The N-terminal methionine excision (NME) pathway is an essential pathway that removes the initial Met from two-thirds of the proteins of any proteome. Methionine aminopeptidase (MAP; EC 3.4.11.18) has been shown to be involved in this process in all organisms studied, and this activity facilitates subsequent protein modification in Eukaryotes (2, 3). In Eubacteria, plastids, and mitochondria, the N-terminal Met residue of nascent polypeptides carries an N-formyl (Fo) group whereas in Eukaryotes and Archaea, nascent proteins synthesized in the cytoplasm start with a free Met (4). The N-terminal Met must be exposed to allow MAP activity in Eubacteria, and this is achieved by systematic removal of the Fo group by peptide deformylase (PDF; EC 3.5.1.88). PDF activity was long considered to be unique to this kingdom (5). Recent studies have demonstrated that PDF orthologs are produced in most eukaryotes, including animals, plants, and many unicellular organisms (6–8). These orthologs have been shown to display peptide deformylase activity in plants, both in vitro and in vivo. All the eukaryotic orthologs have an extended N-terminal domain. The N-terminal domain targets the proteins to the plastids and mitochondria in plants (7, 9–11). Similarly, it has been suggested that the PDF orthologs of Apicomplexan parasites are targeted to the apicoplast, an essential plastid (8, 12, 13). Actinomycin, a natural antibiotic that specifically inhibits PDF, targets both bacterial and plant PDFs (10, 14). Studies carried out in vivo with this drug have shown that PDF is essential in bacteria and plastids (11, 14, 15). In plants plastids, NME plays a crucial role in controlling the half-life of a major organelar protein complex, photosystem II (10). The effect on photosystem II stability was similar regardless of whether Met alone or Fo-Met was retained.

MAPs are part of a large family of metallo-aminopeptidases (16). Two types of MAPs have been described to date (17). MAP2s occur in Archaea and in the cytoplasm of Eukaryotes, whereas MAP1s have been found in Eubacteria, in the cytoplasm of Eukaryotes (MAP1A) and in the organelles of plants and Apicomplexa (MAP1D, Ref. 7). The sequences of MAP2 and MAP1 have weak similarity but these two enzymes display similar active site folding patterns. Organelar MAP1s have a cleavable N-terminal extension that is not found in bacterial MAP1s. This extension targets the catalytic domain to the correct cell compartment. Cytoplasmic MAP1A enzymes also have an extension including a conserved zinc finger. Although not involved in catalytic activity, this additional domain is not...
removed from the mature form and is essential for cell function, possibly allowing interaction with ribosomes (18). PPDFs constitute a growing family of hydrolytic enzymes related to the thermolysin-metazincin HEXXH motif-containing family of metalloproteases (6). PPDFs contain three distinct short stretches of amino acids (Motifs 1–3; Fig. 1) that constitute the active site (19). Motif 3 contains the HEXXH motif (Fig. 1). In most PPDFs, an Fe^{2+} cation is bound by three residues of motifs 2 and 3 and plays a crucial role in hydrolytic activity (20, 21). This ion is highly unstable, and several agents and procedures have been described that preserve deformylase activity (reviewed in Ref. 6). The iron cation may be replaced by zinc, resulting in a stable but weakly active enzyme. Zinc PPDFs have similar substrate specificities to their iron-coordinated counterparts, but lower catalytic constants (22, 23).

Three types of PPDF have been identified on the basis of structural and large scale sequence analysis (6, 13, 24). The three classes differ in several parts of their three-dimensional structures but their active sites are conserved and entirely superimposable.

Type 2 and type 3 PPDF enzymes are found only in Gram-positive bacteria. In contrast to type 2, type 3 PPDF orthologs differ in several parts of their three-dimensional structures but their active sites are conserved and entirely superimposable. Type 2 and type 3 PPDF enzymes are found only in Gram-negative bacteria, bacterial PDF enzymes are found in Gram-negative bacteria, some Gram-positive bacteria and plants. Eukaryotic class 1B PPDFs are targeted to both plastids and mitochondria (7, 9). The three-dimensional structure and enzymatic properties of the class 1B PPDFs of eukaryotes and bacteria are similar (11, 24, 30). Class 1A PPDFs include plant mitochondrial PPDFs and PDF orthologs from animals (9). Biochemical characterization of plant PDF1As has shown that they differ from most previously studied PPDFs, including PDF1Bs and PDF2s, particularly in terms of the optimal metal cofactor: zinc rather than iron (11).

In this work, we provide compelling evidence that the complete machinery, including specific and functional PDF and MAP1D, is expressed and exported to animal mitochondria. Site-directed mutagenesis data, combined with biochemical and structural analyses, showed that HaPDF differs considerably from previously characterized PPDFs in terms of its properties. These differences could be used as the basis for chemical modifications for improving PDF inhibitors for use in the clinical treatment of bacterial infections in humans.

EXPERIMENTAL PROCEDURES

Materials and Preparation of Solutions

All chemicals, including Tris(2-carboxyethyl)-phosphine (TCEP), and enzymes for protein analysis were purchased from Sigma. The peptides used have been described elsewhere (28, 31). Enzymes for DNA manipulation were purchased from New England Biolabs. Oligonucleotides were synthesized by MWG-AG Biotech. Plasmid DNA was purified with mini- and mid-prep kits (Qiagen). For enzyme purification, we used procedures that preserve deformylase activity (see details in Ref. 23).

