Characterization of peptide deformylase homologues from *Staphylococcus epidermidis*

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The emergence of multi-drug-resistant strains of *Staphylococcus epidermidis* emphasizes the need to develop new antibiotics. The unique and essential role of the peptide deformylase (PDF) in catalysing the removal of the N-terminal formyl group from newly synthesized polypeptides in eubacteria makes it an attractive antibacterial drug target. In the present study, both deformylase homologues from *S. epidermidis* (SePDF-1 and SePDF-2) were cloned and expressed, and their enzymic activities were characterized. Co²⁺-substituted SePDF-1 exhibited much higher enzymic activity (k_{cat}/K_m $6.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) than those of Ni²⁺- and Zn²⁺-substituted SePDF-1, and SePDF-1 showed much weaker binding ability towards Ni²⁺ than towards Co²⁺ and Zn²⁺, which is different from PDF in *Staphylococcus aureus* (SaPDF), although they share 80% amino-acid sequence identity. The determined crystal structure of SePDF-1 was similar to that of (SaPDF), except for differences in the metal-binding sites. The other deformylase homologue, SePDF-2, was shown to have no peptide deformylase activity; the function of SePDF-2 needs to be further investigated.

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INTRODUCTION

Staphylococcus epidermidis, an opportunistic pathogen, has become one of most prevalent causes of nosocomial infections, especially in patients with prosthetic medical devices such as indwelling catheters or implanted foreign polymer bodies (Rupp & Archer, 1994; Otto, 2008). *S. epidermidis* infections on these devices are complicated by the formation of biofilms, which render the bacteria more resistant to multiple antibiotics and host defences (Götz, 2002; von Eiff *et al.*, 2002); replacement of the indwelling medical devices is generally necessary. Therefore, there is an urgent need to develop novel antibiotic agents for the treatment of staphylococcal infections. Peptide deformylase

(PDF, EC 3.5.1.88) is an important enzyme catalysing an essential process in bacterial protein synthesis, and it is considered as a potential antibiotic target for drug design (Sharma *et al.*, 2009). Up to now, there have been no reports about the peptide deformylases from *S. epidermidis*.

Unlike the synthesis of cytosolic proteins in mammalian cells, protein synthesis in prokaryotes and eukaryotic organelles (such as mitochondria and chloroplasts) starts with an *N*-formylmethionine, which is formed from methionyl-tRNA by *N*-methionyl-tRNA transformylase. Peptide deformylase catalyses the subsequent removal of the formyl group from the newly synthesized polypeptides (Adams, 1968; Bradshaw *et al.*, 1998; Frotin *et al.*, 2006; Mazel *et al.*, 1994; Meinel *et al.*, 1993). To date, deformylation has been considered to be unique to prokaryotes (Gigione *et al.*, 2000b; Nguyen *et al.*, 2003; Serero *et al.*, 2003), although some peptide deformylases have been found in organelles of eukaryotes (such as *Homo sapiens*, *Arabidopsis thaliana* and *Plasmodium falciparum*) but with low activities, far lower than that of

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Abbreviations: FDH, formate dehydrogenase; f-MAS, *N*-formyl-Met-Ala-Ser; PDF, peptide deformylase; RMSD, root-mean-square distance.

Four supplementary figures are available with the online version of this paper.

PDFs in prokaryotes (Bracchi-Ricard *et al.*, 2001; Dirk *et al.*, 2001; Lee *et al.*, 2004). Deformylation of nascent peptides is essential for bacterial survival, as it has been shown in *Escherichia coli*, *Streptococcus pneumoniae* and *Staphylococcus aureus* that the *def* gene cannot be disrupted (Apfel *et al.*, 2001; Margolis *et al.*, 2000; Mazel *et al.*, 1994; Meinnel & Blanquet, 1994).

