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# Structure-Activity relationship in mutated pyrazinamidases from *Mycobacterium tuberculosis*

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**Abstract:**
The *pncA* gene codes the pyrazinamidase of *Mycobacterium tuberculosis*, which converts pyrazinamide to ammonia and pyrazinoic-acid, the active anti-tuberculous compound. Pyrazinamidase mutations are associated to pyrazinamide-resistant phenotype, however how mutations affect the structure of the pyrazinamidase, and how structural changes affect the enzymatic function and the level of pyrazinamide-resistance is unknown. The structures of mutated pyrazinamidases from twelve *Mycobacterium tuberculosis* strains and the pyrazinamide-susceptible H37Rv reference strain were modelled using homology modelling and single amino acid replacement. Physical-chemical and structural parameters of each pyrazinamidase were calculated. These parameters were: The change of electrical charge of the mutated amino acid, the change of volume of the mutated amino acid, the change of a special amino acid, the distance of the mutated amino acid to the active site, the distance of the mutated amino acid to the metal-coordination site, and the orientation of the side-chain of the mutated amino acid. The variability of the enzymatic activity of the recombinant pyrazinamidases, and the microbiological susceptibility to pyrazinamide determined by BACTEC 460TB, were modelled in multiple linear regressions. Physical-chemical and structural parameters of the mutated pyrazinamidases were tested as predictors. Structural and physical-chemical variations of the pyrazinamidase explained 75% of the variability of the enzymatic activity, 87% of the variability of the kinetic constant and 40% of the variability of the pyrazinamide-resistance level. Based on computer models of mutated pyrazinamidases, the structural parameters explained a high variability of the enzymatic function, and to a lesser extent the resistance level.

**Background:**
According to WHO, tuberculosis disease due to *Mycobacterium tuberculosis* infection caused an estimated 1.3 million deaths, globally. Pyrazinamide (PZA) is an important first-line drug for tuberculosis (TB) and appears to be the most important drug killing *M. tuberculosis* in its latent stage **[1-3]**. The concomitant emergence of strains resistant to PZA represents an important public health problem as the efficacy of primary and standardized re-treatment regimens may be inadequate. PZA-susceptible strains possess a pyrazinamidase (PZAse) that converts PZA to ammonia and pyrazinoic acid (POA). POA is the active compound and is transported outside the Mycobacterium by an efflux pump. If the extracellular environment is acid, POA is protonated, returns to Mycobacterium by passive diffusion, and releases the proton. The acidification of the intracellular environment together with the accumulation of intracellular POA lethally alters the membrane permeability **[4]**. The major mechanism of PZA-resistance is considered to be the loss of PZAse activity linked to mutations in the pncA, the PZAse coding gene. Previous studies showed that mutations in recombinant PZAses decreased the enzymatic activity in about 10 fold, according to the localization of the mutation and the type of substitution **[5, 6]**. In addition, mutations in PZAses have been shown to generate clinical resistance to PZA **[7-9]**. The causal pathway that links pncA mutations with PZA resistance should be: [*pncA* mutations] → [PZAse structural change] → [PZAse functional change] → [PZA resistance level] → ["yes/no" PZA-resistant phenotype]. In order to understand how *pncA* mutations affect the enzymatic function, it is necessary to analyse the three-dimensional structure of mutated PZAses. Previous studies determined structural models of *M. tuberculosis* PZAse for the PZA-resistant reference strain H37Rv.

In these studies the PZAse structure was determined by homology modelling based on the crystallized structures of the homologues *Arthrobacter* N-carbamoylsarcosine aminohydrolase (CHSase) **[5]** and *Pyrococcus horikoshii* PZAse **[10, 11]**. Theoretical structural models serve as good approaches when experimental structure data are not available **[12]**. In a recent study, the PZAse structure of the *M. tuberculosis* H37Rv strain was resolved by crystallography **[13]**, showing that the structure of PZAses are highly conserved among these species. The critical residues associated with PZAse function are those of the active site (D8, A134, C138) and the metal-binding site (D49, H51, H57, and H71). Mutations in these residues and in residues in close proximity to these sites highly affect the PZAse function **[6, 11]**. In the present work, we studied the relationship between structural and physical-chemical characteristics of mutated PZAses with the enzymatic function and PZA-resistance level of *M. tuberculosis* strains.

