Characterization of a Human Peptide Deformylase: Implications for Antibacterial Drug Design†

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ABSTRACT: Ribosomal protein synthesis in eubacteria and eukaryotic organelles initiates with an *N*-formylmethionyl-tRNA$_i$, resulting in N-terminal formylation of all nascent polypeptides. Peptide deformylase (PDF) catalyzes the subsequent removal of the N-terminal formyl group from the majority of bacterial proteins. Deformylation was for a long time thought to be a feature unique to the prokaryotes, making PDF an attractive target for designing novel antibiotics. However, recent genomic sequencing has revealed PDF-like sequences in many eukaryotes, including man. In this work, the cDNA encoding *Homo sapiens* PDF (HsPDF) has been cloned and a truncated form that lacks the N-terminal 58-amino-acid targeting sequence was overexpressed in *Escherichia coli*. The recombinant, Co^{2+}-substituted protein is catalytically active in deformylating N-formylated peptides, shares many of the properties of bacterial PDF, and is strongly inhibited by specific PDF inhibitors. Expression of HsPDF fused to the enhanced green fluorescence protein in human embryonic kidney cells revealed its location in the mitochondrion. However, HsPDF is much less active than its bacterial counterpart, providing a possible explanation for the apparent lack of deformylation in the mammalian mitochondria. The lower catalytic activity is at least partially due to mutation of a highly conserved residue (Leu-91 in *E. coli* PDF) in mammalian PDF. PDF inhibitors had no detectable effect on two different human cell lines. These results suggest that HsPDF is likely an evolutional remnant without any functional role in protein formylation/deformylation and validates PDF as an excellent target for antibacterial drug design.

In prokaryotes and eukaryotic organelles (e.g., mitochondria and plastids), ribosomal protein biosynthesis is initiated with N-formylmethionine (*1*). Consequently, all nascent polypeptides synthesized in bacteria, mitochondria, and chloroplasts bear an N-terminal formyl group. In bacteria, the N-formyl group is subsequently removed from the vast majority of polypeptides by peptide deformylase (PDF),[1] apparently as a co-translational event (*2*−*4*). A fraction of the deformylated polypeptides undergo further N-terminal processing (i.e., removal of N-terminal methionine by methionine aminopeptidase) to give mature proteins (*1*). PDF is a unique and highly unstable metallopeptidase, which contains a ferrous ion (Fe^{2+}) as the catalytic metal (*5*, *6*). PDF is essential for bacterial survival; either deletion of the *def* gene (*7*−*9*) or treatment with a PDF inhibitor (*10*−*13*) prevents bacterial growth. These properties make PDF an attractive target for developing novel antibiotics, and one of the PDF inhibitors is currently in phase I clinical trials for the treatment of upper respiratory tract infections.

Another factor that made PDF an attractive antibacterial drug target was the common belief, in the past, that the enzyme was unique to the bacterial kingdom. Indeed, cytoplasmic protein synthesis in eukaryotes does not involve N-formylation, and therefore, there is no need for deformylation (*1*). Available evidence also suggests that there is no deformylation in the mitochondrion of mammals. For example, the intramitochondrially synthesized proteins from bovine and rat typically retain their N-terminal formyl group or have their N-terminal signal sequences removed (reviewed in refs *2*−*4*). However, genomic sequencing has recently revealed many PDF-like sequences in parasites, plants, and mammals (*2*−*4*). Meinnel and co-workers have shown that the two *def*-like genes in *Arabidopsis thaliana* indeed code for functional PDFs (*14*). We previously reported the cloning and characterization of an eukaryotic PDF from the malaria parasite, *Plasmodium falciparum* (*15*). The presence of PDF-like sequences in the mammalian genome raises several important questions such as the function of PDF in mammals and the suitability of PDF as a novel drug target. To begin to address these questions, we have undertaken the cloning and characterization of human PDF (HsPDF). Biochemical

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[1] Abbreviations: PDF, peptide deformylase; HsPDF, *Homo sapiens* PDF; EcPDF, *E. coli* PDF; PfPDF, *Plasmodium falciparum* PDF; GFP, green fluorescence protein; HEK, human embryonic kidney; GST, glutathione-S-transferase.

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and kinetic analyses show that the recombinant protein is an active PDF, although its activity is considerably lower than that of the bacterial enzyme. In addition, known PDF inhibitors inhibit its catalytic activity in vitro but had little effect on the growth of human cells. The results are consistent with the hypothesis that HsPDF is a mere evolutional remnant of no current function.

MATERIALS AND METHODS

Materials. All Fmoc-protected amino acids, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and 1-hydroxybenzotriazole (HOBT) were purchased from SynPep (Dublin, CA). Rink resin was from Advanced Chemtech (Louisville, KY). Formate dehydrogenase was from Sigma. *Aeromonas* aminopeptidase was purified as previously described (*16*). Other chemicals, including isopropyl-β-D-thiogalactopyranoside (IPTG), phenylmethanesulfonyl fluoride, kanamycin, ethanedithiol and its derivatives, and β-mercaptoethanol, were purchased from Aldrich.

