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Crystallization and X-ray diffraction analysis of a novel surface-adhesin protein: protein E from *Haemophilus influenzae*

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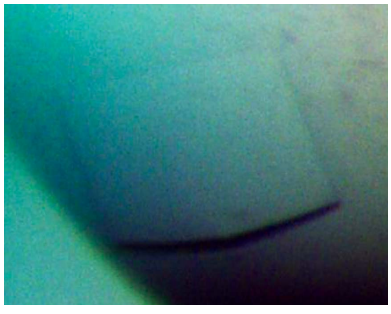
Protein E (PE) is a ubiquitous multifunctional surface protein of *Haemophilus* spp. and other bacterial pathogens of the *Pasteurellaceae* family. *H. influenzae* utilizes PE for attachment to respiratory epithelial cells. In addition, PE interacts directly with plasminogen and the extracellular matrix (ECM) components vitronectin and laminin. Vitronectin is a complement regulator that inhibits the formation of the membrane-attack complex (MAC). PE-mediated vitronectin recruitment at the *H. influenzae* surface thus inhibits MAC and protects against serum bactericidal activity. Laminin is an abundant ECM protein and is present in the basement membrane that helps in adherence of *H. influenzae* during colonization. Here, the expression, purification and crystallization of and the collection of high-resolution data for this important *H. influenzae* adhesin are reported. To solve the phase problem for PE, Met residues were introduced and an SeMet variant was expressed and crystallized. Both native and SeMet-containing PE gave plate-like crystals in space group $P2_1$, with unit-cell parameters $a = 44$, $b = 57$, $c = 61$ Å, $\beta = 96^\circ$. Diffraction data collected from native and SeMet-derivative crystals extended to resolutions of 1.8 and 2.6 Å, respectively.

1. Introduction

Haemophilus influenzae is an important respiratory pathogen that causes acute otitis media in children and exacerbates chronic, obstructive pulmonary disease (COPD; Murphy *et al.*, 2009). Adhesins are surface proteins of pathogens that are used not only for adherence to host cells but also to induce a pro-inflammatory response and thus communication between the host and the pathogen in question (Kline *et al.*, 2009). In recent years, some surface adhesins from *H. influenzae* have been suggested as vaccine candidates as they exhibit protective roles in experimental models (Murphy, 2009). These *Haemophilus* adhesins are multifunctional; in addition to their role in adhesion, some of them are transporters or transmembrane proteins or are secreted during infection of the host. There are 8–10 different surface proteins that have been identified to be adhesins (Murphy, 2009), but only the structures of *Haemophilus* surface fibril and its homologues Hia (Cotter *et al.*, 2005) and high-molecular-weight protein (HMW; Yeo *et al.*, 2007) have partially been determined. Recently, the structure of the *H. influenzae* adhesin protein HAP was revealed (Meng *et al.*, 2011). In general, structural information on adhesins has been useful in providing insights into the host–pathogen relationship.

We recently described the role of the hitherto unknown *H. influenzae* protein E (PE) in interacting with host epithelial cells and its involvement in subverting the host innate immune system. Protein E is a 16 kDa surface lipoprotein from *H. influenzae* that functions as an adhesin and induces a pro-inflammatory response during infection, leading to IL-8 secretion and up-regulation of ICAM-1 (CD54) in both cell lines and primary epithelial cells originating from patients with COPD. Interestingly, immunization of mice with the PE peptide 84–108 showed a protective role in pulmonary clearance (Ronander *et al.*, 2009). By using a detailed peptide-mapping approach, we have described several host protein-binding



regions in PE (Hallström *et al.*, 2009, 2011; Singh *et al.*, 2011). PE was found to be highly conserved among *Haemophilus* spp. and other members of the *Pasteurellaceae* family, with a sequence identity of 34.1–98.8% and a similarity of 55.3–100% (Singh *et al.*, 2010), and thus is likely to also function as an adhesin in other species of this family.

In this paper, we describe the expression of PE in *Escherichia coli* and its purification using various chromatographic steps. The conditions for crystallization were found by using commercial kits and an automated robotic system. A fine grid optimization yielded crystals of good diffracting quality. Since the natural protein does not contain methionine residues, a heavy-atom derivative search to solve the phase problem using MIR or MAD methods was started. This proved to be unsuccessful. To overcome the phasing problem, two methionine residues were introduced into the protein and the phase problem was solved using SeMet-labelled PE. Finally, data were collected to 1.8 Å resolution from native PE crystals and to 2.6 Å resolution from SeMet-labelled PE crystals. These data will be used to solve the structure of PE and will be extremely useful in elucidating the mode of interaction with host proteins.

