

OSAKA UNIVERSITY

**Structure-based Explanation and Prediction of
Resistance to Pyrazinamide in *Mycobacterium
tuberculosis*: a Molecular Modeling Study**

By

Román Jun Sotelo Romero

A thesis submitted in partial fulfillment for the degree of Master of Science
in the
Graduate School of Frontier Biosciences

February 2011

“Part of the inhumanity of the computer is that, once it is competently programmed and working smoothly, it is completely honest.”

Isaac Asimov

OSAKA UNIVERSITY

Abstract

Graduate School of Frontier Biosciences
Laboratory of Protein Informatics

Master of Science

by Román Jun Sotelo Romero

Tuberculosis is a prominent infectious disease caused by the bacterium *Mycobacterium tuberculosis*. The main obstacle in tuberculosis treatment is the rapidly emergence of new multi-drug resistant bacterial strains. Resistance to Pyrazinamide, a very important front-line drug in tuberculosis treatment, is alarmingly growing. Although the structure of the protein interacting with pyrazinamide, pyrazinamidase, is known, the details of the substrate binding and the catalytic mechanism are just partially understood. To explain the molecular mechanism of resistance to pyrazinamide, molecular modeling studies were performed. Molecular dynamics simulations were carried out to calculate the protein-ligand binding free energy in the wild type, sensitive and resistant strains of *Mycobacterium tuberculosis*. Experimental enzyme kinetics data were correlated with the calculated binding free energy. The results offer basis for a potential model to predict resistance to pyrazinamide in new strains of *Mycobacterium tuberculosis*.

Acknowledgements

My most genuine thanks to Professor Haruki Nakamura who decided to give me a chance to perform research at the Laboratory of Protein Informatics in Osaka University on August of 2007. The invaluable opportunity to carry out my study and, at the very same time, to see how the Protein Data Bank Japan (PDBj) operates has broadened my understanding about how scientific knowledge is created, shared and used.

I am vastly indebted to Dr. Daron Standley for helping me from the very moment I entered the laboratory. Thanks for all the interest he put in my research topic, for all his suggestions and explanations regarding molecular modeling, for his example as a scientist, and for his useful and funny tips for living in Japan.

I deeply thank Dr. Yu Takano, Dr. Yoshifumi Fukunishi, Shaq Liu and Dr. Taeho Kim for their support concerning the installation, configuration and use of almost all the software and scripts that were employed in this work.

I am more than thankful to Junko Sato and Yuko Okada for teaching me not only Japanese culture, but also for telling me where, when and how I could learn it. I can not find words to thank them adequately for their kindness.

Very special thanks to my tutors : Koji Umezawa, Jinzen Ikebe and Daisuke Kuroda. They literally saved my life in every way. They were crucial for this project. They were, at the same time, my motivation, medium and reward for learning Japanese. I wish I could have met them earlier in my life. From the bottom of my heart, I wish all the best for them.

Thanks to IRIS, OUISA, OUSSEP, TRUSS, MAFGA, JICA, AMIGOS, TIFA and all the countless institutions that promote cultural exchange and understanding in Japan, specially the organizations that coordinate Homestay Programs for international students. The events that these groups organize have been the richest experiences of my life.

This work was possible thanks to a wonderful scholarship given by the Ministry of Education, Culture, Sports, Science and Technology of Japan. They made my dream come true.

Contents

1. Introduction

2. Overview

2.1 Pyrazinamide Resistance.....	2
2.2 Previous Researches on Pyrazinamidases.....	7
2.3 Structure of Pyrazinamidase from <i>M. tuberculosis</i>	17
2.4 Software and Databases.....	22

3. Modeling of Mutant Proteins

3.1 Objective.....	23
3.2 Methodology.....	23

4. Protein-Ligand Binding Free Energy Calculations

4.1 Objective.....	34
4.2 Methodology.....	34
4.3 Results.....	37

5. Conclusions

Bibliography

Chapter 1

Introduction

In general, this study is about the explanation of drug resistance at the molecular level. Drug resistance is defined as the reduction of the effectiveness of a pharmaceutical drug. Modern medicine has produced wonderful drugs; however, pathogens such as bacteria and virus mutate fast. Given the selection pressure of the drug, eventually resistant strains to the pharmaceutical drug will emerge. The offspring of those resistant strains will be also or maybe even more resistant to the drug. It is the very essence of the concept of evolution.

Specifically, we are going to study tuberculosis. Tuberculosis is one of the most killing infectious diseases of the world. Among all the drugs used in the tuberculosis treatment, there is a very special drug called pyrazinamide. The main property of pyrazinamide is that the World Health Organization recommends its intake in all tuberculosis therapies since it reduces drastically the necessary time for the recovery of the patient. Therefore, it is not surprising that the increasing resistance to pyrazinamide is alarming.

Pyrazinamide offers a good opportunity to carry out studies of drug resistance using molecular modeling studies, since almost all the cases of resistance are correlated with just a single-point mutation in the enzyme that interacts with the drug: Pyrazinamidase. Given its importance, this enzyme has been studied for a long time; nevertheless, its structure has been solved just one year ago. There is still controversy about this protein. For example, different laboratories do not agree about what metal ion is the actual cofactor of this enzyme.

In chapter 2 we will introduce the problematic of resistance to pyrazinamide, previous studies in Pyrazinamidases from other organisms, Pyrazinamide from *Mycobacterium tuberculosis*, and all the software and databases that will be employed in this study.

In chapter 3, Pyrazinamidases of resistant, partially resistant and sensitive strains from *Mycobacterium tuberculosis* will be modeled in order to study their conformational space. Each one of them will go to a simulated annealing process so that the conformation with the lowest potential energy could be found.

In chapter 4, the most important chapter of this study, the enzymatic values of the Michaelis constant, K_M , is correlated with the predicted free energy of binding for each one of the mutants. Finally, we will discuss how these results can be used to predict the degree of resistance in new strains of *Mycobacterium tuberculosis*.

Chapter 2

Background

2.1 Pyrazinamide Resistance

Tuberculosis remains as one of the major serious public health problem globally since it is a leading killing disease. Tuberculosis is regarded as a re-emerging disease which kills about 2 million people per year; more than any other infectious disease. Tuberculosis's pathogen is *Mycobacterium tuberculosis*; a bacteria which has several strains. The wild type strain is called h37rv. Tuberculosis caused by the h37rv strain presents a slightly long, but successful treatment.

Tuberculosis is contagious and spreads through the air. Lung tuberculosis is the most common and most infectious form of tuberculosis. One third of the world's population is estimated to be infected with *Mycobacterium tuberculosis*. The overall lifetime risk of developing tuberculosis disease following infection is estimated to be approximately 10%.

Tuberculosis had been controlled acceptably in developed countries, e.g. Japan, but the situation is now deteriorating because of the spread of HIV infection, which renders its victims susceptible to tuberculosis. In Japan, the incidence of tuberculosis has declined since the 1950s, but now has shown a tendency to increase. The proportion of the elderly in the newly registered TB patients has increased.¹

Pyrazinamide is a first-line anti-tuberculosis drug and is used in short-course treatment in conjunction with other drugs such as rifampin, isoniazid and ethambutol. Its main peculiarity is that exhibits strong activity against semi-dormant bacteria trapped inside the alveolar macrophages. Pyrazinamide is actually a prodrug. A prodrug is a pharmacological substance (drug) administered in an inactive (or significantly less active) form. Once administered, the prodrug is metabolised *in vivo* into an active metabolite, a process termed bioactivation. Pyrazinamide is metabolised *in vivo* by the *Mycobacterium tuberculosis* into pyrazinoic acid, the active metabolite. Pyrazinamide interacts with an enzyme called Pyrazinamidase, PZAase. Pyrazinamidase is coded by a gene denominated *pncA*. The enzymatic mechanism of action of Pyrazinamidase is partially understood.

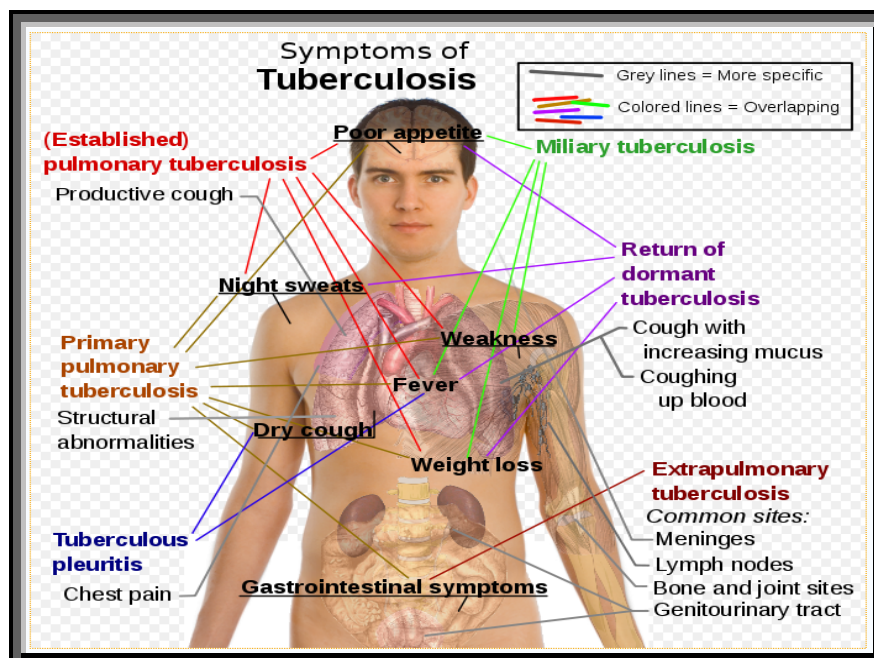


Figure 2.1.1. Main symptoms of tuberculosis.

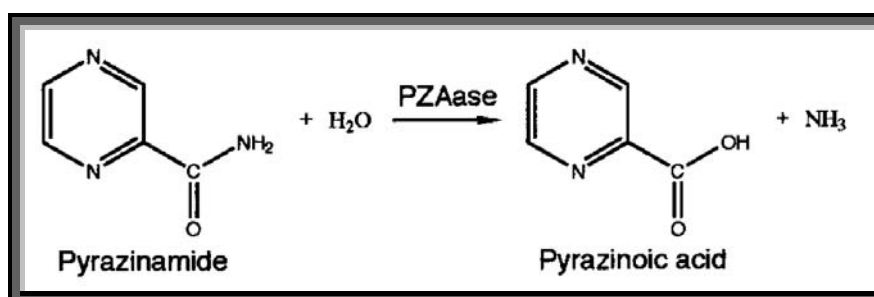


Figure 2.1.2. Hydrolysis of Pyrazinamide into Pyrazinoic acid by Pyrazinamidase, PZAase.

Mycobacterium tuberculosis presents several strains apart from the wild type. The emergence and spread of new antibiotic-resistant strains of *Mycobacterium tuberculosis* constitutes an alarming threat to the control of tuberculosis. Any strain which is resistant to both rifampin and isoniazid is defined as multi-drug resistant tuberculosis, MDR-TB. Extensively drug-resistant tuberculosis (XDR-TB) is a form of TB caused by bacteria that are resistant to at least one of these three injectable (and more toxic) drugs: capreomycin, kanamycin or amikacin, in addition to the previously mentioned rifampin and isoniazid. MDR-TB is expensive and extremely difficult to treat. XDR-TB is untreatable. The true scale of XDR-TB is unknown. It is estimated that there are around 40,000 cases per year. As of June 2008, 49 countries, including Japan, have confirmed cases of XDR-TB.

Mutations on the *pncA* gene are considered to be the main mechanism of resistance to pyrazinamide. These mutations generate single-point mutations on the sequence of the protein PZAase. The loss of PZAase activity is related with single-point mutations in this protein. 72% to 98% of pyrazinamide-resistant strains have at least one mutation in the *pncA* gene. These PZAase mutants are unable to metabolize pyrazinamide. Samples of these *pncA* mutants are being collected, isolated and characterized worldwide.²

Computational structural biology tools allow us to explain with high certainty why *Mycobacterium tuberculosis* mutants can not metabolize pyrazinamide. Since pyrazinamide is the only front line drug that is effective against bacilli in its latent state, resistance to pyrazinamide drastically impairs TB treatment. Therefore, the development of a new generation of new drugs that could replace pyrazinamide in the TB treatment by countering resistance and shortening treatment is more important than ever.

Identifying pyrazinamide resistance requires systematic sequencing the *pncA* gene. This is feasible given its short size. If the gene is found to be identical to the wild-type *pncA* gene, the strain is susceptible to pyrazinamide in most of the cases. However, if the gene presents an unknown mutation, it would indicate a potential susceptibility to pyrazinamide, but the meaning of that specific new mutation is uncertain.



Figure 2.1.3. Distribution of countries reporting at least one case of XDR-TB as of January 2010.

