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A switch in substrate tunnel for directing regioselectivity of nitrile hydratase towards α, ω-dinitriles

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Supplementary information

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Materials and methods

1. Chemical reagents

The 99% pure 5-cyanovaleramide (5-CVAM) was purchased from Toronto Research Chemicals Inc., Canada. Adiponitrile (ADN), adipoamide, malonitrile and malonamide were bought from Sigma-Aldrich Co., USA. Succinonitrile, terephthalonitrile, phthalodinitrile, cyanoacetamide, 4-cyanobenzamide, 2-cyanobenzamide, phthalamide and terephthaldiamide were from Tokyo Chemical Industry., Japan. All other chemicals were obtained commercially.

2. Strains, plasmids and Site-directed mutagenesis

Plasmid pET-24a carrying the NHase genes from *Pseudomonas putida* NRRL-18668 and *Comomonas testosteroni* 5-MGAM-4D were synthesized by Shanghai Gene Core Biotechnologies Co., Ltd.[1] Site-directed mutagenesis to change amino acid residues in the β-subunits of *Pp*NHase and *Ct*NHase was carried out by means of a whole plasmid PCR protocol.[2] For each variant of *Pp*NHase and *Ct*NHase, plasmids carrying *Pp*NHase and *Ct*NHase genes were used as the template respectively. The primers were shown in Table S3. All the PCR products were transformed into *Escherichia coli* JM109.

3. Expression and purification of enzymes

E. coli strain BL21 (DE3) was used for expression of wild-type *Pp*NHase, *Ct*NHase and all their variants. Each strain was grown at 37 ℃ in 2YT medium containing $CoCl_2 \cdot 6H_2O$ (0.05g/L) and kanamycin (50mg/mL). Isopropyl β-D-thiogalactopyranside (IPTG), as an inducer, was added to a final concentration of 0.4mM after the A_{600} reached 0.8. The cells were incubated at 24 ℃ for 16 h, then. All purification steps were carried out at 0℃-4℃ using the AKTA purifier (GE Healthcare UK Ltd). Potassium phosphate buffer (KPB, 10mM, pH 7.4) containing 0.5mM dithiothreitol (DTT) was used in the entire purification process. The cell extraction was prepared through ultrasonics. Centrifugation was performed for 20 min

at 18,000×g, and NHase was partially purified using ammonium sulfate fractionation (40-75%). Desalinized was then carried out by dialysis against KPB buffer; the dialyzed solution was applied to a Hitrap Q HP column (GE Healthcare UK Ltd). The protein was eluted from the column with elution buffer (a linear gradient from 0 to 0.5M KCL in KPB) and then applied to Superdex 200 10/300 GL (GE Healthcare UK Ltd). The fractions containing the enzymes during the whole purification steps were analyzed by SDS-PAGE.

4. Enzymatic assay

The regioselectivity of NHase towards adiponitrile was assayed in a reaction mixture (0.5ml) comprising 10mM KPB (pH 7.5), 20mM adiponitrile, and 10uL of the enzyme with a concentration of 0.2mg/ml. The reaction was performed at 35℃ for 4h and stopped by the addition of 0.5ml of methanol. The amounts of 5-CVAM and ADN were determined using high-pressure liquid chromatography (HPLC) (Hitachi, Tokyo, Japan) with a HITACHI LACHROM C18 (5UM) column. The following solvent system was used: 25mM H_3PO_4 buffer (pH 2.5) and methanol (89.01:10.09 vol/vol), at the flow rate of 1ml/min. The wavelength of 210nm was used for monitoring.[3] The kinetic parameters for ADN and 5-CVAM hydration by wild-type *Pp*NHase, *Ct*NHase and their variants were determined in 10mM KPB at 35℃, and the concentration of the enzyme was 0.3mg/ml. The concentration of ADN and 5-CVAM were 2-100mM and 5-600mM respectively. The results were calculated by Michaelis-Menten equation model. 20mM malonitrile/50mM succinonitrile was added into the same reaction mixture above while determining the regioselectivity of NHase towards malonitrile and succinonitrile. The reaction was stopped by the addition of 0.5ml of acetonitrile. The HPLC solvent system was acetonitrile /5mM K_2HPO_4/ H_3PO_4, pH 2.9, 7:12 (by vol.). The monitoring wavelength was 223nm.[4] The regioselectivity of NHase towards terephthalonitrile and phthalodinitrile was assayed by adding 2mM terephthalonitrile /6mM phthalodinitrile to the reaction mixture described above, the following HPLC solvent system was used:

water/acetonitrile/acetic acid = 2250/750/15 (ml), sodium 1-octanesulfonate 6.45 g. The wavelength of 254nm was used for monitoring.[5]

