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Characterization of pH-dependent structural properties of hydrolase PncA using NMR

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Abstract Catalytic enzyme Pyrazinamidase (PncA) from *Mycobacterium tuberculosis* can hydrolyze substrate pyrazinamide (PZA) to pyrazoic acid (POA) as active form of compound. Using NMR spectroscopy, pH-dependent catalytic properties were monitored including metal binding mode during converting PZA to POA. There seems to be a conformational change through zinc binding in active site from the perturbation of peak intensities in series of 2D HSQC spectra the conformation changes through zinc binding.

Keywords *Mycobacterium tuberculosis*, HSQC, pH-dependent, metal binding, PncA, PZA, POA, NMR

Introduction

PncA from *M. tuberculosis* can convert substrate pyrazinamide (PZA) to pyrazoic acid (POA) as active form.[1-3] The molecular mechanism of catalysis has not been clearly known and hydrolytic reaction of *M. tuberculosis* PncA was inferred from other hydrolase enzymes.[4-6] Experiments of pH dependent activity difference that is thought to be possible from *in vivo* studies were hardly reported yet.

In a previous study, hydrolytic activity of *M. tuberculosis* PncA is increased by about 19-fold with Zn^{2+} metal ion[6]. Other divalent ions tested, including Mg^{2+}, Mn^{2+}, Ca^{2+} and Cd^{2+}, did not increase the activity. Also, temperature and pH dependent effects on *M. tuberculosis* PncA were not reported yet.

As converting PZA to POA, cytoplasm in *M. tuberculosis* is lowered to acidic pH.[1,7] Therefore, there may be a difference of structure or enzymatic activity in *M. tuberculosis* PncA as the change of pH. In this paper, the change of structural properties and hydrolytic activity of *M. tuberculosis* PncA at acidic pH or neutral pH was investigated including metal binding mode. The results were compared with earlier activity reports and were used to predict species-specific catalytic properties of PncA.

Experimental Methods

Protein preparation- PncA protein was expressed

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and purified with the protocols as published previously.[2,8]

Nuclear magnetic resonance (NMR) - For pH titration experiment, A series of 2D [^{15}N-, ^{1}H-] heteronuclear single quantum coherence (HSQC) spectra was recorded in pH 6.7, pH 6.3, pH 6.1, pH 5.9, pH 5.6 and pH 5.3. And metal binding was monitored using 1D ^{1}H-NMR or 2D [^{15}N-, ^{1}H-] HSQC spectra.

Molecular docking of active site - To further investigate the precise mechanism of PZA substrate binding to *M. tuberculosis* PncA, *in silico* docking experiments with AUTODOCK[9-10] was carried out. The coordinates of the substrate PZA were translated away from the PncA protein, and its conformation was energy minimized before AUTODOCK was used to redock the substrate. Every docking experiment began with a random population of 256 different candidate-binding modes. The resulting AUTODOCK docked conformations were clustered into families of similar conformations, and their energies and cluster sizes were compared. The docked positions of PncA into its respective active sites were selected, with lowest energy docked conformation for PZA (-5.88 kcal/mol)

Results

2D HSQC spectra were compared for monitoring structural properties of PncA from pH 5.3 to 6.7 (Fig. 1). Overall residues in possible active site are nearly constant, supporting that active site of *M. tuberculosis* PncA has almost the same electronic environment at neutral and acidic pH. The R2, V163, and D136 signals display chemical shifts changes full pH range. Chemical shift effects at the lower pH end of the titration are seen for the R2, D136 and V163 resonances. This is probably related to the protonation of side chain functional groups or conformational changes in loops connecting β-strand or α-helix. The pH-dependent changes in NMR shifts were reversible.