Buffers. Buffer A, 50 mM Hepes (pH 7.2), 100 mM NaCl, 1 mM β-mercaptoethanol, and 1% Triton X-100; buffer B, 20 mM Hepes (pH 7.2), 100 mM NaCl; buffer C, 50 mM Tris·HCl, 10 mM NaCl, pH 8.0; buffer D, 50 mM Tris·HCl (pH 8.0), 1 mM NaCl; buffer E, 50 mM Hepes (pH 7.0), 150 mM NaCl; buffer F, 20 mM Hepes (pH 7.2), 100 mM NaCl, 0.2 mM β-mercaptoethanol; buffer G, 50 mM Mops (pH 7.0), 10 mM NaCl.

Cloning, Expression, and Purification of HsPDF. On the basis of the reported HsPDF genomic DNA sequence at http://www.ncbi.hlm.nih.gov, two polymerase chain reaction (PCR) primers were designed as follows: 5′-GGGGATCCATATGGCCCGGCTGTGGGGCGCGCTGAGTCTT-3′ and 5′-GGGAATTCTTAGTCATTCACCTTCATCCAATAG-3′. Approximately 0.1 μg of a human fetus Marathon-Ready cDNA library (Clontech, CA) was used in the PCR reaction. The Clontech Advantage-GC PCR mixture was employed to destabilize any secondary structures in G,C-rich DNA (*17*). The PCR reaction (50 μL total volume) contained 800 μM dNTPs, 0.2 μM of each primer, 1.0 M GC melt mixture, and 1 μL of 50× Advantage-GC 2 polymerase mix. The PCR was performed for 32 cycles with the following conditions: 94 °C for 30 s/52 °C for 60 s/68 °C for 70 s. The 0.73 kb PCR product was purified on a Qiaquick column (Qiagen) to remove any free nucleotides, digested with restriction endonucleases *Nde*I and *Xho*I, and cloned into the prokaryotic expression vector pET-22b (Novagen, WI) to produce plasmid pET22b-HsPDF. The entire coding region of HsPDF was sequenced and found to be identical to the published sequence.

N-terminal truncation of HsPDF was carried out by PCR using the above 3′ primer and the following 5′ primers: 5′-GGCCCATGGAACGGCGCTCCTATTGGCGCCA-3′, 5′-GGCCCATGGAACCTGAGGCGTCTGGTGCTGG-3′, and 5′-GGCCCATGGAACCTCCCGAACCGCCGTTCTCG-3′. These primers resulted in the truncation of 44, 52, and 58 amino acids, respectively, from the N-terminus of HsPDF. The PCR products were cloned into the plasmid pET-42b to generate plasmids pET42b-HsPDFΔ44, pET42b-HsPDFΔ52, and pET42b-HsPDFΔ58, respectively. This cloning procedure resulted in the in-frame addition of an N-terminal glutathione-S-transferase (GST) tag along with a Factor Xa cleavage site.

E. coli BL21 (DE3) Rosetta cells (Novagen) carrying the proper plasmid were grown in minimal media containing 60 μg/mL kanamycin and 35 μg/mL chloramphenicol at 37 °C until OD_{600} reached ∼0.9. The culture was supplemented with 100 μM $CoCl_2$ and 100 μM isopropyl-β-D-thiogalactopyranoside and incubated at 30 °C for an additional 3 h. The cells (12 L) were chilled on ice for 1 h and harvested by centrifugation. The cell pellet was suspended in 200 mL of buffer A plus 50 μg/mL phenylmethanesulfonyl fluoride, 0.5% protamine sulfate, 20 μg/mL trypsin inhibitor, and 100 μg/mL lysozyme. The mixture was stirred at 4 °C for 30 min and briefly sonicated (5 × 10 s pulses). The crude lysate was centrifuged to yield a clear supernatant, which was mixed with 10 mL of GST bind resin (Pharmacia). The column was washed with 300 mL of buffer F and eluted with 50 mL of buffer F containing 0.8 mM reduced glutathione. The GST−HsPDF fractions were pooled and concentrated to ∼2 mL using an Amicon YM-3 cellulose membrane filter. The resulting solution was passed through a Pharmacia FPLC Fast-desalting column (eluted with buffer C) to remove the gluthathione and salts. Fractions containing GST−HsPDF were pooled (∼3 mL) and treated with 30 units of Factor Xa (Novagen) at 4 °C for 8 h to cleave the GST tag. HsPDF was purified by passing through a monoQ HR 5/5 anion-exchange column equilibrated in buffer C. The column was eluted with buffer C plus a linear gradient of 10−1000 mM NaCl. Fractions containing HsPDF were pooled and concentrated in an Amicon YM-3 cellulose filter. Protein concentration was determined by Bradford assay using bovine serum albumin as the standard. Typically, ∼1 mg of pure HsPDF was obtained from a 12-L culture. Metal analysis was performed for the GST−HsPDF fusion protein by inductively coupled plasma emission spectroscopy (ICP-ES) at the Chemical Analysis Laboratory of the University of Georgia.