PDF enzymes from different species, including *E. coli*, *S. aureus* and others, have been cloned and both their three-dimensional structures and co-crystal structures with a binding inhibitor have been reported (Baldwin *et al.*, 2002; Cai *et al.*, 2006; Chan *et al.*, 1997; Fieulaine *et al.*, 2005; Guilloteau *et al.*, 2002; Robien *et al.*, 2004; Smith *et al.*, 2003; Zhou *et al.*, 2005; Escobar-Alvarez *et al.*, 2009). Although it has been reported recently that in some species such as *Borrelia burgdorferi*, *Leptospira interrogans* and *Arabidopsis thaliana*, the native PDFs exist as Zn^{2+} -containing forms (Li *et al.*, 2002; Nguyen *et al.*, 2007), most native PDF enzymes in bacteria are unique Fe^{2+} -containing metallohydrolases, which are highly active but unstable *in vitro* due to oxidation (Groche *et al.*, 1998; Ragusa *et al.*, 1998; Rajagopalan *et al.*, 1997). For Fe^{2+} -containing PDFs, by substituting Fe^{2+} with other divalent transition metals such as Zn^{2+} (Chan *et al.*, 1997; Meinnel *et al.*, 1995, 1996), Ni^{2+} (Groche *et al.*, 1998; Ragusa *et al.*, 1998; Wang *et al.*, 2006) or Co^{2+} (Rajagopalan *et al.*, 2000; Dong & Liu, 2008), the enzymes retained their activities but were more stable, permitting further biochemical characterization and X-ray crystallographic analysis. Based on sequence homology and crystallographic data, all bacterial PDF enzymes share three highly conserved motifs that are structurally defined as the active sites of these metallohydrolases: motifs 1 $\text{G}\Phi\text{G}\Phi\text{AA}\Phi\text{Q}$, 2 $\text{EGC}\Phi\text{S}$ and 3 $\text{HE}\Phi\Phi\text{H}$ (Meinnel *et al.*, 1997). The catalytic metal ion is tetrahedrally coordinated with the side chains of two histidines from motif 3, a cysteine from motif 2 and a water molecule hydrogen-bonded to a glutamic acid from motif 3, the major catalytic site of PDF enzyme (Becker *et al.*, 1998; Rajagopalan *et al.*, 2000).

Based on amino-acid sequences, bacterial PDFs have been grouped into type I, II, and III subfamilies. Type I PDFs exist in bacteria and their homologues have been found in eukaryotes, while type II PDFs are only found in bacteria. Type III PDFs are newly defined in Archaea and trypanosomatids, but have not been thoroughly studied yet. Type II PDF of Gram-positive species differs from type I of Gram-negative bacteria mainly in three major amino-acid insertions (from 3 to 12 amino acids) and a distinct C-terminus (Giglione *et al.*, 2000a; Smith *et al.*, 2003). However, some bacteria, especially Gram-positive bacteria, contain both type I and type II PDF homologues. In the genome of *S. epidermidis*, two PDF homologue genes, *def* and *def-2*, were identified. The *def* gene product, SePDF-1 which shares 80 % amino-acid sequence identity with the functional PDF of *S. aureus* (SaPDFB, GenBank Accession no. P68826.1) (Baldwin *et al.*, 2002), is classified into the type II PDF subfamily; while the other PDF homologue, SePDF-2, encoded by *def-2*, is classified into the type I PDF subfamily.

In the present study, we cloned, expressed, and purified both deformylase homologues from *S. epidermidis* (SePDF-1 and SePDF-2). The characteristics and the crystal structure of SePDF-1 were studied. It was found that SePDF-1 has some different enzymic properties compared with PDF from *S. aureus*, though they share similar protein sequences and structures. The other deformylase homologue, SePDF-2, was shown to have no peptide deformylase activity, and its function needs to be further investigated.

METHODS

Protein expression and purification. The SePDF-1-encoding gene, *def* (GenBank Accession no. YP_188262.1), was amplified from the chromosome of *S. epidermidis* ATCC 35984 (American Type Culture Collection) by PCR with primers 5'-GCGCATCCATATGATAAC-AATGAAAGATATTATAAGAG-3' (forward) and 5'-CCGCTCGAG-GACTTCTACGGCCTCTTCATG-3' (reverse), and then cloned into the prokaryotic expression vector pET-22b(+) (Merck) with *Nde*I and *Xho*I restriction sites to produce the recombinant plasmid pET22b-def with a C-terminal six-histidine tag.

The SePDF-2-encoding gene, *def-2* (GenBank Accession no. YP_188364.1), was amplified with primers 5'-GCGGGATCCAAG-GAGCCTAAGTGATGACTGT-3' (forward) and 5'-CCGCTCGAG-CTCATTGTCAAATACGCCT-3' (reverse), and then cloned into the prokaryotic expression vector pET-24 (Merck) with *Bam*HI and *Xho*I restriction sites to produce the recombinant plasmid pET24-def-2 with a C-terminal six-histidine tag.