The relative zinc (II) binding affinity of PncA at pH

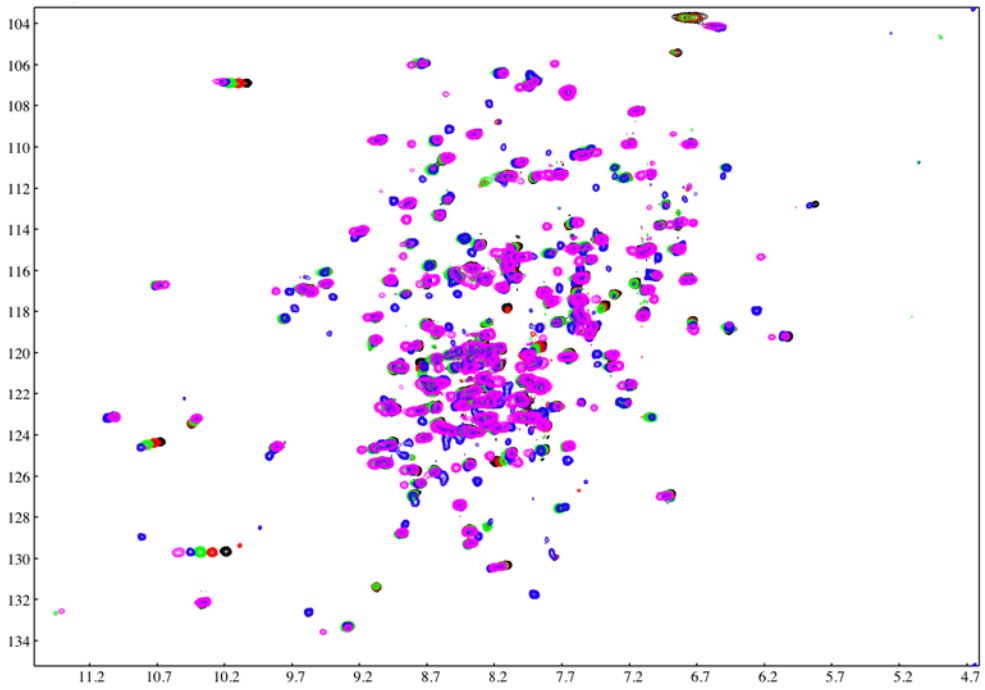

Figure 1. 2D HSQC spectra for pH titration of PncA. 2D HSQC spectra were plotted in various pH (colored by black (pH 5.3), red (pH 5.6), green (pH 5.9), blue (pH 6.1) and magenta (pH 6.7).

6.7 was investigated using ^{1}H-^{15}N HSQC-based zinc (II) titrations (Fig. 2). Chemical shift difference between amide groups of PncA with and without zinc was plotted and relative peak intensity was calculated. To express changes in the chemical shifts of the individual amide pairs a compound chemical shift change (in ppm) was defined as $\Delta\delta_{comp} = [(\Delta\delta_{HN})^2 + (\Delta\delta_N/R_{scale})^2]^{1/2}$, where the chemical shift scaling factor, R_{scale} was determined from the ratio of the average variances, $(\sigma_\delta)_{HN}/(\sigma_\delta)_N$, of the amide nitrogen and proton chemical shifts observed for the 20 common amino acid residues in proteins as deposited with the BioMagResBank. To good approximation, backbone amide ^{15}N and ^{1}H shifts dispersions are independent of amino acid type, resulting in equally narrow distributions: $(\sigma_\delta)_{HN} = 0.66$ (±0.07), and $(\sigma_\delta)_N = 4.30$ (±0.55), giving $R_{scale} = 6.5$.[11]

A relative peak intensity corresponding to a given residue and zinc (II) concentration was normalized to the sharp and well-resolved residue 2 (Fig. 3). Also, the value of relative peak intensity was defined as measured intensity divided by peak intensity corresponding to the same residue measured from spectra at 1 equivalent zinc (II) concentrations. The relative peak intensity (ΔI) between both spectra is given as $\Delta I = (I_2/I_x)_{NoZinc} / (I_2/I_x)_{1eqZinc}$, where I_2 and I_x represent the peak intensities of residue 2 and the analyzed residue, respectively.[12] Electronic environmental change is evident in the zinc (II) binding data in which residues from the loop connecting adjacent β-strands and the possible active site appear to have change of relative peak intensities at 1 equivalent quantities of zinc (II). The conformation changes through zinc binding in active site may be responsible for perturbation of peak intensities and chemical shifts and is currently under investigation.

