



Role of second-sphere arginine residues in metal binding and metallocentre assembly in nitrile hydratases

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ABSTRACT

Two conserved second-sphere β Arg (R) residues in nitrile hydratases (NHase), that form hydrogen bonds with the catalytically essential sulfenic and sulfinic acid ligands, were mutated to Lys and Ala residues in the Co-type NHase from *Pseudonocardia thermophila* JCM 3095 (PtNHase) and the Fe-type NHase from *Rhodococcus equi* TG328-2 (ReNHase). Only five of the eight mutants (PtNHase β R52A, β R52K, β R157A, β R157K and ReNHase β R61A) were successfully expressed and purified. Apart from the PtNHase β R52A mutant that exhibited no detectable activity, the k_{cat} values obtained for the PtNHase and ReNHase β R mutant enzymes were between 1.8 and 12.4 s⁻¹ amounting to <1% of the k_{cat} values observed for WT enzymes. The metal content of each mutant was also significantly decreased with occupancies ranging from ~10 to ~40%. UV-Vis spectra coupled with EPR data obtained on the ReNHase mutant enzyme, suggest a decrease in the Lewis acidity of the active site metal ion. X-ray crystal structures of the four PtNHase β R mutant enzymes confirmed the mutation and the low active site metal content, while also providing insight into the active site hydrogen bonding network. Finally, DFT calculations suggest that the equatorial sulfenic acid ligand, which has been shown to be the catalytic nucleophile, is protonated in the mutant enzyme. Taken together, these data confirm the necessity of the conserved second-sphere β R residues in the proposed subunit swapping process and post-translational modification of the α -subunit in the α activator complex, along with stabilizing the catalytic sulfenic acid in its anionic form.

1. Introduction

Nitrile hydratases (NHase, EC 4.2.1.84) catalyze the conversion of nitriles to amides at ambient temperature and pressure, a marked improvement over the harsh industrial reaction conditions for amide production (either acid or base hydrolysis) that are often incompatible with the sensitive structures of many industrially and synthetically relevant compounds [1,2]. NHases are metalloenzymes that contain either a low spin ($S = 0$) non-corrin Co(III) ion (Co-type) or a low spin ($S = 1/2$) non-heme Fe(III) ion (Fe-type) in the active site [2]. All crystallographically characterized prokaryotic NHases are $\alpha_2\beta_2$ -heterotetramers with a strictly conserved metal binding site located in the α -subunit, where the metal ion is coordinated by three cysteine residues, two backbone amide nitrogen atoms, and a water molecule [2]. The two equatorial cysteine ligands are post-translationally modified to Cys-

sulfenic (-SO₂(H); CSD) acid and Cys-sulfenic (-SO(H); CSO) acid, both of which are catalytically essential [2]. There are various, conflicting proposals regarding the protonation states of the sulfenic and sulfinic acid ligands; however, the most recent work suggests that both are deprotonated in the catalytically active enzyme, with the sulfenic acid ligand functioning as the nucleophile in catalysis [3–5].

Downstream from the structural α/β subunit genes is an activator (ϵ) protein gene that is required for nearly all NHases to be fully metalated and post-translationally modified [6]. While the NHase $\alpha_2\beta_2$ -heterotetramers are quite similar between Fe- and Co-type NHases, the ϵ proteins differ in size and sequence identity, suggesting that Fe- and Co-type NHases may have different mechanisms of metalation and post-translational modification. Fe-type ϵ proteins are ~45 kDa with a known metal binding motif and GTPase activity [49]. On the other hand, the Co-type ϵ proteins are much smaller (~17 kDa) and do not have any

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known metal binding motifs, but can function as an ATPase [2,7,8]. In 2008, Zhou et al. proposed a heretofore unknown ‘subunit swapping’ mechanism for the Co-type NHase from *Pseudonocardia thermophila* JCM 3095 (PtNHase), where the $\alpha_2\beta_2$ -complex first forms as the apo-tetramer while a single α -subunit binds to two Co(II)-containing ϵ proteins to form an $\alpha\epsilon_2$ complex [9]. It was proposed that after insertion of a Co(II) ion into the α -subunit of the $\alpha\epsilon_2$ complex, the cobalt and equatorial cysteines are oxidized to their sulfenic and sulfinic acid states, after which the now functional α -subunits are swapped for apo- α -subunits in the apo- $\alpha_2\beta_2$ -heterotetramer to produce the functional tetrameric protein (Fig. S1) [9–11].

Although not much is known about *how* this subunit swapping process occurs, it is essential to NHase metallocentre assembly and active site post-translational modification [8,9]. One hypothesis is that a salt bridge forms between two highly conserved positively charged second-sphere β Arg (R) residues and the negatively charged active site equatorial sulfenic/sulfinic acid ligands [9]. Based on X-ray crystallography, these two second-sphere β R residues form hydrogen bonds to the sulfenic and sulfinic acid ligands, also suggesting a role in catalysis. Previously, one of these β R residues was mutated to Lys (K), Tyr (Y), and Glu (E) in an Fe-type NHase; however, only the K mutant retained any detectable catalytic activity (~1%) [12]. Herein, we report the mutation of each of these conserved second-sphere arginine residues: β R52 and β R157 in PtNHase and β R61 and β R146 in the Fe-type NHase from *Rhodococcus equi* TG328-2 (ReNHase), to Ala (A) and K residues. Both the PtNHase and ReNHase mutant enzymes were characterized kinetically and spectroscopically; these data, coupled with DFT calculations and X-ray crystal structures of the PtNHase mutants, provide new insights into both the catalytic mechanism of NHases and the proposed subunit swapping process [8,13–16].

2. Materials and methods

2.1. Materials

All reagents were purchased commercially at the highest purity available. Isopropyl- β -D-1-thiogalactopyranoside (IPTG), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), sodium citrate tribasic, glycerol, Luria-Bertani (LB) broth, agar, nitric acid, hydrochloric acid, butyric acid, acetonitrile, acrylonitrile, kanamycin, ampicillin, carbenicillin, imidazole, cobalt chloride, and sodium chloride were all purchased from either Sigma Aldrich or Thermo Fisher scientific. Pre-cast polyacrylamide gel electrophoresis (PAGE) gels, SDS and native PAGE running buffers, sample buffers and ladders were purchased from Bio-Rad Laboratories. NEB and BL21(DE3) *E. coli* competent cells and mutagenesis kits (QuikChange) were purchased from Agilent Technologies, and plasmid purification kits were purchased from Promega. 5 mL IMAC Ni-NTA purification columns were purchased from Qiagen.

2.2. Mutagenesis

The α - and β -subunits of PtNHase were previously inserted into a pET-28a(+) vector with a hexahistidine tag on the C-terminus of the α -subunit [17]. Similarly, the ReNHase α - and β -subunits were inserted into the pET-28a(+) vector with two hexahistidine tags: one on the N-terminus of the α -subunit and the other on the C-terminus of the β -subunit [18]. Both the PtNHase and ReNHase wild type (WT) α/β sequences were used for mutagenic primer design using SnapGene and Integrated DNA Technologies Inc. The forward primer sequences are listed below and used with complementary reverse primer sequences.

- 5'-GACGAGTTCaagTTCGGCATCAG-3' PtNHase β R52K
- 5'-GACGAGTTcgcgTTCGGCATC-3' PtNHase β R52A
- 5'-CACGCCaagCGCGCGCGGTAC-3' PtNHase β R157K
- 5'-AAGGGCCACGCCgcaCGCGCGCGGTACGT-3' PtNHase β R157A

- 5'-GTCGACGAGGTCaagCACGTCGTCGACGC-3' ReNHase β R61K
- 5'-TTCAGTGTGTCGACGAGGTCgctCACGTCGTCG-3' ReNHase β R61A
- 5'-CGTGACGAGTTCTTCGCCGGACACATTaagATGCCTGCGTACTGC CGCGGT-3' ReNHase β R146K
- 5'-TTCGCCGGACACATTgcgATGCCTGCGTAC-3' ReNHase β R146A

Using the polymerase chain reaction (PCR) and the QuikChange site directed mutagenesis kit (Agilent), the bases in lowercase letters were changed in the WT β -subunit of PtNHase or ReNHase. The forward and reverse primers were used to perform two half PCR reactions, and then mixed for another PCR reaction to produce the fully mutated plasmid. The mutant plasmids were transformed into *XL10-Gold* Ultracompetent cells, plated, and grown overnight on LB media. The Promega Wizard SV genomic DNA purification kit was used according to the corresponding protocol to produce plasmids, and mutations were confirmed via sequencing (Azenta).

