Insight to the Molecular Mechanisms of the Osmolyte Effects on *Mycobacterium tuberculosis* Pyrazinamidase Stability using Experimental Studies, Molecular Dynamics Simulations, and Free Energy Calculation

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**Abstract**

**Background:** In this study, we have experimentally investigated the effects of different osmolytes including sucrose, sorbitol, urea, and guanidinium chloride (GdmCl) on the stability and structure of the *Mycobacterium tuberculosis* pyrazinamidase (PZase). PZase converts pyrazinamide to its active form. **Methods:** In addition, in order to gain molecular insight into the interactions between osmolytes and PZase, we have conducted 1000-ns molecular dynamics simulations. **Results:** The results indicated that sucrose and sorbitol increase the stability and compactness of the enzyme, whereas in the presence of urea and GdmCl, PZase loses its stability and compactness. Furthermore, the activity of PZase in the presence of sucrose was more than the other solutions. The energetic analyses imply that the electrostatic and van der Waals interactions are the major factors in the osmolyte–PZase interactions. Sorbitol and sucrose, as protective osmolytes, protect the protein structure by utilizing the van der Waals interaction from denaturation. In addition, urea molecules affect the structure of the protein using the hydrogen bonds and van der Waals interactions. **Conclusion:** The results show that the most important factor in the denaturing effect of GdmCl is the strong interactions of positively charged guanidinium ions with the aspartate and glutamate residues.

**Keywords:** Molecular dynamics simulation, *Mycobacterium tuberculosis*, osmolytes, pyrazinamidase

**Introduction**

Pyrazinamide (PZA) is a critical frontline drug in tuberculosis (TB) treatment.[1] Previous studies have shown that PZA is converted to ammonia and pyrazinoic acid (POA) within the lumen of *Mycobacterium tuberculosis* and which POA is the active antibacterial agent. On the other hand, PZA is a prodrug that is metabolized into the active form, POA, by the *M. tuberculosis* pyrazinamidase (PZase).[1,2] The absence of a PZase-encoding gene in the human genome makes the protein a TB drug target. The previous studies have shown that the PZA-resistant *M. tuberculosis* strains are caused by mutations in the PZase enzyme.[1,2,3,5] Although the general fold of PZase was determined,[6] the structural and functional properties of the enzyme in solution and the influence of osmolytes on the stability of PZase have not been characterized very well. The understanding of the structure–function relationship of PZase can improve the understanding of how TB is treated.

The process of protein folding is one of the great unsolved problems of structural biology with significant medical
implications. Central to this unsolved problem is that the native states of proteins correspond to the conformational states that are most stable under physiological conditions.\(^{17-20}\) Therefore, understanding the mechanisms of protein-folding problem will provide a scheme for predicting a particular three-dimensional protein structure from the one-dimensional protein sequence information, which would have application in industrial and medical biotechnology.\(^{11-14}\) Previous studies have shown that the conformational stability of proteins depends on a proper balance between the protein interactions with itself and its environment.\(^{11,15-18}\) This balance can be modified by addition of osmolytes into aqueous solution of proteins. Osmolytes participate in the interactions, which are responsible for structure, stability, and function of the native-state structure of proteins.\(^{19-23}\) In general, there are two classes of osmolytes; the protecting and the denaturing. The protecting group can push the protein-folding equilibrium toward its native conformation state, for example, osmolytes including trehalose, sorbitol, sucrose, and betaine, while the denaturing agents such as urea and guanidinium chloride (GdmCl) tend to destabilize protein-native states by perturbing the protein structure and function.\(^{19,24-29}\) Therefore, addition of osmolytes to protein solutions can result in a variety of effects such as unfolding and refolding.

A complete understanding of protein stability and a description of the protein structures with industrial and biopharmaceutical applications require experimental and theoretical data.\(^{30,31}\) It is clear that the osmolytes affect the structure and function of proteins, but the mechanisms have not been clarified very well.\(^{19,25,32-36}\) Therefore, it needs to investigate the effects of the osmolytes on the structure and activity of proteins in more detail.

