Yu Hirano,^{a,†} Md. Motarab
Hossain,^{b,†} Kazuki Takeda,^{a,c}
Hajime Tokuda^b and Kunio
Miki^{a,c,*}

^aDepartment of Chemistry, Graduate School of
Science, Kyoto University, Sakyo-ku,
Kyoto 606-8502, Japan, ^bInstitute of Molecular
and Cellular Biosciences, University of Tokyo,
1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032,
Japan, and ^cRIKEN SPring-8 Center at Harima
Institute, 1-1-1 Koto, Sayo, Hyogo 679-5198,
Japan

† These authors contributed equally to this
work.

Correspondence e-mail:
miki@kuchem.kyoto-u.ac.jp

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Purification, crystallization and preliminary X-ray crystallographic analysis of the outer membrane lipoprotein NlpE from *Escherichia coli*

The outer membrane lipoprotein NlpE functions in stress response by activating the Cpx signal transduction pathway. The nonlipidated Cys1Ala mutant of NlpE with a C-terminal His tag from *Escherichia coli* was constructed, overexpressed and purified. Crystals of NlpE were grown in two distinct forms by the sitting-drop vapour-diffusion method at 298 K. The tetragonal crystals diffracted to 2.8 Å resolution and belong to space group $P4_32_12$. The monoclinic crystals diffracted to 3.0 Å resolution and belong to space group $C2$. Initial phases were obtained from a tetragonal crystal of selenomethionylated protein by the MAD method.

1. Introduction

NlpE (new lipoprotein E) from *Escherichia coli* is a 24 kDa lipoprotein possessing three acyl chains at the cysteine residue of position +1 (Snyder *et al.*, 1995). Homologous proteins are widely found in Gram-negative bacteria. The protein is anchored on the periplasmic side of the outer membrane with the acyl chains. The localization is achieved by the Lol proteins (Matsuyama *et al.*, 1995, 1997; Yakushi *et al.*, 2000) owing to the absence of the Lol avoidance signal made up of residues at positions +2 and +3 (Yamaguchi *et al.*, 1988; Terada *et al.*, 2001; Masuda *et al.*, 2002). On the outer membrane, NlpE has been reported to detect envelope stress caused by cell adhesion to hydrophobic surfaces (Otto & Silhavy, 2002). An external copper ion is also thought to be detected by NlpE (Yamamoto & Ishihama, 2005, 2006). These stresses induce the transcription of periplasmic proteins such as the periplasmic protease/chaperone DegP (Danese *et al.*, 1995), the peptidyl-prolyl isomerases PpiA (Pogliano *et al.*, 1997) and PpiD (Dartigalongue & Raina, 1998) and the disulfide oxidase DsbA (Pogliano *et al.*, 1997) through the Cpx pathway. The Cpx pathway, which is one of the two-component signal transduction pathways (Raivio *et al.*, 1999; Raffa & Raivio, 2002; Duguay & Silhavy, 2004; Ruiz & Silhavy, 2005), contains an inner membrane histidine kinase CpxA and a cytoplasmic response regulator CpxR. External stresses induce an autophosphorylation reaction at the cytoplasmic domain of CpxA. After the reaction, the phosphate is transferred from CpxA to CpxR and the phosphorylated CpxR then initiates gene transcription. In addition, a periplasmic protein CpxP has been reported to regulate Cpx signal transduction by binding to the sensor domain of CpxA (Danese & Silhavy, 1998; Raivio *et al.*, 1999, 2000; Buelow & Raivio, 2005; Isaac *et al.*, 2005). However, the molecular mechanism by which NlpE detects the envelope stresses and transmits the signals to the Cpx pathway remains unclear.

