Biochemical Characterization and Computational Identification of Mycobacterium tuberculosis Pyrazinamidase in Some Pyrazinamide-Resistant Isolates of Iran

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Biochemical Characterization and Computational Identification of *Mycobacterium tuberculosis* Pyrazinamidase in Some Pyrazinamide-Resistant Isolates of Iran

Farahnoosh Doustdar\(^1\), Mohammad Pazhang\(^3\), Faramarz Mehrnejad\(^4\), Mehrnoosh Safarzadeh\(^3\), Davod Rabiei\(^3\), Nader Chaparzadeh\(^3\), Hanieh Falahati\(^5\), Mohammad Mir-Derikvand\(^4\)

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Abstract Pyrazinamide (PZA) is one of the first-line anti-tuberculosis drugs that require activation by the pyrazinamidase (PZase). Most PZA-resistant *Mycobacterium tuberculosis* strains have mutations in the *pncA* gene which encoding PZase that result in the reduction or loss of the enzyme activity. Herein, we have examined how various mutations, which have been found from the PZA-resistant *M. tuberculosis* strains in Iran, modify the PZase activity. To elucidate the possible role of these mutations, namely A143T (MUT1), L151S (MUT2), A143T/T168A/E173K (MUT3), in the bioactivity of the enzyme, the PZase and mutant genes were cloned, functionally expressed and biochemically and computationally characterized. In comparison to the PZase enzyme, the enzymatic efficiency of mutant enzymes was decreased, with MUT2 indicating the largest enzymatic efficiency reduction. Homology models of mutants were constructed based on the PZase X-ray crystal structure. Molecular modeling and substrate docking revealed that the wild-type has much stronger binding affinity to PZA than the mutants whereas MUT2 has the weakest binding affinity. In addition, the molecular dynamics simulations and the essential dynamics results illustrated that the positions of the 51st to 71st residues were more dynamics in MUT2 as compared to the other atoms in PZase, MUT1 and MUT3 which could decrease the *K_m* and *k_{cat}* values of the enzymes.

Keywords Pyrazinamidase · *Mycobacterium tuberculosis* · Cloning · Molecular dynamics simulation · Docking

List of symbols

- PZA Pyrazinamide
- PZase Pyrazinamidase
- MUT1 Mutation A143T
- MUT2 Mutation L151S
- MUT3 Mutation A143T/T168A/E173K
- TB Tuberculosis
- MDR-TB Multidrug-resistant tuberculosis
- XDR-TB Extensively drug-resistant tuberculosis
- POA Pyrazinoic acid
- *pncA* Pyrazinamidase coding gene
- RMSD Root mean square deviation
- MD Molecular dynamics
- SPC Simple point charge
- PME Particle Mesh Ewald
- RMSF Root-mean-square fluctuation
- ED Essential dynamics
- MSD Mean square displacements
- OD Optical density
- PDB Protein Data Bank
1 Introduction

Tuberculosis (TB) is an airborne disease caused by the *Mycobacterium tuberculosis*. *M. tuberculosis* takes 1.2–1.5 million lives each year one-third of the global population is latently infected with the bacilli [1]. The number of multidrug-resistant (MDR) TB strains, including extensively drug-resistant tuberculosis (XDR-TB), is increasing globally [2–4]. Therefore, there is an urgent need to design new anti-tuberculosis drugs to combat the disease [5].

Pyrazinamide (PZA), isoniazid and rifampin combination is one of the first-line drugs used for treating of TB [6]. Therefore, prodrug PZA is important part of this shortened chemotherapy because of its activity against the persisting *M. tuberculosis* at an acidic pH [7, 8]. PZA is converted into the active forms, pyrazinoic acid (POA), by mycobacterial pyrazinamidase (PZase) [9]. A major correlation was shown between mutations in PZase coding gene (*pncA*) and PZA resistance ranging from 66.7 to 96.8 %, hence, several studies have suggested that PZA resistance in *M. tuberculosis* is mainly attributed to the mutations in *pncA* gene [10–14]. Interestingly, such mutations are very diverse in different geographical areas, highlighting the need for further characterization of *pncA* mutations.