2.3. Transformation, growth, harvesting, and storage

The plasmids for the mutated α - and β -subunits of PtNHase (pET-28a(+)) and the necessary ϵ protein (pET-21a(+)) were co-transformed into BL21(DE3) cells and plated on carbenicillin (100 μ g/mL)/kanamycin (50 μ g/mL) agar plates. The same protocol was followed for the mutated α - and β -subunits of ReNHase (pET-21a(+)) and ϵ protein (pET-28a(+)). For each mutant, a single colony was used to inoculate 100 mL of LB broth with 50 μ g/mL kanamycin and 100 μ g/mL ampicillin and grown overnight at 37 °C with shaking (200 rpm). These starter cultures were used to inoculate 8 L of LB broth with 50 μ g/mL kanamycin and 100 μ g/mL ampicillin. Cells were grown at 37 °C and 200 rpm until an optical density at 600 nm of ~0.8–1.0 was reached. The cultures expressing mutant enzymes were induced with 0.1 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG), cooled to 20 °C and shaken at 200 rpm for an additional 16 h. Upon induction, 0.25 mM cobalt chloride was added to the PtNHase cultures. Cells were pelleted by centrifugation at 7000 rpm for 15 min and resuspended in 50 mM HEPES with 500 mM NaCl at pH 7.5. Cell paste was stored at –80 °C until purification.

2.4. Purification of mutant PtNHase and ReNHase

The resuspended cell pastes for each PtNHase and ReNHase mutant enzyme were thawed and lysed on ice by ultrasonication (Misonix Sonicator 300 with microtip) in 30 s increments for 20 min at 21 W. The lysate and cell debris were separated by centrifugation at 16,000 rpm for 40 min., and supernatant was collected. Buffers A and B were prepared: (A) 50 mM HEPES, 500 mM NaCl at pH 7.5 and (B) 50 mM HEPES, 500 mM NaCl, 500 mM imidazole at pH 7.5 and filtered through a 0.22 μ m nitrocellulose membrane. Additionally, ReNHase purification requires the addition of 40 mM butyric acid to buffer B to slow the oxidation of the enzyme active site, as previously described [14]. The supernatant containing expressed mutant enzyme was loaded onto a Qiagen IMAC Ni-NTA column that was pre-equilibrated with buffer A. The column was washed with 20 column volumes (CV) of buffer A with 4% buffer B, and subsequently washed with 20 CV of buffer A with 10% buffer B. The protein of interest was eluted with a wash gradient of 60 mL from 10% B to 60% B. Fractions were collected and loaded into a 30 kDa Amicon centrifugation tube where the protein was buffer exchanged back into buffer A and concentrated to a volume of <1 mL. Each purified mutant protein was stored in aliquots at –80 °C until ready for experimental use. Most of the ReNHase mutants did not bind to the Ni-NTA column as they appeared as inclusion bodies, thus, only the β R61A mutant was successfully purified (identity confirmed through SDS-PAGE and circular dichroism (CD)). Native PAGE gels were run on each mutant enzyme with non-denatured samples (prepared with Native Sample Buffer from Bio-Rad) in a Tris/Glycine buffer without SDS.

2.5. Steady-state kinetic assays

The enzymatic activity of NHase utilized acrylonitrile as the substrate and was monitored through product formation at 225 nm (acrylamide; $\Delta\epsilon_{225} = 2.9 \text{ mM}^{-1} \text{ cm}^{-1}$) on an Agilent 8453 UV-visible spectroscopy system with an Agilent 89090 A Peltier temperature-controlled cell holder. Enzyme was added to a 1 mL quartz cuvette containing 50 mM Tris-HCl pH 7.5 at 25 °C. Acrylonitrile concentrations ranged from 0.5 to 480 mM with protein concentrations ranging 100–200 nM. Kinetic parameters k_{cat} and K_M were obtained by fitting these data to the Michaelis-Menten equation using the OriginPro9.0 software (OriginLab) (Table 1).

2.6. Metal analysis, circular dichroism, and electronic absorption spectroscopy

The metal contents of each mutant were determined via inductively coupled plasma atomic emission spectroscopy (ICP-AES) and inductively coupled plasma mass spectrometry (ICP-MS). Each sample was first denatured for 4 h at room temperature with 200 mL of 8 M urea, followed by overnight denaturation with trace metal grade 2% nitric acid and 0.5% hydrochloric acid v/v mixture to a final volume of 10 mL. Denatured samples were centrifuged and run through a 0.2 μm filter to remove precipitation before ICP-AES/ICP-MS analysis (Table 1). The WT ReNHase and ReNHase mutant enzymes were concentrated to 0.5 mg/mL and buffer exchanged into a 20 mM sodium phosphate to collect circular dichroism (CD) spectra. Spectra were collected in a 0.5 mm quartz cuvette on an Applied Photophysics Chirascan Plus CD with a Peltier temperature-controlled cell held at 25 °C. Data analysis was performed using the online software DichroWeb, using the analysis program CONTIN with reference set SMP180 [19]. Each mutant enzyme was concentrated to 300 μM and UV-Vis spectra were collected in 50 mM HEPES buffer, pH 7.5 at 25 °C in a 1 cm quartz cuvette on an Agilent 8453 UV-visible spectroscopy system with an Agilent 89090 A Peltier temperature-controlled cell holder.

2.7. Electron paramagnetic resonance (EPR) spectroscopy

X-band EPR spectra were collected for both WT ReNHase and the βR61A mutant ReNHase enzymes on a Bruker EMX-AA-TDU/L spectrometer. Experimental spectra were recorded at 77 K in a liquid nitrogen cooling system centered around 9.44 GHz with 5 mW microwave power. Spectral analysis and simulations were performed using EasySpin [20,21].

2.8. Computational Methods

Active-site models for computational studies of Fe-type NHase [22,23], were derived from the X-ray structure of the Fe-type NHase from ReNHase-TG328-2 [23]. Constrained geometry optimizations were performed using unrestricted density functional theory (DFT), as implemented in the ORCA 4.0 software package [24,25]. These

calculations utilized the PBE0 functional (i.e., the one-parameter hybrid version of the Perdew-Burke-Ernzerhof functional) and the valence triple- ζ Karlsruhe basis with polarization functions on main-group and transition-metal elements (def2-TZVP) [26–28]. This combination of functional and basis set provided accurate geometries in our earlier studies of Fe-type NHase [22,23]. Fig. S2 illustrates the size and scope of the active-site models examined here, with positions of atoms marked with asterisks belonging to the protein backbone. These positions were fixed during geometry optimizations. For the WT ReNHase enzyme, models included the two conserved arginine residues (βR61 and βR146) that form hydrogen bonds to the αCSO118 and αCSD116 ligands. The position *trans* to the axial Cys donor is occupied by either H_2O or $\text{CH}_3\text{CO}_2\text{H}$; the latter is a truncated version of butyric acid. Models of the βR61A variant were prepared by removing the second-sphere βR61 residue in silico. Because this residue is positively charged, its removal results in an active site with an overall -1 charge. Neutral models in which the αCSO118 ligand is protonated were also generated for the βR61A variant, along with structures that feature an adventitious H_2O molecule in the space formerly occupied by βR61 , as suggested by one of the X-ray structures of Co-type PtNHase (βR52Ala). Each of the structural models was subjected to constrained DFT geometry optimization.

