Membrane-association determinants of the ω-amino acid monooxygenase PvdA, a pyoverdine biosynthetic enzyme from Pseudomonas aeruginosa

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The L-ornithine N²-oxygenase PvdA catalyses the N²-hydroxylation of L-ornithine in many Pseudomonas spp., and thus provides an essential enzymic function in the biogenesis of the pyoverdine siderophore. Here, we report a detailed analysis of the membrane topology of the PvdA enzyme from the bacterial pathogen Pseudomonas aeruginosa. Membrane topogenic determinants of PvdA were identified by computational analysis, and verified in Escherichia coli by constructing a series of translational fusions between PvdA and the PhoA (alkaline phosphatase) reporter enzyme. The inferred topological model resembled a eukaryotic reverse signal-anchor (type III) protein, with a single N-terminal domain anchored to the inner membrane, and the bulk of the protein spanning the cytosol. According to this model, the predicted transmembrane region should overlap the putative FAD-binding site. Cell fractionation and proteinase K accessibility experiments in P. aeruginosa confirmed the membrane-bound nature of PvdA, but excluded the transmembrane topology of its N-terminal hydrophobic region. Mutational analysis of PvdA, and complementation assays in a P. aeruginosa ΔpvdA mutant, demonstrated the dual (structural and functional) role of the PvdA N-terminal domain.

INTRODUCTION

Iron is one of the most important nutrients for bacteria because of its essential metabolic role. To fulfil their nutritional iron demand, many bacteria synthesize, excrete and ingest high-affinity iron chelators, termed siderophores, which bind environmental iron (generally Fe³⁺), and deliver it to the cell through receptor-mediated active transport (Andrews et al., 2003). Pyoverdine is the major siderophore produced by the opportunistic pathogen Pseudomonas aeruginosa, and it plays an important role in P. aeruginosa pathogenicity and ecology (reviewed by Visca et al., 2007).

Structurally, pyoverdine is a chromopeptide consisting of three domains: (i) a fluorescent chromophore, (ii) an acyl side chain, and (iii) a peptide moiety linked via an amide bond to the carboxyl group of the chromophore. Fe³⁺ is bound by pyoverdine with high affinity to form a very stable octahedral complex (dissociation constant approximately 10⁻³² M). In type 1 pyoverdine from P. aeruginosa PAO1, the three bidentate ligands involved in Fe³⁺ coordination are provided by the catecholic hydroxyl groups of the chromophore and two hydroxamic groups of the formylated N³-hydroxynornithine (OHOrn) residues of the peptide moiety. As a rule, the whole pyoverdine molecule is assembled by non-ribosomal peptide synthases, according to the carrier thiotemplate mechanism (Visca et al., 2007).

In P. aeruginosa, L-ornithine (Orn) hydroxylation is catalysed by the Orn N²-oxygenase, which is encoded by the pvdA gene (Visca et al., 1994), and belongs to the ω-amino acid monooxygenase family (EC 1.14.13.–). Several...
lines of evidence indicate that PvdA plays an essential role in pyoverdine biogenesis by P. aeruginosa (Visca et al., 1994, 2007; Putignani et al., 2004). Recently, P. aeruginosa PAO1 PvdA has been purified and biochemically characterized as a monomeric enzyme in solution, and it has been shown to require both FAD and NADPH for activity (Meneely & Lamb, 2007).

The ω-amino acid monoxygenase family is an elusive group of enzymes that contains two putative dinucleotide-binding motifs (GXXGXXG/P and GXGXXG/A for FAD and NAD(P)H, respectively) and a substrate recognition (F/LATGY) domain (Stehr et al., 1998; Putignani et al., 2004). Members of this family catalyse the incorporation of one hydroxyl group into the side-chain amino group of ω-amino acids by reducing molecular oxygen through concomitant oxidation of both NAD(P)H and FADH₂ (Plattner et al., 1989). Interestingly, the only ω-amino acid monoxygenase whose subcellular localization has been investigated to date is the PvdA proximate homologue IucD, which catalyses l-lysine hydroxylation during aerobactin biogenesis in Escherichia coli (Herrero et al., 1988). Pioneer topology studies on IucD have suggested that this enzyme is membrane bound by means of at least one transmembrane (TM) domain that encompasses the putative FAD-binding motif at the N-terminal region (Herrero et al., 1988). Accordingly, Viswanatha and coworkers were unable to obtain a soluble form of IucD, unless a recombinant protein with an altered N terminus was constructed (Thariath et al., 1998). However, the group of Diekmann reported that native IucD could be purified to homogeneity in an active form (Plattner et al., 1989), giving rise to a dispute on the assumption that the membrane-associated form of IucD could be an artefact resulting from the moderate hydrophobicity of dinucleotide-binding domains (Dick et al., 1993). Therefore, the actual cellular localization of bacterial ω-amino acid monoxygenases remains an open issue.

