Electronic Supplementary Material (ESI) for Catalysis Science & Technology.

An anchoring residue adjacent to the substrate access tunnel entrance of nitrile hydratase directs its catalytic activity towards 3-cyanopyridine

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Materials and Methods

Strains, plasmids and chemical reagents

The NHase gene from *Pseudonocardia thermophila* JCM3095 was synthesized and cloned to pET-24a plasmid by GENEWIZ Biotech Co., Ltd. (South Plainfield, NJ, USA). The NHases were heterologously expressed in *Escherichia coli* BL21 (DE3). 3-cyanopyridine and nicotinamide were purchased from TCI (Shanghai) Development Co., Ltd. All other chemicals were obtained commercially.

Construction of mutants

Site-directed saturation mutagenesis was performed following a whole plasmid PCR protocol[1]. PrimeSTAR HS DNA Polymerase from Takara Bio Inc. (Kusatsu, Japan) was used for all PCRs. The PCR mixture was described previously[2]. Primers were shown in Table S2.

Expression and purification of enzymes

The cultivation and expression process were the same as described in our previous study[2]. AKTA purifier (GE Healthcare UK Ltd., Amersham, UK) was used for enzyme purification. A StrepTrap HP column (GE Healthcare UK Ltd.) equilibrated with binding buffer (20 mM $Na_2HPO_4 \cdot 12H_2O$, 280 mM NaCl, 6 mM KCl, pH 7.4) was used for purifying the NHases with a Strep tag at the N terminus of the β subunit. After injecting the supernatant into the column, proteins were eluted using elution buffer (binding buffer containing 25 mM desthiobiotin, pH 7.4). The active fractions

were collected, concentrated to 500 μL by ultrafiltration, and applied to a Superdex 200 10/300 GL column (GE Healthcare UK Ltd.).

Enzymatic assay

The specific activity of NHase was determined by the increase of the nicotinamide. The reaction mixture contained 10 mM kalium phosphate buffer (KPB) buffer (8.02 mL of 1 M K_2HPO_4 buffer and 1.98 mL of 1 M KH_2PO_4 buffer in 1 L ddH_2O) (pH 7.4), 200 mM 3-cyanopyridine, and 10 μL of the appropriate amount of the enzyme solution (enzyme in 10 mM KPB buffer). The reaction was carried out at 30°C for 10 min. The amide concentration was analyzed by HPLC equipped with a HITACHI C18 reverse phase column (solvent: acetonitrile/water = 1:2 (v/v)). The detection wavelength was set to 215 nm. One unit (U) of NHase activity was defined as the amount of enzyme that produced 1 μmol amide product per minute under the above assay conditions.

For the determination of the substrate tolerance of NHase, 100% relative activity was defined as the specific activity of NHase reacted with 0.2 M 3-cyanopyridine, while the substrate tolerance of the target enzyme was defined by the relative activity of the enzyme treated with 1 M 3-cyanopyridine.

The enzyme concentration for the measurement of kinetic parameters toward 3-cyanopyridine was set to 0.2 mg/mL. The reaction mixture contained: 10 μL purified enzyme (concentration: 0.2 mg/mL), 490 μL 3-cyanopyridine diluted in 10 mM KPB buffer (pH 7.4). The concentrations of 3-cyanopyridine were 10, 20, 50, 100, and 200

mM. The reactions were carried out under 30°C for 2 min and were terminated with 500 μL of acetonitrile.

Thermal denaturation analysis

The half-life of target enzymes was determined by pre-incubating enzyme solutions in the absence of a substrate at 60°C for different durations. The residual enzyme activity was then measured under standard conditions described above. The half-life ($t_{1/2}$) was defined as the time point at which the residual activity of NHase retained 50% of its original activity.

The melting temperature (T_m) was defined as the temperature when half of the protein was unfolded. Differential scanning calorimetry (Nano DSC, TA Instruments, New Castle, NY, USA) was used to capture changes in energy during the protein folding and unfolding processes. Enzymes were dialyzed against potassium phosphate buffer at a concentration of 0.3 mg/mL, and the instrument was scanned up and down from 25 to 100 ℃ using a 1 ℃/min scan rate and scanned buffer alone to obtain background measurements. The heat capacity results were analyzed using the Nano DSC analysis software, and the T_m was calculated from a fitted curve.