EPR g -values for $S = 1/2$ NHase models were calculated using three different methodologies, and the results are summarized in Table S1. The application of coupled-perturbed SCF (CP-SCF) equations to DFT calculations [29,30] yielded g -values in generally poor agreement with experiment, as the computed g_3 -values are all >2.0 (instead of below 2.0, as is typical for low-spin Fe(III) ions). We therefore pursued a multiconfigurational approach, referred to here as quantum chemical theory (QCT), that pairs complete active space self-consistent field (CASSCF) calculations with N -electron valence perturbation theory (NEVPT2) [31,32]. Although this combination has provided accurate g -values for a number of low-spin Fe(III) systems [32,33], our previous study of ReNHase found that it greatly overestimates the g -anisotropy of Fe-type NHase models [22]. Those earlier calculations employed the minimal CAS(5,5) active space consisting of five electrons in the five iron 3d orbitals. Here, we expanded the active space to CAS(11,13) to better account for metal-ligand covalency and radial correlation. A minimum of five doublet states were calculated. Because the results of CASSCF/NEVPT2 calculations exhibit only minor basis set dependence, the CAS(11,13) calculations employed the smaller valence double-zeta Karlsruhe basis set with polarization (def2-SVP). Expanding the active site to CAS(11,13) significantly improved agreement with experiment; however, the g_1 -values are still overestimated by approximately 0.15 for WT ReNHase models. Improved results were obtained via a combined approach (henceforth referred to as QCT/Taylor), that employed ab initio CASSCF/NEVPT2 calculations to compute the relative energies of the three lowest energy ligand-field states, which are derived from the parent $^2\text{T}_{2g}$ state in octahedral symmetry. These energies were then used to calculate EPR g -values by applying the set of equations derived originally by Taylor for low-spin Fe(III) systems [34,35]. Calibration of this method using a synthetic nonheme Fe-S/N complex with known g -values provided an optimal spin-orbit coupling parameter (ζ) of 400

Table 1

Kinetic constants (k_{cat} and K_M) for WT and mutant PtNHase and ReNHase with acrylonitrile as substrate in 20 mM Tris-HCl at pH 7.5 and 25 °C, and metal concentration in soluble protein (ICP-MS and ICP-AES), where M^{3+} is cobalt for PtNHase and iron for ReNHase.

	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($\text{s}^{-1} \text{ mM}^{-1}$)	% activity	M^{3+} eq.
WT PtNHase	1600 ± 120	7 ± 1	300	100%	2.0 ± 0.1
PtNHase βR157A	1.8 ± 0.1	3.1 ± 0.4	0.6	0.11%	0.4 ± 0.1
PtNHase βR157K	12.4 ± 0.1	6.0 ± 0.8	2.1	0.78%	0.2 ± 0.1
PtNHase βR52A	nd*	nd*	nd*	nd*	0.4 ± 0.1
PtNHase βR52K	4 ± 1	77 ± 9	0.1	0.25%	0.4 ± 0.2
WT ReNHase	1300 ± 200	31 ± 1	42	100%	2.0 ± 0.1
ReNHase βR61A	3.9 ± 0.1	4.0 ± 0.9	1.0	0.30%	0.8 ± 0.1

nd* = none detected.

Table 2Refinement and data collection statistics for each *Pt*NHase mutant crystal structure.

Data Set	βR157A	βR157K	βR52A	βR52K
Space Group	P3 ₂ 21	P3 ₂ 21	P3 ₂ 21	P3 ₂ 21
Cell Dimensions				
a = b (Å)	66.176	65.91	65.964	65.895
c (Å)	185.671	184.28	186.400	184.943
α = β (°)	90	90	90	90
γ (°)	120	120	120	120
Resolution (Å)	19.47–1.29	19.54–1.56	31.09–1.45	19.55–1.47
R _{merge}	0.070 (0.491)	0.067 (0.737)	0.168 (0.967)	0.066 (0.932)
R _{pim}	0.023 (0.262)	0.019 (0.286)	0.055 (0.336)	0.021 (0.291)
I/sigma	15.7 (2.1)	20.6 (2.2)	8.8 (2.1)	18.4 (2.2)
Completeness (%)	99.87 (99.17)	99.91 (99.85)	99.37 (100.00)	98.79 (98.18)
Multiplicity	9.3 (4.3)	13.1 (7.2)	10.5 (9.1)	10.8 (11.0)
No. Total reflections	1,112,253 (25044)	886,773 (24242)	888,880 (38180)	857,926 (42437)
No. Unique reflections	119,354 (11701)	67,059 (6604)	83,861 (8359)	79,255 (7759)
R _{work}	0.1647 (0.2647)	0.1694 (0.2403)	0.2473 (0.3349)	0.1955 (0.2803)
R _{free}	0.1896 (0.2984)	0.1913 (0.2780)	0.2663 (0.3500)	0.2279 (0.3251)
Twin Law	None	None	-h, -k, l	None
No. of Atoms	4241	4183	4007	4059
No. of Solvent Atoms	713	670	468	550
B-factors (Å ²)				
Overall	21.48	27.71	18.15	27.09
Protein	19.09	25.98	17.20	25.41
Water	33.31	36.74	25.33	37.83
RMSD Bond Length (Å)	0.012	0.005	0.002	0.004
RMSD Bond Angles (°)	1.24	0.83	0.49	0.75
Ramachandran				
Favored (%)	98.10	97.41	97.64	97.64
Allowed (%)	1.90	2.59	2.36	1.65
Outlier (%)	0.00	0.00	0.00	0.71

cm⁻¹ [36]. Computed g-values were required to correspond to a normalized singly occupied 3d orbital. The combined Taylor/QCT approach reduces the gap between the computed and experimental g₁-values to 0.07 or less for WT species (Table S1), and the overall agreement for the three g-values is excellent (root-mean-square deviation of 0.02–0.04).

2.9. Crystallization and X-ray data collection

Crystallizations of the *Pt*NHase mutant enzymes were performed using a sitting drop protocol of 20 mg/mL protein solution mixed with a well solution consisting of 1.2 M sodium citrate tribasic in 0.1 M HEPES at pH 7.5, as previously reported [2,37]. Diffraction quality crystals grew within two weeks and upon harvesting, were submerged in cryoprotectant containing 20% glycerol (v/v), flash frozen, and stored in liquid nitrogen. Diffraction data were collected at beamline 21-ID-G of the Advanced Photon Source at Argonne National Laboratory. The beamline was equipped with a MAR 300 Charged Coupled Device (CCD). Data were collected using an oscillation angle of 0.5° over a range of 180° and an exposure time of 1.0 s per frame. The wavelength was fixed at 0.97856 Å. Data processing and reduction, including indexing, integration, and scaling was done using the autoPROC software [38]. Data processing and refinement statistics are summarized in Table 2. The *Pt*NHase structures were solved via molecular replacement using PHASER within the Phenix software suite [39,40]. The initial search model included the previously published WT *Pt*NHase (PDB: 1IRE). Model building and subsequent refinement was performed using COOT and PHENIX.REFINE respectively [40]. Structural figures were made using UCSF Chimera [41].

3. Results and discussion

Two strictly conserved arginine (R) residues on the β-subunit of prokaryotic NHases (Table S2) form hydrogen bonds with the active site sulfenic and sulfinic acid ligands (Fig. 1) [2]. These residues are βR52 and βR157 in *Pt*NHase and βR61 and βR146 in *Re*NHase [2,42]. In all

NHases, the equivalent βR residue to βR52 in *Pt*NHase forms a hydrogen bond to both the sulfenic-αCSO113 ligand and the sulfinic-αCSD111 ligand (αCSO118 and αCSD116 in *Re*NHase), while the equivalent of βR157 forms two hydrogen bonds to the sulfinic-αCSD111 ligand (Fig. 1) [9,10,12]. It was hypothesized that the function of these βR residues is two-fold: (i) they form a salt bridge between the β-subunit and the α-subunit in the α₂ complex to facilitate α-subunit swapping and (ii) likely play a role in catalysis through hydrogen bond formation to the active site sulfenic and sulfinic acid ligands, the latter of which is proposed to function as a nucleophile towards the nitrile substrate. In an effort to gain insight into the roles of these two conserved βR residues in NHase maturation and catalysis, both were mutated to K and A residues.

