The Crystal Structure of Mitochondrial (Type 1A) Peptide Deformylase Provides Clear Guidelines for the Design of Inhibitors Specific for the Bacterial Forms*

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Peptide deformylase (PDF) inhibitors have a strong potential to be used as a new class of antibiotics. However, recent studies have shown that the mitochondria of most eukaryotes, including humans, contain an essential PDF, PDF1A. The crystal structure of the Arabidopsis thaliana PDF1A (AtPDF1A), considered representative of PDF1As in general, has been determined. This structure displays several similarities to that of known bacterial PDFs. AtPDF1A behaves as a dimer, with the C-terminal residues responsible for linking the two subunits. This arrangement is similar to that of Leptospira interrogans PDF, the only other dimeric PDF identified to date. AtPDF1A is the first PDF for which zinc has been identified as the catalytic ion. However, the zinc binding pocket does not differ from the binding pockets of PDFs with iron rather than zinc. The crystal structure of AtPDF1A in complex with a substrate analog revealed that the substrate binding pocket of PDF1A displays strong modifications. The S1' binding pocket is significantly narrower, due to the creation of a floor from residues present in all PDF1As but not in bacterial PDFs. A true S3' pocket is created by the residues of a helical CD-loop, which is very long in PDF1As. Finally, these modified substrate binding pockets modify the position of the substrate in the active site. These differences provide guidelines for the design of bacterial PDF inhibitors that will not target mitochondrial PDFs.

In all organisms, the protein synthesis machinery requires newly synthesized peptides to start systematically with methionine. In eu-bacteria, mitochondria, and chloroplasts, the N-terminal methionine is N-formylated. This is due to the initiator methionyl-tRNA, the methionine moiety of which must be formylated by a formyltransferase. However, the N-terminal methionine is removed by a methionine aminopeptidase (MAP; EC 3.4.11.18) in about two-thirds of mature proteins (for a review, see Ref. 1). Methionine aminopeptidase activity is essential for cell growth in bacteria, fungi (1), and higher eukaryotes (2, 3). However, methionine aminopeptidase cannot cleave N-formylated peptides (4). The N-formyl group (Fo)3 is removed by peptide deformylase (PDF; EC 3.5.1.88). Fo removal is therefore an essential first step in N-terminal processing.

PDFs behave as monomeric metal cation hydrolases (for a review, see Ref. 5). Amino acid sequence alignments for PDFs from various eubacteria have identified three conserved regions, comprising the catalytic pocket of the enzyme and involved in metal cation and substrate binding: (i) GdGdAXXQ (motif 1), (ii) EGCLS (motif 2), and (iii) HE6DH (motif 3), where φ is a hydrophobic amino acid (6, 7). The Cys of motif 2 and the two His residues of motif 3 are involved in metal ion binding in the active site (8). The fourth ligand of the tetrahedrally coordinated metal ion corresponds to a water molecule. Bacterial PDFs are mononuclear iron enzymes (9, 10). It is noteworthy that this is in contrast to most metalloproteases with a conserved HEXXH motif (part of motif 3 in PDFs), which use zinc instead of iron as catalytic cation (for a review, see Ref. 11). The ferrous cation of PDF is extremely sensitive to oxidation, making the enzyme unstable (9, 12). This cation can be replaced by Ni2+ (9, 13) or Co2+ (14) to give stable variants with little or no loss of catalytic activity. Zinc substitution results in inactive enzymes. A detailed discussion on the topic of metal cation activation of PDF was published recently (5).

PDF folds into a single compact domain consisting of a five-stranded antiparallel β-sheet, a two-stranded β-ribbon, two regular α-helices, and two short 3₁₀ helices (for a review, see Ref. 5). Two types of bacterial PDF, PDF1 and PDF2, have been described based on sequence and structural data (TABLE ONE) (5, 15). These two types can be differentiated on the basis of two insertion sequences around helix α₁ and their C-terminal domain folds (16). However, these differences do not affect folding of the active site. We and others have reported the three-dimensional structures of PDFs from various bacteria, in complex with the catalytic metal cation and/or with a ligand, such as an inhibitor or a product of the deformylation reaction (17–19). No significant differences in overall or active site structure were observed between the two forms. The determination of these structures led to identification of the metal and peptide binding modes and elucidation of a catalytic mechanism for the deformylation reaction (13, 18, 19).