Computational design of NHase

The 3-dimensional structure of the NHase mutant was constructed using the trRosetta online tool[3]. VERIFY3D and PROCHECK were applied to evaluate the constructed model[4, 5]. The docking of 3-cyanopyridine to the active site of WT *Pt*NHase and its mutant were evaluated using Glide[6]. Molecular dynamics (MD) simulations were

performed using NAMD 2.14 with the Charmm27 force field for 100 ns[7]. The proteins were put into a cubic water box (73 × 75 × 88 Å3) with a layer of water of at least 10 Å in each dimension. A cutoff of 12 Å for non-bonded interactions was applied. To account for nonstandard amino acids and cobalt III ions at the active site that cannot fit the Charmm27 force field, our previously determined parameters were used.[2] The substrate access tunnel and the size of tunnel entrance were calculated and analyzed using CAVER analyst 2.0[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 the NHase active site. CaverWeb was used for calculating the binding energies between substrate 3-cyanopyrinde and NHase during substrate transport[9].

Table S1. Interactions between residue βGlu50 and its surrounding residues in WT *Pt*NHase and its βE50L mutant.

WT (4 hbonds)			E50L (3 hbonds)		
Donor	Acceptor	Occupancy[a]	Donor	Acceptor	Occupancy
βGLY54-Main	βGLU50-Main	15.06%	βGLY54-Main	βLEU50-Main	19.90%
βGLY47-Main	βGLU50-Side	13.76%	βLEU50-Main	βGLY47-Main	0.14%
βGLU50-Main	βGLY47-Main	0.08%	βPHE53-Main	βLEU50-Main	0.01%
βGLU50-Main	βASP49-Side	2.59%			

[a] The time occupancy of the hbonds during the whole 100 ns MD simulation.

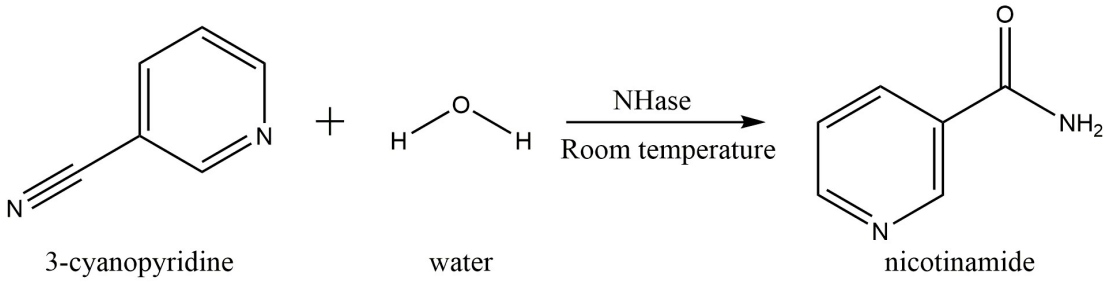
The detailed information of hydrogen bonds was calculated by the 'Hydrogen Bonds' plugin in VMD based on the MD trajectories.

Table S2. Primers used in present study.

Primers	Sequence (5'-3')
βR42G-Foward	GCCATGTTTCCGGCAACCTTT**GGT**GCAGGTTTTAAAGGTCTG
βR42A-Foward	GCCATGTTTCCGGCAACCTTT**GCA**GCAGGTTTTAAAGGTCTG
βR42V-Foward	GCCATGTTTCCGGCAACCTTT**GTT**GCAGGTTTTAAAGGTCTG
βR42L-Foward	GCCATGTTTCCGGCAACCTTT**CTG**GCAGGTTTTAAAGGTCTG
βR42I-Foward	GCCATGTTTCCGGCAACCTTT**ATT**GCAGGTTTTAAAGGTCTG
βR42M-Foward	GCCATGTTTCCGGCAACCTTT**ATG**GCAGGTTTTAAAGGTCTG
βR42C-Foward	GCCATGTTTCCGGCAACCTTT**TGT**GCAGGTTTTAAAGGTCTG
βArg42-Reverse	TTCATCCAGACCTTTAAAACCTGC
βE50G-Foward	GCAGGTTTTAAAGGTCTGGAT**GGT**TTTCGTTTTGGCATTGAA
βE50A-Foward	GCAGGTTTTAAAGGTCTGGAT**GCA**TTTCGTTTTGGCATTGAA
βE50V-Foward	GCAGGTTTTAAAGGTCTGGAT**GTT**TTTCGTTTTGGCATTGAA
βE50L-Foward	GCAGGTTTTAAAGGTCTGGAT**CTG**TTTCGTTTTGGCATTGAA
βE50I-Foward	GCAGGTTTTAAAGGTCTGGAT**ATT**TTTCGTTTTGGCATTGAA
βE50M-Foward	GCAGGTTTTAAAGGTCTGGAT**ATG**TTTCGTTTTGGCATTGAA
βE50C-Foward	GCAGGTTTTAAAGGTCTGGAT**TGT**TTTCGTTTTGGCATTGAA