This study was undertaken to explore the membrane topology of the P. aeruginosa PAO1 PvdA enzyme, with the aim of providing novel insights into the membrane-association determinants of bacterial ω-amino acid monoxygenases. By using in silico topology predictions, alkaline phosphatase (PhoA) translational fusions, cell fractionation assays and mutational analysis, we demonstrate that PvdA is anchored to the cytoplasmic membrane with the core of the protein exposed to the cytosol, and that the PvdA N-terminal hydrophobic domain is important for membrane association. Overall, our results provide a valuable background for further investigations on the pyoverdine multienzyme biosynthetic pathway in P. aeruginosa, and provide a basis for topological studies on biosynthetic enzymes of other hydroxamate siderophores.

METHODS

In silico analyses. Secondary structure and solvent accessibility were inferred by using Jpred algorithms (http://barton.ebi.ac.uk/servers/jpred.html). Java outputs were manually edited. Hydrophobicity profiles were generated by using Hopp–Wood’s Hopp & Doolittle, 1982) functions over a window size of 19 aa (http://www.expasy.org/tools/). Prediction of TM domains was accomplished by combining four software programs: TMpred, DAS and TMHMM (all available on the ExPASy server, www.expasy.org/tools/), and ConPred II (http://bioinfo.ihb.jussieu.fr/ConPred2/). All user-adjustable parameters were left at their default values.

Construction of PvdA–PhoA translational fusions. A series of PvdA–PhoA translational fusions was constructed by PCR amplification, using P. aeruginosa PAO1 genomic DNA as a template. Routine genetic manipulations were carried out according to Sambrook et al. (1989). The sense primer, which annealed to the start codon of the pvdA coding sequence, was paired with antisense primers to generate fusions at appropriate positions. In both sense and antisense primers, the KpnI restriction site was included to allow cloning of each ampiclon into the compatible restriction site of pBAD::phoA (Table 1), upstream of the phoA gene without a signal sequence, which encodes a PhoA variant lacking the N-terminal signal peptide essential for PhoA export into the periplasm (Melpers et al., 1999). Fusions 12P and 22P were obtained by the oligonucleotide adaptor technique (Invitrogen). Primer and oligonucleotide sequences used in this study are given in Table 2. Correct in-frame cloning and ampiclon sequences were verified by automated DNA sequencing. The resultant fusion plasmids were individually introduced into the phoA-null mutant E. coli strain LMG194 (F⁻ lacZΔ74 galE thi rpsL ΔphoA Δara-714 leu::Tn10, Guzman et al., 1995).

Expression of fusion proteins, PhoA assay, and preparation of E. coli subcellular fractions. E. coli LMG194 cells expressing PvdA–PhoA fusion proteins were grown at 37°C in NZYM medium (Sambrook et al., 1989) supplemented with 100 µg ampicillin ml⁻¹. PhoA activity was assayed by measuring the rate of p-nitrophenyl-phosphate hydrolysis using 4-methylumbelliferone as a fluorogenic substrate.

