Journal of the Korean Magnetic Resonance Society **2017**, *21,* 85-89
DOI 10.6564/JKMRS.2017.21.3.085

Identification of Enzymatic Catalysis of PncA using ^{1}H-NMR

Jong-Jae Yi[1,‡], Won-Je Kim[2,‡], Jin-Kyu Rhee[3], Jongsoo Lim[4], Bong-Jin Lee[2], and Woo Sung Son[1,*]

[1]College of Pharmacy, CHA University, 120 Haeyong-ro, Pocheon-si, Gyeonggi-do, 11160, Republic of Korea
[2]College of Pharmacy, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul, 08826, Republic of Korea
[3]Department of Food Science and Engineering, Ewha Womans University, 52 Ewhayeodae-gil, Seodaemun-gu, Seoul, 03760, Republic of Korea
[4]Discovery Technology Team, Dong-A ST Research Institute, 21 Geumhwa-ro 105beon-gil, Yongin-si, Gyeonggi-do, 17073, Republic of Korea

Received Jul 18, 2017; Revised Jul 22, 2017; Accepted Jul 30, 2017

Abstract Pyrazinamidase (PncA) from *Mycobacterium tuberculosis* is the hydrolytic enzyme (hydrolase) that can hydrolyze substrate PZA to active form pyrazoic acid (POA). To investigate hydrolytic reaction of M. tuberculosis PncA, 1D NMR spectra were monitored at various molar ratios of PncA and PZA. The line-width of PZA was changed as PncA was added into PZA with different molar ratios. These results suggested that determination of PncA enzymatic activity could potentially serve as an indirect measure of PZA susceptibility.

Keywords *Mycobacterium tuberculosis*, Enzyme, Substrate, PncA, PZA, POA, NMR

Introduction

Basic sole treatment of tuberculosis is multiple chemotherapies and is widely used short six months course *M. tuberculosis* drug treatment regimen. It uses an initial 2-month phase of daily therapy with isoniazid (INH), rifampicin (RMP), and pyrazinamide (PZA), plus either streptomycin (SM) or ethambutol (EMB). This regimen is followed by daily therapy with INH and one other primary drug for the next four months. Drug-resistant strains emerge when chemotherapy is intermittent or otherwise inadequate.[1,2]

Pyrazinamide (PZA) has remarkable sterilizing activity, and when added to regimens containing RMP, it is responsible for much of the killing of persisting tubercle bacilli during the initial intensive phase of chemotherapy, allowing treatment to be shortened from 9 months to 6 months. Despite its remarkable *in vivo* activity, PZA is not active against *M. tuberculosis* under usual culture conditions at close to neutral pH. The reasons for this paradoxical behavior have been the subject of many studies during the past years, and PZA was found to be active against M. tuberculosis only at an acidic pH, which can occur during active inflammation due to the production of lactic acid by inflammatory cells. Also, PZA is a prodrug whose activity depends on its conversion to pyrazoic acid (POA) by a bacterial amidase. Despite the importance of PZA in the treatment of TB, its mechanism of action is probably the least understood of all anti-tuerculosis drugs.[3-7]

The role of Pyrazinamidase (PncA) is to convert the prodrug PZA to the active form POA[7] (Figure 1). The PncA is located in the cytoplasm of *M. tuberculosis*

‡These authors equally contributed to this work.
* Correspondence to: **Woo Sung Son**, College of Pharmacy, CHA University, 120 Haeryong-ro, Pocheon-si, Gyeon ggi-do, 11160, Republic of Korea, Tel: +82-31-850-9398; Fax: +82-31-850-9398; E-mail: wson@cha.ac.kr

and is expressed constitutively.[8,9] Recent reports that mutations in the pncA gene encoding PncA lead to the loss of PncA activity, have implicated that the loss of PncA activity is highly related with PZA-resistance.[9-11] The studies on physicochemical properties and structure-activity relationship of *M. tuberculosis* PncA were limited to experiments using mutagenesis or one report of X-ray crystallography. [12-13] Here, the evidence of enzymatic catalysis of PncA was presented from the analysis of ^{1}H-NMR.