5. LC-MS analysis of 3-cyanopropionamide

The mass of 3-cyanopropionamide was determined using WATERS ACQUITY UPLC and WATERS MALDI SYNAPT Q-TOF MS instruments. The UPLC analysis was performed using a CSH C18 2.1X100mm 1.7um column. The following solvent system was used: 9 minute for acetonitrile/0.1% formic acid, 1:49 (by vol.), 11 minutes for acetonitrile/0.1% formic acid, 3:7 (by vol.) and 13 minutes for 100% acetonitrile. The flow rate was 0.3ml/min. The wavelength from 200-600nm was used for monitoring. Mass spectrum determination was performed in positive electrospray ionization (ESI) mode. The mass spectrometer was operated with a capillary voltage of 3500 V, the sampling cone at 20 V and the source block at 100℃ and the collision energy (CE) was 6 eV.

6. Circular dichroism measurement and computational design methods

Circular dichroism (CD) spectrum analysis was performed on the MOS-450/AF-CD-STP-A instrument at a protein concentration of 0.2mg/ml in 10mM KPB buffer. Wavelength scans were run from 195 to 250nm to detect the second structure of wild-type NHases and their variants. The 3D-structure model of NHase from *Comomonas testosteroni* 5-MGAM-4D was generated using Modeller software[6] based on the crystal structure of NHase from *Pseudomonas putida* NRRL-18668 (PDB ID: 3QXE). Models of all the mutants were constructed by Modeller as well. Structures with the lowest DOPE scores and the highest GA341 scores (GA341 = 1.000) were selected. CHARMm force field[7] was applied to all the structures and Accelrys Discovery Studio 2.5 was used to minimize structure energy. The substrate access tunnels of NHases were calculated and presented by CAVER analyst 1.0.[8] The Min. probe radius was 0.9 Å and the shell depth was 4 Å. The tunnel starting point was the

geometric center of NHase active site (C-T-L-C-S-C). The surface presentation of NHase was done using Pymol software with the default solvent radius.[9]

7. Supplementary Tables and Figures

8. Tables

Table S1. Regioselectivity of NHases and their variants towards 4-cyanobenzamide and terephthalamide.

Enzyme	4-cyanobenzamide (%)	Terephthalamide (%)
*Pp*NHase-WT	72.6	27.4
L37F	70.4	29.6
L37W	77.6	22.4
L37Y	92.2	7.8
*Ct*NHase-WT	14.9	85.1
F37L	17.0	83.0
F37P	14.9	85.1

Table S2. Regioselectivity of NHases and their variants towards 2-cyanobenzamide and phthalamide.

Enzyme	2-cyanobenzamide (%)	Phthalamide (%)
*Pp*NHase-WT	96.3	3.7
L37F	95.4	4.6
L37W	97.8	2.2
L37Y	97.6	2.4
*Ct*NHase-WT	96.1	3.9
F37L	97.6	2.4
F37P	98.0	2.0

Table S3. PCR primers for cloning and site-directed mutagenesis with mutanted bases undelined. Only the primers used for the selected variants were shown.

Construct	Primer	Sequence (5'-3')
*Pp*NHase-WT		GAAAAAACGGTCATGTCCCTGCTCCCGGCGCTGCTCGCCAACGGC
L37F	LF_up	GAAAAAACGGTCATGTCCCTG**TTT**CCGGCGCTG
	LF_down	GCCGTTGGCGAGCAGCGCCGG**AAA**CAGGGACAT
L37W	LW_up	GAAAAAACGGTCATGTCCCTG**TGG**CCGGCGCTG
	LW_down	GCCGTTGGCGAGCAGCGCCGG**CCA**CAGGGACAT
L37Y	LY_up	GAAAAAACGGTCATGTCCCTG**TAT**CCGGCGCTG
	LY_down	GCCGTTGGCGAGCAGCGCCGG**ATA**CAGGGACAT
*Ct*NHase-WT		GAAAAAACGGTCATGTCCCTGTTTCCGGCGCTGTTCGCCAACGGC
F37L	FL_up	GAAAAAACGGTCATGTCCCTG**CTC**CCGGCGCTG
	FL_down	GCCGTTGGCGAACAGCGCCGG**GAG**CAGGGACAT
F37P	FP_up	GAAAAAACGGTCATGTCCCTG**CCG**CCGGCGCTG
	FP_down	GCCGTTGGCGAACAGCGCCGG**CGG**CAGGGACAT

