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# Characterization of *Mycobacterium tuberculosis* nicotinamidase/pyrazinamidase

Hua Zhang[1,2,3,4], Jiao-Yu Deng[2], Li-Jun Bi[1], Ya-Feng Zhou[2], Zhi-Ping Zhang[2], Cheng-Gang Zhang[3], Ying Zhang[5] and Xian-En Zhang[2]

1 National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China
2 State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, China
3 Shenyang Institute of Applied Ecology, Chinese Academy of Sciences, China
4 Graduate School, Chinese Academy of Sciences, Beijing, China
5 Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD, USA

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**Correspondence**
X.-E. Zhang, Wuhan Institute of Virology, Chinese Academy of Sciences, Xiaohongshan, Wuchang District, Wuhan 430071, China
Fax: +86 10 64888464
Tel: +86 10 64888464
E-mail: zhangxe@sun5.ibp.ac.cn or x.zhang@wh.iov.cn
Y. Zhang, Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins University, 615 N. Wolfe Street, Baltimore, MD 21205, USA
Fax: (410) 955 0105
Tel: (410) 614 2975
E-mail: yzhang@jhsph.edu

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The nicotinamidase/pyrazinamidase (PncA) of *Mycobacterium tuberculosis* is involved in the activation of the important front-line antituberculosis drug pyrazinamide by converting it into the active form, pyrazinoic acid. Mutations in the *pncA* gene cause pyrazinamide resistance in *M. tuberculosis*. The properties of *M. tuberculosis* PncA were characterized in this study. The enzyme was found to be a 20.89 kDa monomeric protein. The optimal pH and temperature of enzymatic activity were pH 7.0 and 40 °C, respectively. Inductively coupled plasma-optical emission spectrometry revealed that the enzyme was an $Mn^{2+}/Fe^{2+}$-containing protein with a molar ratio of $[Mn^{2+}]$ to $[Fe^{2+}]$ of 1 : 1; furthermore, the external addition of either type of metal ion had no apparent effect on the wild-type enzymatic activity. The activity of the purified enzyme was determined by HPLC, and it was shown that it possessed similar pyrazinamidase and nicotinamidase activity, by contrast with previous reports. Nine PncA mutants were generated by site-directed mutagenesis. Determination of the enzymatic activity and metal ion content suggested that Asp8, Lys96 and Cys138 were key residues for catalysis, and Asp49, His51, His57 and His71 were essential for metal ion binding. Our data show that *M. tuberculosis* PncA may bind metal ions in a manner different from that observed in the case of *Pyrococcus horikoshii* PncA.

Pyrazinamide (PZA) is one of the first-line drugs recommended by the World Health Organization for the treatment of tuberculosis [1]. This drug plays a key role in shortening the duration of chemotherapy from 9–12 to 6 months because of its ability to kill the population of persisting tubercle bacilli in an acidic pH environment [2,3]. Despite the importance of PZA in the treatment of tuberculosis, its mechanism of action is probably the least understood of all the antituberculosis drugs. PZA is a prodrug that is converted into

**Abbreviations**
ICP-OES, inductively coupled plasma-optical emission spectrometry; IPTG, isopropyl thio-β-D-galactoside; NAM, nicotinamide; PZA, pyrazinamide; PncA, nicotinamidase/pyrazinamidase.

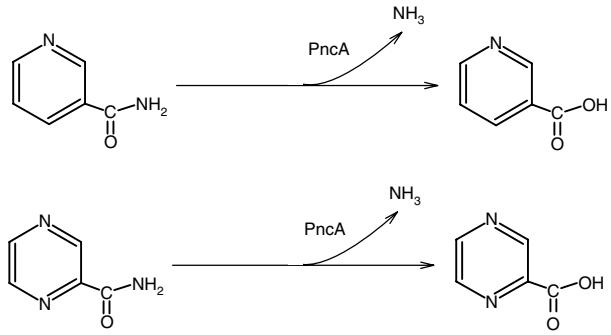


**Fig. 1.** Conversion of NAM and PZA to their acid forms by PncA.

its active derivative, pyrazinoic acid, by bacterial nicotinamidase/pyrazinamidase (PncA) (Fig. 1), which is encoded by the *pncA* gene, for activity against *Mycobacterium tuberculosis* [4,5]. Since mutations in *pncA* associated with PZA resistance were found by Scorpio and Zhang [6], many research groups have identified various mutations in *pncA* that can lead to the loss of PncA activity, and these mutations are thought to be the main reason for PZA resistance in *M. tuberculosis* [7–16].

PncA has been found in many microorganisms, such as *Escherichia coli*, *Flavobacterium peregrinum*, *Torula cremoris* and *Saccharomyces cerevisiae* [17–20]. The enzyme is involved in the conversion of nicotinamide (NAM) to nicotinic acid. The biochemical features of certain bacterial PncAs have been studied, but the *M. tuberculosis* PncA has not been well characterized. In 1998, Boshoff and Mizrahi [21] attempted to characterize the PncA of *M. tuberculosis* using the partially purified enzyme protein. In 2001, Lemaitre *et al.* [22] determined the PncA activity of nine naturally occurring PncA mutants bearing a single amino acid substitution, and speculated that a decrease in PncA activity was correlated with structural modifications caused by mutations in the putative active site Cys138. Residues such as Asp8, Lys96 and Ser104 have been suggested to play a role in the functioning of the PncA catalytic centre, as these three residues are located close to Cys138 and drastically impair the enzymatic activity if mutated. Du *et al.* [23] conducted correlative research and resolved the three-dimensional crystal structure of the *Pyrococcus horikoshii* PncA (37% amino acid sequence identity with *M. tuberculosis* PncA). In their study, they suggested that Asp10, Lys94 and Cys133 (Asp8, Lys96 and Cys138, respectively, in *M. tuberculosis*) were the enzyme catalytic centres, and that Asp52, His54 and His71 (Asp49, His51 and His71, respectively, in *M. tuberculosis*) were the $Zn^{2+}$-binding sites. They also proposed that the Cys133 residue of PncA probably attacks the carbonyl carbon of PZA to form an acylated enzyme via the thiolate after being activated by Asp10, and releases ammonia; zinc-activated water then attacks the carbonyl carbon of the thioester bond. Through the binding of another water molecule, the reactants release pyrazinoic acid. The Lys94 residue is then in a position to form an ion pair with either Asp10 or Cys133 [23].