2. Materials and methods

2.1. Selection of Met positions for phasing

Guidance for the selection of the amino acids to be substituted by methionine residues was obtained from the Dayhoff matrix as well as a putative protein fold based on prediction (Fig. 1). A secondary-structure prediction based on *JPred*3 (Cole *et al.*, 2008) and *PredictProtein* (Rost & Liu, 2003) was used to select low-penalty substitutions placed within secondary-structure elements. The presence of a Met residue in related sequences in a multi-sequence alignment by *ClustalW* was also taken into account.

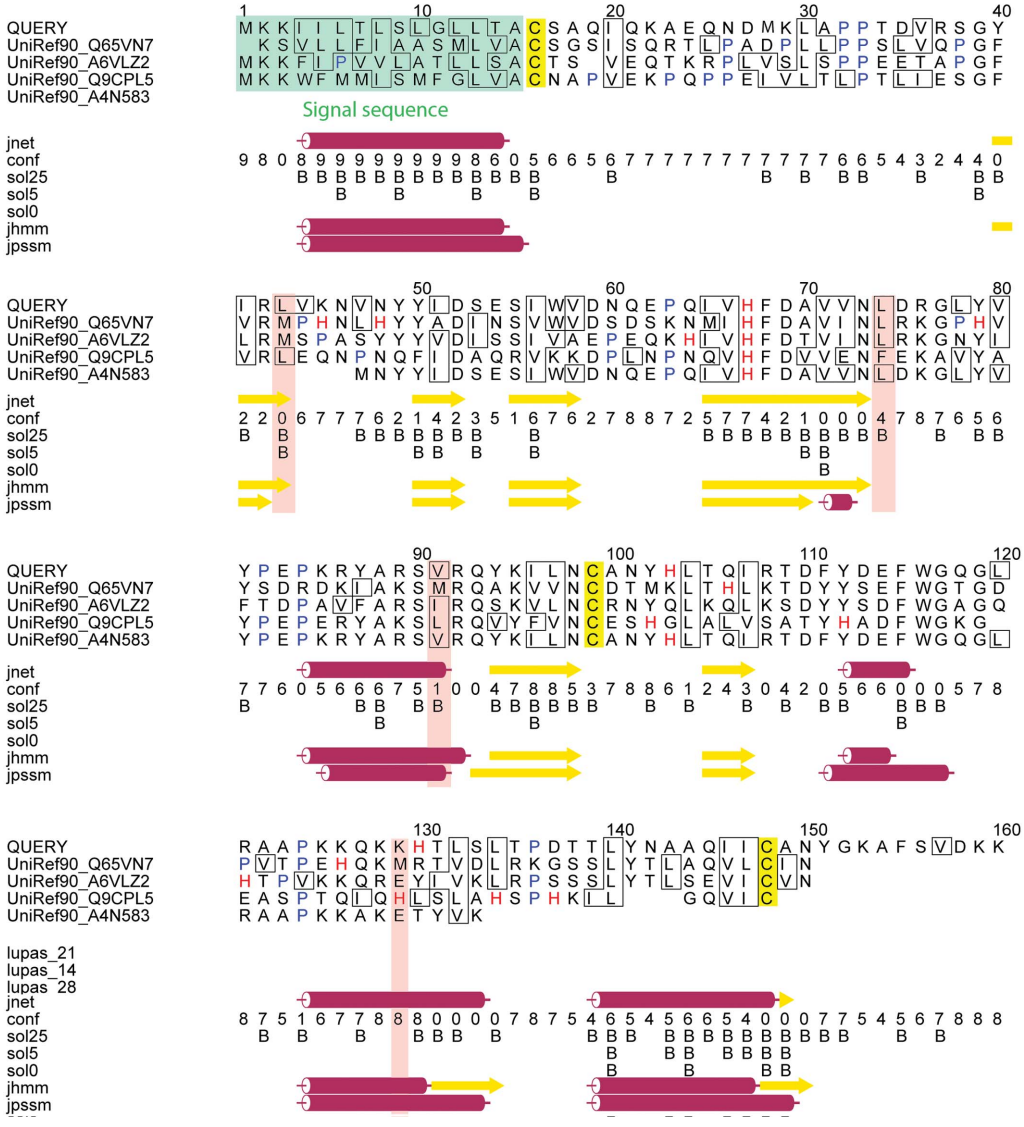


Figure 1
Plan of the site-directed mutagenesis of the PE molecule. The PE amino-acid sequence was analysed for secondary-structure-based alignment using the *JPred*3 software. PE homologues were compared to determine the availability of Met in their sequences. Possible mutations for the introduction of Met are highlighted in magenta at four positions. Leu43 is present as Met in other homologues of PE and thus was selected for mutation. Additionally, the conserved Leu74 residue was mutated to Met. In the alignment scheme, β-sheets are shown as yellow arrows and α-helices are shown as cylinders.

2.2. Construction of vectors, site-directed mutagenesis and expression of PE

The *H. influenzae* gene (HI0178) encoding PE has successfully been cloned and His-tagged PE has been expressed in our laboratory (Ronander *et al.*, 2009; Singh *et al.*, 2011). For crystallization, we produced PE without any tag. The *pe* gene encoding amino acids 17–158 (excluding the signal peptide) was amplified using PCR with the forward primer PE_For, 5′-GCCATGCATC**CATATG**TCTGCTCA-AATCCAAAAGGCTGAACAAAATGA-3′, and the reverse primer PE_Rev, 5′-CCC**GAATTC**TCAATCAACTGAAAATGCTTTACC-ATAATTTGCACA-3′ (*Nde*I and *Eco*RI restriction sites are shown in bold). The insert was ligated into pET26b that had been digested with *Nde*I and *Eco*RI followed by dephosphorylation. Finally, the sequenced and correct vector was transformed into *E. coli* BL21 (DE3). The purpose of this construct was to produce untagged PE in the cytoplasm, which resulted in the formation of inclusion bodies owing to protein misfolding. Purification of the inclusion bodies and protein refolding was performed to obtain optimally purified PE.