9. Figures

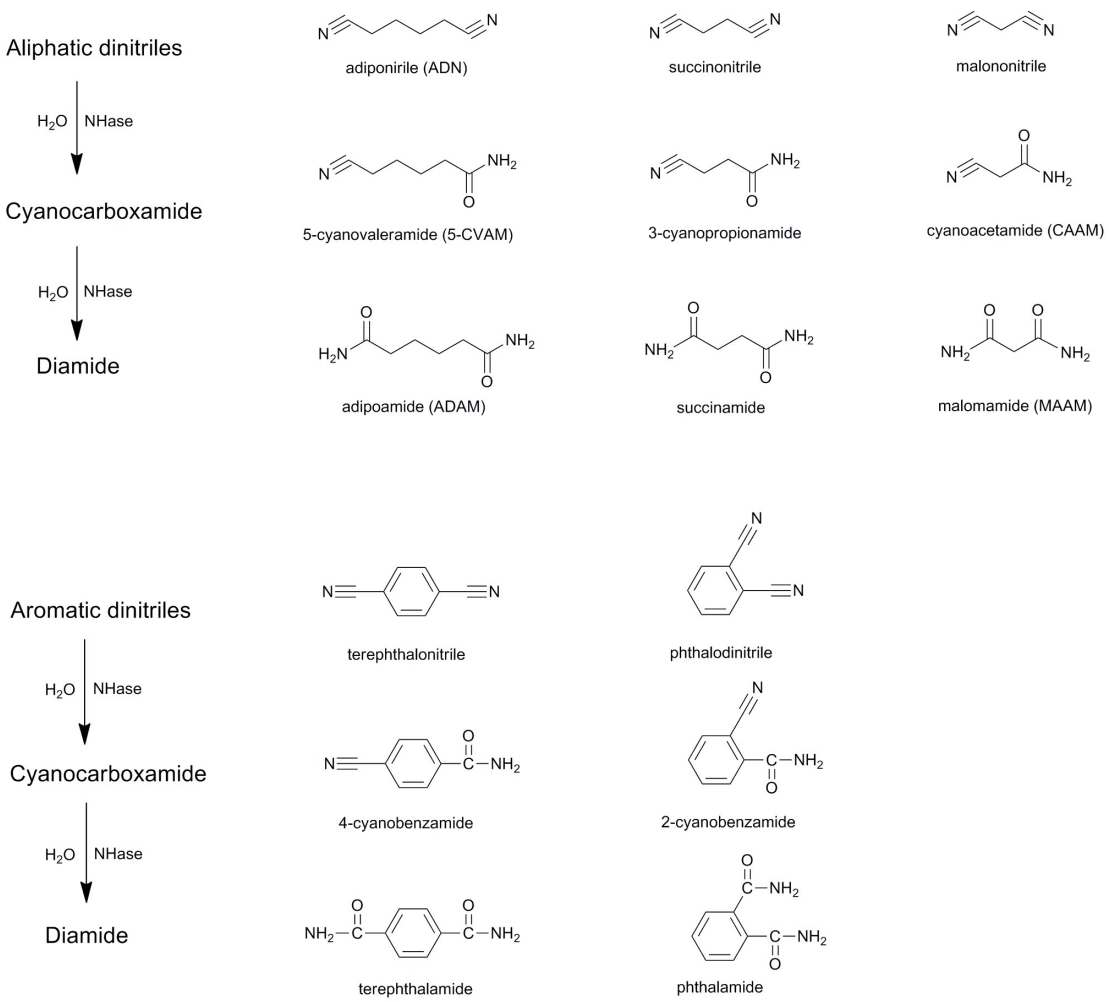

Fig S1. Regioselective hydration of dinitriles to their corresponding amides by NHase. All the substrates and products referred in this paper are shown.

