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Nicotinamidase/pyrazinamidase of *Mycobacterium tuberculosis* forms homo-dimers stabilized by disulfide bonds

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Abstract

Recombinant wild-pyrazinamidase from H37Rv *M. tuberculosis* was analyzed by gel electrophoresis under differential reducing conditions to evaluate its quaternary structure. PZAse was fractionated by size exclusion chromatography under non-reducing conditions. PZAse activity was measured and mass spectrometry analysis was performed to determine the identity of proteins by *de novo* sequencing and to determine the presence of disulfide bonds.

This study confirmed that *M. tuberculosis* wild type PZAse was able to form homo-dimers *in vitro*. Homo-dimers showed a slightly lower specific PZAse activity compared to monomeric PZAse. PZAse dimers were dissociated into monomers in response to reducing conditions. Mass spectrometry analysis confirmed the existence of disulfide bonds (C72-C138 and C138-C138) stabilizing the quaternary structure of the PZAse homo-dimer.

Keywords

pyrazinamidase; nicotinamidase; pyrazinamide; nicotinamide; dimer; multimer; reducing stress; tuberculosis; mycobacterium; drug resistance

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INTRODUCTION

Pyrazinamidase (PZAse) is an essential enzyme in the mechanism of action of pyrazinamide (PZA) in *M. tuberculosis*. PZA is a pro-drug that enters bacteria by passive diffusion. It is converted to pyrazinoic acid (POA) by PZAse [1–3]. POA is expelled from the mycobacterium by an efflux mechanism [4]. In the acidic extracellular space is protonated and reabsorbed into the bacilli where the proton is released. This cycle results in POA accumulation and reduction of intracellular pH leading to a lethal disruption of membrane permeability [3]

Important characteristics of *M. tuberculosis* PZAse have been elucidated from the recently crystallized structure of *M. tuberculosis* PZAse [5] and from crystallized homologous hydrolases (N-Carbamoylsarcosine amidohydrolase from *Arthrobacter*, nicotinamidases from *Pyrococcus horikoshii* and *Acinetobacter baumannii* [6]), as well as from theoretical models of *M. tuberculosis* mutated PZAases [7]. *M. tuberculosis* PZAse has an active site that comprises residues D8, A134, and C138, and a metal binding site that comprises residues D49, H51, and H71 [5]

In this study we analyze the capacity of *M. tuberculosis* PZAse to form homo-dimers *in vitro* stabilized by disulfide bonds.

MATERIALS AND METHODS

Cloning, expression and purification

The DNA from *M. tuberculosis* H37Rv PZA susceptible *pncA* wild type reference strain was extracted and the entire *pncA* gene was amplified and purified (575bp) as described previously [8]. Briefly, for purification, a six-histidine tag was added to the carboxy-terminal end. The *pncA* cloned plasmid was transformed into *E. coli* BL21(DE3)pLysS cells (Novagen) as described above for protein expression. Protein expression was induced with 1mM Isopropyl β -D-thiogalactoside (IPTG) and also purified using Histrap columns as described before [9]. Aliquots of the fractions obtained were analyzed by 12% SDS-PAGE, and those containing pure PZAse (molecular weight: 20.7 kDa) were combined. The purified protein was concentrated 10 times and then washed 3 times with 20 mM Tris-HCl, pH 7.9 by ultra-filtration using cellulose membranes with 10 kDa pore size in an Ultracel Amicon Ultrafiltration system (Millipore, Billerica, MA) at 4°C.

Rabbit anti-PZAse sera

Two 2-month old New Zealand white rabbits, were immunized with recombinant PZAse in four doses given at intervals of four days. The first dose was 5 μ g of antigen with 50 μ g of saponin. The second, third, and fourth doses included 10, 20, and 40 μ g of antigen respectively with 50 μ g of saponin. Pre-immune and post-immune sera were tested in a Western immunoblot against recombinant PZAse to confirm the presence of anti-PZAse antibodies. The animal study component was reviewed and approved by the Institutional Review Board of Universidad Peruana Cayetano Heredia.

Polyacrylamide gel electrophoresis under reducing conditions

The purified PZase (5 mg/ml) was resolved on a SDS-PAGE 12% polyacrylamide gels as described before [9] under reducing and non-reducing conditions. 20 µl of each of these solutions were loaded on each lane. One gel was stained with Coomassie blue method (staining solution: 0.25 g on 100 ml of 45% water, 45% methanol, 10% acetic acid). A second gel (under non-reducing conditions) was stained with Western blot. Proteins were transferred to a nitrocellulose membrane and revealed against the pre and post immune rabbit serum.

