Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 332 (2005) 418–425
BBRC
www.elsevier.com/locate/ybbrc

The carboxy-terminal end of the peptide deformylase from *Mycobacterium tuberculosis* is indispensable for its enzymatic activity

Rahul Saxena, Pradip K. Chakraborti *

Institute of Microbial Technology, Sector 39A, Chandigarh 160 036, India

Received 15 April 2005
Available online 3 May 2005

Abstract

The peptide deformylase in bacteria is involved in removal of *N*-formyl group from newly synthesized proteins. The gene encoding this enzyme from *Mycobacterium tuberculosis* was cloned and expressed in *Escherichia coli*. The enzyme activity of the recombinant protein (mPDF) was insensitive to modulation by common monovalent/divalent cations. Kinetic analysis, using *N*-formylmethionine-alanine as the substrate, yielded K_{cat}/K_m of $\sim$1220 M^{-1} s^{-1}. Actinonin, a naturally occurring antibiotic, and 1,10-*ortho*-phenanthroline strongly inhibited the enzyme activity. The mPDF was very stable at 30 °C with a half-life of $\sim$4 h and exhibited resistance to oxidizing agents, like H_2O_2. Thus, the mPDF achieved distinction in its behavior among any reported iron-containing peptide deformylases. Furthermore, amino acid sequence analysis of mPDF revealed the presence of an unusually long carboxy-terminal end (residues 182–197), which is atypical for any gram-positive bacteria. Our results, through deletion analysis, for the first time established the role of this region in mPDF enzyme activity.

Keywords: Gene expression; Iron-containing peptide deformylase; Metalloprotease; Mycobacteria; Peptide deformylase

Protein synthesis in prokaryotes is initiated with *N*-formyl-methionyl-tRNA leading to formylation of all nascent polypeptides at the amino-terminal end. The *N*-formylmethionine, however, is not retained in mature proteins of eubacteria and has been reported to be deformylated by peptide deformylase [1]. This formylation/deformylation event appears to be a mandatory step in eubacterial protein synthesis [2] and therefore, the importance of this enzyme has long been envisaged. The presence of putative genes (named as *def*) for Peptide deformylase (PDF) is well known throughout the eubacterial lineage [3]. The recombinant proteins following over-expression in *Escherichia coli* were purified, characterized, and their structures from different bacteria have been solved in recent years [4–7]. The role of PDF is still poorly understood in eukaryotes. The existence of similar amino terminal protein processing mechanism has already been reported in eukaryotic organelles (mitochondria and chloroplasts), but not in cytosol [1,8].

PDF is a protease having a metal ion at its coordinating core. Presence of iron (as Fe^{2+}) or Zn^{2+} as the metal ion has been reported in naturally occurring PDFs from different bacteria [5,9,10]. However, for a long time the extreme sensitivity of the Fe^{2+} to environmental oxygen has prevented isolation as well as biochemical characterization of this enzyme from bacteria [11]. In this context, we have focused on PDF enzyme from *Mycobacterium tuberculosis*, the causative agent of the dreadful disease tuberculosis. It is noteworthy that *M. tuberculosis* as a successful pathogen withstands oxidative stress within the host and therefore the nature of PDF enzyme in this pathogen will definitely be interesting. Analysis of its

* Corresponding author. Fax: +91 172 2690585.
E-mail address: pradip@imtech.res.in (P.K. Chakraborti).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.04.142

genome sequence also indicated the presence of a putative *def* open reading frame [12]. However, no report has yet been available on the mycobacterial PDF enzyme as such.

In this communication, we carried out detailed characterization of PDF enzyme from *M. tuberculosis* (mPDF) following its over-expression and purification in *E. coli*. The recombinant mPDF was found to be an iron-containing enzyme. Its activity was very stable at 30 °C and insensitive to modulation by common monovalent/divalent cations. Furthermore, it maintained its distinction by exhibiting resistance to oxidizing agents, like H_2O_2. Sequence analysis revealed the presence of an unusually long carboxy-terminal end of mPDF, which is atypical for other gram-positive bacteria. Our results with deletion mutant unequivocally established the contribution of this region in conferring deformylation ability of the mPDF protein.

Materials and methods

PCR amplification, construction of recombinant plasmids, and generation of PDF-mutants. Oligonucleotides used in this study were custom synthesized (Biobasic, Canada). Restriction/modifying enzymes were obtained from New England Biolabs, USA. Genomic DNA was isolated from *M. tuberculosis* strain H37Ra as described previously [13] and used for PCR amplification of mPDF gene (*def*). Primers (CR1: 5′CATATGGCAGTGGTACCC3′ where *Nde*I site was incorporated and CR3: 5′CCATTAGTGACCGAACGGG3′) used were designed based on *def* (Rv0429c) sequence of published *M. tuberculosis* genome [12]. The *def* open reading frame (594 bp) was PCR-amplified using Expand long template PCR system (Roche) following manufacturer's recommended protocol. Following treatment with DNA polymerase I (Klenow), the PCR-amplified fragment was initially cloned in pUC19 vector (pUC-PDF) and its nucleic acid sequence was determined using an automated sequencer (Applied Biosystems). The construct was subsequently used for subcloning of *def* open reading frame at *Nde*I/*Hin*dIII sites of pET28c [14] and transformed in *E. coli* strain DH5α (pET-PDF). Clones containing gene of interest were confirmed by restriction analysis.