**Methodology:**
**PZAse selection:**
In a previous work, the PZAse of the H37Rv strain and 12 mutated PZAses (D12G, D12A, G24D, Y34D, K48T, D49N, H51R, T76P, G78C, F94L, L116P, and T135P), from *M. tuberculosis* PZA-resistant strains were cloned, expressed, and purified. The catalytic constant $k_{cat}$, and the enzymatic activity

 

were measured for each recombinant enzyme. The PZA-MIC and the growth-index in BACTEC-460TB at 100 µg/mL PZA were measured for each strain **[6]**. Data of PZAse function and PZA-resistance is shown in the **Table 4 (see supplementary material)**.

**Modelling:**
Theoretical structures were obtained by homology modelling and single amino acid replacement. For each PZAse, two theoretical structures were generated. The first set of structures was obtained by single amino acid replacement using as template the crystal structure of the *M. tuberculosis* H37Rv PZAse (PDB 3pl1). The second set was obtained by homology modelling using as template the *P. horikoshii* PZAse crystal structure (PDB 1im5). Modelled structures were refined by energy minimization and molecular dynamics. Energy minimization was performed with GROMACS **[14]**, using the steepest-descent algorithm with a convergence gradient of 10 $KJ\times mol^{-1}\times nm^{-1}$. Structures were solvated in explicit water followed by 1ns molecular dynamics (MD) using GROMACS. The accuracy of the models were evaluated using PROCHECK **[15]**.

**Physical-chemical and structural parameters:**
Parameters estimated for the mutated aminoacid included: (1) change of electrical charge; (2) change of volume; (3) change of a special aminoacid; (4) closeness to the active site; (5) closeness to the metal coordination site (MCS); and (6) orientation of the side chain **(Tables 1 & 2, see Supplementary material)**. Aminoacids were considered as: hydrophilic (Basic: K, R and H; Acidic: D and E; and polar with uncharged side-chain group: S, T, N and Q), hydrophobic (A, V, I, L, M, F, Y and W) and special (C, G and P). The volume change was calculated using Pontius values **[16]**. The closeness of the mutated aminoacid to the active-site and to the MCS, was measured as the distance between the alpha-carbon of the mutated aminoacid and the alpha-carbons of the 3 residues of the active-site (D8, A134, C138) and 3 residues of the MCS (D49, H51, H71). In order to prevent a bias due to over-modelling, amino acid H57 was not considered because its orientation and position in an external loop, showed a low structural stability during the MD run. The distance between the barycentre of the 3 alpha-carbons of the active-site residues and the alpha-carbon of the mutated aminoacid (D_AS) was determined. Similarly, the equivalent distance to the MCS (D_MCS) was also determined.

The orientation of the side-chain of the mutated aminoacid was determined by the distance between the barycentre of the 3 alpha-carbons of the active-site residues and the intersection of the projection of the mutated side-chain with the plane defined by the alpha-carbons of the active-site (d_AS). The projection of the side-chain was defined as the resultant vector connecting the alpha-carbon with each of the atoms of the side-chain. Similarly, we calculated the equivalent distance for the MCS (d_MCS). We calculated the angle between the projection of the mutated side-chain and the normal vector of the plane defined by the alpha-carbons of the active site (α_AS). We calculated the equivalent angle to the plane defined by the alpha-carbons of the MCS (α_MCS). The orientation of the side-chain of the mutated aminoacid was classified accordingly to whether its projection crosses the triangle formed by the 3 alpha-carbons of the active-site (AS_triangle), and to whether its projection aims at the plane defined by the alpha-carbons of the active-site (AS_plane). We classified the orientation of the side-chain of the mutated amino acid against the MCS triangle (MCS_triangle), and the MCS plane (MCS_plane).

