Method	Hanging-drop vapour diffusion
Plate type	24-well
Temperature (K)	295
Protein concentration (mg ml^{-1})	20
Buffer composition of protein solution	10 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl, 2 m*M* β-mercaptoethanol
Composition of reservoir solution	30–40 m*M* ammonium sulfate, 0.1 *M* sodium acetate pH 4.6, 16–18% PEG 4000
Volume and ratio of drop	0.8 µl, 1:1
Volume of reservoir (µl)	500

2.2. Crystallization

NisI$_{22\text{-}C}$ was crystallized using the sitting-drop vapour-diffusion method in 96-well MRC crystallization plates (Molecular Dimensions). Initial crystallization conditions were screened using the sparse-matrix method (Jancarik & Kim, 1991) using commercially available crystallization solutions such as Crystal Screen, Crystal Screen 2, Index and SaltRX (Hampton Research, California, USA) and Wizard Classic 1 and 2 (Rigaku Reagents, Washington, USA). Two protein concentrations, 25 and 50 mg ml^{-1}, were tested for native NisI$_{22\text{-}C}$. For methylated NisI$_{22\text{-}C}$, samples at a concentration of 45 mg ml^{-1} were initially tested. The samples (0.8 µl) and an equal volume of crystallization reagent were dispensed manually and the drops were equilibrated against 70 µl reservoir solution at 295 K. Optimized crystals of methylated NisI$_{22\text{-}C}$ were obtained in crystallization solution consisting of 30–40 m*M* ammonium sulfate, 0.1 *M* sodium acetate pH 4.6, 16–18% PEG 4000 in a VDX plate (Hampton Research) at a protein concentration of 20 mg ml^{-1} at 295 K and grew to dimensions of 0.3 × 0.05 × 0.05 mm within 3–4 d. Crystallization information is summarized in Table 2.

2.3. Data collection and processing

A single crystal was prepared by breaking up a cluster of crystals using a micro-needle tool. The resulting single crystal was transferred into cryobuffer (30–40 m*M* ammonium sulfate, 0.1 *M* sodium acetate pH 4.6, 16–18% PEG 4000, 25% glycerol) for 3–5 s and flash-cooled directly in a nitrogen stream at 100 K. An X-ray diffraction data set consisting of 360 frames was collected from the single crystal using an ADSC Quantum 270 CCD detector on beamline 7A at Pohang Accelerator Laboratory (PAL, Republic of Korea) using 1° oscillations at a wavelength of 1.0000 Å. The data were indexed and scaled with the *HKL*-2000 software package (Otwinowski & Minor, 1997). The crystallographic data statistics are presented in Table 3.

3. Results and discussion

A lipid-free from of NisI (NisI$_{22\text{-}C}$; residues 22–245) was successfully expressed in a soluble form in *E. coli* with an N-terminal TEV-cleavable His$_6$ tag. NisI$_{22\text{-}C}$ was sufficiently purified for crystallization using the following steps: immobilized metal-affinity chromatography (IMAC), cleavage of the

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	7A, PAL
Wavelength (Å)	1.0000
Temperature (K)	100
Detector	ADSC Quantum 270 CCD
Crystal-to-detector distance (mm)	200
Rotation range per image (°)	1
Total rotation range (°)	360
Exposure time per image (s)	1
Space group	$P2_12_12_1$
a, *b*, *c* (Å)	45.99, 76.67, 76.39
α, β, γ (°)	90.00, 90.00, 90.00
Mosaicity (°)	0.862
Resolution range (Å)	30–1.9 (1.93–1.90)
Total No. of reflections	292919
No. of unique reflections	21763 (1032)
Completeness (%)	98.9 (96.6)
Multiplicity	13.5 (13.0)
$\langle I/\sigma(I)\rangle$	58.2 (12.2)
$R_{\rm r.i.m.}$	0.019 (0.085)
$CC_{1/2}$	0.982
Overall *B* factor from Wilson plot (Å^2)	17.9