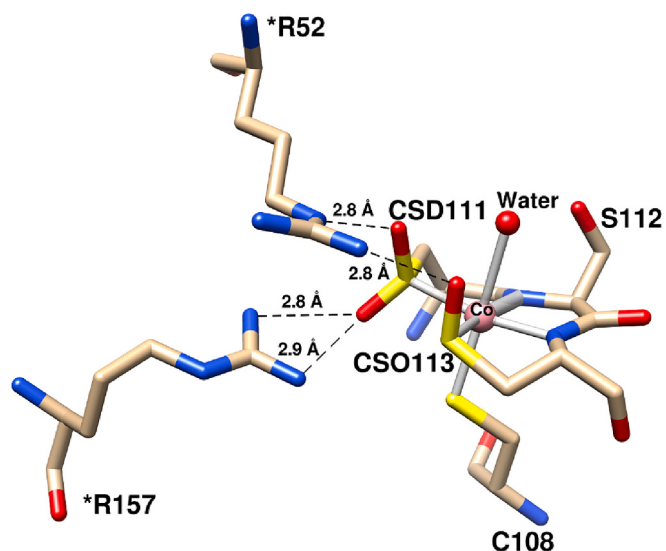


Fig. 1. Active site of WT *Pt*NHase (PDB: 1IRE). Residues that are from the β-subunit are denoted with an *. Key distances in proposed salt bridges are shown in Å.

3.1. Purification, kinetic, and metal analysis

The PtNHase β R52A, β R52K, β R157A, and β R157K mutants were successfully expressed and purified, providing colorless samples. On the other hand, three of the ReNHase Fe-type mutants (β R61K, β R146K, and β R146A) formed inclusion bodies and could not be refolded. Therefore, only the β R61A ReNHase mutant enzyme was purified and exhibited a light green color. CD spectra of the ReNHase β R61A mutant enzyme display α -helix ($\sim 18\%$) and β -sheet ($\sim 31\%$) content similar to WT ReNHase, which exhibits $\sim 20\%$ α -helix and $\sim 30\%$ β -sheet content. Both enzymes contain $\sim 50\%$ random coil content (Fig. S3). These data indicate that the ReNHase β R61A enzyme is folded properly.

The metal content of each mutant was determined through both ICP-AES and ICP-MS and the data from both methods were in good agreement (Table 1). As expected, WT PtNHase and ReNHase had ~ 2 eqs. of metal per tetramer, however, each of the five mutants displayed < 2 eqs. of metal. The PtNHase β R52A, β R52K and β R157A mutants each contained ~ 0.4 eqs. of cobalt per tetramer, while the PtNHase β R157K mutant enzyme had only ~ 0.2 eqs. On the other hand, the ReNHase β R61A mutant enzyme contained ~ 0.8 eq. of iron per tetramer (Table 1). As the metal concentration for PtNHase ranged from 10% to 20% while the ReNHase mutant contained only $\sim 40\%$ of the WT metal content, mutation of the second-sphere β R residues clearly impacts the ability of the active sites to incorporate metal ions, suggesting that these β R residues may indeed play a role in the subunit swapping process.

The catalytic constants for WT and mutant NHases are presented in Table 1. The PtNHase mutants exhibited 0–0.8% of the catalytic activity observed for WT PtNHase, while the β R61A ReNHase mutant exhibited only 0.3% of WT activity. As a result of the decreased k_{cat} values, the catalytic efficiency (k_{cat}/K_M) for each mutant enzyme examined decreased ~ 100 -fold for PtNHase and ~ 42 -fold for ReNHase (Table 1). As the substrate binds directly to the metal center, the diminished catalytic efficiency is not simply due to incomplete maturation of the enzyme. The k_{cat} and K_M values relate to the remaining, small fraction of metalated enzyme as there are no known secondary substrate binding partners and catalysis is unlikely in the apo- form of the enzyme. For the PtNHase β R157A and β R157K mutant enzymes, the K_M values were not distinguishable from those of WT PtNHase, but interestingly, the PtNHase mutant, β R52K mutant enzyme, exhibited a K_M value approximately an order of magnitude greater than that of WT PtNHase. One explanation is that an increase in electron density on the metal center after the loss of the β R52 hydrogen bond diminishes the Lewis acidity of the Co(III) ion. A previous investigation of the β R61K mutant in an Fe-type NHase reported $< 1\%$ activity towards methacrylonitrile with a K_M an order of magnitude less than WT NHase [12]. For the ReNHase β R61A mutant, its observed K_M is also an order of magnitude less than

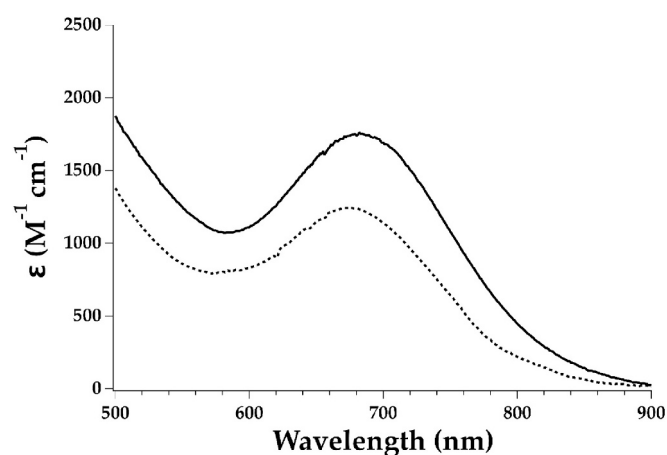


Fig. 2. Absorption spectra from 400 to 900 nm of WT ReNHase (solid trace) and ReNHase β R61A (dashed trace).

that of WT ReNHase. Collectively, the metal binding and kinetic data indicate that the second-sphere β R residues not only impact the ability of the NHase active sites to catalytically hydrate nitriles to amides, but also play a role in the ability of the α -subunit to incorporate metal ions. These conclusions are consistent with the proposal that these β R residues may play a role in the subunit swapping mechanism.

3.2. Electronic absorption and electron paramagnetic resonance spectroscopy

The UV–Vis spectrum of WT PtNHase exhibited a characteristic band at 320 nm due to an axial thiolate $S(\pi)$ -to-Co(III) charge transfer (CT) transition. As a result, the sample exhibited an observable tan (straw) color [16,43]. All four PtNHase β R mutant enzymes were colorless and had no observable ligand-to metal CT (LMCT) band (Fig. S4). The loss of this LMCT band is most likely the result of low Co(III) content in each mutant enzyme; however, no absorption was observed even at high enzyme concentrations (~ 1 mM). The UV–Vis spectrum of the ReNHase β R61A mutant enzyme exhibited an absorption band at 680 nm, assigned to an axial thiolate ($S \rightarrow Fe(III)$) LMCT. This band is blue shifted by ~ 20 nm with respect to the corresponding peak in the WT ReNHase spectrum (Fig. 2). This observation is in good agreement with previous

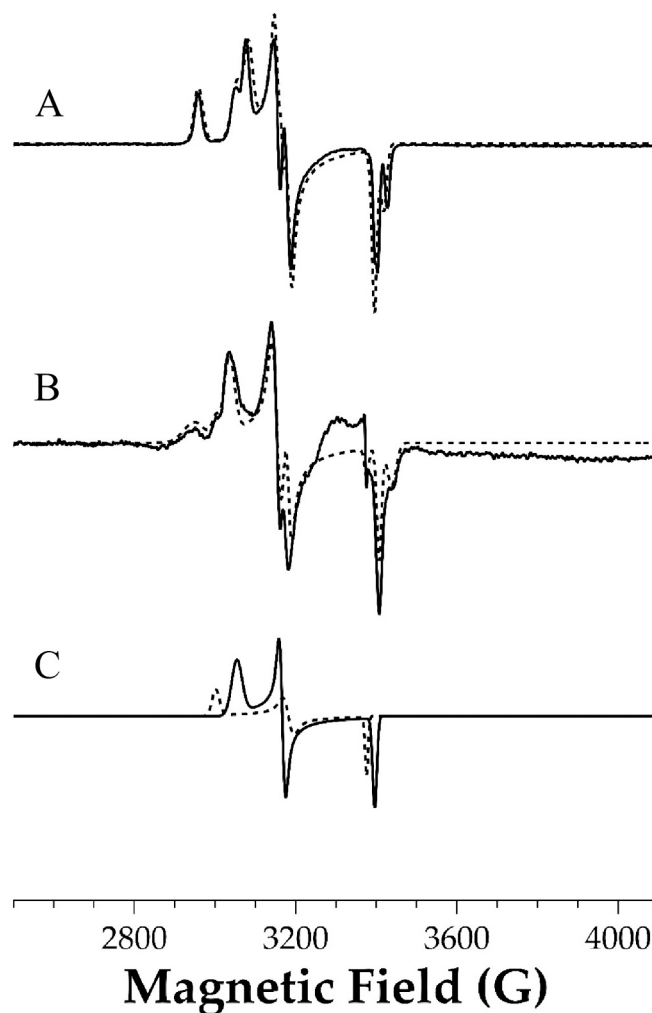


Fig. 3. A. Simulated EPR spectrum of WT ReNHase (dashed trace) overlaid on the experimental EPR spectrum of WT ReNHase with butyric acid (solid trace). B. Simulated EPR spectrum of ReNHase β R61A (dashed trace) overlaid on the experimental EPR spectrum of ReNHase β R61A with butyric acid. C. Isolated simulation species of WT ReNHase^{Aq} (solid trace) and β R61A ReNHase^{Aq} (dashed trace) where β R61A is shifted upfield by $\delta g \approx 0.04$.