**Prediction of PZAse-function and PZA-susceptibility:**
The variability of the growing-index in BACTEC, the enzymatic activity and $k_{cat}$ were modelled in a linear regression. The structural and physical-chemical parameters were tested as predictors. The PZA-MIC was also tested as a dichotomous variable: Low-MIC (MIC< 400µg PZA/mL) versus high-MIC (MIC>=400µg PZA/mL). The variability of PZA-MIC was modelled in a logistic regression.

**Discussion:**
This study presents the relationship of theoretical structure models of *M. tuberculosis* mutated PZAses with a single amino acid substitution with PZAse enzymatic-function and strain PZA-susceptibility. The structural alignment between *P. horikoshii* and H37Rv *M. tuberculosis* crystallized PZAses, shows that both structures are highly conserved (RMSD=0.661 A at backbone level). The MCS for H37Rv *M. tuberculosis* PZAse is proposed to be composed by residues D49, H51, H57, and H71 **[13]** in contrast to the corresponding of *P. horikoshii* that lacks H57 **[6, 11]**. H57 in *M. tuberculosis* PZAse is located in a loop, which shows structural instability as detected by the MD simulation. To prevent any bias during MD simulation, we obviate the H57 for the extraction of structural parameters. After excluding the H57-loop-region, the two predicted structures of each mutated PZAse were highly similar (RMSD = 0.5 A at backbone level). All results described below were similar for the two models. The major structural changes of the mutated PZAses were in the orientation of the amino acid side-chains and in a lesser extent the backbone. The structural and physical-chemical parameters were able to explain a high variability of the PZAse function and in a lesser extent the variability of the PZA-resistance level.

The physical-chemical and structural parameters for the 12 mutated PZAses are shown in **Tables 1 & 2 (see supplementary material)**. In the univariate analysis, the enzymatic activity, the $k_{cat}$, and the Bactec growing index were significantly associated with several structural parameters **(Table 3, see Supplementary material)**. After adjustment, the best multiple linear model to explain the enzymatic activity included the distance d_MCS (P=0.005) with a determination coefficient $R^2$ of 0.76. The best multiple linear model for the catalytic constant $k_{cat}$ included the change of electrical charge (P=0.001), the distance d_A134 (P=0.003), the angle α_MCS (P=0.05), and the indicator variable AS_plane (P=0.024), with a determination coefficient $R^2$ of 0.88. The variability of the growing index in BACTEC was significantly associated with the angle α_MCS (P=0.027) with a determination coefficient $R^2$ of 0.40. The change of electrical charge was the dominant term in the models. Therefore, the most important structural parameters able to explain the variability of the PZAse activity and $k_{cat}$ were related to the physical-chemistry properties of the active site and the MCS. The closeness of the mutation to A134 as a predictor confirms the importance of the active site in the hydrolysis of PZA. As described by Du *et al.* (2001) the initial interaction of PZA with the PZAse active site at the beginning of the hydrolysis occurs very close to A134 **[11]**. The closeness of the mutation and the orientation of the mutated amino acid side chain towards the MCS, confirm the importance of the MCS in the hydrolysis of PZA, and explain the high MIC observed in mutations that are close to the MCS and their side chains aim directly at it. Lemaitre *et al.* in 2001 have previously discussed that the level of activity displayed by PZAse mutants is correlated with the location of the substituted amino acids **[5]**. Those that are close to the catalytic site diminish the activity of the enzyme more than those that are far apart **[5]**. We found that this is not generally true. This study showed that the PZAse structure is a proximal predictor of PZAse function, confirming the well-known structure-function relationship. The lack of a high association between the PZAse structure and PZA-resistance level suggests that an important amount of PZA-resistance is determined by other factors than PZAse structure/function.

**Conclusion:**
Our results showed that the PZAse structure was able to explain a high variability of the enzymatic function, but in a lesser extent the PZA-resistance level in *M. tuberculosis*. The PZAse structural aspects that best explained the variability of the enzymatic activity were those associated to the physical-chemical characteristics of the AS and the MCS. Despite the small number of mutations analyzed, our results confirm that the PZAse structure is a proximal predictor of PZAse function, confirming the well-known structure-function relationship. The lack of an important association between the PZAse structure and the PZA-resistance level suggests that an important amount of PZA-resistance is determined by other factors than PZAse activity.