To determine the susceptibility to drugs of the tolC strain GIF1 and to determine the susceptibility to drugs of the tolC strain GIF1, we transformed the pBAD construct and selected on Luria-Bertani (LB) medium supplemented with 50 μg/ml ampicillin, 3.4 μg/ml chloramphenicol, and 0.5% glucose. Bacteria were cultured overnight in this medium at 37 °C and the culture was then diluted 1:100 and used to inoculate 5 ml of medium. When the OD_{600} reached 0.9, we diluted the suspension appropriately and layered 2 × 10^{10} of bacteria in 100 μl on 30 ml of solid LB supplemented with 50 μg/ml ampicillin, actinonin (0.1–3 μM), and either glucose (0.5%) or arabinose (0.0002–0.2%) in Petri dishes. The minimum inhibitory concentration was defined as the lowest concentration of actinonin causing no growth after 18 h of incubation at 37 °C.

Cell Biology, Transient Expression of GFP in Homologous and Heterologous Systems

Homologous Studies—Mammalian cell lines were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) with Glutamax-I (Invitrogen) at 37 °C, 5% CO₂. HEK293 cells were plated on poly-L-lysine (Sigma)-coated cover slips 12 h before transfection and cultured in 6-well plates in fresh medium at 37 °C, 5% CO₂. Cells were transfected in the 6-well plates according to the calcium phosphate transfection protocol (CaPhos nucleofection kit, Lonza) with 2 μg of plasmid DNA per well. Cells were washed with phosphate-buffered saline after 7 h to remove plasmid DNA and fixed in 4% formaldehyde/4% sucrose 48 h post-transfection for localization studies. Fixed cells were permeabilized with 0.05% Triton X-100 for 5 min and stained with 4′,6-diamidino-2-phenylindole (DAPI, 15 mg/ml; 1:1000 dilution; Molecular Probes) for nuclear DNA. Coverslips were mounted in Vectashield (Vector Laboratories, Inc. CA) for further examination at the confocal microscope. Cells were imaged by a Leica SP confocal microscope through a ×100 1.4 NA Planapochromat oil immersion objective. eGFP was excited by a 488 line of an Argon laser and RFP by a 543 nm line of Green HeNe laser. In order to avoid bleed-through, the fluorophores were excited sequen-

Bacterial Strains and Plasmids, Molecular Biology

For the sake of clarity, all PDF sequences were numbered similar to the EcPDF sequence, as previously suggested (24). A substitution of residue X in HaPDF or AtPDF1A indicates that the substitution concerns the residue corresponding to amino acid X in EcPDF, as shown in the alignment in Fig. 1. Residues upstream from Ser^{1} of EcPDF are numbered relative to position 1 and take a negative sign.

General Methods—Strain PAL2412T (fmsΔ, galK::prL, recA56, srl-300::Tn10) and CAG1284 (Δ−10, tolC210::Tn10, rph−) have been described elsewhere (32, 33). Strain G1F1 was derived from CAG1284 and contains a chloramphenicol-resistant plasmid, pLYS5, supplying tRNAs for AGG, AGA, AUA, CUA, CCC, and GGA. The human fetus RACE library was purchased from Clontech. HsMAP1D was amplified with one 5′-terminal primer and transformed with the pBAD construct and selected on Luria-Bertani (LB) medium supplemented with 50 μg/ml ampicillin, 3.4 μg/ml chloramphenicol, and 0.5% glucose. Bacteria were cultured overnight in this medium at 37 °C and the culture was then diluted 1:100 and used to inoculate 5 ml of medium. When the OD_{600} reached 0.9, we diluted the suspension appropriately and layered 2 × 10^{10} of bacteria in 100 μl on 30 ml of solid LB supplemented with 50 μg/ml ampicillin, actinonin (0.1–3 μM), and either glucose (0.5%) or arabinose (0.0002–0.2%) in Petri dishes. The minimum inhibitory concentration was defined as the lowest concentration of actinonin causing no growth after 18 h of incubation at 37 °C.
filter. Stacks of conical sections separated by 0.2 μm increments were taken and images analyzed by Metamorph 5.0 software (Universal Imaging Corporation).

Heterologous Studies—We bombarded onion epidermal cells with DNA constructs using the PDS-1000/He instrument (Bio-Rad) as previously described (7). Transient GFP production was examined with an up-right Axioskop 2 imaging fluorescence microscope (Zeiss) with interference contrast (excitation 488 nm; barrier filter, 510 nm). Protein samples (200 μl) consisting of 20 μm sodium phosphate buffer pH 7.3 plus 10 μm 2-mercaptoethanol. The samples were subjected to sonication, and cell debris removed by centrifugation. The supernatant (5–15 ml) was applied to a Hi-Trap chelating HP (0.7 by 2.5 cm; Amersham Biosciences) nickel affinity column equilibrated in buffer B (buffer A plus 0.1 M imidazole). The sample was eluted at a flow rate of 0.5 ml/min in five 15-ml steps, with buffer C (buffer B plus 0.5 M imidazole) at concentrations of 0.17 and 0.5 M imidazole, respectively. The purity of the protein was monitored by SDS-PAGE (13%) and the final yield was 3 mg for a 400 ml culture. The pooled purified protein preparation (5 ml) was first dialyzed against buffer A for 12 h and then against buffer A plus 55% glycerol for 24 h before storage at –20 °C. Protein concentration was measured with the Bio-Rad protein assay kit. Bovine serum albumin was used as the protein standard.