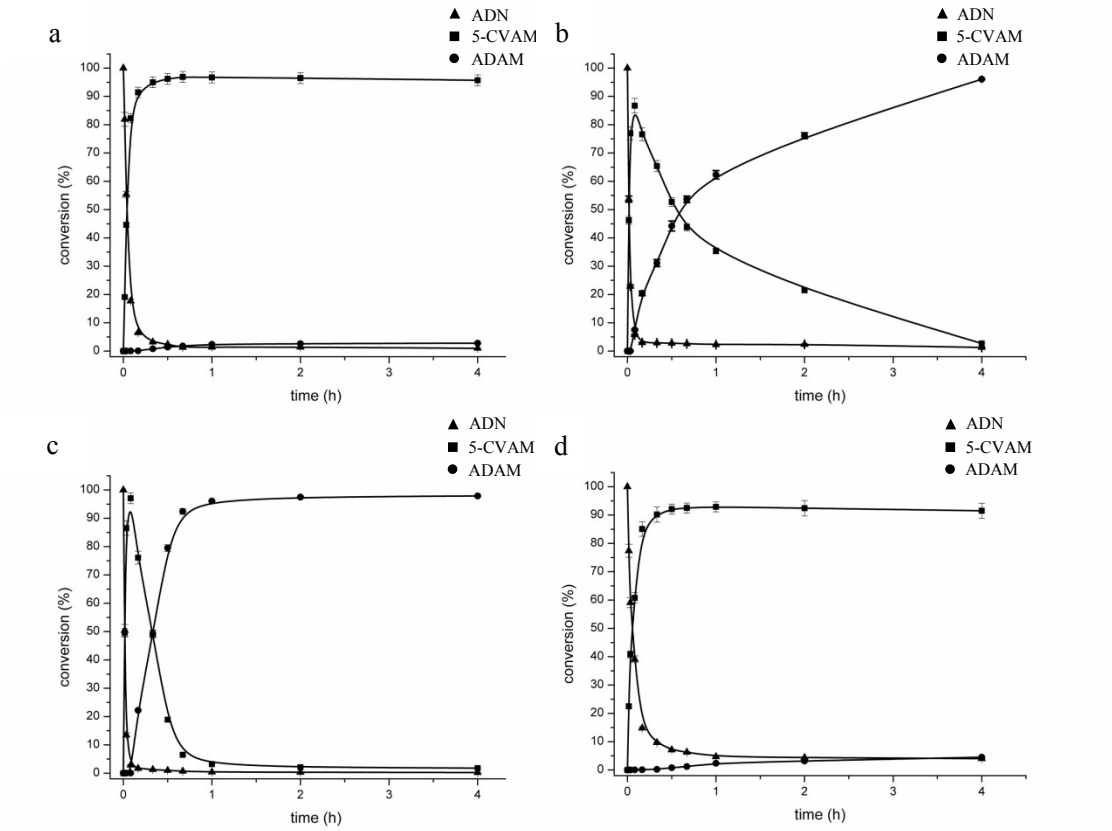

Fig S2. Conversion of ADN at 35℃ by purified NHase in KPB buffer (pH 7.4, 10mM). a. Conversion of ADN by the wild-type *Pp*NHase; b. Conversion of ADN by the L37Y mutant of *Pp*NHase; c. Conversion of ADN by the wild-type *Ct*NHase; d. Conversion of ADN by the F37P mutant of *Ct*NHase. ADN was added in a concentration of 20mM. Samples were analyzed by HPLC. The percentage of products 5-CVAM and ADAM is related to the concentration of ADN as 100%.

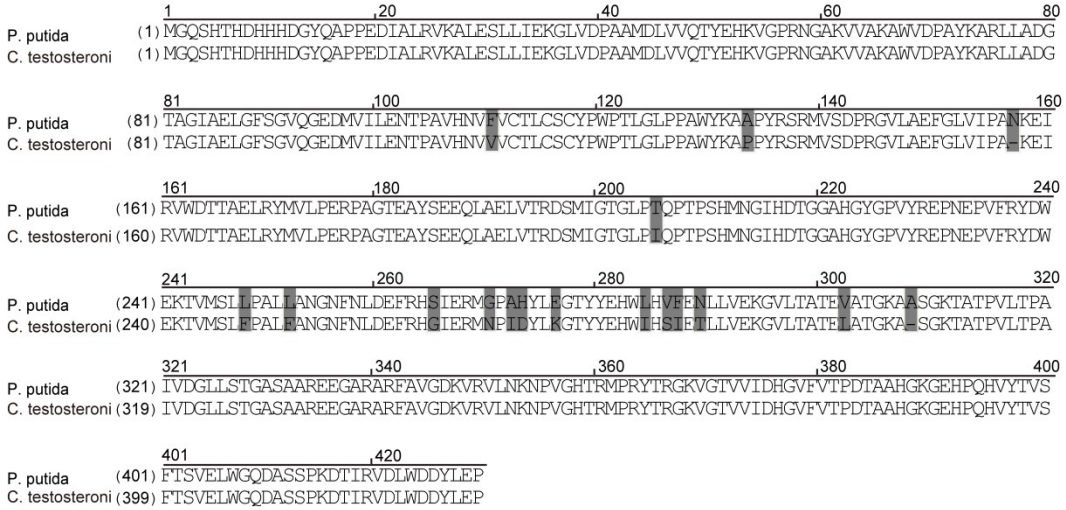

Fig S3. Sequence alignment of NHases from *P. putida*NRRL-18668 and *C.testosteroni* 5-MGAM-4D. Gaps in the aligned sequences were indicated by lines. Seventeen distinct amino acids were highlighted in grey.