Residues Leu43 and Leu74 were mutated to methionine using a site-directed mutagenesis approach as described previously (Singh & Röhm, 2008; Singh *et al.*, 2011). The forward primer L43M_For, 5′-GCGGATATATACGT**A**TGGTAAAGAATGTG-3′, and the reverse primer L43M_Rev, 5′-CACATTCTTTACCA**T**ACGTATATATCC-GC-3′, were used to mutate Leu43 to Met; a subsequent second mutation was made in this template using the primers L74M_For, 5′-GCAGTGGTGAATA**T**T**G**GATAAGGGATTG-3′, and L74M_Rev, 5′-CAATCCCTTATC**C**A**T**ATTCACCACTGC-3′. The bases that were mutated are shown in bold. QuikChange site-directed mutagenesis was performed using high-fidelity *PfuTurbo* DNA polymerase (Stratagene, La Jolla, California, USA). Finally, the sequenced vector was transformed into *E. coli* 834 (DE3). For selenomethionine labelling of the $PE^{L43M,L74M}$ variant, a SelenoMet Medium Base and SelenoMet Nutrient Mix expression-media kit (Molecular Dimensions, Athena Enzyme Systems, Baltimore, Maryland, USA) was used and solutions were prepared according to the manufacturer's instructions. For labelling, a single colony of *E. coli* 834 (DE3) containing $pET26PE^{L43M,L74M}$ was grown overnight in 100 ml SelenoMet Medium supplemented with L-methionine containing 50 µg ml^{-1} kanamycin. Cells were spun down at 4000*g*, washed three times with 100 ml sterile water and resuspended in 5 ml sterile water. This culture was subsequently inoculated into 1 l pre-heated (310 K) SelenoMet Medium containing L-selenomethionine and grown for 2 h at 310 K with 200 rev min^{-1} shaking. The expression of protein was induced by the addition of 1 m*M* IPTG and further growth was performed for 6 h at 310 K with 200 rev min^{-1} shaking. Bacteria were harvested and resuspended in 25 ml PBS. The expression of unlabelled PE was performed using a routine laboratory protocol as described in Singh *et al.* (2011). In brief, a single colony of *E. coli* containing pET26bPE was inoculated into 5 ml LB containing 100 µg ml^{-1} kanamycin and incubated overnight at 310 K at 200 rev min^{-1}. Thereafter, the bacterial culture was transferred to 1 l fresh LB containing 50 µg ml^{-1} kanamycin and incubated at 310 K at 200 rev min^{-1} until the OD reached 1.0. Expression was induced by the addition of 1 m*M* IPTG followed by 6 h further incubation using the same conditions. Finally, the bacterial cells were harvested and resuspended in 25 ml PBS.