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3 The abbreviations used are: Fo, N-formyl; PDF, peptide deformylase; AtPDF1A, A. thaliana PDF1A; EcPDF, E. coli PDF; PePDF, peptide deformylase inhibitor(s); r.m.s., root mean square; HsPDF1A, Homo sapiens PDF1A; PEG, polyethylene glycol; UPDF, L. interrogans PDF; Mes, 4-morpholineethanesulfonic acid; MKE, monomethyl ether.
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For 3 decades, it has been widely accepted that eukaryotes have no peptide deformylase (20, 21). Since this enzyme is essential in bacteria, bacterial PDF was therefore recognized as an attractive target for the design of new antibacterial agents (5, 21, 22). Since PDF was also shown to be present in several human parasites, such as the causative agent of malaria, it was also considered a good target for antiparasitic agents (23). The structural comparison of several PDFs in complex with an inhibitor has provided guidelines for the design of high affinity PDF inhibitors (PDF-In) (16, 19). Actinonin, a pseudotripeptide hydroxamate derivative, has been shown to target peptide deformylases specifically and therefore to have natural antibiotic activity (24, 25). Many actinonin derivatives have been synthesized and shown to display potent antibiotic activity (26). Phase I clinical studies were recently completed for two such potent peptide deformylase inhibitors derived from actinonin (27–29), which have now gone on to phase II and III trials. PDF-In therefore appear promising as a class of new antibiotics.

Recent studies, together with the release of complete genome sequences for differentiated organisms, have led to the identification of peptide deformylases in eukaryotes; these enzymes are targeted to the plastids and mitochondria of plants and to animal mitochondria (15, 30). Two PDFs have been identified in plants and one in humans. Since they do not contain the two insertions typical of PDF2 molecules, all eukaryotic PDFs are unambiguously of type 1 (PDF1; TABLE ONE). However, the amino acid sequence of the PDF specific to mitochondria differs from those of other PDFs in a number of specific features. There is a long insertion between \( \beta \)-strands \( \beta_1 \) and \( \beta_2 \) and systematic amino acid changes in the vicinity of the active site (see an alignment in Ref. 31). PDF1 molecules therefore form two classes: PDF1A and PDF1B (TABLE ONE). PDF1Bs correspond to bacterial and plastid PDFs, whereas PDF1As correspond to mitochondrial PDFs. Unlike PDF1B, which is specific to plants and Apicomplexa, PDF1A is found in almost all eukaryotes. Both classes of PDF1 are functional in deformylation and potently inhibited by actinonin (31, 32). Deformylation has been shown to be an essential process in eukaryotes (33, 34). Since PDF1As are very sensitive to actinonin and probably also to most of the derivatives currently in clinical trials, this necessity for deformylation represents a major obstacle to the use of PDF-In as antibiotics. Indeed, the inhibition of mitochondrial PDF by PDF-In could be extremely toxic in the medium term, as reported for other antibiotics with a mitochondrial target. Unlike eukaryotic PDF1Bs, which do not differ significantly from bacterial PDFs in terms of their biochemistry (32) or three-dimensional structures (35), PDF1As have a number of specific features. They are active and stable with a zinc cation bound at the active site (TABLE ONE). Enzymatic studies have also shown that PDF1As from plants and animals differ from bacterial PDFs in a number of ways. We investigated these differences, with a view to developing guidelines for the design of PDF-In devoid of anti-PDF1A activity (31, 32). As actinonin derivatives inhibit cell proliferation by inhibiting human PDF1A (HsPDF1A), HsPDF1A has been proposed as a target for new anticancer agents (34). It is therefore important to determine the three-dimensional structure of PDF1A.

We describe here the structure of the representative PDF1A, that from *Arabidopsis thaliana*, free and in complex with the tripeptide Met-Ala-Ser. Our findings provide guidelines for the design of PDF-In specific for bacterial enzymes.