Site-Directed Mutagenesis. Mutation of Glu-173 to leucine in HsPDF was performed by the Quick-Change mutagenesis method using the following primers: 5′-CCGAGGGCTGCGCTAGCGTCGCCGGCT-3′ and 5′-AGCCGGCGACGCTAGCGCAGCCCTCGG-3. The DNA amplification reaction contained 800 μM dNTPs, 0.1 μg of the plasmid pET42b-HsPDFΔ58 DNA, 0.2 μM of each primer, 1.0 M GC melt mixture, and 2.5 units of *Pfu*Turbo DNA polymerase (Stratagene). Twenty cycles were performed as follows: 95 °C for 30 s/54 °C for 60 s/72 °C for 14 min. The identity of the mutants was confirmed by DNA sequencing. The mutant was expressed and purified in the same manner as the wild-type enzyme. Mutation of Leu-91 of EcPDF to a glutamate was similarly carried out with the following primer pair: 5′-GAAGAAGGTTGCGAGTCGATCCCTGAACAACGTG-3′ and 5′-CACGTTGTTCAGGGATCGACTCGCAACCTTCTTC-3′, except that the plasmid pET22b-EcPDF (*18*) was used as the template. Expression and purification of L91E EcPDF were carried out as previously described (*19*).

Peptide Synthesis. All peptides were prepared by solid-phase synthesis on Rink resin as previously described (*18*). HPLC analysis showed generally >85% purity. The identity of the peptides was confirmed by matrix-assisted laser desorption ionization mass spectrometry.

Synthesis of PDF Inhibitors. PDF inhibitors **1**−**4** were synthesized from commercially available starting materials. The synthetic details are described in the Supporting Information.

PDF Assays. Two different methods were employed to assay for PDF activity. Method A employed f-ML-pNA as substrate which, upon deformylation by PDF, is further processed by *Aeromonas* aminopeptidase (AAP) to release *p*-nitroaniline (*20*). The assay reaction (total volume of 1.0 mL) typically contained buffer E, 1 mM tris(2-carboxyethyl)-phosphine (TCEP), 0−200 μM f-ML-pNA, and 2 units of AAP. The reaction was initiated by the addition of 1−10 μg of HsPDF, and the reaction progress was monitored at 405 nm on a UV−vis spectrophotometer. The initial reaction rate was calculated from the early, linear region of the progress curve (0−45 s). Method B was used for all other N-formylated substrates. It couples the PDF reaction with formate dehydrogenase, which oxidizes formate into carbon dioxide while reducing NAD^+ to NADH (*18*, *21*). A typical reaction (total volume of 500 μL) contained buffer G, 1 mM TCEP, and 0−2 mM N-formylated peptide. The reaction was initiated by the addition of 1−10 μg of HsPDF, and allowed to proceed for 30 min at room temperature before being quenched by heating at 95 °C for 10 min (the inactivation process is usually complete within the first 30 s). After cooling to room temperature, the amount of released formate was quantified as previously described (*18*, *21*). Inhibition assays (method A) were carried out with 150 μM f-ML-pNA as substrate, 0−400 μM inhibitor, and 1.1 μg of HsPDF. The reaction was quenched by heating at 95 °C for 10 min and cooled to room temperature. After the addition of AAP (1.0 unit) and incubation for 15 min, the absorbance increase at 405 nm was measured. For all end-point assay reactions, the substrate to product conversion was kept at <20%.

Fluorescence Microscopy. A PCR was performed with plasmid pET22b-HsPDF as template and primers 5′-TTGCTCGAGATGGCCCGGCTGTGGGGCGCGC-3′ and 5′-GCCGGATCCTCATTCACCTTCATCCAATA-3′. The resulting full-length HsPDF cDNA was digested with *Xho*I and *Bam*HI and cloned into a mammalian expression vector pEGFP(N1) (Clontech). This cloning procedure resulted in the in-frame fusion of the enhanced green fluorescence protein (EGFP) at the C-terminus of HsPDF to produce the plasmid pEGFP-HsPDF. Approximately 800 000 human embryonic kidney (HEK293) cells in 2 mL of Dulbecco's minimum essential medium (DMEM) with high glucose containing 10% fetal bovine serum (FBS) were plated onto a sterile 35 mm plastic plate and incubated at 37 °C overnight. Transient transfection of pEGFP−HsPDF into HEK cells was performed according to LipofectAMINE 2000 protocol (Life Technologies). The transfected cells were incubated at 37 °C in a CO_2 incubator for 48 h and were subsequently trypsinized and plated onto glass cover slips. After 24 h, the cells were stained with MitoTracker Red CMXRos (Molecular Probes), and the glass cover slip was removed with the adhered HEK cells. Cells were fixed with 4% paraformaldehyde. Visualization was performed under a Bio-Rad MRC-1024 confocal laser scanning unit equipped with a krypton/argon laser, a photomultiplier tube, and an upright Nikon microscope. Images were taken with a 60× oil objective at an Iris setting of 3. The 488 and 568 nm laser line was used separately to acquire images for the GFP and MitoTracker, respectively. Merged images were generated using the Confocal Assistant software (Bio-Rad).