Size exclusion chromatography

One ml of purified PZase (5 mg/ml) was resolved in a G75 Sephadex column (120 ml, 75 cm length), and equilibrated with sodium phosphate buffer (20 mM, 0.25 M NaCl and pH 7.4). Buffer (150 ml) was passed at a flow rate of 0.2 ml/minute and fractions of 2 ml were collected. The absorbance profile at 280 nm was monitored. Aliquots (1.5 ml) were freeze-dried and resuspended in 100 µl of water for further analysis..

PZase activity

Each eluted fraction from the size exclusion chromatography was tested for PZase activity with the Wayne test as described before [9]. Each eluted fraction was read in duplicate. The overall experiment was repeated two times. The specific activity was estimated as the amount of µmolar POA produced in a 1 min reaction divided by the total amount of enzyme as described before [9].

Mass spectrometry analysis

Three different bands obtained by polyacrylamide gel electrophoresis stained with Coomassie blue and with molecular masses estimated as 20.5 kDa, 39.7 kDa and 64 kDa (Figure 1) were individually analyzed by mass spectrometry.

In-gel tryptic digestion

In-gel digestion was performed using Trypsin Gold (Promega, Madison, WI). Briefly, excised spots were destained with destaining solution (100 µl of 100 mM ammonium bicarbonate/acetonitrile, 1:1, vol/vol), followed by reduction with 10 mM DTT/100 mM ammonium bicarbonate for 30 min at 60 °C and alkylation with 55 mM iodoacetamide/100 mM ammonium bicarbonate for 20 min in the dark. The gel plugs were washed twice with 50% acetonitrile (ACN)/100 mM ammonium bicarbonate, 20 min each wash, and dehydrated with 100% ACN for 20 min. The gel pieces were subsequently dried and digested overnight in 25 µl of 10 ng/µl trypsin at 37°C. Tryptic peptides were then extracted twice, first with 50% ACN for 15 min, followed by 70% ACN for another 15 min. The extracted solutions were pooled together into a clean tube and dried using a vacuum centrifuge.

Nanoflow HPLC-MS/MS

The tryptic peptide mixture was analyzed by online nanoflow liquid chromatography tandem mass spectrometry (LC-MS/MS) on Accela system (1/20 split-flow) connected to the LTQ

Orbitrap Velos instrument (Thermo Fisher Scientific, Bremen, Germany) through a Thermo nanoelectrospray ion source. 5 μ l of the peptide mixture was sampled directly onto a capillary column in-house packed with 3- μ m C₁₈ beads with a 90-min gradient from 5% to 50% acetonitrile in 0.5% formic acid. To prevent repetition in the detection of peptides due to capillary column overloading, the system was rinsed thoroughly and different samples were intercalated among analyzes. The effluent from the capillary column was directly electrosprayed into the mass spectrometer.

For high-resolution CID and HCD MS/MS top10 method, full scans MS spectra (from m/z 300–1800) were acquired in the Orbitrap analyzer after accumulation to a target value of $1e6$ in the linear ion trap. Resolution in the Orbitrap was set to $r = 60,000$ for full scan MS and $r = 30,000$ for MS/MS detection. The ten most intense signals with only charge state 2^+ and 3^+ were sequentially isolated and fragmented in the high-pressure linear ion trap by low energy Collision-Induced Dissociation (CID) with normalized collision energy of 35% and wideband-activation enabled. The same ten most intense peaks doubly and triply charged were also fragmented in parallel in the High-Energy Collision Dissociation (HCD) collision cell with normalized collision energy 40%. The resulting ion fragments from both CID and HCD were detected in the Orbitrap system. The ion selection thresholds were 500 counts for CID and 5,000 counts for HCD, accumulation time was 500 ms for full scans and 250 ms for both dissociation methods. A dynamic exclusion window of 0.5 Da was applied that prevented the same m/z from being selected for 180 s after its acquisition. Standard mass spectrometric conditions for all experiments were: spray voltage, 2.2 kV; no sheath and auxiliary gas flow; heated capillary temperature, 200 °C; predictive automatic gain control (AGC) enabled, and an S-lens RF level of 50–60% [10].