Mutations on pncA and PZA resistance of *M. tuberculosis* complex

High frequency zone	Sensitive strain	wt				Nucleotide position	Substitution				N° of isolates	Phenotype		Pyrazinamidase		Publication	Origin of isolates	
		codon	nucleotide	AA	AA		Nucleotide substitution	AA substitution	AA	AA		Phenotype: S sensitive; R resistant	Method: 1 Proportion; 2 Rapid broth; 3 BACTEC 400; 4 MGIT 960	Pyrazinamidase activity	Method: 1 Wayne 2 other test			
51	H51Q		CAC	C	His	H	153	A	CAA	Gln	Q	2	R	3			Somoskovi A. J. Clin. Microb. 07	USA
51	H51Q	microarray	CAC	C	His	H	153	A	CAA	Gln	Q	1	R	3	negative	2	Derkin S. J Med. Microbiol. 05	USA
51	H51Q	microarray	CAC	C	His	H	153	A	CAA	Gln	Q	1	R	3			Wade M.M. Diag. Microbiol. Infect. Dis. 04	USA
51	H51Q		CAC	C	His	H	153	A	CAA	Gln	Q	1	R	3	negative	1	Scorpio A. Antimicrob. Agents Chemother. 97	USA
52	no published mutation		ATC	Ile	I													
53	D53N, frameshift		GAC	G	Asp	D	157	A	AAC	Asn	N	1	R	2	negative	1	Sekiguchi J.I. J. Clin. Microb. 07	Japan
	Frameshift			ins			349	CAC	TG									Japan
53	D53A		GAC	A	Asp	D	158	C	GCC	Ala	A	1	R	1	negative	1	Cheng S-J. Antimicrob. Agents Chemother. 00	South Korea
53	D53A		GAC	del	Asp	D	158	del	A			1	R		negative	1	Lee K.W. J Korean Med Sci 01	South Korea
54	P54S		CCG	C	Pro	P	160	T	TCG	Ser	S	1	R	3			Jurén P. Antimicrob. Agents Chemother. 08	Sweden
54	P54T		CCG	C	Pro	P	160	A	ACG	Thr	T	1	R	1 ou 3			Sreevasan S. Antimicrob. Agents Chemother. 97	USA
54	P54T		CCG	C	Pro	P	161	A	ACG	Thr	T	1	R	1,3			Hou L. Epidemiol. Infect. 00	China
54	Frameshift		CCG	del	Pro	P	161	del	C			1	R		negative	1	Lee K.W. J Korean Med Sci 01	South Korea
54	P54Q		CCG	C	Pro	P	161	A	CAG	Glu	Q	1	R	1	negative	1	Barco P. J Antimicrob. Chemother. 06	Brazil
54	P54L		CCG	C	Pro	P	161	T	CTG	Leu	L	1	R	3			Jurén P. Antimicrob. Agents Chemother. 08	Sweden
54	P54L		CCG	C	Pro	P	161	T	CTG	Leu	L	1	R	4			Louw G.E. Int. J. Tuberc. Lung Dis. 06	South Africa

Figure 2.1.4. Worldwide report on mutations on pncA and pyrazinamide resistance of *Mycobacterium tuberculosis*

2.2 Previous Researches on Pyrazinamidases

About 10 years ago there was no structural information concerning the enzyme Pyrazinamidase.³ Du determined the crystal structure of Pyrazinamidase from *Pyrococcus horikoshii*. The main features of the structures were:

- Pyrazinamidase is a metalloenzyme. In the case of Pyrazinamidase from *Pyrococcus horikoshii*, the ion is Zn^{+2} . The ion binding zone is made up of Asp52, His54, His 71 and 3 water molecules

- There is a cis-peptide in the structure of Pyrazinamidase which forms a potential oxyanion hole theorized to be the place where pyrazinamide binds to Pyrazinamidase.

The structure was deposited on the Protein Data Bank, code 1IM5, with a resolution of 1.65 Å.

⁴Zhang characterized several properties of Pyrazinamidase from *Mycobacterium tuberculosis*. The enzyme was found to be, as expected, a 20.89 kDa monomeric protein. The optimal pH and temperature for enzymatic activity were 7.0 and 40°C, respectively. They found that Pyrazinamidase from *Mycobacterium tuberculosis* was a 0.5:0.5 metalloenzyme of Mn^{+2} and Zn^{+2} , meaning that, according to the experimental data, Pyrazinamidase from *Mycobacterium tuberculosis* binds to these 2 ions with no preference between them. Mutagenesis assays showed that the residues Asp8, Lys96 and Cys138 were key residues for catalysis. Furthermore, residues Asp49, His51, his57 and His 71 were essential for metal ion binding.

⁵Zimic analyzed the distribution of mutations on Pyrazinamidase in clinically reported resistant strains of *Mycobacterium tuberculosis*. A consensus clustering of mutation was observed in some regions, while in other regions the number of mutations was few. The highest number of mutations was found on the 50-90 region of the primary structure of the enzyme.

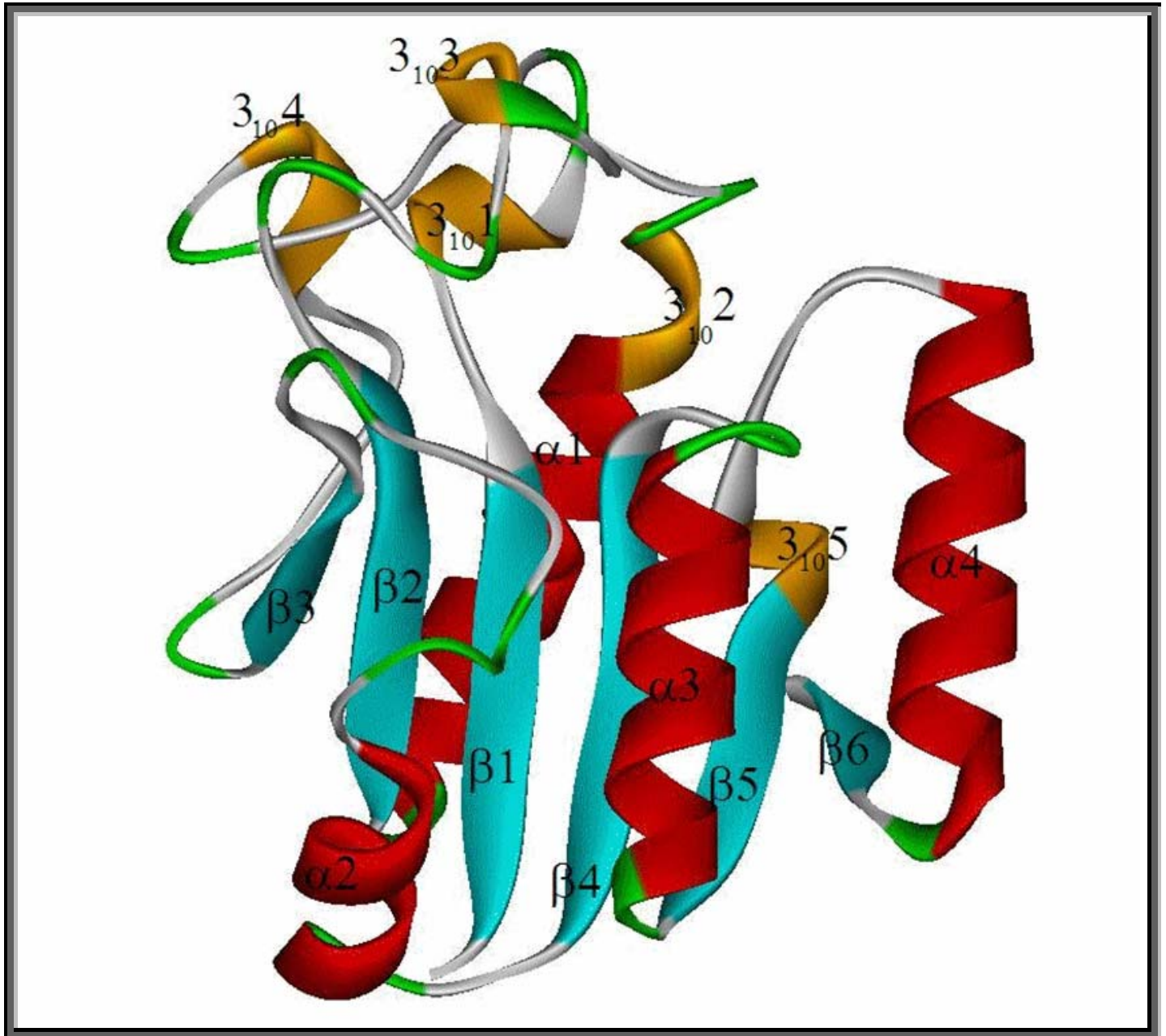


Figure 2.2.1. Topology of Pyrazinamidase from *Pyrococcus horikoshii*.

	1	3	7	25	30	43	50	90	94	117	122	126	131	140	149	152	157	166	178	181	183
MIT	A3P	D8E	A25E	Y34D	H43P	H51N	I90L	K96E	N118T	R123L	V128G	G132V	R140S	LI51S	T153N	L159P	T167I	V180A	LI82S		
MII	A3E	D8N	A26G	Y34S	V45G	H51Y	I90S	K96Q	W119R	R123P	V130G	G132A	Q141P		R154G	L159R	T168N	V180F			
R2R	L4S	D8Y	L27P	L35P	A46V	H51P	V93L	K96T	L120P	V125F		G132D	T142M		V155G	L159V	A171T				
	L4W	D8G	L27R	L35R	A46E	H51Q	F94P	K96N	L120R	V125G		G132S	T142K		V155A	T160P	A171E				
	I5N	D8H	A28D	A36V	T47A	H51R	F94S	G97S	R121P	V125D		I133N	T142P		L156Q	A161P	A171V				
	I5S	V9A	A28E	Y41H	T47P	D53A		G97C				I133S	T142A		V157G	G162D	A171P				
	I6T	V9G			T47S	D53N		G97D				I133T	A146T			V163R	L172A				
	V7A	Q10K			K48E	P54T		Y99D				A134V	A146V		S164P	L172P					
	V7F	Q10P			D49A	P54L		A102T				A134F	R148S			M175V					
	V7D	Q10R			D49V	P54Q		A102V				T135P				M175T					
	V7G	D12A			D49G	P54R		Y103H				D136H									
	V7I	D12G				P54S		Y103C				D136N									
		D12N				H57D		Y103S				D136G									
		F13S				H57P		S104R				D136Y									
		C14R				H57R		S104C				H137R									
		C14Y				F58L		G105D				H137P									
		C14W				S59P		G108D				C138R									
		G17D				T61P		K111Q				C138S									
		G17S				P62T		T114P				C138Y									
		L19P				P62R		L116R				V139L									
		G23V				P62H						V139M									
						P62L						V139G									
						D63A						V139A									
						D63G															
						Y64D															
						S66P															
						S67P															
						T68R															
						W68R															
						W68G															
						W68L															
						W68S															
						P69L															
						P69R															
						H71Y															
						H71D															
						H71R															
						H71E															
						H71P															
						H71T															
						C72R															
						C72W															
						T76P															
						T76I															
						G78C															
						G78D															
						G78V															
						A79V															
						H82D															
						H82R															
						H82L															
						L85P															
						L85R															
						T87M															

Mutations detected in
the World

Figure 2.2.2. Worldwide distribution of mutations of Pyrazinamidase from *Mycobacterium tuberculosis*.

⁶Sheen calculated the kinetic parameters, k_M and k_{cat} , for the wild-type Pyrazinamidase from *Mycobacterium tuberculosis* and the following mutants : H51R, T76P, L116P, D12A, D12G, G24D, G78C and K48T. The resistance of each mutant ranges from sensitive, partially resistant to resistant. The data is summarized in **Table 2.1**.

Mutant	kM (mM)		Wayne activity Resistance
	Mean	95% CI \pm	
H51R	1.41	0.18	Resistant
T76P	0.31	0.05	Resistant
L116P	1.56	0.15	Resistant
D12A	0.99	0.16	Partially Resistant
D12G	0.55	0.06	Partially Resistant
G24D	0.42	0.25	Partially Resistant
G78C	1.07	0.14	Partially Resistant
K48T	0.44	0.05	Sensitive
Wild Type	1.24	0.44	Sensitive

Table 2.1. Michaelis constant for Pyrazinamidase from *Mycobacterium tuberculosis* and mutants.

⁷Fyfe resolved the crystal structure of Pyrazinamidase from *Acinetobacter baumannii*. The structure was deposited on the Protein Data Bank, code 2WTA, with a resolution of 1.70 Å. The main importance of this job is that the protein was crystallized in complex with an analog of pyrazinamide: pyrazinoic acid. The crystal showed that the ligand binds to the metallic ion (in this case, Zinc).