The recombinant plasmids pET22b-def and pET24-def-2 were transformed into *E. coli* BL21(DE3) for expression of recombinant proteins. In order to obtain SePDF-1 and SePDF-2 (SePDFs) with unique metal ions, a chemically defined medium was used and the metal ion (Co^{2+} , Ni^{2+} or Zn^{2+}) was added separately. The chemically defined medium contained the following (per litre): 2.5 g glucose, 10.8 g K_2HPO_4 , 5.5 g KH_2PO_4 , 10.0 g NaCl, 1.0 g ammonium sulfate, 2 mg thiamine, 124 mg MgSO_4 , 74 μg CaCl_2 , 20 μg MnCl_2 , 31 μg H_3BO_3 , 1.2 μg $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ and 1.6 μg CuSO_4 and was fortified with 50 $\times$ amino acids solution (Sigma-Aldrich) (Rajagopalan *et al.*, 2000). *E. coli* BL21(DE3) containing recombinant plasmids was inoculated into 250 ml chemically defined medium with 100 μg ampicillin ml^{-1} and 100 μM CoCl_2 (or NiCl_2 or ZnCl_2) and grown at 37 °C until the OD_{600} reached 0.6. IPTG (Merck) was added at a final concentration of 0.8 mM, and the bacteria were further cultivated at 25 °C for 12 h. After centrifugation, the pellets were resuspended and sonicated. The recombinant SePDFs were purified by Ni-NTA (Qiagen) affinity chromatography, and all purification procedures were performed at 4 °C.

The purification buffers for SePDFs expressed using Ni^{2+} -fortified medium were compensated with 100 μM NiCl_2 to avoid possible Ni^{2+} competition between Ni-NTA and SePDFs. After the purification, the recombinant SePDFs with different metal ions were dialysed against dialysis buffer (buffer D: 20 mM Tris/HCl, pH 8.0, 500 mM NaCl) to remove imidazole and free metal ions. The concentration of the purified proteins was determined by Bradford assay.

The metal content of SePDF-1 was determined as p.p.m. by using an AA-220 atomic absorption spectrometer (Varian) with Co^{2+} -specific absorption at 240.7 nm, Ni^{2+} -specific absorption at 232.0 nm and Zn^{2+} -specific absorption at 213.9 nm, and then converted to mol l^{-1} . The molar ratios for Co^{2+} -, Ni^{2+} - and Zn^{2+} -substituted SePDF-1 were calculated by dividing the metal contents by enzyme concentrations, to indicate the number of metal ions bound per protein. The metal content of SePDF-1 stripped by EDTA was measured as a negative control.

Oligomeric state analysis. The oligomeric state of SePDF-1 was studied by gel-filtration chromatography using an AKTA purifier system (GE Healthcare). The recombinant Co-SePDF-1 was used in the following experiments. The protein sample was chromatographed on a HiLoad 16/60 Superdex 75 prep-grade column (GE Healthcare) at a flow rate of 1 ml min⁻¹ with buffer D. The Superdex 75 column was calibrated with the following low-molecular-mass standards: conalbumin (75.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa) and RNase A (13.7 kDa) (GE Healthcare).

Enzymic activity assay. The enzymic activities of the recombinant SePDFs with different metal ions were evaluated by the PDF/FDH (formate dehydrogenase, EC 1.2.1.2) coupled assay (Chen *et al.*, 2000; Lazennec & Meinnel, 1997). During the assay, the formate released by PDF from f-MAS (N-formyl-Met-Ala-Ser) was oxidized by FDH, thereby reducing one molecule of NAD⁺ to NADH. All assays were carried out in a 96-well plate spectrophotometer at 37 °C (Beckman Coulter DTX880) by measuring the increase in absorbance at 340 nm ($\epsilon=6180 \text{ M}^{-1} \text{ cm}^{-1}$). The conditions, including concentrations of FDH, PDF and the substrates, used in the assays were optimized based on the protocols of Lazennec & Meinnel (1997) and Chen *et al.* (2000). The optimized assay solutions (total volume 200 µl) contained 50 mM HEPES, pH 7.5, 10 mM NaCl, 0.2 mg BSA ml⁻¹ (Sangon), 8 mM NAD⁺ (Fluka), 0.5 U FDH ml⁻¹ (Fluka) and f-MAS (Scipteptide). The reactions were initiated by adding diluted SePDF enzymes to the assay solutions.

SePDF-1 crystallization and data collection. Prior to crystallization, the purified recombinant Co-SePDF-1 was concentrated to 10 mg ml⁻¹. An initial screening was performed at 4 °C by the hanging-drop vapour-diffusion method using Crystal Screen kits 1 and 2 (Hampton Research). Drops were prepared by mixing 1 µl protein solution with 1 µl reservoir solution and equilibrated against 500 µl reservoir solution, following the procedure described by the manufacturer. The conditions yielding small crystals were further optimized by varying the buffer pH and precipitant concentration. Diffraction-quality crystals were grown in a reservoir solution containing 0.2 M ammonium acetate, 0.1 M trisodium citrate dihydrate pH 5.6 and 30% (v/v) PEG 4000.