Cell Growth Inhibition Assay. To facilitate the monitoring of growth rate using a fluorescence plate reader, HEK 293 cells were transfected with the mammalian expression vector pEGFP-IRESneo (Clontech) as described above. Cells were grown on a 60 mm polystyrene plate with 4 mL of DMEM + FBS media supplemented with 400 μg/mL G418. To maintain a stable cell line, the transfected cell line was grown to >95% confluency (typically 3 days), trypsinized, and subcultured for 8 weeks. To test the effect of PDF inhibitors, cells were plated onto a polyornithine-treated 96-well polystyrene plate in 150 μL of DMEM+FBS (~20 000 cells per well), and grown overnight. The media was removed and replaced with fresh DMEM+FBS containing 0−128 μM PDF inhibitor **1**. Fluorescence signal was measured every 8 h over a 3 day period using the FLEXstation (Molecular Devices) at an excitation wavelength of 485 nm and an emission wavelength of 525 nm at 23 °C.

DNA Synthesis Inhibition Assay. The cell line used, L5178YD10/R cells, is an acute lymphoblastic leukemia cell line resistant to L-asparaginase (obtained from ATCC). Cells were maintained in DMEM with high-glucose-containing 10% FBS, 1% antibiotic/antimycotic and 1% L-glutamine. Cells in log phase were seeded in a 96-well plate at a dilution of 5×10^5 cell/200 μL in three wells, which was served as the negative control. Log phase cells at a concentration of 2.5×10^6 cells /ml were then mixed with ^{3}H-thymidine (1 μCi/mL of cell suspension) and added to the wells (200 μL/well). Cells in individual wells were treated with PDF inhibitor **1** or **2** at the specified concentrations (in triplicate) ranging from 50 nM to 150 μM and incubated at 37 °C for 18 h in a CO_2 incubator. At the end of incubation, cells were harvested in a cell harvester (Tomech Harvester), and labeled DNA was captured on filter membranes. Membrane-bound DNA was washed in distilled water and air-dried for 3 h. Radioactivity retained on the membrane was measured in a liquid scintillation counter (Beckman).

RESULTS

Overexpression and Purification of HsPDF. A BLAST search of the human genomic sequence stored at the NCBI website (http://www.ncbi.hlm.nih.gov/) with EcPDF sequence as query resulted in a single PDF-like homologue, located on chromosome 16 (GenBank accession number AF23915). On the basis of the predicted protein sequence (*14*), we cloned the full-length cDNA, which encodes a protein of 243 amino acids, from a human fetal tissue cDNA library by PCR. Sequence alignment shows that HsPDF contains a catalytic domain, which shares ~30% sequence identity to EcPDF, and a 61-amino acid extension at the N-terminus (Figure 1). The N-terminal extension resembles that of *P. falciparum* PDF (PfPDF) (*15*). We anticipated that the full-length HsPDF would be difficult to express as a soluble protein in *E. coli*; therefore, we constructed various truncation mutants that lack the N-terminal 44 (ΔN44), 52 (ΔN52), and 58 (ΔN58) amino acids. We first cloned the truncation mutants in prokaryotic expression vector pET22b and attempted to overexpress them in *E. coli* BL21(DE3) cells. Unfortunately, neither the mutants nor the full-length protein could be expressed at significant levels. After

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Hs    --------------------MARLWGALSLWPLWAAVPWGGAAAVGVRACSSTAAPDGVE   40
Pf    --------------------MLMYYSLFLFNLIICCNVTSIYGYIHNVRSLEPYIKNDQI   40
At    MGLHRDEATAMETLFRVSLRLLPVSAAVTCRSIRFPVSRPGSSHLLNRKLYNL-PTSSS    58
Sa    ------------------------------------------------------------
Bs    ------------------------------------------------------------
Ec    ------------------------------------------------------------

Hs    GPALRRSYWRHLRRLVLGPPEPPPFSHVCQVGDPVLRGVAAPVERAQLGGPELQRLTQRLV  100
Pf    KNYSSNIKQKRKGSLYLLKNEKDEIKIVKYPDPILRRRSEEVTNFD---DNLKRVVRKMF   97
At    SSLSTKAGW--LLGLGEKKKKVDLPEIVASGDPVLHEKAREVDPGEIGSERIQKIIDDMI  116
Sa    ----------------------MLTMKDIIRDGHPTLRQKAAELELPL--TKEEKETLIAMR   38
Bs    ----------------------MITMENIVRDGHPALRETAEPVELPP--TDAEKQQLADMI   38
Ec    ----------------------MSVLQVLHIPDERLRKVAKPVEEVN---AEIQRIVDDMF   36

Hs    QVVRRRRCVGLSAPQLGVPRQVLALELPEALCRECPPRQRALRQMEPFPLRVFVNPSLRV  160
Pf    DIMYESKGIGLSAPQVNISKRIIVWN-------------ALYEKRKEENERIFINPSIVE  144
At    KVMRLAPGVGLAAPQIGVPLRIIVLEDTKEYISYAPKEEILAQERRHFDLMVMVNPVLKE  176
Sa    EFLVNSQDEEIAAKRYGLRSGVGLAAPQINISKRMIAVLIPDDGSGKSYDYMLVNPKIVS   97
Bs    EFVKNSQNPELAAEKYKLRPGVGLAAPQINIKKRMIAVHA-EDASGKLYSYALFNPKIVS   96
Ec    ETMYAEEGIGLAATQVDIHQRIIVID-------------V---SENRDERLVLINPELLE   80