**EXPERIMENTAL PROCEDURES**

Protein Expression and Purification—AtPDF1ADH is a 197-amino acid-long protein. It corresponds to full-length AtPDF1A (259 residues) deleted of residues 2–68 and 258–259 (i.e. 191 residues). AtPDF1ADH
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 displays an artificial Ser residue followed by a His6 tag fused to the C-terminal Gly. AtPDF1AΔH was expressed from derivative-containing plasmid (pQdefAtD was described in Table 3 of Ref. 30). Bacteria expressing AtPDF1AΔH were cultured to an A$_{600}$ of ~0.5 at 20°C in 1 liter of Luria-Bertani medium supplemented with 50 µg/ml ampicillin, as previously described (Fig. 2 of Ref. 30). Cells were induced at 20°C by incubation with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside overnight. The cells were harvested by centrifugation (15 min, 6000 rpm) and resuspended in 25 ml of lysis buffer (20 mM Hepes buffer, pH 7.5, 0.1 M NaCl). We added 100 µM lysozyme and 10 mM β-mercaptoethanol and incubated the cells for 15 min at room temperature. The sample was subjected to sonication, and cell debris was removed by centrifugation (30 min, 15,000 rpm). The supernatant (~30 ml) was applied to a 5-ml Hi-Trap chelating HP (Amersham Biosciences) nickel affinity column equilibrated in buffer A (20 mM Hepes buffer, pH 7.3, 0.5 M NaCl). The sample was eluted with a linear gradient from 0 to 40% of buffer B (20 mM Hepes buffer, pH 7.3, 0.5 M NaCl, 1 M imidazole) in 10 column volumes. This purification step was carried out at room temperature, with the buffers on ice. Samples of the collected fractions were analyzed by SDS-PAGE in 14% acrylamide gels. The buffer of the pooled purified AtPDF1AΔH preparation was exchanged using a PD-10 desalting column (Amersham Biosciences) to give a solution of protein in 20 mM Hepes buffer, pH 7.5, 0.1 M NaCl. The protein was concentrated on a Centriprep YM-10 and then on a Microcon YM-10 centrifugal concentrator (Millipore Corp.). The resulting AtPDF1AΔH preparation was frozen in aliquots and stored at ~80°C. The typical yield was 10–15 mg of AtPDF1AΔH/liter of culture. 15N-Labeled AtPDF1AΔ was produced in culture and purified as previously described (17, 30).

Protein Analysis—Protein concentration was monitored spectrophotometrically, using a theoretical extinction coefficient of 12,210 M⁻¹ cm⁻¹. We carried out a coupled assay of peptide deformylase and formate dehydrogenase activity, as previously described (36).

Amino acid sequence was determined by Edman degradation with a gas phase sequencer (Applied Biosystems model 492). The phenylthiohydantoin amino acid derivatives generated at each sequencing cycle were identified and quantified online with an Applied Biosystems model 140C high pressure liquid chromatography system, using the data analysis system for protein sequencing from Applied Biosystems model 610A (software version 2.1). The phenylthiohydantoin-derivative standard kit (PerkinElmer P/N 0310040) was used and reconstituted according to the manufacturer’s instructions (900776 Rev D). The procedures and reagents used were as recommended by the manufacturer. Chromatography was used to identify and quantify the amino acid derivative removed at each sequencing cycle. Retention times and integration values of peaks were compared with the chromatographic profile obtained for a standard mixture of amino acid derivatives.

Gel permeation experiments were performed on a Superdex75 HR 10/30 column (Amersham Biosciences) equilibrated with 20 mM Hepes, pH 7.5, 0.1 M NaCl. Samples with a protein concentration of 2 or 10 mg/ml were centrifuged (10 min, 14,000 rpm at 4°C), and 200 and 100 µl respectively, was loaded onto the column, which had been calibrated with molecular weight markers (Amersham Biosciences). Proteins present in the eluted fractions were analyzed and characterized by SDS-PAGE.

Crystallization, Soaking Experiments, and X-ray Diffraction—Crystallization conditions were screened by a robot, using the sitting drop vapor diffusion method. Two different crystallization conditions were obtained and optimized at 20°C: (i) 16% polyethylene glycol (PEG) 5000 MME, 0.1 M MES buffer, pH 5.5; (ii) 12% PEG 6000, 0.1 M MES buffer, pH 5.5. Crystals were obtained in a hanging drop formed by a 1:1 mix-