In this study, *M. tuberculosis* PncA was cloned and overexpressed in *E. coli*. The purified enzyme was used to investigate the enzymatic activity, optimum pH and temperature, and ion dependence. In order to elucidate the reaction mechanism of the PncA enzyme, nine mutants were constructed by site-directed mutagenesis. These mutants were further subjected to studies on substrate comparison, CD spectral analysis and determination of the metal ion content. The results are presented herein.

## Results

### Purity and molecular weight of *M. tuberculosis* PncA

After induction by 0.4 mM isopropyl thio-β-D-galactoside (IPTG), the PncA protein was found in the soluble fraction of the *E. coli* BL21 (λDE3)/pET-20b(+)-*pncA* cell extract. A two-step chromatographic protocol, nickel chelate chromatography and molecular sieve, was adopted for PncA purification. The purity of the purified enzyme protein was assessed by SDS-PAGE. A single band was found in the molecular weight range 18.4–25.0 kDa. Using analytical ultracentrifugation and mass spectrometry, the molecular weight of PncA was further estimated to be 22.2 and 20.89 kDa, respectively (supplementary Fig. S1). As the theoretical molecular weight is 20.69 kDa, it is concluded that the *M. tuberculosis* PncA enzyme is a monomeric protein.

### Optimal pH and temperature

The experiments were performed using NAM as the substrate. Fig. 2 shows the effects of pH and temperature on enzyme activity. The optimal pH of the PncA enzyme was found to be close to pH 7.0. The enzyme activity decreased rapidly below pH 6.0 or above pH 8.0. The PncA enzyme exhibited its maximum activity at a temperature close to 40 °C. Below 25 or above 70 °C, the enzyme lost its activity rapidly.

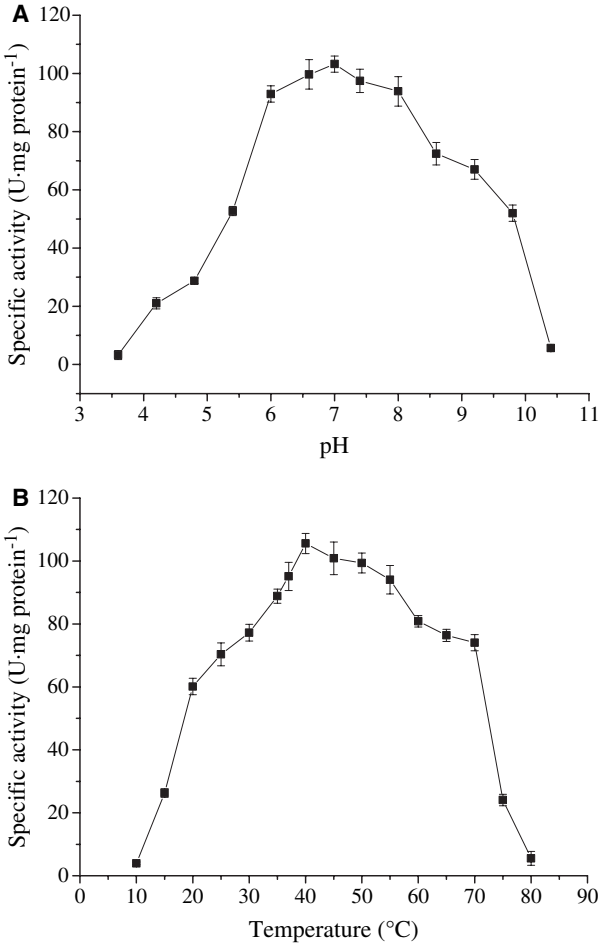


**Fig. 2.** Effects of pH and temperature on *Mycobacterium tuberculosis* PncA. (A) pH profile of the hydrolysis of NAM. Acetic acid/sodium acetate (pH 3.6–6.0), disodium hydrogen phosphate/sodium dihydrogen phosphate (pH 6.0–8.0) and glycine/sodium hydrate (pH 8.6–10.4) were used for the measurements, and the buffer concentrations were controlled to 100 mM. (B) Temperature profile of PncA. Disodium hydrogen phosphate/sodium dihydrogen phosphate buffer (100 mM, pH 7.5) was used as the solvent.

## Selection of the conserved residues and site-directed mutagenesis

The PncA sequence of *M. tuberculosis* H37Rv was compared with those of *P. horikoshii*, *Mycobacterium smegmatis* and *E. coli*, and the conserved residues were selected (Fig. 3A). As a large number of residues were conserved, only those that were likely to participate in enzyme activity and metal ion binding, as suggested by previous studies [22,23], were considered. These residues were located on the cave surface of the *P. horikoshii* PncA structure and were polar residues (Fig. 3B). On the basis of these criteria, nine residues were chosen for further study (Table 1), including the His57 residue (a mutation at this site leads to natural PZA resistance in *Mycobacterium bovis* [6]) and the Ser59 residue (a residue that binds metal ions in the presence of water molecules [23]). Ala was introduced into PncA at these selected sites by site-directed mutagenesis, resulting in the substitution mutations D8A, D49A, H51A, H57A, S59A, H71A, K96A, S104A and C138A.