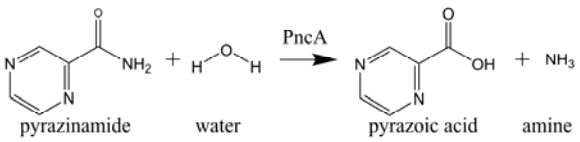

Figure 1. PncA and PZA. Mode of action of PZA is initiated by POA hydrolyzed using PncA.

Experimental Methods

Protein Preparation - PncA protein was expressed and purified with the protocols as published previously.[14,15] All materials were purchased from certified vendors in molecular biology grade for the reliability of results. Host (*E. coli* BL21 (DE3) Codon Plus-RIL™) and vector system (pET-15b) were purchased from Novagen Inc. (Darmstadt, Germany). All reagents except isotopes were from Sigma Chemical Company (St. Louis, U.S.A.). Isotopes such as ^{15}N sources ($(^{15}NH_4)_2SO_4$, $^{15}NH_4Cl$) were purchased from ISOTEC Inc. (Ohio, U.S.A.). Ni^{2+}-agarose column (His bind® Resin) was purchased from Novagen Inc. (Darmstadt, Germany). Chelating Sepharose Fast flow® from Amersham Pharmacia Biotech Inc. (Uppsala, Sweden) were used for the purification of proteins. The sonic oscillator, Sonifier 450, for cell lysis was from Branson Ultrasonics Corporation (Connecticut, U.S.A.). The centrifugation was carried out by Beckman Instruments Inc. (California, U.S.A) J2-MC, and the Fraction collector was purchased from Bio-Rad Laboratories Inc. (California, U.S.A.). CentriPrep® and CentriCon® from Millipore Corporation (Massachusetts, U.S.A.) were used to exchange buffers and to concentrate the sample.

Nuclear magnetic resonance (NMR)- NMR experiments for backbone assignment were conducted with the methods as reported previously .[14,15]

Predicted 1H-NMR spectrum - Proton Chemical shifts of PZA were predicted ChemNMR module in ChemOffice Ultra 2000.[16]

^{1}H NMR - Pyrazinamide was dissolved in 500 µl D_2O 99.998%, in the concentration range 10 µM – 5 mM. All proton spectra were recorded at 313K on the Bruker DRX500. The standard spectra were recorded using standard presaturation of water resonance with 64 K data points and a spectral width of 8000 Hz. Number of scans and receptor gain were adjusted to the sample concentration, from NS = 32 and RG = 32 (5 mM) to NS = 4800 and RG = 32000 (10 µM). Pyrazinamidase was used with lyophilized sample dissolved in D_2O.

Results

Proton chemical shifts of PZA with respect to tetramethylsilane (TMS) were estimated using ChemNMR module (Figure 2). Three protons of 2-pyrazine ring were calculated as major peaks in NMR spectrum corresponding to the chemical shift of 9.96, 9.03, and 8.89, respectively. Protons of primary amide were clear one peak at 8.18 ppm in proton NMR spectrum.

Experimental NMR methods were applied for the analysis of interactions between PncA and PZA. During catalysis, enzyme protein and substrate undergo structural perturbation which are reflected by changes in a variety of NMR physical parameters or observables including chemical shifts, relaxation parameters (T_1, T_2, and NOEs), dynamic parameters (S_2, Hydrogen/Deuterium exchange), diffusion coefficients, saturation transfer difference, transfer NOE. Substrate line-width (T_2) changes of ^{1}H-NMR

signals in an enzymatic reaction with PncA were monitored. As PncA was titrated into a substrate PZA, the PZA line-width broadened supporting that PZA binds PncA. Especially, aromatic protons were broadened apparently and signals of aliphatic protons in POA which was converted from PZA during hydrolysis (Figure 3).