Table 1. Plasmids and encoded proteins used in this study

<table>
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<tr>
<th>Plasmid or construct*</th>
<th>Encoded protein</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>pBAD::phoA</td>
<td>PhoA⁺</td>
<td>Melchers et al. (1999)</td>
</tr>
<tr>
<td>pBAD::pvdA12E</td>
<td>12P</td>
<td>This study</td>
</tr>
<tr>
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<tr>
<td>pUCP19::pvdAΔ30</td>
<td>Δ30PvdA</td>
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*Construct names report the number of encoded PvdA N-terminal residues and the one-letter symbol of the amino acid at the fusion joint. The amino acid substitutions resulting from the introduction of the KpnI site are indicated in bold (D→E, I→M and S→T). †PhoA enzyme lacking its signal peptide sequence.
Table 2. Oligonucleotides used in this study

<table>
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<th>Oligonucleotide</th>
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<tr>
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<tr>
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<td>ΔpvdARVΔFW†</td>
<td>CCCCCGTACCTACCTCAACGACACGCGCTGGTGGTTCTAGGACACGAGGCTCGGGGG</td>
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*Restriction sites are underlined.
†This primer carries a single nucleotide substitution (G→A) that generates the amino acid substitution G→D in the Δ9PvdA(G15→D) protein.

phosphate hydrolysis in permeabilized cells (Michaelis et al., 1983), and it was reported in arbitrary units (Melchers et al., 1999). Periplasmic, cytoplasmic and membrane fractions from *E. coli* cells were prepared as described (Neu & Heppel, 1965), using ice-cold 1 mM MgCl₂ instead of water to reduce the amount of shock-released cytoplasmic proteins (Hantash & Earhart, 2000). Immunoiodotests of PhoA fusion proteins were performed with rabbit anti-PhoA polyclonal antibody (Abcam). Immunodetection was performed with goat anti-rabbit alkaline phosphatase-conjugated polyclonal antibody (Abcam). Protein concentration was determined using the DC protein assay kit (Bio-Rad). Immunoblot analysis was carried out by using Quantity One software and a Gel Doc 2000 CCD camera (Bio-Rad).}

**Generation of the *P. aeruginosa* PAO1 ΔpvdA mutant.** Site-specific excision of the entire *pvdA* coding sequence was performed using a *sacB*-based strategy described by Hoang et al. (1998). Two regions of approximately 800 bp upstream and downstream of the *pvdA* gene were generated by PCR with specific primer pairs (Table 2). Upstream and downstream fragments were digested with appropriate restriction enzymes, and directionally cloned into the *pEX* IIC suicide vector (Hoang et al., 2000). Immuoniodotests of PhoA fusion proteins were performed with rabbit anti-PhoA polyclonal antibody (Abcam). Immunodetection was performed with goat anti-rabbit alkaline phosphatase-conjugated polyclonal antibody (Abcam). Protein concentration was determined using the DC protein assay kit (Bio-Rad). Densitometric measurements were obtained by using Quantity One software and a Gel Doc 2000 CCD camera (Bio-Rad).

**Construction of *pvdA* deletions and point mutations.** The DNA sequences encoding the whole PvdA protein and three N-terminal-deleted PvdA derivatives [Δ9PvdA, Δ9PvdA(G15→D) and Δ30PvdA] were PCR amplified with specific primers containing *BamHI* (sense) and *HindIII* (antisense) restriction sites (Table 2), using *P. aeruginosa* PAO1 genomic DNA as a template. The amplicons were directionally cloned into the same restriction sites of the vector pQE60 (Qiagen), and excised by *Xhol/PvuII* digestion to obtain *pvdA*-deletion derivatives under the control of the T5 promoter provided by pQE60. These DNA fragments were ligated to the compatible *SalI* and *SmaI* sites of the *Escherichia–Pseudomonas* shuttle vector pUCP19 (Schweizer, 1991). Correct frame and sequence were verified by DNA sequencing. The resultant constructs were individually introduced into the *PvdA*-defective *P. aeruginosa* mutant PAO1Δ*pvdA* by transformation.

