Supporting Information for

The Active Site Sulfenic Acid Ligand in Nitrile Hydratases can Function as a Nucleophile

Salette Martinez,[1,2‡] Rui Wu,[2‡] Ruslan Sanishvili,[3] Dali Liu,[2*] and Richard Holz[1*]

Contribution from the [1]Department of Chemistry, Marquette University, Milwaukee, Wisconsin 53201, the [2]Department of Chemistry and Biochemistry, Loyola University Chicago, Chicago, Illinois 60660, and the [3] X-ray Science Division, Advanced Photon Source, Argonne National Laboratory 9700 S. Cass Ave., Lemont, 60439.

Materials and Methods

Materials. Acrylonitrile, 1-butaneboronic acid, phenylboronic acid, sodium citrate tribasic dehydrate, and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) were purchased from Sigma-Aldrich. All reagents were of the highest purity available and used as received without further purification.

Expression and Purification of PtNHase. Recombinant *Pt*NHase was expressed and purified according to published methods followed by a gel-filtration chromatography step.[1]

Kinetic Assay. The kinetic constants were determined by measuring the hydration of acrylonitrile to acrylamide in 50 mM Tris-HCl, pH 7.5 at 25 °C. The rate of the acrylonitrile hydration was followed continuously by monitoring the increase in absorbance at 225 nm using an absorption coefficient of $\Delta\varepsilon_{225} = 2.9\ mM^{-1}\ cm^{-1}$. Assays were performed in a 1 mL quartz

cuvette on a Shimadzu UV-2450 PC spectrophotometer equipped with a TCC temperature controller, in triplicate from three independent enzyme samples. The reaction was initiated by adding the enzyme to the reaction mixture (1 mL) containing various final concentrations of the substrate (0 – 60 mM). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the production of 1 μmol of acrylamide per minute at 25 °C.

PtNHase inhibition with 1-butaneboronic acid. For determination of the inhibition constant, the kinetic assay was performed as describe above with the addition of various final concentrations of 1-butaneboronic to the assay mixture and fixed concentrations of acrylonitrile. The reactions were initiated by the addition of *Pt*NHase (final concentration of 3 – 4 nM). The initial velocity data were analyzed by nonlinear regression with the Enzyme Kinetics Module in SigmaPlot 12.3 (Systat Software, San Jose, CA) using the *Single Substrate-Single Inhibitor* function to determine the inhibition mechanism and the K_i constant.

Slow-binding inhibition by phenylboronic acid assay started with enzyme. For determination of the kinetic inhibition constants, the kinetic assay was performed as describe above with the addition of various final concentrations of phenylboronic acid to the assay mixture. The reactions were initiated by the addition of *Pt*NHase (final concentration of 0.56 – 0.7 nM) and monitored continuously for 10 min (Figure S1A).

Slow-binding inhibition by phenylboronic acid assay started with enzyme-inhibitor complex. *Pt*NHase (188 – 237 nM) was preincubated for 60 min at room temperature with phenylboronic acid (0 – 2.4 μM) to form the enzyme-inhibitor complex. Reactions were initiated by the addition of 3 μL of the preincubation mixture to a 1 mL reaction mixture containing the substrate (Figure S1B); this resulted in a 333-fold dilution of the enzyme and inhibitor (0.56 – 0.7 nM *Pt*NHase and 0 – 7.2 nM phenylboronic acid, final concentrations).

Dialysis of the Enzyme-Inhibitor Complex. The reversibility of the *Pt*NHase-PBA was examined. *Pt*NHase (0.2 μM) was preincubated with 20 μM of phenylboronic acid for 1 h at room temperature. A control sample that contained no inhibitor was also prepared. After the 1 h preincubation, 3 μL of each sample was taken and used to initiate a 1 mL reaction containing 8 mM acrylonitrile. The remaining the samples were subjected to overnight dialysis at 4 °C in 50 mM HEPES pH 7.5 and 300 mM NaCl. Dialysis was performed in a 0.5 mL Slide-A-Lyzer® G2 Dialysis Cassette (Thermo Scientific) with molecular-weight cutoff of 10 K. After overnight dialysis, 3 μL of each sample was taken and used to initiate a 1 mL reaction containing 8 mM acrylonitrile. The final concentration of enzyme and inhibitor in the reaction was 0.6 nM and 60 nM, respectively. The dialyzed sampled regained ~100% of its activity showing that phenylboronic acid is a slow-binding reversible inhibitor of *Pt*NHase.