In this study, first, the PZase enzyme has been overexpressed and purified. In addition, the enzyme stability has been studied in the presence of protectant (sorbitol and sucrose) and denaturant (urea and GdmCl) osmolytes using biochemical and biophysical methods and molecular dynamics (MD) simulations. It has been elucidated that since the function of the protein in the treatment of TB is entirely dependent on the stability of its structure, we hope that the results of this work will help researchers to design a suitable drug for the disease, especially for mutated species.

**Methods**

**Cloning, purification, determination of enzyme concentration, and activity assay**

The cloning of the PZase gene in expression vector pET21a (+), protein expression, and purification were performed according to the procedure described previously.\(^{35}\) The PZase concentration was obtained by the Bradford assay (Bio-Rad protein dye reagent). The Wayne test was used for measuring the PZase activity.\(^{37}\) Finally, one unit of the PZase activity was determined as the amount of the enzyme required to produce 1 μmol of POA in 1 min using POA as a standard.

The activity of the enzyme was determined in the absence and presence of 20% (w/v) of sorbitol, sucrose, and 4 M urea and GdmCl at 37°C. The residual enzyme activities were measured after certain periods and incubated on ice for 30 min. Afterward, the residual activities were measured at 37°C. In all experiments, the PZase/osmolyte mixtures and/or the activity of the enzyme solution incubated on ice was considered as control (100%).

**Calculation of half-life and \(k_{in}\)**

To measure the rate constant of the PZase inactivation \(k_{in}\) and the half-life, we have determined the time course of the decrease in the residual activity [Table 1]. Herein, the decreases in the PZase residual activity with respect to the duration of the enzyme incubation are linear. This shows first-order reaction for the inactivation of the enzyme. Thus, we have used the following equations for evaluation of the rate constant of the enzyme inactivation \(k_{in}\) and the half-life, respectively:

\[
\ln (activity) = \ln (activity)_0 - k_{in} t
\]

and

\[
\text{Half life} = \frac{0.693}{k_{in}}
\]

**Spectroscopic studies**

To investigate the effects of the osmolytes on the PZase conformation, the enzyme (final concentration of 0.02 mg/ml) was diluted in buffers containing 20% (w/v) of sorbitol, sucrose, and also 4 M urea and GdmCl and then incubated at 37°C for 5 min. The intrinsic fluorescence spectra were measured on a Cary Varian Eclipse spectrofluorimeter. The excitation wavelength was set to 295 nm and the emission was recorded from 300 to 400 nm for the enzyme samples.

The far ultraviolet (UV) visible circular dichroism (CD) spectra were measured by a Jasco-810 spectropolarimeter. To record far-UV CD spectrum from 190 to 260 nm, the PZase enzyme solutions were loaded to quartz cell with 1 mm path; afterward, the PZase CD spectra were recorded.

**Table 1: \(k_{in}\) and half-life of pyrazinamidase in water and osmolyte solutions**

| System            | \(k_{in}\) \((\text{min}^{-1})\)×10³ | Half-life (min) |
|-------------------|--------------------------------|----------------|
| Control           | 39.5±2.0                       | 17.5±1         |
| Sorbitol          | 31.0±1.0                       | 22.2±1         |
| Sucrose           | 27.0±1.0                       | 24.8±1         |
| Urea              | 41.0±1.0                       | 16.6±1         |
| GdmCl             | 44.0±1.0                       | 15.5±1         |