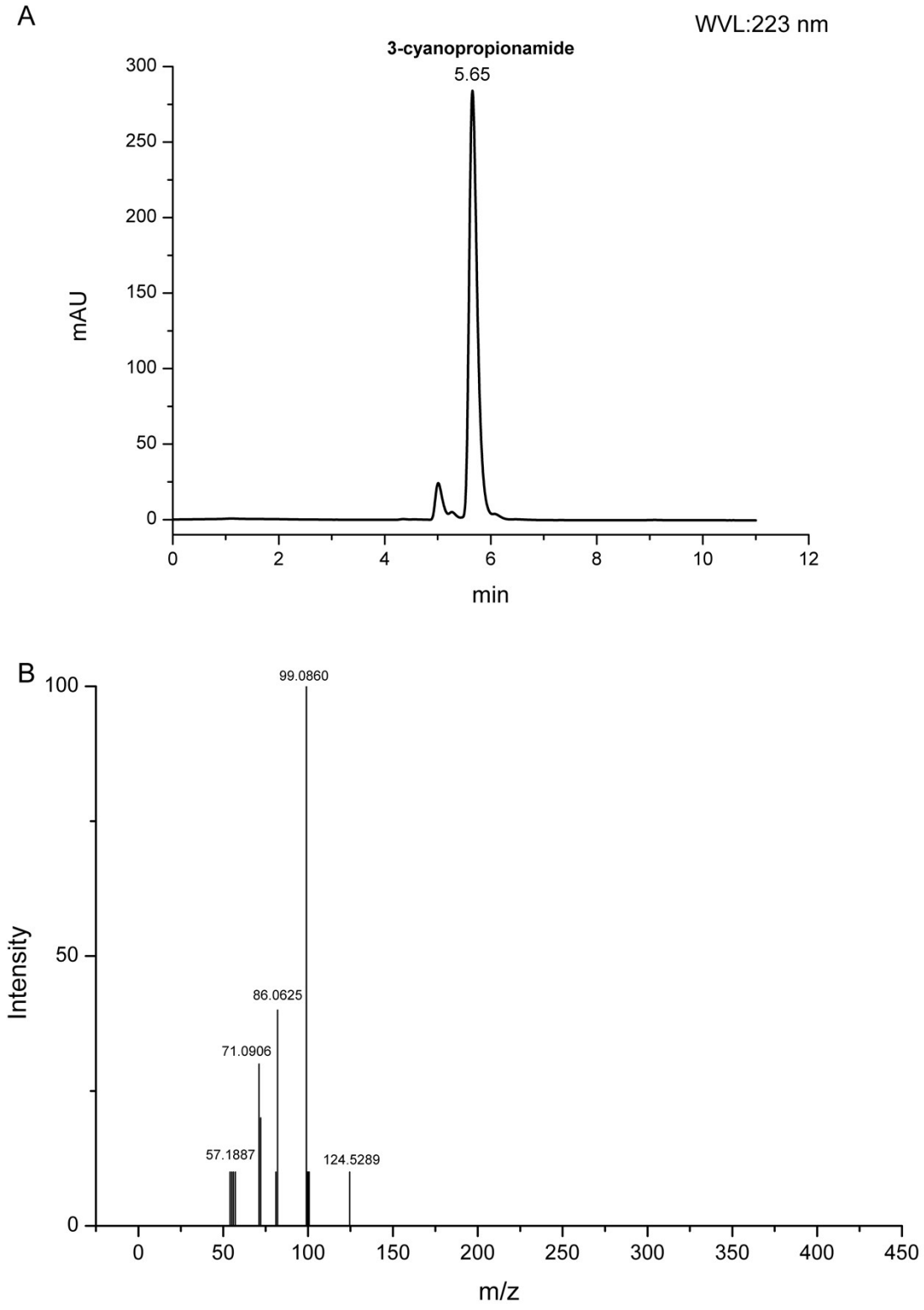

Fig S4. Identification of 3-cyanopropionamide. A: HPLC analysis of product of succinonitrile by wild-type *Pp*NHase. B: TOF MSMS ESI+ chromatogram of 3-cyanopropionamide. The product of succinonitrile by wild-type *Pp*NHase was characterized by LC-MS since we failed to obtain 3-cyanopropionamide commercially. The electrospray ionization mass spectrometry analysis indicated the presence of 3-cyanopropionamide because of the observation of a fragment at *m*/*z* 99.0860 (the mass of 3-cyanopropionamide, $C_4H_6ON_2$, equals to 98 approximately).

