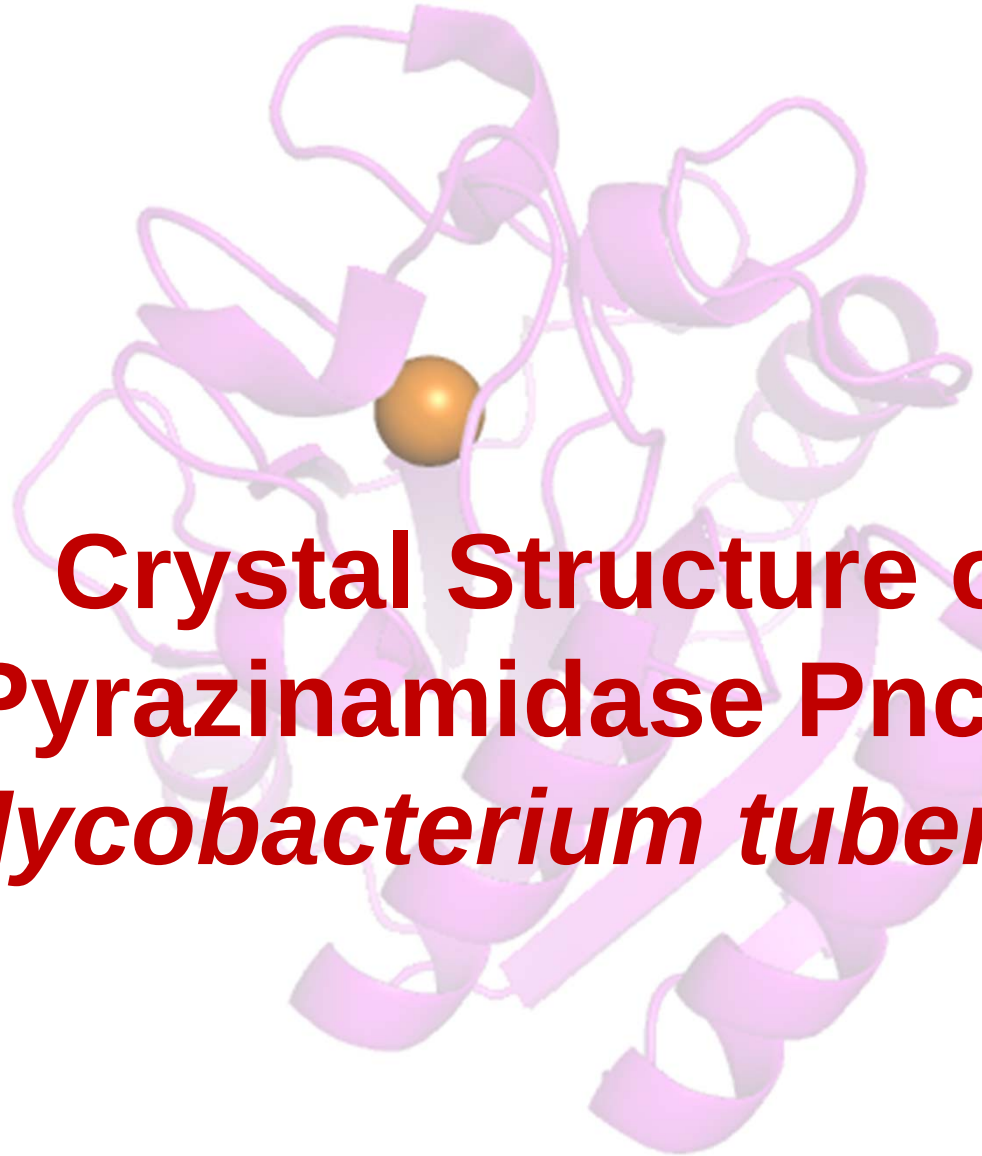

Crystal Structure of the Pyrazinamidase PncA from *Mycobacterium tuberculosis*

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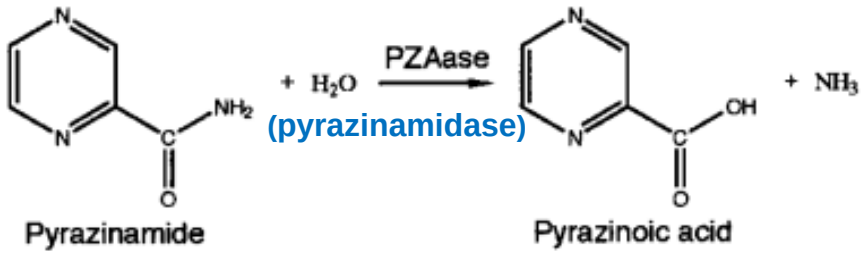
BACKGROUND

- **PZA is a prodrogue:** the *M.tuberculosis* nicotinamidase encoded by the *pncA* gene is a pyrazinamidase which converts the prodrug pyrazinamide (PZA) to pyrazinoic acid which is active against *Mycobacterium tuberculosis*.