Hs    LDSRLVTFP--EGCESVAG-FLACVPRFQAVQLSGLDPNGEQVVWQASGWAARIIQHEMD  217
Pf    QSLVKLKLI--EGCLSFPG-IEGKVERPSIVSLSYYDINGYKHLKILKGIHSRIFQHEFD  201
At    RSNKKALFF--EGCLSVDG-FRAAVERYLEVVVTGYDRQGKRIEVNASGWQARILQHECD  233
Sa    HSVQEAYLPTGEGCLSVDDNVAGLVHRHNRITIKAKDIEGNDIQLRLKGYPAIVFQHEID  157
Bs    HSVEKSYLTSGEGCLSVDEAIPGYVPRYARIRVKGTTLEGENIDIRLKGFPAIVFQHEID  156
Ec    KSG-ETGIE--EGCLSIPE-QRALVPRAEKVKIRALDRDGKPFELEADGLLAICIQHEMD  136

Hs    HLQGCLFIDKMDSRTFTNVYWMKVND--------------   243
Pf    HLNGILFIDKMTQVDKKKVRPKLNELIRDYKATHSEEPAL   241
At    HLDGNLYVDKMVPRTFRTVDNLDLPLAEGCPKLGPQ----   269
Sa    HLNGVMFYDHIDKNHPLQPHTDAVEV--------------   183
Bs    HLNGVMFYDHIDKENPFKEPENAIAIER------------   184
Ec    HLVGKLFMDYLSPLKQQRIRQKVEKLDRLKARA-------   169
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FIGURE 1: Sequence alignment of various PDF's from eukaryotic and prokaryotic organisms: *Plasmodium Falciparum* (*Pf*), *Escherichia coli* (*Ec*), *Homo sapiens* (*Hs*), *Bacillus subtilis* (*Bs*), *Staphylococcus aureus* (*Sa*), and *Arabidopsis thaliana* (*At*). Alignment was performed by inputting PDF sequences into ClustalW software located at http://clustalw.genome.ad.jp/ website.

considering the different codon bias between human and bacterial systems, we next constructed HsPDF as a GST fusion protein and employed an *E. coli* Rosetta strain that carries several rare tRNA's normally found in *E. coli*. The GST fusion would also facilitate its purification on a glutathione column.

Previous studies have shown that bacterial PDF contains a catalytic Fe^{2+} ion and is highly sensitive to molecular oxygen and/or H_2O_2 in solution (*22*). Replacing the ferrous ion with Co^{2+} or Ni^{2+} results in a stable enzyme of essentially wild-type activity (*6*, *19*, *23*). Therefore, we produced HsPDF in the Co^{2+}-substituted form by growing *E. coli* cells in minimal medium supplemented with 100 μM $CoCl_2$. The ΔN44 and ΔN52 variants did not produce any detectable protein on SDS−PAGE gels. However, we were able to express the ΔN58 variant and partially purify it on a glutathione column. The ΔN58 protein thus obtained still contained multiple bands, most of them smaller than the 45-kDa GST−ΔN58 fusion protein (Figure 2, lane 4). We believe that the smaller species are due to proteolysis of the HsPDF portion of the fusion. Consistent with this notion, when cells were induced for >6 h, the intensity of the 45-kDa band actually decreased (data not shown). Metal analysis of the GST−HsPDF fusion revealed that the protein contained approximately 0.67 mol of Co^{2+} and 0.33 mol of Zn^{2+}, but no significant amounts of any other divalent metals. Cleavage of the GST fusion with protease Factor Xa, followed by anion-exchange chromatography, produced Co^{2+}-HsPDF of apparent homogeneity (Figure 2, lane 6). Using the above procedure, ~1.0 mg of HsPDF was obtained from 12 L of *E. coli* cells.

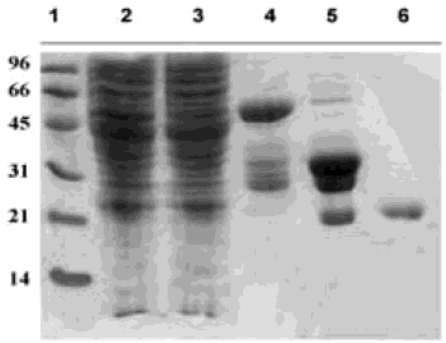


FIGURE 2: A 15% SDS−PAGE gel showing the purity of HsPDF-(ΔN58) during different stages of purification. Lane 1, molecular-weight markers; lane 2, crude cell lysate; lane 3, flow-through fraction of the GST-bind column; lane 4, proteins eluted from the GST-bind column; lane 5, after factor Xa cleavage; and lane 6, HsPDF after purification on Mono-Q column. Molecular weight standards (kD) are indicated on the left side of the gel.

