Investigation of the Active Site Maturation and Catalytic Mechanism of Nitrile Hydratases (NHase, E.C.4.2.1.84)

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INVESTIGATION OF THE ACTIVE SITE MATURATION AND CATALYTIC MECHANISM OF NITRILE HYDRATASES (NHASE, E.C.4.2.1.84)

By

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A Dissertation submitted to Faculty of Graduate School, Marquette University, in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

Milwaukee, Wisconsin.

August 2019
ABSTRACT

Investigation of the Active Site Maturation and Catalytic Mechanism of Nitrile Hydratases (NHase, E.C.4.2.1.84)

Irene Ogutu, B.Ed-Sc.

Marquette University, 2019

Nitrile hydratase (NHase, E.C.4.2.1.84) is a metalloenzyme that catalyzes the hydration of nitriles into their corresponding amide under ambient conditions. NHase enzymes contain either a non-heme Fe$^{3+}$ ion or a non-corrin Co$^{3+}$ ion in their active site. NHase enzymes have industrial applications as biocatalysts in the large-scale production of acrylamide and nicotinamide, though it’s catalytic and biochemical properties are not fully understood. This research project provides insight into the active site maturation process and catalytic mechanism of NHase using peptide model complexes, site-directed mutagenesis and synthesis of deuterated proteins.

Insight into the sequential maturation of the NHase active site has been provided in chapter 2. An eight amino acid peptide mimic of the metal binding motif of PtNHase was prepared and the metal bound complexes were analyzed using optical, EPR and MCD spectroscopy. These metallopeptide mimics clearly revealed that the metal binds into the active site in a high spin divalent state, then the equitorial cysteine residues are oxidized and finally the metal ion is oxidized to the trivalent ion. In chapter 3, the catalytic role of the axial cysteine ligand was examined through site directed mutagenesis, optical spectroscopy, kinetic analysis and X-ray crystallography. The role of the activator protein and the metal ion in active site maturation process was examined by expressing NHase in the absence and presence of Co$^{2+}$ and/or activator protein and then elucidated through X-ray crystallography and kinetic analysis. This investigation strongly supports the role of activator protein in metal ion insertion and the maturation of the active site in conjunction with the metal ion. The axial ligand has been shown to be necessary for catalysis and metal ion insertion. A neutron diffraction investigation into the protonation states of active site residues supports the fact that a large and well ordered protein crystal is key to full data set collection for neutron diffraction. PtNHase is stable in its perdeuterated and deuterated states and large crystals, that diffract X-ray to >1.9 Å can be obtained. The perdeuterated protein has 60% activity of the wildtype and is able to grow into large crystals.
ACKNOWLEDGMENTS

Irene Ogutu, B. Ed-Sc.

My appreciation goes to God for His guidance, love, and protection throughout my life and studies.

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My sincere thanks also to Dr. St. Maurice for training and guiding me on the use of X-ray diffractometer and solving crystal structures. His availability and patience helped me to achieve a great deal in my research. I am grateful to our collaborators Dr. Lui and Dr. Mascarenhas who have helped with part of the X-ray crystallographic work, as well as Dr. Dean, Dr. Ruslan and Dr. Weiss with whom I have worked on the neutron diffraction project. I am grateful to Dr. Bennett for sharing skills in EPR and for helping with EPR-related research. His contribution and guidance throughout my studies have been invaluable. I am also grateful to Dr. Fiedler for the MCD work, his patience, availability and for his contributions in my research and Dr. Stein for her support and advice.
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Finally, I am grateful to Jesus my spouse and for being all that I can ever need in life and dedicate this work to the Blessed Virgin Mary my mother and role model.
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CHAPTER 1

INTRODUCTION

1.1. General introduction

1.2. Nitrile biosynthesis and biodegrading pathways

Nitriles are organic compounds that are essential in nature and important industrially. Nitriles are used in organic synthesis as starting materials and intermediates in the preparation of various chemicals (carboxylic acids, amides, amines, ketones, esters, aldehydes and other heterocyclic compound)\(^1,\)\(^{1b}\) They are also used as solvents, extractants and pesticides.\(^1b,\)\(^2\) In nature, nitriles are synthesized and hydrated by plants, fungi and bacteria (Figure 1), they are also present in low concentrations in some of the living organisms.\(^3\) The starting material is an amino acid from which nitriles are synthesized and then hydrated into various organic products (organic acids, aliphatic hydrocarbons, amides), which are broken down into other chemical substances such as ammonia and carbon dioxide.\(^4\) The synthesis and degradation of nitriles by living organisms is made possible due to the presence of the various enzymes (aldoxine dehydratase, nitrile hydratase, amidase, and nitrilase), which catalyze these reactions; compounds from these biosynthetic pathways are sources of defense mechanisms for these organisms.\(^5\) Nitrile degradation has become of interest in contemporary society due to the extensive use of nitrile containing chemical substances in the pharmaceutical and
agricultural industries. This, therefore, has led to the introduction of nitrile-toxic waste into water and soil through surface runoffs and industrial wastes.\textsuperscript{1b, 6}

![Figure 1. Metabolic pathways of nitrile synthesis and degradation in nature.\textsuperscript{4}](image)

1.3. Nitrile degrading enzymes.

Nitriles are synthesized in nature from aldoxine in the presence of the enzyme aldoxine dehydratase (EC 4.99.1.5). The hydration of nitriles into their corresponding organic acid and ammonia can be achieved through two different pathways: i) direct hydration to an organic acid and ammonia by nitralase (EC 3.5.5.1) or ii) hydration to an amide by nitrile hydratase (NHase, EC 4.2.1.84) followed by hydration by an amidase (EC 3.5.1.4) (Figure 1).\textsuperscript{4,7b} In most cases, microorganisms either contain nitralase (\textit{Bacillus sp. OxB-1}) or the NHase-amidase system (\textit{Rhodococcus sp. YH3-3, Rhodococcus sp. N-771, Rhodococcus globerulus A-4} and \textit{Pseudomonas chlororaphis})
B23) for nitrile hydration; *Rhodococcus erythropolis* AJ270 contains a gene cluster encoding for all three enzymes.\(^8,8b,9,10\)

The gene encoding for nitrile degrading enzymes has been isolated from various organisms. Howden and Preston proposed that the enzymes may be involved in nutrient metabolism, hormone synthesis, nitrile detoxification and nutrient assimilation.\(^5\) Nitrile degrading enzymes have a wide range of synthetic nitrile substrates, making them useful in organic synthesis, industrial processes, and bioremediation.

**1.4. Nitrile hydratases (NHase, EC 4.2.1.84)**

NHases are intracellular metalloenzymes that catalyze the hydration of nitriles into their corresponding amides under ambient temperature and physiological pH.\(^11,11b\) Conventional chemical methods require acidic or basic conditions with high temperatures, pressures and synthetic catalysts generating unwanted by-products and toxic wastes. NHases have, therefore, become important for organic synthesis within the chemical and pharmaceutical industries because of their ability to hydrate a broad range of nitriles under mild conditions. NHases are used in the production of acrylamide (more than 30,000 tons per year) and nicotinamide, and is considered the most successful biocatalyst.\(^12\)

NHases have been isolated from many microorganisms across a diverse genera of phyla Proteobacteria, Actinobacteria, Cyranobacteria and Firmicutes; Molds (*Trichoderma sp.* and *Myrothecium verrucaria*) and yeast (*Aureobasidium pullulans*,...
Candida spps. and Cryptococcus flavus) have been reported to harbor NHase though the catalytic efficiency, biochemical and structural properties have not been investigated.\textsuperscript{4,13,13b} Some reports have revealed that there is an NHase encoding gene in Monosiga brevicollis, which is a marine chanoflagellate.\textsuperscript{14} Though there is no report of NHases isolated from plants or animals, phylogenetic studies suggest that the NHase gene is present in Ricinus communis.\textsuperscript{4} These organisms, from which the NHase genes were isolated, are found in varied habitats such as agricultural fields, waste water, mountain sediments and forest soil.\textsuperscript{4} NHases have been isolated from both mesophilic (Rhodococcus, Brevibacterium, Nocardia etc.) and thermophilic organisms ((Geo) bacillus and Pseudonocardia) and can therefore, function over a large temperature range of 4-40 °C and 45-65 °C respectively.\textsuperscript{15}

NHases have widely been categorized into two major groups as either iron-type (Fe-NHase) or cobalt-type (Co-NHase) since they contain either a low spin Fe\textsuperscript{3+} or Co\textsuperscript{3+} ion, respectively, in their active sites.\textsuperscript{16,17,18,19} Though most NHases have iron or cobalt ions in their active sites, a unique NHase from Rhodococcus sp. RHA1 was reported to contain one cobalt, two copper and one zinc ion per functional enzyme;\textsuperscript{4,20} however, the relative orientation of these four metal ions in a holoenzyme is unknown. NHases are made up of two non-homologous sub-units (α and β); the sub-units differ from each other depending on the organisms from which they are isolated ranging from 20-35 kDa.