Preparation of Cell Extracts—Exponentially growing human cells were collected and frozen at –80 °C. Cells were then resuspended in 200 μl of 50 mM Hepes (pH 7.5), 150 mM NaCl, 15 mM MgCl2, 1% Triton, 0.17 and 0.5 M imidazole, respectively. The purity of the protein was monitored by SDS-PAGE (13%) and the final yield was 3 mg for a 400 ml culture. The pooled purified protein preparation (5 ml) was first dialyzed against buffer A for 12 h and then against buffer A plus 55% glycerol for 24 h before storage at –20 °C. Protein concentration was measured with the Bio-Rad protein assay kit. Bovine serum albumin was used as the protein standard.

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Immunochemical Methods—The rabbit antisera against A. thaliana AtPDF1A used has been described elsewhere (10). Rabbit antisera against HsPDF were raised at Eurogentech (Herstal, Belgium) and AtPDF1A used has been described elsewhere (10). Rabbit antisera against MmPDF homologs were identified in human and mouse of cDNAs encoding homologs of PDF1A (HsPDF and MmPDF) corresponding to a single gene located on chromosome 16 in human (locus NT_019608; Unigene Hs.130849) and 8 in mouse (locus NT_039467; Unigene Mm.246236, previously Mm.34708). We used the sequences corresponding to HsPDF and MmPDF to screen various metazoan cDNA libraries. We identified and annotated cDNAs encoding HsPDF homologs in most major vertebrates species (4 different homologs in fish, 2 in amphibians, 1 in birds, 3 in mammals). We also identified 4 HsPDF homologs in invertebrates: 3 in insects and one in Hydra magnipapillata (Cnidaria). We identified no PDF homologs in nematodes and fungi. The various sequences were aligned and compared with other PDF sequences (Fig. 1; see “Experimental Procedures” for numbering of PDfs). The various animal PDF sequences were very similar (shown in pink in Fig. 1) and all belonged to the PDF1A class, which includes mitochondrial PDfs from plants and PDfs from Actinobacteria (9).

The occurrence of expressed PDF homologs in most animal genomes suggested that there should be a mitochondrial NME. If this were indeed the case, then we would expect dedicated MAPs to be identified in the same organism. It should be borne in mind that (i) cytoplasmic MAPs (MAP1As) occur in all eukaryotes and have a long N-terminal extension with a zinc finger domain involved in ribosome tagging (18) and (ii) organelar MAPs (MAP1D) have been identified in plants, in Apicomplexa and in Mycetozoa; they resemble MAP1As but have no zinc-finger in their N-terminal domain (7). We used plant organelar MAP1Ds to screen various cDNA libraries and identified MAP1D homologs in vertebrates (humans, pigs, cattle, mice, frogs, fish, and chickens) and in insects. The full-length cDNA for human MAP1D (HsMAP1D) was cloned from a fetal cDNA library. Its ORF (GenBank™AY374142; see “Supplementary Materials” Fig. 1) was very similar to that of a full-length cDNA recently isolated from mouse (AK010067) and identical to that deduced from a single gene identified on human chromosome 2 (locus NT_005348; Unigene Hs.406338). Similarly, a single MAP1D gene was identified in the complete genomes of the fish Fugu rubripes and the mouse Mus musculus (chromosome 2; locus NT_039208; Unigene Mm.27075). All known MAP1D genes map to loci different from those encoding MAP1A homologs (chromosome 4 in human and 3 in mouse). One MAP1 pseudogene was also identified in each of the human (chromosome 12) and mouse (chromosome 9) genomes. We used the full-length MAP sequence and our previously identified MAP1As and bacterial MAPs (classified here as MAP1Bs) to construct a phylogenetic tree (Fig. 2). We identified three main branches: (i) bacterial MAP1Bs, found in all bacterial groups (ii) cytoplasmic MAPs (MAP1A) found in all eukaryotes, as previously reported (7) and (iii) MAP1D, found in most eukaryotes but not in fungi (Ascomycota) and nematodes. Actinobacteria has at least two MAP1Bs: one related to bacterial MAPs (MAP1B) and the other strongly related to MAP1D. This phylogeny is similar to that of PDF1A (see Fig. 1 and Ref. 9). These data suggest that most animal genomes express genes encoding specific machinery for an NME other than the cytoplasmic NME.

Both HsPDF and HsMAP1D Are Located in the Mitochondria—Using the available prediction software at www.expasy.org/tools/, we identified a clear putative mitochondrial targeting signal in the amino acid sequence of HsMAP1D, at position 1–43 (leader peptide LP). We investigated the location of the protein and whether the LP did indeed target the protein to the organelle by fusing sequences encoding the LP and the full-length HsMAP1D to that encoding GFP. The resulting constructs were used to transfet cells in a heterologous expression systems (Fig. 2; see “Experimental Procedures”).
system. Both fused proteins were detected in the mitochondria (Fig. 3C), confirming that the putative sequence LP-HSMAP, identified by consensus structural motifs in the predicted amino acid sequence, is genuinely responsible for protein targeting to mitochondria.