**Complementation and membrane-association assays.** *P. aeruginosa* PAO1, or *P. aeruginosa* PAO1Δ*pvdA* cells expressing PvdA, Δ9PvdA, Δ9PvdA(G15→D) or Δ30PvdA, were grown to late-exponential phase at 37 °C in DCAA medium (Visca et al., 1993). When necessary, the medium was supplemented with 150 μg carbenicillin ml⁻¹. Pyoverdine production was assayed by absorbance at 405 nm of culture supernatants (Tiburzi et al., 2008) and by UV fluorescence on cetrimide (*Pseudosel*; Acumedia) agar plates. *P. aeruginosa* cells were spheroplasted by the lysozyme/sucrose method (Robles-Price et al., 2004) to release periplasmic proteins. Spheroplasts were then disrupted by sonication, and cell debris was removed by low-speed centrifugation (10 min at 3000 g). Cytosolic and membrane fractions were separated by ultracentrifugation at 55 000 g for 2 h at 4 °C. The specificity of subcellular fractions was verified by measuring relative isocitrate dehydrogenase and lactate dehydrogenase activities, as previously described (Tiburzi et al., 2008). Separation was considered acceptable when subcellular fractions showed less than 2% cross-contamination between enzyme markers of the different fractions. Immunoblot analysis was carried out by using Quantity One software and a Gel Doc 2000 CCD camera (Bio-Rad).
out using the mouse anti-PvdA monoclonal antibody 3H6D12 raised against the C-terminal region of PvdA (Putignani et al., 2004), a mouse anti-RpoD monoclonal antibody (Neoclone), and a rabbit anti-XcpY polyclonal serum (Michel et al., 1998).

The nature of PvdA association with P. aeruginosa PAO1 membranes was assessed by means of different chemical treatments. Membrane samples were treated with 1.0 M NaCl, 0.1 M NaOH, 1.5 M urea, 40 % sucrose, 1.5 mM EDTA, 33 mM Tris/HCl (pH 8) and 5 µg lysozyme ml−1. After 10 min on ice, and 10 min at 37 °C, spheroplasts were stabilized by the addition of MgCl2 at 10 mM final concentration. Spheroplasts were then incubated for 1 h on ice in the presence and absence of 50 µg proteinase K ml−1. PMSF (2 mM) was added to terminate the reaction, and proteins were precipitated with 10 % trichloroacetic acid, and suspended in SDS-PAGE loading buffer (Sambrook et al., 1989), while proteins in the supernatants were precipitated with 10 % trichloroacetic acid, and suspended in SDS-PAGE loading buffer.

**Proteinase K accessibility assay.** Proteinase K accessibility experiments were performed as described (Arts et al., 2007). Briefly, P. aeruginosa PAO1 cells were grown to late-exponential phase at 37 °C in DCAA medium. Cells were spheroplasted by incubation in ice-cold 40 % sucrose, 1.5 mM EDTA, 33 mM Tris/HCl (pH 8) and 5 µg lysozyme ml−1. After 10 min on ice, and 10 min at 37 °C, spheroplasts were stabilized by the addition of MgCl2 at 10 mM final concentration. Spheroplasts were then incubated for 1 h on ice in the presence and absence of 50 µg proteinase K ml−1. PMSF (2 mM) was added to terminate the reaction, and proteins were precipitated with 10 % trichloroacetic acid, and suspended in SDS-PAGE loading buffer.

**RESULTS**

**PvdA hydrophobicity, TM domain and secondary structure predictions**

Hydropathy plots of the PvdA amino acid sequence, computed by both Kyte–Doolittle (Kyte & Doolittle, 1982) and Hopp–Woods (Hopp & Woods, 1983) algorithms, showed a highly hydrophobic peak encompassing amino acid residues 10–30 (Fig. 1a). Analyses with TMpred, TMHMM, DAS and ConPred II algorithms predicted a single putative TM domain overlapping this hydrophobic region (Fig. 1b, and data not shown), although this showed a moderate probability score with TMHMM. Analysis of the residues flanking the predicted TM region highlighted a net negative charge (−1) on the left site (D12) and a zero charge (E31 plus R32) on the right site. This feature could represent an extension of the positive inside rule (von Heijne, 1992), thus resembling protein behaviour in eukaryotes in which cytoplasmic location is conferred by the more positive flanking sequence rather than by a positive charge per se (Goder & Spiess, 2001). Indeed, the Nout/Cin TM orientation for this presumptive TM domain was strongly suggested by TMpred (Fig. 1b) and ConPred II (the latter probed against eukaryotic databases). The PvdA secondary structure was predicted by the Jpred PSIBLAST algorithm, following multiple alignment with putative or confirmed hydroxylase/monooxygenase homologues (supplementary Fig. S1, available with the online version of this paper). All homologues belong to pyridine nucleotide-disulphide oxidoreductase (Pfam00070) or flavin-binding monooxygenase-like (Pfam00743) families, which are members of the FAD/NAD(P)H-binding Rossmann fold superfamily. The multiple alignment showed the highest amino acid conservation over an extended N-terminal region of approximately 110 residues (10–120 aa relative to PvdA) and over two shorter amino acid stretches located in the central and C-terminal regions (210–225 and 345–356 aa relative to PvdA, respectively). Solvent accessibility algorithms predicted the longest stretch of contiguous buried residues at the PvdA N terminus, overlapping the FAD signature (Fig. S1), and corroborated the inferred high hydrophobicity of this region. Finally, PvdA secondary structure predictions highlighted two β-strand folds (residues 9–46 and 210–240), followed by three consecutive β-strands (Fig. S1); this structure resembles a variant of the typical mononucleotide-binding fold (Dym & Eisenberg, 2001).