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TABLE TWO

Crystallographic data and refinement statistics

| Values in parentheses are for the outer resolution shell. |
|---------------------------------------------------------|
| **Crystallographic conditions** | **PEG 5000 MME** | **PEG 6000** | **PEG 5000 MME** |
| Ligands | | | |
| Ligand used for soaking | | | Fo-Met-Ala-Ser |
| Ligand observed | | | Met-Ala-Ser |
| Data | | | |
| Wavelength (Å) | 0.979 | 0.920 | 0.978 |
| Resolution (Å) | 50.0–2.8 (2.97–2.80) | 50.0–2.9 (3.07–2.90) | 50.0–3.0 (3.18–3.00) |
| Space group | P2₁2₁2₁ | P2₁2₁2₁ | P2₁2₁2₁ |
| Unit cell (Å) | \(a = 51.2, b = 73.8, c = 109.4\) | \(a = 51.5, b = 76.8, c = 109.3\) | \(a = 51.6, b = 76.3, c = 109.3\) |
| No. of protein subunits per asymmetric unit | 2 | 2 | 2 |
| Measured intensities | 51,694 | 52,465 | 63,340 |
| Unique reflections | 10,552 | 10,137 | 9,219 |
| Completeness (%) | 99.0 (94.8) | 99.3 (96.8) | 99.2 (95.1) |
| R𝟏(%) | 13.2 (5.9) | 21.2 (6.0) | 17.9 (5.0) |
| Rvironments (%) | 10.5 (35.0) | 5.0 (22.1) | 9.4 (41.5) |
| Rwork (%) | 23.0 | 24.3 | 22.0 |
| Rfree (%) | 28.4 | 28.3 | 24.7 |
| Mean B factor (Å²) | | | |
| Mol A | 26.5 | 65.9 | 49.5 |
| Mol B | 27.9 | 68.1 | 51.8 |
| r.m.s. deviation from ideal geometry | | | |
| Bond angles (degrees) | 1.432 | 2.225 | 1.849 |
| Ramachandran statistics | | | |
| Most favorable (%) | 86.9 | 88.7 | 86.4 |
| Allowed (%) | 13.1 | 14.3 | 13.6 |
| Generously allowed/Disallowed (%) | 0.0/0.0 | 0.0/0.0 | 0.0/0.0 |
| Protein Data Bank accession code | 1ZXZ | 1ZX0 | 1ZX1 |

* Values in parentheses are for the outer resolution shell.

mature protein (without the His tag) was defined, excluding the N-terminal methionine, which was not visible on electron density maps. This residue was detected in the protein sample by N-terminal Edman sequence determination. Two of the six C-terminal histidine tags could be identified in the structural model. Molecules A and B of the two models were superimposed, giving a root mean square (r.m.s.) deviation of less than 0.6 Å for 100% of the Cα positions. Molecules A and B from the second model (i.e. crystal grown in PEG 6000) were also superimposed on molecule A from the first model (i.e. crystal grown in PEG 5000 MME), and no significant differences were found between the two models (r.m.s. deviation less than 0.4 Å for 100% of the Cα positions).

AtPDF1A\(\text{H}\) has a folding pattern essentially identical to that of other known PDF three-dimensional structures (supplemental Fig. S1). The protein is composed of four α-helices, nine β-strands, and four 3_10 helices, as shown by DSSP (48). Helix \(\alpha_1\) is central in the compact structure and is surrounded by the nine β-strands, organized into three antiparallel β-sheets. This helix plays a key role in the function of the protein, since it carries the conserved HEXXH motif 3 (HECDH\((\text{I})\); homologous to residues \(^{129}\)HEMDH\(^{136}\) in E. coli PDF (EcPDF)), which is critical for metal coordination and substrate activation (8). The other two conserved motifs (motif 1 (\(^{157}\)GVGLAAPQ\(^{154}\); \(^{43}\)GIGLAAATQ\(^{50}\) in EcPDF) and motif 2 (\(^{109}\)EGCLS\(^{113}\); \(^{88}\)EGCLS\(^{95}\) in EcPDF)) are arranged next to each other in space and define the active site crevice. The Gln of motif 1 (Gln\(^{43}\); Gln\(^{50}\) in EcPDF), which is crucial for catalysis, is located on the second 3_10 helix, as observed in other PDFs (16, 49, 50). Two of the four 3_10 helices are located in the unstructured N- and C-terminal regions.