Different mutants of mPDF were generated using pUC-PDF as the template following PCR-based methods [15]. For the deletion mutant (TD), PCR was carried out with primers CR1 and CR23 (5′TTAAACGCCCCAGCCATG3′, designed eliminating 48 bases from the carboxy terminal end, see Fig. 7A). For other mutants (C106S, H148A, and H152A), two external (CR26: 5′GGA ATTCCATATGGCAGTCGTACCC3′ and CR27: 5′CCCAAGCT TTTAGTGACCGAACGG3′) and different internal primers (CR44: 5′AACCGACAGTGAGCCTTCGTC3′ and CR45: 5′GACGAA GGCTCACTGTCGGTT3′ for C106S; CR28: 5′ATGCTGCAGG CAGAAACCGGG3′ and CR29: 5′CCCGGTTTCTGCCTGCAGC ATCCG3′ for H148A; and CR32: 5′GAAACCGGGGCACTTGA TGGA3′ and CR33: 5′TCCATCAAGTGCCCCGGTTTC3′ for H152A) were used. PCR products containing desired mutations were initially cloned in pUC19, digested with *Sac*II/*Hin*dIII, and incorporated in the corresponding sites of pET-PDF to obtain either pET-TD or pET-C106S/-H148A/-H152A constructs.

Expression and purification of recombinant proteins. The pET-PDF or different mutants were transformed into *E. coli* strain BL21(DE3) for over-expression. For purification of proteins, overnight culture of these colonies (~15 h at 37 °C in LB broth containing 50 μg/ml of kanamycin) was re-inoculated and grown until OD_{600} of ~0.8. Cells were then induced with 0.4 mM IPTG at 25 °C, harvested after 12 h, and suspended in lysis buffer (20 mM phosphate buffer, pH 7.4, containing 5 mM DTT, 10 μg/ml catalase, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, and 1 μg/ml leupeptin). Cells were sonicated and the pellet fraction (~12,000*g* for 30 min at 4 °C) was resuspended in lysis buffer containing 3 M urea and 2% Triton X-100. Following centrifugation, supernatant fraction was dialyzed (14 h at 4 °C) to remove urea and purified on Ni–NTA column (Qiagen) following manufacturer's recommended protocol. Finally, mPDF or different mutants were eluted in elution buffer (20 mM phosphate buffer, pH 7.4, containing 300 mM NaCl, 250 mM imidazole, and 10 μg/ml catalase) and protein concentration was estimated following Bradford's method [16]. Recombinant PDF was also obtained as soluble protein following co-transformation of pET-PDF and pKY206 [17] in *E. coli* strain BL21(DE3). The protein concentration of the purified mPDF stock was maintained at 3.5 mg/ml and stored at −80 °C until used. Unless mentioned otherwise, mPDF was diluted in dilution buffer (20 mM phosphate buffer, pH 7.4, containing 1 mg/ml BSA and 10 μg/ml catalase) adjusting protein concentration at 3.5 μg/ml prior to use in assays.

Peptide deformylase activity. The ability of mPDF to deformylate N-formylmethionine was assessed in a spectrophotometric assay following the method described elsewhere [18] with slight modification. Briefly, in 50 μl reaction volume mPDF protein (usually 70 ng) in 1× assay buffer (100 mM phosphate buffer, pH 7.4, containing 100 μg/ml catalase) was incubated with the substrate (0–80 mM *N*-formyl-Met-Ala, Sigma, USA) at 30 °C for 30 min. The reaction was terminated by addition of 50 μl of 4% $HClO_4$ and further incubated (37 °C for 2 h) with TNBSA reagent (0.01% in 0.1 M $NaHCO_3$ buffer, pH 8.4). Following addition of 10% SDS (250 μl) and 1 N HCl (125 μl), the highly chromogenic derivative generated due to reaction of primary amine with TNBSA was measured at 335 nm [19]. The values obtained were corrected by subtracting the blank (all ingredients except mPDF enzyme) readings. Standard curves were prepared with known amounts (0–42.8 nmol) of methionine and the enzyme activity of mPDF was expressed as nanomoles of free amino group produced/min/mg protein. Finally, the data were presented in the form of means ± SD from at least three independent experiments.

Western blotting. Anti-his tag monoclonal antibody (Amersham Biosciences) was mainly used to monitor the expression of mPDF and mutant proteins in Western blotting. Samples (~1 μg protein/slot) were resolved in SDS–PAGE and transferred to nitrocellulose membrane (0.45 μm) using Tris–glycine–SDS buffer (48 mM Tris, 39 mM glycine, 0.037% SDS, and 20% methanol, pH ~8.3). Blots were probed with primary and secondary (horseradish peroxidase conjugated anti-mouse IgG) antibodies and processed with ECL detection system (Amersham Biosciences) as described elsewhere [20].

Bioinformatic analysis. Nucleotide derived amino acid sequence of mPDF was compared with 'nr' database in BLAST-P program using mail server at NIH [21]. The multiple sequence alignments of the retrieved sequences were carried out using the Clustal X 1.81 program [22]. Neural network based method was used for prediction of secondary structures of mPDF [23].

Results and discussion

The PDF enzyme catalyzes the deformylation of *N*-formylmethionine of the nascent polypeptide chains in the cytoplasm of prokaryotes [1]. Since amino-terminal peptidases are unable to utilize formylated peptides as substrates, therefore, importance of PDF was realized for a long time [1]. Interestingly, *def* has already been reported to be an essential gene in several bacteria and is currently being considered to be an appropriate target