Reports have shown that Fe-/Co-type NHases exhibit some substrate preferences \textit{i.e.} Co-type NHases have shown high affinity for aromatic nitriles while Fe-type NHase have high affinity for aliphatic nitriles.\textsuperscript{4} Among all the NHases characterized to date, the sub-units have a high similarity in amino acid sequence.\textsuperscript{21}
1.5. Expression of NHases and the role of the activator protein

The overexpression of recombinant proteins in *E. coli* often results into aggregation resulting in inclusion bodies. This can be a hinderance in obtaining soluble and active protein. The expression of a fully functional NHase is induced by substrate or product concentrations in wild-type organisms, which seem to occur during transcription thus, regulating the assembly of the α- and β-subunits.\(^{22,17}\) For successful expression of an active and soluble Fe-type or Co-type NHase enzyme in *E. coli*, co-expression with an activator protein or molecular chaperones is essential for most NHase enzymes.\(^{17,23}\) The gene for the activator protein is downstream from the NHase gene operon (Figure 2).\(^{17}\) The NHase from *Comamonas testosterone* Ni1 (*CtNHase*) is an exception as it has successfully been expressed in the absence of an activator protein.\(^{23c,24}\) The role of the activator protein has not been fully defined but has been proposed to: i) insert the metal ion into the active site, ii) modify the active site during protein expression, iii) enable the proper folding of the protein, and iv) bind to the α-subunit and insert the metal ion.\(^{23a,25,4}\)

<table>
<thead>
<tr>
<th>α</th>
<th>β</th>
<th>Activator</th>
</tr>
</thead>
<tbody>
<tr>
<td>β</td>
<td>α</td>
<td>Co-type, <em>Pseudonocardiathermophilia</em> JCM3095</td>
</tr>
<tr>
<td>α</td>
<td>β</td>
<td>Fe-type, <em>Rhodococcus equi</em> TG328-2</td>
</tr>
<tr>
<td>α</td>
<td>β</td>
<td>Fe-type, <em>Comamonas testosteroni</em> Ni1</td>
</tr>
</tbody>
</table>

**Figure 2.** Three open reading frames for the NHase genes and the activator.
1.5.1. The Cobalt and Iron activator protein

The activator protein for Co-type and Fe-type NHases have no homology. The Co-type NHase activator is <17 kDa and has some similarity with the β-subunit while the Fe-type NHase activators are >40 kDa with a highly conserved CXCC metal binding motif and has no homology to either of the two subunits. The activator proteins for Co/Fe-NHases selectively insert metal ion into the active site and this has been proposed to be due to the difference in their homology. The Fe-NHase activator’s CXCC metal binding motif has not been observed in any of the Co-activator proteins.

1.5.1.1. Iron activator protein

The Fe-NHase activator protein contains a conserved CXCC motif which has been identified as the metal binding motif. The CXCC motif is conserved in Fe-type NHase activator proteins (Table 1). Cysteine rich regions in proteins are known to play important roles in metal binding, transportation, and protein-protein interactions. Similar cysteine rich motifs have also been identified in Saccharomyces cerevisiae with CXCC/MTCXXC/CXC motifs that have been proposed to assist in trafficking copper within the system. Mutations to the CXCC motif in Fe-NHase activator led to a drastic decrease in protein activity indicating that the cysteine rich region is involved in metal ion delivery to the active site.
Table 1. Conserved CXCC motif in Fe-type NHase activators from different organisms.\textsuperscript{29}

<table>
<thead>
<tr>
<th>Name</th>
<th>Species</th>
<th>Region with the putative binding motif</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHase activator</td>
<td><em>Rhodococcus sp.</em> N-771</td>
<td>69MTNGCICCTLR79</td>
</tr>
<tr>
<td>P44K protein</td>
<td><em>Rhodococcus sp.</em> AJ270-6</td>
<td>68MTNGCICCTLR78</td>
</tr>
<tr>
<td>P47K</td>
<td><em>Pseudomonas chloraphis</em> B23</td>
<td>72MTNGCICCTLR82</td>
</tr>
<tr>
<td>KB15K</td>
<td><em>Rhodococcus equi</em> TG328-2</td>
<td>68MTNGCICCTLR78</td>
</tr>
</tbody>
</table>

The Fe-NHase activator has been proposed to ensure metal ion delivery through guanosine-5'-triphosphate (GTP)-dependent iron trafficking, the presence of an SKTD sequence like NKED guanine-bonding motif, which is common in GTPases, is adjacent to a Walker A-binding site which is associated with phosphate binding; with the pattern G-X(4)-G-[TS]. The presence of Walker A supports the proposal of it functioning as a GTPase (Figure 3).\textsuperscript{32} Kinetic studies have shown that the ReNHase TG328-2 activator exhibits GTPase activity, which is dependent on the addition of a divalent ion.\textsuperscript{32} This was further supported by CD spectroscopy, which showed significant conformational change in the activator with the addition of GTP.\textsuperscript{32} Apart from the activator protein, there are other molecular chaperones in NHases, but they are not as efficient as the NHase activator protein.\textsuperscript{33} Xiong and co-workers showed that when GroEL-GroES was
used for co-expression of the NHase from *Pseudoxanthomonas sp.* AAP-7, the protein did not exhibit maximum protein activity.\textsuperscript{33}

**Figure 3.** Homology model for the *Re*NHase TG328-2 activator. The residues making up the Walker A and B motifs highlighted along with the proposed thiolate metal-binding site and the guanine-binding motif. (B) An expanded view of the CXCC metal-binding site where it is clear that two of the cysteine residues (Cys 72 and Cys 74) are on the same side of the β-sheet.\textsuperscript{32}
1.5.1.2. The Cobalt activator protein

The activator proteins for Co-NHases have significant sequence identity to the NHase β-subunit. For example, the activator P14K protein from *Pseudomonas putida* NRRL-18668 exhibits 23% similarity with the β-subunit while the Nh1E activator protein of the lower molecular weight NHase from *Rhodococcus rhodochrous* J1 has 31% similarity. The Co-NHase activator proteins in the presence of Co(II) ions have been characterized using electronic absorption spectroscopy. The two activator proteins used (P14K and Nh1E) exhibited absorption maxima around 420 nm which was attributed to a coordination sphere of coordinated Co(II) ions. The Co(II) binding to the activator protein was further examined via isothermal titration calorimetry (ITC), which revealed that Co(II) was able to bind to the activator protein with a $K_d$ of 1.5 nM and 9.84 nM for Nh1E and P14K respectively. Kobayashi and co-workers examined the pathway through which Co(II) is delivered to the NHase active site. Their findings strongly supported that Co(II) is inserted, through a self-subunit swapping mechanism, via exchange between apo-α-subunit of the L-NHase and a Co(II) containing mediator (holo-Nh1AE).34 Therefore, Nh1E is referred to as a self-subunit swapping chaperone (Figure 4). Another investigation proposed that the metallochaperones play a role in the oxidation of the post-translationally modified cysteine residues in the NHase active site.35
Figure 4. A proposed model for the process of cobalt incorporation into L-NHase. Mg$^{2+}$ uptake systems are designated as Mg$^{2+}$ uptake systems (which transport cations such as Co$^{2+}$, Zn$^{2+}$, and Mg$^{2+}$ into the cell). The cobalt ion is shown as a closed circle. MUS, Mg$^{2+}$ uptake system.$^{34}$

Recent studies by Zhou and co-workers strongly support the previously proposed modes of Co(II) insertion into the active site.$^{36}$ They further explored metal insertion into a fused αβ-subunit for the NHase from *Pseudomonas putida*. Interestingly, P14K was found to be important for expression of an active protein but it was not clear on how metal insertion occurred without the possibility of α-subunit swapping. A further investigation using a subunit fused gene for low molecular weight NHase (L-NHase) from *Rhodococcus rhodochrous* J1, revealed that the fused βα-subunit expressed without Nh1E had very low protein activity while there was an increased catalytic rate when the protein was co-expressed with the activator protein.$^{36}$ These results have given new insight onto cobalt insertion into the NHase α-subunit by demonstrating that Co-NHase
activator proteins, can bind and transfer metal ions to apo-NHases acting as metallochaperones.

There has been no metal binding motif identified for the Co-NHase activator protein but Zhou and co-workers, through mutational studies on the C-terminus, suggested a possible binding site for Co(II). They observed reduced enzyme activity when the NHase was co-expressed with a mutant gene of the P14K and Nh1E and suggested the cobalt binding site shown in Figure 5.
Figure 5. Protein structures of P14K (yellow) and NhIE (blue). (A) De novo models predicted by QUARK, and the conserved α-helices presented with the same alphabet. (B) The residues of potential cobalt binding site located at C-terminus (shown as gray transparent surface) of activator models. (C) The possible cobalt binding site orientation of activators after molecular dynamics simulations and annealing simulations. The residues of potential cobalt binding sites are showed as blue sticks, and the distance (Å) is showed in a dashed line.  

Expression of the Co-type NHase from *Pseudonocardia thermophila* JCM 3095 (PrNHase) in the absence of the metal ion yielded an apoenzyme that exhibited no catalytic activity. The equatorial cysteine ligands, which are always post-translationally modified in the holoenzyme, were not oxidized and the axial cysteine (αC108) and the equatorial αC113 formed a disulfide bond. The enzyme could not be
reactivated even after incubation in the presence of Co\(^{3+}\) ion and a reducing agent.\(^{11a}\) These data suggest that the activator protein without metal ion cannot activate the oxidation of the equatorial cysteine residues, which are essential for catalysis; the metal ion therefore, appears to be required along with the activator for the modification of the cysteine residues into cysteine-sulfenic (Cys-SO(H)) acid and cysteine-sulfinic acid (Cys-SOOH).