GdmCl: Guanidinium chloride
Molecular dynamics simulations

The MD simulations were conducted by the GROMACS 5.1.2 package [38,39] The starting structure of PZase was taken from the protein data bank (PDB ID: 3PL1) [Figure 1a]. [40] The systems were solvated with simple point-charge (SPC) model. [41] All systems were placed in a cubic large enough to contain PZase and 1 nm of solvent on all sides. Counterions (Na⁺ and Cl⁻) were added to the cells to neutralize all MD simulation systems. The pressure (with coupling time constant of \( \tau_p = 0.5 \) ps) and the temperature (with coupling time constant of \( \tau_T = 0.1 \)) were retained close to the intended values (1 bar and 310 K, respectively) using the Berendsen algorithm. [42] The chemical bond lengths were maintained constant using the LINCS algorithm. [43] The long-range electrostatic interactions were treated by the particle mesh Ewald method. [44] Subsequently, all systems were minimized by steepest descent energy minimization method. Afterward, the MD simulation systems were run for 200 ps under constant volume conditions, and the equilibration was completed with a 1000 ps of NPT simulation. Minimum donor–hydrogen–acceptor angles of 135° and maximum hydrogen–acceptor distance cutoff of 0.25 nm were applied for defining of hydrogen bond. Finally, five independent 200-ns MD simulations were carried out for PZase in the pure water, sorbitol, sucrose, urea, and GdmCl solutions. The Molecular Mechanics (MM)-Poisson–Boltzmann Surface Area approach was used to calculate the total contributions of free energy using g_mmpbsa tools developed by Kumari et al. [45] A summary of the all MD simulation systems are shown in Table 2.

Table 2: Summary of the molecular dynamics simulations

| System   | Temperature (K) | Number of water molecules | Number of osmolyte molecules | Duration (ns) |
|----------|-----------------|---------------------------|-------------------------------|---------------|
| Water    | 310             | 10887                     | 0                             | 200           |
| Sorbitol | 310             | 9412                      | 170                           | 200           |
| Sucrose  | 310             | 9502                      | 90                            | 200           |
| Urea     | 310             | 9468                      | 500                           | 200           |
| GdmCl    | 310             | 9201                      | 500                           | 200           |

GdmCl: Guanidinium chloride

Results and Discussion

Cloning, expression, and purification of pyrazinamidase

To analyze the activity and stability of the enzyme, the PZase gene was cloned into expression vector pET21a (+). For the recombinant enzyme overexpression, the Escherichia coli BL21 (DE3) cells were transformed by the constructed vector. After overexpression, the Ni-NTA sepharose was used to purify the recombinant enzyme with a His₆-tag on its C-terminal, and then, the purity of the PZase has been analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Our results indicate that the purified enzyme has a molecular mass of 20.6 kDa.

Effects of osmolytes on the pyrazinamidase activity

The enzymatic activity of the purified PZase in the osmolytes solutions was determined at 37°C [Figure 1b]. The normal activity of PZase kept in the presence of sorbitol and sucrose, whereas in the presence of urea and GdmCl, its enzymatic activity was completely lost.

Figure 1: (a) Schematic diagram of the crystal conformation of pyrazinamidase. (b) The enzymatic activity of the pyrazinamidase at 310 K in the presence of 20% of sorbitol and sucrose and 2.5 M of urea and guanidinium chloride. The pyrazinamidase activity in the absence of osmolytes is considered as 100%. (c) Thermostability of pyrazinamidase in the osmolyte solutions. The residual enzyme activity was applied by the Wayne test. The activity of pyrazinamidase was characterized using a 50 ml of a solution containing 100 mM Tris (pH 6.5), 2 mM mercaptoethanol, and 40 mM pyrazinamide.
Effect of osmolytes on the thermostability of pyrazinamidase
In order to examine the stability of the enzyme, PZase was incubated in the presence and absence of osmolytes during different times at 45°C. Thereafter, the residual activity of the enzyme was determined under these conditions. As shown in Figure 1c, the stability of PZase has been decreased and the half-life was 20 min. Sorbitol and sucrose have increased the PZase half-life to 20.3, 24.8, and 22.2 min, respectively [Table 1]. Therefore, sorbitol and sucrose slightly raised the enzyme stability. Moreover, the half-life of the enzyme was decreased in the urea and GdmCl aqueous solutions to approximately 16.5 and 15.5 min, respectively [Figure 1c].