## Enzyme activity

Enzyme specific activities of wild-type and mutant PncA were determined by HPLC, performed using excess substrate concentration, and the data were obtained when the concentration of the reacted substrate was < 10% of the total substrate (Table 2). The results were obtained at pH 7.5 and 37 °C, the same conditions as described previously for the purpose of comparison [21,24,25]. The wild-type PncA enzyme exhibited 89.6 U·mg$^{-1}$ protein of nicotinamidase activity and 81.9 U·mg$^{-1}$ protein of pyrazinamidase activity. Mutants D8A, D49A, H51A, H57A, H71A, K96A and C138A showed a significant decrease in enzyme activity, whereas mutants S59A and S104A showed only a partial loss of enzyme activity (Table 2).

## CD spectra

As shown in Fig. 4, the CD spectra of the wild-type and mutant PncA (D49A, H51A, H57A, S59A, H71A, S104A) were virtually the same. These CD spectra revealed that each of these enzymes contained almost identical percentages of α-helices, β-sheets, turns and random coils, indicating that they had uniform secondary structures. However, although the D8A, K96A and C138A PncA mutants displayed similar secondary structures, about 8% of their α-helices were transformed to β-sheets.

## Metal ion contents

The presence of metal ions in PncA was determined using inductively coupled plasma-optical emission spectrometry (ICP-OES), and the metal ion contents were calculated using the calibration curve obtained for each metal ion (11–30 in the Periodic Table, also including molybdenum and palladium) after subtracting the background signal in the blank buffer. The results indicated that PncA contained manganese and iron in a molecular ratio of 1 : 1 ([$Mn^{2+}$] : [$Fe^{2+}$]) (Table 3) and a low concentration of nickel (5 μM). We believe that this low concentration of nickel is a