In contrast to MAP1D, the prediction software failed to identify a classical mitochondrial signal peptide in HsPDF. However, such software did identify a consensus mitochondrial localization motif composed of the first ~20 amino acids in all the other mammalian PDFs. We therefore tried to express full-length (FL) and various C-terminal-deleted forms of HsPDF (Fig. 3A) fused to GFP in a heterologous expression system (i) to demonstrate clear mitochondrial location of the protein and (ii) to define the minimal structural motif controlling HsPDF localization. In transient expression studies, FL-HsPDF/GFP and the C-terminal-deleted forms LP4-HsPDF/GFP, LP5-HsPDF/GFP and LP1-LP2-HsPDF/GFP (Fig. 3A) were located primarily within mitochondria (Fig. 3B). In contrast, the LP1-HsPDF/GFP fusion protein, containing the predicted mitochondrial leader peptide clearly displayed diffuse

**Fig. 1.** Alignment of the amino acid sequences of PDFs from animals and comparison with plant PDF1As. The HsPDF and MmPDF sequences (7) were used as input probes for BLAST (59) searches of the data at NCBI. Matching cDNA sequences were compared and aligned if an overlap was detected. The genomic fragments were analyzed with various software packages for the prediction of intron-exon splice sites and amino acid sequence similarity, alignment, and comparison of the cDNAs. The deduced amino acid sequences are shown. Translated sequences were aligned with ClustalX (60) and then by eye. A series of question marks indicates that the corresponding nucleotide sequence is missing. The numbering of each of the three amino acid sequences is indicated below the sequences for each block of 100 residues. Strictly conserved residues within the catalytic core are shown in red or in green for the ligands of the catalytic metal. Residues in pink are conserved in PDF1As.
cytoplasmic staining (Fig. 3B). We therefore concluded that the first 44 amino acids were not sufficient to target the protein to the organelle and that the minimal fragment containing all the information required for the efficient targeting of HsPDF to mitochondria corresponded to the first 69 amino acids (LP1/H11001LP2-HsPDF). We characterized the leader peptide further by investigating whether the region between amino acids 44 and 69 (LP2-HsPDF) was sufficient for correct localization by creating a construct encoding the LP2-HsPDF fragment fused to GFP. In this case, we obtained a cytoplasmic signal (Fig. 3B). Taken together, these data show that the region encompassing at least LP1+LP2-HsPDF is necessary and sufficient for mitochondrial localization.

The results obtained with the heterologous system guided us to set up the localization experiments in a homologous expression system. Human embryonic kidney (HEK) cells were transfected with constructs encoding GFP fusions with (i) the full-length MAP1D and PDF and (ii) the LP-HsMAP1D and the LP1/H11001LP2-HsPDF sequences (Fig. 4). A number of control experiments were carried out in both cases: (i) labeling of the nucleus with DAPI (Fig. 4, A–B) and (ii) cotransfection with a construct encoding a mitochondrial control fused to red fluorescent protein (mt/RFP; Fig. 4C). No bleed-through was observed as confirmed by the absence of colocalization if cells are not cotransfected with mt/RFP (Fig. 4D). This confirmed the colocalization shown in Fig. 4C. Both HsPDF and HsMAP1D were clearly specifically targeted to the mitochondria by their N-terminal extensions, LP-HsMAP1D and LP1+LP2-HsPDF, respectively.

Expression in Escherichia Coli of the Human Peptide Deformylase Homolog, HsPDF—The catalytic centers of animal MAP1Ds resemble those of their counterparts in bacteria and plants (Supplementary Figs. 1 and 16) and is therefore expected to display MAP activity. In contrast, animal PDFs differ from phylogenetically related plant PDF1As in several features in addition to the conserved N-terminal extension (Fig. 1). We decided therefore to investigate the functional impact of these features. Prior to this, purified HsPDF was needed to raise antibodies against it and analyze the protein form accumulating in vivo.

The full-length ORF of HsPDF was fused to a His6 tag in...
various E. coli plasmids. We were unable to detect the protein in either the soluble or the insoluble fraction in all conditions tested. The first 380 base pairs (codons 1–126 of 244, corresponding to amino acids 62 to 64 in HsPDF) of the HsPDF ORF have a G+C content of 77% and include 18 codons corresponding to the rarest tRNAs in E. coli (40). Bacteria overproducing a large number of rare tRNAs were therefore transformed with pET derivatives encoding the HsPDF ORF. Unfortunately, this new system did not increase HsPDF production. We therefore redesigned the HsPDF ORF fragment to create a new sequence encoding the same polypeptide, but with codon usage optimized for E. coli and a lower G+C content and fewer long G+C stretches (see details under “Experimental Procedures”). Time-course analysis of expression of modified HsPDF ORF in E. coli revealed that the 25 kDa full-length protein was produced, but was subject to proteolysis. Two shorter, more stable fragments of 23 and 20 kDa accumulated in the cell extract throughout induction (Fig. 5, A and C), the shorter predominating in the long term (data not shown). Western blot analysis with anti-His tag antibodies revealed that these fragments corresponded to polypeptides with deletions of 20–50 N-terminal residues (Fig. 5A). We carried out deletion analysis to characterize these fragments in more detail and to obtain a single, stable form. Constructs encoding HsPDF with N-terminal deletions of 19, 39, 45, 62, and 78 residues (designated ΔN19, ΔN39, ΔN45, ΔN62, and ΔN78, respectively; see Fig. 1) were expressed. In all cell extracts except that for ΔN78, the corresponding protein accumulated normally in the soluble fraction of the crude extract (Fig. 5A). As residue 62 can be aligned with residue 1 of EcPDF, the catalytic domain of

FIG. 4. The leader peptides of HsPDF1A and HsMAP1D target these proteins to human mitochondria. The analysis was similar to that in Fig. 3 except that we transfected human HEK293 cells and used constructs encoding the indicated leader peptides of HsPDF and MAP1D fused to GFP. The dimension of the bar represents 10 μm. Panels A and B, confocal sections of transiently transfected HEK 293 cells expressing LP1+LP2-HsPDF/GFP (panel A) and LP-HsMAP/GFP (panel B). GFP is shown in green and DAPI staining of nuclear DNA in blue. Panels C and D, HEK 293 cells expressing LP1+LP2-HsPDF/GFP and mt/RFP. Confocal sections are shown for GFP (green, left panels), RFP (red, middle panels), and the co-localization of GFP and RFP in the overlay (yellow, right panels).
HsPDF was predicted to start immediately after this residue. In EcPDF, N-terminal deletions of more than 3 residues result in incorrect folding and inactivation of the enzyme (27). Consistent with this, the ΔN78 form proved to be unstable and did not accumulate significantly in the soluble fraction (Fig. 5A).