All diffraction data were collected in-house on a Rigaku rotating-anode X-ray generator operated at 50 kV and 100 mA ($\lambda=1.5418 \text{ Å}$). Diffraction images were recorded by a Rigaku R-Axis IV++ imaging-plate detector with an oscillation step of 1°. The crystal-to-detector distance was set to 150 mm. The crystals were soaked briefly in a cryoprotectant made by adding 10% glycerol to the original reservoir solution and flash-cooled in liquid nitrogen for data collection at 100 K. The data were integrated by MOSFLM (Leslie, 1992) and scaled to 2.3 Å by SCALA (Evans, 1993) in the CCP4 program suite. The R_{merge} of the highest resolution shell is high (58%), but considering the acceptable redundancy (4.3) and I/σ (2) of that shell, we still processed the diffraction data to 2.3 Å. Complete statistics of the data are summarized in Table 1.

Determination of the SePDF-1 structure. Crystals of SePDF-1 belong to space group C222₁ with one monomer in the asymmetrical unit. Molecular replacement was carried out by MOLREP (Vagin & Teplyakov, 1997) using the structure of SaPDFB (PDB code: 1LQW) (Guilloteau *et al.*, 2002) as the search model. The structure was refined by Refmac (Murshudov *et al.*, 1997) and CNS 1.1 (Brünger *et al.*, 1998) and included simulated annealing, energy minimization and B factor refinement. Manual building of the model was carried out with the program Coot (Emsley & Cowtan, 2004). After several cycles of model building and refinement, most of the residues, including two C-terminal vector residues and the cobalt ion, were clearly positioned in the $2F_o-F_c$ map. But three residues from the flexible CD loop (Asn80–Asn82) were disordered in the structure and could not be modelled. Water molecules were added automatically with CNS using 3σ peaks in the F_o-F_c map and hydrogen bonding

Table 1. Statistics of diffraction data and model refinement

Diffraction data	
Space group	C222 ₁
Cell length (Å) (a, b, c)	99.288, 121.463, 45.781
Cell angles (°) (α , β , γ)	90, 90, 90
Resolution range (Å)	76.92–2.30 (2.42–2.30)
No. of observed reflections ($I/\sigma>0$)	55 639 (7867)
No. of unique reflections	12 458 (1817)
Redundancy	4.5 (4.3)
I/σ	17.8 (2.0)
Completeness (%)	98.0 (100.0)
R_{merge} (%)	7.1 (58.0)
Refinement and model	
Resolution range (Å)	30.00–2.30
No. of reflections ($F_o \geq 0\sigma(F_o)$)	12 427
Free R set	625
R factor (%)	22.7
Free R factor (%)	25.3
No. of residues	182
No. of protein atoms	1458
No. of water molecules	62
No. of cobalt ions	1
Mean B factor of all atoms (Å ²)	47.3
Protein main chain atoms	46.4
Protein side-chain atoms	48.6
Water molecules	47.3
Cobalt ion	46.5
RMSD bond lengths (Å)	0.006
RMSD bond angles (°)	1.3
RMSD dihedral angles (°)	23.6
RMSD improper angles (°)	0.81
Ramachandran plot (%)	
Most favoured regions	92.4
Allowed regions	7.0
Generously allowed regions	0.6

requirements, and problematic waters were manually removed if their definition in the electron density map was poor, or their B factors were too high. The final R factor is 22.7% (free R factor 25.3%) according to CNS, and statistics show that the final model of SePDF-1 is well refined (Table 1).

Site-directed mutagenesis of SePDF-2. Site-directed mutagenesis of SePDF-2 was performed with the QuikChange site-directed mutagenesis kit (Stratagene) using the plasmid pET24-def-2 as template and following the manufacturer's instructions. The mutation sites and the primers for mutagenesis are listed in Table 2. The mutant plasmids were confirmed by DNA sequencing before being transformed into *E. coli* BL21(DE3). The methods for expression and purification of the mutated proteins were the same as that of the wild-type SePDFs.

RESULTS

The deformylase-encoding genes in *S. epidermidis*

Based on the genomic sequence of *S. epidermidis* ATCC 35984 (GenBank Accession no. NC_002976.3), two deformylase homologue genes were identified. The first of these,