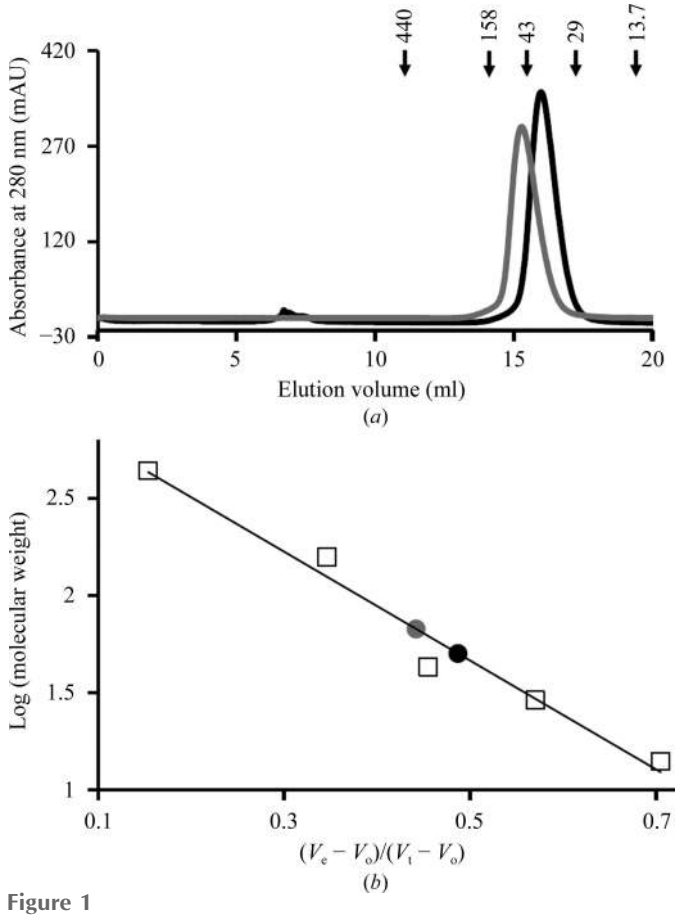

Figure 1
(*a*) Analytical size-exclusion chromatography of native NisI$_{22\text{-}C}$ (black) and methylated NisI$_{22\text{-}C}$ (grey). The molecular weights of known standards are marked on the elution profile in kDa. (*b*) Standard curve obtained using proteins of known molecular weight. The positions of the proteins of known molecular weight (ferritin, 440 kDa; aldolase, 158 kDa; ovalbumin, 44 kDa; carbonic anhydrase, 29 kDa; ribonuclease A, 13.7 kDa) are marked with open black rectangles. The positions of native NisI$_{22\text{-}C}$ and methylated NisI$_{22\text{-}C}$ are marked with black and grey dots, respectively.

N-terminal His_6 tag using TEV protease and size-exclusion chromatography. The final yield of $NisI_{22\text{-}C}$ was ~50 mg per litre of cell culture. $NisI_{22\text{-}C}$ eluted as a monodisperse form with an elution volume that corresponded to a molecular weight of ~50 kDa using an analytical size-exclusion column calibrated with proteins of known molecular weight, suggesting that $NisI_{22\text{-}C}$ may exist as a multimer such as a dimer. However, the presence of one molecule in the asymmetric unit as estimated from the Matthews coefficient (V_M; see below) suggests that $NisI_{22\text{-}C}$ may exist as a monomer but may elute earlier than the expected elution volume corresponding to the size of a monomer (Fig. 1*a*).

The initial crystallization experiments were attempted using the sitting-drop vapour-diffusion method at a concentration of 25 mg ml^{-1} at 295 K, but most of the crystallization drops were clear (Fig. 2*a*). Accordingly, further crystallization trials were performed with the protein solution at a concentration of 50 mg ml^{-1} at 295 K to create conditions that facilitate precipitation. However, the crystallization of $NisI_{22\text{-}C}$ was unsuccessful. As a salvage step for crystallization, reductive methylation of the lysine residues of $NisI_{22\text{-}C}$ was performed to obtain methylated $NisI_{22\text{-}C}$ (Walter *et al.*, 2006). The methylation step during purification had no profound effect on the final yield, so that ~40 mg of methylated $NisI_{22\text{-}C}$ per litre of cell culture could routinely be obtained. Moreover, methylated $NisI_{22\text{-}C}$ also eluted from the analytical size-exclusion column at a similar position, suggesting that methylated $NisI_{22\text{-}C}$ has similar solution properties to native $NisI_{22\text{-}C}$ (Fig. 1).