1.6. The structure of NHase

The structural and electronic properties of NHases from several different microorganisms have been elucidated using various techniques such as X-ray crystallography, electron paramagnetic resonance (EPR), Fourier transform infrared spectroscopy (FT-IR) and UV-visible absorption spectroscopy.\(^{37}\) These have revealed that NHase is made up of α- and β-subunits with a low spin Co\(^{3+}/Fe^{3+}\) ion inserted within the active site.\(^{38,39,40,37d}\) The active site is found at the interface between the α and the β-subunit of the dimer (Figure 6); the two subunits have noncovalent interactions with each other forming an αβ-heterodimer.\(^{38,39}\) In some organisms, the NHase enzyme is made up of four subunits forming an α\(_2β_2\)-heterotetramer (\textit{Pseudonocardia thermophila}-NHase),\(^{38}\) while others contain a three sub-unit heterotrimer (αβγ) (Toyocamycin-NHase).\(^{41,42}\) For the NHases with α\(_2β_2\) structures, the αβ dimers have loose interactions with each other forming the tetramer (Figure 6).
Figure 6. A crystal structure of α₂β₂ heterotetramer of NHase from PtNHase JCM 3095 (PDB: 1IRE). The α subunit is in green or yellow, and the β subunit in blue or red. The magenta spheres are cobalt ions within the active site. The green and blue strands represent the α and β subunits of one heterodimer, and the other two represent the second heterodimer, respectively. Two heterodimers are arranged in a crystallographic two-fold symmetry.  

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Figure 7. Superimposition of the Co-type NHase from *PtNHase* JCM 3095 (PDB: 1IRE) and the Fe-type NHase from *Rhodococcus* sp. N-771. The α (green) and β (blue) subunits of the Co-type NHase and the α (purple) and β (orange) subunits of Fe-type are shown. The magenta sphere is the cobalt ion in the active site, and the cyan sphere is an iron ion. (A) Active sites of the Co-type and the Fe-type NHase. The side chain residues of the Co-type NHase are represented by thick sticks and those of the Fe-type NHase by thin sticks.\(^3\)

Even though the Fe-type and the Co-type NHases have high levels of sequence identity, there are significant differences between them. The residues between β95 and β138 of the Co-type enzyme differ from those in the Fe-type enzyme and interact with the α-subunit.\(^3\) The residues β111-β125 in the Co-type enzyme also has interactions with the α subunit unlike the Fe-type enzyme (Figure 7).\(^3\) There are also significant differences around the active site especially in the second sphere residues. These differences have been proposed to play a role in determining the substrate preferences observed for the Fe/Co-type enzymes. Substrate preference is highly dependent on βTrp\(^{72}\), βLeu\(^{48}\) and βPhe\(^{51}\) in the Co-type (*PtNHase*) enzyme.\(^3\) In the metal binding motif there are residues that are strictly conserved in the Fe-type enzyme such as Ser\(^{110}\) and Thr\(^{115}\) while in Co-type enzymes they are replaced with Thr\(^{109}\) and Tyr\(^{114}\) at the third
and eighth amino acid residues in the metal binding domain (Figure 8); this has been suggested to contribute to metal ion selectivity by the enzymes.\textsuperscript{38, 43, 43b}

1.6.1. NHase active site

Both the Co- and Fe-type NHase active sites contain a highly conserved amino acid sequence within the metal binding motif of Cys-Thr/Ser-Leu-Cys-Ser-Cys-Tyr/Thr (Figure 8).\textsuperscript{38, 39} The metal binding motif for all NHases are found in the α-subunit, the active site residues interact with arginine residues contributed from the β-subunit, which are strictly conserved in all NHases. These Arg residues form hydrogen bonds with the active site sulfinic and sulfenic acid residues (Figure 9).\textsuperscript{37c, 44, 37c, 38}

\textbf{Figure 8.} The NHase metal binding motif of Co-NHase from \textit{PtNHase-JCM 509} PDB:1IRE.
Figure 9. NHase Active site (A) Co-type NHase from *Pseudonocardia thermophila* PDB:1IRE and (B) Fe-type NHase from *Rhodococcus sp.* N-771.
The metal ion is coordinated in a N₂S₃ ligand environment from four different amino acid residues (3 cysteine residues, 1 serine) with the sixth ligand from either a water molecule or hydroxide ion for Co-type NHases or NO for Fe-type NHases resulting in a tetragonally-distorted octahedral ligand field (Figure 9). In some Fe-type NHases (for example, the NHase from *Comamonas testosteroni* Ni1 (CtNHase)) the sixth ligand position is unoccupied. Two of the cysteine residues are post-translationally modified into cysteine-sulfenic acid (Cys-SOH) and cysteine-sulfinic acid (Cys-SO₂H), respectively (Figure 9). These modified cysteines are in equatorial positions as are two amide backbone nitrogen atoms from the cysteine-sulfenic acid and serine residues. A third, unmodified cysteine residue, is in the proximal axial position trans to the sixth ligand. Based on DFT calculations, the two amide nitrogen atoms are proposed to be deprotonated, giving the C-N bond double-bond character, and therefore have imidate character when bonded to a metal ion. The amide nitrogen atoms are therefore good σ-donors to the metal ion. The oxidation of the two equatorial cysteine ligands make them poor π-donors to the metal ion; the lost π-donation of the two cysteines is compensated for by the axial cysteine residue through π-back donation to the metal ion. The two oxidized cysteines form hydrogen bonds with the two conserved arginine residues from the β-subunit as shown in Figure 9. Hydrogen bonding to the arginine residues is proposed to stabilize the active site and is essential for catalytic activity. Mutation of βArg56 in Fe-NHase, which hydrogen bonds to the oxygen atom of the sulfenic acid, to a Lys yielded enzyme with low catalytic activity of <1% of that observed for wild type enzyme. This residue has been proposed to position the oxygen atoms of the sulfenic and sulfinic acids through hydrogen bonding thus tuning the
active site.\textsuperscript{50} Similarly, it can therefore be inferred from the literature that βArg157, which forms hydrogen bonds with the sulfinic acid, plays the same role in NHases. Disruption of these hydrogen bonding interactions with the active site residues offsets the electron density distribution on the metal ion and subsequently, the ligands leading to the catalytic activity loss as reported by Inoue and Co-workers.\textsuperscript{50}

NHases have a relatively unusual active site compared to most other metalloenzymes due to the nature of the ligands and the oxidation states of the active site Fe\textsuperscript{3+} and Co\textsuperscript{3+} centers (Figure 9). Mononuclear non-heme enzymes typically have an Fe\textsuperscript{2+} ion, which is redox active unlike NHases. The presence of a Co\textsuperscript{3+} ion in the active site is also unusual, as cobalt has been shown to be required by relatively few enzymes (cobaltamine, ATP sulfurylases in Desulfovibrio and related bacteria, some prolidases and lysine-2,3-aminomutase).\textsuperscript{54} Moreover, the metal ion is ligated to deprotonated amide nitrogen atoms from the peptide backbone, which have been observed in few enzymes (the P-cluster of nitrogenase, the A cluster of acetyl CoA synthase, and virtually all of the copper-binding proteins involved in neurodegenerative diseases, including prions, amyloids, and synucleins).\textsuperscript{37a} The other unusual characteristic is the presence of two post-translationally modified cysteine residues at the active site, the modification of cysteine residues observed in other metalloenzymes is typically only a single residue.\textsuperscript{37a} The unusual active site of NHase enzymes was termed a “claw-setting”.\textsuperscript{47} This unique active site has also been reported to be present in thiocyanate hydrolases (SCNase), which converts the SCN\textsuperscript{−} anion into NH\textsubscript{3} and SCO.\textsuperscript{55,56,57} It is clear that NHase and SCNase enzymes are only active when the two modified cysteine residues are in the correct oxidation states as it has been shown that there is a direct correlation between the
catalytic rate and the amount of cysteine-sulfenic acid present. Apart from SCNase, other proteins with Cys-SO$_2$H and/or CysOH modifications include peroxiredoxins, hydrogenases, and NADH peroxidases.