Table 2. Primers used in the site-directed mutageneses of SePDF-2

Name	Sequences (5'–3')	Mutation site	Function
S90C	F: ctgacttagaaggctgtatcagtttacctc R: gaggtaaactgatacagccttctaagtcag	tca-tgt	S→C
I91L	F: ctgacttagaaggctcactgagtttacctcatatatg R: catatatatgaggtaaactcagtgagccttctaagtcag	atc-ctg	I→L
SI-90,91-CL	F: ctgacttagaaggctgtctgagtttacctcatatatg R: catatatatgaggtaaactcagacagccttctaagtcag	tca-tgt atc-ctg	S→C and I→L
A44G	F: cattatatcatgaggaaggcgctggaattagtcacaccaaattg R: caatttgaggtgcactaattccagcgcttctcatgatataatg	gcc-ggc	A→G
A46G	F: cattatatcatgaggaaggcgctggaattagtcacaccaaattg R: caatttgaggtgcactaattccagcgcttctcatgatataatg	gca-gga	A→G
L131Q	F: gatatcgacgcatgattcaacatgagattgatcatcttaattg R: ccattaagatgatcaatctcatgttgatcatgctgcgataatc	tta-caa	L→Q
M133E	F: gatatcgacgcatgattcaacatgagattgatcatcttaattg R: ccattaagatgatcaatctcatgttgatcatgctgcgataatc	atg-gag	M→E
I47L	F: catgaggaaggcgctggacttgctgcacaccaaattggagtag R: ctactccaatttgaggtgcagcaagtcagcgcttctcatg	att-ctt	I→L
S48A	F: catgaggaaggcgctggacttgctgcacaccaaattggagtag R: ctactccaatttgaggtgcagcaagtcagcgcttctcatg	agt-gct	S→A
A45V	F: cattatatcatgaggaaggcgctggacttgctgcacaccaaattggag R: ctccaatttgaggtgcagcaagtcacgccttccatgatataatg	gct-gtt	A→V

def, encodes a protein containing 183 amino acids (SePDF-1). According to amino-acid sequence alignment of PDFs from five species (*S. epidermidis*, *S. aureus*, *E. coli*, *Helicobacter pylori* and *Homo sapiens*) (see Supplementary Fig. S1, available with the online version of this paper), SePDF-1 contains three of the most conserved motifs structurally defined as the active sites of the PDF enzymes, and is 80 % identical to the functional PDF in *S. aureus* (SaPDFB), a representative of the type-II PDF subfamily (Guilloteau *et al.*, 2002; Margolis *et al.*, 2000). This suggested that the *def* gene product is the functional deformylase in *S. epidermidis*.

The other deformylase homologue gene in *S. epidermidis* is *def-2*. The *def-2* gene product, SePDF-2, shows only 24.5 % amino acid identity with SePDF-1, and lacks the consensus cysteine and histidine residues that have been shown to be essential for deformylase activity as a metal cofactor binding site (Becker *et al.*, 1998; Meinnel *et al.*, 1997; Rajagopalan *et al.*, 2000). The *def-2* gene of *S. epidermidis* is considered unlikely to encode a protein with deformylase activity.

Biochemical characterization of SePDF-1, the *def* gene product

In order to identify the functional peptide deformylase, both of the deformylase-coding genes (*def* and *def-2*) were cloned and expressed in *E. coli*; only SePDF-1 (*def* coding) was shown to have deformylase activity.

PDFs from most bacteria represent a family of metallohydrolases containing a catalytic Fe^{2+} ion. Replacing the Fe^{2+} ion with other divalent transition metals, such as

Co^{2+} , Ni^{2+} and Zn^{2+} , resulted in a stable enzyme activity similar to that of PDF with Fe^{2+} (Groche *et al.*, 1998; Meinnel *et al.*, 1995, 1996; Ragusa *et al.*, 1998; Rajagopalan *et al.*, 1997, 2000). To determine the effects of different metal cations on the catalytic activity of SePDF-1, and to obtain stable and high enzyme activity for subsequent experiments, we prepared Co^{2+} -, Ni^{2+} - or Zn^{2+} -substituted SePDF-1 by growing *E. coli* cells in a chemically defined medium supplemented with 100 μM CoCl_2 , NiCl_2 or ZnCl_2 , respectively. The recombinant SePDF-1 coupled with a C-terminal six-histidine tag was purified to apparent homogeneity by Ni-NTA affinity chromatography (Supplementary Fig. S2). The molecular mass of the recombinant SePDF-1 was confirmed by LC/ESI-MS, and its oligomeric state was analysed by gel filtration. The size-exclusion chromatography of SePDF-1 showed only one peak between carbonic anhydrase (29.0 kDa) and RNase A (13.7 kDa), as shown in Supplementary Fig. S3. This indicated that the SePDF-1 existed as a monomer in solution state *in vitro*, whose molecular mass was 21.8 kDa.