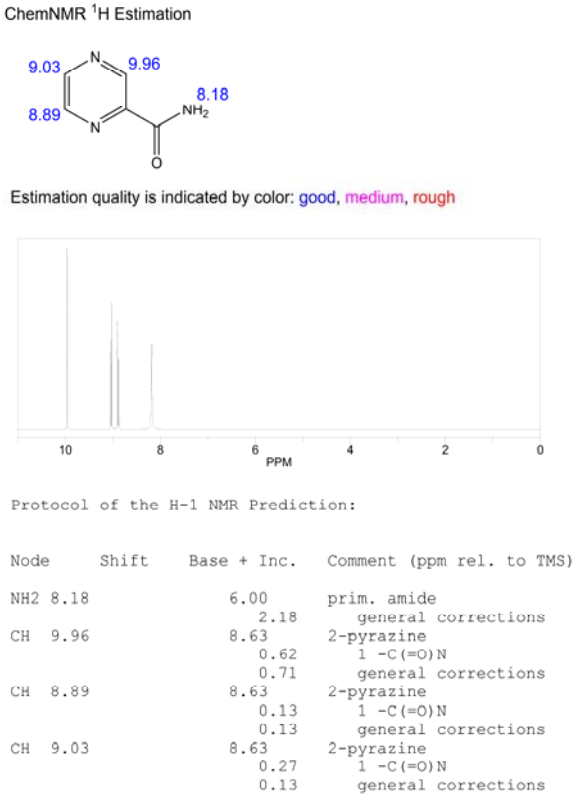

Figure 2. Predicted proton chemical shift of PZA. Good estimated values of chemical shifts were represented with blue color. Total 4 values of chemical shits of PZA were predicted including three protons of 2-pyrazine ring and protons of primary amide.

Discussion

To predict chemical shifts of PZA, heuristically-driven protocol was used with ChemNMR module. Prediction method of chemical shifts can be used with almost compounds even for large proteins. However, this prediction depends on fixed set of parameters corresponding to atom types and subgroups. Therefore, the calculated results could be reliable when built-in to the parameters are valid for predicting molecule.

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 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 simple factor.

In case of *M. tuberculosis* PncA, the fact that neutral pH is optimum pH seems to have relation with self-defense mechanism. 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. Therefore, conversion of prodrug PZA to active form POA is inhibited like feedback inhibition mechanism with accumulated POA in acidic pH.

Because of the good correlation of loss of PncA activity and PZA resistance, determination of PncA activity could potentially serve as an indirect measure of PZA susceptibility. Generally, there are three types of PncA enzymatic assays: the Wayne method is based on detection of POA, which forms a brownish or pink colored compound with ferrous ammonium sulfate. The Russell method is based on detection of ammonia, which reacts with phenol-hypochlorite to form a blue colored compound. The McClatchy method depends on different mobilities of PZA and POA on thin-layer chromatography (TLC) plate in a particular solvent system. Similarly, high performance liquid chromatography (HPLC) has also been adapted to measure PncA activity based on the different mobilities of PZA and POA.

While the measurement of PncA enzymatic activity is a good indicator of PZA resistance, the results have not been accepted for determining PZA susceptibility, largely because of the poor sensitivity of the conventional Wayne assay, which can give false resistance results. Despite this, the PncA assay is

often performed in parallel with PZA susceptibility testing as an independent measure of PZA susceptibility. To overcome these limitations in PZA enzymatic assay, ^{1}H-NMR was used for enzyme activity assay of *M. tuberculosis* PncA. In ^{1}H-NMR spectra, conversion from PZA to POA could be identified in the proton atom level.

In conclusion, enzymatic properties of PncA from *M. tuberculosis* using ^{1}H-NMR, suggested the initial point of understanding catalysis of PZA in molecular level. Also, it is proposed that simple proton NMR method could be used to identify enzymatic reaction and binding mode of substrate in enzymatic reaction.