Table 3Experimental and Computed EPR g-values for WT and β R61A ReNHase.

NHase species	Axial ligand	C118-SO _x (H)	Outer-sphere H ₂ O?	Method	g ₁	g ₂	g ₃	Δg
WT ReNHase ^{Aq}	H ₂ O	CysSO ⁻	No	QCT/Taylor	2.257	2.118	1.969	0.288
				Exper.	2.206	2.131	1.987	0.219
β R61A ReNHase ^{Aq}	H ₂ O	CysSO ⁻	No	QCT/Taylor	2.305	2.141	1.956	0.349
	H ₂ O	CysSOH	No	QCT/Taylor	2.287	2.125	1.962	0.325
	H ₂ O	CysSO ⁻	Yes	QCT/Taylor	2.279	2.129	1.963	0.316
				Exper.	2.248	2.142	1.998	0.250
WT ReNHase ^{BA}	CH ₃ CO ₂ H	CysSO ⁻	No	QCT/Taylor	2.302	2.126	1.959	0.343
				Exper.	2.281	2.150	1.974	0.307
β R61A ReNHase ^{BA}	CH ₃ CO ₂	CysSOH	No	QCT/Taylor	2.486	2.125	1.905	0.581
	CH ₃ CO ₂ H	CysSOH	No	QCT/Taylor	2.322	2.116	1.956	0.366
	CH ₃ CO ₂	CysSOH	Yes	QCT/Taylor	2.447	2.116	1.921	0.526
				Exper.	2.289	2.123	1.963	0.326
WT ReNHase ^{Ox}	H ₂ O	CysSO ₂ ⁻	No	QCT/Taylor	2.250	2.108	1.972	0.278
				Exper.	2.180	2.114	1.998	0.182
β R61A ReNHase ^{Ox}	H ₂ O	CysSO ₂ ⁻	No	QCT/Taylor	2.320	2.126	1.955	0.365
				Exper.	2.223	2.142	1.981	0.242

Exper. = experimental data.

work on the Fe-type NHase β R61K mutant enzyme, which also exhibited a blue-shift of ~ 20 nm [12]. A similar hypsochromic blue shift was also observed when NO or N₃⁻ was added to an Fe-type NHase. As this LMCT band is due to the axial thiolate ligand donating into the unoccupied e_g^{*} orbital of the Fe(III) center, the observed blue-shift was attributed to greater donation from the axial thiolate cysteine to compensate for the π -accepting nature of the NO or N₃⁻ [12,14]. For the ReNHase β R61A mutant enzyme, the loss of the hydrogen bonding interaction between β R61 and the equatorial sulfenic acid ligand increases the electron density on the ligand, thereby destabilizing the Fe-based 3d orbitals and increasing the energy of the LMCT band. The increase in electron donation to the metal center for both Co- and Fe-type NHases also decreases the Lewis acidity of the active site metal ion, impacting the lability of the axial water molecule and binding affinity of the substrate [16]. These data indicate that the hydrogen bonds from the PtNHase β R52 and ReNHase β R61 positions play an important role in tuning the Lewis acidity of the active site metal ion, as well as the nucleophilicity of the sulfenic acid ligand.

The EPR spectrum of WT ReNHase corresponds to those previously reported and contains signals from three species: active ReNHase (ReNHase^{Aq}), inactive and oxidized ReNHase (ReNHase^{Ox}), and the butyric acid (BA)-bound ReNHase (ReNHase^{BA}) (Fig. 3A) [23]. The spectrum of ReNHase β R61A also contains three species and was simulated using ReNHase^{Aq}, ReNHase^{Ox}, and ReNHase^{BA} (Fig. 3B; Table 3). One species in the EPR spectrum of the ReNHase β R61A mutant enzyme, with an isolated peak at 2950 G ($g_1 = 2.289$) and attributed to the BA complex due to the high value of g_1 and large g -anisotropy, is interesting as this resonance is much broader ($\Delta F_{\text{WHM}} \approx 45$ G) than in WT ($\Delta F_{\text{WHM}} \approx 20$ G). The broadening is likely indicative of a less constrained coordination geometry (g -strain), consistent with the loss of the second-sphere β R hydrogen bonding interaction. The other two species exhibited low-field resonances at 3000 G ($g_1 = 2.248$) and 3035 G ($g_1 = 2.223$), respectively. The g_1 value of 2.223 is close to that (2.206) of WT ReNHase^{Aq} but such an assignment would imply a shift in the g_1 value δg of 0.08 for the oxidized species. A more likely explanation is that the g_1 values of both species increased by about 0.04, such that the $g_1 = 2.248$ species corresponds to the active form and the $g_1 = 2.223$ species corresponds to the oxidized form of the ReNHase β R61A mutant enzyme (Fig. 3C, Table 3).

3.3. X-ray crystal structures of the PtNHase β R52 and β R157 mutant enzymes

X-ray quality, colorless crystals of each of the PtNHase β R mutant enzymes were obtained using identical crystallization conditions to those previously reported for WT PtNHase [2,37]. The lack of any

detectable color is consistent with low Co(III) content within the active sites (Table 1). The crystals diffracted between ~ 1.3 and ~ 1.6 Å and detailed crystallographic parameters are presented in Table 2. All four X-ray structures confirm the mutations, and their overall structures were nearly identical to the WT PtNHase structure with RMSD ranging from ~ 0.27 to ~ 0.45 Å.

The β R157K and β R157A active sites are presented in Fig. 4A and B. No metal density was observed in either active site, so the collected data was solved as the apo-form; however, densities $<20\%$ are often poorly resolved via X-ray crystallography so we cannot exclude the possibility of low metal occupancy in the active site. The active site equatorial cysteine ligands α C111 and α C113 are fully reduced in the β R157K structure; each occupies two different orientations in an $\sim 50:50$ mixture (Fig. 4A). Substitution of β R157 with Lys allows a single hydrogen bonding interaction, which occurs between the Lys-based N atom and the α C111 sulfur atom (3.8 Å), suggesting a weak hydrogen bonding interaction as the average hydrogen bonding distances between NH and S is 3.5 Å (Fig. 4A, Table 4) [44,45]. Substitution of the β R157 position with Ala removes the hydrogen bonding interaction to the CSD111 ligand that is observed in WT PtNHase (Fig. 1). Interestingly, the structure of the β R157A enzyme revealed that the equatorial α C111 ligand is properly oxidized but, surprisingly, the axial α C108 ligand is partially oxidized to α CSO108 ($\sim 50\%$) (Fig. 4B). The other equatorial α C113 ligand is fully reduced (Fig. 4B). For the β R157A structure, an hydrogen bond from the primary amine to an oxygen atom of the oxidized α CSD111 ligand is observed (3.2 Å; Fig. 4B) and also to the reduced equatorial α C113 ligand sulfur atom (3.6 Å) (Fig. 4B). However, an hydrogen bond is not observed between the secondary amine of β R52 and the α CSD111 equatorial ligand, which is observed in the WT structure (Fig. 1) as its too far from the secondary amine of β R52 (4.0 Å, Table 4). It is unclear why the α CSD111 and α C108 ligands are oxidized in the β R157A mutant enzyme but not in the β R157K structure. Understanding this oxidation is complicated by the proposed subunit swapping mechanism, but an appealing explanation is the loss of salt bridge formation, which impedes active site maturation and consequently subunit swapping. This explanation is also consistent with the depletion of cobalt in the active site of both β R157A and β R157K structures. Then, perhaps, in the absence of any hydrogen bonds, the α C111 sulfur is more susceptible to oxidation, as the redox potential of a cysteine ligand would be impacted with the loss of the β R hydrogen bonding interactions [46,47]. Taken together, these data further support the proposal that β R157 plays a role in the proposed subunit swapping mechanism.