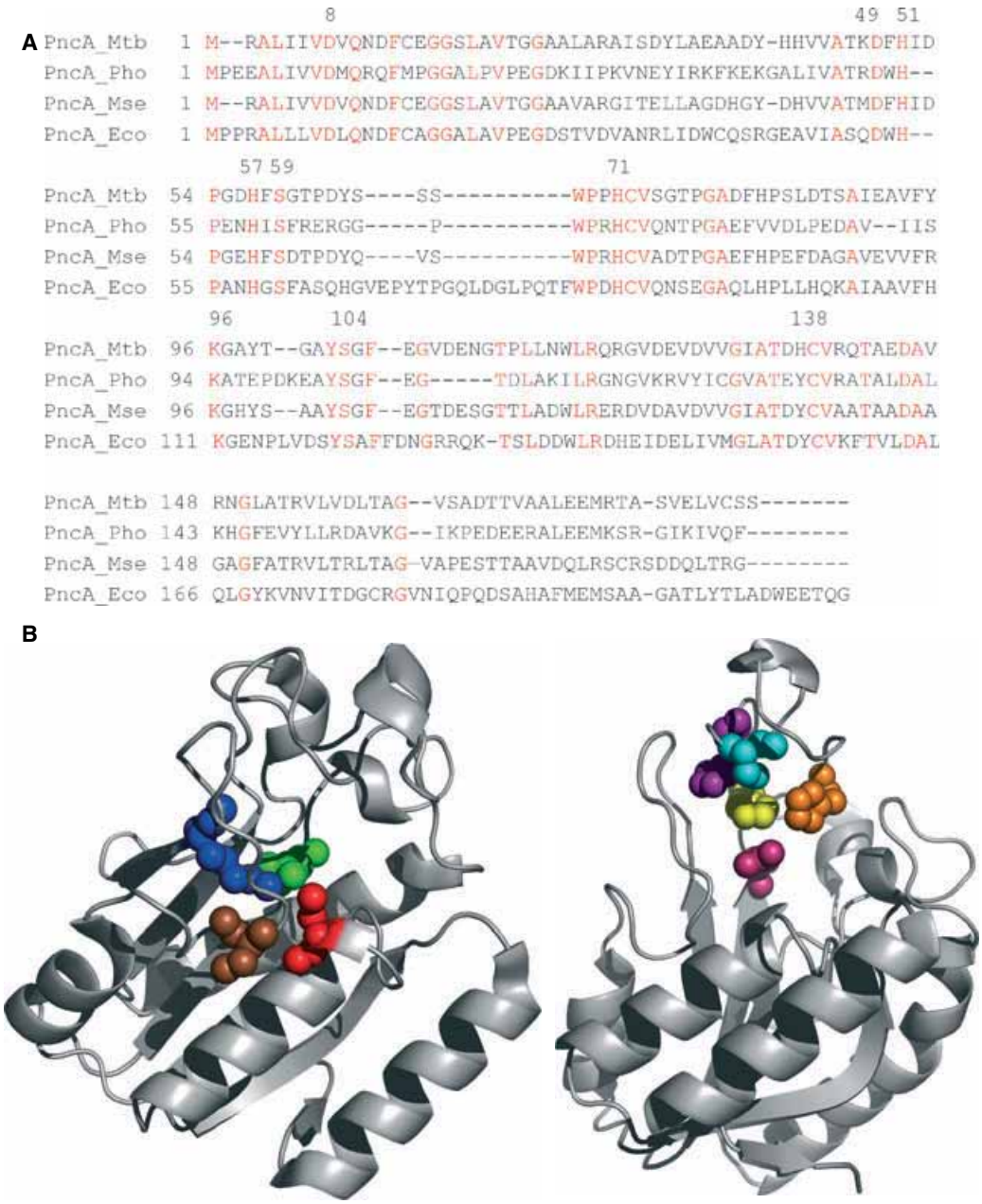


**Fig. 3.** Selection of the conserved residues in PncA. (A) Multiple sequence alignment of PncA from *Mycobacterium tuberculosis* (Mtb), *Pyrococcus horikoshii* (Pho), *Mycobacterium smegmatis* (Mse) and *Escherichia coli* (Eco). The alignment of the four PncAs was made using the MEGALIGN program (CLUSTALW). The residues conserved in the enzyme are coloured in red. Numbers above the alignment indicate the sites of selected conserved amino acids. (B) A cartoon diagram of *P. horikoshii* is shown. The nine highly conserved amino acids are Asp10 (Asp8 in Mtb, green), Asp52 (Asp49 in Mtb, pink), His54 (His51 in Mtb, yellow), His71 (His71 in Mtb, orange), Lys94 (Lys96 in Mtb, blue), Cys133 (Cys138 in Mtb, red), Ser60 (Ser59 in Mtb, cyan), Ser104 (Ser104 in Mtb, brown), and the site of mutation in *M. bovis* is His58 (His57 in Mtb, purple).

result of His-tag purification, as it was not detected when e-tag purification was performed (data not shown). Thus, it is of particular interest that the *M. tuberculosis* PncA is an enzyme that contains manganese or iron (or both), and is not a zinc-binding protein as observed in the case of *P. horikoshii* PncA [23]. A micro-quantity of $Mn^{2+}$ and $Fe^{2+}$ was observed in the mutants D49A, H51A, H57A and

**Table 1.** Highly conserved residues selected from PncA enzymes from different bacterial species.

| Strain | Selected conserved residues | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *Mycobacterium tuberculosis* | D8 | D49 | H51 | H57 | S59 | H71 | K96 | S104 | C138 |
| *Pyrococcus horikoshii* | D10 | D52 | H54 | H58 | S60 | H71 | K94 | S104 | C133 |
| *Mycobacterium smegmatis* | D8 | D49 | H51 | H57 | S59 | H71 | K96 | S104 | C138 |
| *Escherichia coli* | D10 | D52 | H54 | H58 | S60 | H86 | K111 | S121 | C156 |

**Table 2.** Relative activities of wild-type PncA (WT) and the nine mutants. Enzyme reaction mixtures, which contained 20 mM PZA (or NAM) and 160 μg PncA in 30 mM Tris/HCl buffer at pH 7.5 in a total volume of 200 μL, were incubated at 37 °C. Each enzyme (including the wild-type and nine mutant enzymes) was tested in three independent experiments with 15 s intervals during the enzyme reaction.

| | Enzyme specific activity[a,b] ($U \cdot mg^{-1}$ protein) | |
|---|---|---|
| Proteins | NAM | PZA |
| WT | 89.6 ± 3.1 | 81.9 ± 2.3 |
| D8A | 0 ± 0.01 | 0 ± 0.05 |
| D49A | 0.03 ± 0.002 | 0.2 ± 0.01 |
| H51A | 8.7 ± 0.03 | 3.8 ± 0.06 |
| H57A | 0.8 ± 0.06 | 0.5 ± 0.02 |
| S59A | 37.3 ± 0.4 | 33.6 ± 0.7 |
| H71A | 0.9 ± 0.02 | 0.7 ± 0.06 |
| K96A | 0 ± 0.02 | 0 ± 0.01 |
| S104A | 18.3 ± 0.6 | 26.7 ± 0.8 |
| C138A | 0 ± 0.03 | 0 ± 0.02 |

[a] The data are presented as the mean ± standard deviation of triplicate tests. [b] One unit of pyrazinamidase or nicotinamidase was defined as the amount of enzyme required to produce 1 μmol of pyrazinoic acid or nicotinic acid per minute.

H71A, and the total amount of the two ions in each of the mutants D8A, K96A, S59A, S104A and C138A was similar to that in wild-type PncA. Interestingly, D8A, K96A, S59A and S104A were observed to bind $Fe^{2+}$ to a greater extent than $Mn^{2+}$.

**Table 3.** Metal ion contents of wild-type and mutant PncA. The protein concentration used was 100 μM. Purified proteins (800 μL, 2.0 $mg \cdot mL^{-1}$) were digested with nitric acid (200 μL) and then diluted to 4 mL. The metal ions in the samples were detected by ICP-OES.

| | Metal ion concentration[b] (μM) | |
|---|---|---|
| Proteins[a] | $Mn^{2+}$ | $Fe^{2+}$ |
| WT | 44.2 ± 2.8 | 46.7 ± 3.5 |
| D8A | 12.0 ± 1.8 | 69.0 ± 0.1 |
| D49A | 0.04 ± 1.2 | 0.01 ± 0.9 |
| H51A | 0.1 ± 2.2 | 2.3 ± 1.9 |
| H57A | 0.05 ± 0.8 | 0.04 ± 0.3 |
| S59A | 30.8 ± 1.7 | 52.2 ± 0.6 |
| H71A | 0.05 ± 3.2 | 0.09 ± 2.1 |
| K96A | 19.4 ± 4.3 | 51.5 ± 2.5 |
| S104A | 0.5 ± 2.3 | 87.2 ± 3.2 |
| C138A | 56.6 ± 2.4 | 43.2 ± 3.2 |

[a] The protein concentrations were all 100 μM. [b] The data are presented as the mean ± standard deviation of triplicate tests.