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## Supplementary material:

**Table 1:** Variation of the physical-chemical characteristics of the mutated amino acid

| Mutation | **Change of electrical charge** | **Change of volume** ($A^3$) | Change of a special amino acid |
|---|---|---|---|
| D12A | Polar charged (acid)-Non polar | 43.7 | - |
| D12G | Polar charged (acid)-Non polar | 67.7 | Gain of Special G |
| G24D | Non polar-Polar charged (acid) | -67.7 | Loss of Special G |
| Y34D | Non polar-Polar charged (acid) | 74.6 | - |
| K48T | Polar charged-Polar uncharged | 36.5 | - |
| D49N | Polar charged-Polar uncharged | -3.1 | - |
| H51R | No | -32.9 | - |
| T76P | Polar uncharged-Non polar | 2.6 | Gain of Special P |
| G78C | Non polar-Polar uncharged | -48.4 | Gain of -SH (C) |
| F94L | No | 22.69 | - |
| L116P | No | 40 | Gain of Special P |
| T135P | Polar uncharged-Non polar | 2.6 | Gain of Special P |

Change of electric charge, change of special amino acid and change of volume ($A^3$) for all mutations studied.

**Table 2:** Structural parameters of the mutated pyrazinamidases

| Mutation | d_D8[1] | d_A134[1] | d_C138[1] | A_AS[2] | d_AS[3] | D_AS[4] | AS Triangle[5] | AS Plane[6] | d_D49[1] | d_H51[1] | d_H71[1] | α_MCS[2] | d_MCS[3] | D_MCS[4] | MCS Triangle[5] | MCS Plane[6] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| D12A | 9.48 | 13.74 | 16.09 | 2.67 | 5.124 | 12.38 | No | No | 10.02 | 11.17 | 5.1 | 1.053 | 9.21 | 8.07 | No | No |
| D12G | 8.53 | 12.21 | 14.71 | 2.5 | 3.111 | 12.37 | No | No | 9.58 | 11.25 | 4.62 | 0.876 | 7.67 | 8.03 | No | No |
| G24D | 11.2 | 10.51 | 15.92 | 2.06 | 9.643 | 10.57 | No | No | 16.66 | 20.71 | 14.11 | 0.922 | 13.38 | 14.75 | Yes | No |
| Y34D | 18.27 | 20.8 | 24.39 | 1.05 | 10.09 | 20.68 | No | Yes | 24.38 | 30.12 | 26.14 | 1.005 | 15.52 | 26.64 | No | No |
| K48T | 5.25 | 14.07 | 15.49 | 2.05 | 7.56 | 10.34 | No | No | 3.84 | 9.51 | 10.6 | 0.209 | 7.16 | 7.35 | No | No |
| D49N | 0 | 6.31 | 8.09 | 2.6 | 6.566 | 9.471 | No | No | 6.4 | 13.12 | 11.58 | 1.462 | 4.06 | 4.06 | Yes | Yes |
| H51R | 6.29 | 0 | 7.88 | 2.67 | 7.045 | 14.14 | No | No | 12.25 | 17.16 | 15.28 | 1.623 | 3.91 | 3.91 | Yes | No |
| T76P | 15.38 | 20.65 | 21.65 | 2.66 | 4.984 | 18.74 | No | Yes | 12.73 | 10.19 | 8.87 | 1.016 | 7.75 | 9.81 | Yes | Yes |
| G78C | 10.53 | 17.24 | 18.56 | 2.55 | 6.496 | 15 | No | Yes | 9.4 | 9.91 | 7.67 | 0.899 | 9.95 | 7.94 | Yes | Yes |
| F94L | 10.15 | 16.34 | 13.24 | 2.11 | 6.378 | 12.48 | No | Yes | 4.55 | 6.96 | 11.97 | 1.544 | 45.57 | 7.22 | No | No |
| L116P | 13.01 | 18.76 | 16.03 | 1.06 | 4.376 | 15.28 | No | Yes | 13.54 | 18.83 | 21.1 | 1.526 | 46.56 | 17.58 | No | No |