As the second-sphere β R52 residue forms hydrogen bonds with both the α CSD111 and α CSO113 acid ligands in the WT PtNHase structure (Fig. 1), the latter of which functions as the nucleophile during catalytic

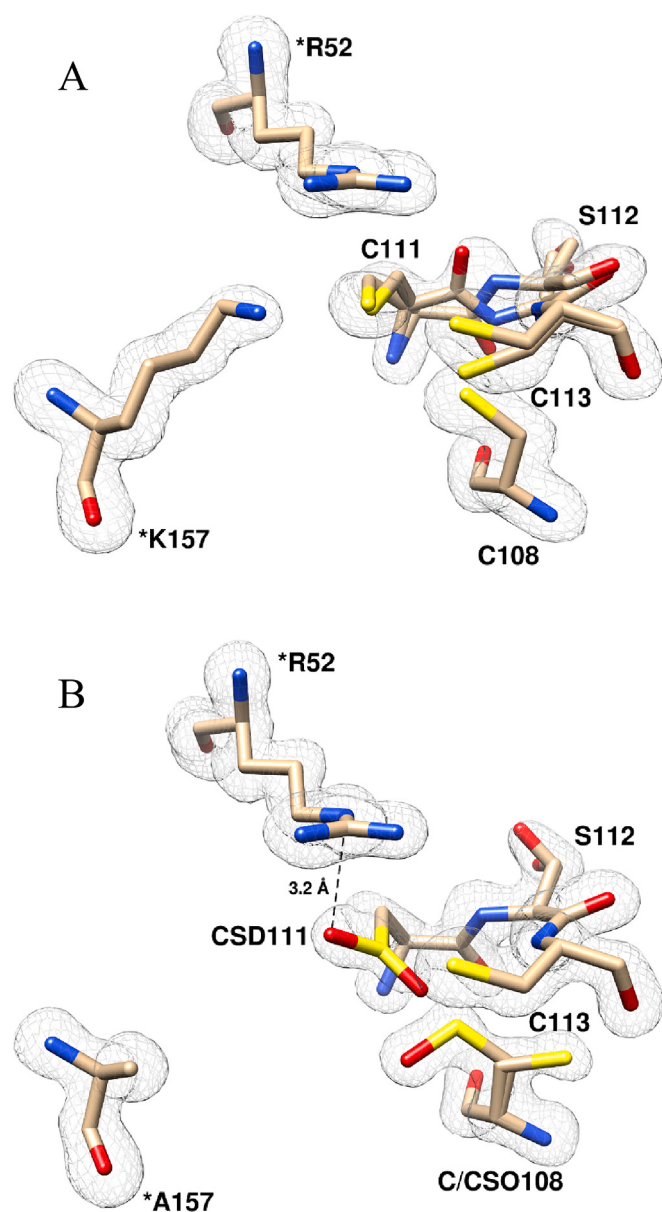


Fig. 4. A) Active site of β R157K with second-sphere 52 and 157 positions from the β -subunit shown. No metal was observed in the active site. Residues from the β -subunit are denoted with an *. Only hydrogen-bonding distances (<3.5 Å) are shown. α Cys111, α Ser112 and α Cys113 are built in two alternate conformers respectively based on electron density (Polder map at 4.5 sigma level). B) Active site of β R157A with second-sphere 52 and 157 positions from the β -subunit shown. No metal was observed in the active site. Residues from the β -subunit are denoted with an *. Key hydrogen bonding distances between the α and β subunits are shown in Å. α Cys108 and α Ser112 are built in two alternate conformers respectively based on electron density (Polder map at 4.0 sigma level). One of the alternate conformers of α Cys108 is modeled as sulfenic acid (CSO). α Cys111 is modeled as sulfinic acid (CSD).

turnover, mutation of the β R52 residue to a K or A residue provides insight into the catalytic mechanism as well as the subunit swapping mechanism. Interestingly, in the β R52K structure, neither of the active site equatorial α Cys ligands (α C111 and α C113) are oxidized, similar to the β R157K structure (Fig. 5A); however, two different α C108, α C111, and α C113 sulfur atom conformations are observed in an $\sim 60:40$ ratio. Hydrogen bonds are observed between the β R52K primary amine N atom and the α C111 sulfur atom (3.3 Å) and the α C113 sulfur atom (3.1 Å). Substitution of β R52 with an A residue, which eliminates hydrogen

Table 4

Hydrogen bonding distances between first- and second-sphere ligands with distances in Å.

Bonds	Species					
	WT	APO-WT	R52K	R52A	R157K	R157A
R157/K157 Primary NH ₂ – S-C111	3.9	3.3	–	3.3	3.8	–
R157 Secondary NH ₂ – S-C111	4.0	4.0	–	2.8	–	–
R52/K52 Primary NH ₂ – S-C111	–	4.7	3.3	–	4.3	4.6
R52/K52 Primary NH ₂ – S-C113	3.8	5.4	3.1	–	3.7	3.6
R52 Secondary NH ₂ – S-C111	3.9	4.1	–	–	3.8	4.0
R52 Secondary NH ₂ – O-CSD111	2.8	–	–	–	–	3.2

WT PtNHase (PDB: 1IRE) and Apo-WT PtNHase (PDB: 1UGQ) [2,37].

bonding, results in the α C113 ligand being partially oxidized to α CSO113 ($\sim 60\%$) but the α C111 ligand remains fully reduced (Fig. 5B). It is also notable that the α C113 ligand is oxidized in the β R52A structure but not in the β R52K structure, which is the opposite to that observed for the β R157A structure. This observation supports the suggestion that the salt bridges proposed to form between the β R157 and β R52 residues with the α_2 complex are important for the post-translational maturation of the NHase active site as well as the subunit swapping process.

3.4. Computational studies

Additional insights into the spectroscopic and crystallographic features of the NHase active site were obtained using computational methods. Our previous studies of WT ReNHase determined that the functional active site (ReNHase^{Aq}) features a six-coordinate Fe(III) center bound to an axial H₂O ligand, which participates in hydrogen bonding interactions with the anionic (i.e., unprotonated) α CSO118 and α CSD116 donors [23]. Removal of the β R61 residue in silico, followed by constrained geometry reoptimization using DFT, causes only minor changes in the overall structure; however, it's clear that loss of the β R61 residue strengthens the hydrogen bond between the axial H₂O ligand and the deprotonated α CSO118 ligand, as evident in the shortening of the α CSO118 anion SO $\cdots$ H₂O distance (from 1.61 in WT to 1.47 Å in β R61A) and elongation of the O–H bond by 0.04 Å. Thus, it appears that eliminating the hydrogen bond with the second-sphere β R61⁺ residue causes the deprotonated α CSO118 ligand to become significantly more basic in the β R61A variant. This conclusion is further supported by DFT calculations of the BA-bound ReNHase β R61A mutant enzyme (ReNHase^{BA}), in which the BA molecule is truncated to acetic acid in DFT calculations for simplicity. In the WT enzyme, the carboxylic acid of BA coordinates as a neutral ligand, stabilized by hydrogen bonding between the carboxylate (CO₂H) functional group and the α CSO118 anionic ligand. Interestingly, geometry optimizations of the β R61A site converge to a structure featuring an acetate ligand and a protonated α CSO118 unit (Fig. 6).