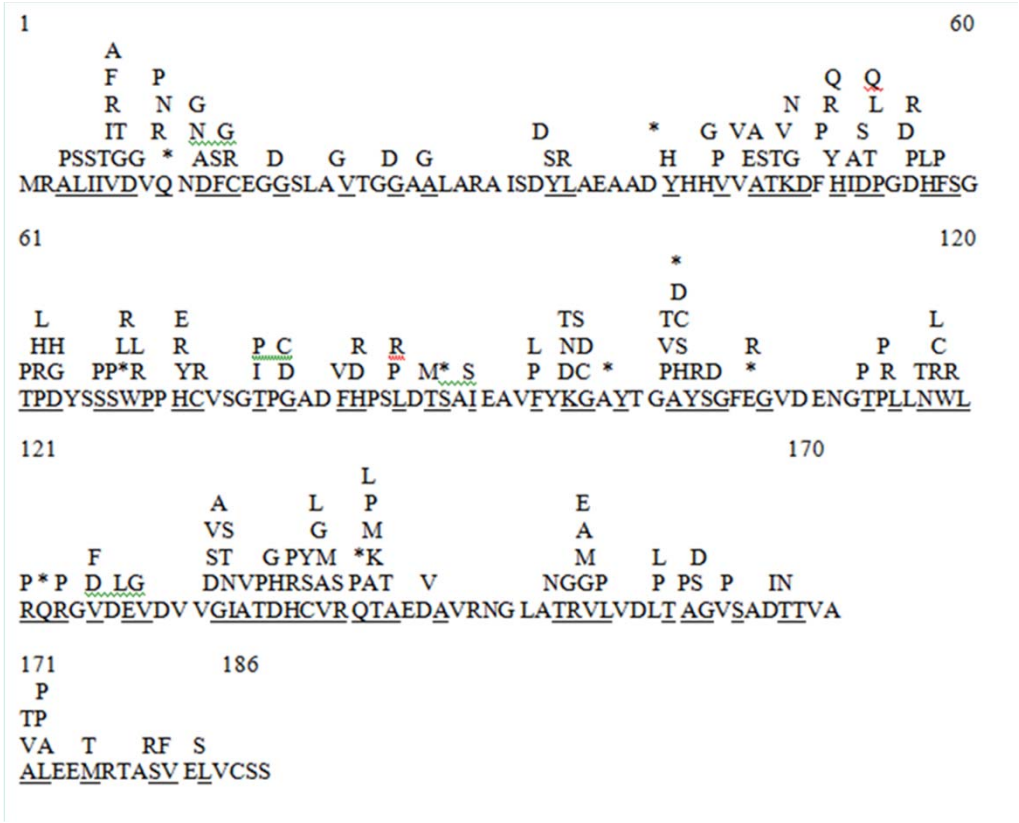


- **The *M. tuberculosis* pyrazinamidase PncA :**
  - Cysteine amido hydrolase
  - Also active against nicotinamide
  - Monomeric enzyme of 186 amino acids (19,6 kDa)

Characterization of New Mutations in Pyrazinamide-Resistant Strains of Mycobacterium tuberculosis and Identification of Conserved Regions Important for the Catalytic Activity of the Pyrazinamidase PncA (Lemaitre N et al., AAC 1999)

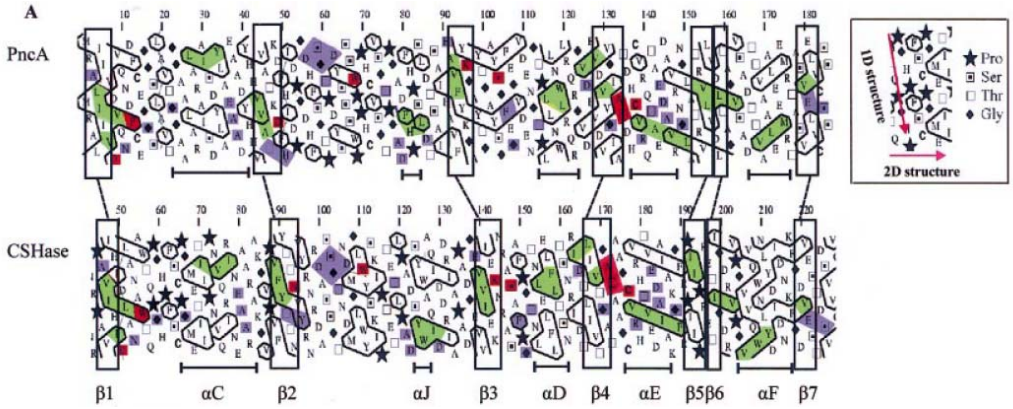
Although the mutations found in *pncA* are scattered along the gene, 54% of the amino acid substitutions reported in the literature occurred preferentially in 3 distinct regions (positions 3 to 17, 45 to 76, and 132 to 142

these regions could be structurally and/or catalytically important for the activity of the enzyme against PZA.

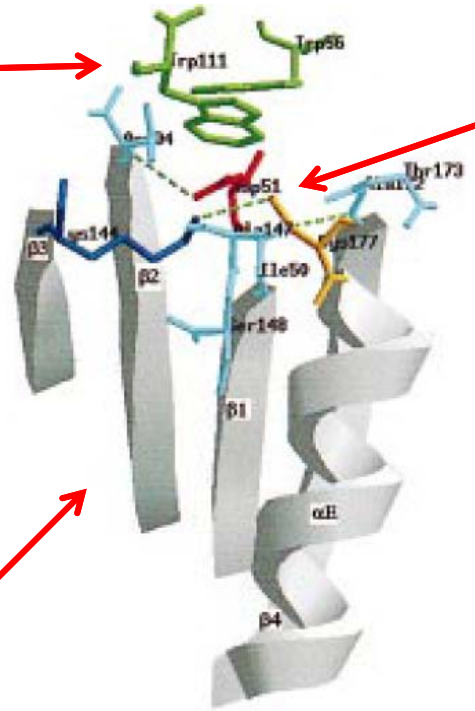


Study of the structure–activity relationships for the pyrazinamidase (PncA) from Mycobacterium tuberculosis (Lemaitre N. et al., Biochem J 2001)

Model of the 3-D structure of the wild-type PncA protein constructed by homology modelling from an optimized alignment obtained using the 2D HCA method with the CSHase (N-carbamoylsarcosine amidohydrolase) from Arthrobacter sp as template.