Compared with Co-SePDF-1, the enzymic activity of Ni^{2+} - or Zn^{2+} -substituted SePDF-1 showed a fourfold decrease (Fig. 1). In contrast, earlier studies found that while the Zn^{2+} -substituted PDFs from *E. coli* and *S. aureus* showed reduced activity, the Co^{2+} - or Ni^{2+} -substituted PDFs retained rather high activity and essential stability (Baldwin *et al.*, 2002; Ragusa *et al.*, 1998; Rajagopalan *et al.*, 2000; Smith *et al.*, 2003). The Michaelis–Menten parameters of Co-SePDF-1 were determined from the slope and intercept values of the linear fit in a Lineweaver–Burk plot. The kinetic parameters K_m , k_{cat} and the second-order rate constant k_{cat}/K_m for PDF enzymes from four species are listed in Table 3. The k_{cat}/K_m value of Co-SePDF-1 was

Fig. 1. Enzymic activities of SePDF-1 substituted by different metal cations. SePDF-1 was prepared from cells grown in chemically defined medium supplemented with 100 μM Co^{2+} , Ni^{2+} or Zn^{2+} . The enzymic activities were determined by FDH-coupled assay. The relative enzymic activity of the different enzyme forms (Co^{2+} , Ni^{2+} and Zn^{2+}) of SePDF-1 was determined by comparing the initial velocities, taking Co-SePDF-1 as 100%.

$6.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, indicating that the recombinant SePDF-1 has high catalytic efficiency. When stored at 4 °C at a concentration of $>5 \text{ mg ml}^{-1}$ during enzyme purification and kinetic experiments, Co^{2+} -containing SePDF-1 was quite stable and retained similar activity for weeks.

Weak nickel-binding ability of SePDF-1

In order to study why Ni^{2+} -substituted SePDF-1 was less active, we determined the nickel content in Ni-SePDF-1. Atom absorption spectrum analysis showed that no nickel binding was detectable in SePDF-1 expressed using Ni^{2+} -fortified medium, while cobalt or zinc was normally detected in SePDF-1 expressed using medium containing Co^{2+} or Zn^{2+} , respectively (Table 4). Considering that the Ni-NTA agarose may bind the Ni^{2+} ion in the solution in competition with SePDF, additional Ni^{2+} ions (to a final concentration of 100 μM) were added to the purification buffers. However, SePDF-1 purified using Ni^{2+} -containing buffers still showed no nickel binding. No upregulation of enzyme activity was observed, even when additional Ni^{2+} was directly added to the enzymic activity assay buffer. These results showed that SePDF-1 had a weak Ni^{2+} -binding ability, although it shared high amino-acid sequence identity (80 %) with SaPDFB, which has a high enzymic activity in the Ni^{2+} form (Table 3) (Smith *et al.*, 2003).

SePDF-1 binds more Zn^{2+} (1.36 mol Zn per mol protein) than Co^{2+} (0.87 mol Co per mol protein), although the activity of Zn-SePDF was much lower. The zinc detected in Co^{2+} - or Ni^{2+} -form SePDF-1 expressed in chemically defined medium may be from the environment.

Crystal structure of SePDF-1

In order to study the metal ion binding mechanism, and explore the reason for the weak nickel-binding ability of SePDF-1, we crystallized the recombinant protein using the hanging-drop vapour-diffusion method, and analysed the overall structure of SePDF-1. Because of the lack of nickel binding to SePDF-1 and the low activity of Zn-SePDF-1, Co^{2+} substituted SePDF-1 was used for the crystallography studies.

The crystal structure of SePDF-1 was analysed by molecular replacement using the structure of SaPDFB (PDB code: 1LQW) (Guilloteau *et al.*, 2002) as the search model. Similar to the amino-acid sequences, the crystal structure of SePDF-1 is also similar to that of SaPDFB (Baldwin *et al.*, 2002), with a root-mean-square distance (RMSD) of 0.603 Å superimposing 180 C_α atoms out of 183 (Asp80–Asn82 were not modelled in SePDFs because of missing electron density). SePDF-1 contains three α -helices, four 3_{10} helices and eight β -strands (Fig. 2). The N-terminus of the protein is a cluster of helices, followed by two three-stranded anti-parallel β -sheets wrapping around a central α -helix where the catalytic site is located. A three-stranded mixed β -sheet is positioned at the C-terminus and the tail of its last strand folds back toward the entrance of the substrate-binding pocket. The Co^{2+} ion is tetrahedrally coordinated by His154 and His158 from the motif HE Φ DH, Cys111 from the motif EGC Φ S, and a water molecule held in place by the catalytically active residue Glu155.

Site-directed mutagenesis of SePDF-2

The *def-2* gene product, SePDF-2, was expressed and purified with Co^{2+} , Ni^{2+} or Zn^{2+} , and the enzymic activity was evaluated using the same method as for SePDF-1. Distinct from SePDF-1, none of the Co^{2+} -, Ni^{2+} - or Zn^{2+} -substituted SePDF-2 proteins showed peptide deformylase activity.