Based on these data, active-site structures of the ReNHase β R61A mutant enzyme in which removal of β R61 is accompanied by protonation of the anionic α CSO118 ligand to give the protonated α CSO118 form, were also considered. In the resulting ReNHase^{Aq} model, a weak hydrogen bond is established between the adjacent protonated α CSO118 and the α CSD116 anion ligands during geometry optimization, which reduces the (O₂)S-Fe-S(OH) angle from 94.3° (in WT) to 89.6°. Protonation also disrupts the hydrogen bond between α CSO118 and the axial H₂O ligand that is observed in both WT and unprotonated β R61A structures. Not surprisingly, the protonated α CSO118 group is incapable of deprotonating the carboxylic acid moiety of the BA-bound model of

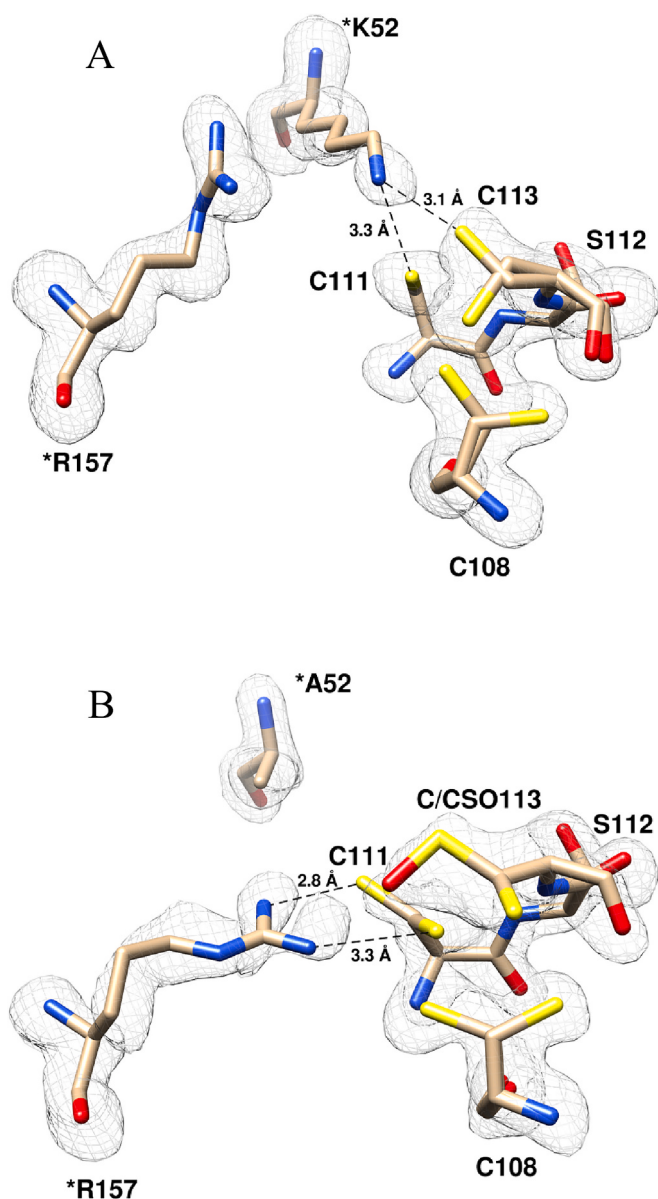


Fig. 5. A) Active site of β R52K with second-sphere 52 and 157 positions from the β -subunit are shown. No metal was observed in the active site. Residues from β subunit are denoted with an *. Key hydrogen bonding distances between α and β -subunit residues are shown in Å. α Cys108 and α Cys113 are built in two alternate conformers respectively based on electron density (Polder map at 3.0 sigma level). B) Active site of β R52A with second-sphere 52 and 157 positions from the β subunit are shown. No metal was observed in the active site. Residues from the β -subunit are denoted with an *. Key hydrogen-bonding distances between α and β -subunit residues are shown in Å. α Cys108, α Cys111, α Ser112 and α Cys113 are built in two alternate conformers respectively based on electron density (Polder map at 3.0 sigma level).

β R61A. Instead, the resulting structure features a hydrogen bond between the neutral $\text{CH}_3\text{CO}_2\text{H}$ and αCSO118 ligands, similar to the arrangement found in WT models of $\text{ReNHase}^{\text{BA}}$. Finally, active-site models of ReNHase β R61A that contain an adventitious H_2O molecule in the space formerly occupied by β R61 were generated. After geometry optimization, the added H_2O assumes a position in the optimized structures that maximizes hydrogen bonding interactions with both the αCSO118 and αCSD116 donors. In the BA-bound structure, the model optimizes to a structure featuring an anionic carboxylic acid ligand and protonated αCSO118 unit, suggesting that the outer-sphere H_2O

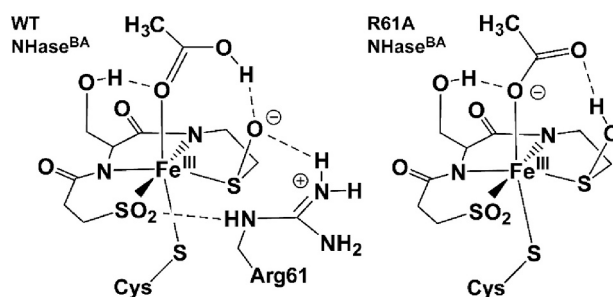


Fig. 6. Changes in ligand protonation states in DFT models of the $\text{ReNHase}^{\text{BA}}$ active site.

molecule does not fully compensate for loss of hydrogen bonding from the β R61 residue.

Calculations of EPR g -values were also used to assess the relative validity of these competing models of the WT ReNHase and the ReNHase β R61A active sites. As described in the Computational Methods section, quantum chemical theory (QCT) based on ab initio CASSCF/NEVPT2 calculations were used to determine the relative energies of the three lowest energy ligand-field states, which are derived from the parent $^2T_{2g}$ state in octahedral symmetry. According to the theoretical framework developed by Taylor and others, the mixing of these states via spin-orbit coupling (SOC) is responsible for the anisotropy of the g -tensor [34,35]. Table 3 compares experimental g -values to those computed using QCT for different ReNHase active-site models. The combined QCT/Taylor approach tends to overestimate the overall anisotropy of the g -tensor, defined as $\Delta g = g_1 - g_3$, by 0.05–0.10. Yet the calculations accurately reproduce the trend in g -anisotropy across the WT series, which follows the order $\text{ReNHase}^{\text{BA}} > \text{ReNHase}^{\text{Aq}} > \text{ReNHase}^{\text{Ox}}$. Significantly, the computed g -values of the three β R61A $\text{ReNHase}^{\text{Aq}}$ models are each slightly more anisotropic than the WT model, consistent with the shifts observed experimentally upon removal of β R61 (Table 3). The best agreement with experiment is provided by $\text{ReNHase}^{\text{Aq}}$ models in which the αCSO118 ligand is either protonated ($\alpha\text{CSO118-SOH}$) or hydrogen bonded to an outer-sphere H_2O molecule ($\alpha\text{CSO118-SO}^-/\text{H}_2\text{O}$), although the variations of computed g -values across the β R61A $\text{ReNHase}^{\text{Aq}}$ series are fairly small.

More dramatic differences are observed between the computed g -tensors of β R61A $\text{ReNHase}^{\text{BA}}$ models. Those containing an axial carboxylate anion (CH_3CO_2^-) yielded computed g_1 -values of 2.49 and 2.45 (Table 3), far greater than the experimental g_1 -value of 2.289. Thus, based on the computational data, deprotonation of the BA-derived ligand in the β R61A $\text{ReNHase}^{\text{BA}}$ active site is expected to be accompanied by large changes in the EPR spectrum, which is not observed experimentally. Instead, better agreement with experiment is observed for the β R61A $\text{ReNHase}^{\text{BA}}$ model that features a neutral carboxylic acid and a protonated αCSO118 ligand ($g_1 = 2.322$). The computed g -tensor of this model is only slightly more anisotropic than the corresponding WT tensor, which is consistent with the minor shifts in g -values observed experimentally between WT and β R61A $\text{ReNHase}^{\text{BA}}$.

Collectively, our computational results suggest that the deprotonated αCSO118 unit of ReNHase may compensate for the loss of the β R61 residue by gaining a proton to generate a protonated αCSO118 ligand. Because the O-atom of αCSO118 engages in nucleophilic attack of the nitrile substrate in the NHase mechanism, protonation of this ligand may account for the lack of activity of the β R61A mutants (Fig. 6). A neutral αCSO118 moiety would be far less nucleophilic than an anionic αCSO118 unit, thereby hindering formation of the proposed cyclic intermediate. Thus, the second-sphere β R61 residue likely facilitates catalysis by stabilizing the αCSO118 ligand in its deprotonated (anionic) state via hydrogen bonding interactions.