## Effect of metal ions on PncA activity

The effect of metal ions on the hydrolytic activity of PncA was investigated systematically. The metal ions were pre-removed from the enzyme protein by dialysis. ICP-OES showed that manganese and iron were completely removed from PncA. $Mg^{2+}$, $Mn^{2+}$, $Ca^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Ni^{2+}$, $Fe^{2+}$ and $Fe^{3+}$ ions, at a final concentration of 2 mM, were added to the wild-type enzyme and apo-PncA solutions. The complexes were incubated at 4 °C for 24 h prior to the determination of the enzyme activities. The enzyme activities were determined using HPLC, and the results are summarized in Table 4. The wild-type enzyme was unaffected by $Mg^{2+}$, $Mn^{2+}$, $Ca^{2+}$, $Ni^{2+}$ and $Fe^{2+}$, but was inhibited by $Cu^{2+}$, $Zn^{2+}$ and $Fe^{3+}$. The hydrolytic activity was eliminated completely on removal of the

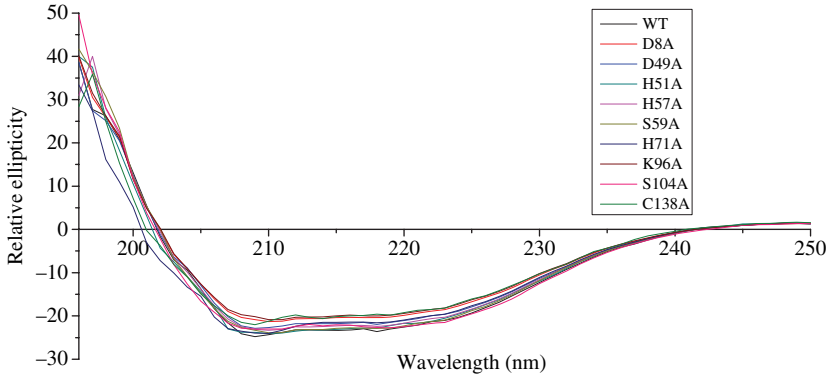


**Fig. 4.** CD spectra of the wild-type and mutant PncA. Purified protein (100 μL of 0.3 $mg \cdot mL^{-1}$) in 20 mM sodium phosphate buffer (pH 7.5) was determined from 190 to 240 nm using a Jasco J-720 CD spectrometer, and the results from 195 to 240 nm are presented.

**Table 4.** Effect of metal ions on the enzymatic activity of PncA. $Mg^{2+}$, $Mn^{2+}$, $Ca^{2+}$, $Cu^{2+}$, $Ni^{2+}$, $Zn^{2+}$, $Fe^{2+}$ and $Fe^{3+}$ ions, at a final concentration of 2 mM, were added to the holoenzyme and apo-PncA solutions. The complexes were incubated at 4 °C for 24 h prior to the determination of the enzyme activities. The enzyme activities were determined using HPLC.

| | Enzyme activity[b] (%) | |
|---|---|---|
| Metal[a] | NAM | PZA |
| Effects of metal ions on the activity of holoenzyme[c] | | |
| Wild-type | 100 | 100 |
| $Mg^{2+}$ | 98.9 ± 2.6 | 97.2 ± 3.2 |
| $Mn^{2+}$ | 99.2 ± 2.8 | 97.9 ± 1.5 |
| $Ca^{2+}$ | 98.9 ± 3.2 | 98.2 ± 3.7 |
| $Zn^{2+}$ | 5.7 ± 1.5 | 9.1 ± 1.8 |
| $Cu^{2+}$ | 6.7 ± 1.3 | 8.3 ± 2.4 |
| $Ni^{2+}$ | 95.6 ± 2.5 | 95.4 ± 3.2 |
| $Fe^{2+}$ | 84.9 ± 4.7 | 136.3 ± 2.3 |
| $Fe^{3+}$ | 5.4 ± 2.2 | 3.3 ± 1.5 |
| Effects of metal ions on the recovery of activity for apoenzyme[c] | | |
| apo-PncA | 0.05 ± 0.1 | 0.4 ± 0.1 |
| $Mg^{2+}$ | 1.2 ± 0.9 | 0.3 ± 0.07 |
| $Mn^{2+}$ | 91.2 ± 2.8 | 89.4 ± 1.2 |
| $Ca^{2+}$ | 1.0 ± 0.2 | 0.8 ± 0.06 |
| $Zn^{2+}$ | 0 ± 0.04 | 0 ± 0.07 |
| $Cu^{2+}$ | 0 ± 0.2 | 0 ± 0.4 |
| $Ni^{2+}$ | 0.4 ± 0.03 | 0.8 ± 0.09 |
| $Fe^{2+}$ | 80.1 ± 3.2 | 124.9 ± 1.9 |
| $Fe^{3+}$ | 0 ± 0.03 | 0 ± 0.08 |

[a] Final concentration, 2 mM. [b] The data are presented as the mean ± standard deviation of triplicate tests. [c] The protein concentrations are all 15 μM.