Tryptophane over the biding site

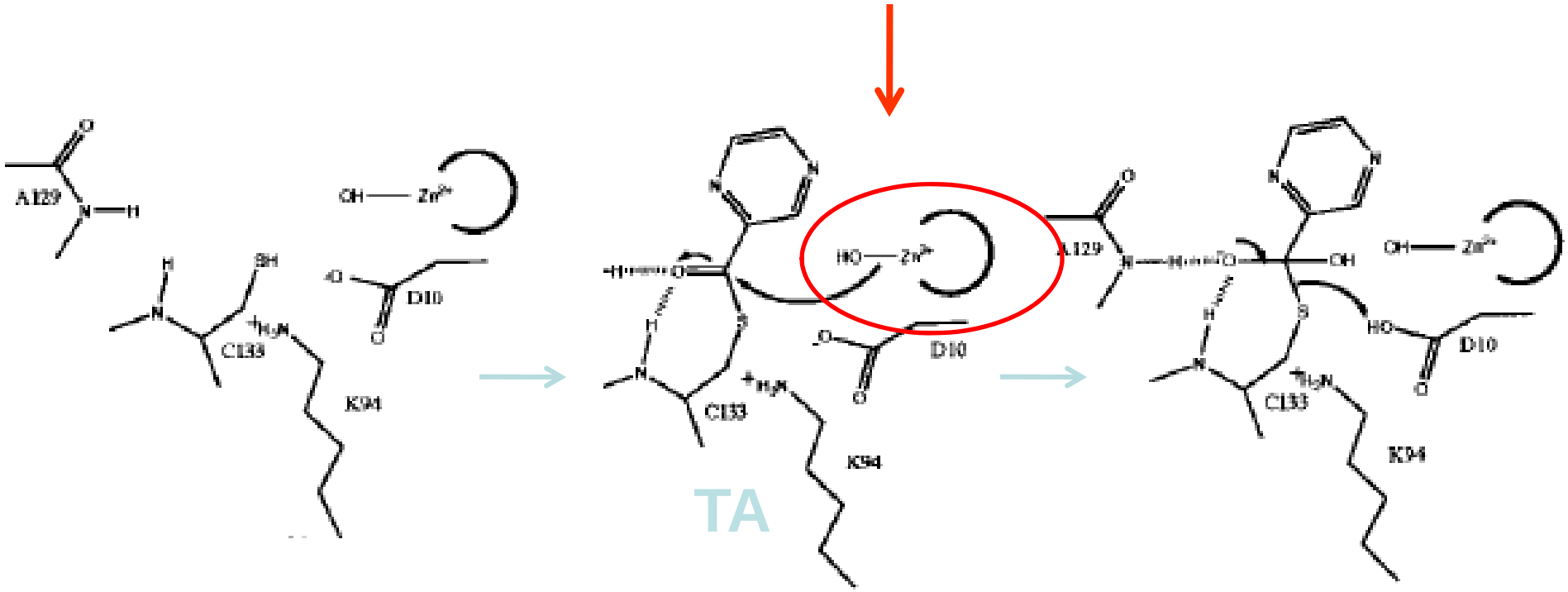


Catalytic triad : Cys-Asp-Lys

Rossmann motif

Crystal Structure and Mechanism of Catalysis of a Pyrazinamidase from Pyrococcus horikoshii (Du X et al., Biochemistry 2001)

- Catalytic triad (D10, K94, and C133)
- A metal ion-binding site in complex with **Zn^{2+}** (but crystals growth with 5 mM Z_nCl_2)
- **Proposed mechanism: catalytic role for Zn^2: the Zn^{2+} bound water molecule would attack the carbonyl-carbon of the thioester bond in the acylenzyme**

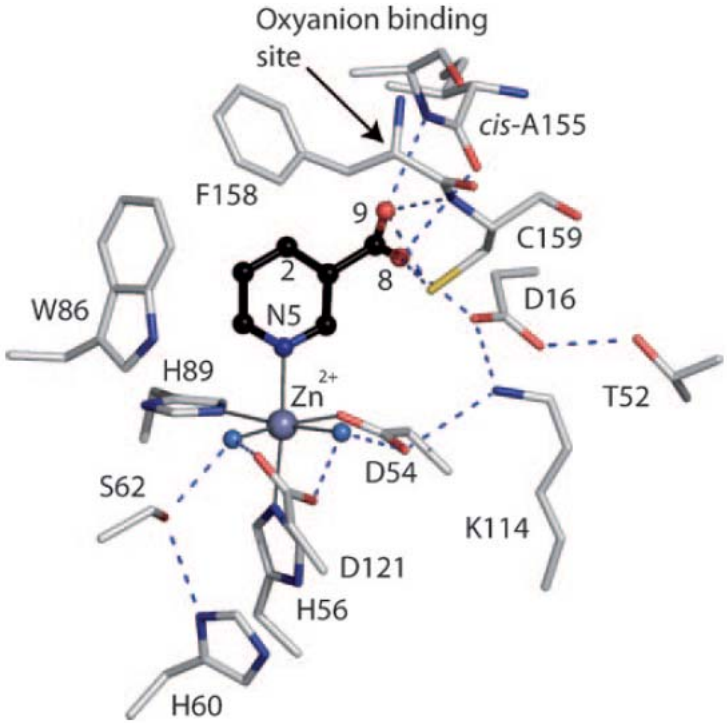


TA

Specificity and Mechanism of Acinetobacter baumanii Nicotinamidase: Implications for Activation of the Front-Line Tuberculosis Drug Pyrazinamide (Fyfe et al, Angew. Chem. Int. Ed. 2009)

High-resolution crystal structures of *Acinetobacter baumanii* PncA complexed with nicotinic acid

-Inductively coupled plasma-atomic emission spectrometry (ICP-OES) identified that AbPncA contained Fe^{2+} and Zn^{2+} ions in a 1:1 ratio
-Anomalous dispersion measurements were consistent with a higher occupancy of Zn^{2+} at the active site
-Coordination of Zn^{2+} ion involves Asp54, His56, and His89, two water molecules and nicotinic acid N5
- Nicotinic acid is tethered to the cation and positioned between hydrophobic residues



Conclusion:
-Zn^{2+} plays a structural role provided by octahedral coordination
- contributes to the precise placement of the substrate)

Characterization of *Mycobacterium tuberculosis* nicotinamidase/pyrazinamidase (H. Zang et al, FEBS Journal 2008)

- Inductively coupled plasma-optical emission spectrometry (ICP-OES) revealed that the enzyme was an Fe2+/ Mn2+ -containing protein with a molar ratio of manganese to iron of 1 : 1
  - When the metal ion was removed from PncA, the hydrolytic activity was completely lost. The activity could be restored by Mn^{2+} and Fe^{2+}, but not by Zn^{2+}.

Kinetics and Inhibition of Nicotinamidase from *Mycobacterium tuberculosis* (Derrick R. Seiner et al, Biochemistry 2010)

Metal ion content of PncA:

-EPR (electron paramagnetic resonance) spectroscopy indicated the presence of Mn^{2+} bound to the enzyme

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PLoS one

Crystal Structure of the Pyrazinamidase of *Mycobacterium tuberculosis*: Insights into Natural and Acquired Resistance to Pyrazinamide

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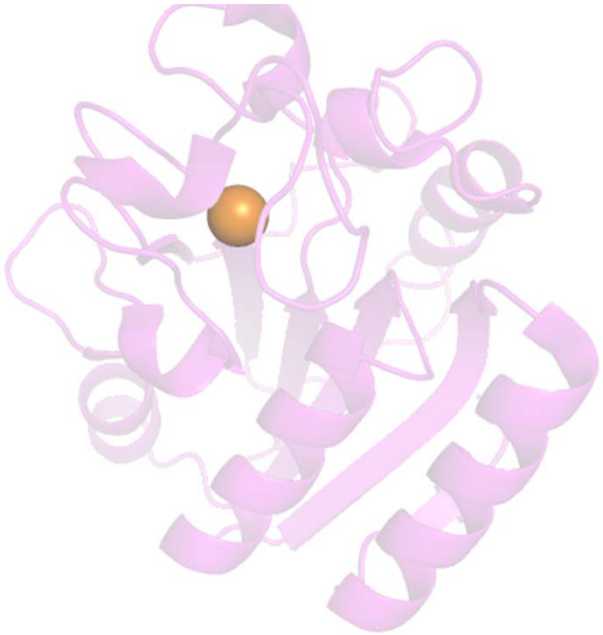
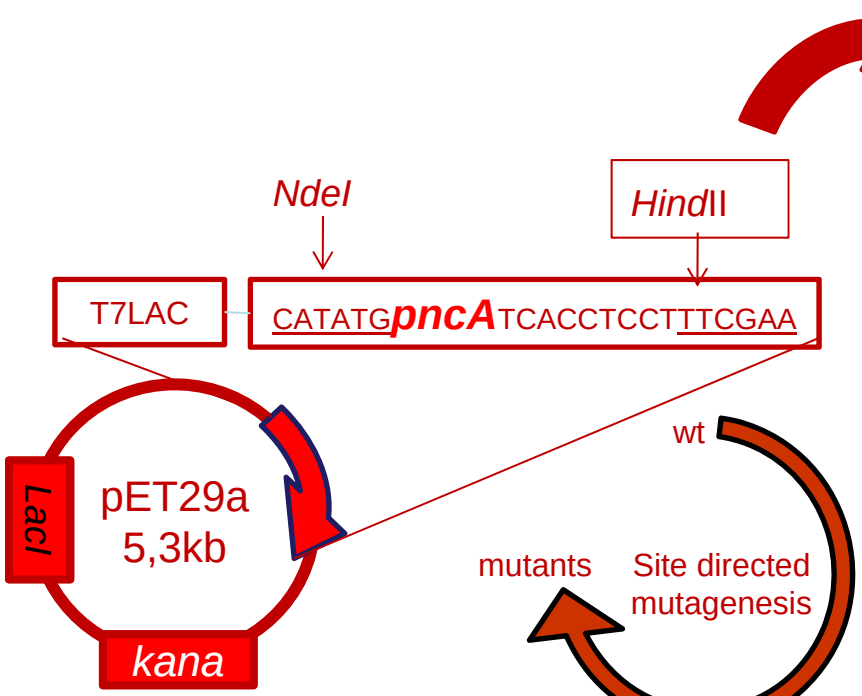