The axial thiolate ligand in the NHase active site has been proposed to compensate for the decrease in electron density on the metal ion, due to oxidation of the equatorial cysteines, through π-back donation. This electronic buffering effect helps to maintain a relatively constant redox potential, thus stabilizing iron and cobalt ions in the 3+ oxidation state. Oxidation of the equatorial Cys residue to a sulfenic acid has been suggested to play a role in tuning the donor strength of the axial cysteine similar to heme proteins. In heme-proteins, the thiolate donor ligand strength modulates the porphyrin (π) → Fe$^{3+}$ charge transfer bands, as they are good π-donors, via π-back donation. The thiolate donor ligand strength was shown to be affected by hydrogen bonding networks with nearby residues. The sulfinic acid was shown to exist in its protonated state by FTIR and K-edge X-ray absorption spectroscopy (XAS) in combination with density functional theory (DFT) calculations. Unlike the sulfinic acid, the sulfenic acid protonation state remains controversial; FTIR assigned it as deprotonated while XAS assigned it as protonated. The protonation states of the active site residue have not been determined experimentally, addressing this would be of great importance in proposing a more detailed NHase catalytic mechanism.
1.6.2. NHase active site maturation

The post-translational modification of the NHase active site is essential for the catalytic activity of the enzyme. The different oxidation states of the three coordinating cysteine residues, the deprotonation of the ligated nitrogen atoms and the low spin state of the metal ion are vital to nitrile hydration. Therefore, the unique nature of the NHase active site has been of great interest in order to understand how the enzyme achieves its functional state. Proposals have been made on the sequence within which the active site maturation process occurs. The activator protein has been shown to be required for most NHases to bind metal ion, which has been proposed as the initial step in active site maturation. The following steps require oxidation of the two equatorial cysteine residues and the metal ion. The oxidation of the Cys-sulfur atoms in the NHases has been proposed to occur through reaction of the sulfur atoms with molecular oxygen in vivo.
Figure 10. Comparison of the cysteine modifications in rSCNasep, rSCNasem and rSCNasei.
Close-up views around the metallocenters of (a) rSCNasep, (b) rSCNasem and (c) rSCNasei at resolutions of 2.25, 1.9 and 1.78 Å, respectively. The backbone carbons are indicated in green, cyan, or magenta, nitrogens in blue, oxygens in red, sulfurs in orange and cobalts in pink. The 2Fo - Fc electron density map and the nonbiased Fo - Fc omit map are overlaid on the models. Oδ1, Oδ2 of γCys131-SO2-, Oδ atom of γCys133-SO(H) residues and the water ligand are omitted from the calculations of Fo - Fc map. The 2Fo - Fc map is contoured with 1.5 σ in gray surfaces, and the Fo - Fc map is contoured with 4.5 σ in red mesh.57
An investigation done using SCNase demonstrated the sequential oxidation of the equatorial Cys residues (Figure 10). The enzyme catalytic activity increased during storage at -80 ºC after 8 months but when the enzyme was stored under anaerobic conditions at 20 ºC for 4 months, there was complete loss of catalytic activity. The crystal structures of the enzyme at different conditions were solved, interestingly after purification only a small fraction of Cys-SO(H) was present and this increased during storage, thus the increased catalytic activity of the enzyme. On the other hand, the enzyme active site was double sulfinated leading to the loss of activity when stored under anaerobic conditions. The double sulfination and loss of protein activity has been reported in Fe-NHase when stored under aerobic conditions.

The sequential oxidation of the equatorial cysteine residues is supported by recent studies done using metallopeptides. Shearer and co-workers synthesized the first functional metallopeptide mimic of a Co-type NHase. They utilized a 12 amino acid sequence that contained an N\textsubscript{2}S\textsubscript{3} ligand environment, the results obtained support the proposal that the presence of sulfenic and sulfinic acid in the equatorial positions is essential for catalysis. They further presented evidence that the sulfenate was protonated based on XAS. A closely related study by Jones and co-workers utilized a seven amino acid sequence known as SODA with a coordination geometry of N\textsubscript{2}S\textsubscript{2}. They in turn demonstrated that metal ion oxidation occurs after the oxidation of the equatorial cysteine residues into sulfenic and sulfinic acid and suggested that the slow step in active site maturation is the oxidation of the Co\textsuperscript{2+} to Co\textsuperscript{3+}. The findings from these two recent investigations supports the idea that the formation of the sulfinic acid precedes that of the sulfenic acid.
1.7. Mutational, structural, kinetic and spectrometric analyses on nitrile hydratases

The Fe-type NHase contains a low-spin Fe$^{3+}$ ion which exhibits an S=1/2 spin state, thus enabling its characterization using spectroscopic techniques such as EPR. On the other hand, the Co-type NHase contains a low-spin Co$^{3+}$ ion with an S=0 ground state rendering it EPR silent. The Co-type NHases have therefore been characterized mostly through X-ray crystallography and kinetic studies. Co-type NHases are generally more air stable than Fe-type NHases and remain catalytically active for longer periods of time (days to months) when stored at 4 °C unlike Fe-type NHases, which lose catalytic activity quickly (hours to days) due to oxidation of the cysteine-sulfenic acid (Cys-SOH) to cysteine-sulfinic acid (Cys-SO$_2$H). When the Fe-type NHase is stored in the presence of the weak inhibitor butyric acid the oxidation process is markedly slowed.$^{11a,44,81}$

Some Fe-type NHases have been purified in an inactive form where NO is ligated to the active site Fe$^{3+}$ ion at the distal axial ligand position.$^{47}$ These enzymes are activated through photoirradiation. With NO at the distal axial position, Fe-type NHases exhibit an absorption band at 370 nm, which is a characteristic feature of an Fe$^{3+}$$\rightarrow$N metal-to-ligand charge transfer (MLCT) band.$^{37b}$ This band is due to strong back donation of the low-spin Fe$^{3+}$ center to the N $\pi^*$ orbital of nitrogen of the NO, which is then compensated for through strong $\pi$-donation by the axial thiolate ligand to the metal ion.$^{37b,82}$ The Fe-type NHase in its resting state has an intense absorption near 700 nm, which has been assigned as a S$\rightarrow$Fe$^{3+}$ LMCT band due to the $\pi$-donation from the axial thiolate ligand to the low-spin Fe$^{3+}$ ion.$^{83,83b,83c}$ The Co-type NHase has a significant absorption band within the visible region between 300 – 450 nm, which is a S$\rightarrow$Co$^{3+}$
LMCT band due to the π-donation from the axial thiolate ligand to the low-spin Co$^{3+}$ ion.\textsuperscript{84,85,37a} Mutation of some of the residues around the active site, which interact directly or indirectly with the active site residues have led to shifting of these charge transfer bands, an indication that the mutations perturb the active site environment and most particularly the π-donation from the axial thiolate ligand to the metal ion.\textsuperscript{86,21,50,87}

Mutational studies that have been performed on both Fe-type and Co-type NHase enzymes have shed light on the some of the catalytically important active site amino acid residues. For example, the strictly conserved βR157, αH80A, αH81A, αH80A/αH81A, αH80W/αH81W in CtNHase and the βY68F, αT109S, αY114T have been mutated in PtNHase as summarized in Table 2. Single mutations of the three active site cysteines to alanine residues led to catalytically inactive enzyme while triple mutations did not yield NHase.\textsuperscript{37b} This clearly shows that the three cysteines residues are required for catalytic activity.
Table 2. Mutations to various amino acid residues for both Fe-/Co-type NHases.\textsuperscript{23c 21}

<table>
<thead>
<tr>
<th>NHase identity</th>
<th>Mutant</th>
<th>$k_{cat}$ (s(^{-1})) &amp; % activity</th>
<th>$K_m$ (mM)</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>$PrNH$ase (Co-NHase)</td>
<td>$\alpha Y114T$</td>
<td>33.9 (1.7%)</td>
<td>9.9</td>
<td>Low cobalt content, 2Cys not modified; no disulfide bond formation. increased distance between the Co(^{3+}) ion and the ligands.</td>
</tr>
<tr>
<td></td>
<td>$\beta Y68F$</td>
<td>15.2 (1%)</td>
<td>58</td>
<td>No detectable Co ion</td>
</tr>
<tr>
<td></td>
<td>$\alpha T109S$</td>
<td>621 (32.5%)</td>
<td>107</td>
<td>Decreased Co content</td>
</tr>
<tr>
<td>$Rhodococcus$ sp. (Fe-NHase)</td>
<td>$\beta R56K$</td>
<td>1%</td>
<td></td>
<td>Changes in UV-Vis absorption band</td>
</tr>
<tr>
<td>$CtNH$ase-N1 (Fe-NHase)</td>
<td>Wildtype</td>
<td>1110</td>
<td>250</td>
<td>Loss of the hydrogen bonding to the cysteine residues Exibited a red shift in absorption band The hydrogen bond to the cysteines was present. Exhibited a red shift in absorption band</td>
</tr>
<tr>
<td></td>
<td>$\beta R157A$</td>
<td>10</td>
<td>204</td>
<td>Metal ion insertion was successful There was a red shift in absorption band</td>
</tr>
<tr>
<td></td>
<td>$\beta R157K$</td>
<td>32</td>
<td>239</td>
<td>Ion content was not disturbed Exhibited a red shift in absorption band</td>
</tr>
<tr>
<td></td>
<td>$\alpha H80A$</td>
<td>220</td>
<td>187</td>
<td>Metal ion insertion was successful There was a red shift in absorption band</td>
</tr>
<tr>
<td></td>
<td>$\alpha H81A$</td>
<td>77</td>
<td>179</td>
<td>Ion content was not disturbed Exhibited a red shift in absorption band</td>
</tr>
<tr>
<td></td>
<td>$\alpha H80A/\alpha H81A$</td>
<td>132</td>
<td>213</td>
<td>Had blue shift with increase in molar absorptivity</td>
</tr>
<tr>
<td></td>
<td>$\alpha H80W/\alpha H81W$</td>
<td>79</td>
<td>232</td>
<td>Had blue shift with increase in molar absorptivity</td>
</tr>
</tbody>
</table>
1.8. Proposed reaction mechanisms

There are several proposed reaction mechanisms for NHases and in all cases, the metal ion is proposed to be directly involved in catalysis, which has been strongly supported by EPR, resonance Raman spectroscopy, UV-vis absorption spectroscopy and X-ray crystallography.\textsuperscript{37c,88,88b} Three different catalytic mechanisms were proposed by Huang and co-workers (Figure 11, Figure 12, Figure 13). In the inner sphere mechanism (Figure 11), the substrate binds directly to the metal ion followed by nucleophilic attack on the nitrile carbon by a nearby water molecule. Two protons are transferred to the nitrile nitrogen from a nearby base and the other from the water molecule which attacks the nitrile carbon leading to formation of the amide. The product is then released from the active site.