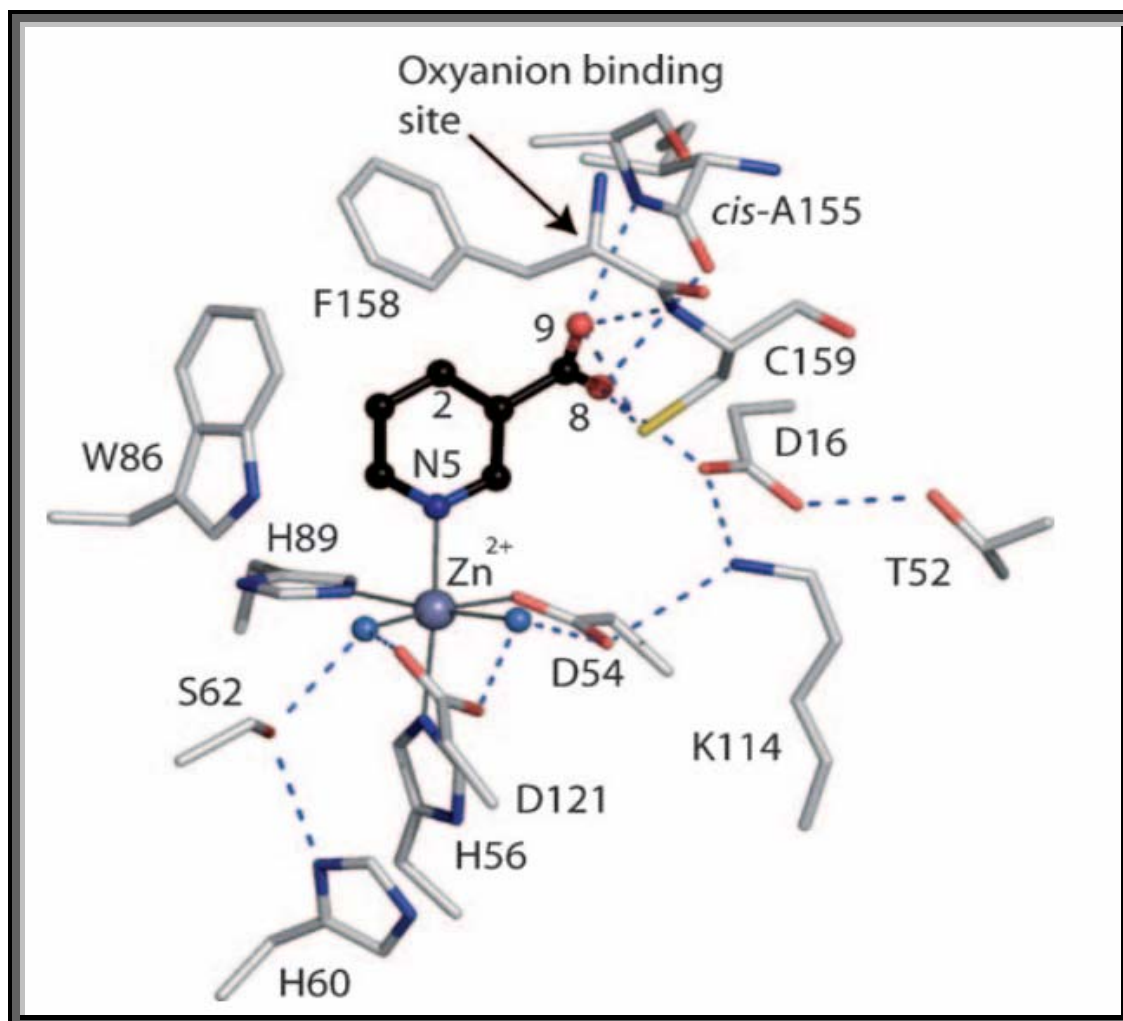


Figure 2.2.3. Binding site of Pyrazinamidase from *Acinetobacter baumannii*. It can be appreciated that the ligand, pyrazinoic acid, binds to the metal ion, Zn⁺².

Using the structures of Pyrazinamidase from *Pyrococcus horikoshii* and Pyrazinamidase from *Acinetobacter baumannii*, ⁸Unissa developed a homology modeling model of Pyrazinamidase from *Mycobacterium tuberculosis*. He tried to explain resistance to pyrazinamide, but got contradictory results. It was shown later that the homology model wrongly predicted the conformation of the loops due to its long length.

⁹French crystallized Pyrazinamidase from *Streptococcus pneumoniae* unbound and bound to pyrazinamide. It was the first time in which the docking pose of the pyrazinamide-Pyrazinamidase complex could be appreciated. Also, it was revealed that the residues that form part of the loop between $\beta 2$ and $\alpha 1$ are the ones that drastically change their position upon bonding of pyrazinamide.

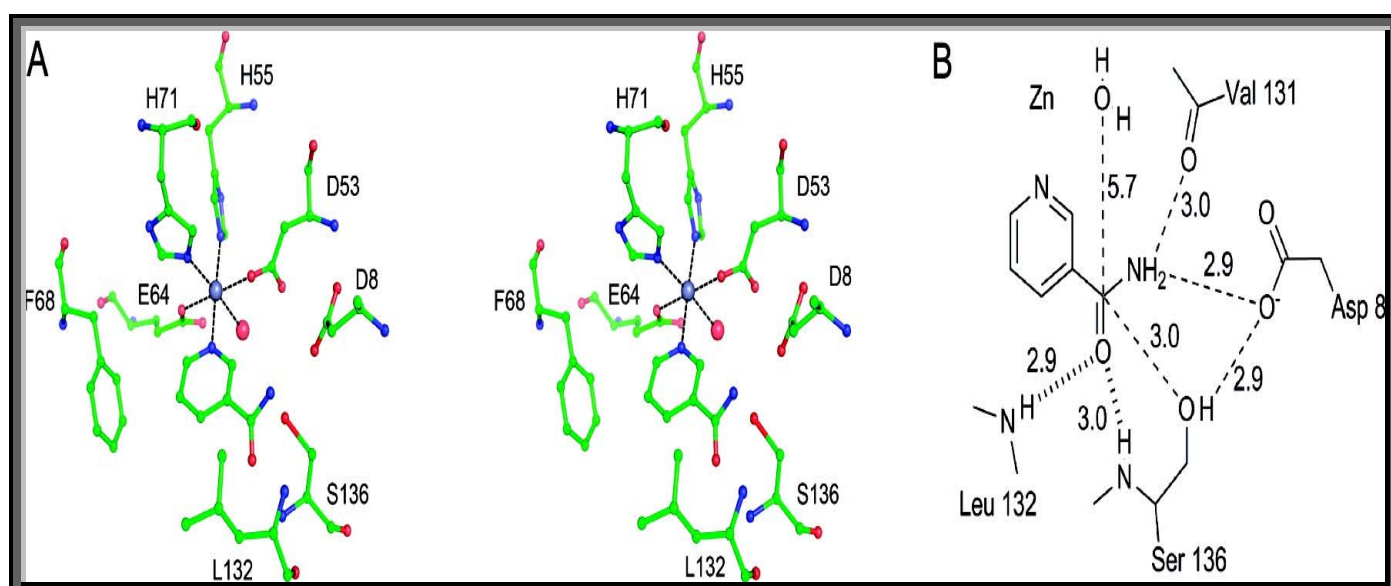


Figure 2.2.4. Binding site of Pyrazinamidase from *Streptococcus pneumoniae*. It can be appreciated that pyrazinamide binds to the metal ion, Zn⁺², as well as the potential oxyanion hole made by the cis-peptide.



Figure 2.2.5. Comparison of the unbound and bound conformation of Pyrazinamidase from *Streptococcus pneumoniae*. The loop between $\beta 2$ and $\alpha 1$ is highlighted.

Recopiling all the previous information, ⁹Sauve proposed 2 probable enzymatic mechanism of reaction of Pyrazinamidase. They are described below.

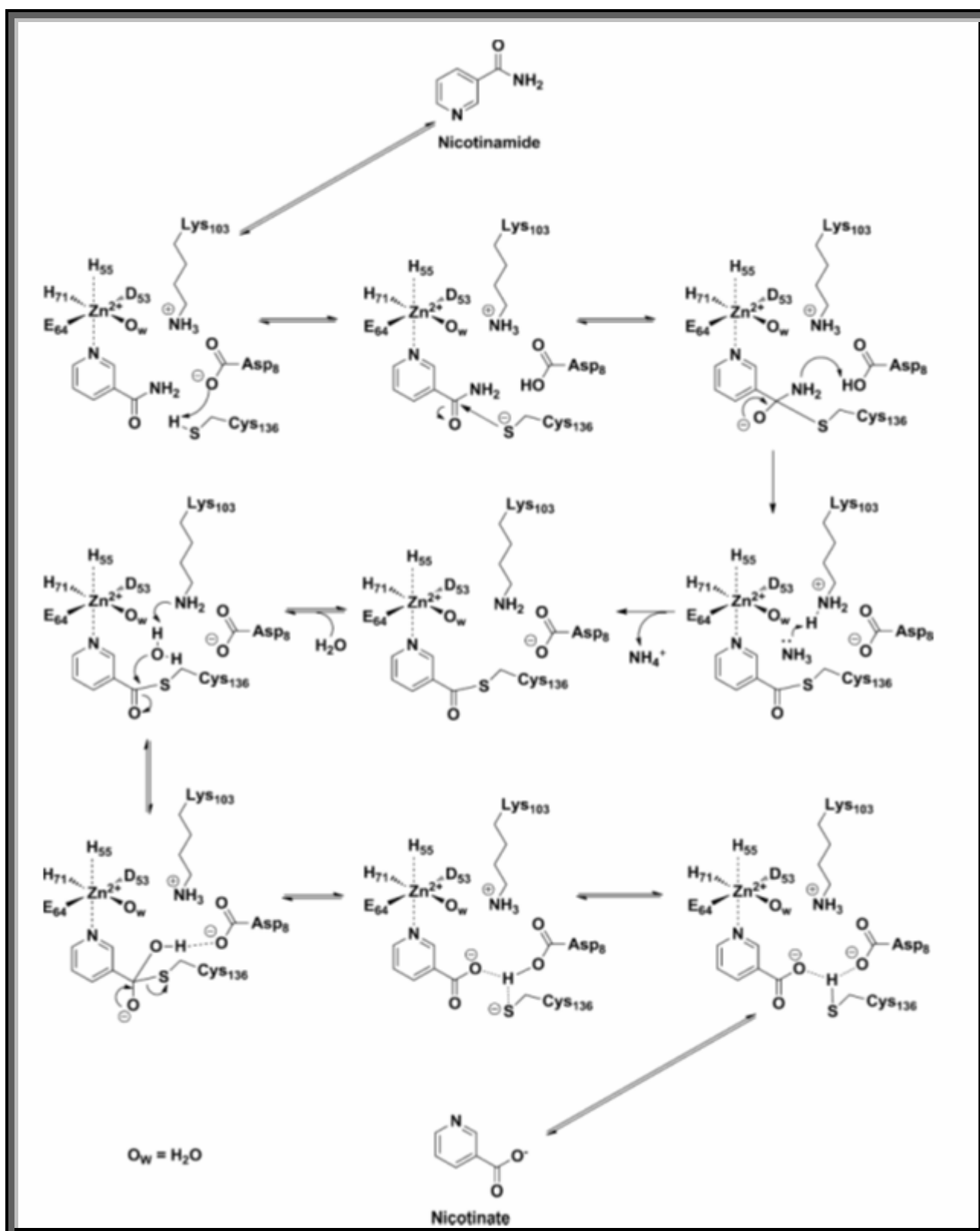


Figure 2.2.6. Ammonia generated in the first half of the mechanism deprotonates the Lysine, which in turn act as a base to activate the water molecule.

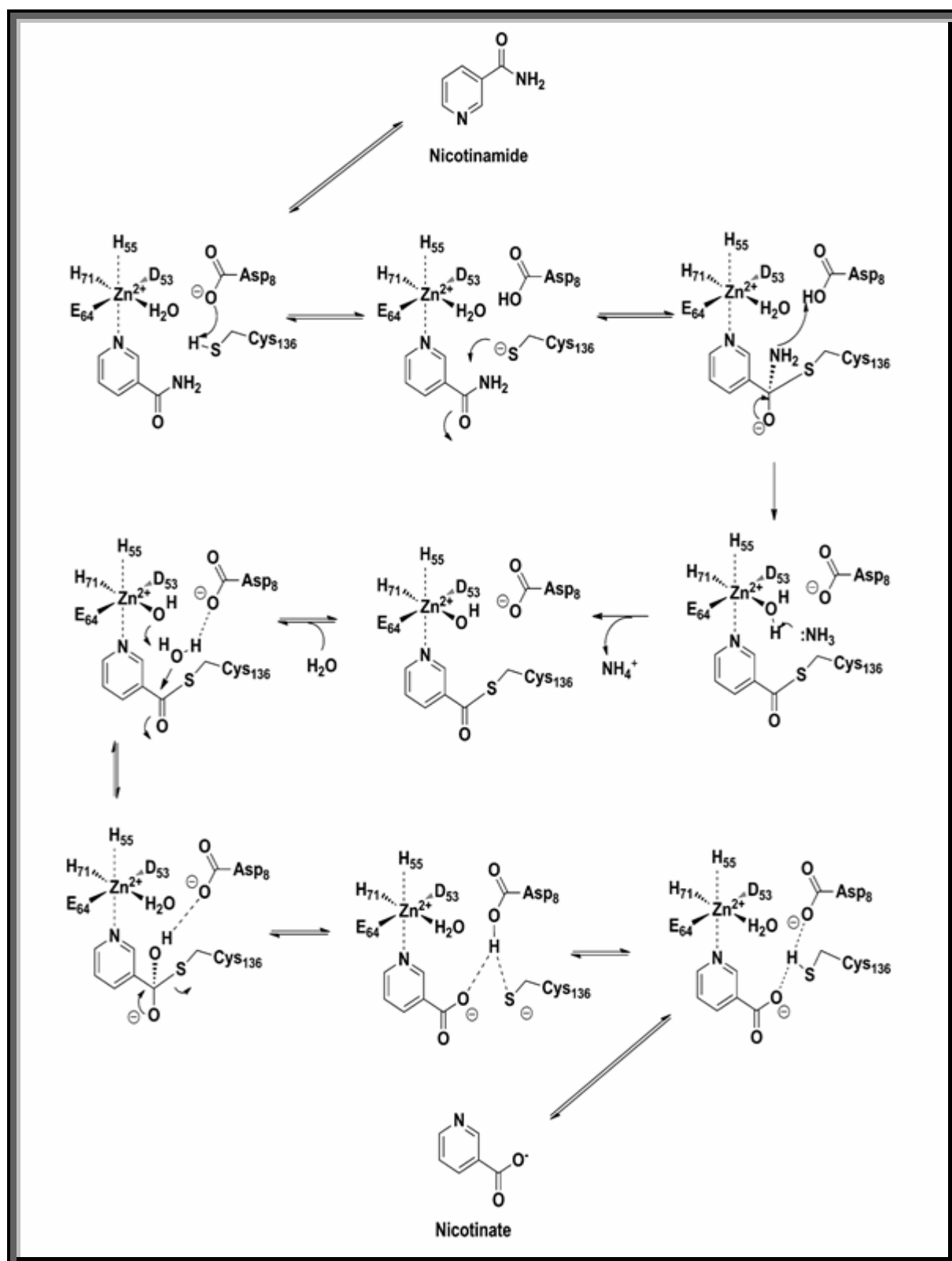


Figure 2.2.7. Ammonia generated in the first half generates a Zn⁺²-hydroxide, which in turn act as a base to activate the water molecule.