TOOLS and METHODOLOGY



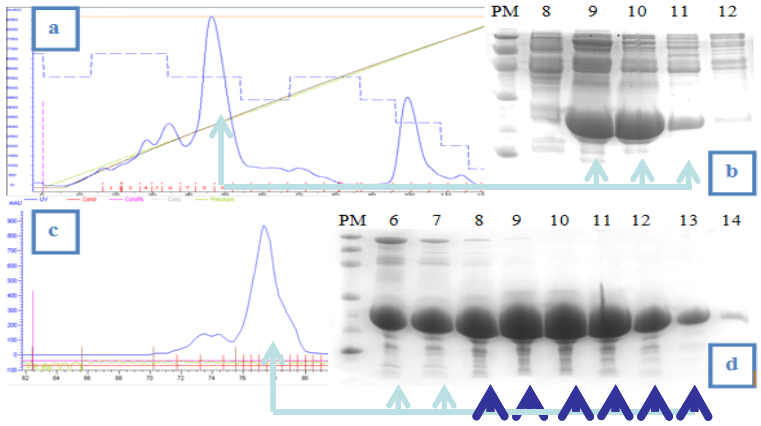
Transformation of pET29a-*pncA* in *E.coli* BL21

Induction by IPTG 0,4mM of 4 x 250ml of culture

Purification :

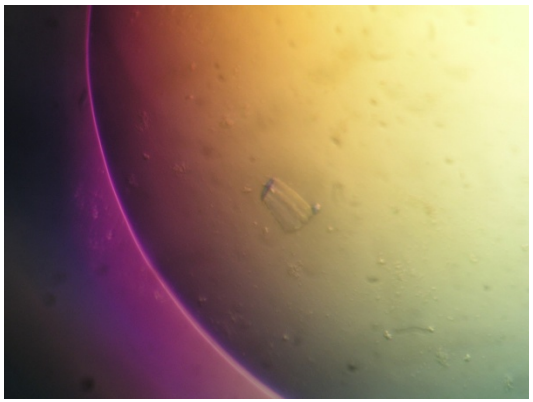
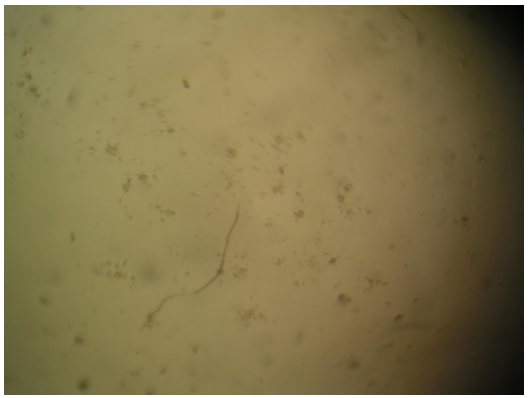
- Q sepharose Fast Flow in Bis-Tris pH 5.9, eluted by NaCl gradient
- Gel filtration on Superdex 75

Protein concentrated to 20 mg/ml



Crystallization and structure determination

- PncA concentration = 20 mg/ml
- Hampton Research® Cristal Screen® I et II (98 solutions)
- 30 solutions of MES 0,1M ; $MgSO_4$ =1 to 2M; pH = 6 to 6,9
- PncA crystals were obtained using sitting drops against a mother liquor containing 1.6 M of $MgSO_4$ and 0.1 M of MES at pH 6.6.

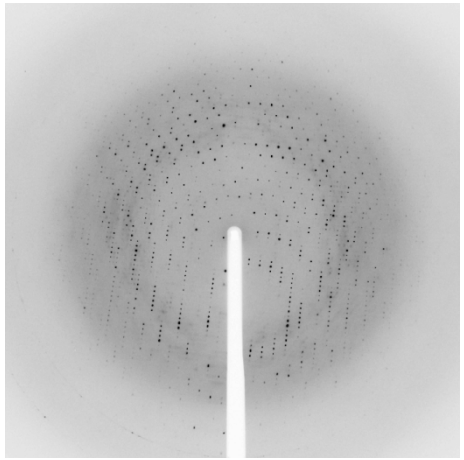



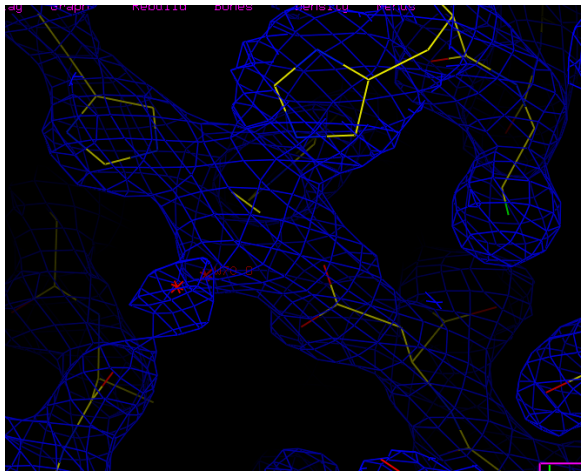

Crystallization and structure determination (2)

Data collection:

- crystals were frozen by a 100 K nitrogen gas stream without cryoprotection
- data collection at the ESRF (European Synchrotron Radiation Facility, Grenoble, France, ID23-2 beamline Micro-focus).
- phasing by molecular replacement with the crystal structure of PncA of *Pyrococcus horikoshii* as search model
- Refinement with CNS and Refmac