Primers	Sequence (5’-3’)
βGlu50-Reverse	CATCTGTTCAATGCCAAAACGAAA
βR82G-Foward	ACCTATATTCATCATGGCGTG**GGT**ACCGGTAAAATTGATCTG
βR82A-Foward	ACCTATATTCATCATGGCGTG**GCA**ACCGGTAAAATTGATCTG
βR82V-Foward	ACCTATATTCATCATGGCGTG**GTT**ACCGGTAAAATTGATCTG
βR82L-Foward	ACCTATATTCATCATGGCGTG**CTG**ACCGGTAAAATTGATCTG
βR82I-Foward	ACCTATATTCATCATGGCGTG**ATT**ACCGGTAAAATTGATCTG
βR82M-Foward	ACCTATATTCATCATGGCGTG**ATG**ACCGGTAAAATTGATCTG
βR82C-Foward	ACCTATATTCATCATGGCGTG**TGT**ACCGGTAAAATTGATCTG
βArg82-Reverse	TTCTTCCAGATCAATTTTACCGGT
βE114G-Foward	CCGCGCCATGAACAGAAACCG**GGT**CTGATTGAATTTGTGAAT
βE114A-Foward	CCGCGCCATGAACAGAAACCG**GCA**CTGATTGAATTTGTGAAT
βE114V-Foward	CCGCGCCATGAACAGAAACCG**GTT**CTGATTGAATTTGTGAAT
βE114L-Foward	CCGCGCCATGAACAGAAACCG**CTG**CTGATTGAATTTGTGAAT
βE114I-Foward	CCGCGCCATGAACAGAAACCG**ATT**CTGATTGAATTTGTGAAT
βE114M-Foward	CCGCGCCATGAACAGAAACCG**ATG**CTGATTGAATTTGTGAAT

Primers	Sequence (5’-3’)
βE114C-Foward	CCGCGCCATGAACAGAAACCG**TGT**CTGATTGAATTTGTGAAT
βGlu114-Reverse	GGCCTGATTCACAAATTCAATCAG



3-cyanopyridine water nicotinamide

Scheme S1 Hydration of 3-cyanopyridine for the production of nicotinamide with NHase.

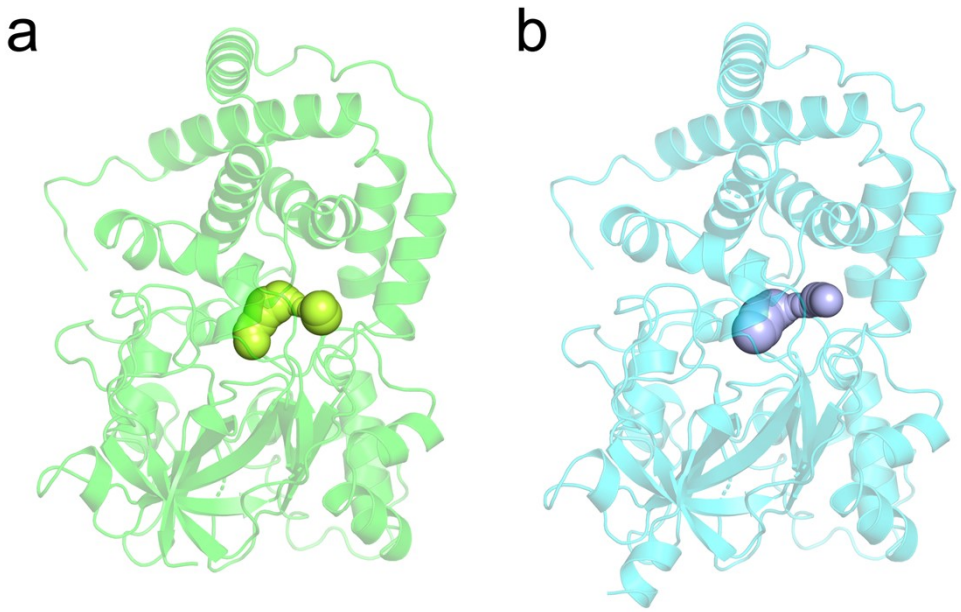


Fig. S1 Cartoon model of the substrate access tunnel of a) WT *Pt*NHase and its b) βE50L mutant. Tunnels are shown as spheres.