Catalytic Properties of HsPDF. We first evaluated the catalytic properties of HsPDF against an artificial substrate, f-ML-pNA. HsPDF is a catalytically active enzyme, with a k_{cat} of 0.17 s^{-1}, a K_M of 27 μM, and a k_{cat}/K_M of 5.9×10^3 $M^{-1}s^{-1}$ (Table 1). This activity is comparable to that of another eukaryotic PDF, PfPDF, but is ~200-fold lower than that of EcPDF (*15*, *19*). Since HsPDF is located in the human mitochondrion (vide infra), its physiological substrates, if any, would be the mitochondrially synthesized proteins. The human mitochondrial DNA encodes 13 proteins (*24*). We synthesized six N-formylated hexapeptides corresponding to the N-terminal sequences of six of the human mitochondrial

Table 1: HsPDF Activity against f-ML-pNA and Human Mitochondrial Peptides

substrate	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($M^{-1}s^{-1}$)
f-ML-pNA	0.17 ± 0.06	27 ± 6	5900
f-MAHAAQ[a]	0.57 ± 0.08	2500 ± 310	230
f-MTHQSH[b]	1.5 ± 0.5	1850 ± 230	810
f-MNPLAQ[c]	ND	ND	ND
f-MTMHTT[d]	2.2 ± 0.9	4000 ± 800	545
f-MPQLNT[e]	ND	ND	ND
f-MTPMRK[f]	NA	NA	NA

[a] These peptides correspond to the N-terminal sequences of human mitochondrial proteins: *a*, cytochrome *c* oxidase II; *b*, cytochrome *c* oxidase III; *c*, NADH dehydrogenase subunit II; *d*, NADH dehydrogenase subunit V; *e*, ATP synthase F0 subunit 8; and *f*, cytochrome *b*. ND, activity not determined due to insolubility. NA, no detectable activity. All assays were performed in triplicates and data reported represent the mean ± SD.

proteins (cytochrome *c* oxidase II and III, NADH dehydrogenase subunit II and V, ATP synthase F0 subunit 8, and cytochrome *b*) (Table 1). HsPDF exhibit low but detectable activity to three of the peptides (f-MAHAAQ, f-MTHQSH, and f-MTMHTT), with k_{cat}/K_M values in the range of 200−800 $M^{-1}s^{-1}$. These activities are again 20−50-fold lower than those of EcPDF against the same substrates (Table 2). Two of the peptides had very poor solubility and accurate activity measurement was not possible for either EcPDF or HsPDF (f-MNPLAQ and f-MPQKNT). HsPDF showed no detectable activity toward the remaining peptide (f-MTPMRK).

A striking difference between HsPDF (also mouse PDF) and bacterial PDF is that the leucine in the highly conserved EGCLS motif is mutated into a glutamic acid in mammalian PDF (Glu-173 in HsPDF). The leucyl side chain is involved in hydrophobic interactions with the P_2' side chain of a substrate (*25*−*27*). To determine whether this mutation is responsible for the low activity of HsPDF, we mutated Glu-173 of HsPDF back to leucine and the corresponding leucine (Leu-91) in EcPDF to a glutamic acid. When assayed against the above set of substrates, the E173L mutant HsPDF showed consistently higher activity than the wild-type enzyme (2−4-fold). Vice versa, mutation of Leu-91 to Glu resulted in a 100-fold reduction of catalytic activity in EcPDF (Table 2). Thus, mutation of the conserved leucyl residue is at least partially responsible for the low catalytic activity of mammalian PDF.

Intracellular Localization of HsPDF. The function of the N-terminal extension of HsPDF was assessed by several prediction software, including signalP (*28*) and Predotar (http://inra.fr/Internet/Produits/Predotar/). Both programs predicted that the N-terminal sequence contain organelle targeting signals. Also, since the mitochondrion is the only place where N-formylated proteins are synthesized, one would expect that HsPDF be localized in the mitochondrion, if its function were to deformylate proteins. To determine the subcellular localization of HsPDF, we fused the green fluorescence protein to the C-terminus of the full-length HsPDF. The fusion protein was transiently expressed in HEK cells. Confocal laser scanning microscopy revealed that the fusion protein is localized in the mitochondria (Figure 3). As a control, we transfected the HEK cells with the expression vector pEGFP(N1) alone, which produces only GFP in the cells. Green fluorescence was observed throughout the cytoplasm (data not shown).

Inhibition of HsPDF. Since PDF is being pursued as a target for antibacterial drug design, an important issue is whether the inhibitors designed against bacterial PDF will also inhibit HsPDF. BB-3497 (compound **1** in Table 3) is a potent inhibitor against bacterial PDF (K_I = 7 nM against EcPDF) and an orally available, broad-spectrum antibacterial agent (*13*). Its N-formylhydroxylamine moiety binds to the catalytic metal of PDF in a bidentate fashion (*13*). To address the issue, we synthesized BB-3497 and three analogues (compounds **2**−**4** in Table 3) and tested them against both HsPDF and EcPDF. All of the compounds showed potent inhibition of both enzymes, with K_I values in the low to medium nM range (Table 3). Among them, BB-3497 is the most potent, showing competitive inhibition pattern (Figure 4) and K_I values of 8 and 11 nM (this work) against HsPDF and EcPDF, respectively. Further, with the exception of

Table 2: Catalytic Activity of Wild-Type vs Mutant HsPDF and EcPDF

	k_{cat}/K_M ($M^{-1}s^{-1}$)			
substrate	Wt HsPDF	E173L HsPDF	Wt EcPDF	L91E EcPDF
f-ML-pNA	5900 ± 1100	21800 ± 3000	(1.2 ± 0.1) × 10^6	12000 ± 2000
f-MAHAAQ	230 ± 55	810 ± 60	12000 ± 1600	ND
f-MTHQSH	810 ± 65	1200 ± 400	15000 ± 1300	ND
f-MTMHTT	540 ± 50	1320 ± 450	29000 ± 5800	ND
f-MTPMRK	NA	NA	2390 ± 230	ND

ND, not determined; NA, no detectable activity.