**Topogenenic determinants of PvdA**

The presence of possible topogenenic determinants in the PvdA amino acid sequence was investigated by means of PhoA fusions (Melchers et al., 1999). PvdA coding regions were...
fused in-frame with a truncated phoA gene encoding a PhoA enzyme lacking the native signal peptide. The eventual presence of topogenic determinant(s) in the fused protein fragment may target PhoA, lacking a signal peptide sequence, across the inner membrane to the periplasm, where PhoA folds and becomes enzymically active (Manoil et al., 1990).

Based on computational analyses, we fused the PhoA reporter enzyme, lacking a signal peptide sequence, with eight different N-terminal fragments of PvdA extending from position 1 to positions 12, 22, 31, 40, 210, 220, 240 and 443 (Fig. 2a). The resulting fusion proteins were named 12P, 22P, 31P, 40P, 210P, 220P, 240P and 443P, respectively (Fig. 2a, Table 1). The enzymic activity of each fusion protein was measured in E. coli LMG194, as summarized in Fig. 2(a). The highest PhoA activity value was observed for the 12P fusion, whereas the other fusions showed activities comparable with the PhoA enzyme, lacking a signal peptide sequence (i.e. 22P, 31P and 40P), with the host strain (i.e. 210P, 220P, 240P and 443P) (Fig. 2a). The expression level of each PhoA fusion protein was determined by immunoblot analysis of E. coli LMG194 whole-cell lysates with an anti-PhoA antibody. All fusion proteins showed the expected molecular mass, indicating that none was processed by the heterologous E. coli host (Fig. 2b). However, expression levels were variable and extremely low for 12P (Fig. 2b). When PhoA activities were normalized by the protein expression levels, as measured by densitometric analysis, the relative PhoA activity of the 12P fusion increased by at least 40-fold with respect to all the other fusions (data not shown). To further assess the subcellular localization of fusion proteins, cell fractionation experiments were performed. Immunoblot analysis with the anti-PhoA antibody on E. coli LMG194 subcellular fractions showed that 22P, 31P, 40P, 210P, 220P, 240P and 443P localized in the membrane fraction, while only 12P was released in the periplasmic fraction (Fig. 2c). As expected, the PhoA control, lacking a signal peptide sequence, was detected in the cytosolic fraction (Fig. 2c). These results indicate that the first 12 residues of PvdA drive the export of PhoA across the inner membrane of E. coli, and that a minimum of 22 residues at the PvdA N terminus is able to retain PhoA on the cytosolic leaflet of the membrane. Overall, topological studies in the heterologous host E. coli suggested that one single N-terminal domain anchors PvdA to the inner membrane, with the bulk of the protein plausibly exposed to the cytosol.

Fig. 2. Enzymic activity and expression profile of PvdA–PhoA fusion proteins. (a) Linear representation (not to scale) of PvdA and PvdA–PhoA fusions. FAD, NADPH and LATGY domains are represented as white, light grey and dark grey boxes, respectively, and their amino acid positions are indicated in full-length PvdA. The PhoA, lacking a signal peptide sequence (grey ellipse), and the E. coli LMG194 host strain were included as negative controls. The histogram shows mean PhoA activity values (+ SD) of three independent experiments. (b) Coomassie Blue staining (upper panel) and immunoblot analysis (lower panel) of cell extracts (5 and 10 μg protein, respectively) from E. coli LMG194 cells expressing different PvdA–PhoA fusions with the anti-PhoA antibody. (c) Cell fractionation immunoblot analysis from E. coli LMG194 cells as reported in (b). A 3 μg quantity of protein for whole-cell extract (W), periplasmic (P), cytosolic (C) and membrane-bound (M) fractions was loaded onto gels. Molecular mass standards (kDa) are indicated on the left. The immunoblots are representative of three experiments showing similar results.
Membrane association of PvdA