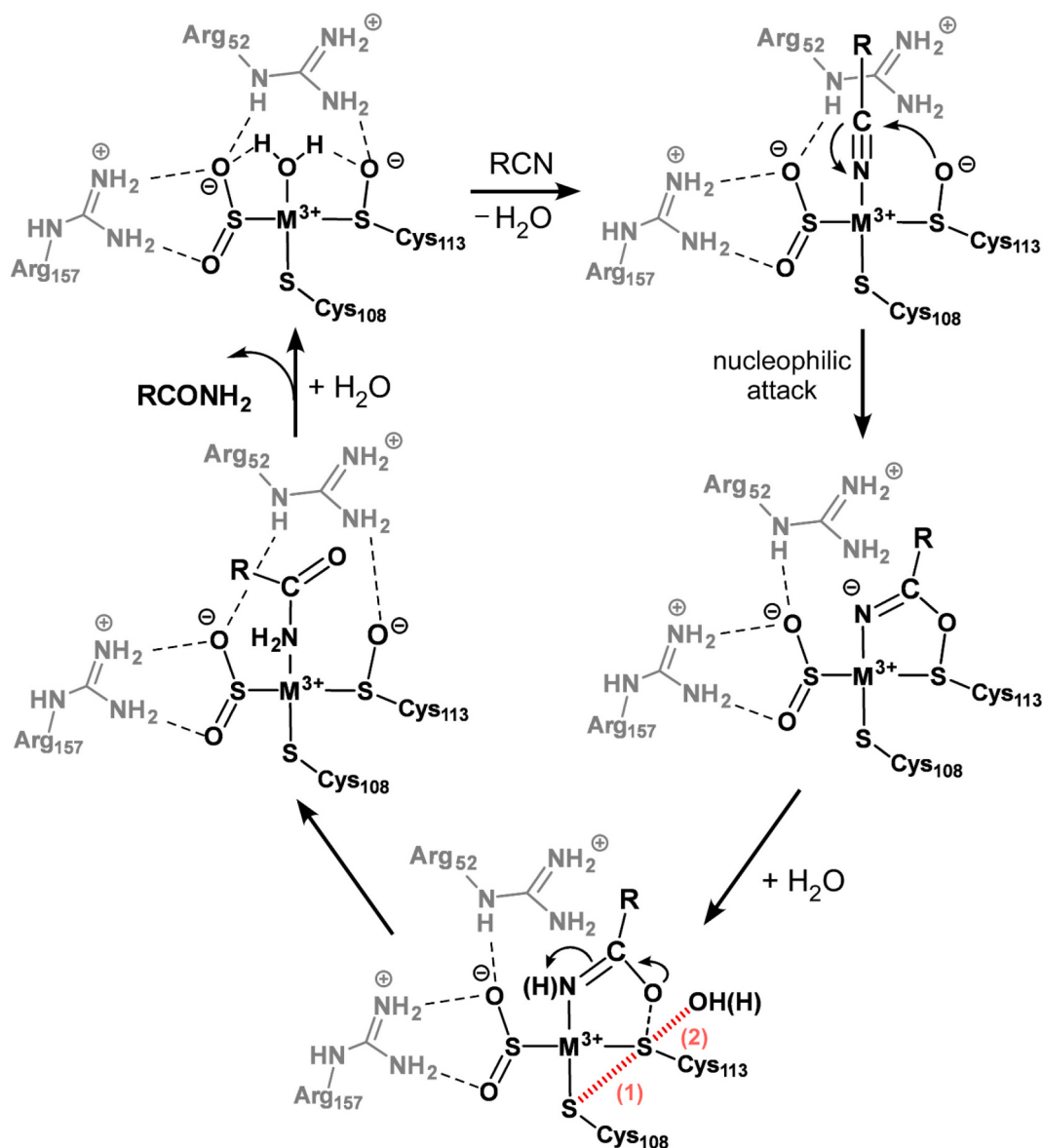


Fig. 7. Proposed mechanism of catalysis, consistent with previous publications, with the second-sphere arginine residues included. Red hashed lines indicate potential stabilizing disulfide bond (1) or the stabilization of the sulfur through the reformation of the sulfenic acid with a water molecule (2). Ligand numbering refers to *PtNHase* system. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Conclusions

The data described herein supports the proposed salt bridge formation between the two highly conserved, positively charged second-sphere active site β R residues and the negatively charged active site equatorial sulfenic/sulfinic acid ligands in the α -subunit of the $\alpha\epsilon_2$ complex, which drives subunit swapping [9–11]. Additionally, these data support the previously proposed catalytic mechanism for NHases and lend new insights into the second-sphere hydrogen bonding network that is integral to catalysis. Specifically, the hydrogen bond from the β R52 and β R61 positions in *PtNHase* and *ReNHase*, respectively, tune the Lewis acidity of the active site metal ion as well as the basicity of the sulfenic acid ligand. The combination of DFT calculations, EPR spectroscopy and X-ray crystallographic data reported herein support the suggestion that both of the sulfenic and sulfinic acid ligands are deprotonated in the active enzyme, and that the anionic forms are stabilized by hydrogen bonds from the conserved second-sphere β R residues. As the O-atom of the sulfenic acid ligand (SO⁻) is required to function as the nucleophile in the catalytic mechanism, protonation of

the sulfenic acid (SOH) ligand may account for the lack of activity of the β R52/61Ala mutant enzymes.

These findings allow the proposed catalytic mechanism to be updated to include two integral second-sphere residues (Fig. 7). Salient features of the proposed mechanism that remain the same include (i) direct coordination of the nitrile substrate to the active site metal center activating the nitrile bond towards nucleophilic attack, (ii) nucleophilic attack of the bound nitrile carbon by the sulfenic acid ligand, forming a cyclic intermediate, and (iii) the transfer of two protons upon product release, which is the rate-limiting step based on kinetic isotope studies [14,48]. The anionic and deprotonated nucleophilic equatorial sulfenic acid ligand is stabilized in the more nucleophilic -SO⁻ form through the hydrogen bonding of β R52 in *PtNHase* and β R61 in *ReNHase*. After nucleophilic attack, once the cyclic intermediate is formed, tautomerization occurs to form the iminol. This intermediate state necessitates stabilization of a reduced α C113 ligand, which has been proposed to occur either through a disulfide bond intermediate or through a water molecule that reforms the sulfenic- α C113 ligand (shown through red hashed lines in Fig. 7) [16]. The suggested disulfide intermediate has

only been proposed from theoretical studies. Time-resolved X-ray crystallographic studies failed to detect a disulfide species, and mutation of the axial Cys ligand to His still resulted in active enzyme [16]. Therefore, the formation of a disulfide bond between the axial Cys ligand and the equatorial sulfenic acid is not catalytically required. However, a recent EPR study reported a catalytically relevant intermediate that was tentatively assigned to a disulfide intermediate [22]. Finally, when the amide product is released and the nucleophilic sulfenic acid is reformed, the second-sphere β R52 residue is able to stabilize the α CSO113 anion through hydrogen bonding. Therefore, both second-sphere arginine residues are important in stabilizing the equatorial cysteine ligands in their oxidized and deprotonated forms, which is essential for catalysis. This work also confirms the necessity of the conserved second-sphere β R residues for the subunit swapping process and post-translational modification of the α -subunit in the $\alpha\epsilon_2$ complex along with stabilizing the catalytic nucleophile, α CSO113, in its anionic form through hydrogen bonding.

CRedit authorship contribution statement

Callie Miller: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Delanie Huntoon:** Investigation, Formal analysis, Data curation. **Nicholas Kaley:** Formal analysis, Data curation. **Irene Ogutu:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Adam T. Fiedler:** Writing – review & editing, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis. **Brian Bennett:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Dali Liu:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Richard Holz:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

The manuscript is accompanied by Supporting Information which contains a scheme of the proposed subunit swapping process, the DFT geometry-optimized model of WT N_{Hase}^{Aq}, circular dichroism spectra of WT and β R61A ReN_{Hase}, and UV–Vis absorbance spectra of the PtN_{Hase} mutants. Supplementary data to this article can be found online at [<https://doi.org/10.1016/j.jinorgbio.2024.112565>].

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