Crystallographic analysis of NosA, which catalyzes terminal amide formation in the biosynthesis of nosiheptide

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Nosiheptide is a member of the thiopeptide family of antibiotics which demonstrates potent activities against various bacterial pathogens. The formation of its C-terminal amide is catalysed by NosA in an unusual strategy for maturing certain thiopeptides by processing precursor peptides featuring a serine extension. Here, a recombinant C-terminally truncated selenomethionine-derivatized NosA_{1–111} variant from *Streptomyces actuosus* consisting of residues 1–111, named SeMet NosA_{1–111}, was crystallized using the sitting-drop vapour-diffusion method. Diffraction data were collected to 2.40 Å resolution using synchrotron radiation. The crystals belonged to the primitive cubic space group *P*₄₁32, with unit-cell parameters *a* = *b* = *c* = 143.3 Å. Assuming the presence of three molecules in the asymmetric unit, the calculated Matthews coefficient was 3.94 Å³ Da^{−1} and the corresponding solvent content was 40.3%.

1. Introduction

Thiopeptides are a well known class of sulfur-rich, highly modified peptide antibiotics that are active against various drug-resistant bacterial pathogens. Interestingly, these antibiotics share a common ribosomal synthesis paradigm in their biosynthesis pathway, which features conserved post-translational modifications of a precursor peptide to afford a characteristic framework with a nitrogen-containing, six-membered ring which is central to multiple azoles and dehydroamino acids. Many thiopeptides, such as the bimacrocylic nosiheptide and thiostrepton, generally contain a terminal amide moiety, the formation of which, however, can proceed via completely different biosynthetic routes. During thiostrepton biosynthesis (Liao & Liu, 2011; Liao *et al.*, 2009), formation of the terminal amide involves the incorporation of an exogenous amino group arising from Gln, a precursor peptide-independent residue, by an asparagine synthetase-like protein. In contrast, the amino group of the terminal amide moiety in nosiheptide is endogenous and is derived from an extended Ser residue of the precursor peptide NosM (Yu *et al.*, 2009, 2010). Dehydration of this residue at an early stage in the nosiheptide biosynthetic pathway produces an enamide, and subsequent dealkylation requires the activity of NosA (Fig. 1), which was characterized as a new terminal amide synthase, leading to a C^α–N bond cleavage for nosiheptide maturation with release of the co-product pyruvate (Yu *et al.*, 2009, 2010). The reaction catalysed by NosA is obviously

Table 1

NosA_{1–111} production information.

Source organism	<i>S. actuosus</i> (ATCC 25421)
DNA source	Genomic DNA
Forward primer†	CTAGCTAGCATGACCGAACACCCCGCAGCA
Reverse primer‡	CCCAAGCTTCTAGCGCGACGGCGGGGCCCTCG
Cloning vector	pSL4001 (pOJ446-based <i>S. actuosus</i> genomic library 7 cosmid containing the entire nosiheptide biosynthesis gene cluster)
Expression vector	pET-28a(+)
Expression host	<i>E. coli</i> BL21(DE3)
Complete amino-acid sequence of the construct produced§	MGSSHHHHSSGLVPRGSH ₃ M ₂ A ₁ S ₀ M ₁ T ₂ E-HPAQQLYCTVVLWDLRSAAATVASLRAYLRDH-AVDAYTTVPGLRQKTWISSTGPEGEQWGAAYL-WDSPEAAAYGRPPGVSKVVELIGYRPTERRYYS-VEAATEGPAAAA

† The NheI site is underlined. ‡ The HindIII site is underlined. § This His₆ tag and thrombin site are underlined.

different from those of known endogenous C-terminal amide-forming proteins, which typically catalyse the oxidative cleavage of C-terminally Gly-extended peptides accompanied by glyoxylate production (Bradbury *et al.*, 1982; Weinig *et al.*, 2003; Kulathila *et al.*, 1999; Eipper *et al.*, 1983). The structural basis of how NosA catalyzes this reaction is still unclear.