In the second and third proposed reaction mechanisms (Figure 12, Figure 13), there is no direct interaction between the metal ion and the substrate. Instead, the metal ion activates a water molecule that acts as a nucleophile to attack the nitrile carbon, which is followed by proton transfer, amide formation, and then release of the product.\textsuperscript{37c}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{inner_sphere.png}
\caption{Inner-sphere/ first shell mechanism.\textsuperscript{39}}
\end{figure}
The outer-sphere/second shell mechanism (Figure 12) proposed that a hydroxide ligated to the metal ion is deprotonated by an active site base after nucleophilic attack on the C of the nitrile. The intermediate formed abstracts a proton from this base forming an amide, which is then released from the active site.

The second outer-sphere mechanism (Figure 13) suggests that a water molecule is deprotonated by the oxygen atom of the hydroxide bound to the active site metal ion. The hydroxide generated then acts as a nucleophile and attacks the nitrile carbon, this activates the nitrile to abstract a proton from a nearby base leading to formation of an amide and subsequently product release.
The inner-sphere mechanism has received much support from DFT studies (Figure 14). The nitrile is proposed to coordinate directly to the metal ion, but due to the metal ion being low-spin and in an electron rich environment, the metal ion is a weak Lewis acid. This led to Cys114-SO\(^-\) being proposed to function as a catalytic base, abstracting a proton from a water molecule thus activating it for a nucleophilic attack on the nitrile carbon. The metal ion, therefore, is suggested to provide electrostatic stabilization to the imidate intermediate, thereby lowering the reaction barrier for nucleophilic attack (Figure 14).\(^{89}\) The involvement of the cysteine-sulfenic acid ligand in catalysis has also been proposed, based on theoretical studies conducted on the second sphere mechanism, to function as a base which abstracts a proton from the metal bound water molecule activating it for nucleophilic attack on the nitrile carbon (Figure 15). Interestingly, the sulfenic acid ligand was also suggested to act as a nucleophile to the nitrile carbon atom, based on synthetic model compounds (Figure 16).\(^{37f}\)

![Figure 14. First-shell mechanism of NHase, based on DFT calculations.](image-url)
Figure 15. Outer sphere mechanism, involving attack of a metal-bound hydroxide ion on the nitrile.

Figure 16. Outer sphere mechanism involving Cys-SO$_2^-$ as a nucleophile.

To further elucidate the reaction mechanism, Holz and Mitra examined the solvent isotope effect, pH and temperature dependance of the kinetic constants $k_{cat}$ and $K_m$ for PtNHase. The study provided the first experimenal evidence that two protons are transferred in the rate limiting step.$^{89}$ A water proton was proposed to transfer to the nitrile nitrogen and $\alpha$Ser112 was proposed to shuttle a proton from $\beta$Tyr68, which gets protonated during the reaction.$^{89}$ Two ionizable groups were identified: one group is protonated while the other is deprotonated depending on the pH. The proposed residues included $\beta$Arg52, $\beta$Arg157 or $\alpha$Ser112, which are protonated at pH 7.5 and $\beta$Tyr68 or the metal-bound Cys-SO$_2^-$ ligand, which are deprotonated.$^{89}$

Reversible binding of the substrate to the metal ion was realized from single turnover stopped-flow spectrophotometry of the Fe-type $Re$NHase TG328-2 with
methacrylonitirile as the substrate. The pre-steady state behaviour of the reaction indicated two reaction steps. The first is the fast phase (substrate binding) while the second slow phase step is product release, which is the rate-limiting step. A minimal three-step kinetic model was proposed (Figure 17), which allows for i) reversible substrate binding, ii) the presence of an intermediate and iii) product formation. Rapid formation of Fe$^{3+}$-nitile species converts the resting Fe$^{3+}$ state to the substrate bound state along with an intermediate and product bound form, this was evident in the four spectrally unique species identified from singular value decomposition. The observed absorption bands are due to S→Fe$^{3+}$ LMCT bands and the observed changes are the result of changes in the back donation from the low-spin Fe$^{3+}$ center to the nitrine π* orbitals, which is similar to NHase-NO and Fe$^{3+}$-N$_3$ or –NO model complexes. These data confirmed for the first time the direct binding of a nitrile substrate to the metal ion.

![Figure 17. Proposed reaction model from single turn-over stopped flow experiment.](image)

The X-ray crystal stucture of 1-Butane boronic acid (BuBA), a competitive inhibitor, complexed with the PtNHase was obtained. BuBA is coordinated to the metal ion through an oxygen atom, but surprisingly, the oxygen atom of αCys113-SO(H) is covalently bound to the boron atom (Figure 18a). This interaction is due to nucleophilic
attack by the Cys-sulfenic acid oxygen on the empty P₂ orbital of the boron atom leading
to the loss of one of the boronic acid oxygen atoms (Figure 18b). A similar interaction of
an inhibitor and the PtNHase active site (Figure 18a) was also obtained through co-
crystallization of Phenyl-boronic acid (PBA) and PtNHase. These data strongly support
the first shell/inner sphere mechanism. Based on these data, Holz and Co-
workers proposed a catalytic mechanism that involves the Cys113-O(H) functioning as
the nucleophile during catalysis (Figure 19).
Figure 18. The crystal structure of the PtNHase complexed with inhibitors. A) Stereo view of PtNHase bound by BuBA after soaking in a cryoprotectant containing BuBA and B) Stereo view of PtNHase bound to BuBA after co-crystallization with BuBA (PDB: 4OB1). 

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Combination of the stopped-flow data and X-ray crystallographic data obtained from the PtNHase-boronic acid inhibitor complexes suggests that, the substrate binds directly to the metal ion leading to the formation of a substrate-enzyme complex. The metal bound water/hydroxide is displaced from the active site. The direct binding of the nitrile to the metal ion activates the nitrile carbon for nucleophilic attack by Cys113-SO(H) leading to the formation of a cyclic intermediate. This is then followed by the transfer of two protons. The first proton transfer is proposed to occur between
Cys113-SO(H) and the nitrile N-atom while the second proton is from the water molecule (proposed to regenerate the sulfenic acid ligand) and the nitrogen atom of the imidate. This proposed mechanism is therefore, consistent with the solvent isotope effect results. The sulfenic acid has been proposed to be protonated based on S K-edge XAS and DFT studies and the analysis of model metallopeptides. The second proton transfer is followed by tautomerization of the imidate upon nucleophilic attack by an active site water molecule to either the Cys-sulfenic acid or to the carbon atom of the imidate. The amide group is then displaced by a water molecule leading to the release of the product from the active site. The source of the amide oxygen atom had not been determined when this mechanism was proposed but was suggested to be the Cys113-SO(H) oxygen atom.

Quantum chemical studies support the Cys-sulfenic acid functioning as a nucleophile. These studies proposed that the amide is formed and released from the active site without the direct involvement of a water molecule as shown in Figure 20. Nucleophilic attack by the Cys-sulfenic acid and the first protonation step was proposed to be followed by formation of a disulfide bond between the Cys-sulfenic sulfur ligand and the axial cysteine (Figure 20).
Figure 20. A full reaction mechanism for NHase, involving the cyclic intermediate cleaved through an unexpected disulfide formation with the axial cysteine, without the involvement of a water molecule.\textsuperscript{37f}

Disulfide bond formation leads to a diminished interaction between the Cys-sulfenic acid and the cyclic intermediate.\textsuperscript{37f} The formation of the disulfide bond is highly unlikely given the proposals from the previous theoretical studies on the role of the axial thiolate ligand and the other proposed mechanisms.\textsuperscript{37b, 49, 88a, 91} The second proton from βTry72 is transferred to the intermediate via an active site serine residue\textsuperscript{37f, 89} and the oxygen atom from the Cys-sulfenic acid ligand is incorporated into the amide.\textsuperscript{37f, 88a} The active site is regenerated through proton transfer by βTry72 and a water molecule, which attacks the disulfide bond leading to its cleavage followed by proton transfer to βArg56.\textsuperscript{37f} Similar disulfide formation was recently proposed by Solomon and co-workers\textsuperscript{85} but this still remains to be determined experimentally.
Recently, an Fe-type ReNHase was characterized via time-resolved X-ray crystallography using the nitrile substrate pivalonitrile (PivCN). These data further support the direct coordination of the substrate to the active site metal ion and the Cys-sulfenic acid ligand functioning as a nucleophile during catalysis (Figure 21).\textsuperscript{37f, 88a, 91} FTIR analysis of nitrile hydration by wild-type ReNHase revealed that the oxygen atom of the Cys-sulfenic acid does not exchange with the water molecules around it when incubated in O\textsuperscript{18}-labelled water. Oxygen atoms from the nearby water molecule had been proposed to be incorporated into the Cys-sulfenic acid ligand during catalysis. These data suggest that the oxygen atom from the Cys-sulfenic acid is incorporated into the product.\textsuperscript{37f, 88a, 91} The active site is then regenerated through nucleophilic attack from a water molecule activated by the nitrile nitrogen, which abstracts a proton from it. The second proton transfer was proposed to take place from the regenerated Cys-sulfenic acid to the nitrogen of the nitrile (Figure 22).\textsuperscript{37f, 88a, 89, 91}