Table 3. Kinetic parameters of SePDF-1 compared with PDFs from other bacteria

SaPDF, PDF from *S. aureus*; EcPDF, PDF from *E. coli*; HpPDF, PDF from *H. pylori*.

PDF	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1} \text{ s}^{-1}$)	Reference
Co-SePDF-1	2.227	140	6.3×10^4	Present study
Ni-SaPDFB	2.6	152	5.8×10^4	Smith <i>et al.</i> (2003)
Ni-EcPDF	9.1	103	1.1×10^4	Smith <i>et al.</i> (2003)
Co-HpPDF	1.7	3.4	0.2×10^4	Han <i>et al.</i> (2004)

Table 4. Metal contents and enzymic properties of SePDF-1 substituted by different metal cations

Ion-SePDF	Metal content*			K_m (mM)	k_{cat} (s ⁻¹)
	Co	Ni	Zn		
Co-SePDF-1	0.87	<0.01	0.08	2.277	140
Ni-SePDF-1	<0.01	<0.01	0.30	NA	NA
Zn-SePDF-1	<0.01	<0.01	0.99	NA	NA
EDTA-treated SePDF-1	NS	NS	NS	—	—

*The metal contents in the table are given as molar ratios, which were calculated by dividing the metal contents (determined by atomic absorption spectrometry as p.p.m., and then converted to mol l⁻¹) by the enzyme concentrations (mol l⁻¹). NS, No signal: no significant signal was obtained.

NA, Not analysed, because of low enzymic activity.

Protein sequence analysis showed that SePDF-2 belongs to the type I PDF family, and it has several amino-acid mutations in the three regions which are highly conserved in all PDFs. The cysteine and histidine residues in the metal-binding domain of PDF enzymes are crucial for the peptide deformylase activity, and lack of those conserved residues might result in the inactivity of SePDF-2.

Fig. 2. Cartoon representation of the overall structure of SePDF-1. The whole molecule is coloured in blue to red from its N-terminus to its C-terminus. The secondary structures were assigned by DSSP and are labelled (Kabsch & Sander, 1983). The cobalt ion is drawn as a magenta sphere, and the dashed green line stands for disordered residues missing in the CD loop. The graphic was generated by Pymol.

Based on the sequence alignment of essential motifs in SePDF-2, SePDF-1 (type II PDF family), BsPDF-1 (type I PDF family, an active PDF in *Bacillus subtilis* whose *def* gene products are both proved to be active: Haas *et al.*, 2001), and EcPDF (PDF from *E. coli*, type I PDF family) (Fig. 3), the essential sites of SePDF-2 were changed by a series of site-directed mutagenesis (A44G, A45V, A46G, I47L, S48A, S90C, I91L, L131Q, M133E) to form a mutant protein with the same active centre as the active BsPDF-1. However, the final mutant with all the sites mutated still showed no peptide deformylase activity in the Co²⁺, Ni²⁺ or Zn²⁺ forms.

DISCUSSION

Distinct characteristics of SePDF-1

Since PDF was considered as a potential target for antimicrobial drug discovery, there have been more and more reports of PDFs from various bacteria. In the present study we have investigated the characteristics of PDF from *S. epidermidis*, and found that the functional PDF in *S. epidermidis* (SePDF-1) has some distinct characteristics compared with that in *S. aureus* (SaPDFB), although they share 80 % amino-acid sequence identity. The recombinant Co²⁺-substituted SePDF-1 was highly active towards the peptide substrate formyl-MAS, with a k_{cat} of 140 s⁻¹, K_m of 2.227 mM, and k_{cat}/K_m of 6.3×10^4 M⁻¹ s⁻¹, but the Ni²⁺-substituted SePDF-1 was less active. Earlier studies have found that Ni²⁺-substituted PDFs from *E. coli* and *S. aureus* retained rather high activity (Table 3) (Baldwin *et al.*, 2002; Ragusa *et al.*, 1998; Rajagopalan *et al.*, 2000; Smith *et al.*, 2003), while the enzymic activity of Ni²⁺-substituted SePDF-1 was much lower, and SePDF-1 showed a weak Ni²⁺-binding ability (Table 4).

To investigate the basis of the weak Ni²⁺-binding ability of SePDF-1, a structural comparison between SePDF-1 and SaPDFB was performed by the Pymol program, and this

	Motif 1	Motif 2	Motif 3
SePDF-2	...AAAIAPQ...	EGSIS...	LHMDHL...
BsPDF-1	...GVGLAAPQ...	EGCLS...	QHMDHL...
SePDF-1	...GVGLAAPQ...	EGCLS...	QHEIDHL...
EcPDF	...GIGLAATQ...	EGCLS...	QHMDHL...
	. . . : : * .	* : : * .	* : : *
mSePDF-2	...GVGLAAPQ...	EGCLS...	QHEIDHL...