	PncA
Space group	$P6_122$
Resolution (Å)	42 - 2.2 (2.32-2.2)
Rcrystal (%)	19.7
Rfree (%)	24





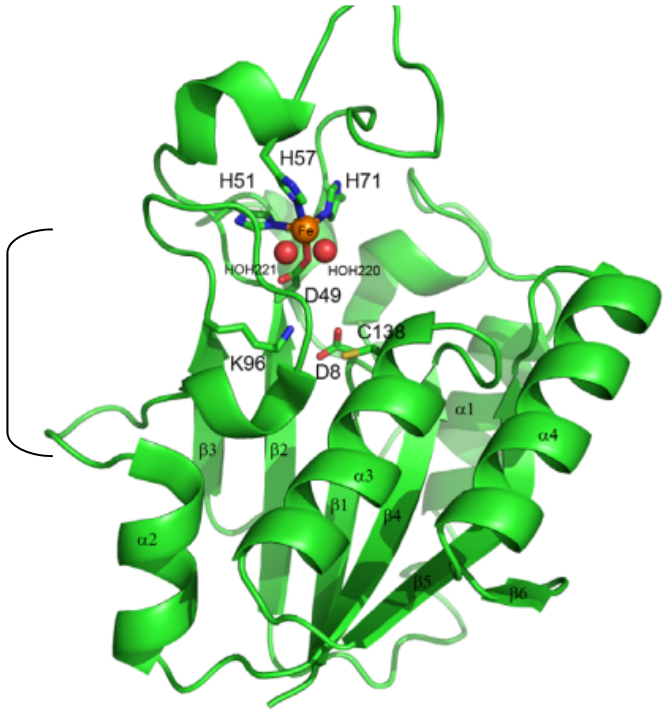
Structure of the PncA of *Mycobacterium tuberculosis*

❖ Rossman fold

6-stranded parallel beta sheet

Metal binding site: Asp49, His51, His57, His71

Catalytic triad: Cys138, Asp8, Lys96

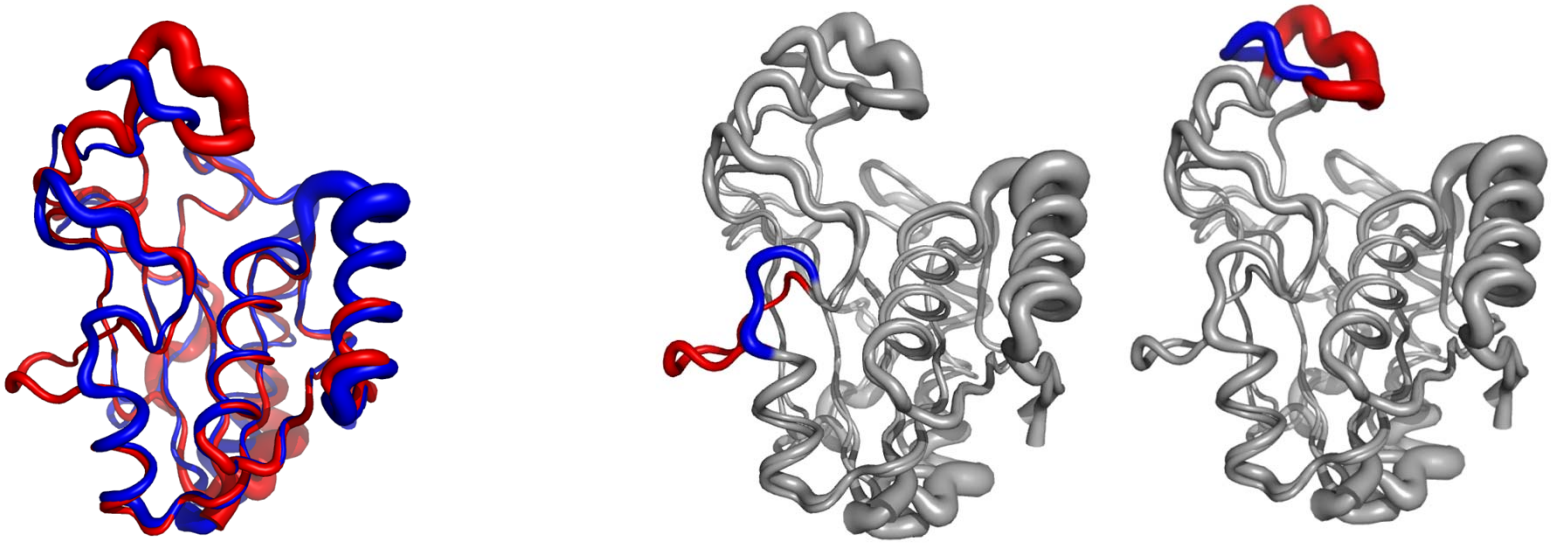


Determination of the metal ion in PncA

- Inductively coupled plasma-mass spectrometry (ICP MS) was used for metal-ion identification
- ICP-MS analysis showed that PncA contained iron at a concentration of 180 mM /260 mM. Low concentrations of zinc and manganese (42 mM and 16 mM, respectively) were also detectable.

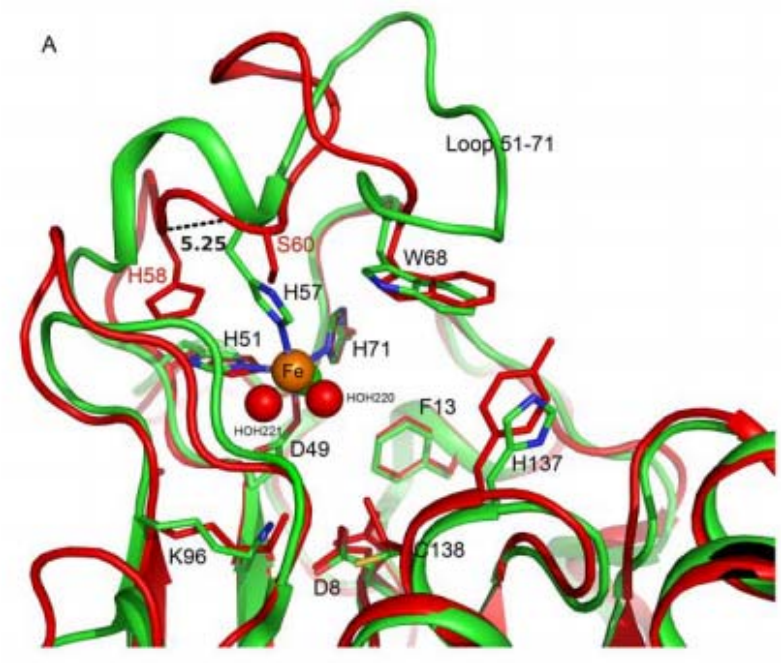
Superposition of the PncA structures

- Structural alignments revealed a high level of similarity between the structure of PncA from *M. tuberculosis* and the two crystal structures of PncA from *P. horikoshii* and *A. baumanii* (rmsds of 0.96 Å and 2.8 Å, respectively).
- Structural differences in 2 regions of PncA :
  - •insertion of a stretch of 5 amino acids, VDENG, between residues Gly108 and Thr114 in MtPncA
  - •loop extending from residues His51 to His71 (local rmsd of 3 to 5 Å)