[1]Distance (Angstrom) between the alpha carbon of the mutated amino acid and the alpha carbons of the residues of the AS/MCS; [2] Angle between the projection of the mutated side chain and the normal vector of the plane defined by the alpha carbons of the AS/MCS; [3] Distance (A) between the barycenter of the 3 alpha carbons of the AS/MCS residues and the intersection of the projection of the mutated side chain with the plane defined by the alpha carbons of the AS/MCS; [4] Distance (A) between the barycenter of the 3 alpha carbons of the AS/MCS, and the alpha carbon of the mutated amino acid; [5] Classification of the side chain mutated amino acid orientation accordingly to whether its projection crosses the triangle formed by the 3 alpha carbons of the AS/MCS; [6] and to whether its projection aims at the plane defined by the alpha carbons of the AS/MCS.

**Table 3:** Single and multivariable regression models for the prediction of the PZA susceptibility and the PZAse kinetic parameters.

| | Logistic regression | | | | Linear regression | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | MIC | | Activity | | $K_M$ | | $k_{cat}$ | | Growth % | |
| Covariate | SVM | BMM | SVM | BMM | SVM | BMM | SVM | BMM | SVM | BMM |
| Charge change | -0.916 | - | -15.497 | - | -0.698 | - | -495.63 | -726.711 | -1.889 | - |
| | *(0.512)* | - | *(0.097)* | - | *(0.203)* | - | ***(0.044)*** | ***(0.001)*** | *(0.914)* | - |
| Volume change | 0.006 | - | 0.158 | - | -0.001 | - | 4.038 | - | 0.008 | - |
| | *(0.672)* | - | *(0.094)* | - | *(0.854)* | - | *(0.120)* | - | *(0.966)* | - |
| Special change | -1.386 | - | 3.815 | - | -0.653 | - | 85.612 | - | -1.833 | - |
| | *(0.258)* | - | *(0.659)* | - | *(0.166)* | - | *(0.716)* | - | *(0.903)* | - |
| d_D8 | -0.293 | - | 1.185 | - | -0.092 | - | 25.953 | - | 1.030 | - |
| | *(0.149)* | - | *(0.190)* | - | *(0.062)* | - | *(0.300)* | - | *(0.527)* | - |
| d_A134 | -0.348 | - | 1.300 | - | -0.035 | - | 31.580 | 90.919 | -1.442 | - |
| | *(0.088)* | - | ***(0.032)*** | - | *(0.357)* | - | *(0.060)* | ***(0.003)*** | *(0.205)* | - |
| d_C138 | -1.859 | - | 0.993 | - | -0.072 | - | 19.360 | - | -1.285 | - |
| | *(0.272)* | - | *(0.206)* | - | *(0.099)* | - | *(0.376)* | - | *(0.357)* | - |
| α_AS | 1.732 | - | -15.716 | - | 0.091 | - | -384.285 | - | 4.831 | - |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *(0.191)* | - | ***(0.017)*** | - | *(0.834)* | - | ***(0.039)*** | - | *(0.714)* | - |
| d_AS | 0.052 | - | -1.097 | - | -0.018 | - | -30.711 | - | 1.855 | - |
| | *(0.700)* | - | *(0.247)* | - | *(0.743)* | - | *(0.232)* | - | *(0.261)* | - |
| D_AS | -0.376 | - | 1.461 | - | -0.046 | - | 29.867 | - | -0.038 | - |
| | *(0.130)* | - | *(0.183)* | - | *(0.477)* | - | *(0.327)* | - | *(0.985)* | - |
| AS_triangle | - | - | - | - | - | - | - | - | - | - |
| | - | - | - | - | - | - | - | - | - | - |
| AS_plane | -2.303 | - | -16.140 | - | 0.035 | - | 409.573 | -681.649 | -9.000 | - |
| | *(0.099)* | - | ***(0.042)*** | - | *(0.944)* | - | *(0.062)* | ***(0.024)*** | *(0.553)* | - |
| d_D49 | -0.140 | - | 0.292 | - | -0.049 | - | 0.034 | - | 1.795 | - |
| | *(0.276)* | - | *(0.712)* | - | *(0.264)* | - | *(0.999)* | - | *(0.172)* | - |
| d_H51 | -0.090 | - | 0.312 | - | -0.015 | - | 2.089 | - | 1.164 | - |
| | *(0.382)* | - | *(0.648)* | - | *(0.700)* | - | *(0.911)* | - | *(0.317)* | - |
| d_H71 | -0.148 | - | 1.025 | - | 0.016 | - | 23.170 | - | 0.360 | - |
| | *(0.236)* | - | *(0.135)* | - | *(0.692)* | - | *(0.223)* | - | *(0.775)* | - |
| α_MCS | 3.386 | - | -1.279 | - | 0.600 | - | 9.843 | 303.592 | 27.128 | 27.128 |
| | *(0.124)* | - | *(0.873)* | - | *(0.166)* | - | *(0.964)* | ***(0.050)*** | ***(0.027)*** | ***(0.027)*** |
| d_MCS | -0.014 | - | 0.768 | 0.635 | 0.014 | - | 22.609 | - | -0.258 | - |
| | *(0.736)* | - | ***(0.001)*** | ***-0.005*** | *(0.416)* | - | ***(0.000)*** | - | *(0.626)* | - |
| D_MCS | -0.207 | - | 0.921 | - | -0.038 | - | 18.836 | - | 0.448 | - |
| | *(0.154)* | - | *(0.154)* | - | *(0.310)* | - | *(0.109)* | - | *(0.704)* | - |
| MCS_triangle | -0.693 | - | -13.891 | - | 0.217 | - | -386.537 | - | 4.029 | - |
| | *(0.560)* | - | ***(0.089)*** | - | *(0.664)* | - | *(0.081)* | - | *(0.792)* | - |
| MCS_plane | -0.916 | - | 9.103 | - | 0.476 | - | 272.334 | - | -5.222 | - |
| | *(0.512)* | - | *(0.353)* | - | *(0.397)* | - | *(0.305)* | - | *(0.764)* | - |