The full-length NosA from *Streptomyces actuosus* consists of a total of 151 amino acids. In our experience, full-length NosA could not be crystallized, which is probably owing to the sequence (residues 106–151) at its C-terminus. The C-terminal sequence of NosA has the potential to form a flexible loop based on secondary-structure analysis using *PSIPRED* (<http://bioinf.cs.ucl.ac.uk/psipred/>). Thus, in order to address how NosA acts in nosiheptide biosynthesis, it was necessary to truncate NosA. We constructed pET-28a(+) plasmids containing the genes for NosA_{1–140} (*i.e.* residues 1–140), NosA_{1–130} (*i.e.* residues 1–130), NosA_{1–120} (*i.e.* residues 1–120) and NosA_{1–111} (*i.e.* residues 1–111). We overexpressed these NosA variants with different lengths in an *Escherichia coli* expression system and purified them using chromatography columns. Data on the expression, purification and biological activities of the NosA variants NosA_{1–140}, NosA_{1–130} and

NosA_{1–120} will be published elsewhere, as will the determination of the active sites Lys49 and Glu101 located in the N-terminus of NosA. Finally, we found that only the N-terminal fragment NosA_{1–111} could be easily crystallized. Here, we report its overexpression, purification, crystallization and preliminary X-ray analysis.

2. Materials and methods

2.1. Protein expression and purification

The strain (*S. actuosus* ATCC 25421), plasmids and other materials used in this study are summarized in Table 1. DNA isolation and manipulation in *E. coli* and *S. actuosus* were performed according to standard methods (Kieser *et al.*, 2001; Sambrook & Russell, 2001). To construct the plasmid expressing NosA, we amplified DNA fragments encoding NosA from genomic DNA based on previous studies (Liao & Liu, 2011). PCR amplifications were carried out using a thermal cycler (Eppendorf AG 22331, Hamburg, Germany) using *LA Taq* DNA polymerase (Takara).

NosA_{1–111} (residues 1–111) was engineered into a pET-28a(+) vector with an N-terminal His₆ tag using NheI and HindIII restriction sites and the primers listed in Table 1. The restriction enzymes used in the study were purchased from Takara Biotechnology Co. Ltd (Dalian, People's Republic of China). The construct was verified by DNA sequencing and the plasmid was transformed into *E. coli* BL21(DE3) competent cells. Primer synthesis and DNA sequencing were performed at Shanghai Invitrogen Biotech Co. Ltd, People's Republic of China. The transformed cells were grown at 37°C in Luria broth (LB) medium containing 50 µg ml^{−1} kanamycin. The cells were induced by addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when the OD₆₀₀ reached 0.5–0.6. Expression was carried out for 24 h at 18°C. The cells were harvested, resuspended in lysis buffer (50 mM phosphate pH 7.5, 500 mM NaCl) and lysed with 10 µg ml^{−1} PMSF by sonication on ice. The lysate was clarified by centrifugation (30 min, 16 000 rev min^{−1}). The soluble protein was purified

Figure 1

The nosiheptide maturation catalysed by NosA. The C^α–N bond cleavage site is highlighted in pink and by a wavy line.

Table 2 Crystallization of SeMet NosA_{1–111}.

Method	Vapour diffusion
Plate type	XtalQuest (screening and production)
Temperature (K)	291
Protein concentration (mg ml ^{−1})	50
Buffer composition of protein solution	25 mM phosphate pH 7.5, 50 mM NaCl
Composition of reservoir solution	0.02 M magnesium chloride hexahydrate, 0.1 M HEPES pH 6.0–9.0, 22% (w/v) polyacrylic acid 5100
Volume and ratio of drop	2 µl final volume, 1:1 protein:reservoir ratio
Volume of reservoir (ml)	0.5

by nickel-affinity chromatography (GE Healthcare), and the protein was eluted with a linear gradient of 0–250 mM imidazole in lysis buffer. Protein fractions were collected and dialyzed twice against lysis buffer at 4°C. To remove the His₆ tag, the fusion protein was digested with thrombin protease (Sigma–Aldrich, USA) overnight at 4°C, followed by a second round of nickel-affinity chromatography. The target NosA_{1–111} was present in the flowthrough fractions.

The fractions were collected and further purified by gel-filtration chromatography on a Superdex 75 column (GE Healthcare) with buffer consisting of 25 mM phosphate pH 7.5, 50 mM NaCl. Finally, fractions containing C-terminally truncated NosA_{1–111} were concentrated to 50 mg ml^{−1} using an ultracentrifugal filter tube (Millipore) and used for crystallization. The protein concentration was estimated from the absorbance at 280 nm using an absorption coefficient of 33 920 M^{−1} cm^{−1}.