Effects of osmolytes on the structure of pyrazinamidase
The effects of the aforementioned osmolytes on the secondary and the tertiary structures of PZase have been measured by far-UV CD and intrinsic fluorescence spectra. The CD spectrum results show two negative bands at 208 and 222 nm wavelengths, representing α-helical contents of the enzyme and a negative band between 210 and 222 nm wavelengths indicating the β-sheet strands of PZase [Figure 2a]. The results also indicate an increase in the α-helical and β-sheet contents in the presence of sorbitol and sucrose, with respect to the aqueous protein solution [Figure 2a]. Furthermore, the magnitude of negative ellipticity at 216 and 222 nm in the presence of urea and GdmCl osmolytes was decreased. Our results also showed that in the presence of GdmCl, the secondary structure contents were more decreased than urea. For better understanding the effects of the osmolytes on the native conformation of PZase, we have also studied the intrinsic fluorescence spectra by the excitation of the enzyme at 290 nm [Figure 2b]. It can be observed that the fluorescence intensity was gradually increased in the presence of the sorbitol and sucrose solutions, respectively. The results have also shown that the enzyme in the water solution had a fluorescence emission at 332 nm, and the most fluorescence intensity was assigned to the sucrose aqueous solution [Figure 2b].

It seems that in the presence of the sucrose and sorbitol solutions, the enzyme gets more compact than the water solution, whereas in the presence of the urea and GdmCl aqueous solutions, the enzyme begins to lose its native tertiary structure, especially in the GdmCl aqueous solution. Thereby, the tryptophan residues of the enzyme gradually became accessible to the solution. According to our results, the largest and the smallest values in the intrinsic fluorescence spectra were allocated to sucrose and GdmCl, respectively. For better indicating the surface accessibility of the tryptophan residues to the solutions, we also calculated the average solvent accessible surface area (SASA) for the tryptophan residues of PZase in the MD simulation systems [Figure 3a]. As can be seen, compared with the aqueous protein solution, the SASA value of the tryptophan residues in the sucrose solution was decreased; conversely, this value in the urea and GdmCl solutions was increased. Therefore, the tryptophan residues become more accessible to the aqueous solution in urea and GdmCl than in sucrose.

In order to investigate the compactness of PZase in the osmolytes, we also calculated the radius of gyration (Rg) of the enzyme in the osmolyte solutions [Figure 3b]. Figure 3b shows that in the presence of sucrose, the enzyme has been gotten a more compact structure than that of the others, which is in good agreement with our fluorescence spectrum results. In addition, the Rg values for the sucrose were slightly decreased. However, the Rg values of PZase were significantly increased in the urea and GdmCl solutions, and hence, the enzyme has been losing its compactness. The root-mean-square deviation (RMSD) of the Cα of PZase has also been calculated to measure the stability of the enzyme in the presence of the osmolytes. Figure 3c shows that the Cα RMSD in the sucrose solution is lower than the other osmolytes, whereas the largest value was

Figure 2: (a) Far-ultraviolet circular dichroism spectra of pyrazinamidase in water and the osmolyte solutions at 310 K. (b) The effect of incubation at 310 K on fluorescence spectra of pyrazinamidase in the osmolyte solutions
allocated to the GdmCl aqueous solution. The MD analyses confirm the experimental results and indicate that compared with GdmCl and urea, the sucrose and sorbitol solutions could increase the stability.

**Free Energy Contributions**

To complement the structural analysis, the binding affinity of osmolytes to PZase has been computed using the MM-Generalized Born Surface Area method. The 1000 snapshots from the last 10 ns of the MD trajectories with interval 10 ps have been collected. The detailed energy contribution has been shown in Table 3. The results have shown that the van der Waals and the electrostatic interactions are the major contributors in the protein–osmolyte interactions. In the case of sorbitol and sucrose, the energy results have indicated that although they have very good electrostatic and van der Waals interactions with the enzyme, their total binding energy is low. According to previous studies, the protecting osmolytes have a lower direct interaction with proteins and only increase the protein structural stability through accumulation in the hydrophobic regions of proteins.\(^{145-47}\) The energy results obtained from the study also confirmed the same. Sorbitol and sucrose molecules have the most interactions with the hydrophobic amino acids [Figure 4]. When the molecules are present in water, they are energetically interested in interacting with themselves. Therefore, they accumulate around the hydrophobic amino acids; on the one hand, by interacting with the hydrophilic residues, they reduce the interactions of the protein with water. However, because of their low tendency to interact with water at the same site of aggregation, they stay and prevent the protein instability.