The structure of PZase was resolved by Petrella et al [15] who showed that the PZase structure contains 185 residues, which are composed of a six stranded parallel β sheet with four helices packed from both sides. The active site cysteine residue, Cys138, is positioned in the N-terminal side of the α3 helix (Fig. 1). The study of the mutations causing significant loss of PZase activity has revealed alterations in the active or metal-binding sites [15–17]. A common method used for mapping the structural features of an enzyme’s active site is to follow the effect of various mutations on the kinetics of the enzyme. By comparing the rates of deamination of PZA it is possible to define the essential structural feature of the active site that is involved in binding to the substrate.

Although PZA is used as a frontline drug to treat TB in Iran, structural and functional data on PZA resistance-related mutant PZases are rare [18]. In this study, the wild-type and three mutant PZases isolated from different PZA-resistant *M. tuberculosis* strains in Iran [19], were cloned, expressed, and purified. The effects of these mutations were investigated experimentally by comparing the kinetic parameters and the thermostability of these mutant enzymes to the wild-type PZase. In addition, we perform computational molecular dynamics simulations to establish further understanding of the effect of such mutations. This extensive experimental and computational study provides mechanistic insights into the enzymatic activity of PZase.

2 Materials and Methods

2.1 Reagents and Bacterial Strains

We have used *E. coli* DH5α (Basingstoke, Hampshire, UK) and *E. coli* BL21 (DE3) (Novagen, Madison, WI, USA) for the routine isolation and purification of plasmid DNA and for the expression of the recombinant enzymes, respectively. All gel, PCR purification and plasmid extraction kits were purchased from Bioneer (Korea). For expression, the pET-28a (+) vector (Novagen, Madison, WI, USA) was used. All solutions for PCR, Taq DNA polymerase, restriction enzymes and T4 DNA ligase were purchased from Fermentas, Lithuania. Ni–NTA super flow was purchased from QIAGEN, USA. Acrylamide, N, N′-methylenebisacrylamide, Tris, ammonium persulfate, and TEMED were obtained from Merck, Germany. Genomic DNA was isolated from *M. tuberculosis*.
2.2 Gene Cloning and DNA Sequencing

The cloning and transformation methods used were adopted from Sambrook et al. [20]. The 558 bp genes, PZase and mutants genes, were amplified by using the following primers: forward (5′-GTCGTTCATGTTGCG-GATCG-3′) and reverse (5′-GCTTTGCAGCGAGGC TCCA-3′) [21]. The PCR products were digested with NdeI/BamHI. The purified PCR products were cloned into the digested expression vector pET-28a (+). The constructed recombinant vectors were transformed into the E. coli BL21 (DE3) cells, made competent by the calcium chloride procedure. All cloned genes have been confirmed by DNA sequencing [22]. For homology searches, we have used BLASTP and BLASTN through the NCBI server [23]. The amino acid and gene sequences of PZase were derived from the SwissProt databases and EMBL/GenBank; respectively [24, 25]. Multiple sequence alignment has been done by The CLUSTALW [26] and ESPript [27].

2.3 Expression and Purification

A single E. coli BL21 (DE3) colony, harboring the recombinant pET-28a (+) plasmid, was inoculated in Luria–Bertani (LB) medium containing 50 μg/ml ampicillin grown overnight. When the bacterial cells had been cultured at 37 °C with shaking at 250 rpm to an optical density (OD 600) of 0.4–0.6, the culture was inoculated into 500 ml of the fermentation media (with 50 μg/ml ampicillin) at a ratio of 5 % (v/v) for recombinant enzyme production at various cultivation conditions. The expression of wild-type and mutants enzymes was induced by isopropylthio-D-galactoside (IPTG) and the cells were incubated overnight at 22 °C, 200 rpm. The cells in 30 ml culture were centrifuged at 4000 rpm for 20 min, re-suspended in 20 mM Tris–HCl (pH 6.0). The bacterial cell disruption has been done by sonication at 4 °C for 5 min, and protein purification was carried out as previously described [15]. To analysis the purity and molecular weight of the proteins, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used [28]. We have measured protein concentration according to the method of Bradford [29] using bovine serum albumin as the standard.