The NosA_{1–111} protein has two Met residues in its amino-acid sequence. Thus, selenomethionine-derivatized (SeMet) NosA_{1–111} was expressed in M9 medium using the methionine-

Table 3

Data collection and processing.

Values in parentheses are for the highest resolution shell.

Diffraction source	SSRF
Wavelength (Å)	0.9794
Temperature (K)	100
Detector	MAR MX225 CCD
Crystal-to-detector distance (mm)	400
Rotation range per image (°)	1
Total rotation range (°)	360
Exposure time per image (s)	1
Space group	<i>P</i> 4 ₁ 32
<i>a</i> , <i>b</i> , <i>c</i> (Å)	143.3, 143.3, 143.3
α , β , γ (°)	90.0, 90.0, 90.0
Mosaicity (°)	0.358
Resolution range (Å)	50.0–2.4 (2.59–2.40)
Total No. of reflections	1483177
No. of unique reflections	18105
Completeness (%)	100.0 (100.0)
Multiplicity	81.9 (81.8)
Average <i>I</i> / σ (<i>I</i>)	90.0 (17.6)
<i>R</i> _{merge} † (%)	9.2 (46.4)
Solvent content (%)	40.3
No. of molecules in asymmetric unit	3
Average <i>B</i> factor (Å ²)	48.4

$$\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}.$$

pathway inhibition protocol (Khandekar *et al.*, 2000; Van Duyn *et al.*, 1993) and purified using the same procedure as used for native NosA_{1–111}. The homogeneity of the SeMet NosA_{1–111} was examined by SDS–PAGE and further identified by electrospray mass spectrometry, as shown in Fig. 2. The molecular weight of native NosA_{1–111} is 12.695 kDa including the additional Gly-Ser-His-Met sequence from thrombin protease cleavage. The measured molecular weight of SeMet NosA_{1–111} is 12.839 kDa, indicating that the Met residues in NosA_{1–111} were successfully replaced by selenomethionine

Figure 2

Identification of purified SeMet NosA_{1–111} using an SDS–PAGE gel and MALDI–TOF MS analysis. Lane 1, protein marker; lane 2, SeMet NosA_{1–111}.

and that the Se atoms in SeMet NosA_{1–111} were partially oxidized during the MALDI–TOF experiment.

2.2. Protein crystallization

Initial crystallization trials were carried out at 291 K with the Crystal Screen HT and Index HT kits (Hampton Research) in 96-well plates using the sitting-drop vapour-diffusion method. For refinement of the crystallization conditions, 1 µl protein solution was mixed with an equal volume of reservoir solution and was equilibrated against 0.5 ml reservoir solution at 291 K in 24-well plates. Crystals of SeMet NosA_{1–111} suitable for X-ray analysis (Fig. 3) were obtained under the following conditions: 0.02 M magnesium

Figure 3
Crystals of SeMet NosA_{1–111}.

Figure 4
X-ray diffraction pattern of an SeMet NosA_{1–111} crystal.

chloride hexahydrate, 0.1 M HEPES pH 6.0–9.0, 22%(w/v) polyacrylic acid 5100. Detailed information on the crystallization of SeMet NosA_{1–111} is summarized in Table 2.

2.3. Data collection and processing

The crystals were picked up in a nylon loop (Hampton Research) and mounted for flash-cooling in liquid nitrogen. X-ray diffraction data were collected on beamline BL17U at Shanghai Synchrotron Radiation Facility (SSRF), People's Republic of China using a MAR MX225 CCD detector. The wavelength of the radiation was 0.9794 Å and the distance between the crystal and the detector was 400 mm. The exposure time for each frame was 1 s with 1° oscillation, and 360 frames were collected. The data were indexed, integrated and scaled using the *HKL-2000* program suite (Otwinowski & Minor, 1997). The final crystal parameters and data-collection statistics are summarized in Table 3.