Ribbon representation of the superimposed polypeptide chains. In red: PncA from *M. tuberculosis*. In blue : nicotinamidase from *P. horikoshii*

Superposition of the PncA structures (2)



In green: *M. tuberculosis*.
In red : *P. horikoshii*

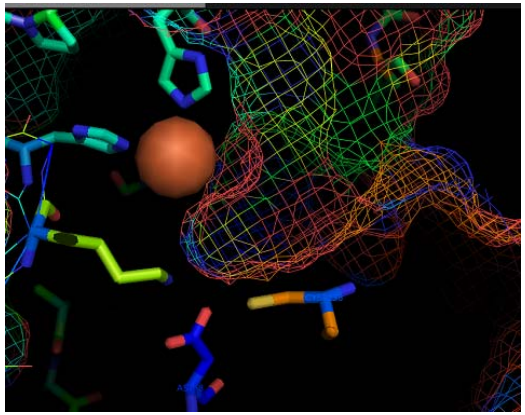
The loop extending from residues His51 to His71 :

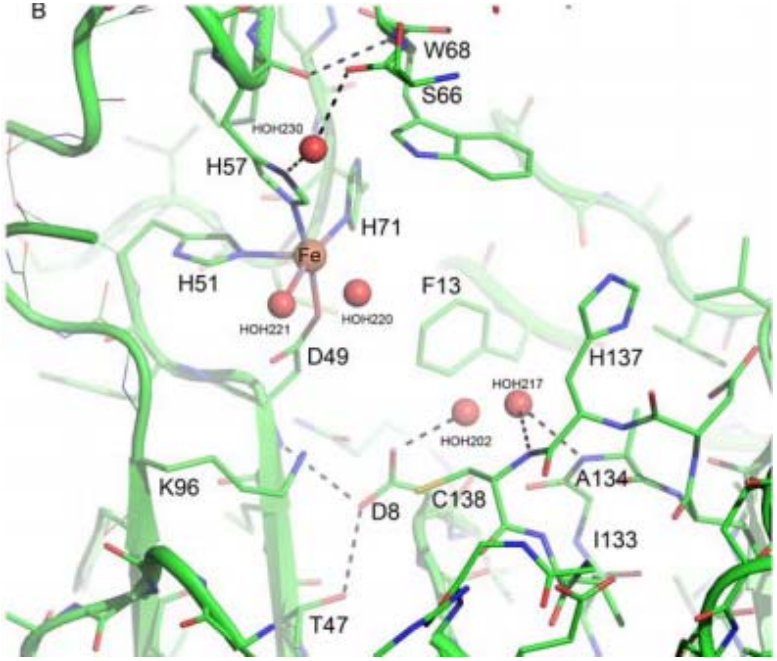
- Lid of the binding cavity
- His51 and His71: hinge of the loop
- Extensive displacements when compared to *P.horikoshii* :
  - *M.tuberculosis*: His57 in axial position, establishes a coordination bond with Fe^{2+} (locks the loop above the binding cavity)
  - Fe^{2+} was coordinated in a distorted tetragonal bipyramidal arrangement by His51, His71 and two water molecules, HOH220 and 221 in equatorial position, and by Asp49 and His57 in axial position
  - *P.horikoshii*: His58 is shifted by >5 Å => cannot participate to the binding of the metal ion
  - His57 is replaced by Asp in *M.bovis* which is a species naturally resistant to PZA.

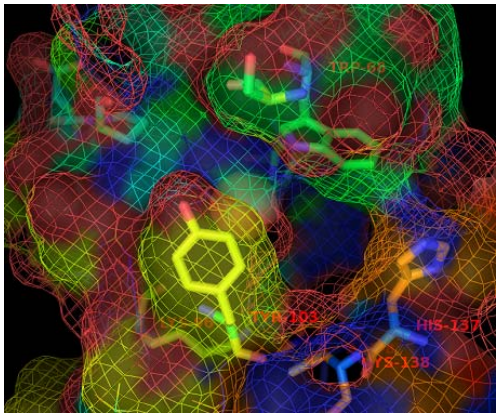
Detailed description of the substrate binding cavity in PncA of *M. tuberculosis*

➢Substrate binding cavity :

- •small cavity of 10 Å deep and 7 Å wide
- •delineated on one side by a catalytic triad made of residues Lys96, Asp8 and Cys138
- •on the opposite side, His51, His57 His71, and Asp49 hold the Fe ion on, leaving room in the middle of the cavity for PZA binding
- •cis-peptide bond between Ile133 and Ala134 → amide nitrogen atoms of Ala134 and Cys138 form an oxyanion hole
- •Trp68, is positioned above the catalytic cleft and is maintained by a conserved H-bond between His57(CO) and Trp68(NH). Trp68 limits, together with Tyr103 and His137, the access to the binding pocket.