2.4 Enzymes Assays

The wild-type and mutants activities were obtained by a modification of the Wayne test [30]. The reaction mixture has contained a 50 μl of a solution containing 40 mM PZA, 100 mM tris (pH 6.5) and 2 mM mercaptoethanol. After incubation at 37 for 3 min, 890 ml of 0.1 M Gly–HCL (pH 3.4) and 10 ml of 20 % FeNH₄(SO₄)₂ were added, and the precipitates were then collected by centrifugation (13,000 rpm for 10 min), and OD₄₈₀ of the supernatant was calculated. To determination of the concentration of POA, a standard curve was used. In order to estimate the kinetic parameters, all assays were measured in different concentrations of PZA. The steady-state kinetic parameters were calculated and the experimental data were studied by Lineweaver–Burk curve (“Appendix”).

2.5 The Homology Modeling and Molecular Docking

The initial model of computational studies came from X-ray crystal structure (Fig. 1) of PZase (Protein Data Bank ID: 3PL1) [15]. This structure was used as template to model the 3D structure of MUT1 (A143T), MUT2 (L151S), and MUT3 (A143T/T168A/E173K). The model of enzymes were constructed by using the MODELLER 9v10 program [31]. The programs of ProCheck (http://nih server.mbi.ucla.edu/SAVS/), Protein Structure Quality Score (PSQS) (http://www1.jcsg.org/psqs), Verify3D and ERRAT were used to check the protein model quality.

In order to study the enzymes and PZA interactions, PZase and the mutant models have been docked with PZA using the server of PatchDock (http://www.dockingserver.com) [32, 33]. The 3D structures of enzymes and PZA, in PDB format, have been uploaded in the server of PatchDock with a clustering root mean square deviation (RMSD) criterion of 4.0 Å. The candidate complexes have been chosen according to the highest geometric shape complementarity score. The geometry of enzymes and PZA structures were then energy minimized by using 1000 steps of the steepest descent and 1000 steps of the conjugate gradient methods, respectively. The GROMACS 4.5.5 software was used to prepare the energy minimization [34, 35].

2.6 Molecular Dynamics Simulation

For all MD simulations, we have used the GROMACS 4.5.5 package [34, 35]. The wild-type and mutated enzymes were solvated with simple point charge (SPC) water [36] and placed in a cubic box containing the protein and 1 nm of solvent on all sides. Na⁺ and Cl⁻ ions were added by replacing water molecules to neutralize the simulation cells. In all MD simulations, the pressure and the temperature were maintained close to the intended values (1 bar and 310 K) by using the algorithm of Berendsen [37] with coupling time constant of τₚ = 0.5 ps for pressure and τₜ = 0.1 for temperature. The lengths of all bonds were maintained constant by using the LINCS algorithm [38]. The initial velocities of atoms were taken randomly from a Maxwell–Boltzmann distribution at the desired initial temperature. For all nonbonded interactions, we used...
a short-range spherical cutoff of 0.9 nm and while the Particle Mesh Ewald (PME) method was used for the long-range interactions [39]. All of the MD simulations were then energy-minimized by steepest descent energy minimization. The systems were then simulated for 100 ps under constant volume conditions, and finally the equilibration was completed with a 1000 ps of NPT simulation. Finally, each system was simulated at 300 K for 120 ns.