To verify the ability of PvdA to associate with the membranes in vivo, we performed experiments of cell fractionation in the homologous P. aeruginosa host. Bacteria were grown in iron-depleted DCAlA medium to induce PvdA expression. Cells were spheroplasted to release the periplasmic protein fraction, and the resulting spheroplasts were lysed by sonication. Soluble (cytosolic) and membrane proteins were separated by ultracentrifugation. Isovolumes of each fraction (or appropriate dilutions) were resolved by SDS-PAGE to determine the distribution of PvdA between the cytosolic and membrane compartments of P. aeruginosa cells. Immunoblot analysis with an anti-PvdA monoclonal antibody showed that almost the whole cellular pool of PvdA co-sedimented with P. aeruginosa membranes, corroborating the membrane localization of PvdA in P. aeruginosa PAO1 cells (Fig. 3a). Detection of the vegetative sigma factor RpoD and the inner-membrane protein XcpY almost entirely in the cytosolic and membrane fractions, respectively, confirmed the selectivity of the fractionation procedure (Fig. 3a).

To investigate the biochemical nature of the membrane association of PvdA, membranes from P. aeruginosa PAO1 were treated with chemical agents that disrupt different types of protein bonding with membranes (Fig. 3b). PvdA was released from membranes by alkali (0.1 M NaOH) and denaturant (1.5 M and 5.0 M urea), both of which solubilize peripheral membrane proteins, while allowing integral membrane proteins to remain anchored to the lipid bilayer (Rosenberg, 2005 and references therein). High salt (1.0 M NaCl) treatment, which is known to affect protein–protein interactions (Rosenberg, 2005), caused only partial solubilization of PvdA from membranes. In contrast, the integral inner-membrane protein XcpY was not solubilized by any of these treatments, confirming that membrane integrity was preserved upon alkali, urea and salt treatments. Finally, both PvdA and XcpY were retrieved in the soluble fraction after treatment of membranes with Sarcosyl (2 %), which is a detergent that selectively disrupts the inner membrane without affecting the stability of the outer membrane (Filip et al., 1973), and this suggests that PvdA is localized at the inner membrane level. Overall, these results indicate that PvdA behaves as a peripheral protein, and rule out any unspecific co-sedimentation of PvdA with the P. aeruginosa membranes.

While in silico topology and PhoA fusion assays suggested that the PvdA N-terminal hydrophobic region (amino acid residues 10–30) could be responsible for PvdA association with membranes, solubilization assays argued against the existence of a canonical TM domain by which PvdA can actually cross the lipid bilayer. To verify whether PvdA is entirely exposed to the cytosolic leaflet of the inner membrane, a proteinase K accessibility assay was performed on spheroplasts obtained from iron-starved P. aeruginosa cells. As expected, treatment of the spheroplasts with proteinase K resulted in substantial signal reduction for the type II bitopic inner-membrane protein XcpY (Fig. 4), which exposes a large region to the periplasmic space (Bleves et al., 1996). In contrast, PvdA was protected from the protease in spheroplasts, while it was completely digested upon treatment of the P. aeruginosa whole-cell lysate with proteinase K (Fig. 4). The SDS-PAGE mobility of PvdA from proteinase-K-treated spheroplasts was identical to that of the native protein from untreated spheroplasts, while it differed from that of the Δ9PvdA deletion derivative, which lacks the first 9 aa at the PvdA N terminus (see below); the Δ9PvdA deletion derivative was used as size control in case of N-terminal proteolysis (Fig. 4).