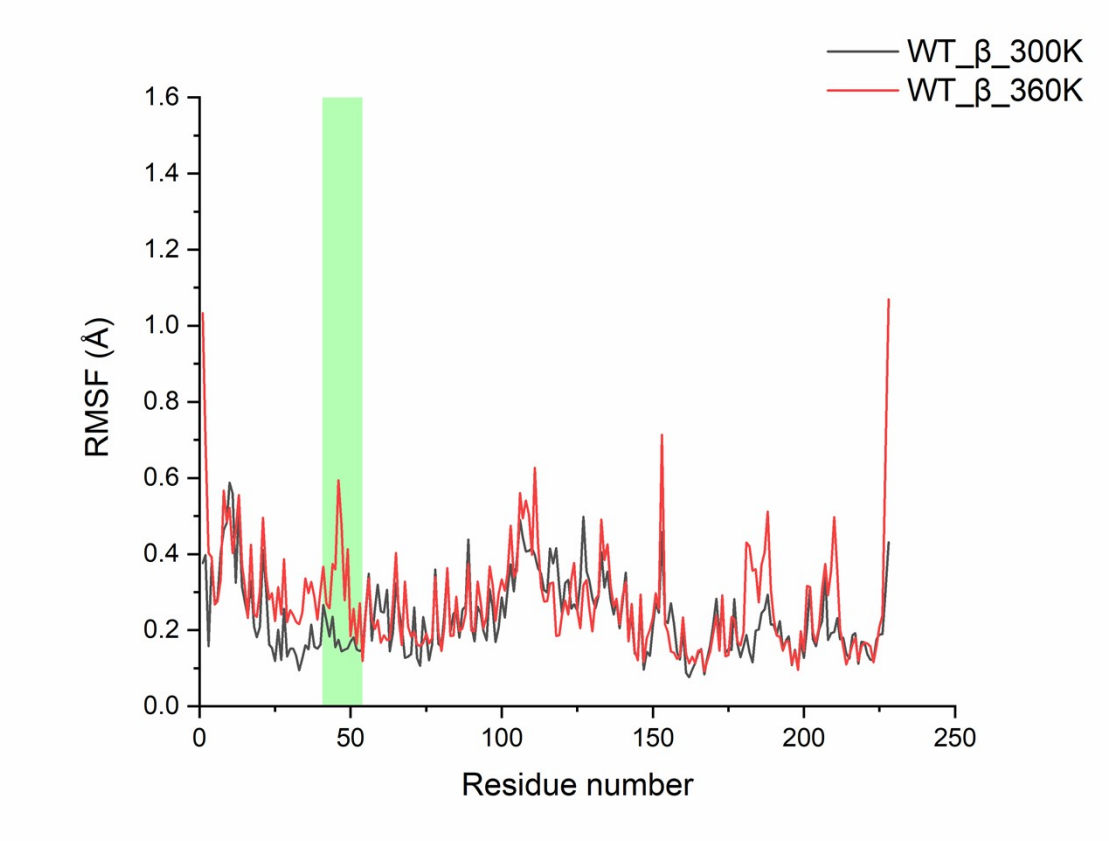


Fig. S2 Comparison of RMSF values of the β subunit of *Pt*NHase under different temperatures. The loop region was highlighted in green.

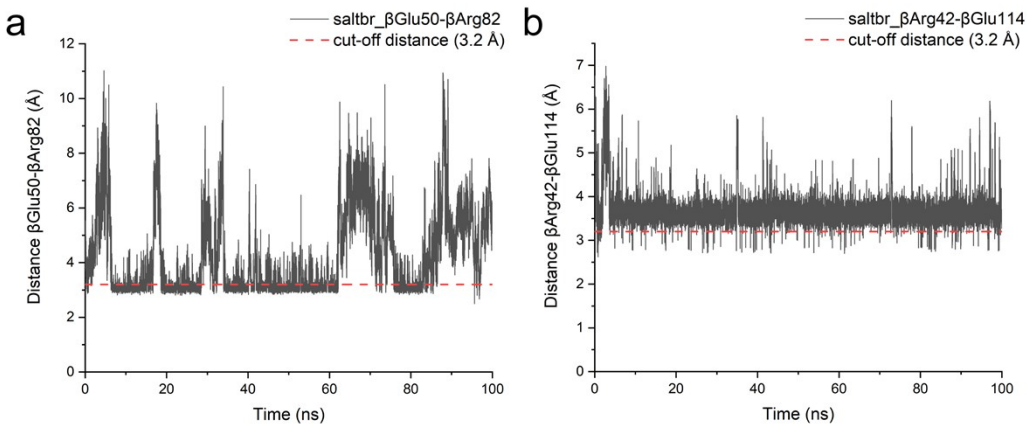


Fig. S3 Salt bridge analysis. a) The distance between βGlu50 and βArg82 of *Pt*NHase during 100 ns MD simulation. b) The distance between βArg42 and βGlu114 of *Pt*NHase during 100 ns MD simulation.