\textbf{Figure 21.} Structure surrounding the non-heme Fe\textsuperscript{3+} center and the βR56K complexed with PivCN a) PivCN approaching the active site, b) PivCN just before the nucleophilic attack, c) PivCN bonded to the Cys-sulfenic acid.
It is clear that the reaction mechanism of NHases has not been clearly determined, the mechanism that is greatly supported by the existing data in the literature currently is shown in Figure 23.
Figure 23. The scheme of the current proposed reaction mechanism for NHases.
1.9. Summary

Characterization of NHases has led to proposals for the catalytic roles of the active site residues and the maintenance of a suitable catalytic environment. Several catalytic mechanisms have been proposed with a clear indication and strong support for the inner sphere/first shell mechanism (i.e. the direct coordination of the substrate to the metal ion). The axial thiolate ligand is of great importance in tuning the active site and stabilizing the trivalent low spin state for the active site metal ion. The reported data from theoretical studies clearly proposes the role of the axial thiolate ligand as having an electronic buffering effect by maintaining a relatively constant redox potential and stabilizing the 3+ oxidation of the metal.\textsuperscript{37b, 49} This is achieved through its strong $\pi$-back donation to the metal ion, which compensates for the lost electron density due to oxidation of the equatorial cysteine ligands.\textsuperscript{49} The importance of this interaction at the metal ion enviroment makes it unlikely that a disufide bond can form during catalysis as proposed by Hopmann and co-workers and supported by Solomon and co-workers, because this distabilizes the low spin state of the metal ion, which has been shown to be important for catalysis.\textsuperscript{37b, 37f, 49}

The most likely mechanism for nitrile hydration is the one proposed by Holz and co-workers through path A, which has been supported by FTIR analysis.\textsuperscript{88a, 91} This mechanism has also been highly supported by theoretical studies, time-resolved X-ray crystallography, single turn-over stopped-flow experiments and is in line with the proposed inner-sphere mechanism.\textsuperscript{37b, c, 37f, 49, 88, 91} Therefore, i) the active site metal ion acts as a Lewis acid activating the nitrile for nucleophilic attack, ii) the Cys-sulfenic acid
ligand functions as a nucleophile during catalysis and iii) the oxygen atom from Cys-sulfenic acid is incorporated into the product. The active site maturation is achieved most efficiently for NHases in the presence of both activator protein and metal ions. The maturation process is initiated by insertion of the metal ion into the active site, which is followed by the formation of the Cys-SO$_2^-$ and subsequently the Cys-SO(H). The oxidation of the metal is proposed to occur after that of the equatorial cysteine residues. However, the proposed catalytic mechanisms, the active site maturation and the role of activator protein and Co ion in the maturation process still leave several unanswered questions: i) the source of the two protons transferred during catalysis, ii) the mode of product release from the active site and the regeneration of the active site, and iii) the protonation states of the post-translationally modified active site cysteine ligands, iv) the maturation process of the active site in vivo v) the role of the axial thiolate ligand in metal ion insertion and its oxidation, and vi) the essence of the activator protein, Co ion active site maturation. Gaining an understanding of these questions will significantly clarify our understanding of the catalytic mechanism of NHases.
1.10. Research aims

Nitrile hydratases have attracted significant interest for industrial applications because of their ability to hydrate nitriles under ambient conditions and physiological pH. They are currently used in the large-scale production of acrylamide and nicotinamide. NHases have been employed as bioremediation agents and they have become increasingly recognized for their reaction specificity. Even so, the active site maturation process, the role of the activator protein, Co ion and the axial thiolate in the maturation process, and the reaction mechanism is not fully understood. Characterization of Co- and Fe-type NHase enzymes has shed some light on its catalytic mechanism, but the role and protonation states of several catalytically important active site residues remains unclear. Therefore, in this thesis I examined the NHase active site maturation process, the role of the axial thiolate ligand, Co and the activator protein, the protonation states using the Co-type NHase from *Pseudonocardia thermophila* JCM 3095 (PtNHase) and the Fe-type NHase from *Rhodococcus equi* (ReNHase)-TG328-2. Specifically, I have:

i) Proposed the active site maturation sequence through mutagenic studies, NHase active site mettaloepptide mimic and expression and crystallization of NHases under different conditions.

ii) Investigated the role of the axial thiolate ligand and the nearby residues through site-directed mutagenesis.

iii) Synthesized and prepared deuterated protein and crystals to determine the protonation states of catalytically relevent active site residues via neutron diffraction.
CHAPTER 2

A Peptide Based Model Complex of the Nitrile Hydratase Active Site

2.1. Introduction

Nitrile hydratases (NHases, EC 4.2.1.84) are metalloenzymes involved in the carbon and nitrogen pathways of some bacteria and are useful industrial biocatalysts for conversion of nitriles into amides under mild conditions.\textsuperscript{37a,54b,93} They are metalloenzymes with a unique active site where a low-spin Fe(III) or Co(III) ions resides in a distorted octahedral geometry with ligands made up of two nitrogen atoms from the amide backbone, three cysteine sulfur atoms and a HO(H) as the source of the sixth ligand.\textsuperscript{38} Two cysteine residues are in the equatorial plane and are posttranslationally modified to sulfenic and sulfinic acids.\textsuperscript{38} The other two equatorial ligands are amide backbone nitrogen atoms that are proposed to be deprotonated with significant double bond character in the CN bond with the axial ligands being a Cys sulfur atom and a water molecule.\textsuperscript{68} The ligands therefore make the NHase active site electron rich, which is unusual for metal ions in a low-spin electronic state, but has been attributed to the oxidation of the equatorial cysteine residues and the tautomerization of the coordinated amides to their imidate forms.\textsuperscript{37a} This unique active site has also been reported to also be present in thiocyanate hydrolases (SCNase), which converts the SCN\textsuperscript{−} anion into NH\textsubscript{3} and SCO.\textsuperscript{55} It is clear that NHase and SCNase enzymes are only active when the two modified cysteine residues are in the correct oxidation states as it has been shown that there is a direct correlation between the catalytic rate and the amount of cysteine-sulfenic
acid present.\textsuperscript{44,58} X-ray crystallographic studies have provided clear evidence for the importance of the postranslational modification of the two cysteine residues with the cysteine-sulfenic acid proposed to function as a nucleophile during catalysis.\textsuperscript{70,91} Even though the active site structure of NHase and SCNase enzymes are ubiquitous and significant structural data exists, the catalytic role of the active site amino acids is not well understood.\textsuperscript{58b}

Complementing this enzymatic work on NHases is the design and synthesis of small molecule model complexes that mimic the NHase active site, some of which are capable of hydrolyzing nitriles to amides.\textsuperscript{79,74} Metallopeptide mimics, for example, based on the Nickel containing superoxide demultase (NiSOD) from \textit{Streptomyces coelicolor} was used and a histidine residue was replaced with cysteinethus providing the correct N\textsubscript{2}S\textsubscript{3} NHase coordination sphere based on ESI-MS, FTIR, Co K-edge X-ray absorption, electronic absorption, and CD spectroscopies.\textsuperscript{79} Reaction of this peptide with Co(I) in air produced an NHase-like active site that was capable of converting acrylonitrile to acryloamide. Like NHase and SCNase enzymes, over oxidation to the bis-sulfinic acid form resulted in inactivation of the catalyst. A similar study on a seven amino acid peptide based on SODA that contained an N\textsubscript{2}S\textsubscript{2} coordination sphere revealed oxidation of the equitoral Cys residues occurs in a stepwise fashion, however, this NHase peptide model complex was not catalytically competent.\textsuperscript{80}

Clearly, metallopeptide NHase model complexes are key components to understanding not only the NHase active site maturation process but also the catalytic mechanism. However, to date, no metallopeptide studies have appeared using the amino acid sequence directly from the NHase active site. In all known NHases, each \(\alpha\)-subunit
has a highly homologous amino acid sequence (CXYCSCX) that forms the metal binding site. Therefore, it was hypothesized that an eight amino acid peptide based on the metal binding motif of the Co-type NHase from *Pseudomonocardia thermophila* JCM 3095 (PtNHase) (Figure 24), would provide an NHase active site that contains the appropriate low-spin, octahedral trivalent iron or cobalt center and the properly oxidized equatorial and axial Cys ligands. Based on UV-Vis, magnetic circular dichroism (MCD) and electron paramagnetic (EPR) spectra, the eight amino acid peptide based on the metal binding motif of PtNHase generates an NHase active site and provides insight into the Cys oxidation process.
Figure 24. The eight amino acid residue metal binding motif from PtNHase PDB:1IRE.
2.2. Materials and methods

2.2.1. Materials

All reagents were purchased commercially and were the highest purity available.

2.2.2. Methods

2.2.2.1. Design and synthesis of the NHase active site peptide

The peptide VCTLCSCY was designed from the *PtNHase* gene sequence using the ApE software. It was synthesized by GenScript Co. with an acetyl group attached to the N-terminal to enhance solubility, and provided as a lyophilized powder of >98.5% purity. Peptide samples were used as provided.