Determination of disulfide bonds

PZase was digested with Trypsin Gold without any previous treatment with reducing or alkylating agents. The tryptic digestion was performed in Tris-HCl under slightly acidic conditions (pH 6.8) for 12 h at 37°C. The presence of four cysteine residues in the PZase produces four peptides containing only one cysteine residue after the trypsin digestion: (i) DFHIDPGDHFSGTPDYSSSWPPHC₇₂VSGTPGADFHPSLDTSIAIEAVFYK, (ii) ALIIVDVQNDFC₁₄EGGSLAVTGGAALAR, (iii) GVDEVDDVVGIA TDHC₁₃₈VR, and (iv) TASVELVC₁₈₄SS. After trypsin digestion, the exact masses of the possible heterodimers composed by the combination of any pair of the four peptides described above were theoretically calculated. Theoretical molecular masses were convoluted in a charge envelope and the m/z values found were searched against experimental m/z values generated by LC-MS/MS to verify possible combinations and determine the disulfide bond arrangements. All peptides, monomers and possible dimers, were submitted to CID/HCD dissociation and partial amino acid sequence was determined for each dimer.

MS Data Analysis

Tandem mass spectra in Thermo RAW files were used to search a combined forward and reverse pyrazinamidase/nicotinamidase *pncA* (PZase encoding gene) database from *Mycobacterium tuberculosis* H37Rv by Protein Discoverer 1.3 software containing the Sequest algorithm. The spectral search parameters considered doubly and triply charged

tryptic peptides ions with a 50 ppm precursor mass window and 10 ppm tolerance was set to fragment ions. Carbamidomethylation (+57.02146) of cysteine was set as a static peptide modification and oxidation of methionine (+15.9949) was set as a variable peptide modification. Candidate peptides were filtered to a 1% protein FDR.

Structural model of the PZase homo-dimer

Quaternary structure docking was performed by HADDOCK web server [31]. The PZase structure was obtained from the Protein Data Bank (ID: 3PL1) [25]. The complex structure model was refined using molecular dynamics (MD) with NAMD 2.9. The structure file and a parameter and parameters were generated in VMD 1.9 with CHARMM27 protein topology and parameters. More specific details are shown in the supplementary material.

RESULTS

The H37Rv PZase was expressed and purified as a soluble protein at a final concentration of 5 mg/ml. The SDS-PAGE of the purified H37Rv PZase stained with coomassie blue and Western blot revealed under non-reducing conditions. The migration pattern of the purified PZase revealed bands corresponding to molecular weights of 20.5 kDa, 39.7 kDa and 64 kDa. However, under reducing conditions, a single band of 20.5 kDa was observed predominant (Figure 1).

The chromatogram from the size exclusion chromatography and the profile of PZase activity, showed peaks (p16, p27 and p37) associated to a 39.7 kDa band and a more intense band of 20.5 kDa (Figure 2). This evidence suggests that the peak p37 is likely to be the PZase monomer, while peaks p27 and p16 are likely to be predominantly PZase homo-dimers. The specific PZase activity of fraction p37 (a mixture of monomers and to a lesser extend dimers) was estimated to be $30.1 \mu\text{moles POA mg}^{-1} \text{PZase min}^{-1}$, which is within the range of previously reported activity (median $38.4 \mu\text{moles POA mg}^{-1} \text{PZase min}^{-1}$) [9]. The specific PZase activities of fraction p27 (likely to be pure dimer) and p16 were 26.7 and $5.7 \mu\text{moles POA mg}^{-1} \text{PZase min}^{-1}$ respectively.

LC-MS/MS analysis identified PZase with at least 2 unique peptides per sample with probability greater than 99%. Sample of 20 kDa were identified with 33.87% sequence coverage whereas samples of 40 kDa and 60 kDa were both identified with 19.35% sequence coverage. These results unequivocally identified PZase in their multimeric forms. Confirming this finding, the *de novo* sequencing of the generated peptides that were not linked by a disulfide bond confirmed the identity of the PZase. The combination of peptides linked by a disulfide bond were (i)+(iii) (6971.1 Da), and (iii)+(iii) (3563.72 Da). The MS/MS fragmentation of these dimers was sequenced *de novo*, confirming once again that a disulfide bond links these peptides (Figure 4 in supplementary material). Therefore it appears that at least disulfide bonds C72-C138 and C138-C138 stabilize the dimers.

The modeling of the PZase dimer structure for the C72-C138 214 dimer, found that the best cluster had 50 models with an average Van der Waals stabilization energy (VdW_E) of -52.8 ± 2.0 kcal/mol, an average electrostatic energy (El_E) of -197.3 ± 16.2 kcal/mol, and an average desolvation energy (Des_E) of -6.9 ± 5.5 kcal/mol. The best scored cluster for

the C138–C138 dimer had 68 models with VdW_E of -62.9 ± 4.1 kcal/mol, El_E of -212.1 ± 19.8 kcal/mol and Des_E of 8.3 ± 4.1 kcal/mol. The MD simulation found a distance between the disulfide cysteines sulphur atoms for the C72-C138 and the C138-C138 dimers as 2.05 Å and 2.11 Å respectively. The orientation of the disulfide bond of the type C₁72-C₂138 allows the active site of the first subunit to be exposed to the solvent in contrast with the C₁138-C₂138 dimer, where the catalytic residue C138 is blocked in both subunits, lacking PZase activity (Figure 3).