Three regions of the amino-acid sequence of NlpE from *E. coli* are responsible for the lipoprotein's function. Firstly, the sequence 31-CADC is suggested to bind divalent cations such as Zn^{2+} and Cu^{2+} or to be involved in redox regulation. Indeed, NlpE has also been identified as the copper homeostasis protein CutF (Gupta *et al.*, 1995). Secondly, the sequence 124-MTPMTRLRGMYFYM including four methionines is similar to the copper-binding motif of the copper-trafficking protein CopC, as mentioned previously (Arnesano *et al.*, 2003). Thirdly, the sequence 99-MLDREGNPIESQFNLYTL resembles the serine protease inhibitor signature that exists in the N-terminus of inhibitors (Mares *et al.*, 1989). In the crystal structure of soybean trypsin inhibitor complexed with porcine trypsin, the

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absorption spectrum. Low-energy and high-energy remote wavelengths were selected at 0.9900 and 0.9700 Å, respectively. The crystal-to-detector distance was 300 mm. Each data set consisting of 180 frames was collected with a rotation angle of 1° and an exposure time of 20 s per frame. All data were processed and scaled using the *HKL-2000* program package (Otwinowski & Minor, 1997). Pseudo-precession photographs for the determination of screw axes were displayed using *XtalView* (McRee, 1999). The program *SHELXD* was used to determine Se-atom sites (Schneider & Sheldrick, 2002). Phase calculation and subsequent density modification were performed using the program *CNS* (Brünger *et al.*, 1998). Initial electron-density maps were displayed with *XtalView*.

3. Results and discussion

Proteins suitable for crystallization were obtained by cobalt-affinity and gel-filtration chromatography (Fig. 2*a*). The molecular weight of mNlpE was determined to be 24 597 Da by MALDI-MS experiments (Fig. 2*b*), while the calculated molecular weight of mature mNlpE is 25.6 kDa. Therefore, a truncation of a further ten residues may be occurring at the N-terminus. This additional N-terminal truncation is also supported by the retention of the C-terminal His tag on examination by Western blotting analyses (data not shown). These results indicate that the signal sequence is absent in the purified sample.

Approximately 30 mg pure protein was obtained per 12 l of culture. Two crystal forms (form I and form II) were obtained for the unlabelled protein, corresponding to the presence (form I) and absence (form II) of zinc in the reservoir solution. Form I crystals grew to average dimensions of $0.4 \times 0.2 \times 0.1$ mm in one week (Fig. 3*a*) and the crystals started dissolving after one month (Fig. 3*b*). Therefore, we collected X-ray images within three weeks of crystallization setup. These crystals diffracted X-rays to a maximum of 7 Å resolution. Two cycles of annealing improved the diffraction limit from 7 Å resolution to approximately 3 Å resolution and the unit-cell parameters were reduced from $a = b = 124.9$, $c = 86.4$ Å to $a = b = 121.2$, $c = 83.9$ Å. These crystals belong to the tetragonal space group $P4_12_12$ or $P4_32_12$. Assuming the presence of two or three molecules in the asymmetric unit, the Matthews coefficient V_M was calculated to be 3.1 or $2.1 \text{ Å}^3 \text{ Da}^{-1}$, respectively. No samarium sites were found in anomalous difference Patterson maps. Form II crystals reached average dimensions of $0.2 \times 0.1 \times 0.1$ mm in one week and the crystals tended to be severely clustered (Fig. 3*c*). The obtained diffraction images were indexed and scaled to 3.0 Å resolution. Form II crystals belong to the monoclinic space group $C2$, with unit-cell parameters $a = 183.5$, $b = 47.2$, $c = 182.9$ Å, $\beta = 91.8^\circ$. Assuming the presence of 5–10 molecules in the asymmetric unit, the corresponding V_M values were calculated to be 3.4 – $1.7 \text{ Å}^3 \text{ Da}^{-1}$ (Matthews, 1968). Crystallization of the selenomethionylated protein was performed under the same conditions as for the unlabelled protein. Although form I crystals of

Figure 2

(*a*) Each purification step of mNlpE from *E. coli* was analyzed by SDS-PAGE. Lane 1, periplasmic fraction from *E. coli* MC4100 cells; lane 2, 250 mM imidazole fraction from a TALON column; lane 3, peak fractions from an HA column; lane M, molecular-weight markers (kDa). (*b*) MALDI-MS spectrum of purified mNlpE. The +1 and +2 charge states of mNlpE are indicated.