2.2.2.2. Peptide-metal ion complex formation.

The NHase peptide has a MW of 933.13 g and is very soluble in degassed 10 mM N-ethyl morpholine (NEM) buffer at pH 8.0. All peptide samples were prepared under anaerobic conditions in a Coy soft-sided glove box. The molar absorptivity of the peptide was calculated to be $1,096 \text{ M}^{-1}\text{cm}^{-1}$ at 280 nm. A 7.6 mM stock solution of apo-peptide, which was colorless, was prepared and confirmation of free thiols was obtained using the Ellman’s method. Iron(II)sulfate heptahydrate was used as a source of Fe(II) and cobalt(II) chloride hexahydrate was used as the Co(II) source.
2.2.2.3. Physical measurements

UV-Vis spectra were obtained using a Shimadzu UV-2600 spectrophotometer in 1 mL quartz cuvettes at room temperature for both anaerobic and aerobic samples in 10 mM N-ethyl morpholine (NEM) buffer at pH 8.0. UV-Vis spectra were obtained for both the reduced and oxidized samples and a time-course following divalent metal ion oxidation to the trivalent form were performed in a soft-sided Coy glove box with the sample exposed to air at 1 hr. intervals. To confirm metal ion binding and oxidation of the cysteine residues, a 1 mg/mL sample of Fe-bound and Co-bound peptide solution that had been exposed to air for 3 hrs were aliquoted into vials and analysed using a high-MS direct inject method on a Shimadzu LC-MS 2020.

The Magnetic circular dichroism (MCD) data were collected using a Jasco model J-715 spectropolarimeter in conjunction with an Oxford Instrument SM-4000 8T magnetocryostat at a magnetic field of 7 T and temperatures of 4, 10, and 25 K. Potential artifacts due to glass strain were eliminated by taking the difference between spectra collected with the magnetic field aligned parallel and antiparallel to the direction of light propagation. A 3 mM sample of reduced Co-bound and Fe-bound peptide was prepared with 50% v/v glycerol in 10 mM NEM buffer pH 8.0. The samples were injected into the MCD cells under anaerobic conditions.

X-band parallel-mode (\(B_0\parallel B_1\); 9.37 GHz) and perpendicular-mode (\(B_0\perp B_1\); 9.63 GHz) EPR spectra were recorded at 10 – 30 K on an updated Bruker EMX-AA-TDU/L spectrometer equipped with an HP 5350B microwave counter for precise frequency measurement. Cryogenic temperatures were maintained with a ColdEdge/Bruker Stinger
S5-L recirculating helium refrigerator, and an Oxford ESR900 cryostat and Mercury ITC temperature controller. L-band EPR data were obtained at 1.3 GHz, 15 K, on a house-assembled instrument (National Biomedical EPR Center, Medical College of Wisconsin, Milwaukee, WI)\(^9\) consisting of a home-built solid-state low-phase-noise 50 mW L-band 1 - 2 GHz octave bridge,\(^6\) with a 70 kHz AFC lock and a low-noise amplifier in the receiver, a Varian V-7200 magnet and V-7700 power supply, a Bruker BH-15 field controller, and a 4 mm I.D. loop-gap resonator with 100 kHz field modulation.\(^7\) Temperature was maintained at 15 K using a modified Air Products Heli-Tran system. For both Co- and Fe-type peptides samples, 0.76 mM solutions were prepared in 10 mM NEM buffer pH 8.0 and transferred into EPR tubes under anaerobic conditions. These samples were then flash-frozen in liquid nitrogen.

2.3. Results

2.3.1. Optical spectroscopy of the Co-NHase peptide complex

An anaerobic aqueous solution of Co(II) was titrated stepwise into a buffered (10 mM NEM buffer pH 8.0) colorless apo-peptide solution in a Coy softsided glove box until one equivalent of the Co\(^{2+}\) was added, providing a green solution (Figure 25 I)\(^8\). Two distinct electronic absorption bands were observed at 685 nm and 750 nm, which arise from Co(II) ligand-field (d-d) transitions (Figure 26).\(^9\) With exposure to air, these two absorption bands are lost and new bands appeared between 310-450 nm where the 310-350 feature is characteristic of a S-to-Co(III) ligand-to-metal charge
transfer (LMCT) band (Figure 26).\textsuperscript{85 37a} The band around 310 to 350 nm is similar to that observed for the Co-type PtNHase.

\textbf{Figure 25.} 0.76 mM NHase peptide solution in 10 mM NEM buffer pH 8.0 at room temperature under anaerobic conditions. (I) NHase peptide solution with no metal, (II) reduced Co-bound NHase, (III) oxidized Co-bound NHase with 5 min exposure to air and (IV) oxidized Co-bound NHase 3-hour exposure to air.
Figure 26. UV-Vis absorption of 0.76 mM NHase active-site peptide in 10 mM NEM buffer pH 8.0 at 25 °C (solid) NHases peptide under anaerobic conditions with no metal ion, (dotted) Co-bound NHase peptide in anaerobic conditions, and (dashed) Co-bound peptide exposed to air for 5 hrs.
Table 3. UV-Vis absorption data for Co-bound NHase peptide

<table>
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<tr>
<th>Sample</th>
<th>Electronic absorption</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>$\lambda$ (nm)</td>
</tr>
<tr>
<td></td>
<td>$\varepsilon$ (M$^{-1}$ cm$^{-1}$)</td>
</tr>
<tr>
<td>Reduced Co-bound NHase peptide</td>
<td>274 (3835)</td>
</tr>
<tr>
<td></td>
<td>322 (2861)</td>
</tr>
<tr>
<td></td>
<td>333 (2831)</td>
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<tr>
<td></td>
<td>685 (426)</td>
</tr>
<tr>
<td></td>
<td>740 (365)</td>
</tr>
<tr>
<td>Oxidized Co-bound NHase peptide</td>
<td>274 (4468)</td>
</tr>
<tr>
<td></td>
<td>350 (2765)</td>
</tr>
<tr>
<td></td>
<td>460 (1489)</td>
</tr>
<tr>
<td>Reduced Fe-bound NHase peptide</td>
<td>279 (1995)</td>
</tr>
<tr>
<td></td>
<td>317 (1461)</td>
</tr>
<tr>
<td>Oxidized Fe-bound NHase peptide</td>
<td>277 (4496)</td>
</tr>
<tr>
<td></td>
<td>306 (4665)</td>
</tr>
<tr>
<td></td>
<td>437 (2332)</td>
</tr>
<tr>
<td></td>
<td>490 (2445)</td>
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</table>

The metal binding properties of the NHase peptide were also analyzed through optical spectroscopy under anaerobic conditions. Co(II) was added dropwise to a 0.76 mM buffered (10 mM NEM buffer pH 8.0) solution of the peptide while monitoring the absorbance at 685 nm. The fraction of saturated metal binding sites ($f_a$) was determined using the equation $1^{98}$. 
\[ f_a = \frac{(y - y_f)}{(y_b - y_f)} \]

1

where \( y \) is the change in the absorbance at 685 nm, \( y_f \) is the absorption at 685 nm when the binding sites are unoccupied, and \( y_b \) is the absorption when the binding sites are fully occupied. The binding function \((r)\) was then calculated using equation 2 assuming a \( p \) value of 1:

\[ f_s = \frac{r}{p} \]

2

where

\[ r = \frac{(\bar{C}_s - C_s)}{\bar{C}_A} \]

3

with \( \bar{C}_s \) equal to the total amount of ligand added, \( C_s \) is the molar concentration of the free ligand, and \( \bar{C}_A \) is the total amount of bound peptide. A plot of \( f_a \) vs. \( \bar{C}_s/\bar{C}_A \) was fit to the Michaelis-Menten equation and the stoichiometry was inferred by extrapolating the two linear segments of the graph to their point of intersection, which represents the endpoint of the titration (Figure 27). The data provide a binding constant (\( K_d \)) of 0.68 ± 0.16 µM. These data are consistent with metal binding constants reported for the Co-
NiSOD and Co-SODA peptide complexes and suggest that a single divalent metal ions binds moderately tightly to the NHase active site peptide.\textsuperscript{79-80}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure27.png}
\caption{Evaluation of stoichiometry of Co(II) to peptide interaction by stoichiometric titration. The curve has been generated using Michaelis-Menten equation.}
\end{figure}

The MCD spectra of the reduced Co-NHase peptide complex exhibited features of two sets of ligand field transitions centered around 710 nm (14,100 cm\textsuperscript{-1}) and 490 nm (20,410 cm\textsuperscript{-1}) as shown in Figure 28. Ligand-to-metal charge transfer bands (likely S-to-Co) are evident at shorter wavelengths while d-d transitions appear around 710 nm. The MCD results strongly support the existence of two species in the anaerobically prepared Co-bound peptide samples.
Figure 28. MCD spectra of 3 mM Co-bound NHase prepared under anaerobic conditions recorded at a magnetic field of 7 T and temperatures of 4, 10, and 25 K.