Data analysis: Slow-binding inhibition by phenylboronic acid. The slow-binding inhibition properties of phenylboronic acid were analyzed assuming the mechanism in Scheme 1, where K_i ($K_i = k_1/k_2$) is the equilibrium inhibition constant for the formation of the initial complex, EI, k_1 and k_2 are the forward and reverse rate constants for the equilibrium between the EI and the EI* complex.[2] The overall inhibition constant (K_i^*) is described by $K_i^* = K_i\, k_4/k_3 + k_4$.[2]

Scheme 1

$$\mathrm{E + I} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{EI} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{EI^*}$$

The reaction time courses (Figure S1) were fit to Equation 1 using OriginPro 9.0 (OriginLab,

$$A = v_s\, t + \frac{(v_0 - v_s)(1 - e^{-k_{obs}t})}{k_{obs}} + A_0 \qquad (1)$$

Northampton, MA), where A is the change in absorbance at 225 nm at any time t, v_0 is the initial velocity, v_s is the final steady-state velocity, k_{obs} is the apparent first-order rate constant for the interconversion between v_0 and v_s, and A_0 is included to correct any possible deviation from the baseline. The initial and final steady-states velocities were then analyzed separately by nonlinear regression with the Enzyme Kinetics Module in SigmaPlot 12.3 (Systat Software, San Jose, CA) using the *Single Substrate-Single Inhibitor* function to determine the inhibition mechanism and the K_i and K_i^* constants, respectively.

Crystallization and Data Collection. Crystallization of *Pt*NHase was performed following the previously reported protocol with slight modifications in freezing and soaking conditions; the native crystals grew in the published condition containing 1.4 M sodium citrate tribasic and 0.1 M HEPES, pH 7.5.[3] For co-crystallization with inhibitors, 10 µL of 25 mg/mL PtNHase was mixed with a 10 µL reservoir solution and 2 µL of 100 mM inhibitor, 1-butaneboronic acid (BuBA) or phenylboronic acid (PBA). Diffraction quality crystals grew within two weeks and belonged to the space group $P3_221$ with one copy of heterodimer in the asymmetric unit. For X-ray data collection, these crystals were briefly transferred to the reservoir solution containing 10 mM inhibitor and 20% (v/v) glycerol as cryo-protectant before flash-cooling by plunging in liquid nitrogen. The crystals of NHase with both BuBA and PBA were also obtained by soaking a native crystal for ~20 seconds in reservoir solution containing 10 mM inhibitor and 20% (v/v) glycerol. The crystals were then flash-cooled. Crystallographic

data were collected on beamlines 23ID-B or 23ID-D of GM/CA@APS of the Advanced Photon Source (APS) using X-rays of 0.99Å wavelength and Rayonix (formerly MAR-USA) 4×4 tiled CCD detector with a 300 mm^2 sensitive area. All data were indexed, integrated, merged and scaled with HKL2000.[4] Crystal parameters along with data collection and processing statistics are given in Table S1.

Phasing, model building, and refinement. Phasing of native NHase crystals was carried out with molecular replacement using the program Phaser from the CCP4 software suite. The structure of *Pt*NHase (PDB code 1IRE)[3], without any water molecules and ligands except for the cobalt ion, was used as the starting search model. Initial R_{free} and R factor for the correct solution were 27.0 and 26.9, respectively. Rigid body refinement was followed by restrained refinement with Refmac5[5] and further manual model inspection and adjustments with Coot.[6] When refinement converged, solvent molecules were added over several rounds. The R_{free} and the R factor values of the final model of the native structure were 20.2% and 17.5%, respectively (Table S1).

The NHase structures in complex with the BuBA and PBA inhibitors were phased by molecular replacement using the refined coordinates of native NHase as the search model. Refinement of the complex structures was carried out following the same protocol as the native structure. To avoid model bias, no ligand coordinates were included until the later stages of the refinement. The f_o-f_c difference maps (experimental maps), before incorporation of inhibitors in each structure show well-defined electron density for the corresponding inhibitor (Figures S2, S3 and S4A).

Inhibitor structures were created in Chemdraw (a "Mol" file); the molecules were regularized and chemical restraints were generated in JLigand. The inhibitors were placed into

residual electron density in COOT after the rest of the structure, including most of the solvent molecules, were refined. Final rounds of refinements were conducted in PHENIX[7] to include occupancy and simulated-annealing refinements. The R_{free} and R factor for the NHase structures with soaked BuBA, co-crystallized BuBA, and co-crystallized PBA were 18.3 % and 15.2 %, 16.6 % and 14.2 %, and 18.6 % and 15.2 %, respectively (Table S1).