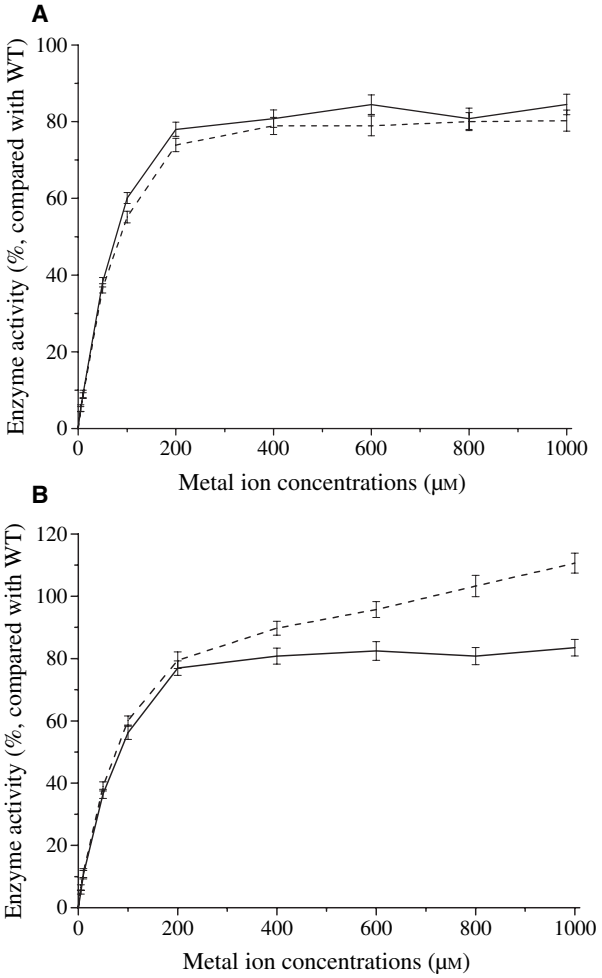


**Fig. 5.** Reconstitution of apo-PncA with $Mn^{2+}$ and $Fe^{2+}$. Metal ions at a final concentration ranging from approximately 0 to 1000 μM were added to the apo-PncA solutions. The complexes were incubated at 4 °C for 24 h prior to the determination of the enzyme activities by HPLC. (A) NAM; (B) PZA; full line, $Mn^{2+}$; broken line, $Fe^{2+}$.

$Mn^{2+}$ and $Fe^{2+}$ ions, and could be restored to 80–90% by $Mn^{2+}$ and $Fe^{2+}$, but not by $Ca^{2+}$, $Mg^{2+}$, $Ni^{2+}$, $Cu^{2+}$, $Zn^{2+}$ and $Fe^{3+}$. Indeed, the protein in the reaction mixture containing $Cu^{2+}$, $Zn^{2+}$ and $Fe^{3+}$ precipitated after centrifugation at 12 000 ***g*** (data not shown). Furthermore, apo-PncA was titrated with $Mn^{2+}$ and $Fe^{2+}$ concentrations in the range 0–1000 μM as the enzyme concentration was 150 μM. Enzyme activities were determined using HPLC, and the results are summarized in Fig. 5. The maximum restoration of activity was attained using approximately 200 μM of metal ion. In the presence of $Fe^{2+}$, however, the restoration of enzyme activity when using PZA as substrate was much higher than that obtained when using NAM as substrate.

## Discussion

In this study, *M. tuberculosis* PncA was cloned, overexpressed, purified and characterized. The enzyme is a 20.89 kDa monomer similar to the PncA enzyme from *P. horikoshii* [23]. The optimal pH and temperature of the enzyme activity were pH 7.0 and 40 °C, respectively.

Previous studies have shown that the nicotinamidase activity of *M. tuberculosis* PncA is much higher than its pyrazinamidase activity [24,25]. However, no such difference was observed in the current study (Table 2). One reason for this is that, in the previous study, enzyme activities were measured using cell extracts or partially purified enzymes, whereas, in the current study, purified enzyme proteins were used; this produced a significant difference in the results. In addition, the enzyme activities measured in this study were much higher than those in the previous study (NAM: 89.6 μmol·min$^{-1}$ in this study; 47.5 nmol·h$^{-1}$ in

a previous report (30)]. This was again a result of the use of purified enzyme proteins.

The ICP-OES data revealed that there were two types of metal ion, $Mn^{2+}$ and $Fe^{2+}$, in *M. tuberculosis* PncA (Table 3), whereas only one metal ion, i.e. $Zn^{2+}$, was found in *P. horikoshii* PncA [23]. It is suggested that *M. tuberculosis* PncA has only one metal centre for the following reasons. First, *P. horikoshii* PncA has one metal centre, as revealed by the structure. Second, the $[Mn^{2+}]/[Fe^{2+}]$ ratio in *M. tuberculosis* PncA is 1 : 1 and the total concentrations of $[Mn^{2+}]$ and $[Fe^{2+}]$ are equal to the concentration of PncA protein, which is a monomeric protein. The binding of PncA to $Mn^{2+}$ and $Fe^{2+}$ can be attributed to the metal content of the growth medium, the dissociation constants of the ions and the rates of metal ion penetration into the cells. Third, manganese and iron are transition elements, both can form four or six coordination bonds in the divalent state, and their covalent radii are the same, i.e. 1.17 Å; therefore, they can be substituted for each other. We believe that PncA binds iron in the natural state, as the mutant is prone to losing manganese. The enzymatic activity of apo-PncA could be restored by 80–90% using either $Mn^{2+}$ or $Fe^{2+}$ (Table 4), and wild-type PncA activity could be inhibited by $Fe^{3+}$ because of protein deposition in the presence of $Fe^{3+}$; these results indicate that both $Mn^{2+}$ and $Fe^{2+}$ may be prosthetic groups of *M. tuberculosis* PncA. The results of the titration of apo-PncA with $Mn^{2+}$ and $Fe^{2+}$ suggest that low concentrations of these ions can restore enzyme activity. The maximum enzyme activity can be acquired at a metal ion concentration of 200 μM with a protein concentration of 150 μM (Fig. 5). In addition, in the presence of $Fe^{2+}$, the restoration of enzyme activity was much higher when PZA rather than NAM was used as a substrate. The enhancement of PZase activity by $Fe^{2+}$ is an interesting finding that is consistent with our previous observation that $Fe^{2+}$ can enhance the antituberculous activity of PZA [26].