2.3 Structure of Pyrazinamidase from *M. tuberculosis*

¹⁰Petrella solved the structure of Pyrazinamidase from *Mycobacterium tuberculosis*. The structure was deposited on the Protein Data Bank, code 3PL1, with a resolution of 2.20 Å. The structure of Pyrazinamidase from *Mycobacterium tuberculosis* consists of 6 β -sheets surrounded by 4 α -helices, which is known as a α/β domain. The β strand order is 3-2-1-4-5-6. α 1, α 2 and α 3 are packed against one side of the central sheet. α 4 helix is packed against the other side of the central sheet.

As expected, Pyrazinamidase from *Mycobacterium tuberculosis* shares all the characteristics of the previously mentioned Pyrazinamidases. Its main structural characteristics are shown in **Figure 2.3.3**.

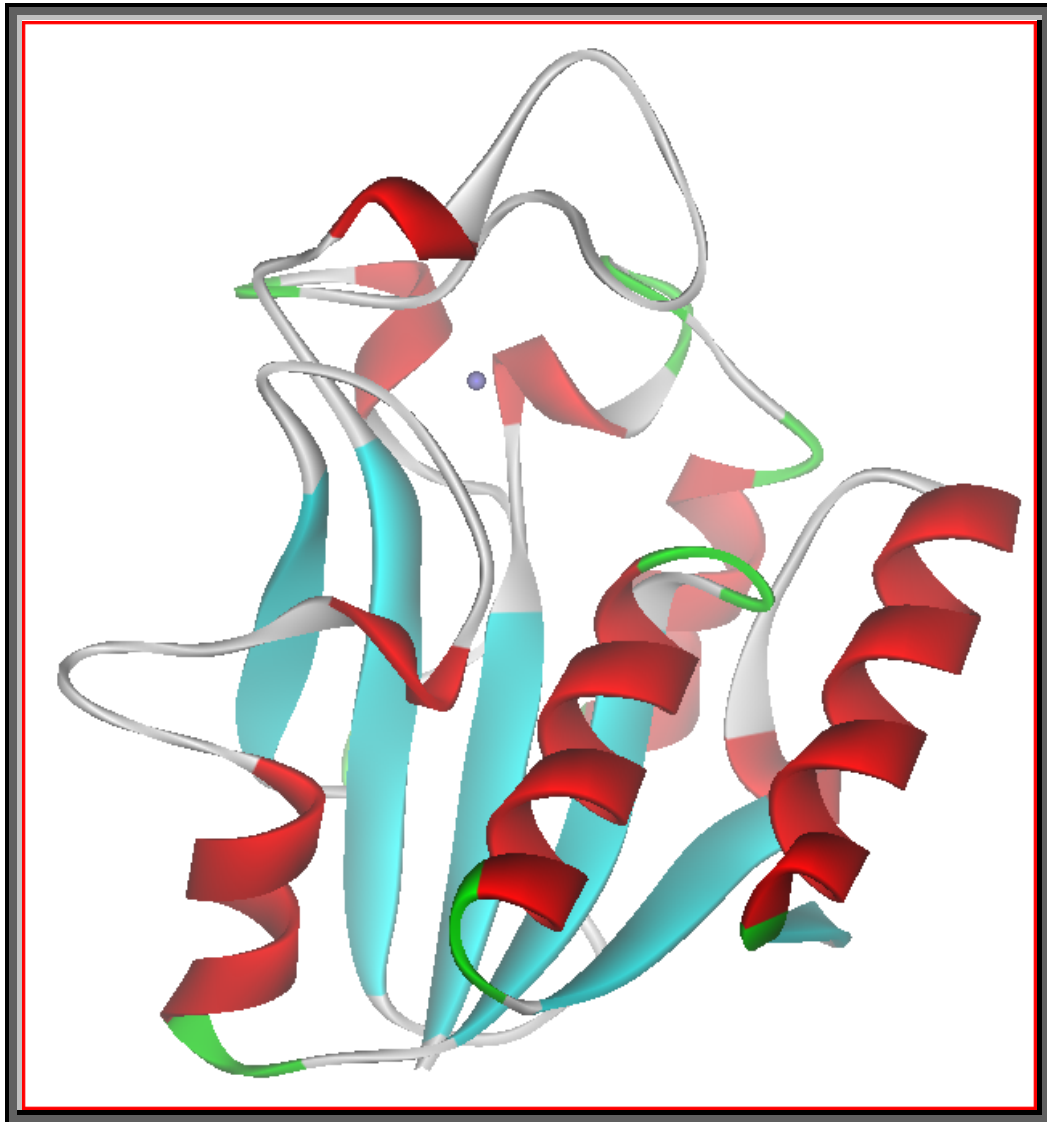


Figure 2.3.1. Structure of Pyrazinamidase from *Mycobacterium tuberculosis*.

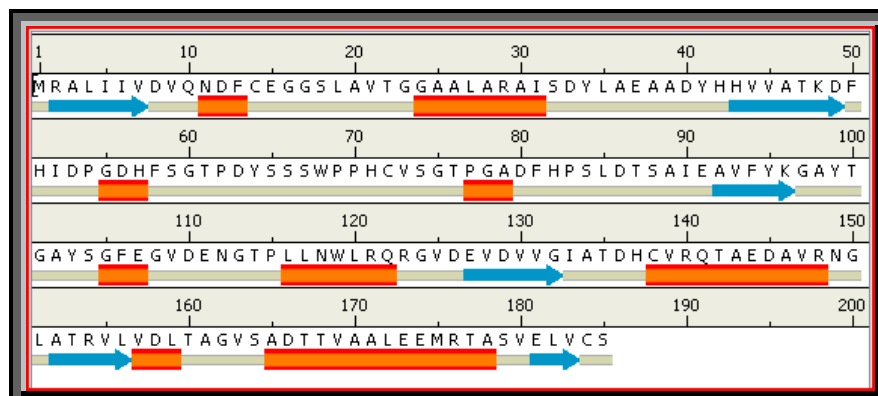


Figure 2.3.2. Sequence of Pyrazinamidase from *Mycobacterium tuberculosis*.

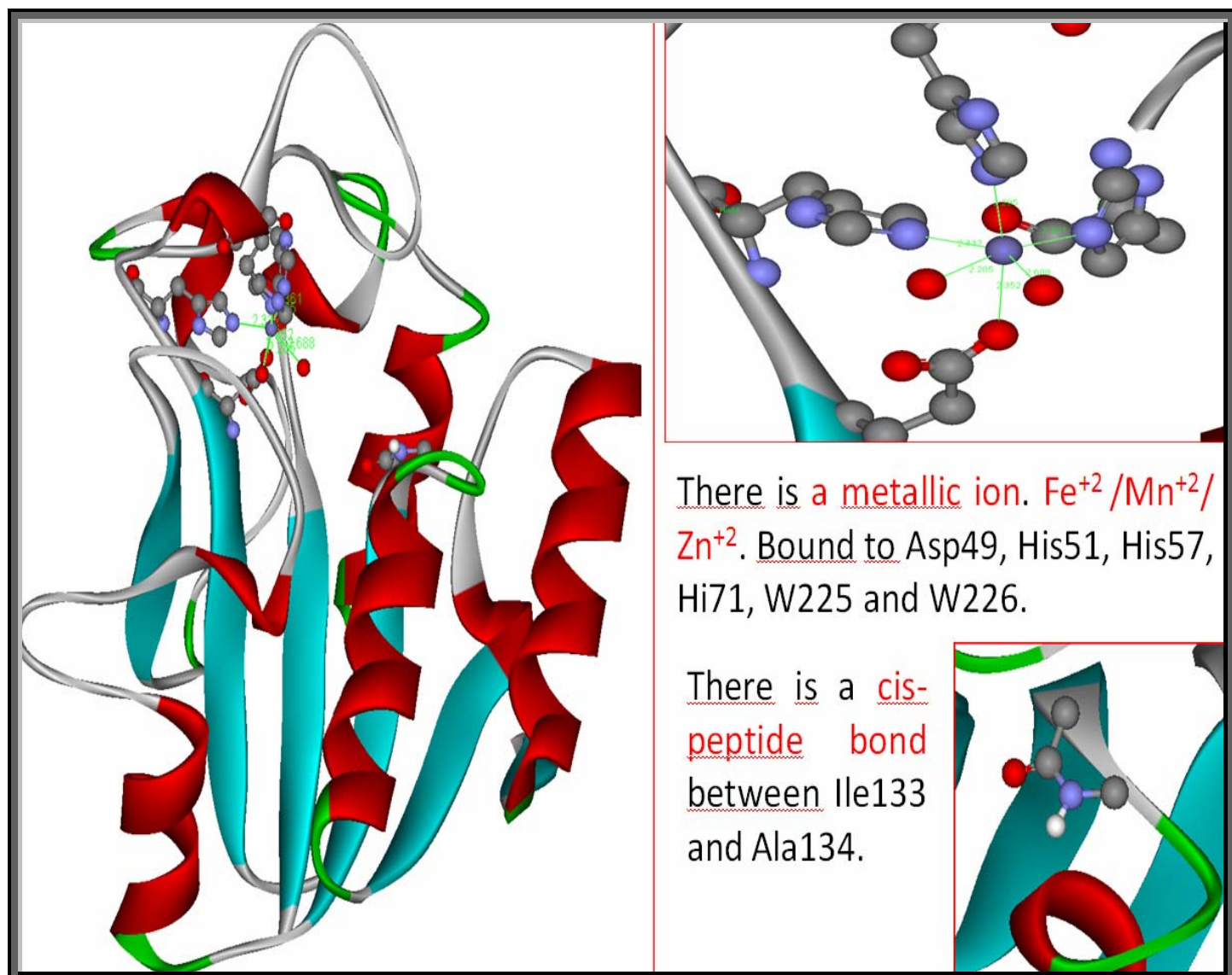


Figure 2.3.3. Structure elements of Pyrazinamidase from *Mycobacterium tuberculosis*.

According to the B-factor analysis, most of the protein residues are “cold”, but the region from residue 50 to residue 56 is “hot”. They are just exactly the residues that face the binding cavity of the protein. Therefore, they might play an important role in ligand binding and product release in the protein binding site.

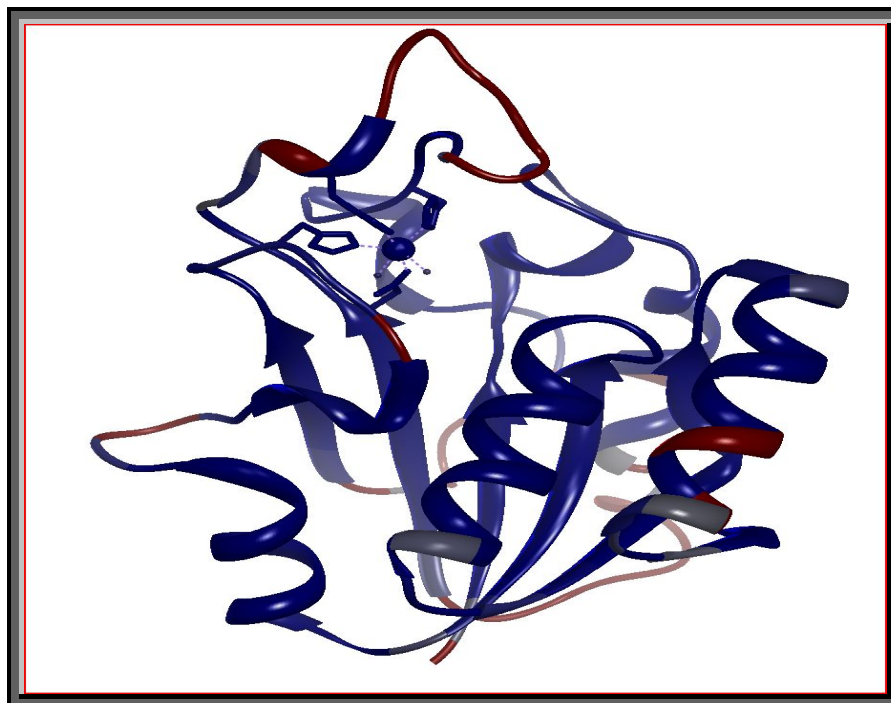


Figure 2.3.3. Structure elements of Pyrazinamidase from *Mycobacterium tuberculosis* colored by B-factor.

Finally, **Figure 2.3.4.** summarizes all the residues of the binding site of Pyrazinamidase from *Mycobacterium tuberculosis*, as well as their role in the binding of the substrate.