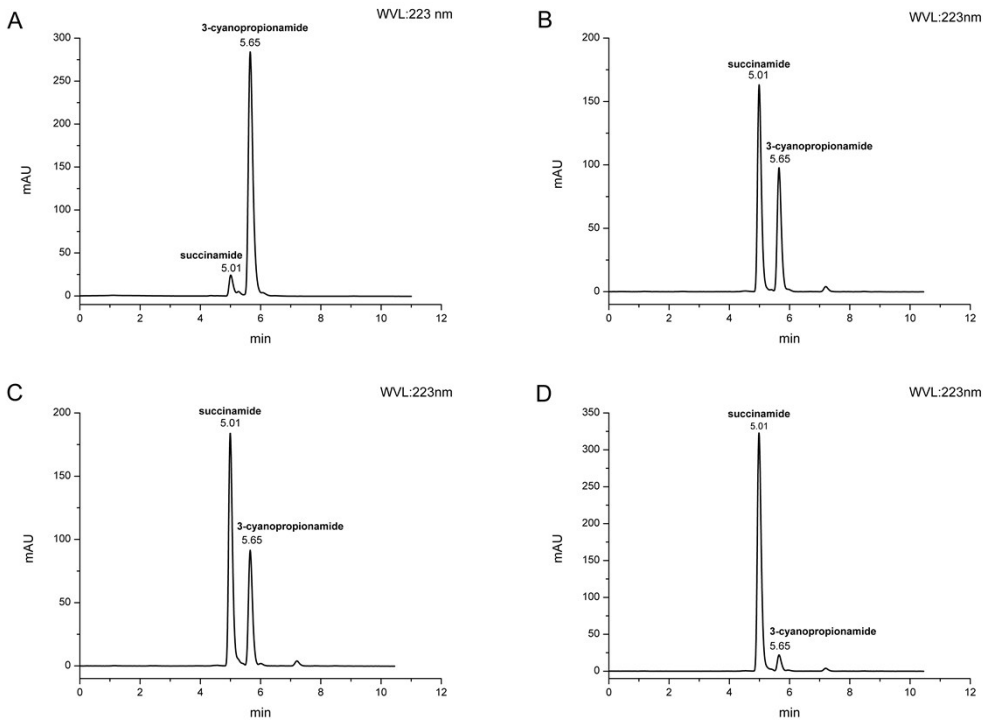

Fig S5. **HPLC analysis of enzyme regioselectivity of wild-type *Pp*NHase (A) and its variants L37W (B), L37F(C) and L37Y (D) towards succinonitrile.** Column C18; flow rate 1ml/min; mobile phase: acetonitrile /5mM K_2HPO_4/ H_3PO_4, pH 2.9, 7:12 (by vol.); succinamide = 5.01min, 3-cyanopropionamide = 5.65min.

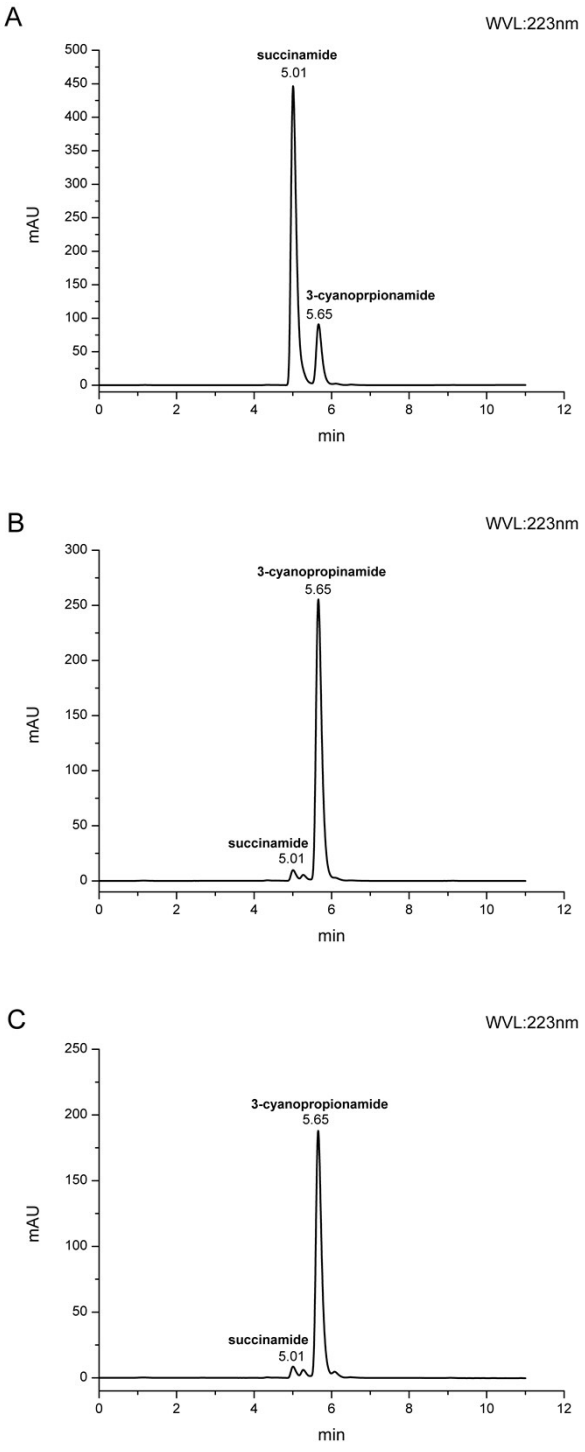

Fig S6. **HPLC analysis of enzyme regioselectivity of wild-type *Ct*NHase (A) and its variants F37L (B), F37P (C) towards succinonitrile.** Column C18; flow rate 1ml/min; mobile phase: acetonitrile /5mM K_2HPO_4/ H_3PO_4, pH 2.9, 7:12 (by vol.); succinamide = 5.01min, 3-cyanopropionamide = 5.65min.