Figure 3

Crystals of the outer membrane lipoprotein mNlpE from *E. coli*. The black bars indicate 0.1 mm. (*a*) Tetragonal crystal (form I) of the unlabelled protein with approximate dimensions of $0.4 \times 0.2 \times 0.1$ mm. (*b*) Tetragonal crystals (form I) of the selenomethionylated protein incubated for one month; the crystals showed a tendency to dissolve within one month. Crystals of the unlabelled proteins also tended to dissolve within two months. (*c*) Monoclinic crystal (form II) of the unlabelled protein with approximate dimensions of $0.2 \times 0.1 \times 0.1$ mm.

Table 1
Data-collection statistics for the two crystal forms and for the form I crystal of the selenomethionyl-substituted protein.
Values in parentheses indicate statistics for the highest resolution shell.

	Unlabelled		SeMet MAD (form I)			
	Form I	Form II	High remote	Peak	Edge	Low remote
Space group	$P4_32_12$	$C2$	$P4_32_12$			
Unit-cell parameters (Å, °)	$a = 121.2, b = 121.2,$ $c = 83.9$	$a = 183.5, b = 47.2,$ $c = 182.9, \beta = 91.8$	$a = 121.6, b = 121.6, c = 84.5$			
Wavelength (Å)	1.6000	1.0000	0.9700	0.9792	0.9797	0.9900
Resolution range (Å)	40–2.8 (2.90–2.80)	50–3.0 (3.10–3.00)	50–3.1 (3.21–3.10)			
Observed reflections	119885	87996	101612	101865	99952	101191
Unique reflections	15130	29475	11615	11657	11529	11637
$R_{\text{sym}}^\dagger$ (%)	8.1 (30.6)	7.3 (24.2)	11.7 (29.3)	12.1 (30.6)	11.3 (30.2)	11.1 (30.8)
$I/\sigma(I)$	22.6 (2.4)	15.6 (2.1)	16.7 (2.8)	16.4 (2.8)	16.0 (2.8)	16.2 (2.6)
Completeness (%)	95.0 (75.3)	92.4 (67.9)	96.7 (84.0)	96.4 (83.6)	96.0 (80.3)	95.7 (79.9)

$^\dagger R_{\text{sym}} = \sum_{hkl} \sum_i |I_{hkl,i} - \langle I_{hkl} \rangle| / \sum_{hkl} \sum_i I_{hkl,i}$, where $I_{hkl,i}$ is the i th measured diffraction intensity and $\langle I_{hkl} \rangle$ is the average of the intensity. Bijvoet pairs of the SeMet data were kept separate but scaled simultaneously and R_{sym} values were calculated with the Bijvoet pairs merged.

the selenomethionylated protein were obtained, form II crystals were never observed. The annealing method was also applied to form I crystals of the selenomethionylated protein in order to improve the diffraction limit. To prevent radiation damage while collecting the MAD data sets, the four data sets were collected with shorter exposure times than for the data sets of the unlabelled protein. Each data set was indexed and scaled to 3.1 Å resolution. The crystallographic statistics for all data sets are listed in Table 1. 13 of a possible 16 selenium sites for two NlpE molecules in the asymmetric unit were found using the anomalous signals from the peak-wavelength data. The experimental phases were calculated and refined at 4.0 Å resolution. The initial electron-density map was improved by solvent flipping and phase extension was performed to 3.1 Å resolution. The electron-density map calculated in space group $P4_32_12$ indicated a clear boundary between molecules and solvent and displayed some secondary-structure elements, whereas the map calculated in the alternative enantiomorph $P4_12_12$ was not interpretable. Consequently, we unambiguously concluded that the form I crystals belong to space group $P4_32_12$. The values of the overall figure of merit before and after density modification were 0.72 and 0.96, respectively.

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