**Characterization of the HsPDF Form from Human Mitochondria; Comparison with Plant PDF1A**—The various HisPDF deletion variants produced in E. coli were purified and antibodies raised against them. A single cross-reacting band of 22 ± 1 kDa was detected on Western blot analysis of various human cell extracts (Fig. 5B). A band of similar size was also detected in crude extracts from simian kidney cell lines (Fig. 5B), in keeping with the strong conservation of the amino acid sequence of animal PDFs (Fig. 1). The HsPDF that accumulated in human cells corresponded to the ΔN39 form (Fig. 5C). This indicates that part, but not all of the 60-residue presequence required for import into the mitochondrion is cleaved by mitochondrial leader peptidase (shaded region in Fig. 1). Because the ΔN39 form displays an additional 6-His C-terminal tag not present in the native HsPDF, our data suggest that the N-terminus of all animal PDFs (Fig. 1) are thus retained in the form accumulating in the mitochondria.

A different situation was observed for plant PDF1A. Western blot analysis with various plant extracts and antibodies against AtPDF1A revealed (Fig. 5D) that cleavage occurred just downstream, next to the catalytic domain (shaded region in Fig. 1). We therefore concluded that the PDF accumulating in human mitochondria contained an N-terminal domain of ~25–30 residues not present in bacterial PDFs and plant PDF1As.

**HsPDF Has Peptidyl Deformylase Activity in Vivo and in Vitro and Its Extra N-terminal Domain Is Involved in Protein Stability and Catalytic Activity**—We investigated the deformylase activity of HsPDF by trying to complement the ΔN78 phenotype of strain PAL421Tr (32). Complementation was observed with the ΔN39, ΔN45 and ΔN62 forms (Fig. 6A) but not with the full-length form. As a control, we replaced the crucial Glu of motif 3 (HEXXH) by an Ala in ΔN62, which prevented complementation. This effect is similar to that observed with bacterial PDFs (26). The full-length HsPDF did not accumulate in the bacterium, whereas the shorter deletion mutants did (Fig. 6B), accounting for the lack of complementation observed with the full-length form. The full-length form was found to be toxic to the bacterium in the long term.

The full-length, ΔN19 ΔN39, ΔN45, and ΔN62 forms were characterized. The full-length, ΔN19, and ΔN39 variants were very sensitive to proteolysis and the most active variant was the ΔN39 form, corresponding to the form that accumulates in vivo (Table I). Proteolysis of the N terminus led to cleavage in the −10/−15 region. Flanking sequences containing Lys or Arg, preceding regions containing Pro-Glu-Ser-Thr, are commonly found in proteins with high turnover (41). The extra N-terminal domain of HsPDF also displays such a structure (Fig. 1). We therefore concluded two ΔN39 variants with substitutions of the two conserved twin-Arg (Table I). These substitutions...
resulted in forms that displayed increased resistance to N-terminal proteolysis in vivo (Fig. 5E).

The purified enzyme was found to have a modest catalytic activity (Table I). We noticed that the final specific activity was improved by the addition of preservative agents such as nickel anion salts (23), cobalt (42) or a mixture of the strong reducing agent TCEP and catalase (21) during purification (Table I). Despite testing the effects of many different conditions, including incubation with various metal cations, we were unable to preserve full activity, as already reported for some bacterial and plant PDF1Bs (ThPDF and LePDF; Refs. 11 and 23). The purified ΔN39 form contained zinc bound in a stoichiometric manner, suggesting that zinc had replaced the physiological metal cation, which was probably iron, as in PDF1Bs (11, 20, 43).

Thus, functional HsPDF contains an unstable metal cation and an additional N-terminal domain that is retained in the mature protein that accumulates in mitochondria. This domain is important for activity and import into mitochondria.

Site-directed Mutagenesis of HsPDF and Plant PDF1As Reveals the Importance and Functional Linkage of Changes at Residues 43 and 91—We next addressed the distinction in metal binding between animal and plant PDF1As, using HsPDF and AtPDF1A as prototype examples. The most striking difference concerns the strictly conserved motifs. First, motif 1 (Gly45-Φ-Gly-Φ-Ala47-Ala-X-Gln56, where Φ is a hydrophobic amino acid) is not conserved: the Gly is replaced by a phobic amino acid) is not conserved: the Gly is replaced by a

We therefore conclude that the physiologically active form of HsPDF coordinates an iron cation, rather than the zinc ion coordinated by plant PDF proteins. The evolutionary substitutions at positions 43 and 91 in animal PDFs are responsible for this effect.

Substrate Specificity of HsPDF and Sensitivity to Common PDF Inhibitors Including Actinomycin; Comparison with Other PDFs and Role of Residue 125—The substrate specificity of HsPDF was investigated and compared with those of AtPDF1A, and PDF1Bs (i.e. AtPDF1B and EcPDF) (Table III). Both HsPDF and AtPDF1A hydrolyzed peptides with bulky P1' chains much less efficiently than EcPDF. This lower efficiency probably results from the smaller size of the S1’ pocket including the conserved residue specific to all PDF1As, Trp 125. This conclusion is consistent with the behavior of the mutant forms at position 125 (Table II and Table III). However, like PDF1Bs and unlike plant PDF1As, animal PDF was insensitive to the nature of hydrophobic bulky P2’ chains (Table III). This effect probably results from the differential impact of residue 87 (one of the two residues, together with Arg7, involved in the S3’ subsite; Ref. 24) in both PDF1A subclasses. Indeed, residue 87 is a conserved bulky hydrophobic Phe in plant PDF1As and a small Pro in animal PDFs; Pro imposes no specific structural restraint on the subsite whereas Phe, which is much larger, does (Fig. 1).