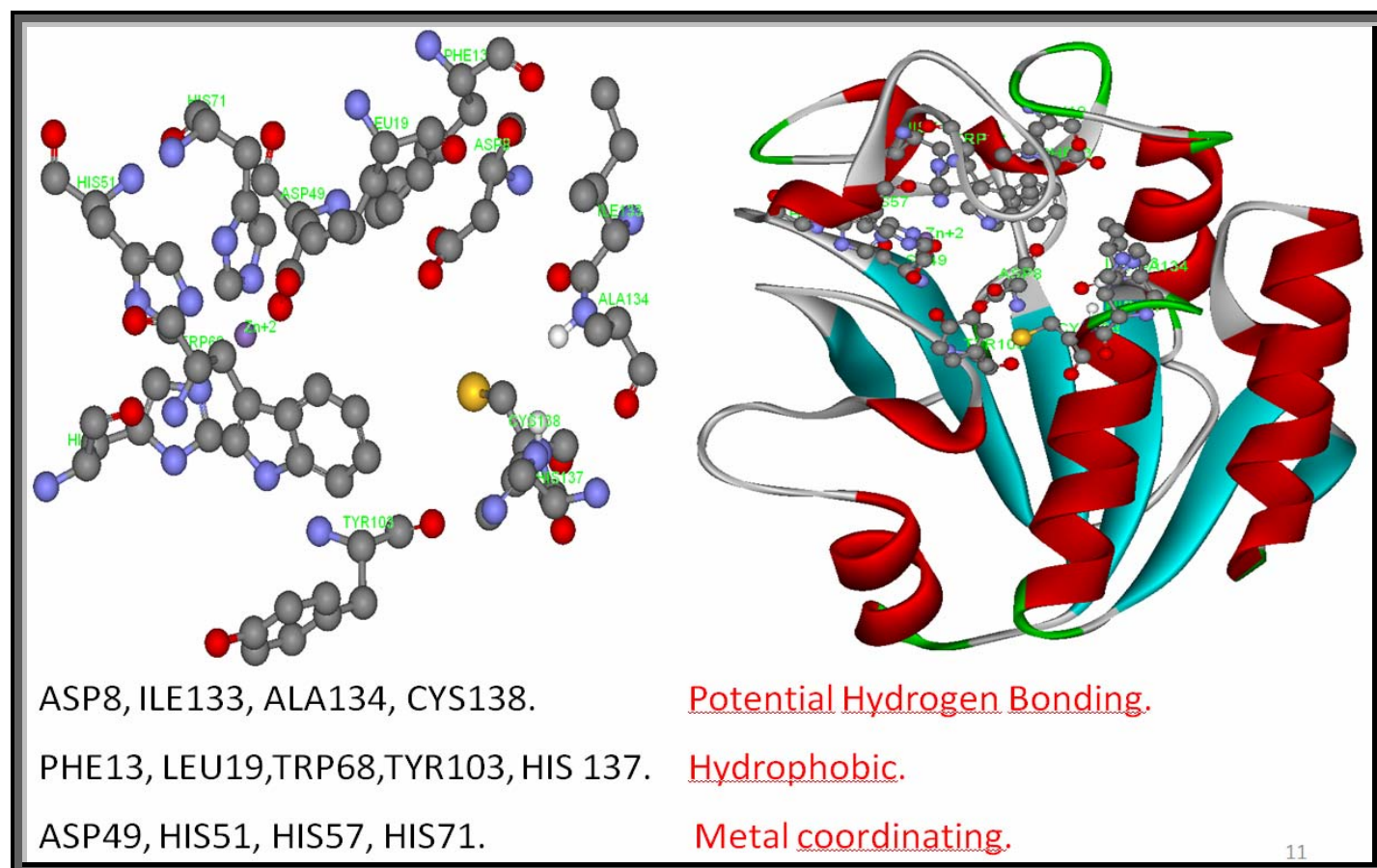


Figure 2.3.4. Binding site of Pyrazinamidase from *Mycobacterium tuberculosis*.

2.4 Software and databases

Several molecular programs and databases have been employed in this study. A list of the most used programs, as well as a brief description of them is shown below.

1. **MyPresto.** A molecular simulation system for structure-guided molecular development. myPresto consists of about 50 programs and databases.
2. **Cosgene.** A molecular dynamics simulation program. "cosgene" can perform several kinds of generalized ensemble methods, like multicanonical MD (McMD)/Force Biased McMD
3. **Sievgene.** A protein-compound docking program.
4. **PocketFinder.** A protein pocket detection algorithm.
5. **Gaussian.** A computational chemistry program.
6. **R.E.D.IV.** Allows computational biologists involved in AMBER force field based biological studies to automatically derive RESP and ESP charge values for small non-standard molecules.
7. **PoseView.** Generates high-quality 2D structure-diagrams of protein-ligand complexes provided as 3D-input. Such input may come directly from crystal structures or be computed for example by a docking program.
8. **DS Visualizer** Generates high-quality 2D structure-diagrams of protein-ligand complexes provided as 3D-input. Such input may come directly from crystal structures or be computed for example by a docking program.
9. **Cambridge Database.** Contains crystal structure information, chemical, and bibliographic data for about 400000 organic and metal-organic compounds.
10. **PDBj.** A repository for the 3-D structural data of large biological molecules, such as proteins and nucleic acids.

Chapter 3

Modeling of Mutant Proteins

3.1 Objective

To model the structure of several mutants of Pyrazinamidase from *Mycobacterium tuberculosis* from the structure of the wild-type strain.

The interaction of each Pyrazinamidase with pyrazinamide will be taken into account. A simulated annealing procedure will be used, and molecular dynamics simulations will be used to sample the structure of every mutant. Finally, an analysis of how each single-point mutation affects the global structure will be done.

3.2 Methodology

First, we need to assign charges to the pyrazinamide molecule. The atoms coordinates of the pyrazinamide molecule were gotten from the Cambridge Compound Database. Partial charges were calculated using restricted electrostatic point charge (RESP), and the electrostatic potential was calculated using Gaussian03. Angles and torsions were frozen at the moment of the calculation. The B3LYP/6-31G* basis set was employed because it works better with aromatic molecules such as pyrazinamide. The topology was assigned using the General Amber Force-Field 8 GAFF8.

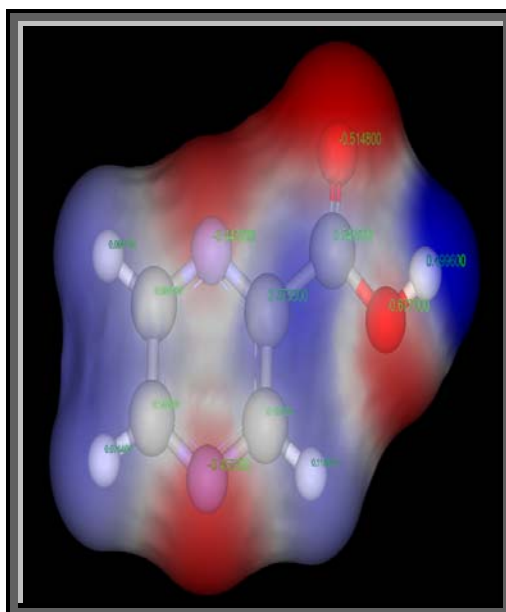


Figure 3.2.1. Electrostatic surface of pyrazinamide.

The mutation of amino acids often impacts protein function and structure. In this study, we are working with single-point mutations of Pyrazinamidase from *Mycobacterium tuberculosis*. The main assumption in this approach is that the secondary structure of Pyrazinamidase from each one of the mutants does drastically change with respect to the secondary structure of the wild type. ⁴Zhang carried out circular dichroism experiments to validate this assumption. He compared the circular dichroism spectra of the wild type with the following mutants: D49A, H51A, H57A, S59A, H71A and S104A. Each of the enzymes contained almost identical percentages of α -helices, β -sheets, turns and random coils; indicating that they have uniform secondary structures. The results are shown below.

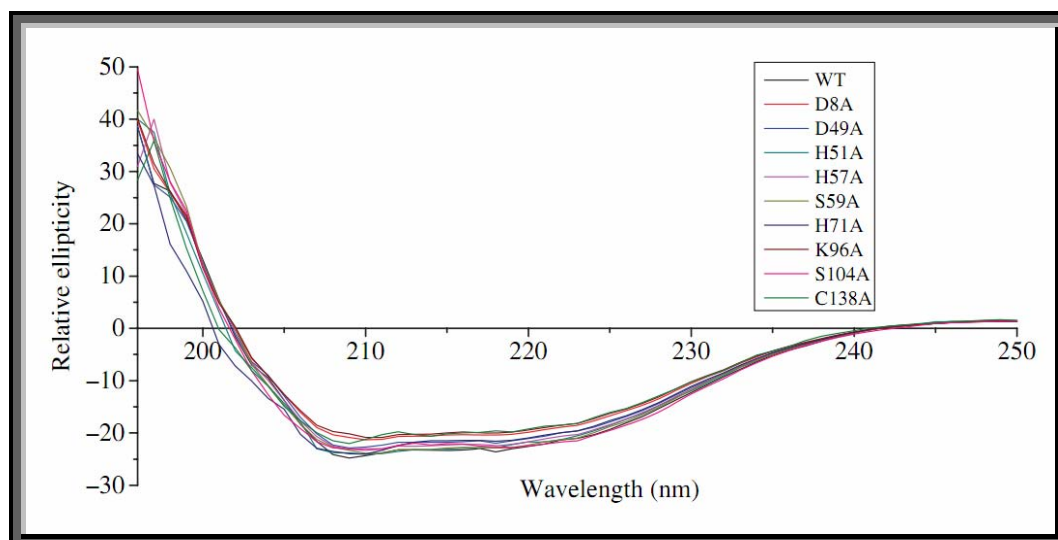


Figure 3.2.2. Circular dichroism spectra of the wild type Pyrazinamidase and selected mutants.

Apparently the crystal structure of Pyrazinamidase from *Streptococcus pneumoniae* can be used to “dock” the pyrazinamide into the structure of Pyrazinamidase from *Mycobacterium tuberculosis*. However, Pyrazinamidase from *Mycobacterium tuberculosis* presents a larger cavity. A direct “transplant” of the docking pose causes the pyrazinamide to be very far from the metallic ion, as shown in **Figure 3.2.3**.



Figure 3.2.3. Pyrazinamidase from *Mycobacterium tuberculosis* presents a larger cavity compared to the other Pyrazinamidases.

The methodology to sample conformations and perform the simulated annealing procedure is as follows:

- The system is composed of the protein, the metallic ion, 1 water molecule and pyrazinamide.
- No additional water is considered. Implicit water is modelled as a distance-dependent dielectric constant

$$\epsilon = 4 \cdot d$$

- Since a detailed interaction of the metallic ion with the water molecule and the pyrazinamide would require a special study [Klein], those interactions will be modeled by using mild distance restraints based on the bibliography.
- The AMBER96 force field will be employed for topology assignment of the protein atoms.
- Canonical ensemble, NVT.
- No cutoff is used.
- The initial conformation of pyrazinamide was got after structural alignment of of Pyrazinamidase from *Streptococcus pneumoniae* with Pyrazinamidase from *Mycobacterium tuberculosis*.
- 1 of the 2 water molecules that coordinate the metallic ion was removed, so that pyrazinamide could bind the metallic ion.

ENERGY MINIMIZATION :

- Conjugate gradient method.
- Root Mean Square Summation of Force less than 0.01.

VELOCITY EQUILIBRATION :

- The temperature of the system was increased for 10 ps of 10000 iterations of MD loop for raising the temperature to 300K. Hoover-Evans Gaussian constraint method.

MOLECULAR DYNAMICS :

- Several mild positions and distance restraints were used. **Figure 3.2.4.**
- 200 ps of molecular Dynamics simulation.
- Coordinates were saved every 21ps.

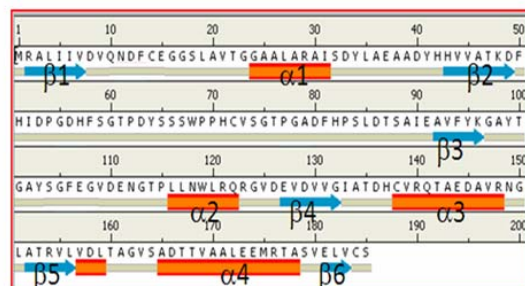
Finally, each one of the 10 saved conformations goes to 10 ps of 10000 iterations of MD loop for cooling down the temperature to 0.1K. Hoover-Evans Gaussian constraint method.

The very same procedure is repeated again for each one of the mutants.

POSITION RESTRAINTS.

Basically the backbone of almost all the secondary structure elements.

2-10, 26-32, 43-46, 88-93, 118-123, 127-160, 165-183.



DISTANCE RESTRAINTS.

Mild restraints were applied to keep the Zn^{+2} ion, the water molecule and the ligand in the binding site.

5 of the 6 atoms coordinating the Zn^{+2} ion (including PZA) was restrained by $\langle 2.2\text{\AA}-2.4\text{\AA} \rangle$.

Asp49 was not considered.

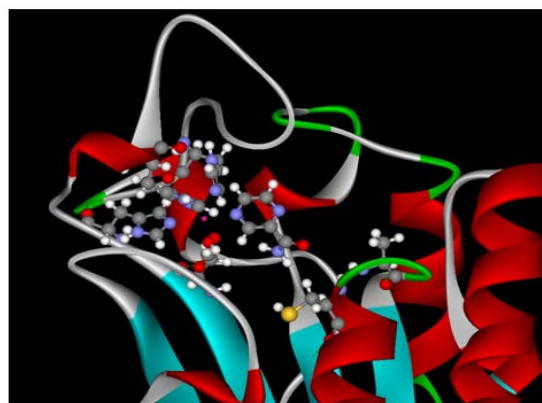


Figure 3.2.4. Description of the positions and distance restraints employed.

The whole sampling methodology is summarized in **Figure 3.2.5**.