A dual role for the PvdA N-terminal region

We have previously generated by chemical mutagenesis the pyoverdine defective PALS124 P. aeruginosa mutant, and...
shown that it is unable to produce OHOrn (Visca et al., 1992). We observed by immunoblotting that this mutant expressed a full-length (apparent mass approx. 49 kDa) PvdA under conditions of iron starvation (data not shown), suggesting that PvdAPALS124 could carry an amino acid replacement that compromises its enzymic activity. Sequencing of the whole pvdA gene revealed that PALS124 carries a single mutation at codon 215 (GGC→A) of pvdA, resulting in a single amino acid substitution (G215→A) in the NADPH-binding motif (G214GGQSA219 in wild-type PvdA). This finding corroborates the importance of the G residues in the dinucleotide-binding motif, in line with their role in the formation of the tight turn necessary for protein binding to the pyrophosphate group of FAD and NAD(P)H (Dym & Eisenberg, 2001).

Since the N-terminal region of PvdA appears to be endowed with both structural (membrane-interaction) and functional (FAD-binding) roles, three PvdA deletion derivatives [Δ9PvdA, Δ9PvdA(G15→D) and Δ30PvdA] were expressed in the PvdA-defective P. aeruginosa strain PAO1ΔpvdA. The Δ9PvdA and Δ30PvdA proteins were PvdA derivatives lacking the first 9 and 30 aa at the N terminus, respectively, while the Δ9PvdA(G15→D) protein originated from Δ9PvdA by a single amino acid substitution (G→D at position 15) in the FAD-binding motif (Fig. 5a). The full-length PvdA protein was used as positive control (Fig. 5a). While all proteins were efficiently expressed in PAO1ΔpvdA cells, only PvdA and Δ9PvdA were able to complement the ΔpvdA mutation by restoring pyoverdine production. Conversely, PvdA derivatives deleted of, or mutated in, the FAD signature did not restore pyoverdine production (Fig. 5b). The subcellular localization of these PvdA derivatives was also investigated by means of cell fractionation experiments, and by immunoblot analysis with an anti-PvdA antibody. As shown in Fig. 5(b), both Δ9PvdA and Δ9PvdA(G15→D) sedimentsed with cell membranes as their native counterpart. On the other hand, a large amount of Δ30PvdA was detected in the soluble cytosolic fraction (Fig. 5b), indicating that deletion of the PvdA N-terminal...
hydrophobic region results in a redistribution of the enzyme from the membrane to the cytosolic compartment. Taken together, these results indicate that, although PvdA retains its activity upon deletion of the nine N-terminal residues, it requires a functional FAD-binding motif for the catalytic activity. In addition, the hydrophobic region encompassing amino acid residues 10–30 is essential for membrane association of PvdA, and this confirms the dual role of the PvdA N-terminal domain.

**DISCUSSION**

In past years, pyoverdine biosynthesis has been extensively studied in light of the role of this siderophore in *P. aeruginosa* virulence, initiation of infection and pathogenesis (reviewed by Visca *et al.*, 2007). It is now well established that pyoverdine synthesis relies on four non-ribosomal peptide synthases that direct the synthesis of the peptide backbone, and on at least three accessory enzymes responsible for synthesis of the non-proteinogenic amino acid precursors. Nevertheless, the subcellular localization of pyoverdine biosynthetic enzymes remains uncertain.

Herein, we provide evidence for the membrane-bound nature of PvdA, which is a key enzyme of the pyoverdine biosynthetic pathway (Visca *et al.*, 1994, 2007). Similar to its close homologue lucD (Herrero *et al.*, 1988), PvdA possesses a major hydrophobic region overlapping the putative FAD-binding motif, and *in silico* analyses predicted a single TM domain at the N-terminal region (Fig. 1b) flanked by a charge bias rather than a cytosolic net positive charge typical of bacterial TM domains (von Heijne, 1992). On this basis, the membrane topology of PvdA was experimentally tested by means of a series of PvdA–PhoA translational fusions in the heterologous host *E. coli*. The results indicate that the 12 N-terminal residues of PvdA are able to target the reporter PhoA protein through the inner membrane into the periplasm, while the following hydrophobic residues seem to be responsible for anchoring the enzyme to the inner membrane (Fig. 2). Given the lack of other topogenic determinants, we expect the bulk of the protein to be exposed to the cytoplasm. In view of this, PvdA could have a membrane topology reminiscent of a eukaryotic reverse signal-anchor (type III) membrane protein that translocates its N-terminal end across the membrane in an N<sub>out</sub>/C<sub>in</sub> orientation (Goder & Spiess, 2001). According to this model, PvdA co-sediments with *P. aeruginosa* membranes in cell fractionation experiments, and its N-terminal hydrophobic region appears to be essential for membrane association (Figs 3a and 5b). However, we demonstrated that native PvdA can be released from membranes by chemical treatments that do not affect membrane stability (Fig. 3b), thus excluding the existence of a canonical TM domain in the PvdA N-terminal region. Accordingly, PvdA was resistant to treatment of *P. aeruginosa* spheroplasts with proteinase K (Fig. 4), and this suggests that PvdA lacks a protease-accessible periplasmic domain. Overall, these observations are compatible with the finding that a recombinant soluble form of PvdA can be obtained by hexahistidine tagging the N terminus, but not the C terminus (Ge & Seah, 2006; Meneely & Lamb, 2007), and this suggests that the hexahistidine tag could provide the N-terminal domain with a hydrophilic character capable of counteracting its intrinsic hydrophobicity.