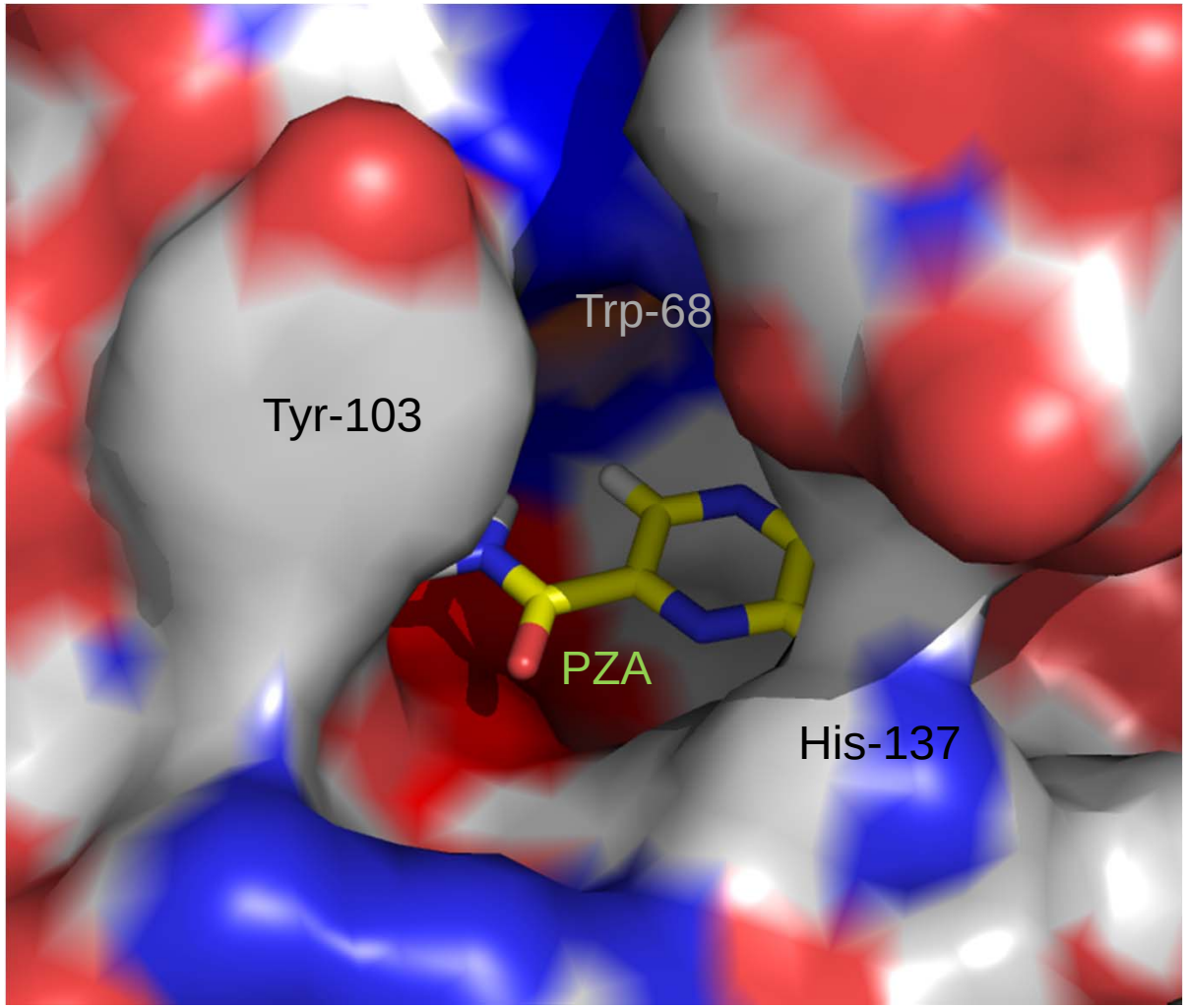






Detailed description of the substrate binding cavity in PncA of *M. tuberculosis* (2)

Entrance of the PZA binding cavity

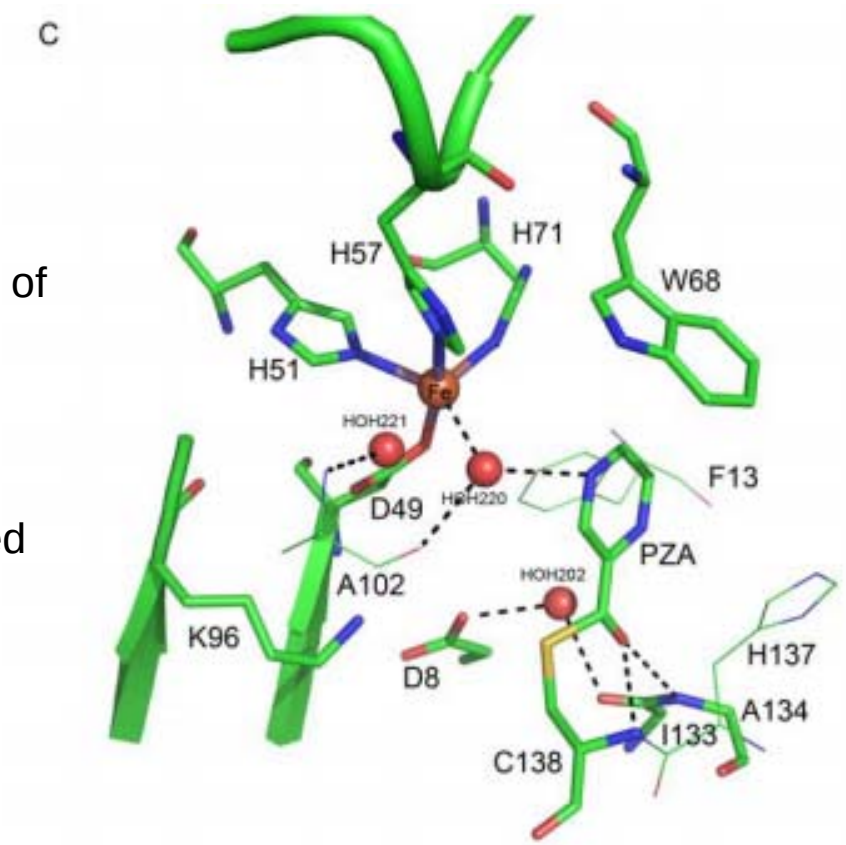


Structure of the acyl-enzyme complex formed between PncA and pyrazinamide

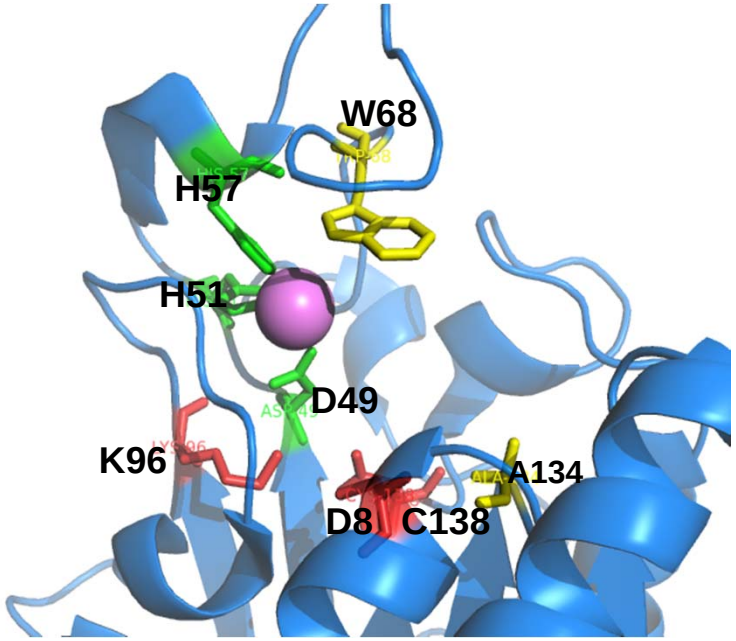
- The carbonyl oxygen atom of PZA is ensconced in the oxyanion hole (between the NH-main chain groups of Cys138 and Ala134)