2.7 The MD Simulations Analysis

Two independent trajectories were analyzed and the simulation data plotted in all figures contain analysis from one of the two trajectories. Hydrogen bonds are calculated using a geometric criterion: a maximum hydrogen acceptor distance of 0.25 nm and a minimum donor-hydrogen-acceptor angle of 135°. Energies of hydrogen bonds (\(E_{\text{HB}}\)) were estimated using the empirical function established by Espinosa et al. [40]

\[ E_{\text{HB}} = 2.5 \times 10^4 \exp(-3.6 \times d(H - O)), \]

where \(d\) (H–O) denotes the distance between hydrogen atom and acceptor atom.

2.8 The Essential Dynamics Analysis

Essential dynamics (ED) analyses have been done according to the protocol [41] within the GROMACS software. The analyses were focused on the motions of the 285 \(C_\alpha\) atoms of the enzymes. The 855 × 855 covariance matrix was built based on the MD trajectories after removal of the translational and rotational movements and then diagonalized to calculate the eigenvectors and eigenvalues. The movements of the enzymes in the essential subspace have been projected along the most important eigenvectors from the analysis.

3 Results

3.1 Sequence Analysis, Molecular Cloning, Expression and Purification

A 558 bp fragment was amplified by PCR with specific primers designed on the basis of gene sequences of the wild-type and mutants PZases from the standard strain and the clinical isolates of \(M.\) \(\text{tuberculosis}\), respectively. The genes were cloned into the pET-28a (+) vector. The wild-type gene sequence was taken from GenBank (The accession number: JX303228). The amino acid sequences, consisting of 185 residues, for the three mutants were compared to the wild-type PZase (Fig. 2) using BLASTP. The amino acid sequences of the wild-type PZase showed sequence identity of 99.46, 99.46, and 98.38 with MUT1, MUT2 and MUT3, respectively. Following the enzymes purification, the recombinant proteins were analyzed by using SDS-PAGE. For each isolated enzyme, one homologous band has been observed with a molecular mass of about 21 kDa (The data not shown).

3.2 Characterization of the Enzymes

The enzymatic activity of PZase and the mutants was analyzed using PZA as the substrate. The kinetic parameters of the PZase and mutant enzymes are given in Table 1. These results showed that \(k_{\text{cat}}\) and \(K_m\) of the mutant enzymes have decreased relative to the wild type while the catalytic efficiencies of the mutants MUT1 and MUT3 have not changed significantly. MUT2 had the lowest \(K_m\) and \(k_{\text{cat}}\), namely 0.431 (mM) and 43.51 (min\(^{-1}\)), respectively. Besides, in the case of MUT3, mutation has not profoundly affected on the values of \(k_{\text{cat}}\) and \(K_m\), the decrease in \(k_{\text{cat}}\) and \(K_m\) of the mutants suggests structural changes in the mutants.

For further characterization of the recombinant enzymes, their thermostabilities were obtained by measuring their activity for different durations of incubation at 40 °C. By plotting the \(\ln\) (% residual activity) against time we have calculated \(k_{\text{inactivation}}\) and \(t_{1/2}\) (half-life) for each of the wild-type and mutant proteins (Fig. 3; Table 2). Our results exhibited that the thermostability of the wild-type enzyme is similar to MUT1 while it decreased in MUT2 and MUT3.

3.3 Models Construction and Molecular Docking

The structural models of the mutant proteins were initially established using of PZase as the template (Fig. 4). A high level of sequence similarity has been shown by the models, and catalytic amino acids have been remained conserved. In order to study the effect of the mutations on the molecular docking, the binding pocket volumes of the PZase and the mutant enzymes have been evaluated. The calculated pocket volume of the wild type, MUT1, MUT2 and MUT3 proteins were 652.4, 636.7, 583.8 and 620.7 (Å\(^3\)), respectively (Table 3), showing considerable differences. These data revealed that the pocket volume of the mutant enzyme is smaller than the wild-type PZase. This suggests that the weak activities of the mutants in comparison with wild-type PZase are probably due to their smaller volume.