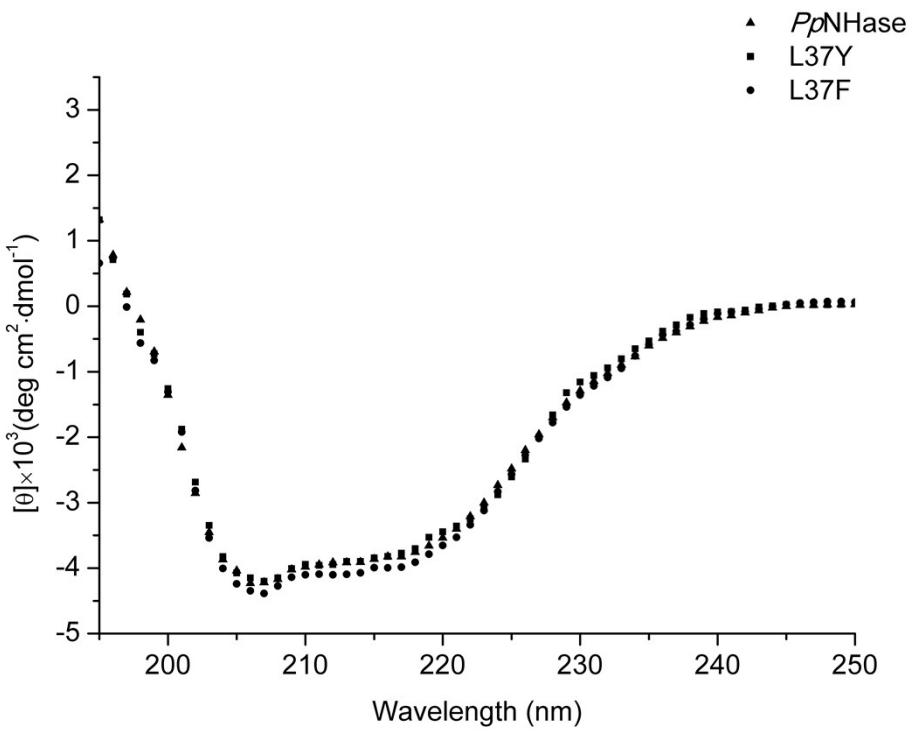

Fig S7. **CD spectra of wild-type *Pp*NHase, and its variants L37F and L37Y.** The data monitored in the far UV region (195-250nm) are shown.

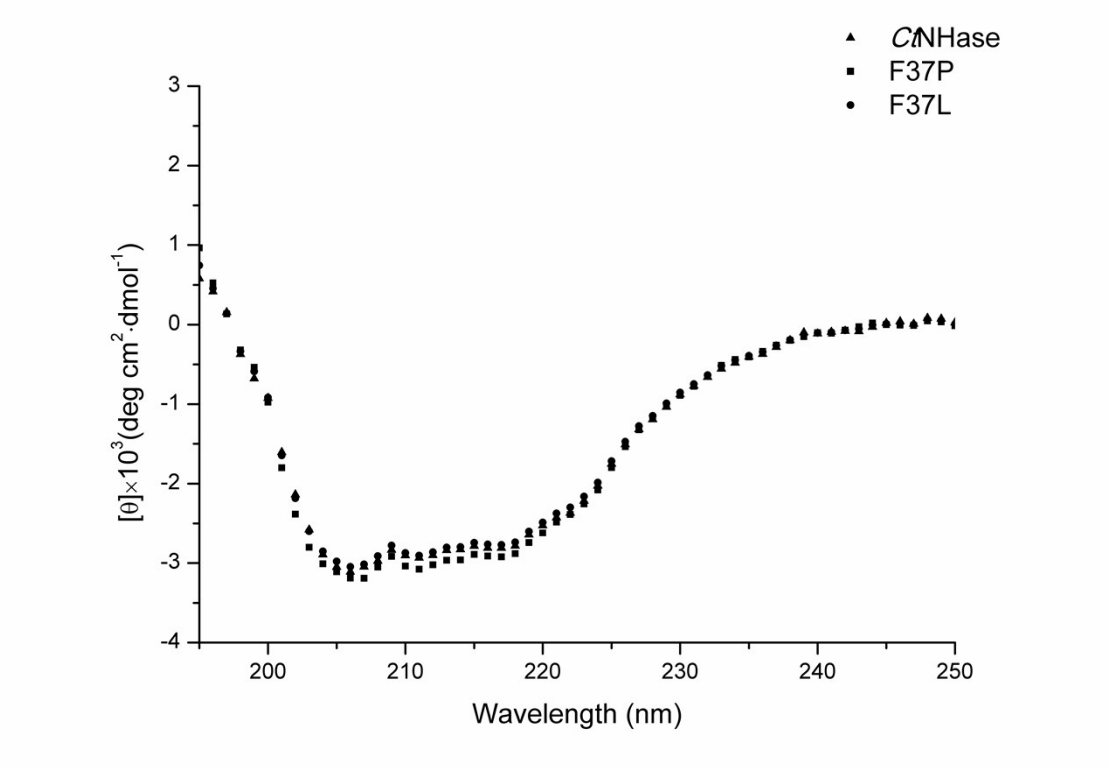

Fig S8. **CD spectra of wild-type *Ct*NHase , and its variants F37P and F37L.** The data monitored in the far UV region (195-250nm) are shown.