Interestingly, crystallization trials using methylated $NisI_{22\text{-}C}$ at a concentration of 45 mg ml^{-1} resulted in crystals from several conditions, including No. 20 (0.2 *M* ammonium sulfate, 0.1 *M* sodium acetate pH 4.6, 20% PEG 4000) of Crystal Screen from Hampton Research (Fig. 2*a*). Finally, crystals that were suitable for X-ray diffraction experiments were obtained using 30–40 m*M* ammonium sulfate, 0.1 *M* sodium acetate pH 4.6, 16–18% PEG 4000 with a protein concentration of 20 mg ml^{-1} (Fig. 2*b*). A recent NMR study showed that NisI consists of structurally similar N- and C-terminal domains, and that the loop linking the two domains in full-length NisI is very flexible, suggesting the possibility of the crystallization of only the N-terminal or C-terminal domain after proteolysis of the loop (Hacker *et al.*, 2015). Therefore, the sample obtained after dissolving several crystals in 20 m*M* Tris pH 8.0, 1 m*M* EDTA was analyzed by SDS–PAGE. The sample migrated at a similar position to that of methylated $NisI_{22\text{-}C}$, corroborating that full-length methylated $NisI_{22\text{-}C}$ was crystallized (Fig. 2*c*).

The crystals were flash-cooled directly in a stream of liquid nitrogen at 100 K after soaking for 3–5 s in a cryobuffer consisting of the optimized crystallization condition supplemented with glycerol at a final concentration of 25%. The crystals diffracted to a resolution of 1.9 Å using synchrotron radiation on beamline 7A at PAL (Fig. 3). The crystal belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 45.99, b = 76.67, c = 76.39$ Å, $\alpha = \beta = \gamma = 90.0°$. Assuming the presence of one molecule in the asymmetric unit, the estimated Matthews coefficient (V_M) was 2.58 Å^3 Da^{-1} with an estimated solvent content of 52.3%.

Phasing using the molecular-replacement method was performed with the isolated N-terminal (residues 9–96; PDB entry 2n32) and C-terminal (residues 119–224; PDB entry 2n2e) domains (Hacker *et al.*, 2015) as search models using *Phaser* in the *PHENIX* package (McCoy *et al.*, 2007; Adams *et al.*, 2010). The two isolated domains were searched for