DISCUSSION

This is the first report of *M. tuberculosis* homo-dimers stabilized by disulfide bonds retaining enzymatic activity *in vitro*. PZase homo-dimer was only observed under non-reducing conditions.

In agreement with previous studies, the most abundant form of the PZase *in vitro* is the monomeric form [11]. With a lesser abundance we found evidence of PZase homo-multimers. A similar result has been found on previous reports of homologous nicotinamidases from *Leishmania infantum*, *Acinetobacter baumannii* and *Oceanobacillus iheyensis* that are able to organize as dimers [6, 12, 13].

This study demonstrates that disulfide bonds stabilize the PZase dimer. There are several ways in which the subunits can accommodate to form multimers, however only C72-C138 and C138-C138 were detected experimentally. According to the X-Ray crystalline structure of the H37Rv *M. tuberculosis* PZase (PDB code: 3PL1), C184 is a naturally exposed cysteine likely to form disulfide bonds with itself and C72 or C14, although C14 was not found to establish disulfide bonds.

The interaction between C72 and C138 does not show steric or electrostatic impairments associated with this disulfide bond. Participation of C138 impairs PZase activity of the particular subunit because C138 is a catalytic aminoacid. The enzymatic activity of the multimer complexes interacting with C138 is associated with the specific activity of the PZase subunits that do not bind through C138, which explains the observed PZase activity in the multimeric complexes. The estimation of the specific activity on the eluted protein fraction of the monomer is slightly higher than the corresponding eluted protein fraction of the dimer.

Only one crystal structure of monomeric *M. tuberculosis* PZase has been reported [5]. Despite multiple efforts and the importance of further understanding the structure of mutated PZases, no other group had succeeded in its crystallization. The fact that no dimers were observed in the only *M. tuberculosis* PZase crystal structure is probably due to the specific conditions used in the crystallization process. Eventually the possibility of PZase homo-oligomers formation could be the reason why PZase crystals are not easily reproduced.

M. tuberculosis PZase has nicotinamidase activity. It hydrolyzes nicotinamide to nicotinic acid and ammonia in the β -Nicotinamide adenine dinucleotide (NAD⁺) salvage pathway [14]. A function of NAD is the modulation of anti oxidation and oxidative stress, as the NADH/NAD⁺ ratio is a measure of cellular reducing potential. Intracellular redox changes in

M. tuberculosis has been previously reported [15]. Cultures of *M. tuberculosis* in presence of certain antibiotics were associated to a NADH/NAD⁺ ratio change of 2.65 fold compared to untreated mycobacterium.

Studies in mice liver indicate that excessive intracellular NADH can produce 'reductive stress', which may result from its capacity to induce release of ferrous iron from ferritin, an enzyme which is present in *M. tuberculosis*

Any evolutionary advantage or biological role of the PZAse homo-dimers *in vivo* might be associated to its specific nicotinamidase activity. To clarify this, further studies are necessary.

Conclusion

This study demonstrates that the *M. tuberculosis* PZAse is able to form homo-dimers with PZAse activity *in vitro*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Complexes formation of the H37Rv PZase. (A) SDS PAGE of soluble PZase under reducing (1% 2-mercaptoethanol) and non-reducing (-) conditions. (B) Identification of the H37Rv PZase multimeric complexes by Western immunoblot with pre and post immune sera of rabbit immunized with recombinant PZase.

Figure 2.

Separation of protein multimeric complexes by size exclusion chromatography (SEC) (**A**) Protein distribution detected by absorbance at 280 nm (—); and PZase activity (Δ) detected by the Wayne assay of the purified recombinant PZase. (**B**) SDS-PAGE of protein fractions associated to the 3 PZase activity peaks collected during the size exclusion chromatography (p16, p27, p37).

Figure 3.

The most favorable structures found by the HADDOCK server: **(A)** C138-C138 dimer after 1ns of molecular Dynamics. Both metal binding sites are shown in black, as well as the catalytic sites in white. Both of the active sites are inactivated as the catalytic cysteines shown in color form part of the disulfide bond and the **(B)** C138-C72 dimer after 1ns of molecular Dynamics. Both metal binding sites are shown in black, as well as the catalytic sites in white. The catalytic site from the PZase, which binds through C72, is complete and permeable to the solvent. The disulfide bond between C72-C138 is also shown in color.