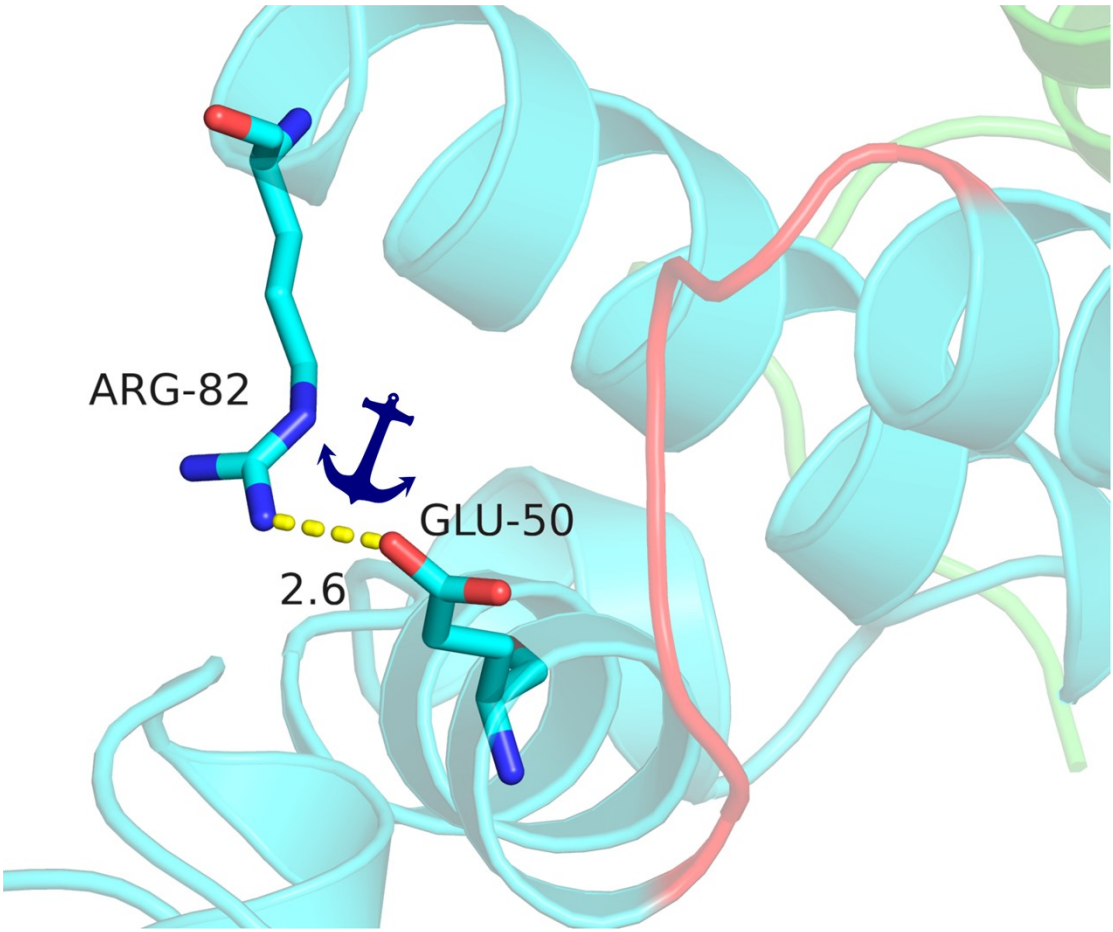


Fig. S4 Salt bridge formed between the anchoring residue βGlu50 and its surrounding residue βArg82 of the WT *Pt*NHase. Salt bridge was shown in yellow dashed line.