We assessed the sensitivity of HsPDF to various inhibitors in vitro (Table IV). HsPDF was not inhibited by compound FRN but was efficiently inhibited by actinomycin. This is consistent with animal PDF displaying restricted selectivity for bulky P1’ side chains. We investigated whether actinomycin inhibition could be observed in vivo. The HsPDF ORF was inserted into an arabinose inducible vector, which was then used to transfect tolC actinomycin-permeable cells. We therefore concluded that the physiological activity of HsPDF was increased in vivo and that the resistance of the bacterium to actinomycin (data not shown).

Thus, HsPDF, like AtPDF1As (11), is the primary target of actinomycin in vivo.

**DISCUSSION**

The Two Components of the Mammalian NME Have Unusual but Similar Phylogenies—In this study, we found that most animal cells produce the two enzymes required for mitochondrial N-terminal methionine excision and that the corresponding mature forms are localized to the correct cell compartment. These two enzymes are a PDF and a dedicated methionine aminopeptidase (MAP1D). At the time we submitted this article, similar data concerning the ΔN62 form of

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**TABLE I**

| Enzyme variant | Preserving conditions | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ | Relative $k_{cat}/K_m$ |
|----------------|----------------------|----------|-------|--------------|----------------------|
| FL$^a$         | TCEP-catalase         | nm$^b$   | nm    | 27 ± 3       | 11                   |
| ΔN19           | TCEP-catalase         | 0.10 ± 0.02 | 2.4 ± 0.7 | 42 ± 5       | 17                   |
| ΔN39           | TCEP-catalase         | 0.06 ± 0.01 | 1.5 ± 0.7 | 38 ± 7       | 15                   |
| ΔN39/R-13Q     | TCEP-catalase         | 1.0 ± 0.2  | 3.9 ± 1.0 | 250 ± 20    | 100                  |
| ΔN39/R-10Q/R-11Q | TCEP-catalase      | ND$^c$   | ND    | 193 ± 16     | 77                   |
| ΔN45           | TCEP-catalase         | 0.057 ± 0.010 | 1.5 ± 0.5 | 38 ± 6       | 15                   |
| ΔN45           | TCEP-catalase         | nm       | nm    | 84 ± 7       | 34                   |
| ΔN62           | TCEP-catalase         | 0.11 ± 0.01 | 1.5 ± 0.5 | 73 ± 9       | 30                   |
| ΔN62           | NiCl$_2$             | 0.26 ± 0.04 | 3.6 ± 0.9 | 72 ± 7       | 30                   |
| ΔN62           | 0.030 ± 0.005        | 1.6 ± 0.4  | 18 ± 2  | 7           |

$^a$ The substrate was Fo-Met-Ala-Ser. The value of $k_{cat}/K_m$ is given as a percentage of the value obtained with variant ΔN39 purified in the presence of TCEP-catalase.

$^b$ A mixture of various forms including the full-length (FL) and N-terminally truncated forms (see Fig. 5, panels A and C) occur in this protein preparation.

$^c$ nm, not measurable because $K_m$ value is too high (>10 nm).

$^d$ ND, not determined.
NiCl₂ was added. The respective concentrations of each compound were described previously (11, 21).

Thus, both mitochondrial NME genes probably originate from an ancestor of eukaryotic cells. This event may be similar to a duplication and fusion to a ribosome-targeting domain. A mammalian mitochondrial N-terminal methionine excision machinery cleaving the initial Met (7) and (ii) the compilation of the branching pattern of the phylogenetic tree (Fig. 2), cytochrome c oxidase subunits 2 and 3 and cytochrome b. The putative cleavage sites for MAP1D are conserved in all forms found in Actinobacteria, although mitochondrial genes are thought to have originated from Rickettsiae bacteria (45).

Both animal PDFs and MAP1Ds strongly resemble enzyme forms found in Actinobacteria, although mitochondrial genes are thought to have originated from Rickettsiae bacteria (45). Thus, both mitochondrial NME genes probably originate from an ancient horizontal gene transfer from an actinobacterium to an ancestor of eukaryotic cells. This event may be similar to that generating the second type of glutamine synthetases, as the phylogenies determined were similar (46), but is probably unrelated to mitochondrial gene transfer to the nucleus. Our present identification of a mitochondrial MAP (MAP1D) confirms the predictions of Keeling and Doolittle (47). According to the branching pattern of the phyllogenetic tree (Fig. 2), cytoplasmic MAP1Ds were probably derived from MAP1D by gene duplication and fusion to a ribosome-targeting domain.