Discussion

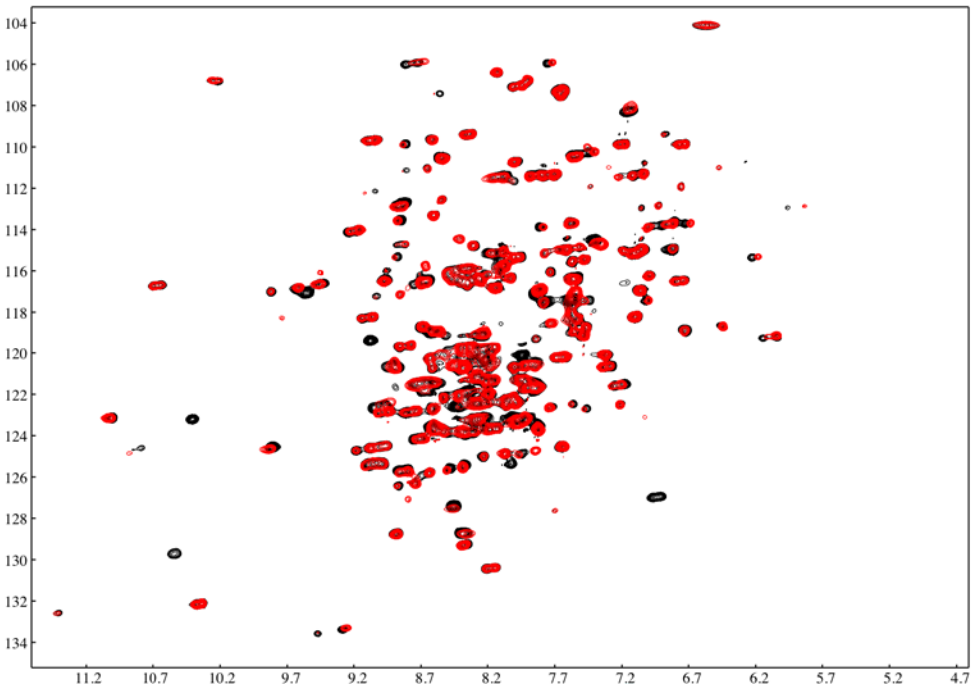

Figure 2. 2D HSQC spectra of zinc titration of PncA. One equivalent of zinc (red) was added to native PncA sample in pH 6.7 (black).

PncA from *M. tuberculosis* had different hydrolytic activity in acidic pH and neutral pH. It was more optimum and effective at neutral pH (6.7) than acidic pH (5.3). Also, in the aspect of thermal and denaturant stability, neutral pH grants more endurance in rigorous conditions. Many enzymes have its optimum pH and effects of pH change cannot be explained by a simple factor.

PncA from *M. tuberculosis* seems to have a change of self-defense mechanism at different pH. Accumulated active form POA has lowered the cytoplasm of *M. tuberculosis* as PncA enzymatic reaction progress with substrate PZA. As pH is more acidic, hydrolytic activity of PncA has decreased.