Table S1. Data collection and refinement statistics.

Data set	Native	*Pt*NHase BuBA Soaking	*Pt*NHase BuBA Co-crystal	*Pt*NHase PBA Co-crystal
Space Group	$P3_221$	$P3_221$	$P3_221$	$P3_221$
Cell Dimensions				
a = b (Å)	65.82	65.6	65.9	65.7
c (Å)	185.61	185.7	186.2	185.7
α = β (°)	90	90	90	90
γ (°)	120	120	120	120
Resolution (A)	54.0-1.9	36.0-1.5	36.1-1.6	31.0-1.2
[a]R_{merge} (%)	12.7(40.6)	8.7(82.4)	6.0(20.7)	8.5 (67.9)
I/sigma	8.4(3.8)	16.3(1.5)	18.2(7.6)	9.4 (1.4)
Completeness (%)	99.8(99.8)	99.7(99.2)	100(100)	95.4(71.0)
Multiplicity	5.0(4.4)	4.5 (4.4)	6.6(6.3)	6.2(2.9)
No. Total reflections	176494	323576	390048	859631
No. Unique reflections	35437	72603	59389	139129
[b]R_{work} / [c]R_{free} (%)	17.5/20.2	15.2/18.3	14.2/16.6	14.5/18.5
No. of Atoms	4005	4175	4315	4169
No. of Solvent Atoms	460	543	748	629
B-factors (Å^2)				
Overall	23.3	20.4	15.8	15.7
Protein	22.2	17.9	13.2	13.8
Boronic Acid Ligand	ND	10.4-33.7	22.1-28.7	13.3-17.1
Water	31.8	30.9	27.8	27.8
[d]RMSD Bond Length (A)	0.02	0.02	0.02	0.02
[d]RMSD Bond Angles (°)	1.09	1.14	1.83	1.50
Ramachandran				
Favored (%)	98.0	96.3	96.2	97.7
Allowed (%)	1.5	2.3	2.4	1.8
Outlier (%)	0.5	1.4	1.4	0.5

The values for the highest resolution bin are in parentheses.
[a]Linear $R_{merge} = \Sigma|I_{obs}-I_{avg}|/ \Sigma I_{avg}$
[b]$R_{cryst} = \Sigma|F_{obs}-F_{calc}|/ \Sigma F_{obs}$.
[c]Five percent of the reflection data were selected at random as a test set and only these data were used to calculate R_{free}.
[d]RMSD, root mean square deviation.
ND, Not Determined/Calculated by the program