Comparison of AtPDF1A with Class 1B and with Type 2 Peptide Deformylases—About 30 three-dimensional structures of PDFs from different bacterial species and of PDF1B from the unicellular eukaryotic parasite Plasmodium falciparum (see Refs. 35 and 51) are currently available. We therefore compared these sequences with that of AtPDF1A\(\text{H}\) (for a review, see Ref. 5). Type 2 PDFs differ from type 1B PDFs (including P. falciparum PDF) in having two sequence insertions in the N-terminal part of the protein (Fig. 1A) (16). The C-terminal region of PDF1Bs is folded as an α-helix, whereas the hydrophobic C-terminal region of type 2 PDFs consists of a loop followed by a β-strand (Fig. 2). The C-terminal regions of type 1 and 2 PDFs run in opposite directions. PDF1As, as shown by this study of the enzyme from A. thaliana, differ significantly from both types of bacterial PDFs in having a long insertion between β-strands \(\beta_5\) and \(\beta_6\) (residues 68–87 of AtPDF1A; Fig. 1A). This 20-residue insertion folds into a loop containing two small α-helices that fold back on themselves, burying the opening of the active site, which is otherwise largely open (Fig. 2). Closing of the substrate-binding site has also been observed in the L. interrogans PDF (LiPDF; see Ref. 40). The second major structural difference between bacterial and plant PDFs concerns the C-terminal region. This region in plant PDFs is not structured like its counterpart in bacterial PDFs (i.e. type 1B or type 2) and lies in a third, intermediate position (Fig. 2). This orientation is similar to that of LiPDF, although the C-ter-
minal domain of LiPDF is shorter. The three classes of PDFs (PDF1As, PDF1Bs, and PDF2) therefore differ in terms of both sequence and structure.

Quaternary Structure—All crystal forms of AtPDF1A/H9004 contain two molecules per asymmetric unit (TABLE TWO). The two monomers are linked by a noncrystallographic 2-fold axis (supplemental Fig. S2). The dimerization surface of AtPDF1A/H9004 involves two types of interactions: (i) a strong hydrophobic patch, including residues Val114, Phe117, Ala119, Leu162, Tyr163, Val164, Met165, and Phe172; (ii) a network of hydrogen bonds or salt bridges, involving residues Ser9, Asp159, Asn161, Asp165, Arg170, Thr174, and Asn177. Most of these residues belong to the C-terminal region (Fig. 1), and are conserved (i.e. identical or similar side chains) in all plant PDF1As (Fig. 1A; see also Ref. 30). In the dimer, subunit contacts bury 1060 Å²/monomer, corresponding to 10% of the solvent-accessible surface area.

We investigated whether A. thaliana PDF1AΔH behaved as a dimer in solution by carrying out size exclusion chromatography with protein samples of different concentrations. In each case, AtPDF1AΔH eluted

FIGURE 1. Alignment of PDF sequences. A, eukaryotic mitochondrial PDFs from higher plants (PDF1As; A. thaliana and Lycopersicon esculentum) are compared with bacterial type 1 (PDF1Bs; E. coli and Pseudomonas aeruginosa) and type 2 (PDF2s; Staphylococcus aureus and Bacillus stearothermophilus) PDFs. As previously described (16), this sequence alignment is based on the superimposition of structures. The AtPDF1A secondary structures at the top were predicted by DSSP (48). B, comparison of A. thaliana and L. intermedium PDF sequences. C, alignment of the A. thaliana and human PDF sequences. Motifs 1 (47GVGLAAPQ54), 2 (109EGCLS113), and 3 (153HECDH157) of AtPDF1A are indicated. The blue frames indicate conserved residues, white characters in red boxes indicate strict identity, and red characters in white boxes indicate similarity. This figure was created with ESPript (60).

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from the column as a single peak, corresponding to an apparent molecular mass of 42 ± 3 kDa (Fig. S2C). NMR with 15N-labeled AtPDF1AΔH showed that the associated line width is fully consistent with a molecule of 40 ± 3 kDa (data not shown). Our data strongly suggest that AtPDF1A behaves as a dimer. The unusual PDF from the pathogenic bacterium L. interrogans (LiPDF) is the only other PDF reported to behave as a dimer in solution (40, 52). Superimposition of the three-dimensional models of AtPDF1AΔH and LiPDF (Protein Data Bank entry 1Y6H) showed dimerization binding to be very similar in the two molecules (Fig. S2A). In each case, the dimer interface is strongly hydrophobic, involving some structurally conserved and homologous aromatic residues. However, if the A molecules of AtPDF1AΔH and LiPDF are superimposed (with a r.m.s. deviation of about 1.4 Å for 162 Cα atoms), the B molecules are rotated by ~10°. This movement between monomers A and B results in a minor change in protein quaternary structure that may be induced by crystal packing. Finally, in the LiPDF structure, single “closed” and “open” substrate-binding pockets were observed in the two monomers (40). However, these two conformations were not observed in the AtPDF1AΔH structure.