Structural parameters and physical-chemical characteristics against kinetic parameters of recombinant pyrazinamidases used to generate Single Variable Models (SVM) and Best Multiple Models (BMM). P-values, in parenthesis, and coefficients are reported. Significant values are in bold.

**Table 4:** Pyrazinamide susceptibility of *Mycobacterium tuberculosis* isolates and kinetic parameters of their corresponding recombinant pyrazinamidase.

| *pncA* mutation | PZA MIC[1] | BACTEC 460TB[2] | Activitiy[3] | $k_{cat}$[4] |
|---|---|---|---|---|
| T135P | >800 | 98 | 0.02 | 0.45 |
| H51R | >800 | 90 | 0.006 | 0.17 |
| D49N | 400 | 74 | 0.045 | 1.53 |
| T76P | 200 | 89 | 8.99 | 202.98 |
| L116P | 100 | 46 | 50.15 | 1324.5 |
| D12A | 400 | 77 | 9.24 | 245.17 |
| D12G | 400 | 70 | 14 | 368.46 |
| F94L | 400 | 70 | 21.19 | 712.92 |
| G24D | 200 | 68 | 4.28 | 100.13 |
| Y34D | 100 | 78 | 20.58 | 386.4 |
| G78C | 100 | 27 | 6.96 | 105.16 |
| K48T | ≤50 | 20 | 10.45 | 241.82 |
| Wild type | ≤50 | 1 | 38.4 | 1005.41 |

[1]PZA MIC (µg PZA/ml); [2]BACTEC 460TB, PZA Growth index; [3]Activity, µmol POAmin$^{-1}$mg$^{-1}$ PZAse; [4]$k_{cat}$, min$^{-1}$.