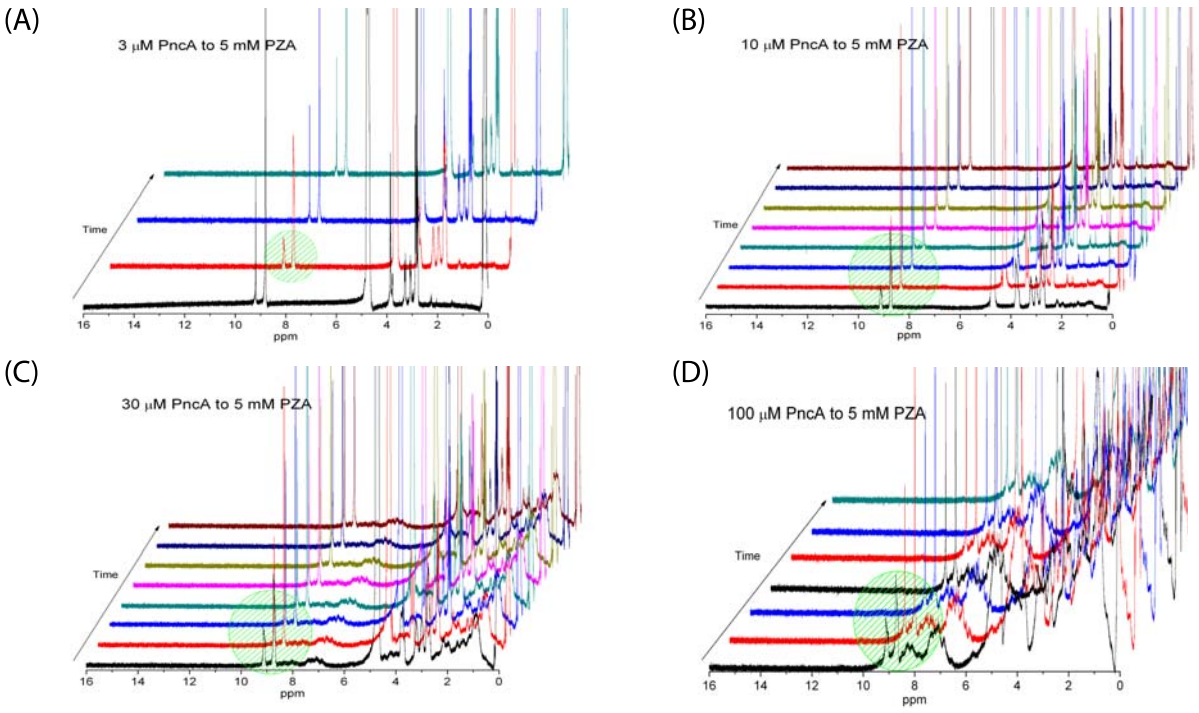

Figure 3. 1D proton NMR spectra of PZA in present of PncA. A series of spectra over a period of time with various molar ratios of PZA to PncA. Each spectra was shown as different color including black, red, blue, cyan etc. Also, lined circles were used to emphasize the change of peak height in proton chemical shifts of 2-pyrzaine ring.

Acknowledgements

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2017R1D1A1B03030723). This study also partly supported by the GRRC program of Gyeonggi province (GRRC-CHA2017-A01, Discovery and Analysis of Regional Specialized Resources and Operation of Regional Research Service Center).

References

1. World Health Organization, *WHO report 2006* (2006)
2. British Thoracic Society, *Br. J. Dis. Chest*. **78**, 330 (1984)
3. Y. Zhang, and D. Mitchison, *Int. J. Tuberc. Lung. D.* **7**, 6 (2003)
4. A. Somoskovi, L. M. Parsons, and M. Salfinger, *Respir. Res.* **2**, 164 (2001)
5. M. S. Tarshis, and W.A. Weed, *Am. Rev. Tuberc.* **67**, 391 (1953)
6. D. W. Mc, and R. Tompsett, *Am. Rev. Tuberc*. **70**, 748 (1954)
7. K. Konno, F. M. Feldmann, and W. McDermott, *Am. Rev. Respir*. Dis. **95**, 461 (1967)
8. Y. Zhang, A. Scorpio, H. Nikaido, and Z. Sun, *J. Bacteriol*. **181**, 2044 (1999)
9. C. Raynaud, M. A. Laneelle, and R. H. Senaratne, *Microbiology* **145**, 1359 (1999)
10. Y. Zhang, and D. Young, *J. Antimicrob. Chemoth*. **34**, 313 (1994)
11. A. Scorpio, and Y. Zhang, *Nat. Med.* **2**, 662 (1996)
12. N. Lemaitre, I. Callebaut, F. Frenois, V. Jarlier, and W. Sougakoff, *Biochem. J.* **353**, 453 (2001)
13. X. Du, W. Wang, R. Kim, H. Yakota, H. Nguyen, and S. Kim, *Biochemistry* **40**, 14166 (2001)
14. J. Yi, J. K. Yoo, J. K. Kim, and W. S. Son, *J. Kor. Magn. Reson. Soc.* **17**, 30 (2013)
15. W. Kim, J. Rhee, J. Yi, B. Lee, and W. S. Son, *J. Kor. Magn. Reson. Soc*. **18**, 36 (2014)
16. D. Strack, *Phytochemistry* **57**, 144 (2001)