2.3. Inclusion-body preparation and refolding of PE

The bacterial cells were lysed mechanically by sonication (1 min cycle at 10×), keeping the samples in an ice bath. The DNA from the cell lysate was digested by the addition of 1 mg DNase I (Sigma, Missouri, USA) followed by incubation for 30 min at 310 K. The lysate was then centrifuged at 10 000*g* at 277 K for 20 min. The pellet, which consisted of inclusion bodies, was washed with 5 *M* urea and dissolved in 10 ml 8 *M* urea with constant stirring for 6 h at 277 K. The refolding of PE was performed by a dilution method, in which 10 ml PE (in 8 *M* urea) was added to 200 ml refolding buffer (50 m*M* Tris–HCl pH 7.8, 500 m*M* NaCl, 5 m*M* DTT, 0.005% Tween-20, 2 *M* urea) at 1 ml h^{-1} at room temperature. The refolded protein solution was centrifuged at 10 000*g* for 30 min at 277 K. The supernatant was dialysed against 50 m*M* Tris–HCl pH 7.8 containing 135 m*M* NaCl and 2 m*M* DTT. Finally, the dialysed PE solution was centrifuged at 10 000*g* for 30 min at 277 K to remove aggregated proteins. The supernatant containing folded PE was used in further purification steps.

2.4. Ion-exchange and gel filtration

Q-Sepharose Fast Flow anion-exchange resin (GE Healthcare Biosciences, Uppsala, Sweden) was packed into XK-16 glass columns (GE Healthcare Biosciences) attached to an ÄKTAprime plus FPLC system (GE Healthcare Biosciences). The column was washed with several volumes of degassed double-distilled H_2O and equilibrated with 50 m*M* Tris–HCl pH 7.8 buffer containing 135 m*M* NaCl and 2 m*M* DTT. The refolded PE solution was loaded onto the column at 0.5 ml min^{-1} using Superloop (GE Healthcare Biosciences). PE is a basic protein and did not interact with the anion-exchange resin, but other contaminant proteins bound to the resin. Therefore, the flow-through collected from the column was concentrated and used in the next purification step. The flowthrough was added to 500 m*M* NaCl and concentrated to 2–3 ml in volume using a 5000 Da molecular-weight cutoff Vivaspin concentrator (Sartorius Stedim Biotech, Göttingen, Germany). A Superdex 200 gel-filtration column (GE Healthcare Biosciences) was connected to the FPLC system and equilibrated with 50 m*M* Tris–HCl pH 7.5 buffer containing 500 m*M* NaCl and 2 m*M* DTT. Samples (250–300 µl) were injected and the proteins were separated at 0.5 ml min^{-1}. Fractions were collected at 1 ml and the purity of the proteins was assessed by SDS–PAGE stained with Coomassie Blue R250. Concentrations were measured using a Nanodrop spectrophotometer and the BCA method according to the manufacturer's recommendations (Pierce, Rockford, Illinois, USA).

2.5. Crystallization of proteins

For the production of native crystals, PE was finally concentrated to 10 mg ml^{-1} in gel-filtration buffer (50 m*M* Tris–HCl pH 7.5, 500 m*M* NaCl, 2 m*M* DTT). PACT Premier Screen, JCSG+, Structure Screens 1 and 2 (Molecular Dimensions, Newmarket, England), Crystal Screen and Crystal Screen 2 (Hampton Research, Aliso Viejo, California, USA) were used for initial screening. Drops consisting of 200 nl reservoir solution and 200 nl protein solution at a concentration of 5–10 mg ml^{-1} were produced using a Mosquito robot (TTP LabTech, Melbourn, England) in 96-well MRC plates at the MAX IV laboratory crystallization facility (Lund University, Sweden). The plates were stored at 298 K and photographed using a CrystalPro camera and the data were managed using *CrystalLims* software (both from TriTek Corporation, Sumerduck, Virginia, USA). PACT Premier kit condition A3, consisting of 100 m*M* SPG buffer pH 6.0, 25%(*w*/*v*) polyethylene glycol (PEG) 1500, produced small rod-shaped crystals. From the same kit, condition F3 [100 m*M* bis-tris propane pH 6.5, 200 m*M* NaI, 20%(*w*/*v*) PEG 3350] produced thin plate-like crystals. Additionally, Crystal Screen 2 condition No. 26 [100 m*M* MES pH 6.5, 200 m*M* ammonium sulfate, 30%(*w*/*v*) poly-