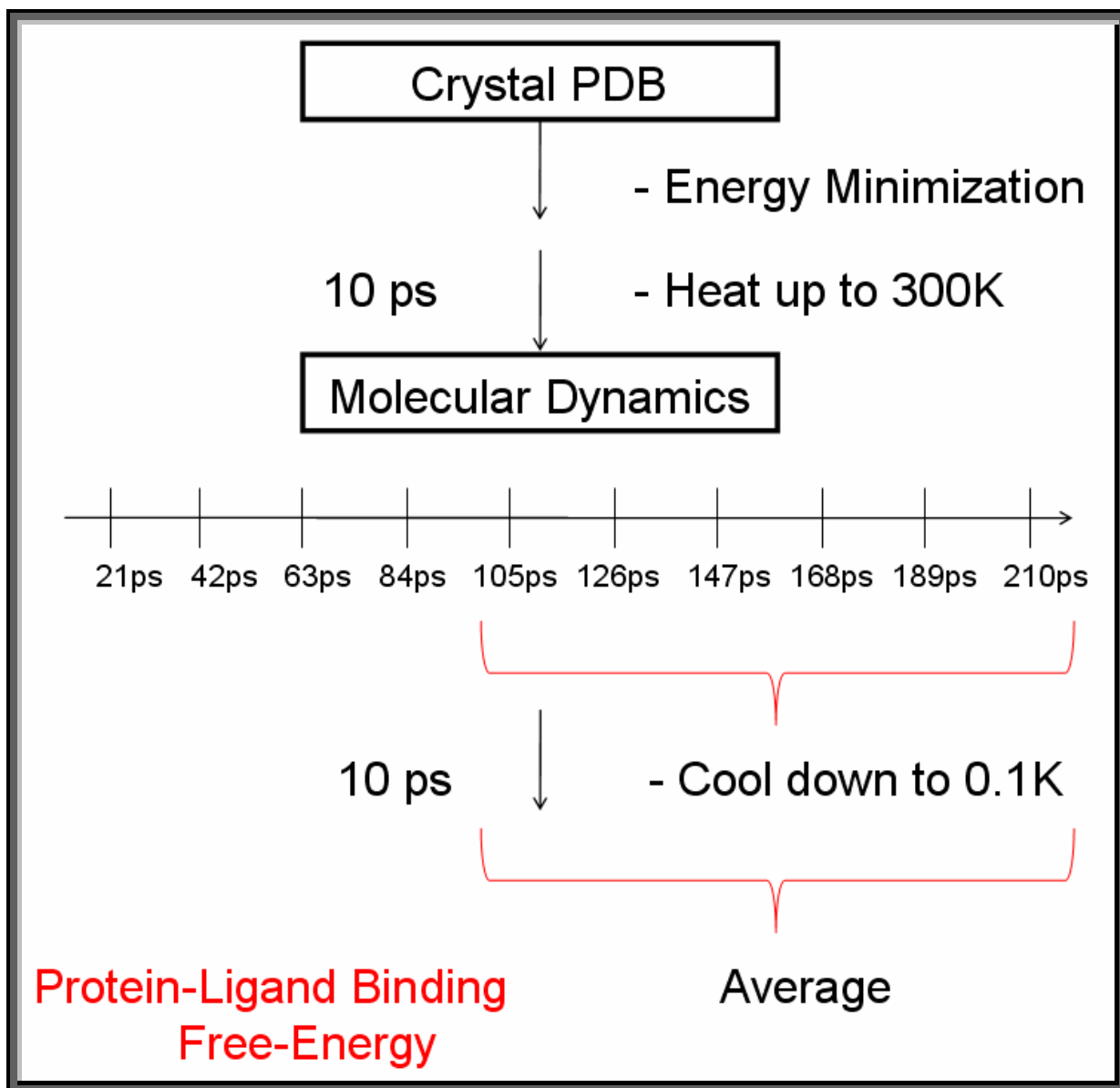


Figure 3.2.5. Sampling methodology employed in this study.

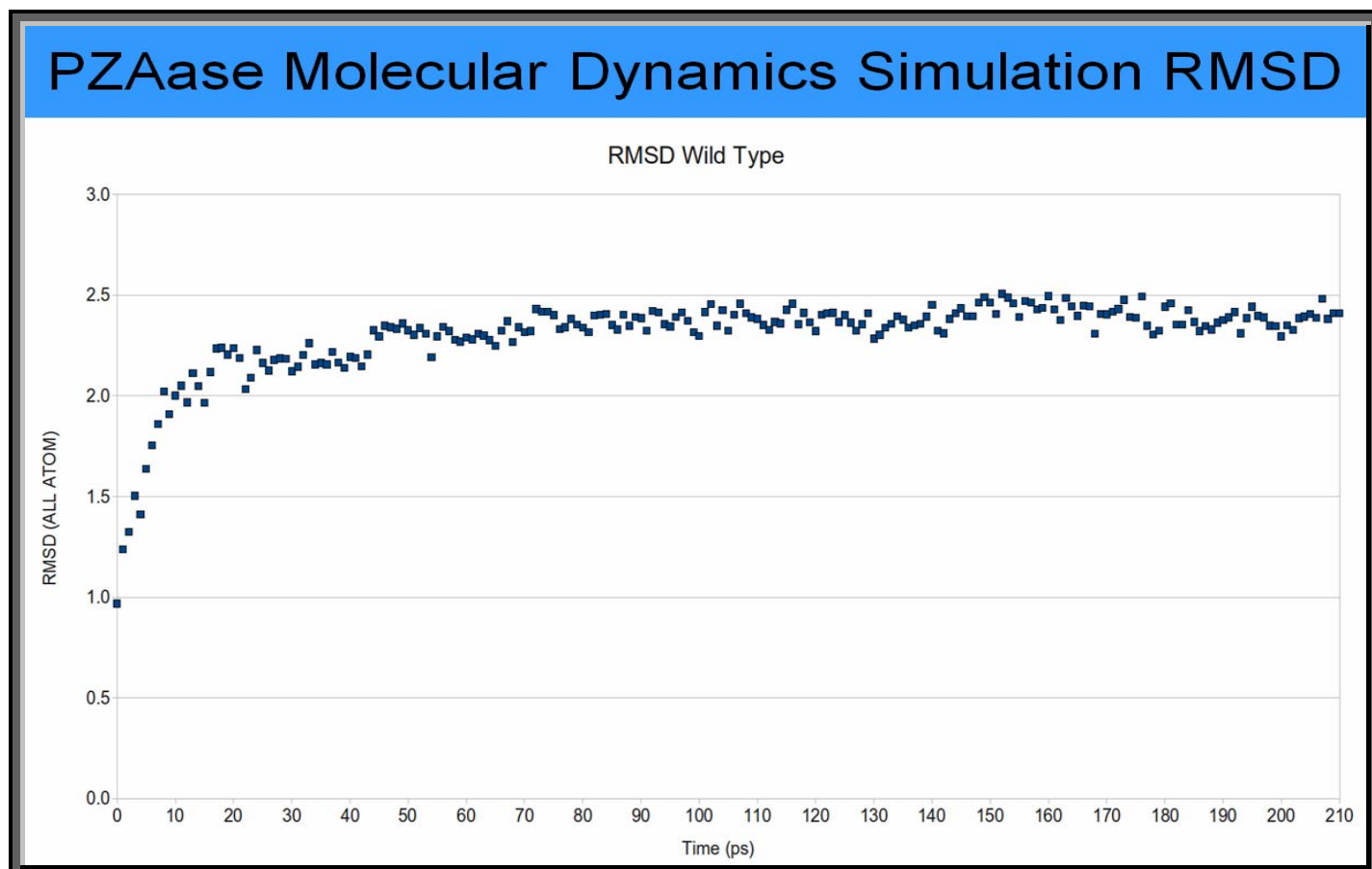


Figure 3.2.6. RMSD of the MD simulation of Pyrazinamidase from *Mycobacterium tuberculosis*.

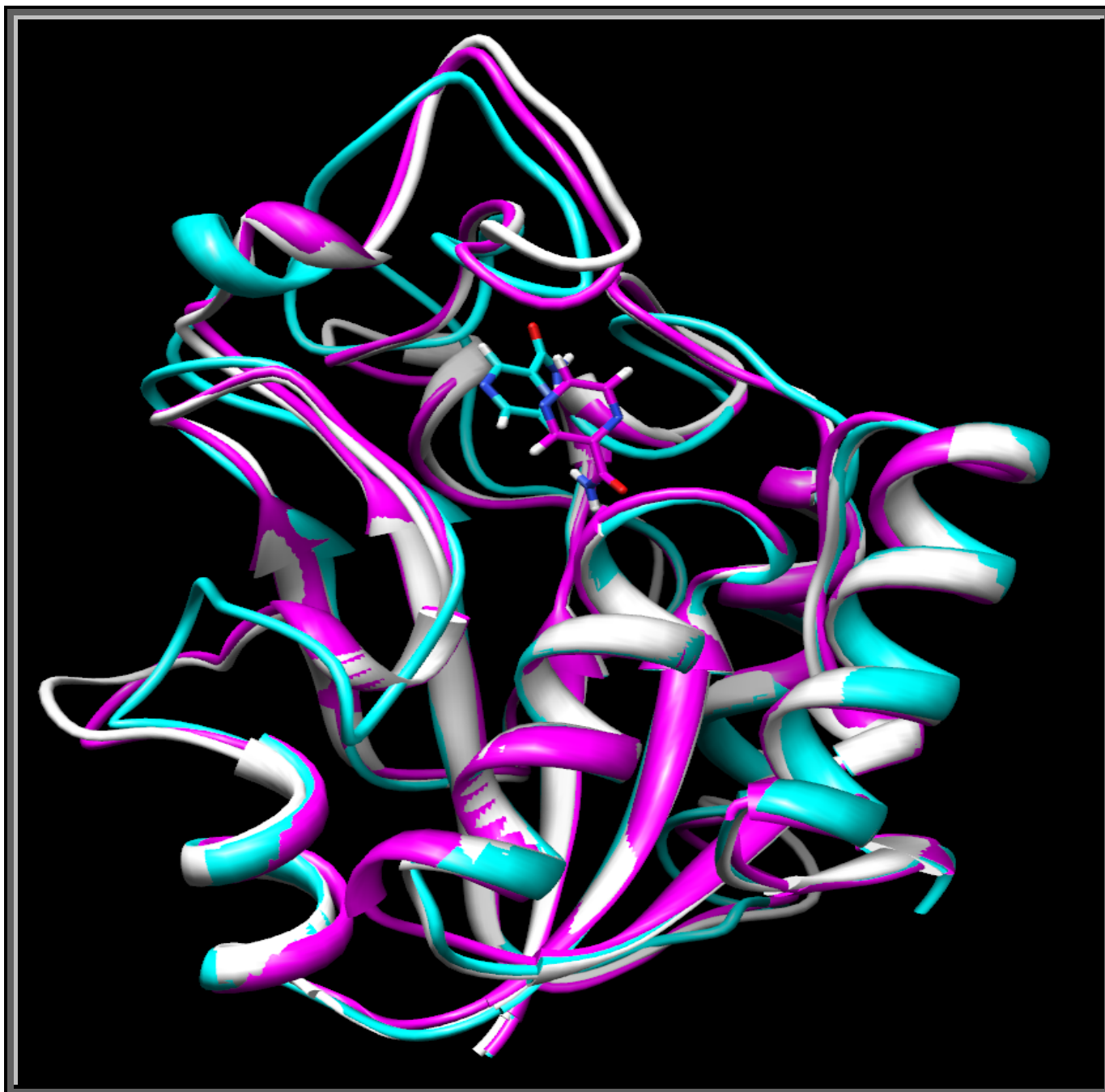


Figure 3.2.7. Differences between Pyrazinamidase from *Mycobacterium tuberculosis* in several cases. Crystal (white), minimized structure (pink), after MD (green).

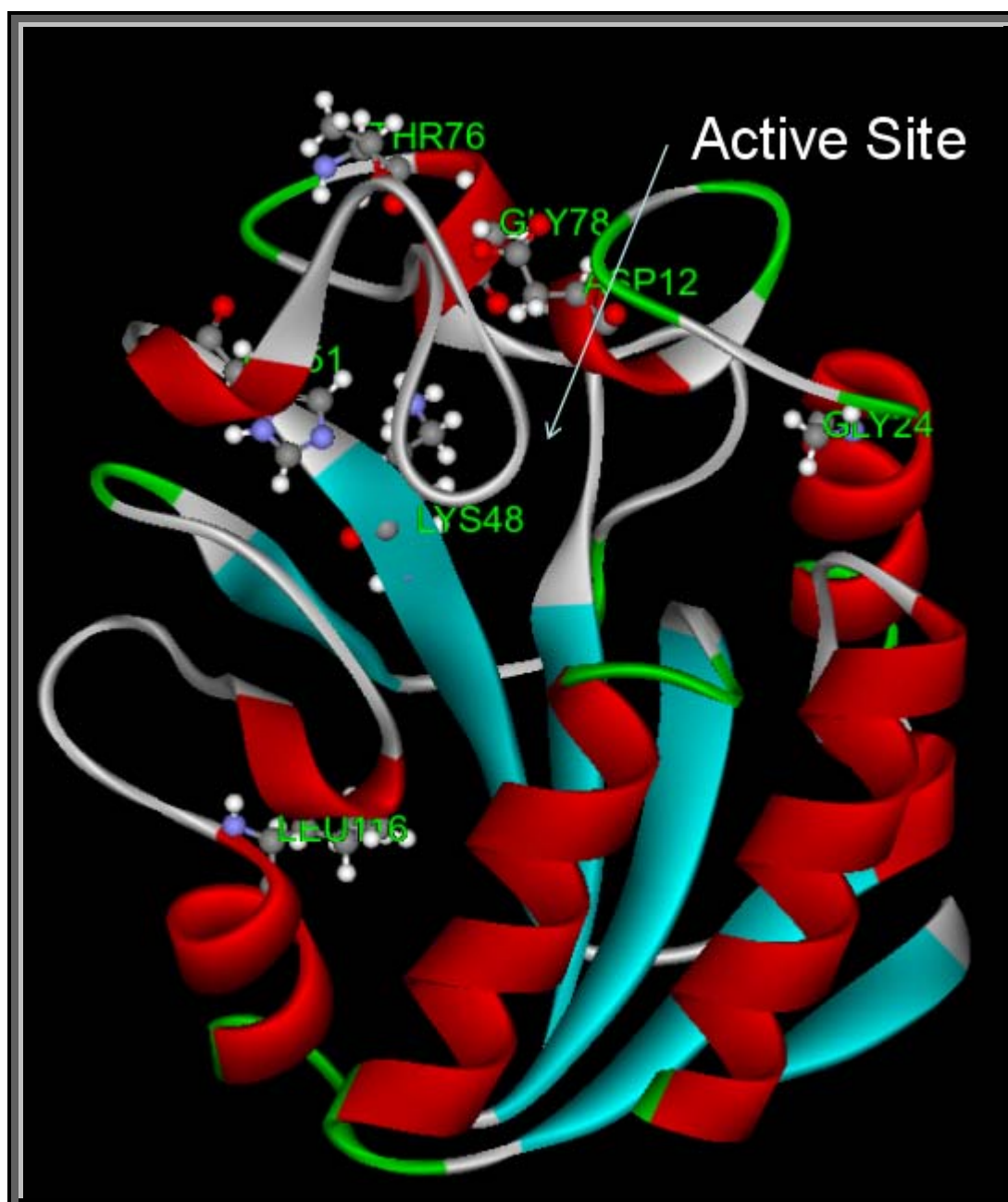


Figure 3.2.8. Distribution of the single-point mutations in the structure of Pyrazinamidase from *Mycobacterium tuberculosis*. D12A, D12G, K48T and H51R are close to the binding site. In contrast, G78C, T76P, G24D and L116P are far from the active site.