In order to investigate the active sites and metal ion-binding site of the *M. tuberculosis* PncA enzyme, site-directed mutagenesis of selected conserved amino acid residues was performed. As expected, all substitutions led to a decrease in the hydrolytic activities of both PZA and NAM. In particular, the substitutions D8A, D49A, K96A and C138A resulted in an almost complete loss of enzyme activity (Table 2). Of these, the Asp8, Lys96 and Cys138 residues also play crucial roles in *P. horikoshii* PncA, as reported by another group studying natural PZA-resistant mutants [22]. These results suggest that these residues are essential for PncA enzyme activity. CD spectral analysis revealed that the D8A, K96A and C138A mutants were no different from each other, although different from wild-type PncA (Fig. 4). Furthermore, the metal ion contents of the mutants D8A, K96A and C138A were not significantly different from that of wild-type PncA (Table 3). These data confirm the previous speculation that Asp8, Lys96 and Cys138 are not the binding sites for metal ions, but crucial residues for substrate binding or catalysis [23]. With regard to D49A, there is nearly no detectable manganese or iron in this mutant; therefore, it is probably one of the crucial residues for metal ion binding; this is also consistent with the results of Du *et al.* [23]. The substitutions H51A and H71A also resulted in low metal ion content, in combination with low enzyme activity, suggesting that the residues His51 and His71 are part of the metal ion-binding sites of *M. tuberculosis* PncA. Interestingly, the data also showed that, in addition to Asp49, His51 and His71, His57 is also crucial for metal ion binding. The mutation H57A led to total suppression of metal ion binding and a drastic decrease in enzymatic activity (Tables 2 and 3). Moreover, H57D, a naturally occurring mutant of *M. bovis* that is highly resistant to PZA, exhibited almost the same enzyme activity and metal ion content as H57A (data not shown). This is in sharp contrast with the findings obtained in the case of *P. horikoshii* PncA, in which the zinc ion is fixed in place by the Asp52, His54 and His71 residues, and the corresponding His58 (His57 in *M. tuberculosis*) residue is not involved in metal ion binding [23]. Furthermore, the enzymatic activity of *M. tuberculosis* PncA can be inhibited by an excess of $Zn^{2+}$ (Table 4). This indicates that $Zn^{2+}$ may compete with $Mn^{2+}/Fe^{2+}$ for the same metal-binding site, but not serve as the activating factor of the enzyme. Considering that Asp49, His51 and His71 (Asp52, His54 and His71 in *P. horikoshii* PncA), plus two water molecules, are the metal-binding residues of *P. horikoshii* PncA, and the mutation H57A results in an almost complete loss of both metal-binding and enzyme catalytic activities, it is possible that His57 is directly involved in metal binding and alters the metal-binding specificity. However, this needs to be confirmed after resolving the three-dimensional structure of the enzyme. A significant decrease in PncA activity was also observed in the two remaining mutants S59A and S104A. Their metal ion contents were the same as that of wild-type PncA; this suggests that neither Ser59 nor Ser104 is a metal ion-binding site.

In conclusion, *M. tuberculosis* PncA is a monomeric $Fe^{2+}/Mn^{2+}$ protein with similar hydrolytic activity for the substrates PZA and NAM. The three-dimensional structure and drug resistance caused by mutagenesis need to be investigated in follow-up studies.

## Experimental procedures

### Materials and chemicals

The PZA, NAM, $MnCl_2$, $FeCl_2$, $FeCl_3$, $ZnSO_4$, $NiCl_2$, $CaCl_2$ and $MgCl_2$ were obtained from Sigma Chemicals (St Louis, MO, USA). 2-(*N*-morpholino)-ethanesulfonic acid (MES) buffer was purchased from Amresco Inc. (Solon, OH, USA). Nickel chelate and Sephadex G-75 medium were supplied by Amersham Bioscience (Piscataway, NJ, USA). All other reagents were of analytical grade.

### Strains and plasmids

*Escherichia coli* DH5α was used as the host cell for cloning purposes. *E. coli* strain BL21 (λDE3) was used for protein expression. The plasmid pET-20b(+) (Novagen, Darmstadt, Germany) was used to construct vectors for the overexpression of *M. tuberculosis* PncA.

### Construction of *pncA* overexpression vector

The *pncA* gene was amplified by PCR from the genomic DNA of *M. tuberculosis* H37Rv (obtained from Wuhan Institute for Tuberculosis Prevention and Treatment, Wuhan, China) and ligated into pET-20b(+). The resulting plasmid pET-20b(+)-*pncA* was sequenced and confirmed to be identical to the *M. tuberculosis pncA* sequence in the GenBank database (accession number GI: 888260).

### *In vitro* mutagenesis

To identify the enzyme activity sites, site-directed mutations were introduced into the selected sites in the *pncA* gene by overlap PCR [27,28]. All fragments were ligated into pET-20b(+). and were subsequently sequenced to confirm the presence of the site-directed mutations.