The Metal Cation-binding Site—AtPDF1AΔH was previously shown to tightly bind a zinc cation, and there is no need to add zinc salts in the buffers to maintain it at the catalytic site during purification (32). The active sites of both subunits of the three structural models of AtPDF1AΔH displayed a high electron density that could reasonably be interpreted as a metal ion by comparison with other known PDFs. X-ray fluorescence studies showed that the metal cation in the active site was a zinc (Fig. S3), as previously demonstrated in solution (32). This Zn2+ ion is tetrahedrally coordinated, by the side chains of Cys111 (the conserved Cys of motif 2, homologous to Cys60 in EcPDF), His153, and His157 (the two conserved His residues of motif 3, homologous to His32 and His138 in EcPDF), with a water molecule as the fourth ligand (corresponding to the water molecule W1 described in Ref. 18). This coordination shell is identical to those of other known PDFs and therefore does not account for differences in enzymatic activity with respect to iron PDFs.

The Substrate-binding Site—Crystals of AtPDF1AΔH grown in PEG 5000 MME were soaked in a solution containing a peptide deformylase substrate analog, Fo-Met-Ala-Ser. Electron density maps revealed that one tripeptide molecule had bound each of the two monomers of the asymmetric unit. However, the tripeptide was clearly no longer N-formylated (Fig. 3A), indicating that the enzyme was active in the crystal.

The overall structure of AtPDF1AΔH in complex with Met-Ala-Ser is almost identical to that of the free enzyme (r.m.s. deviation less than 0.5 Å for 100% of the 190 Cα positions). The N-terminal amino group of the tripeptide forms hydrogen bonds with the side chain of Glu34 from motif 3 (homologous to Glu133 in EcPDF), the carbonyl group of Gly49 from motif 1 (homologous to Gly45 in EcPDF) and a water molecule corresponding to W2 as described by Becker et al. (18). It has been suggested that this water molecule is replaced by the carbonyl oxygen of the substrate formyl group during the enzymatic reaction. The tripeptide is also held in the peptide-binding site by hydrogen bonds formed by the carbonyl and nitrogen amide groups of the main chain of the protein and peptide backbones. However, tripeptide-protein interactions differ from those of the EcPDF-zinc-MAS complex (Protein Data Bank entry 1BS8 in Ref. 18), because the side chain of the third residue (i.e. Ser) is rotated by 80° and shifted by 2.6 Å (Fig. 3B). This results in a shift in the position of the tripeptide in the substrate-binding site of AtPDF1AΔH.

The Met side chain of the tripeptide fits into a hydrophobic pocket (known as S1’ as defined by Ref. 53) formed by Val36, Glu109, Ile150, and His153 (which correspond to Ile64, Glu49, Cys129, and His132 in EcPDF), as previously described (18). Unlike that of bacterial PDFs, the S1’ pocket of AtPDF1AΔH is completed by the side chains of residues Phe107, a residue of strand β8 (located just before motif 2), Trp146, and Arg149 from helix α2. The bulky side chains of Phe107, Trp146, and Arg149 are unique to PDF1As and close the floor of the S1’ cavity, making it significantly narrower than in other enzymes (Fig. 3C). The side chains of residues Phe107 and Trp146 interact via hydrophobic contact. The side chain of Arg149 is buried and its position is maintained by several hydrogen bonds with the side chain of Glu109 from motif 1 and the main chain carbonyls of residues Ala105, Phe107, and Val121. The side chain of Arg149 makes therefore a supplementary salt bridge with the unusual buried salt bridge already involving residue Arg135 with residues Glu109 and
Asp^{156} (corresponding to Arg^{102}, Glu^{88}, and Asp^{135} in EcPDF) in all bacterial PDFs (54). This complex network of interactions forms a very solid and very precisely delimited S1’/H11032 pocket that cannot be deformed by ligand binding. This is in contrast with the S1’ pocket of bacterial PDFs.