Fig. 3. Sequence alignment of essential motifs in SePDF-2 and PDFs from other species. The most conserved motifs in SePDF-2, SePDF-1, BsPDF1 (PDF1 from *B. subtilis*, GenBank Accession no. P94462.1), EcPDF (PDF from *E. coli*, GenBank Accession no. P0A6K3.2) were aligned by CLUSTAL W. The conserved residues are marked with an asterisk. Conserved substitutions are represented by (:), and semi-conserved substitutions by (.). The sequence of SePDF2 was mutated to be similar to active BsPDF-1 and SePDF-1, and the mutant was named mSePDF2.

Fig. 4. Schematic representation of superimposed PDF structures. Structures of Zn-SaPDFB (PDB code: 1LQW) (Guilloteau *et al.*, 2002), Ni-SaPDFB (PDB code: 2AI9) (Smith *et al.*, 2003) and Co-SePDF-1 were superimposed by using the Pymol program. Carbons in Zn-SaPDFB, Ni-SaPDFB and Co-SePDF-1 are coloured green, blue and pink, respectively. The cations of Zn, Ni, and Co in the structures are represented as brown, blue, and red spheres, respectively.

indicated some structural differences between the two proteins. The first difference was found in the metal-ion-binding domains. Although the metal-binding pattern of SePDF-1 is quite similar to that of SaPDFB, the position of the cobalt ion in SePDF-1 is about 1 Å away from that of zinc and nickel in SaPDFB when their structures are superimposed, which is closer to the catalytic centre of PDF and may be ascribed to subtle displacement of the sidechain of the conserved Cys in the pocket (Fig. 4). Another difference between SePDF-1 and SaPDFB occurs at the CD loop (Pro78–Ser85, in which Asn80–Asn82 was disordered), with an RMSD of 2.11 Å. This is in agreement with the intrinsic flexibility of this loop, which is located at the entry point for the substrate. The final difference is in the part of the loop preceding the last 3_{10} helix (Asp169–Leu174, RMSD 1.49 Å), probably due to its exposure to the solvent. These structural differences may result in the different nickel-binding pattern between SePDF-1 and SaPDFB, but the detailed reason for the weak Ni^{2+} binding remains to be further investigated.

Two deformylase homologues

In the genome of *S. epidermidis* strain RP62A, two deformylase homologues, *def* and *def-2*, were identified. Although both genes were transcribed consistently through all growth phases of *S. epidermidis* (Supplementary Fig. S4), only the *def*-encoded protein had deformylase activity.

In most Gram-negative bacteria, the gene coding for the functional peptide deformylase is located next to the formyl Met-tRNA transferase-encoding gene *fmt*. However, the *def-2* gene of *S. epidermidis* located next to the *fmt* ORF encodes a protein without deformylase activity, like *def-A* in *S. aureus*.

Bacillus subtilis is considered to be a model organism for Gram-positive bacteria. Both of the *def* gene products in *B. subtilis* have been shown to have peptide deformylase activity (Haas *et al.*, 2001). We were curious about whether *def-2* in *S. epidermidis* might also have encoded an active PDF during an earlier stage of its evolution, and

the inactivation of this gene product was due to mutations in the active centre of the protein. Therefore, we mutated *def-2* in *S. epidermidis* to investigate whether the activity of this gene product can be recovered by site-directed mutagenesis.

Protein-sequence analysis showed that SePDF-2 has several single point mutations in the highly conserved activity centres present in all PDF enzymes. Previous studies have demonstrated that the cysteine and histidine residues in the metal-binding domain of PDF enzymes are crucial for the peptide deformylase activity. We therefore assumed that the absence of the key residues in the active centres of SePDF-2 might result in the inactivation of its deformylase activity. The SePDF-2 was changed by a series of site-directed mutagenesis, and the final mutant had the same active centre as the active BsPDF-1, which also belongs to the type I PDF subfamily. However, none of the SePDF-2 mutants showed deformylase activity. Thus, there must be multiple reasons for the inactivation of SePDF-2. Besides the absence of the crucial residues, the conformational differences between these two proteins may also result in different enzyme characteristics; Co^{2+} , Ni^{2+} or Zn^{2+} may not serve as the appropriate metal co-factors for SePDF-2, and the lack of the appropriate metal co-factors (e.g. Fe^{2+}) may lead to the inactivation of SePDF-2 as well. In addition, *def-2* in *S. epidermidis* may have some other functions that have not yet been identified.

Taken together, our present work may contribute to a better understanding of the SePDF catalytic mechanism and help to discover potential PDF inhibitors.

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