ethylene glycol monomethyl ether 5000] also produced thin plate-like crystals. Usually, all of these crystals appeared in drops after 3–4 d of setup. The conditions described above [100 m*M* SPG buffer and 25%(*w*/*v*) PEG 1500 or 100 m*M* bis-tris propane buffer, 200 m*M* NaI and 20%(*w*/*v*) PEG 3350] were used to reproduce crystals manually using the sitting-drop method. Single crystals were obtained that could be used for data collection.

SeMet PE^{L43M,L74M} did not produce crystals under the native PE crystallization conditions, so the four kits mentioned above were screened again to obtain initial conditions. PACT Premier condition B7 [100 m*M* MES pH 6, 200 m*M* NaCl, 20%(*w*/*v*) PEG 6000] and condition B8 [100 m*M* MES pH 6, 200 m*M* NH_4Cl, 20%(*w*/*v*) PEG 6000] produced microcrystals. The pH, salt and precipitant concentrations were optimized based on these conditions, but this did not improve the sizes of the crystals. Hence, microseeding was used to improve the crystal size. Microseeds were prepared using a Seed Bead kit (Hampton Research). In brief, 400 nl of a microcrystal-containing drop was mixed with 15 µl seed-crystal stabilizing solution [100 m*M* MES pH 6.0, 300 m*M* NaCl, 20%(*w*/*v*) PEG 6000], vortexed and dilutions were made according to the kit manual. Four different reservoir solutions, (i) 100 m*M* MES pH 6, 100 m*M* NH_4Cl, 20%(*w*/*v*) PEG 6000; (ii) 100 m*M* MES pH 6, 200 m*M* NH_4Cl, 20%(*w*/*v*) PEG 6000; (iii) 100 m*M* MES pH 6, 100 m*M* NaCl, 20%(*w*/*v*) PEG 6000 and (iv) 100 m*M* MES pH 6, 200 m*M* NaCl, 20%(*w*/*v*) PEG 6000, were pipetted into MRC 96-well plates in duplicate rows using a Freedom EVO 150 liquid-handling workstation (Tecan, Männedorf, Switzerland). Serial dilutions of seeds were made in up to eight steps. In each drop, 100 nl seed solution, 300 nl reservoir solution and 200 nl protein solution were pipetted using a Mosquito robot. Conditions (ii) and (iv) with seeds produced crystals of approximate dimensions 100 × 150 × 10 µm. These crystals were used to collect MAD data sets at the MAX IV Laboratory, Lund University, Sweden.

2.6. Data collection

Crystals were quickly cooled with a cryoprotectant solution in a mitotic loop using liquid nitrogen before exposing them to the X-ray beam. Several cryoprotectants such as glycerol, *m*-phenylenediamine (MPD) and PEG 400 were tried at different concentrations before a suitable one was identified. For native PE crystals, a universal cryosolution consisting of 32%(*w*/*v*) glycerol, 32%(*w*/*v*) ethylene, 36%(*w*/*v*) sucrose and 2%(*w*/*v*) glucose was used to flash-cool the crystals. This universal cryosolution was added in a 1:1 ratio to the protein drop before picking up crystals using a mitotic loop and placing them in a cryogenic N_2-gas stream. 15%(*w*/*v*) PEG 400 in the reservoir conditions acted as the best cryoprotectant for the SeMet PE crystals.