### Protein overexpression and purification

The wild-type and mutants of PncA were overexpressed and purified by the same procedure. Typically, *E. coli* BL21 (λDE3)/pET-20b(+)-*pncA* was induced by 0.4 mM IPTG at $A_{600} = 0.6$ for 4 h at 25 °C. The cells were harvested by centrifugation, resuspended in binding buffer (20 mM Tris/HCl, pH 7.9, 500 mM NaCl and 5 mM imidazole), and then disrupted using an ultrasonic cell disruptor (VCX 750, Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China). The cell lysate was centrifuged and the supernatant was loaded on to a nickel chelate column pre-equilibrated with the binding buffer. The column was washed initially with washing buffer (20 mM Tris/HCl, pH 7.9, 500 mM NaCl and 60 mM imidazole), and the histidine-tagged protein was eluted with an elution buffer (20 mM Tris/HCl, pH 7.9, 500 mM NaCl and 120 mM imidazole). According to the purity determined by SDS-PAGE, the peak fractions were concentrated by ultrafiltration with phosphate buffer (30 mM Tris/HCl buffer, pH 7.5) and loaded on to a Sephadex G-75 molecular sieve column equilibrated with phosphate buffer. The peak fractions whose purity was determined by SDS-PAGE were concentrated by ultrafiltration. The proteins were centrifuged at 20 000 ***g*** for 15 min and the supernatant was stored at − 20 or − 80 °C. The protein concentration was measured by the bicinchoninic acid protein assay kit (Beyotime Biotechnology, Beijing, China) with bovine serum albumin as a standard, according to the manufacturer's protocol.

### Enzyme activity assay

The PncA activity was assayed by HPLC (CoulArray®, ESA Biosciences, Inc., Chelmsford, MA, USA) according to previous reports [24,29]. The enzyme reaction mixtures contained 20 mM PZA (or NAM) and 160 μg PncA in 30 mM Tris/HCl buffer at pH 7.5 in a total volume of 200 μL; they were incubated at 37 °C for 1 min. This resulted in a substrate conversion of 0–10%. The incubation time was increased to 30 min for mutants with almost no activity. The reaction was terminated by the addition of 20 μL of trichloroacetic acid (80%, w/v). The precipitates were removed by centrifugation (13 000 ***g*** for 10 min), and 40 μL of the reaction mixture was diluted in 1 mL of 30 mM Tris/HCl buffer. Samples were filtered (filter pore size, 0.45 μm), and 20 μL aliquots were separated on an XTerra® MS $C_{18}$ column (150 × 3.9 mm) with a 5% methanol elution buffer. Substrates and products were detected at 254 and 280 nm, respectively. At a flow rate of 1 mL·min$^{-1}$, nicotinic acid was eluted at 1.55 min, NAM at 4.30 min, pyrazinoic acid at 1.44 min and PZA at 3.98 min. The wild-type and the nine mutant enzymes were tested in three independent experiments. During the enzyme reaction, samples were taken at 15 s intervals and subjected to HPLC. All data were the averages of triplicate assays.

### Analytical ultracentrifugation

The molecular weight experiment was performed using an XL-I analytical ultracentrifuge (Beckman Coulter, Fullerton, CA, USA) equipped with a four-cell An-60 Ti rotor. The purified PncA protein (0.8 mg·mL$^{-1}$) in 100 mM Tris/HCl buffer (pH 7.5) was centrifuged at 4 °C and 262 000 ***g*** for 4 h, with Tris/HCl buffer as the control. In order to determine the molecular weight of the protein, the data were analysed using the software SEDFIT [30] from http://www.analyticalultracentrifugation.com/download.htm.

### Mass spectrometry

The mass spectrometric assay was performed using AXIMA-CFR Plus (Kratos, Manchester, UK). Purified PncA protein (0.1 mM) in 10 mM Tris/HCl buffer (pH 7.5) was used as a sample for the assay.

### Determination of optimum pH and temperature

The effects of pH and temperature on the hydrolysis of NAM by PncA were determined at pH 3.6–10.4 and 15–80 °C. The following buffers (100 mM) were used for the measurements: acetic acid/sodium acetate (pH 3.6–6.0), disodium hydrogen phosphate/sodium dihydrogen phosphate (pH 6.0–8.0) and glycine/sodium hydrate (pH 8.6–10.4). In order to assess temperature stability, PncA was incubated at each temperature for 5 min prior to the assay of enzyme activity. Disodium hydrogen phosphate/sodium dihydrogen phosphate buffer (100 mM, pH 7.5) was used as the solvent for optimum temperature determinations. The thermostability of PncA was determined by incubating the enzyme at optimal temperature for 2 h. The residual activity was assayed every 20 min by HPLC.

### CD analysis

CD spectra (190–240 nm) of the wild-type enzyme and mutants were obtained using a Jasco J-720 CD spectrometer (Jasco Inc., Easton, MD, USA). All samples were tested using 100 μL of 0.3 $mg{\cdot}mL^{-1}$ protein in 20 mM Tris/HCl buffer (pH 7.5).

### Determinations of metal ion content

The metal ion contents in the wild-type PncA and the mutants were determined using ICP-OES (Optima 2000, Perkin-Elmer, Waltham, MA, USA). Purified proteins (800 μL, 2.0 $mg{\cdot}mL^{-1}$) were digested with nitric acid (200 μL) and diluted to 4 mL. The metal ion content in the purified proteins was determined by ICP-OES with the metal ion standard solution (GSB 04-1766-2004, General Research Institute for Nonferrous Metals, Beijing, China). To investigate the effect of metal ions on enzyme activity, the ions were pre-removed from the enzyme proteins by dialysis. The purified wild-type PncA was dialysed against MES buffer (20 mM, pH 6.5) to which 2 mM EDTA and 2 mM 1,10-phenanthroline had been added for 1 day, and then against MES buffer alone to remove the remaining EDTA and 1,10-phenanthroline.

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## Supplementary material

The following supplementary material is available online:

**Fig. S1.** Molecular weight determination of *Mycobacterium tuberculosis* PncA: (A) analytical ultracentrifugation; (B) mass spectrometry.

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