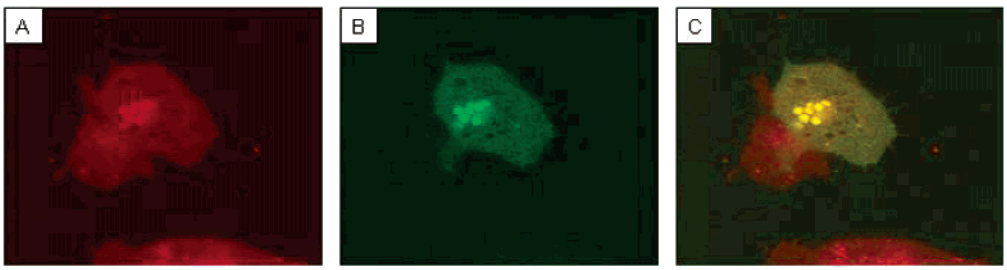
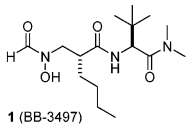
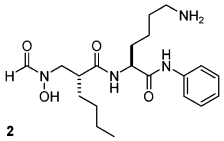
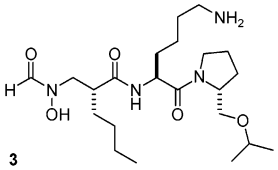
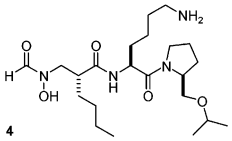


FIGURE 3: Intracellular localization of HsPDF−GFP fusion in HEK cells. Fluorescence signal was visualized under a confocal microscope. (A) Fluorescence signal of mitochondria stained by MitoTracker (red). (B) Fluorescence signal of GFP (green). (C) An overlay of panels A and B.

Table 3: Inhibition Constants (K_I, nM) of EcPDF and HsPDF

	HsPDF	EcPDF
 **1** (BB-3497)	8.0 ± 1.3	11 ± 1
 **2**	150 ± 17	18 ± 1
 **3**	36 ± 2	29 ± 3
 **4**	45 ± 2	59 ± 10

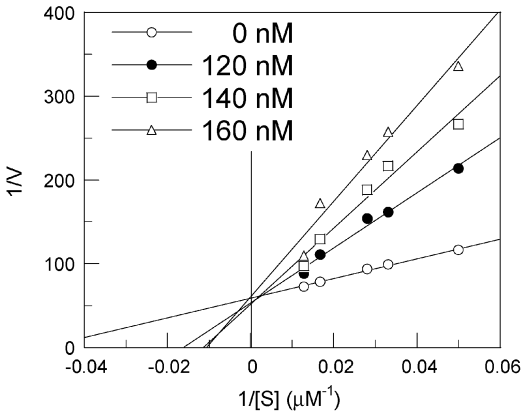


FIGURE 4: Lineweaver−Burk plot for the inhibition of HsPDF by BB-3497. Assay reactions (total volume of 500 μL) contained buffer E, 1.1 μg of HsPDF, 0−160 nM inhibitor, and 0−80 μM f-ML-pNA.

compound **2**, each of the inhibitors was essentially equipotent against both enzymes.

Effect of PDF Inhibitors on the Growth of Human Cells. BB-3497 was employed to test the effect of PDF inhibitors on human cells. Initially, we used a stably transfected HEK cell line that expresses GPF and monitored cell growth by following the fluorescence intensity of the cells. We treated the cells with various concentrations of the inhibitor (up to 128 μM) and monitored cell growth over a period of 3 days. The overall fluorescence intensity increased with time, but no significant difference was observed between the treated versus control cells (no inhibitor) (Figure 5A). Direct cell counting also revealed no difference between treated versus untreated cells. No obvious morphological difference could be observed either. Finally, we examined the effect of BB-3497 and inhibitor **2** on DNA synthesis by monitoring the incorporation of [^{3}H]thymidine using a leukemia cell line. Again, neither inhibitor (up to 150 μM) had any significant effect on the rate of DNA synthesis (Figure 5B).