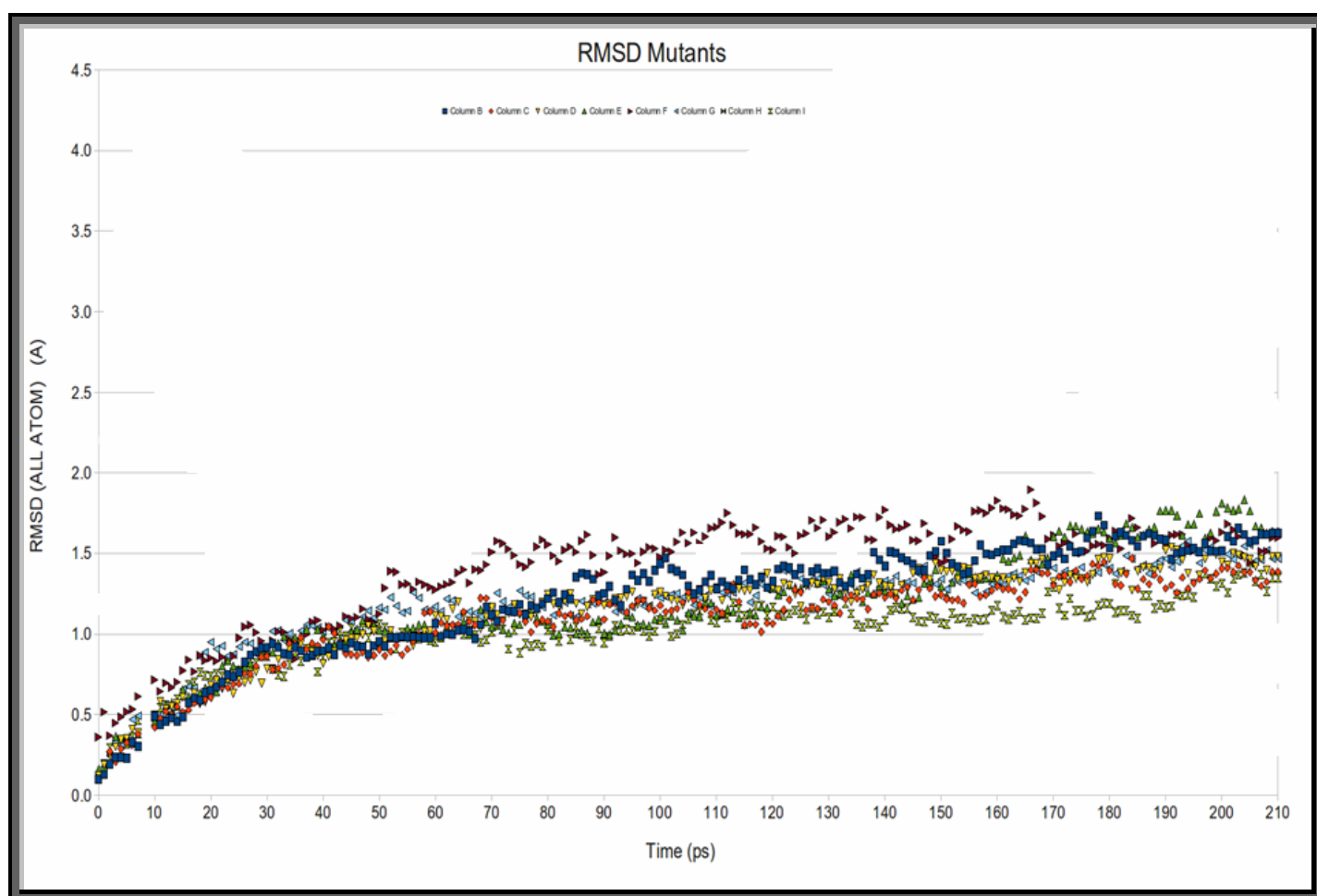


Figure 3.2.9. RMSD of the MD simulation of mutants of Pyrazinamidase from *Mycobacterium tuberculosis*.

Chapter 4

Protein-Ligand Binding Free Energy Calculations

4.1 Objective

The differences in the values of K_M , Michaelis constant, for *Mycobacterium tuberculosis* and some mutants will be correlated with the theoretical value of binding free-energy for each protein-ligand complex. Molecular modeling studies will be carried out to rationalize the differences or lack of differences in the K_M values for each mutant with respect to the wild type Pyrazinamidase considering the influence of each single-point mutation in the mutant Pyrazinamidases.

4.2 Methodology

Traditionally, protein-ligand binding free energy calculations have been performed using molecular docking simulations. Briefly explained, given the structure of a protein which is kept rigid, the ligand will undergo several changes in its position, rotation and conformation inside the binding cavity of the protein so as to optimize a score function which measure directly or indirectly the interaction between the ligand and the protein. Since this score function is supposed to have its maximum value when the conformation with the lowest protein-ligand binding free energy is reached, the value of the score function is converted into a theoretical value of protein-ligand binding free energy. This conversion is done mainly using experimental values of protein-ligand binding free energy as reference.

In this study, we use a software called SievGene which is part of the myPresto suite (Medicinally Yielding Protein Engineering Simulator). However, we are just going to use the software for, given a specific binding pose of the protein-ligand complex, predicting the protein-ligand binding free energy.

The Sievgen score calculates the protein-ligand binding free energy according to the following equation :

$$\Delta G = C_{\text{rot}} \cdot N_{\text{rot}} + C_{\text{ASA}} \cdot E_{\text{ASA}} + C_{\text{vdW}} \cdot E_{\text{vdW}} + C_{\text{ele}} \cdot E_{\text{ele}} + C_{\text{hyd}} \cdot E_{\text{hyd}} + C_{\text{intra-vdW}} \cdot E_{\text{intra-vdW}}$$

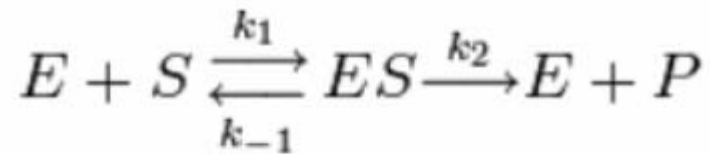
The description of each term is as follows :

- Nrot = number of rotatable bonds
- Easa = hydrophobic energy due to the accessible surface area
- EvdW = van der Walls energy
- Eele = electrostatic energy
- Ehyd = hydrogen bond energy
- Eintra-vdW = intramolecular van der Walls energy of the ligand

The C_i values before each of the previous mentioned terms are coefficients that were optimized after correlating the calculated values of binding free energy with experimental ones. They were estimated by an empirical free energy function that was calibrated by using a set of 50 structurally known protein-ligand complexes with experimentally determined binding constants. [Fukunishi]

Pocket-finder is used to define the probe points of the protein binding site so that the energy grid could be evaluated.

kM values for the wild-type and mutants of Pyrazinamidase will be used as relative measure of substrate affinity for each protein-ligand complex. The calculations will be done for each one of the 5 last saved conformations of the wild-type and mutants since it is when most of them have already reached equilibrium.



$$K_M = \frac{k_{-1} + k_2}{k_1}$$

if $k_2 \ll k_{-1}$

$$K_M \approx \frac{k_{-1}}{k_1} = \frac{[E][S]}{[ES]} = K_d$$

in an equilibrium :

$$\Delta G \approx -R \cdot T \cdot \ln(K_{eq})$$

$$\Delta G \approx R \cdot T \cdot \ln(K_d)$$

$$\Delta G \approx \text{constant} \cdot \ln(K_M)$$

4.3 Results

The values of the binding free energy for each one of the complex got in the previous chapter are summarized in **Table 4.1**.

	nWT	D12A	D12G	L116P	T76P	G24D	G78C	H51R	K48T
21 ps	3.87	5.00	4.45	4.41	4.48	4.76	4.51	5.32	4.33
42 ps	3.33	5.13	4.63	4.44	4.49	4.58	4.47	5.27	4.35
63 ps	4.22	4.46	4.39	4.62	4.40	4.84	4.35	5.17	4.58
84 ps	4.45	4.16	4.22	4.53	4.46	4.48	4.44	5.45	4.55
105 ps	4.94	4.49	4.25	4.40	4.62	4.86	4.38	5.49	4.35
126 ps	5.08	4.63	4.46	4.57	4.38	4.46	4.15	5.05	4.39
147 ps	5.46	4.67	4.42	4.70	4.75	3.90	4.58	5.01	4.53
168 ps	5.01	4.84	4.33	4.64	4.55	4.83	4.42	5.14	4.34
189 ps	5.49	4.70	4.66	5.04	4.84	4.76	4.60	4.97	4.24
210 ps	4.98	4.62	4.27	4.65	4.92	4.77	4.45	5.13	4.24
MEAN	5.16	4.66	4.40	4.67	4.68	4.60	4.43	5.13	4.35
kM	1.24	0.99	0.55	1.56	0.31	0.42	1.07	1.41	0.44
ln(kM)	0.22	-0.01	-0.60	0.44	-1.17	-0.87	0.07	0.34	-0.82

Table 4.1. Values of $-\Delta G$ for each strain at the respective time in which the protein coordinates were saved and then cooled down.

In **Table 4.1**, MEAN is the average of binding free energy from 105ps to 210ps. As can be seen in **Figure 3.2.8**, the structures have already reached equilibrium. We decided to plot the values of $-\Delta G$ versus the $\ln(K_M)$. As can be appreciated there is just correlation in the **Figure 4.2**, and **Figure 4.3**. These mutations are the ones that are close to the binding site.

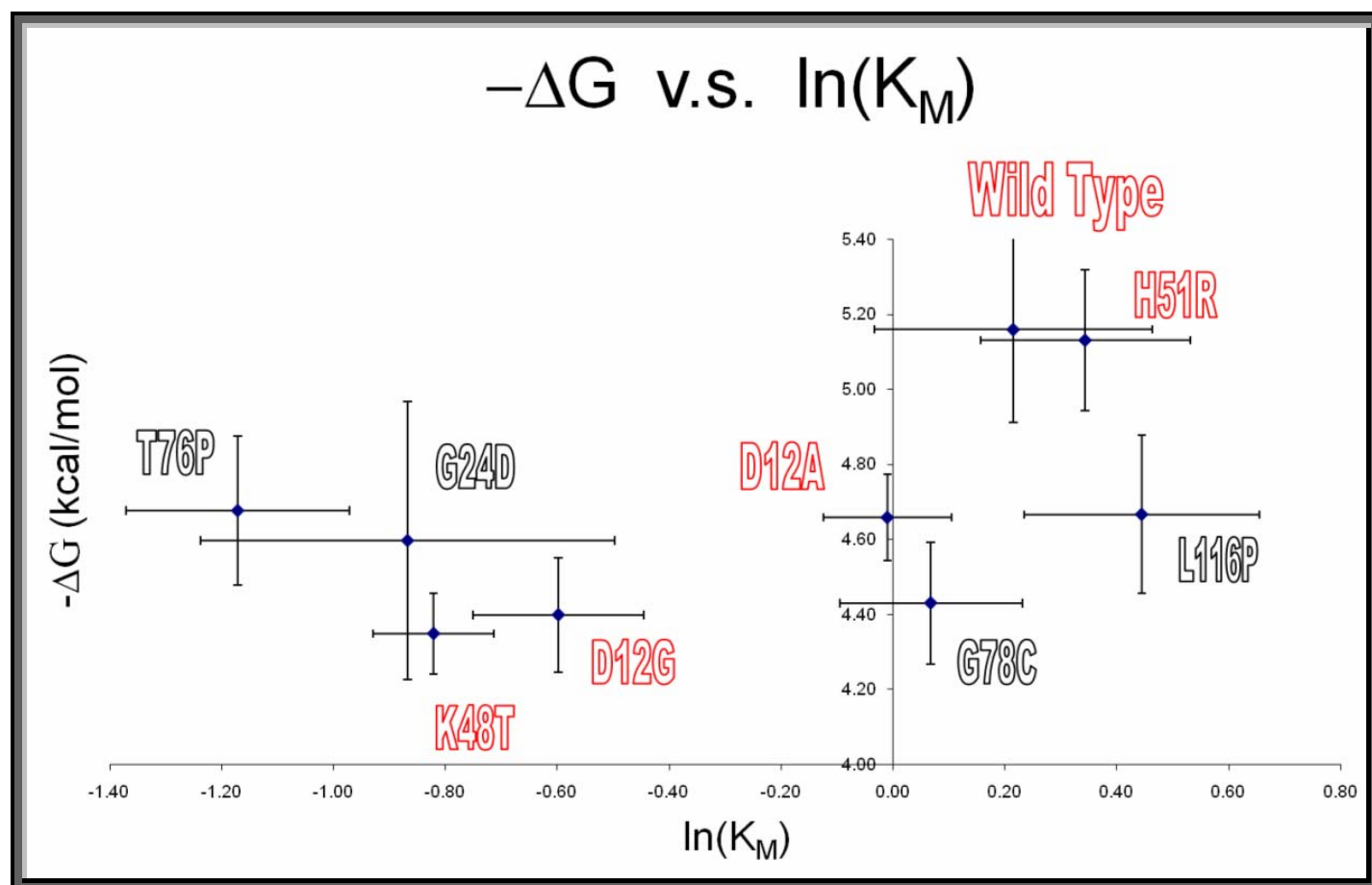


Figure 4.1.