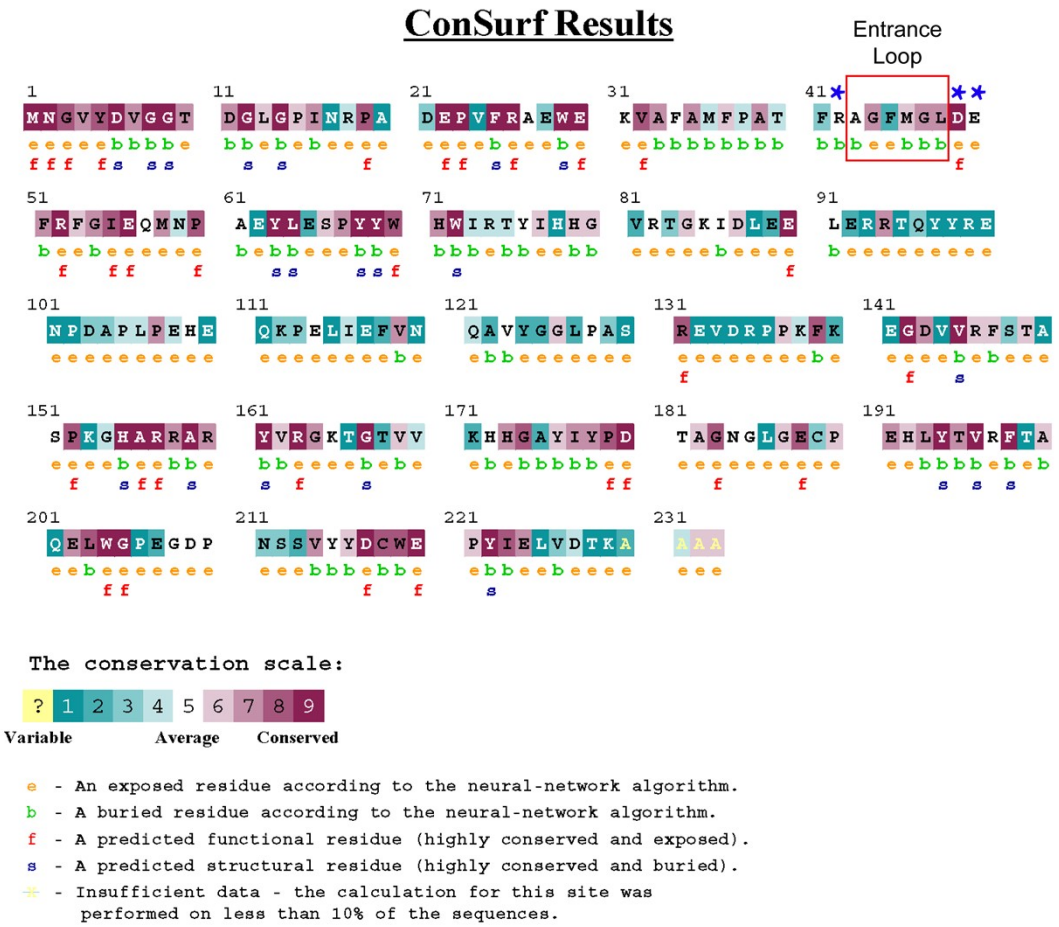


Fig. S5 Residue conservative analysis of β subunit of *Pt*NHase using Consurf. The substrate access tunnel entrance loop region was circled in red. The potential anchoring residues were marked as blue stars.

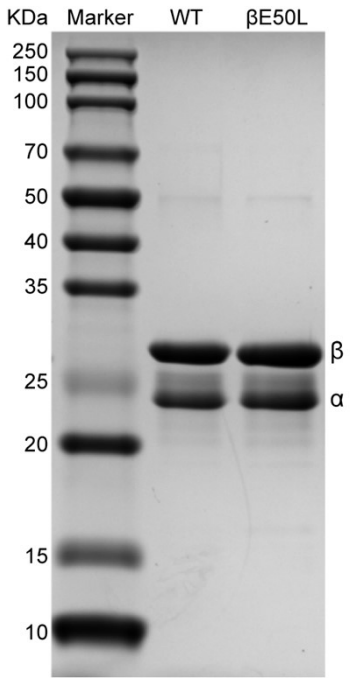


Fig. S6 SDS-PAGE of the purified WT PtNHase and its βE50L mutant.

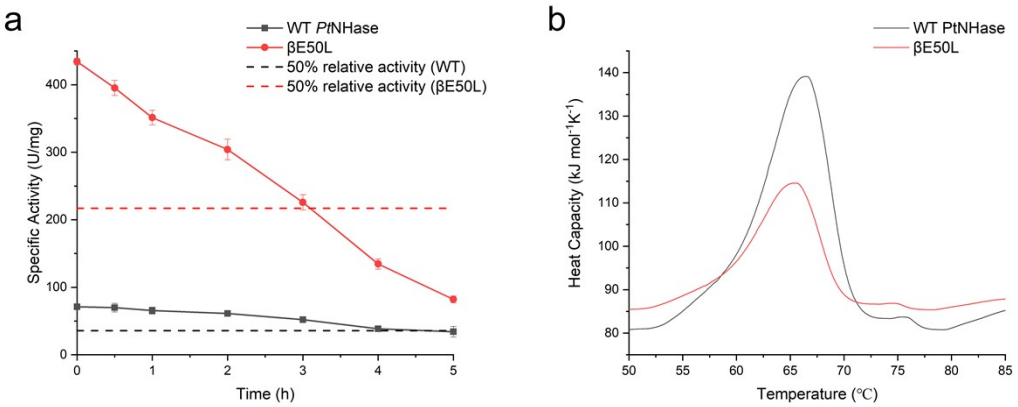


Fig. S7 Determination of the a) half-life time and b) melting temperature of the WT *Pt*NHase and its βE50L mutant. The half-life of target enzymes was determined by pre-incubating enzyme solutions (10 mM kalium phosphate buffer (8.02 mL of 1 M K_2HPO_4 buffer and 1.98 mL of 1 M KH_2PO_4 buffer in 1 L ddH_2O, pH 7.4)) in the absence of a substrate at 60°C for different durations.