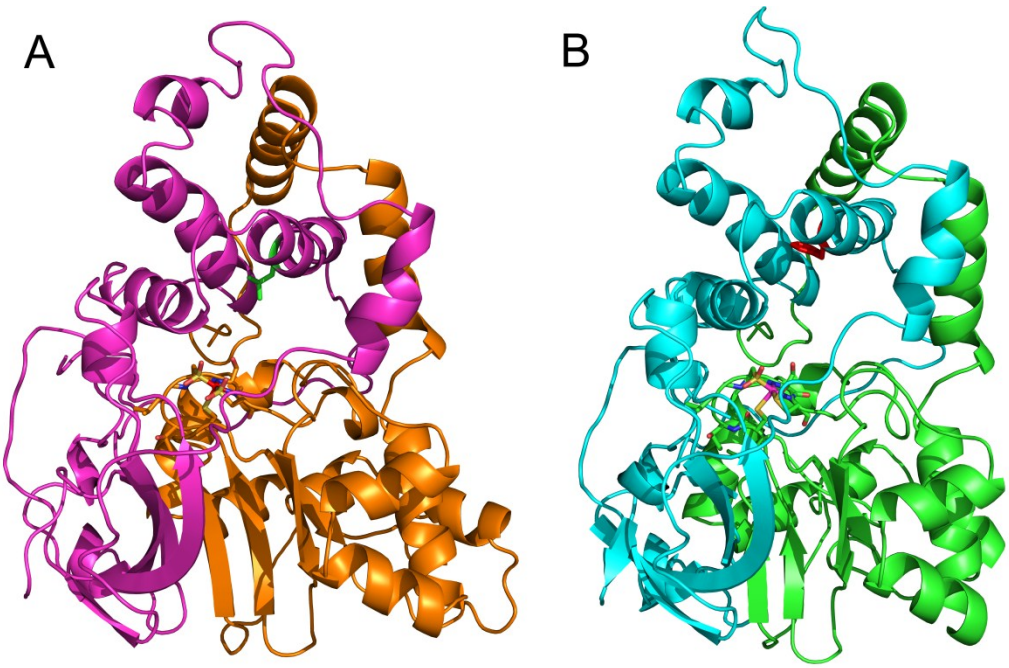

Fig S9. Overall structures of *Pp*NHase and *Ct*NHase. The active sites were shown in sticks. A: Crystal structure of *Pp*NHase (PDB ID: 3QXE). Orange cartoon represented α subunit of *Pp*NHase, β subunit was colored in magenta. The βLeu37 residue was shown as green sticks. B: Modeled structure of *Ct*NHase. Cyan cartoon representedα subunit of *Pp*NHase, β subunit was colored in green. The βPhe37 residue was shown as red sticks.

10.References

1. Y. Cui, W. Cui, Z. Liu, L. Zhou, M. Kobayashi and Z. Zhou, *Biochemical and Biophysical Research Communications*, 2014, **450**, 401-408.
2. M. Laible and K. Boonrod, *Journal of Visualized Experiments : JoVE*, 2009, DOI: 10.3791/1135, 1135.
3. Y. Shen, M. Wang, X. Li, J. Zhang, H. Sun and J. Luo, *Journal of Chemical Technology & Biotechnology*, 2012, **87**, 1396-1400.
4. T. Nagasawa, K. TAKEUCHI and H. Yamada, *European Journal of Biochemistry*, 1991, **196**, 581–589.
5. *SHOWA DENKO KK (JP) Pat.,* EP0989115, 2000.
6. A. Fiser and A. Šali, in *Methods in Enzymology*, Academic Press, 2003, vol. Volume 374, pp. 461-491.
7. B. R. Brooks, R. E. Bruccoleri, B. D. Olafson, D. J. States, S. Swaminathan and K. Martin, *Journal of Computational Chemistry*, 1983, **4**, 187-217.
8. M. Petřek, M. Otyepka, P. Banáš, P. Košinová, J. Koča and J. Damborský, *Bmc Bioinformatics*, 2006, **7**, 164-193.
9. L. M. Surhone, M. T. Tennoe and S. F. Henssonow, *PyMOL*, Betascript Publishing, 2012.