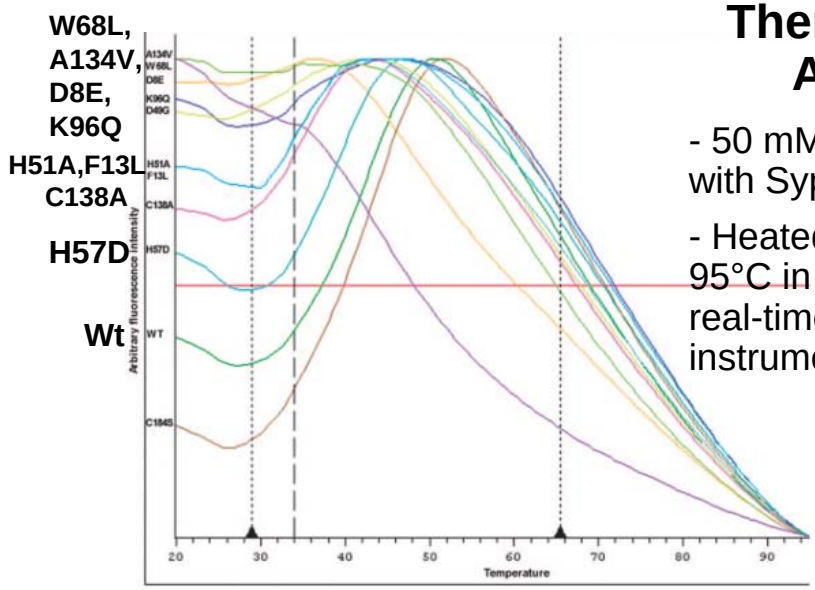
- HOH202, lies beneath the carbonyl carbon atom (ideally positioned for nucleophilic attack of the acyl bond)

- The 6-membered ring of PZA is embedded between residues Trp68, Phe13 and His137, and the positioning of the substrate is stabilized by one H-bond formed between the nitrogen atom N7 of PZA and HOH220 (itself bound to CO of Ala102 and to the Fe^{2+} ion.



Thermal stability and catalytic behaviour of PncA mutants





Thermal shift Assay :

- 50 mM PncA mixed with Sypro Orange
- Heated from 20 to 95°C in a PTC-200 real-time PCR instrument

PncA proteins	Yield of protein (mg)	TSA results IFI [a]	Tm [b]	Specific activity [c]
Wt	27	1	43	24
C184S	18.5	0.5	43	22
D8L	pp [d]			
D8E	1.4	2.3	32	0.020
K96Q	6.2	2.2	35	0
A134V	1.8	2.5	nd [e]	0.010
C138A	6.8	1.5	38	0
D49G	1.9	2.2	33	0
H51A	2.7	1.9	35	0.25
H57D	2.4	1.5	39	0.005
W68L	8.4	2.5	nd [e]	0.005
F13L	17	1.9	35	0.02

[a] IFI, initial fluorescence intensity, expressed comparatively to that of the wild-type enzyme set to 1.

CONCLUSIONS

Overall architecture of PncA of Mtb similar to that of *P.horikoshii* and *A.baumanii*

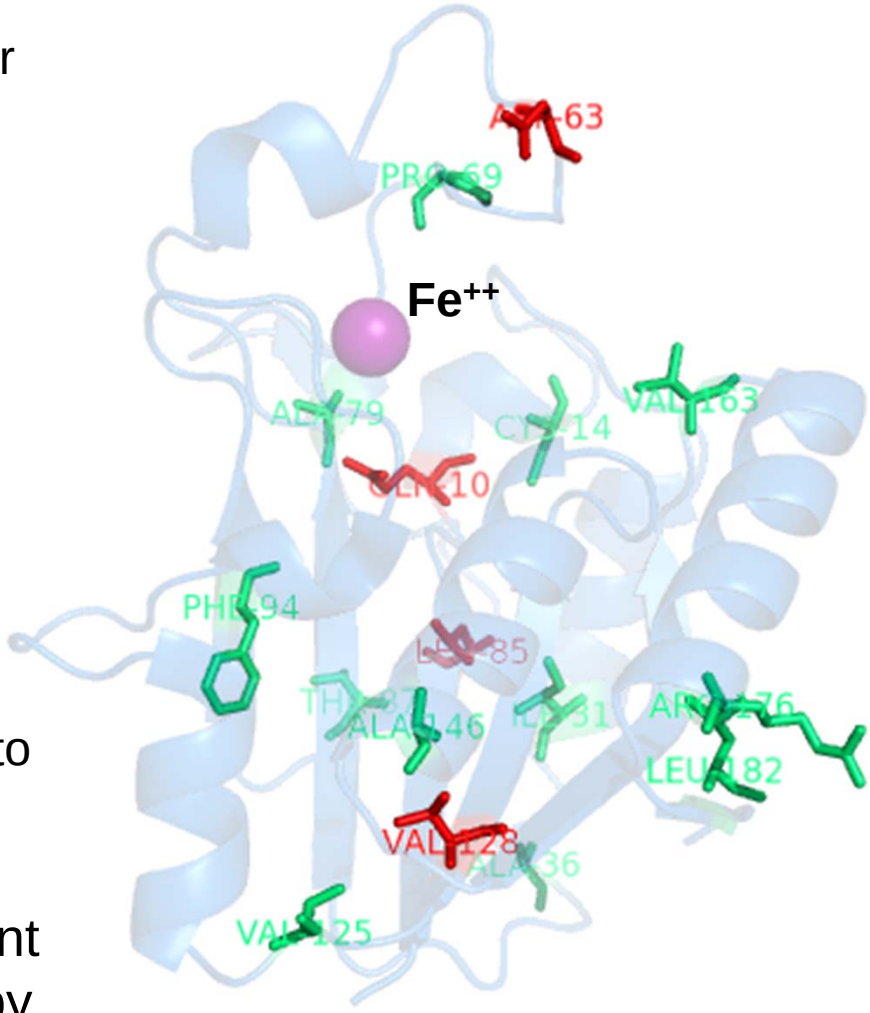
Structural variations:

-Iron-containing enzyme

Peculiar conformation of the 51-71 loop occluding the binding cavity

Specific positionning of His57 involved in the coordination of Fe^{2+}

Essential contribution of thermal stability to the activity of PncA mutants : Predicting the structure-activity relationships for PncA mutants should take into account not only the kinetics effects induced by the mutations, but also their effects on protein folding and stabilization.



Acknowledgements

Stéphanie Petrella, Nathalie Gelus-Ziental, Arnaud Maudry, Caroline Laurans, Rachid Boudjelloul, Vincent Jarlier

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Catalytic mechanism of the M.tuberculosis pyrazinamidase

Kinetics and Inhibition of Nicotinamidase from Mycobacterium tuberculosis
(Derrick R. Seiner et al, Biochemistry 2010)

Scheme 3: Proposed Mechanism of Catalysis of *Mt*PncA