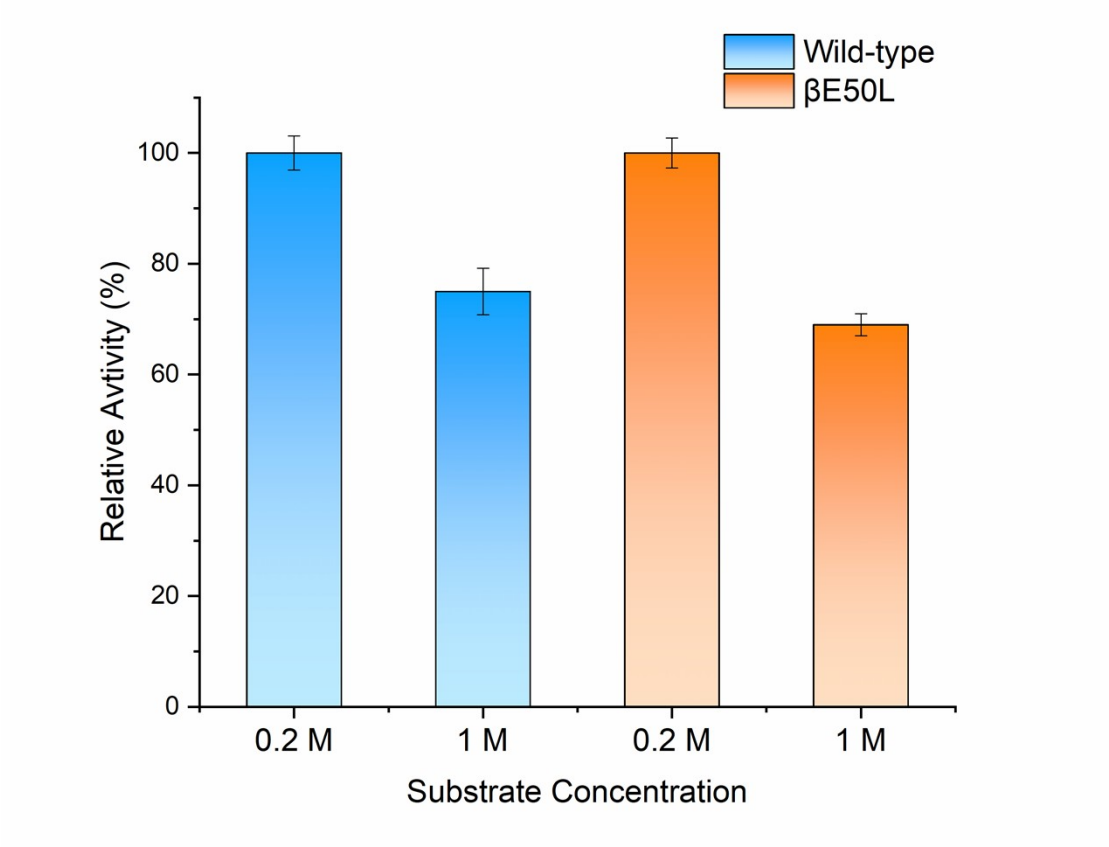


Fig. S8 Relative activity of the WT *Pt*NHase and its βE50L mutant under the treatment of 1 M 3-cyanopyridine.