The contribution of the electrostatic interaction and polar solvation in the GdmCl simulation are larger than other osmolytes. In all simulations, the electrostatic and van der Waals interactions play an important role in the interactions of proteins and osmolytes. The results have

### Table 3: Binding free energy (kcal/mol) of pyrazinamidase-osmolyte complexes computed by the MM-GBSA method

| Osmolyte | Sorbitol | Sucrose | Urea | GdmCl |
|----------|----------|---------|------|-------|
| van der Waals energy | −1094.61 | −1248.29 | −1348.44 | −506.64 |
| Electrostatic energy | −148.498 | −1452.59 | −341.903 | −3908.8 |
| Polar energy | 937.99 | 2662.799 | 1143.187 | −1215.55 |
| SASA energy | −193.372 | −2662.799 | −1143.187 | −1537.25 |
| Binding energy | −124.623 | −190.411 | −209.327 | −518.02 |

SASA: Solvent accessible surface area, GdmCl: Guanidinium chloride
shown that the guanidinium ions have the highest amount of interactions with the enzyme, and then, urea molecules interact very much with PZase. Therefore, the strong interactions can be a major reason for the denaturing effect of these compounds. In the urea simulation, the van der Waals value is higher than other osmolytes. This property is a hallmark of urea, which, by creating a hydrophobic environment, causes the protein to be denatured, and this is illustrated by previous studies. The simulation results also indicated that the electrostatic interactions and polar solvation are the most important factors for the interaction of the protein with the guanidinium ions. The result can be a significant reason for the rapid denaturing effect of this compound. Previous studies have shown that direct interactions of denaturants with proteins are one of the main factors of denaturing effect of GdmCl and urea, and the energy results of the present work confirm this effect in the enzyme.

These results have revealed that urea molecules interact most effectively with positively charged and hydrophobic amino acids; therefore, it could be suggested that interacting with these amino acids can be one of the reasons for destabilization of the protein structure. The free energy binding analysis of amino acids has shown that the negatively charged residues have the strongest interactions with positively charged guanidinium ions, and it can be concluded that these highly strong interactions between the protein residues and guanidinium ions disturb the protein conformation. As can be seen in Figure 4, all the negatively charged residues of the protein interact electrostatically with the guanidine positive ions with a significant difference in numerical value compared to the other residues.

**Conclusion**

Herein, MD simulation and experimental methods were used to study the PZase behavior in the presence of sorbitol, sucrose, urea, and GdmCl. The spectroscopy results and MD analyses have indicated that the stability and the compactness of PZase increase in the presence of the sucrose and sorbitol solutions. In contrast, in the presence of urea and GdmCl, the structure of the enzyme is denatured and the function is decreased. In addition, the free binding energy calculations have also demonstrated that the main factors in the protection of the structure and function of PZase in the presence of sorbitol and sucrose were the van der Waals and hydrophobic interactions between the proteins and osmolytes. In addition, the enzyme structure and function have been decreased by the electrostatic and van der Waals interactions in the presence of urea and GdmCl, respectively. In summary, the results demonstrated that sucrose is a good stabilizer of structure and function of PZase and GdmCl acts as the strongest denaturant for PZase. This study, based on data from the structure and function of the *M. tuberculosis* PZase in different environmental conditions, can provide good challenges and opportunities in developing novel drugs for TB.

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**Conflicts of interest**

There are no conflicts of interest.

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