Most membrane proteins span the bilayer with long α-helical stretches. However, the predicted secondary structure of the PvdA N-terminal region consists of a short β-sheet (from H<sup>11</sup> to V<sup>16</sup>) and an α-helix (from S<sup>21</sup> to Q<sup>30</sup>) separated by two residues generally involved in α-helical breaking (G<sup>19</sup> and P<sup>20</sup>). P and G residues are widely distributed in TM domains of many integral membrane proteins (Williams & Deber, 1991; Landolt-Marticorena *et al.*, 1993), and their helical propensity is greatly enhanced in lipid bilayers (Li *et al.*, 1996; Deber *et al.*, 1999). Nevertheless, the N-terminal hydrophobic region of PvdA overlaps the putative FAD-binding motif. We showed that this motif is essential for PvdA activity, since a single amino acid substitution in the FAD-binding motif of PvdA abrogated the genetic complementation (i.e. restoration of pyoverdine synthesis) in the PvdA-defective *P. aeruginosa* mutant (Fig. 5b). This confirms the crucial role of the FAD signature in PvdA activity and function.

It is difficult to reconcile the inclusion of a functional FAD signature within a structural membrane-anchoring domain. We can only speculate that the PvdA N-terminal hydrophobic domain interacts with the lipid bilayer by forming a U-shaped or re-entrant loop aided by contiguous G<sup>19</sup>–P<sup>20</sup> residues without actually crossing the membrane. Then, part of the FAD-binding domain may interact with the membrane, concomitantly leaving exposed residues to the intracellular milieu. Hence, the observed export of the 12P translational fusion into the periplasm of *E. coli* cells should plausibly be an artefact due to the lack of constraints otherwise imposed by the downstream structure(s). Notably, a similar cryptic signal peptide unmasked upon fusion to the PhoA reporter enzyme has been detected in the HMWP2 protein of *Yersinia* spp. (Guilvout *et al.*, 1995). In view of this, it is worth noting that the first 9 aa at the N terminus of PvdA are not required for either membrane association or enzymic activity (Fig. 5). The dual role of the PvdA N-terminal hydrophobic domain is very intriguing, and deserves further studies to refine residues responsible for membrane anchoring, and to elucidate the actual secondary structure of this domain.

Overall, the membrane localization for PvdA is biologically meaningful. Since PvdA is involved in iron metabolism, and requires oxygen for oxygenase activity, the membrane location would facilitate oxygen recruitment from the outer environment, and thereby contribute to the maintenance of a reducing intracellular milieu. On the other hand, the putative substrate-binding pocket (LATGY) would be localized on the cytoplasmic side, and as result it would be accessible to the substrate (Orn). To the best of our knowledge, in *P. aeruginosa*, OHOrn is exclusively
utilized for pyoverdine biosynthesis. Moreover, because of its high instability at neutral pH (Akers & Neilands, 1973), cytoplasmic OHOrn should promptly be formylated and/or sequestered into the nascent pyoverdine backbone by the pyoverdine biosynthesis machinery. In this scenario, our work provides the basis for future studies aimed at investigating the subcellular localization of the whole pyoverdine biosynthesis machinery.

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REFERENCES


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