We next used PatchDock algorithm, which is used for structure prediction of PZase–PZA complexes as well as the shape complementarity of PZA with the enzyme. The docking score of complex of PZA with the wild-type, MUT1, MUT2 and MUT3 was 2522, 2394, 2374 and 2494, respectively (Table 3). Consequently, the wild-type
enzyme has the best complementarity as indicated by the highest docking score. In addition, the enzyme-PZA van der Waals, the enzyme-PZA electrostatic and the total enzyme-PZA binding energies were calculated from the docking analysis (Table 3). The wild-type enzyme had the highest electrostatic, van der Waals and total binding

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**Table 1** Kinetic parameters of PZase, MUT1, MUT2 and MUT3

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>$k_m$(mM)</th>
<th>$K_m$(min$^{-1}$)</th>
<th>PZase efficiency (mM$^{-1}$ min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PZase</td>
<td>0.790 ± 0.01</td>
<td>120.0 ± 0.1</td>
<td>151.89</td>
</tr>
<tr>
<td>MUT1</td>
<td>0.460 ± 0.02</td>
<td>64.52 ± 0.1</td>
<td>140.26</td>
</tr>
<tr>
<td>MUT2</td>
<td>0.431 ± 0.02</td>
<td>43.51 ± 0.1</td>
<td>100.95</td>
</tr>
<tr>
<td>MUT3</td>
<td>0.680 ± 0.01</td>
<td>100.23 ± 0.1</td>
<td>147.39</td>
</tr>
</tbody>
</table>

**Table 2** $k_{inactivation}$ and half-life of PZase, MUT1, MUT2, and MUT3

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>$k_{in}$ (1/min) $\times 10^3$</th>
<th>Half-life (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PZase</td>
<td>4 ± 0.08</td>
<td>173.25 ± 8</td>
</tr>
<tr>
<td>MUT1</td>
<td>3 ± 0.05</td>
<td>201.00 ± 12</td>
</tr>
<tr>
<td>MUT2</td>
<td>10 ± 0.3</td>
<td>69.30 ± 4</td>
</tr>
<tr>
<td>MUT3</td>
<td>20 ± 0.5</td>
<td>34.65 ± 2</td>
</tr>
</tbody>
</table>
energy values for the wild-type were $-0.26$, $-4.78$ and $-4.62$ kcal/mol, respectively. The MUT1-PZA complex has shown van der Waals and electrostatic energy levels of $-3.43$ and $-0.16$ kcal/mol, respectively. The total binding energy of MUT1-PZA was $-3.13$ kcal/mol. The van der Waals and electrostatic energies of the MUT2-PZA complex were $-3.25$ and $-0.08$ kcal/mol, respectively, and the total binding energy of this complex was $-2.96$ kcal/mol. The MUT3-PZA complex has indicated the van der Waals and electrostatic energy levels of $-4.14$ and $-0.25$ kcal/mol, respectively, and the total binding energy of complex was $-3.99$ kcal/mol (Table 3). Based on these energies, the native enzyme has much stronger binding interaction with PZA than the mutants, and also MUT2 has the weakest binding energy with PZA.

### 3.4 The Effects of Point Mutations on PZase Structure and Dynamics

To study the effects of reported point mutations on PZase structure and dynamics, we performed all-atom MD simulations of the wild-type and three PZase mutants. In Fig. 5, the $C_{\alpha}$ root-mean-square fluctuation (RMSF) of the average was examined as a function of residue number. As can be seen, the fluctuations in the mutants are higher than the wild-type in almost all residues. A further comparison of the MD results has indicated that MUT1, MUT2 and MUT3 have shown more flexibility in the residues of positions 51–71 (the 51–71 loop). In MUT2 the 51–71 loop exhibited the highest degree of fluctuation compared to the PZase, MUT1 and MUT3 conformations (Fig. 5). The fluctuations of the binding residues (Asp8, Lys96, Cys 138) were also lowest in the wild-type compared to the mutant enzymes (Fig. 5).