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### Table II

Comparison of the enzymatic parameters of HsPDF, AtPDF1A, and other PDF variants

| Enzyme* | Variant | \(k_{cat}\) | \(K_m\) | \(k_{cat}/K_m\) | Relative \(k_{cat}/K_m\) |
|---------|---------|-------------|---------|-----------------|---------------------|
| HsPDF   | —       | 0.030 ± 0.005 | 1.6 ± 0.4 | 18 ± 2 | 100 |
| C43G    | ND*     | —           | —       | —               | —                   |
| S47A    | 0.4 ± 0.15 | 4.1 ± 2.3 | 100 ± 5 | 555 |
| E91A    | 0.20 ± 0.02 | 1.1 ± 0.2 | 100 ± 10 | 555 |
| E91L    | 0.07 ± 0.01 | 2.6 ± 0.6 | 26 ± 3 | 144 |
| E91M    | 0.04 ± 0.01 | 0.6 ± 0.1 | 69 ± 8 | 383 |
| W125L   | 0.015 ± 0.0002 | 1.0 ± 0.3 | 16 ± 3 | 89 |
| E135A   | nm      | —           | —       | <0.08 | <0.5 |
| C43G/S47A/E91L | 2.1 ± 0.3 | 0.9 ± 0.2 | 2,300 ± 250 | 12,800 |
| S47A/E91L | 0.17 ± 0.02 | 1.2 ± 0.2 | 138 ± 12 | 770 |
| C43G/S47A/E91L | 18 ± 4 | 2.9 ± 1.0 | 6,200 ± 600 | 34,440 |
| AtPDF1A | —       | 22 ± 2 | 0.25 ± 0.07 | 88,800 ± 150 | 100 |
| G43C(Ni) | 0.20 ± 0.02 | 0.10 ± 0.02 | 2,000 ± 300 | 2.3 |
| G43C    | 0.19 ± 0.03 | 0.25 ± 0.08 | 740 ± 100 | 0.8 |
| L91E    | 0.023 ± 0.003 | 1.60 ± 0.60 | 14.5 ± 3.0 | 0.016 |
| G43C/L91E | 0.11 ± 0.02 | 0.7 ± 0.2 | 470 ± 47 | 0.53 |
| W125L   | —       | —           | —       | —    | —   |
| AtPDF1A | —       | 5.6 ± 1.5 | 70 ± 20 | 80 | 0.15 |
| L91A    | —       | 210 ± 20 | 3.9 ± 1.0 | 54,000 | 100 |
| PPFDF   | —       | —           | —       | —    | —   |

### Table III

Comparison of the substrate specificity of the two forms of eukaryotic PDF1As and the effect of Trp 125

| Substrate | HsPDF | AtPDF1Aa | AtPDF1A W125L | EcPDFb |
|-----------|-------|----------|---------------|--------|
| Fo-Met-Ala-Ser | 100 | 100.0 | 100 | 100 |
| Fo-Met-Ser-Asn-Glu | 46 | 5.0 | ND | 60 |
| Fo-Met-Leu-Glu | 10 | 0.2 | ND | 24 |
| Fo-Met-Ala-OCH₃ | 9 | 29.0 | ND | 267 |
| Fo-Nva-Ala-Ser | 6 | 2.0 | ND | 15 |
| Fo-Nle-Arg-(NH₂) | 11 | 13.5 | ND | 839 |
| Fo-Phe-Ala-Ser | ≤1 | 0.02 | 134 | 35 |
| Fo-Met-Leu-pNA | 350 | 1.1 | 2100 | 3519 |

a Data from Ref. 11.

b Data from Ref. 28 and 31.

c ND, not determined.

Fo-Met-Asp-Arg-Phe-Ser was added. The respective concentrations of each compound were described previously (11, 21).

### Table IV

Comparison of the binding of several compounds to HsPDF and EcPDF

| Inhibitor compound | HsPDF | EcPDFb |
|--------------------|-------|--------|
| Actinomycinb | 3.7 ± 0.4 | 0.018 ± 0.003 |
| TNRc | 48 ± 10 | 2.5 ± 0.5 |
| FRNd | 4470 ± 740 | 95 ± 10 |

a Data from Ref. 31.

b Data from Ref. 31.

c In either case, the IC₅₀ corresponded to about half the concentration of the enzyme, indicating tight binding.

d FRN, NH₂-Phe-Arg-β-naphthylamide.
of a number of N-terminal protein sequencing data (reviewed in Ref. 9). Although phylogenetically related to plant PDF1As (Fig. 1 and Refs. 7 and 9), the possible existence of an active peptide deformylase in animals has been a controversial issue (6, 7, 13, 44). One objection raised was that the sequences of mitochondrially encoded proteins from mammals indicate that their Fo groups are retained, whereas this is not the case for the corresponding proteins of plant mitochondrial genomes (see data compiled in Ref. 13). This suggested that there was no PDF and MAP activity in animal mitochondria. However, the same tissues (adult bovine heart) were always used to prepare mitochondria. Moreover, the method used to determine whether an Fo group was present was indirect. It was suggested that the protein was N-formylated because mild acid treatment of the N terminus exposes the terminal residue for Edman sequencing. Direct mass spectrometric analysis of the N termini of mitochondrial proteins from several tissues would be required to draw definitive conclusions. The identification in this study of MAP and PDF enzymes in animals strongly suggests that NME is functional in mitochondria. Interestingly, the crystal structure data of cytochrome bc1 (48) lack the electron density of the N-terminal Met of the mitochondrially encoded cytochrome b, suggesting Fo-Met removal. This result is consistent with NME activity as the cytochrome b sequence starts with Fo-Met-Thr, which strongly favors Met removal (1).

In this study, we provide compelling evidence that HsPDF is active in vivo, as it allows complementation of a PDF gene (def) defect, and in vitro, although its activity is highly unstable (Table I).