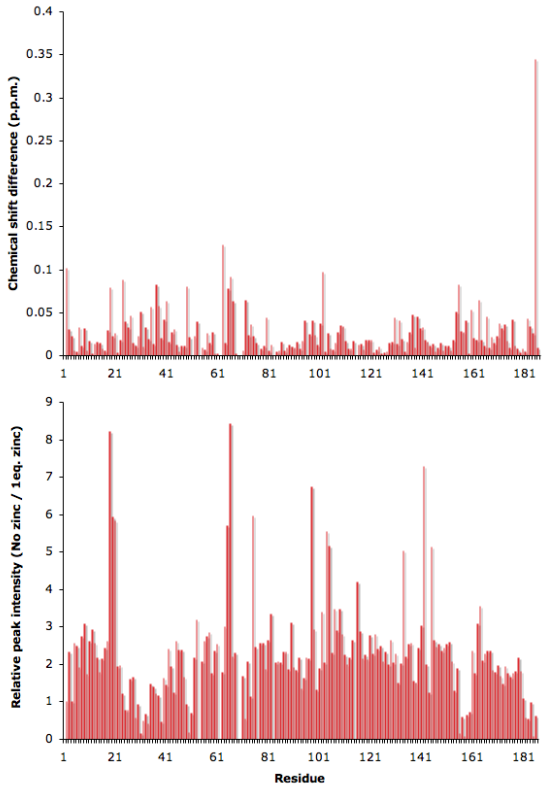

Figure 3. Analysis of ^{1}H-^{15}N HSQC spectra of PncA with and without zinc ion at 313 K, pH 6.7. Chemical shift differences (top) for all clearly detectable peaks and relative peak intensity (bottom) of two HSQC spectra with and without zinc ion were shown.

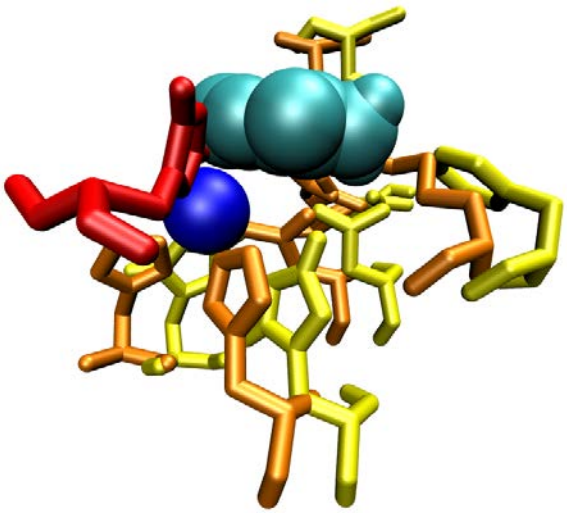

Figure 4. Active site comparison of PncA. Active site residues of *P. horikoshii* PncA was colored by orange and that of *M. tuberculosis* by yellow. Histidine 57 was colored by red, substrate PZA by cyan and zinc ion by blue.

Therefore, conversion of prodrug PZA to an active form POA is inhibited such as feedback inhibition mechanism with accumulated POA in acidic pH.

Unlike earlier reported *P. horikoshii* PncA, *M. tuberculosis* PncA had little effect on enzymatic activity by Zn^{2+}. Other divalent metals (Mg^{2+}, Cd^{2+} and Cu^{2+}) had also little effect on enzymatic activity like in case of Zn^{2+}. Zinc led to motional changes with aromatic residues, especially, in tryptophan residue but did not make a remarkable change in hydrolytic reaction rate. There seems to be well-formed active site that progresses enzymatic reaction without Zn^{2+}.

Transient metal ions such as Zn^{2+}, is necessary to many catalytic reactions, which coordinate with amino acids and enhance catalysis at the active site. They act as cofactor having a directional or template effect. In case of *M. tuberculosis* PncA, zinc as this cofactor acting is not much effective on transition state of enzymatic reaction. There seems to be another stabilizing factor of *M. tuberculosis* PncA transition state of hydrolytic reaction.

PncA from *M. tuberculosis* has high sequence identity with *P. horikoshii* PncA (37%). Two

structures have similar global shape and secondary structure motif pattern is also similar. Also, conserved region has high sequence identity and possible active site was well conserved. Therefore, possible active site of PncA from *M. tuberculosis* seems to be at almost identical position with that of *P. horikoshii*. Catalytic triad and Zn^{2+} binding site are highly conserved region. The volume of active site was almost the same with two PncA proteins. Compared with PncA from *P. horikoshii*, that from *M. tuberculosis* is likely to have more flexible residues were existed around active site (Fig. 4).

In conclusion, active site of PncA from *M. tuberculosis* had many aromatic residues that affect reaction rate with increase of orientation effect and stability in transition state. Also, it is likely that H57 in PncA from *M. tuberculosis* could play key role in enzymatic reaction and zinc binding coordination and structural properties of active site of PncA from *M. tuberculosis* may lead to features of species-specific enzymatic function.

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