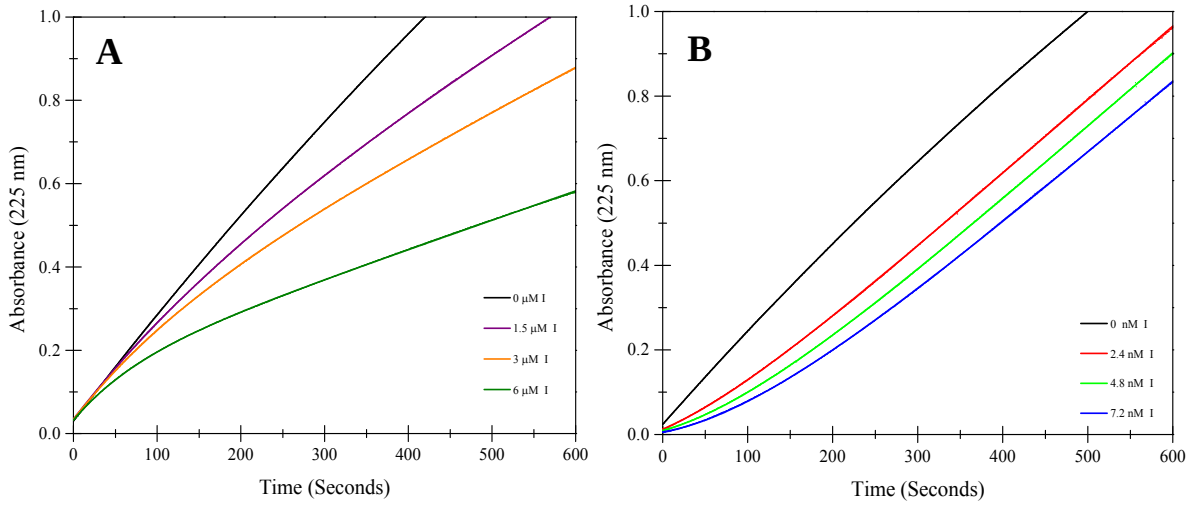

Figure S1. Example of progress curves for the slow-binding inhibition of *Pt*NHase by PBA. (A) Reaction progress curves (monitored at 225 nm for 10 min) for the hydration of 10 mM acrylonitrile by 0.56 nM *Pt*NHase in the presence of the indicated concentration of PBA in 50 mM Tris-HCl pH 7.5 at 25 °C. The reactions were initiated by the addition of *Pt*NHase. (B) Reaction progress curves for the hydration of acrylonitrile by reactivated *Pt*NHase. *Pt*NHase (237 nM) was preincubated with various concentrations of PBA (0 – 2.4 μM) for 60 min at room temperature. The preincubated *Pt*NHase/inhibitor complex was diluted 333-fold into reaction buffer containing 10 mM acrylonitrile to initiate the hydration reaction under the same conditions as in panel A. The reaction time courses were fit by nonlinear regression to Equation 1 to obtain the v_0, v_s, and k_{obs} values.

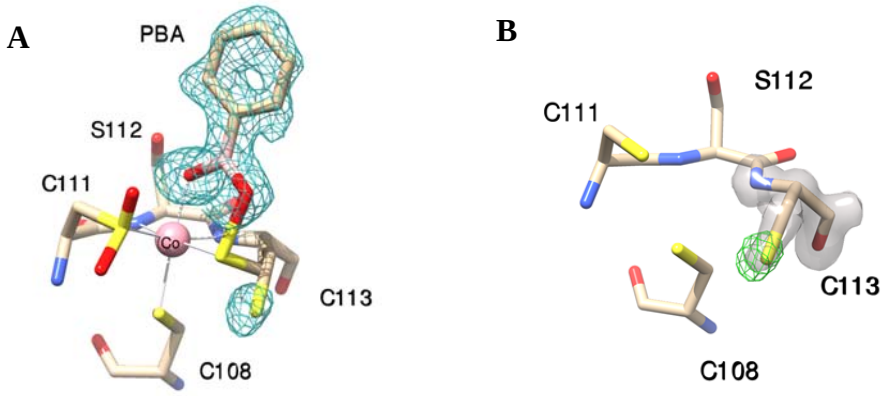

Figure S2. *Pt*NHase bound by PBA at 1.2 Å resolution obtained *via* co-crystallization of WT *Pt*NHase and 10 mM PBA. A) The experimental difference (f_o-f_c) map is shown around the major alternate conformation (~80%) around later built PBA and the minor apo-structure (~20%) as a cyan mesh at 2.6 sigma level. B) The $2f_o$-f_c map around the minor alternate conformation of αCys^{113} is shown in transparent grey surface at 1.4 sigma level, the simulated-annealing omit (f_o-f_c) map for the minor conformation of αCys^{113} is shown in green mesh at 2.8 sigma level.

References

(1) Martinez, S.; Kuhn, M. L.; Russell, J. T.; Holz, R. C.; Elgren, T. E. *Journal of Molecular Catalysis B: Enzymatic*, **2014**, *100*, 19-24.

(2) Morrison, J. F.; Walsh, C. T. *Adv Enzymol Relat Areas Mol Biol* **1988**, *61*, 201.

(3) Miyanaga, A.; Fushinobu, S.; Ito, K.; Shoun, H.; Wakagi, T. *Eur J Biochem.* **2004**, *271*, 429.

(4) Otwinowski, Z.; Minor, W. *Methods Enzymol.* **1997**, *276*, 307.

(5) Murshudov, G. N.; Vagin, A.; Dodson, J. *Acta Crystallogr. D* **1997**, *53*, 240.

(6) Emsley, P.; Cowtan, K. *Acta Crystallogr D Biol Crystallogr* **2004**, *60*, 2126.

(7) Adams, P. D.; Grosse-Kunstleve, R. W.; Hung, L.-W.; Ioerger, T. R.; McCoy, A. J.; Moriarty, N. W.; Read, R. J.; Sacchettini, J. C.; Sauter, N. K.; Terwilliger, T. C. *Acta Crystallogr. D* **2002**, *58*, 1948.