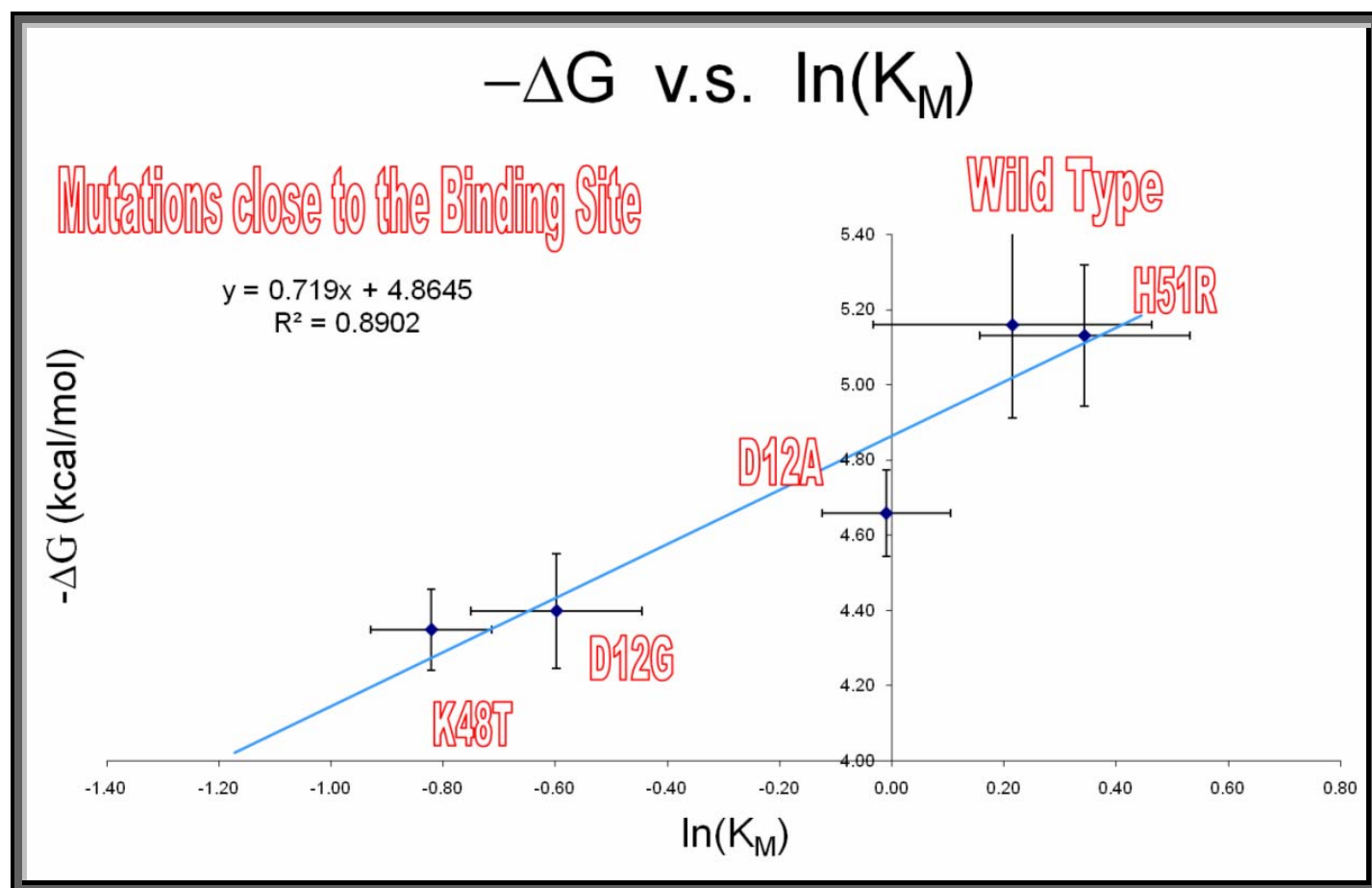


Figure 4.2.

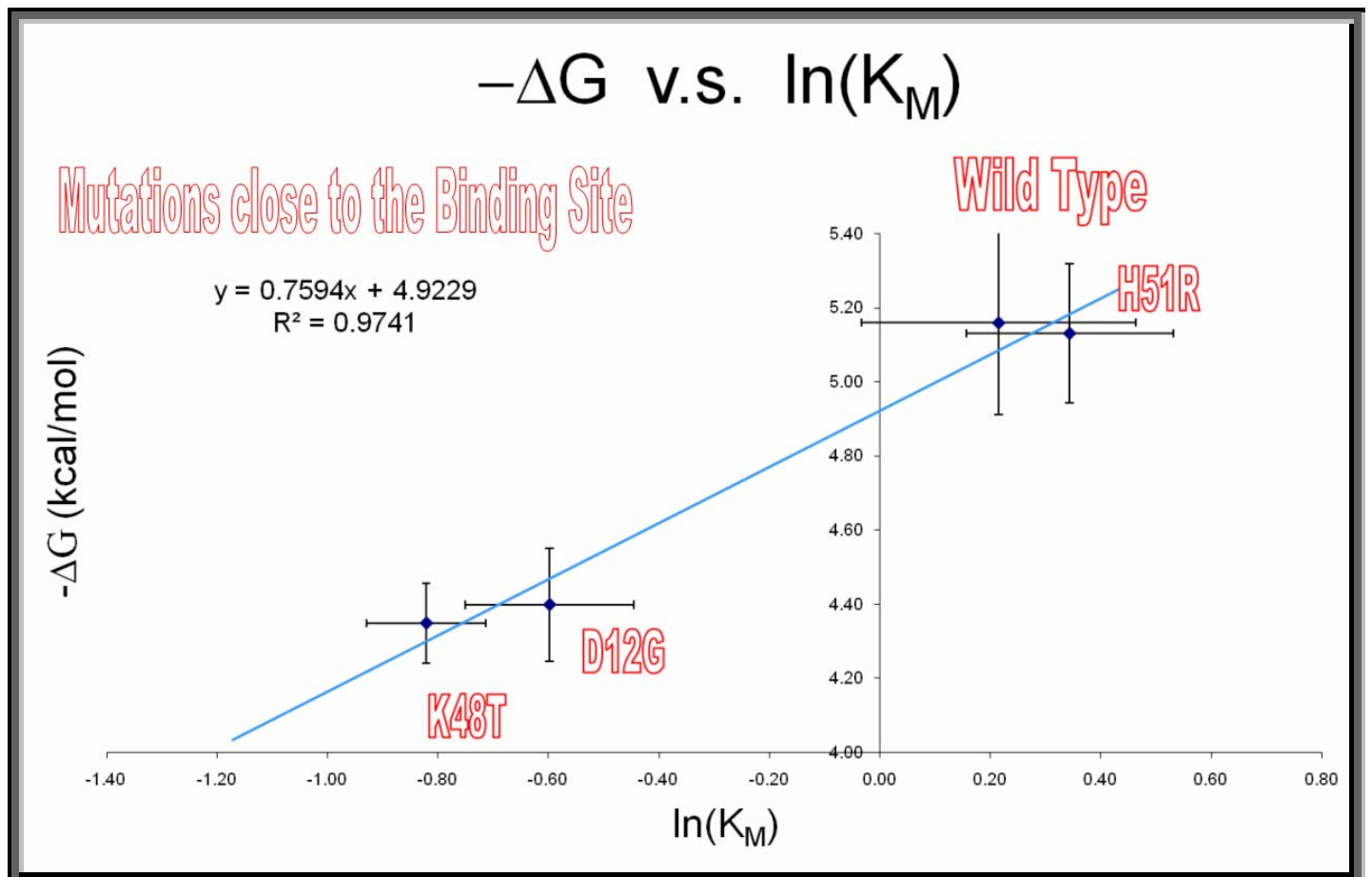


Figure 4.3.

Chapter 5

Conclusions

Sampling properly the conformational space of a protein is a very expensive task. Not only because there is no certainty that the global minimum of energy was reached, but also because the global minimum for a specific force-field can not correspond to the global minimum for another force-field. In general, conformational samplings simulate about 10ns.

In this work, using the simulated annealing approach, we managed to get acceptable results by just simulating for about 200 ps. Explicit water was not employed, reducing drastically the computing time. The influence of water was simulated as a parameter that weakens the Coulomb interaction proportionally to the distance of two 2 atoms.

The objective of this study is to study the influence on single-point mutations in the pyrazinamide-Pyrazinamidase complex. A correlation was found between a experimental value, the Michaelis constant, and a theoretical value, the pyrazinamide-Pyrazinamidase binding free energy. Pyrazinamide interacts directly with the amino acids that form the binding cavity, and not with the amino acids that are far from there. This might be the reason why the previous correlation could not be applied to the mutations that were far from the binding site.

An approach to study the role of those mutations might require explicit water, longer simulation times, as well as a re-parametrization of the topology of the metallic ion and the atoms that coordinate it.

Although now we have a tool to predict the experimental values of K_m for new strains of *Mycobacterium tuberculosis* (as long as the mutation is close to the binding site), both the binding free-energy and k_M do not correlate with the yes/no phenotypic category of pyrazinamide resistance. Part of this failure might be to the reason that pyrazinamide it is not an inhibitor, but rather a pro-drug. It must to be metabolized by Pyrazinamidase in order to become the substance that will eventually kill *Mycobacterium tuberculosis*. So, if the binding free energy is very large, the enzymatic reaction might not occur to to the rigidity of pyrazinamide inside the binding cavity of Pyrazinamidase. In the same way, if the binding free-energy is very low, pyrazinamide might not remain enough time inside the binding cavity of of Pyrazinamidase, and the reaction can also no occur.

Right now, new protein candidates for inhibition are being searched in *Mycobacterium tuberculosis*, but still, research in the Nicotinamidase/Pyrazinamidase protein continues.

Bibliography

1. Ando, H., *et al.* Pyrazinamide resistance in multidrug-resistant *Mycobacterium tuberculosis* isolates in Japan. *Clin Microbiol Infect* **16**, 1164-1168 (2010).
2. Boshoff, H.I., *et al.* Biosynthesis and recycling of nicotinamide cofactors in *Mycobacterium tuberculosis*. An essential role for NAD in nonreplicating bacilli. *J Biol Chem* **283**, 19329-19341 (2008).
3. Du, X., *et al.* Crystal structure and mechanism of catalysis of a pyrazinamidase from *Pyrococcus horikoshii*. *Biochemistry* **40**, 14166-14172 (2001).
4. Zhang, H., *et al.* Characterization of *Mycobacterium tuberculosis* nicotinamidase/pyrazinamidase. *FEBS J* **275**, 753-762 (2008).
5. Zimic, M., Sheen, P., Quiliano, M., Gutierrez, A. & Gilman, R.H. Peruvian and globally reported amino acid substitutions on the *Mycobacterium tuberculosis* pyrazinamidase suggest a conserved pattern of mutations associated to pyrazinamide resistance. *Infect Genet Evol* **10**, 346-349 (2010).
6. Sheen, P., *et al.* Effect of pyrazinamidase activity on pyrazinamide resistance in *Mycobacterium tuberculosis*. *Tuberculosis (Edinb)* **89**, 109-113 (2009).
7. Fyfe, P.K., Rao, V.A., Zemla, A., Cameron, S. & Hunter, W.N. Specificity and mechanism of *Acinetobacter baumannii* nicotinamidase: implications for activation of the front-line tuberculosis drug pyrazinamide. *Angew Chem Int Ed Engl* **48**, 9176-9179 (2009).
8. Unissa, A.N., Selvakumar, N. & Hassan, S. Insight to pyrazinamide resistance in *Mycobacterium tuberculosis* by molecular docking. *Bioinformation* **4**, 24-29 (2010).
9. French, J.B., *et al.* Characterization of nicotinamidases: steady state kinetic parameters, classwide inhibition by nicotinaldehydes, and catalytic mechanism. *Biochemistry* **49**, 10421-10439 (2010).
10. Petrella, S., *et al.* Crystal structure of the pyrazinamidase of *Mycobacterium tuberculosis*: insights into natural and acquired resistance to pyrazinamide. *PLoS One* **6**, e15785 (2011).