A recent study showed that human cells are resistant to the PDF-specific inhibitor actinonin and its derivatives, if used at concentrations with antibacterial effects (49). However, if used at higher concentrations, these inhibitors have a toxic, antiproliferation effect in vivo and in vitro, the intracellular target of which is unknown (50, 51). Possible effects of inhibition of the cell surface zinc aminopeptidase CD13 and other metzincins has been excluded. The most likely target is therefore HsPDF, suggesting that PDF is functional in humans. The PDF gene is expressed in many mammalian cell types (Fig. 5B) and a similar gene is expressed in most animal genomes (Fig. 1). In all these genomes, genes encoding a dedicated mitochondrial MAP (MAP1D) can be identified (Fig. 2). As the function of PDF is to facilitate MAP activity (52), these data indicate the genetic and functional linkage of the two components of mitochondrial NME in animals. Finally, it was recently shown that the PDF gene is part of a cluster that is tightly regulated in skeletal muscle regeneration in mice (53), with down-regulation after injury and up-regulation during cell differentiation and re-entry into the cell cycle (see H3113G11 in cluster 7). This is consistent with NME activity being tightly controlled in animal mitochondria.

Animal PDFs Are Unusual PDF1s with Non-random Substitutions in the Conserved Motifs—Unlike HsMAP1D, animal PDFs differ markedly from the many other PDFs characterized to date. HsPDF has an additional 20-amino acid N-terminal extension (Figs. 1 and 5). This extension is strongly conserved, predicted to fold as an α-helix (www.embl-heidelberg.de/predictprotein/predictprotein.html, Ref. 54), has a very high pl due to the presence of five conserved arginines (Fig. 1). It is separated from the rest of the molecule by a region with high local concentrations of Pro, Glu, Ser, and Thr. This suggests a high turnover of the protein in vivo. Interestingly, all the arginine residues occur on one side of the helix and the hydrophobic residues occur on the other. The N-terminal domain may fold back toward the E1 extended structure of the enzyme stabilizing the active site. This would provide a role for this domain in deformylase activity. However, we cannot exclude the possibility that this domain is also involved in binding to mitoribosomes, like the additional N-terminal domain of MAP1As to cytosolic ribosomes. All animal PDFs display systematic substitutions altering crucial amino acids in motifs 1 and 2: Gly43, Ala47, and Leu91 (Fig. 1). Our results have shown that the Cys43 and Glu91 substitutions are responsible for the unusual behavior of HsPDF, as the replacement of these two residues by Gly and Leu, respectively, makes HsPDF as active as plant PDF1As (Table II). The side chains of residues 43 and 91 are located in close proximity (within 4.5 Å) in the crystal structure of known PDF1s (24). They are involved in recognition of the Fo group and are located close to the P2’ binding site. There is unlikely to be a true S2’ binding pocket because PDF has no marked specificity for a given P2’ side chain (28, 55). The other residue close (3.7 Å) to residues 43 and 91 is residue 41, a Glu in E. coli and an Arg in human PDF. We further investigated the three-dimensional structure of this region, by constructing a model of the active site of HsPDF (Fig. 7). This model suggests that a salt bridge could be created in HsPDF between the side chains of residues 41, 43, and 91. The alignment in Fig. 1 shows that if residue 91 is a Glu, then residue 41 is positively charged. Conversely, if residue 91 is another non-charged residue, as in amphibians, then residue 41 is uncharged. We conclude that the substitutions of residues 41, 43, and 91 in animal PDFs are not random and that these substitutions are interdependent. This may make it possible to fine tune PDF activity in mitochondria, possibly facilitating adaptation to metal availability or to the highly oxidative environment in this organelle. Finally, we cannot rule out the possibility that these three residues interact with the N-terminal extension of HsPDF.

Implications of the Existence of Human PDF for PDF Inhibitor-based Antibacterial Treatments—Bacterial PDF inhibitors (PDFI) constitute one of the most promising families of new antibiotics (6, 56, 57). However, our characterization of a functional PDF in humans raises a major concern as to the use of PDFI in human medicine. Clearly, the unambiguous mitochon-
drial location of HsPDF lessons this concern to some extent and accounts for the high concentration of PDFI required to obtain a toxic effect. Millimolar amounts of PDFI are also required to inhibit plant growth (11). Mitochondrial membranes are less permeable to small molecules than most other membranes and act as an efficient supplementary filter for many drugs. For this reason, a number of antibiotics (tetracyclines, macrolides, etc.) that inhibit mitochondrial targets are nevertheless currently used in human medicine. However, as PDFI toxicity was observed (see above), the use of PDFI might cause side effects in the long term due to inhibition of HsPDF. We therefore think that great care should be taken when designing PDFI to reduce the potency of these molecules against HsPDF. Our data are consistent with the hypothesis that there is a significant degree of structural and functional divergence between PDF1As and PDF1Bs, with the latter being sensitive to preservative agents, indicating that they are highly resistant to chemical head, a formylhydroxylamine as in E.coli toxin, is required to inhibit PDFI but unlike plant PDF1A, are stimulated rather than inhibited by chemical head, a formylhydroxylamine as in E.coli toxin, is required to inhibit PDFI but unlike plant PDF1A, are stimulated rather than inhibited by the presence of a hydrophobic P3’ side chain (Table III). Conversely, animal PDFs resemble plant PDF1A in not tolerating a bulky hydrophobic group at position P1’ (Tables III-IV) This intolerance results from the presence of a Thr rather than a Leu at position 125, causing a decrease in the size of the S1’ pocket of the enzyme (Tables II-IV). Thus, modification of the P3’ group in PDF1 will not result in discrimination between PDF1B and HsPDF. Instead, replacing the P1’ side chain with a bulkier group, such as a phenyl group, dramatically decreased the affinity of PDFI for HsPDF, without affecting their potency with respect to bacterial PDFs.

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