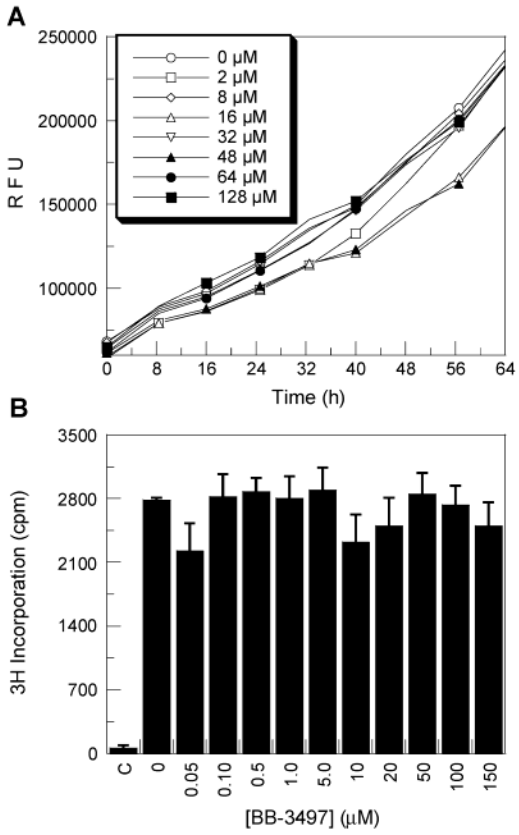


FIGURE 5: Effect of BB-3497 on human cell growth. (A) Fluorescence cell count. HEK cells that stably express GFP were treated with the indicated concentrations of BB-3497, and the total fluorescence of the culture was measured at various times. (B) [^{3}H]-thymidine incorporation. Acute lymphoblastic leukemia cells (L5178YD10/R) were grown in the presence of specified concentrations of BB-3497 (0−150 μM). The amount of radioactivity in the isolated DNA was measured by liquid scintillation counting. RFU, arbitrary fluorescence unit.

DISCUSSION

The question about the roles of mammalian PDF is of both biological and medical significance. Since numerous earlier studies have shown that mammalian mitochondrial proteins do not undergo N-terminal deformylation, what is the physiological function of PDF? As a practical matter, will PDF inhibitors also inhibit human PDF and have intolerable toxicity? In this work, we have shown unambiguously that the human PDF orthologue encodes a functional PDF. The fact that we and others were able to clone the cDNA indicates that transcription of the HsPDF gene took place. Indeed, Meinnel et al. have reported the detection of HsPDF mRNA in a wide variety of human tissues (*14*). Since the plasmid-encoded HsPDF−GFP fusion protein is synthesized and targeted to the mitochondrion, one can assume that the native HsPDF, encoded by chromosome 16, is also expressed and targeted to the mitochondrion. The lack of deformylation of mitochondrial proteins is likely due to one or both of the following factors. First, HsPDF (at least the N-terminally truncated Co^{2+} form) is an inefficient catalyst; its k_{cat}/K_M value is orders of magnitude lower than that of a typical bacterial PDF. Another possibility is that the N-termini of the mitochondrial proteins may not be accessible to PDF due to folding or being imbedded in the membranes. In this regard, it is notable that several bacterial proteins are known to retain their N-formyl group (*29*−*31*).

What is, then, the function of HsPDF? Our view is that HsPDF is a mere remnant of an ancient function that has no current role in mammalian cells, although we cannot rule out the possibility of it having a function totally unrelated to protein deformylation. PDF is an essential activity in bacteria apparently due to the inability of methionine aminopeptidase, another essential activity in all living organisms, to process N-formylated polypeptides (*1*−*4*). Given that the removal of the N-terminal methionine only occurs in a fraction of bacterial proteins, PDF is required probably because a small number of essential proteins cannot function properly unless their N-formylmethionine is removed. The mitochondrion is believed to have evolved from an ancestral endosymbiotic bacterium and shares many of the features of bacterial protein synthesis including translational initiation with N-formylmethionine. Presumably, HsPDF was an essential activity in the mitochondrion early on, when the organelle genome encoded a larger number of proteins, some of which required N-terminal processing for activity. As the mitochondrial genome shrank to encode just 13 proteins, all of which are apparently functional without deformylation, the essentiality of HsPDF was lost. The lack of any observable effect on human cells by BB-3497, a potent inhibitor against the purified HsPDF, is consistent with this notion. The acquisition of the L173E mutation in mammalian PDF during evolution, which significantly reduces its catalytic activity, also supports our view. It should be emphasized here that this notion may not apply to other organelles that have much larger genomes (e.g., chloroplasts and apicoplasts). In those organelles, the likelihood of having a protein that requires N-terminal processing is much higher, and PDF is more likely to be an essential activity (*14*).

There are also several possible explanations for the lack of effect on human cells by PDF inhibitors. One possibility is that HsPDF has no catalytic function, and therefore, its inhibition has no effect on the cell. Other possibilities include the inability of the inhibitor to travel into the mitochondrion and rapid degradation of the inhibitor in human cells. Regardless of the cause, the lack of toxicity to human cells by PDF inhibitors makes PDF an attractive target for designing novel antibiotics.

In conclusion, we have demonstrated that the human mitochondrion contains a PDF. However, it apparently has no functional role due to its poor catalytic activity. Since PDF inhibitors show little toxicity to human cells, PDF is an exciting new target for designing novel antibiotics.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

The synthetic details for PDF inhibitors. This material is available free of charge via the Internet at http://pubs.acs.org.

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