To study the mass-weighted root mean square distance of atoms from their common center of mass, we have calculated the radius of gyration of $C_{\alpha}$ atoms of the PZase...
and mutant enzymes over time (Fig. 6). Based on this method, we have observed a major deviation in MUT2 between 50 and 120 ns and in MUT3 between 90 and 120 ns. These analyses showed that the wild-type and MUT1 conformations are largely preserved throughout the MD simulation time (Fig. 6). The Rg values also showed that MUT2 is more flexible with respect to the other mutants and the wild-type throughout the simulation time.

In order to show the changes in the secondary structure of the enzymes, we used the dictionary of secondary structure of proteins (DSSP) algorithm [42]. Using this method we have observed that the most significant structural changes were a decrease in sheet and increase in helical content, which were observed in MUT2 and MUT3 conformations (Table 4).

To further support the MD simulation results, we have performed an ED analysis to study the differences in the molecular motions between the PZase and mutant enzymes. Using this method, we have calculated the mean square displacements (MSD) of the first 10 eigenvector projections as a function of eigenvector index. For the eigenvectors with indices >1, significant differences were obtained in the MSD of the eigenvector projections, which can be used to study the changes in the protein motions between the mutants and wild-type enzymes (Fig. 7). These results have revealed that the most significant motions for the MUT2 are shown in the subspace spanned by eigenvector 4 (Fig. 7). Therefore, our ED study has confirmed that MUT2 is more flexible than the wild-type and other mutant enzymes (Fig. 8).

### 3.5 Hydrogen Bond Analyses of the 51–71 Loop

To study the reason of increased flexibility in the 51–71 loop after mutations, we have calculated hydrogen bonds existing in this region for the last 20 ns of the MD simulations. Overall, 12 hydrogen bonds existed in this Loop (residues 51–71) of wild-type PZase, 7 hydrogen bonds existed in MUT1, 5 hydrogen bonds existed in MUT2 and 11 hydrogen bonds existed in MUT3 (Table 5). The hydrogen bonds involving S66, S65 and S61 in the wild-type were not observed in MUT1 and MUT2. In the wild-type, the occupancies of hydrogen bonds involving S59 were all above 98 %, much larger than those in MUT1, MUT2 and MUT3 (Table 5). Such weaker hydrogen bonding interactions could contribute to the increased flexibility of the Loop in mutant PZases.

### 3.6 Radial Distribution Function (RDF) Analysis of Water Around Mutant and Native Residues

Water molecules play a crucial role during the folding/unfolding of all proteins. These molecules form a narrow hydration shell on the scale of nanometer around proteins. Previous studies have shown that water molecules in the solvation shell result a specific arrangement of the hydrophilic and hydrophobic residues [34, 43]. Therefore, the hydrophilic residues are exposed and are in direct contact with the solvent while the hydrophobic residues are usually buried into the interior surface. Here, we have computed the radial distribution function (RDF) of the water molecules that solvated the hydrophilic residues. In MUT1 and