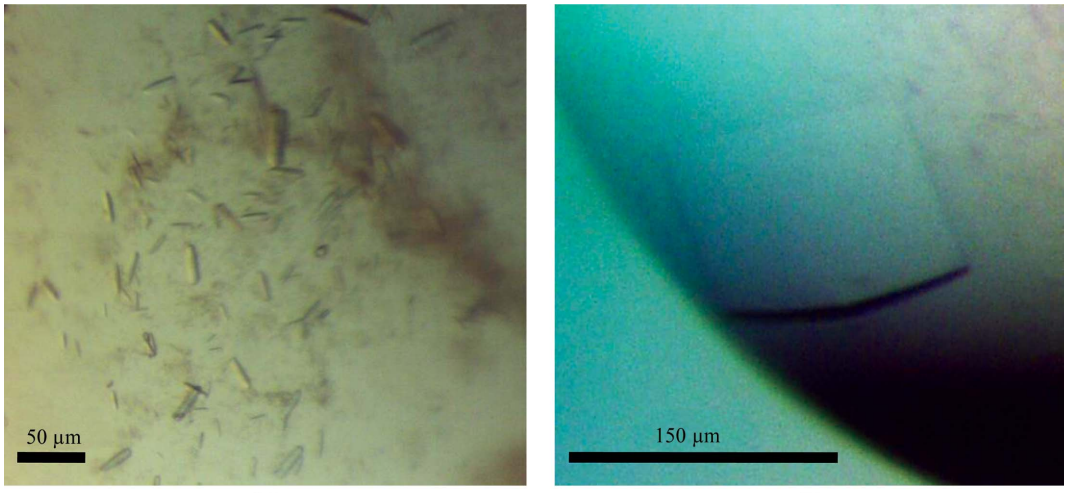
All crystal testing and data collection was performed at station I911-3 at the MAX IV Laboratory (Lund University, Sweden). The data were collected on a MAR 225 CCD detector (MAR Research, Germany) using an MD2 goniostat (Maatel, France). To obtain phase information to solve the structure of PE, three data sets, peak (PK), inflection point (IP) and remote (RM), were collected at the Se *K* edge (∼0.9795 Å) from the same SeMet-containing crystal (see Table 1 for data-collection details and statistics).

Data for PK and IP were collected first, with exposure times such that the influence of radiation damage on the phasing could be

Table 1
Data-processing and phasing statistics.

Values in parentheses are for the last shell.

	Native 1	SeMet PK	SeMet IP	SeMet RM
Space group	$P2_1$	$P2_1$	$P2_1$	$P2_1$
Unit-cell parameters				
a (Å)	44.2	44.1	44.1	44.1
b (Å)	57.3	56.9	56.9	56.9
c (Å)	61.4	61.4	61.4	61.4
β (°)	96.1	96.1	96.1	96.1
Wavelength (Å)	1.000	0.97918	0.97942	0.9700
Maximum resolution (Å)	1.8	2.6	2.7	2.3
Monomers in asymmetric unit	2	2	2	2
V_M (Å^3 Da^{-1})	2.28	2.26	2.26	2.26
Solvent content (%)	46.0	45.7	45.7	45.7
Total observations	115628	62016	60725	101090
Unique reflections	28219	17365	16137	26407
R_{merge} (%)	6.2 (45.7)	5.5 (20.2)	4.7 (15.7)	9.4 (50.4)
Completeness (%)	99.5 (98.6)	94.4 (68.2)	98.8 (94.3)	99.0 (95.1)
Average $I/\sigma(I)$	12.34 (2.64)	16.37 (4.19)	20.45 (6.76)	11.40 (2.85)
Phasing				
FOM	—			0.32
Phasing power (isomorphous)	—	—	0.713 (0.538)	0.331 (0.253)
Phasing power (anomalous)	—	1.079	1.050	0.416
Cullis *R* factor (isomorphous)	—	—	0.617 (0.642)	0.899 (0.976)
Cullis *R* factor (anomalous)	—	0.843	0.826	0.958



(*a*) (*b*)

Figure 2
Native and SeMet PE crystals grown under different conditions. (*a*) Native PE (5 mg ml^{-1}) produced rod-shaped crystals of approximately 10 × 20 × 50 µm in size using 100 m*M* SPG buffer pH 6.0, 25%(*w*/*v*) PEG 1500. (*b*) An SeMet PE crystal (100 × 150 × 10 µm) obtained after microseeding in a solution consisting of 100 m*M* MES pH 6, 200 m*M* NH_4Cl with 20%(*w*/*v*) PEG 6000.

minimized; however, the data for RM were collected with longer exposure times in order to obtain reliable data out to the diffraction limit of the crystals (see Table 1). All data were integrated and scaled using *XDS* (Kabsch, 2010). A first impression of the phasing power was obtained using *HKL2MAP* (Pape & Schneider, 2004), which provides a graphical interface to a set of programs from the *SHELX* suite (Sheldrick, 2008). Six of the eight possible Se positions could be identified using Patterson methods in *SHELXC*. Subsequent refinement and phasing of the structure was achieved with *autoSHARP* (Vonrhein *et al.*, 2007) using data from all three data sets.