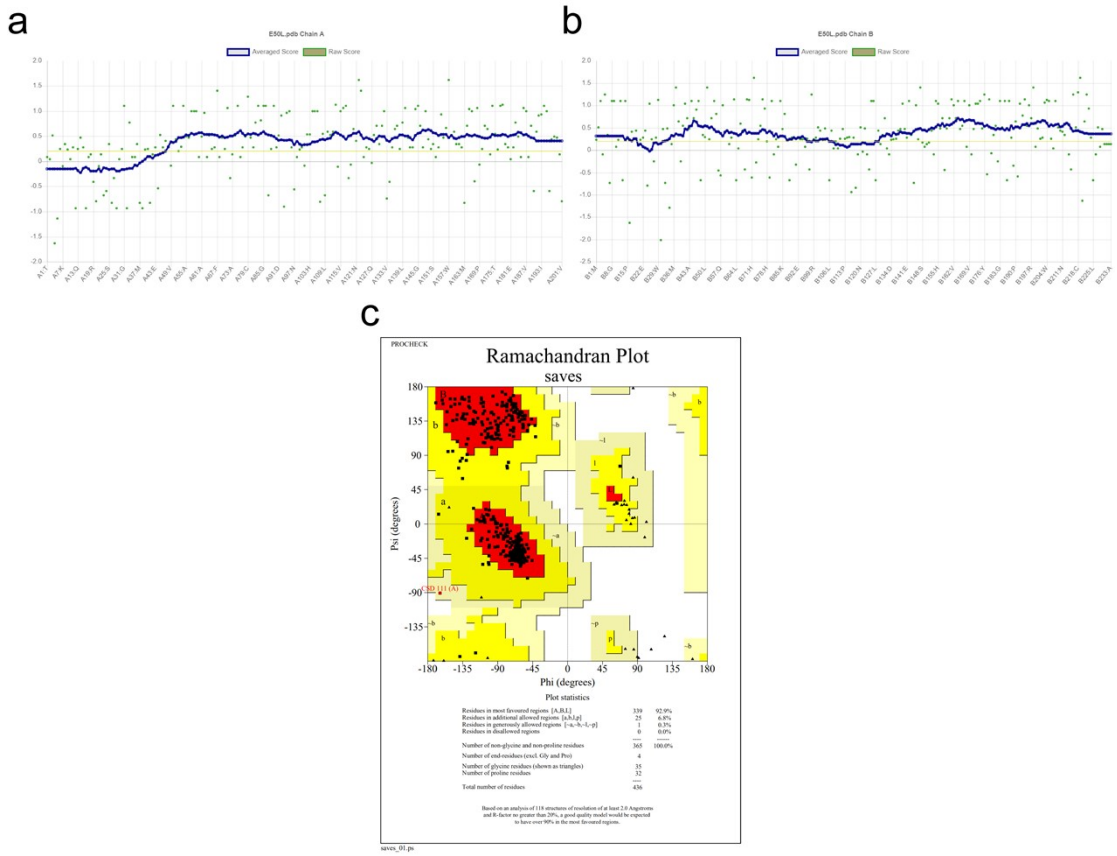


Fig. S9 Structure evaluation of the constructed βE50L mutant model by VERIFY3D on a) the α subunit, b) the β subunit and c) the Ramachandran plot of the model.

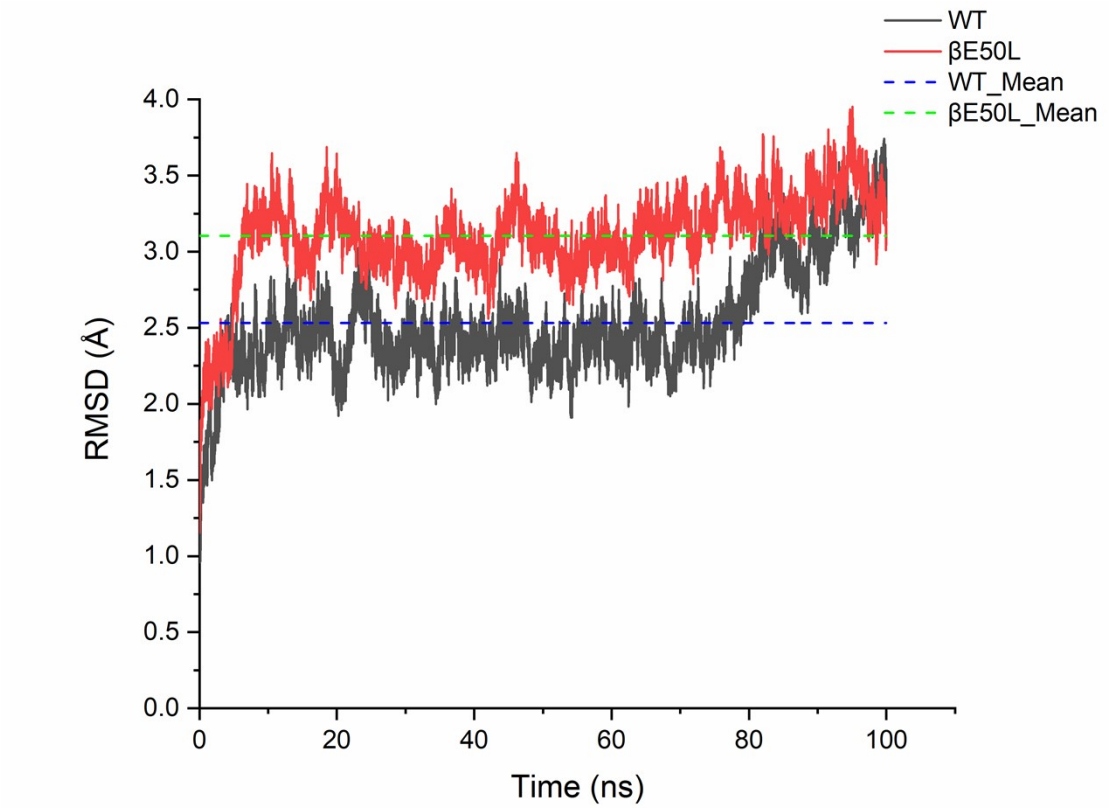


Fig. S10 RMSD values for simulation of WT *Pt*NHase and its βE50L mutant at 300 K

References

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