---

**Table 4** Comparative analysis of protein secondary structures of PZase and mutant enzymes

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>β sheet</th>
<th>α-helix</th>
<th>Coil</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>PZase</td>
<td>24</td>
<td>11</td>
<td>57</td>
<td>8</td>
</tr>
<tr>
<td>MUT1</td>
<td>25</td>
<td>11</td>
<td>54</td>
<td>10</td>
</tr>
<tr>
<td>MUT2</td>
<td>22</td>
<td>12</td>
<td>59</td>
<td>7</td>
</tr>
<tr>
<td>MUT3</td>
<td>22</td>
<td>14</td>
<td>59</td>
<td>5</td>
</tr>
</tbody>
</table>
MUT2, the water molecules can form hydrogen bonds with residues 143 and 151 either via the side-chain hydroxyl group oxygen atom or via the hydrogen atom (Fig. 9). In the first shell of solvation, the first peak was located at r = 0.18 nm, showing the formation of hydrogen bonds. The second peak was located 0.31 nm away from the atom of oxygen, which relates to the second hydrogen atom on the water molecules nearest to Thr143 and Ser151 (Fig. 10). Accordingly, the higher amount of water molecules might be able to penetrate into MUT1 and MUT2 compared to the wild-type. In MUT3, the RDF of Thr143 had a sharp first peak at 0.18 nm and a wider second peak at 0.32 nm indicating the first and the second solvation shells surrounding the hydroxyl group, respectively (Fig. 10). The side chain of Lys173 in MUT3 is less hydrated when compared to Glu173 in the wild-type (Fig. 10).

4 Discussion

In recent years, PZA is an essential first-line antitubercular drug. Activation of PZA by PZase is a necessary step for the treatment of TB. However, previous studies have shown that the decreased activity of mutant PZases correlates well with the structural and dynamical modifications [17, 44, 45]. Here, we have combined the enzymatic and kinetic properties, molecular docking, molecular dynamics simulations and ED to understand the molecular consequences of gene mutations on the structure and function of PZase, as well as its interaction with PZA upon binding. The biochemical analyses have shown a simultaneous decrease in $K_{m}$ and $k_{cat}$ for MUT1, MUT2 and MUT3 when compared to the wild type. Among these mutants, MUT2 has shown the largest reduction in the enzymatic efficiency.
The molecular docking process and the interaction analysis have shown that the wild-type enzyme has a better complementarity and more interactions with PZA compared to the mutant enzymes. The lower docking score of the mutants compared to the PZase suggests that the structure of the active site of the enzyme when forming a complex with the substrate is more loose in the mutants allowing for more thermal fluctuations of the active site.

**Table 5** The percent occupation of the hydrogen bonds existing in the 51–71 loop (residues from 51 to 71) of the wild-type and the mutant proteins during the last 20 ns of MD simulations

<table>
<thead>
<tr>
<th>Residue</th>
<th>Donor atom</th>
<th>Residue</th>
<th>Acceptor atom</th>
<th>Occupancy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PZase</td>
<td>MUT1</td>
<td>MUT2</td>
<td>MUT3</td>
<td></td>
</tr>
<tr>
<td>His51</td>
<td>N–H</td>
<td>His51</td>
<td>ND1</td>
<td>100</td>
</tr>
<tr>
<td>His57</td>
<td>NE2–HE2</td>
<td>Gly60</td>
<td>O</td>
<td>65.30</td>
</tr>
<tr>
<td>His57</td>
<td>NE2–HE2</td>
<td>Ser59</td>
<td>OG1</td>
<td>78.21</td>
</tr>
<tr>
<td>His57</td>
<td>NE2–HE2</td>
<td>Trp68</td>
<td>NE1</td>
<td>73.10</td>
</tr>
<tr>
<td>Phe58</td>
<td>N–H</td>
<td>Pro54</td>
<td>O</td>
<td>76.22</td>
</tr>
<tr>
<td>Phe58</td>
<td>N–H</td>
<td>Gly55</td>
<td>O</td>
<td>73.96</td>
</tr>
<tr>
<td>Phe58</td>
<td>N–H</td>
<td>Asp56</td>
<td>O</td>
<td>83.87</td>
</tr>
<tr>
<td>Ser59</td>
<td>OG–HG</td>
<td>Asp56</td>
<td>OD</td>
<td>81.23</td>
</tr>
<tr>
<td>Ser59</td>
<td>N–H</td>
<td>Asp56</td>
<td>O</td>
<td>97.10</td>
</tr>
<tr>
<td>Ser59</td>
<td>OG</td>
<td>Gly60</td>
<td>N–H</td>
<td>98.36</td>
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<td>Ser59</td>
<td>OG</td>
<td>Thr61</td>
<td>N–H</td>
<td>63.58</td>
</tr>
<tr>
<td>Ser59</td>
<td>OG–HG</td>
<td>Trp68</td>
<td>O</td>
<td>77.78</td>
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<td>Trp68</td>
<td>O</td>
<td>81.65</td>
</tr>
<tr>
<td>Gly60</td>
<td>N–H</td>
<td>Phe58</td>
<td>O</td>
<td>72.31</td>
</tr>
<tr>
<td>Gly60</td>
<td>N–H</td>
<td>His57</td>
<td>ND1</td>
<td>86.32</td>
</tr>
<tr>
<td>Gly60</td>
<td>N–H</td>
<td>Trp68</td>
<td>O</td>
<td>59.20</td>
</tr>
<tr>
<td>Thr61</td>
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![Fig. 9 Radial distribution function (RDF) analysis of water around mutant and native residues in MUT1 and MUT2](image)
atoms that can make it difficult to convert PZA to the active form, POA. Overall, the docking results are consistent with the experimentally observed activity profiles of each of the studied enzymes.