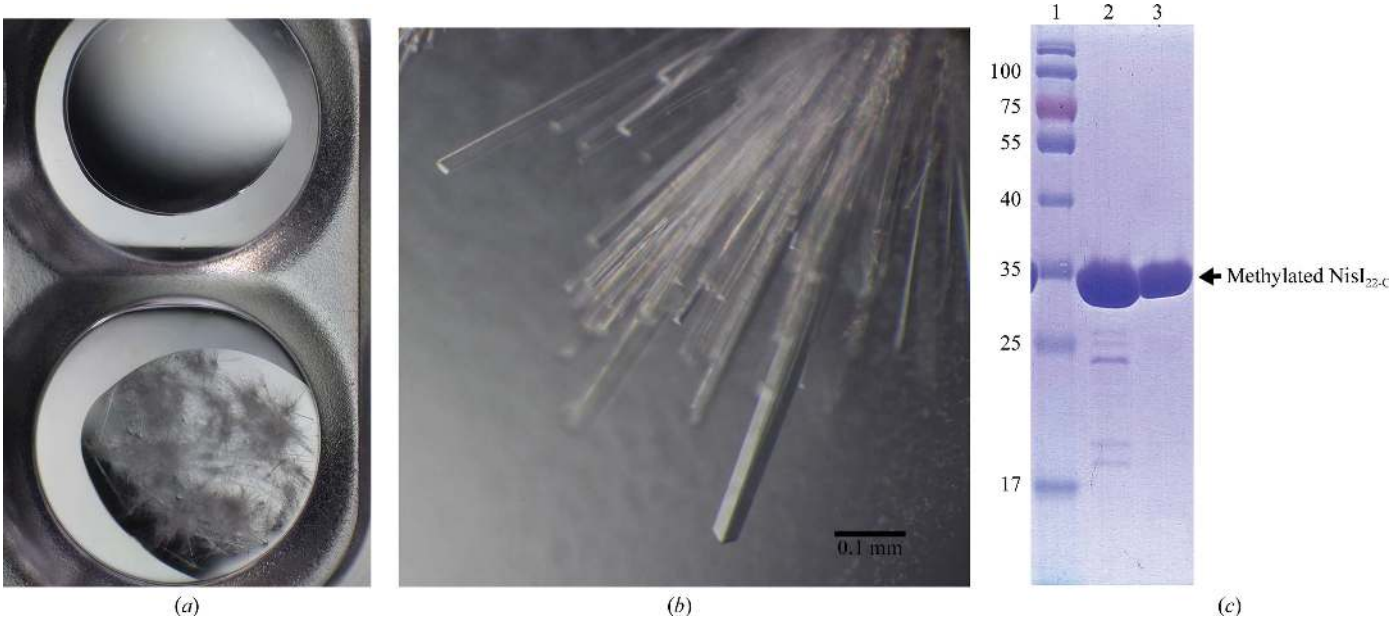

Figure 2
(*a*) An example of the initial crystallization in a 96-well plate using native $NisI_{22\text{-}C}$ (top) and methylated $NisI_{22\text{-}C}$ (bottom). (*b*) Optimized crystals of methylated $NisI_{22\text{-}C}$ crystallized in 30–40 m*M* ammonium sulfate, 0.1 *M* sodium acetate pH 4.6, 16–18% PEG 4000. The maximum dimensions are 0.04 × 0.04 × 0.5 mm. (*c*) SDS–PAGE analysis of crystals of methylated $NisI_{22\text{-}C}$ (lane 1, standard molecular weights, labelled in kDa; lane 2, sample used for crystallization; lane 3, sample obtained by dissolving several optimized crystals).

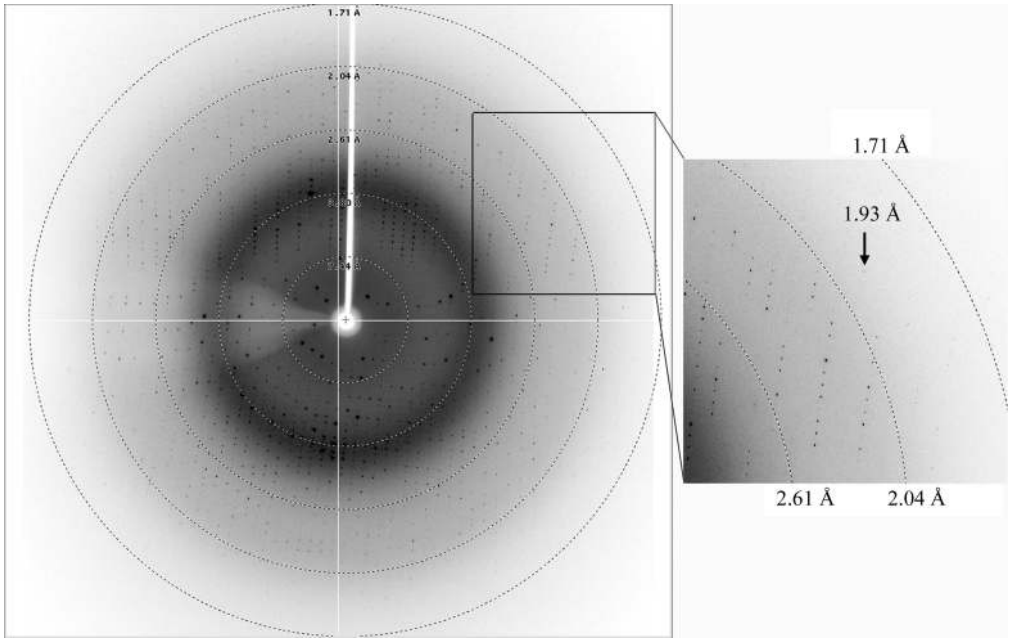

Figure 3
Diffraction image of a crystal of methylated $NisI_{22\text{-}C}$. A diffraction spot at 1.93 Å resolution is visible.

sequentially in the asymmetric unit using default parameters. *Phaser* found one promising solution with a *Z*-score of 7.8, which is considered to be a probable solution. The first round of refinement using the *PHENIX* package produced a model with an R_{work} of 39.8% and an R_{free} of 45.2% (Adams *et al.*, 2010). Further model building and refinement of the structure are still in progress. Structure determination using *de novo* phasing methods with selenium-labelled crystals is also still in progress to unequivocally locate the structurally similar N-terminal and C-terminal domains.

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