The P2’ group of Met-Ala-Ser (Ala side chain) fits into a relatively large, poorly defined S2’ pocket, very similar to that of bacterial type 1 and type 2 PDFs (Fig. 3C). The side chain of Arg^{118} is the only molecule that can force the position of the second residue of the substrate, but the
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FIGURE 4. Stereoview of the substrate-binding site of A. thaliana PDF1A. Tripeptide in complex with AtPDF1AΔH is shown in ball-and-stick format, together with the residues comprising the S1, S2, and S3′ pockets and other important residues of the enzyme. The sticks are shown in yellow, pink, blue, green, and gray, as in Fig. 3C. Solid lines indicate the coordination shell of the metal ion, whereas dashed lines indicate some of the hydrogen bonds observed in the Met-Ala-Ser-AtPDF1AΔH complex.

weak steric environment facilitates conformational changes, making it possible to accommodate any side chain of the substrate. The P3′ group (Ser side chain) fits into a poorly defined, very large S3′ pocket in bacterial PDFs, which can accept all side chains, without true specificity (18). In contrast, AtPDF1AΔH has a more specific, narrow S3′ pocket formed from the side chains of residues Pro46, Val48 (motif 1), Asp66, Tyr70 (which is part of the so-called CD-loop), and Arg84 (Fig. 3C). Bulky substrate side chains can only fit into this pocket if they adopt a very precise position. This pocket may account for the side chain of the third residue (P3′) of the peptide with respect to the EcPDF-MAS complex. In addition, the region formed by residues Tyr73—Glu78 of the third residue (P3′) is shown in yellow, pink, blue, green, and gray, as in Fig. 3C. Solid lines indicate the coordination shell of the metal ion, whereas dashed lines indicate some of the hydrogen bonds observed in the Met-Ala-Ser-AtPDF1AΔH complex.

A detailed view of the substrate-binding site, including all of the aforementioned residues, is shown in Fig. 4.

DISCUSSION

Compared with all currently known bacterial PDF structures, mitochondrial AtPDF1A displays a number of important new properties, at the level of the metal specificity and of the substrate recognition pockets in the active site. This latter feature is conserved in mitochondrial PDFs, including human PDF1A, and thus provides critical information for the design of selective bacterial PDF inhibitors.

Catalytic Mechanism and Importance of the Metal Ion—Comparison of the overall structures of the mitochondrial-specific eukaryotic PDF (AtPDF1A) and bacterial PDFs of types 1B (E. coli) and 2 (B. stearothermophilus) showed conservation of the PDF fold in the bacterial and eukaryotic kingdoms, with the exception of the C-terminal region, which may adopt a number of different structures, such as those in LiPDF (40), PDF1B (7), PDF2s (16), and PDF1A (this study). The active site cavity is also well conserved, especially in terms of metal chelation, and the reaction mechanism has been studied in detail. Based on the observed structural similarities, we suggest that the catalytic mechanism of eukaryotic PDFs is strictly identical to that of bacterial PDFs (13, 18, 19), although PDF1A is highly active as a zinc enzyme, and bacterial PDFs in which the iron is replaced by zinc are inactive.

We have previously shown that PDF1As from higher plants are unique among PDFs in having zinc rather than iron as the catalytic metal ion (32). However, the metal coordination shell and the active site environment in the structure described here are strictly identical to those reported for iron PDFs. It therefore remains unclear why PDF1A from A. thaliana is active with zinc as the cofactor, whereas other PDFs are active with a bound ferrous ion and inactivated with zinc (9, 10, 55). Interestingly, LiPDF also contains a tightly bound zinc ion rather than the ferrous ion usually observed (52). A recent study (56) showed differences in the binding of the metal to formate, one of the two products of the reaction catalyzed by the enzyme. The fourth ligand of the metal is generally a water molecule. This metal-coordinating water molecule is replaced by a formate group in several PDF structures. It has been suggested that, in the presence of iron or cobalt, formate binds to the metal cation in a bidentate fashion, whereas with zinc in the active site, formate binds to the metal in a monodentate fashion. Jain et al. (56) concluded that the metal ion may be penta- or tetracoordinated in the active site at the end of the catalytic reaction. They also suggested that this might explain why bacterial PDFs are active with iron (or cobalt) but not with zinc. However, LiPDF presents the same coordination sphere as inactive bacterial zinc-PDFs but is fully active with a zinc ion (57). We therefore conclude that the model proposed by Jain et al. (56) to explain the weak activity of zinc-EcPDF cannot account for the high activity of the zinc-containing AtPDF1A and LiPDF enzymes.

AtPDF1A Behaves as a Dimer—Our results show that AtPDF1AΔH behaves as a dimer in solution and in the crystal. To date, only one other PDF has been shown to be dimeric, that of L. interrogans (LiPDF). Gong and co-workers (40, 52) were unable to draw firm conclusions concerning the possible biological significance of the dimerization of some peptide deformylases, and our results shed no new light on this question. However, it could be suggested that dimerization, mainly mediated by hydrophobic interactions, may reinforce the hydrophobic environment of the active and substrate-binding sites. Future work should therefore compare the quaternary structures of PDFs by means of several complementary methods.