Molecular dynamics simulation is one of the well-known theoretical techniques, popularly used for assessing the stability of any predicted 3D model. The predicted 3D homology models of mutant enzymes were processed by the MD simulations for a 120 ns time scale. The relative flexibility of each system was characterized by plotting RMSF for the wild-type and the mutant enzymes. The wild-type has the lowest RMSF value indicating the minimum fluctuation. The results of MD simulations of the modeled enzymes depicted that the residues 51–71 were indeed dynamic as compared to the other Cα atoms in the enzymes. Herein, the mutant enzymes have shown more flexibility between the positions of 51st to 71st residues. Therefore, these mutations caused increased flexibility of the binding loop residues, reflecting the loss of the anchoring hydrogen bonds. The highest loop flexibility belonged to MUT 2 (with 5 hydrogen bonds) and the lowest loop flexibility was found for MUT 3 (with 11 hydrogen bonds). On the other hand, kinetic results have indicated that these mutations in PZase decrease $K_m$ and $k_{cat}$ of the enzyme. It has been proved that the 51–71 loop is directly involved in the PZA binding [46]. Our results have demonstrated that the loop flexibility not only has a role in the substrate binding ($K_m$) but also can affect catalysis of the enzyme ($k_{cat}$). This suggests that there is a clear correlation between the activity of different mutants and the loop flexibility. For example, MUT 2 has the highest loop flexibility observed among the mutants, but the lowest PZase activity.

The ED results have shown that PZase mutants obtained more degree of flexibility in the phase space. Interestingly, MUT 2 has displayed measurable differences in the computational and biochemical characteristics when compared to the wild-type enzyme, thus providing supporting data to conclude that the mutation of L151S has altered the structure and function of protein. The residue L151 is located on the $\alpha_3$ ‘gating’ the residue C138, which is critical for PZA substrate recognition.

Thermostability results demonstrated that among the recombinant enzymes, MUT 1 and MUT 3 have the highest and the lowest stability (kinetic stability), respectively. One of the reasons for resistance to PZA can be reduction in the stability of PZase. As noted above, kinetic results showed that catalytic efficiency of MUT 1 and MUT 3 has not been changed significantly. We conclude that in MUT 1, mechanisms other than decreased catalytic efficiency and reduced enzyme stability play a role in resistance to PZA and in the case of MUT 3; the significant decrease in the enzyme stability is involved in the resistance to PZA.

In summary, the good agreement between the experimental data and the computational results suggests that the loss of activity observed for MUT 2 may be due to an alteration in the active site cavity provided by D8, K96 and C138 to the PZA-binding pocket of the PZase enzyme, resulting from mutation of alanine 143 to threonine 143.

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Appendix

See Fig. 11.
Fig. 11 The kinetic date (Lineweaver–Burk curves) of wild type pyrazinamidase and mutants

References