3. Results and discussion

We expressed truncated NosA_{1–111} and its selenomethione derivative and purified them to homogeneity for crystallization. The purified proteins were estimated to have a purity of >95%. Crystals appeared after three weeks and grew in a cubic form with all dimensions approximately 0.3 mm. The diffraction of the SeMet NosA_{1–111} crystals extended to 2.40 Å resolution. Fig. 4 shows an image of the X-ray diffraction data from SeMet NosA_{1–111}. The crystals belonged to the primitive cubic space group *P*4₃2, with unit-cell parameters $a = b = c = 143.3$ Å. The X-ray diffraction data set for SeMet NosA_{1–111} was processed using data in the resolution range 50.0–2.4 Å. After initial data processing, three molecules were predicted to be present in the asymmetric unit. The typical Matthews coefficient and solvent content were estimated to be 3.94 Å³ Da^{−1} and 40.3%, respectively. The three-dimensional structure of NosA_{1–111} will be determined using the single-wavelength anomalous dispersion (SAD) method using the SeMet NosA_{1–111} data set.

After we have obtained the crystal structure of NosA_{1–111}, we will be able to predict the possible binding mode of NosA to its substrate using a *DALI* search (Holm & Sander, 1993). Since the substrate cannot be dissolved in water, it is impossible to obtain crystals of NosA in complex with its substrate. Currently, we have also been unable to obtain crystals of NosA_{1–111} in complex with the C-terminal NosA_{112–151}. Thus, in order to understand how the substrate interacts with NosA, ligand docking, homology modelling and molecular-dynamics (MD) simulations of complexation between full-length NosA and its substrate will be conducted. At the same time, site-directed mutation studies based on the amino-acid sequence alignment of NosA and its homologues and an enzymatic assay on an HPLC system will be performed, which will provide information on the active sites necessary for the catalytic reaction and on whether or not the C-terminal loop NosA_{112–151} is important for the catalytic reaction. Taking all of these results together, we will be able to understand the

molecular mechanism of nosiheptide C-terminal amide formation catalyzed by NosA.

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References

- Bradbury, A. F., Finnie, M. D. & Smyth, D. G. (1982). *Nature (London)*, **298**, 686–688.
- Eipper, B. A., Mains, R. E. & Glembotski, C. C. (1983). *Proc. Natl Acad. Sci. USA*, **80**, 5144–5148.
- Holm, L. & Sander, C. (1993). *J. Mol. Biol.* **233**, 123–138.
- Khandekar, S. S., Konstantinidis, A. K., Silverman, C., Janson, C. A., McNulty, D. E., Nwagwu, S., Van Aller, G. S., Doyle, M. L., Kane, J. F., Qiu, X. & Lonsdale, J. (2000). *Biochem. Biophys. Res. Commun.* **270**, 100–107.
- Kieser, T., Bibb, M., Butter, M., Chater, K. F. & Hopwood, D. A. (2001). *Practical Streptomyces Genetics*. Norwich: The Johns Innes Foundation.
- Kulathila, R., Merkler, K. A., Merkler, D. J. & Kulathila, R. (1999). *Nat. Prod. Rep.* **16**, 145–154.
- Liao, R., Duan, L., Lei, C., Pan, H., Ding, Y., Zhang, Q., Chen, D., Shen, B., Yu, Y. & Liu, W. (2009). *Chem. Biol.* **16**, 141–147.
- Liao, R. & Liu, W. (2011). *J. Am. Chem. Soc.* **133**, 2852–2855.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual*, 3rd ed. New York: Cold Spring Harbor Laboratory Press.
- Van Duyne, G. D., Standaert, R. F., Karplus, P. A., Schreiber, S. L. & Clardy, J. (1993). *J. Mol. Biol.* **229**, 105–124.
- Weinig, S., Hecht, H.-J., Mahmud, T. & Müller, R. (2003). *Chem. Biol.* **10**, 939–952.
- Yu, Y., Duan, L., Zhang, Q., Liao, R., Ding, Y., Pan, H., Wendt-Pienkowski, E., Tang, G., Shen, B. & Liu, W. (2009). *Chem. Biol.* **4**, 855–864.
- Yu, Y., Guo, H., Zhang, Q., Duan, L., Ding, Y., Liao, R., Lei, C., Shen, B. & Liu, W. (2010). *J. Am. Chem. Soc.* **132**, 16324–16326.