Modeling of the Three-dimensional Structure of the Human PDF—Humans also have a PDF1A (30, 31). HsPDF1A is very similar to...
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AtPDF1A but exhibits an extended N terminus, a shorter C terminus, and lower identity levels in the CD-loop (Fig. 1C). We modeled the structure of HsPDF1A, based on that of AtPDF1AΔH, using the program Modeller for satisfying spatial restraints (58). Our model predicted that 165 of 191 residues would occupy identical positions with an r.m.s. deviation value of 0.3 Å for the Ca positions. The positioning of most of the key residues in the substrate- and metal-binding pockets was conserved. However, HsPDF1A presents a number of important differences (Fig. 1C): (i) the first Gly of motif 1 is replaced by a Cys; (ii) the first Ala of motif 1 is replaced by a Ser; (iii) the conserved Leu of motif 2 is replaced by a Glu. These residues are involved in the catalytic mechanism, and the substitution between plant and human PDFs accounts for the biochemical differences observed between human PDF and other known PDF1As (31). Indeed, HsPDF1A appears to differ from AtPDF1A in containing iron but is highly unstable and is less active than EcPDF (30, 31). Nevertheless, the narrow S1′ pocket of AtPDF1AΔH is strictly conserved in HsPDF1A (Fig. 5), including the Phe, Trp, and Arg residues forming this pocket in eukaryote PDF1A (Fig. 1C). The S1′ pocket is therefore predicted to be as rigid as that in AtPDF1AΔH. The S2′ and S3′ pockets differ from those of AtPDF1AΔH (Fig. 5). However, HsPDF1A presents the long insertion specific to mitochondrial PDFs.

Impact of the Three-dimensional Structure of PDF1A on the Design of New PDF Inhibitors—It is now widely accepted that there are two types of bacterial PDFs. They differ in sequence and in structure but have similar metal-binding sites. The unique conformation of the C-terminal region of type 2 PDFs, with an additional β-strand replacing the α-helix of type 1B PDFs and running in the opposite direction, decreases constraints on the active site (5, 16). However, bacterial PDFs of both types display strictly conserved modes of binding to actinonin or Met-Ala-Ser. This feature has facilitated the design of PDF-In and their choice as broad spectrum antibiotics (26). Studies of recently identified eukaryotic PDFs, which are all of type 1, have shown that PDF1 enzymes should be classified into two subtypes: PDF1As (found in most eukaryotes, specifically in the mitochondria, in humans) and PDF1Bs (i.e. the PDFs from bacteria and from the plastids of eukaryotes; see TABLE ONE). As expected from their biochemical properties and amino acid sequences (30, 32, 59), eukaryotic PDF1Bs have three-dimensional structures (P. falciparum PDF) (35, 51) similar to those of bacterial PDF1As. However, PDF1As differ from PDF1Bs and PDF2s in several biochemical aspects (31, 32). The amino acid sequences of PDF1As also differ significantly from those of bacterial PDF1Fs, as they possess a long CD-loop insertion sequence and are active with a zinc cation rather than a ferrous ion. They also differ from bacterial PDF1Fs in terms of substrate specificity with unusual substrates or certain inhibitors, with a lower level of tolerance in the P1′ and P3′ binding pockets (31, 32). For instance, PDF1As cannot accept bulky side chains such as phenyl groups at P1′ or para-nitroanilide or β-naphthylamide at P3′, whereas PDF1Bs can.

Our structural data confirm this observation, by showing narrowing of the S1′ pocket, and extend these findings to the S3′ pocket. The S1′ pocket of bacterial PDF1F is fully open to the outside, allowing large groups to bind at P1′; there is no true S3′ pocket, and there is no delimitation between the S1′ and the pseudo-S3′ pockets (Fig. 6). Hence, in the complex made between EcPDF and a transition analog mimicking Met-Leu-para-nitroanilide, the para-nitroanilide moiety makes a lid burying the Met mimic side chain into a hydrophobic cleft (19). This effect participates in strongly increasing the binding constant of this compound. In mitochondrial PDF1A, the binding of such compound is prevented by (i) the series of additional residues that block the entry of the S1′ pocket and (ii) the occurrence of a true S3′ pocket (Fig. 6). This information should make it possible to develop new antibiosis, specifically active against bacterial PDFs but with little or no activity against mammalian PDFs.

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