3. Results and discussion

We expressed and purified native and SeMet PE 17–158 without any tag with a high purity suitable for crystallization. Approximately 1 l *E. coli* culture yielded 50–60 mg purified protein after ion-exchange and gel-filtration chromatography. The majority of PE was obtained in a dimeric form, while a minor fraction was oligomeric (data not shown). The protein purity was >98% pure as judged by SDS–PAGE. PE was stable at 253 K for more than a year and successfully produced crystals. Different expression, purification and storage batches did not alter the crystallization behaviour of the protein. The results obtained here were thus reproducible. The homogeneity and multimeric association of each protein-preparation batch was further confirmed by dynamic light-scattering (DLS) experiments before proceeding to crystallization experiments. The DLS data also suggested that >85% of PE was present as a dimer, while ∼15% was oligomeric (data not shown).

In order to solve the phase problem, Met residues were introduced (Fig. 1). For success, it was important that the point mutation in the PE molecule did not produce any constraint on the PE molecule and that the structures of the native and the SeMet variant were very similar. The positions of possible mutations were carefully analyzed. A Met residue favours a nonpolar environment in the core of the protein and thus a choice was made both on the mutability and the position of the residues in possible secondary-structure elements. The resulting SeMet PE behaved almost identically to native PE and existed with the majority in a dimeric form.

During screening, two different conditions produced high-quality native PE crystals: (i) 100 m*M* SPG buffer pH 6.0, 25%(*w*/*v*) PEG 1500 and (ii) 100 m*M* bis-tris propane pH 6.5, 200 m*M* NaI and 20%(*w*/*v*) PEG 3350. The first condition produced small rod-shaped crystals with approximately dimensions 10 × 20 × 50 µm (Fig. 2*a*) and after fine-tuning of the conditions produced plate-like crystals with approximate dimensions 100 × 150 × 10 µm. Data sets were collected from both crystal forms and despite their smaller size the rod-shaped crystals (Fig. 2*a*) diffracted as well as the larger plate-shaped crystals. The space groups and details of the data sets are described in Table 1. Finding a suitable cryosolution for the native PE crystals was critical and challenging, since the crystals were easily damaged and the diffraction pattern compromised. After a search for appropriate cryoconditions, the universal cryosolution 32%(*w*/*v*) glycerol, 32%(*w*/*v*) ethylene, 36%(*w*/*v*) sucrose and 2%(*w*/*v*) glucose gave the best results when applied in a 1:2 ratio to the protein drop in which the crystals appeared.

SeMet PE crystals were produced under different conditions and the size of the crystals was improved using the microseeding technique. Two conditions generated SeMet PE crystals: (i) 100 m*M* MES pH 6, 200 m*M* NH_4Cl, 20% PEG 6000 and (ii) 100 m*M* MES pH 6, 200 m*M* NaCl, 20% PEG 6000. These crystals were small in size and were not useful for diffraction. In order to proceed, these microcrystals were used for seed preparation; serially diluted seeds were added to new fresh drops of conditions (i) and (ii). Of these two conditions, 100 m*M* MES pH 6, 200 m*M* NH_4Cl with 20% PEG 6000 produced large plate-like crystals with final dimensions of approximately 100 × 150 × 10 µm (Fig. 2*b*). Before proceeding to X-ray exposure, SeMet PE crystals were soaked in reservoir-condition solution supplemented with 15%(*w*/*v*) PEG 400 as a cryoprotectant. We collected full data sets from a single SeMet PE crystal at the peak (PK), inflection-point (IP) and remote (RM) wavelengths of the Se *K* edge, with the RM data set extending to the diffraction limit of 2.3 Å, while taking care that no major radiation damage occurred during the collection of the first two data sets that could hinder the subsequent phase determination. Therefore, the first two data sets (PK and IP) extended to only 2.6–2.7 Å resolution, while the RM data set, which was collected last with longer exposure times, extended to 2.3 Å resolution. The three data sets collected for this MAD data set were used to obtain the positions of the Se atoms in the structure in Patterson functions and to subsequently solve the structure. During data processing and subsequent calculations for phasing, the PK data set was used as a reference set. Six out of eight positions were found using Patterson searches in *SHELXC*. Using the data set from the rod-shaped crystals (native 1), we managed to extend the PE atomic structure to 1.8 Å resolution, which allowed us to obtain a reliable model of the structure of PE.

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