2.3.2. Optical spectroscopy of the Fe-NHase peptide complex

An anaerobic aqueous solution of Fe(II) was titrated into a buffered (10 mM NEM buffer pH 8.0) NHase peptide sample resulting a colourless solution. Upon exposure to air, the Fe-NHase peptide complex turned light orange within 5 min but the color intensified to an orange-brown within one hour (Figure 29). The UV-Vis spectrum of the aerobic Fe-NHase peptide complex exhibited absorption bands at 490 nm with a shoulder at around 436 nm (Table 3; Figure 30), these bands are characteristic of S-to-Fe(III) charge transfer bands, and similar absorption bands are observed between 425-500 nm for Fe-type NHase enzymes. The observed Fe-NHase peptide complex
absorption bands decreased in intensity with prolonged exposure to air. There was no absorption band observed at ~700 nm, which is characteristic of Fe-type NHases due to S-Fe(III) LMCT bands. MCD spectra of an anaerobic Fe-NHase peptide complex did not exhibit any features in the visible or near-IR regions.

**Figure 29.** 0.76 mM NHase peptide solution in 10 mM NEM buffer pH 8.0 at room temperature under anaerobic conditions. (I) NHase peptide solution with no metal, (II) reduced Fe-bound NHase, (III) oxidized Fe-bound NHase with 5 min exposure to air and (IV) oxidized Fe-bound NHase 3-hour exposure to air.
Figure 30. UV-Vis absorption spectra obtained by titration of the 0.7 mM NHase active-site peptide with Fe (II) in 10 mM NEM buffer pH 8.0 at 25 °C, (dashed) peptide with no Fe, (solid) Fe-bound peptide with 1 equivalent of Fe$^{2+}$ under anaerobic conditions and (dotted) Fe-bound peptide with 1 equivalent Fe$^{2+}$ with 5 hr. exposure to air.

2.3.3. EPR Spectroscopy of anerobically-prepared Co- and Fe-NHase peptide

EPR spectra recorded at X-band on anaerobically-prepared Co-NHase peptide complex exhibited two distinct signals (top panel, Figure 31). The first, with a high amplitude peak at 2986 G ($g = 2.03$), is essentially axial with $g$-values of 2.30, 2.25, and 2.03 and was relatively fast-relaxing, indicative of a low-spin $d > 5$ transition ion system and suggestive of a low-spin Co(II) ion. A symmetric pattern of resonances around $g = 2.03$ suggested a hyperfine manifold but good simulations could not be obtained and even
the best approximations required unprecedented amounts of strain in both $g$ and $A$. An unreasonable $g$-tensor resulted, though non-collinearity of $g$ and $A$ was not exhaustively explored. The assignment of the signal to a low-spin Co(II) was, however, confirmed by EPR at L-band (bottom panel, Figure 31), in which a clear multi-line hyperfine pattern centered around $g = 2.03$ was observed. At low-field, between 1000 and 2000 G, a low-amplitude resonance was observed that, on closer inspection, exhibited resolved turning points at $g' = 5.7$ and 4.8. These values are suggestive of an $S = 3/2$ spin system and, at a temperature of 30 K (Figure 31), the spectrum can be seen to clearly cross the baseline at around 2000 G and the broad absorption continues until around 4000 G. This signal is indicative of high-spin Co(II). Although the signal exhibited low amplitude in the spectrum, integrational analysis indicated that the contributions to the overall spin-density of the low- and high-spin signals are almost equal (51 and 49%, respectively).
Figure 31. EPR spectra of 0.76 mM Co-NHase peptide sample in 10 mM NEM buffer, pH 8.0 of anaerobically-prepared Co-VCTLCSCY. Top panel: X-band (9.63 GHz) EPR spectra of Co-VCTLCSCY recorded at 12 K, 1 mW. The low-field signal corresponds to an $S = \frac{3}{2}$, system, consistent with high-spin Co(II) with $M_S = \pm \frac{1}{2}$, and the high-field signal corresponds to an $S = \frac{1}{2}$, system, consistent with low-spin Co(II). Bottom panel: L-band (1.3 GHz) EPR spectrum of Co-VCTLCSCY recorded at 15 K with 16 dB microwave power attenuation of a nominal 50 mW source. The spectrum is dominated by the low-spin signal and resonances due to high-spin Co(II) are not identifiable.
The X-band perpendicular-mode spectrum of anaerobically-prepared Fe-NHase peptide complex (Figure 32) exhibited no significant or immediately-assignable features and was characterized only by an ill-defined low-field trough extending from zero-field to around 1200 G, with a minimum at \( g' \approx 9 \). These data are perhaps suggestive of an integer-spin system. This suggestion was dramatically confirmed upon recording the parallel-mode spectrum, which revealed an intense resonance at \( g' \approx 9 \) that, in turn, suggested an \( S = 2 \) spin-system by application of the \( g' \approx 4S \) rule\(^{100}\) and, therefore, Fe(II). The envelope of zero-field splitting energies extends beyond the microwave quantum energy, and only one set of non-Kramers' signals is observed, so the spin Hamiltonian parameters are not uniquely determinable. However, a very good simulation was obtained using \( S = 2, g = 2 \), and a zero-field splitting envelope that is partially accessible at 9.37 GHz (\( D = 0.29 \text{ cm}^{-1}, E/D = 0.14, \sigma D = 1.2 \text{ GHz}, \sigma E = 200 \text{ MHz} \)), showing that the signal is at least consistent with the assignment of an Fe(II).

<table>
<thead>
<tr>
<th>Species</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced Co-bound peptide</td>
<td>( g_x = 2.30, g_y = 2.25, g_z = 2.03; g' = 5.7 \text{ and } 4.8 )</td>
</tr>
<tr>
<td>Reduced Fe-bound peptide</td>
<td>( g' \sim 9 )</td>
</tr>
<tr>
<td></td>
<td>( D(\text{cm}^{-1}) = 0.29, E/D = 0.14, \sigma D(\text{GHz}) = 1.2, )</td>
</tr>
<tr>
<td></td>
<td>( \sigma E(\text{MHz}) = 200 )</td>
</tr>
<tr>
<td>Oxidized Fe-bound peptide</td>
<td>( g_\perp (g = 2.04), g_\parallel (g = 2.20, 2.16) )</td>
</tr>
</tbody>
</table>
Figure 32. EPR spectra of 0.76 mM NHase peptide sample in 10 mM NEM buffer, pH 8.0 of anaerobically-prepared Fe-VCTLCSCY. The top two traces show the perpendicular- and parallel-mode EPR spectra, respectively, of Fe-VCTLCSCY. The bottom trace is a computer simulation invoking an $S = 2$ system with a zero-field splitting envelope that is partially accessible by the microwave quantum (9.37 GHz).
2.3.4. Air-oxidation of Co- and Fe-NHase peptide complexes.

Upon exposure of the Co-NHase peptide complex to air, the EPR signal completely disappeared (Figure 33). Comparison with an EPR signal observed for Co(II) in buffer showed (i) that the high-spin Co(II) signal from Co$_\text{aq}$ in buffer was distinct from that in Co-NHase peptide complex, and (ii) that exposure to air did not simply liberate cobalt from the peptide. The only explanation for this behavior, then, is oxidation of Co(II) to Co(III) for both the high-spin and low-spin Co(II) components of the anaerobic Co-NHase peptide complex.
Figure 33. The effect of exposure to air on the EPR signals of 0.76 mM Co-NHase peptide sample in 10 mM NEM buffer, pH 8.0 of Co-VCTLCSCY. The main figure shows the EPR spectra of anaerobically-prepared Co-VCTLCSCY (top trace) and the spectrum of the sample after exposure to air for 30 minutes (bottom trace). The inset shows the low-field region of the spectra, and the signal from Co(II) added to the buffer system is overlaid, showing that the $S = 3/2$ signals from anaerobically-prepared Co-VCTLCSCY and Co(II)$_{aq}$ in buffer are distinct. Spectra were recorded at 30 K, 2 mW microwave power.
Upon exposure of the Fe-NHase peptide complex to air, more complicated changes to the EPR spectrum resulted (Figure 34). After a 3 h exposure, the $S = 2$ parallel-mode signal due to Fe(II) was diminished by a factor of almost three. In the perpendicular mode spectrum, a high-spin signal appeared that was clearly absent from the perpendicular-mode spectrum of the anaerobically-prepared Fe-NHase peptide complex (top trace, Figure 32). This high-spin signal exhibited a low-field resonance at $g' = 6$, as expected for an axial $M_S = \pm \frac{1}{2}$ signal of an $S = \frac{5}{2}$ high-spin Fe(III) ion. The dominant signal, though, appeared to exhibit a rhombic signal with a low-field peak at $g' = 5.2$, a broad derivative with an inflection at $g' \sim 3.3$ (~2100 G), and a high-field broad resonance in the region of $g' = 2$ that extended to > 4000 G. A set of $S = \frac{5}{2}$ simulations employing a systematic parameter search was employed to explain the spectra of model compounds for nitrile hydratase that exhibited high-spin Fe(III) signals in earlier work. Given the sometimes non-intuitive dependence of these simulations on the spin Hamiltonian parameters, particularly strains in the zero-field splittings, and that the simulations do not take into account relaxation behavior that can lead to predicted resonances not being observed experimentally, one is reluctant to definitively rule out $S = \frac{5}{2}$ as a source of the $g' = 5.2, 3.3, \sim 2$ signal observed here. However, these parameters are immediately recognizable as being consistent with, and sufficient to completely define, a single $S = \frac{3}{2}, M_S = \pm \frac{1}{2}$ spin system with $g$ slightly larger than 2.0 and $E/D \sim 0.2$. 
**Figure 34.** The effect of exposure to air on the EPR signals of 0.76 mM Co-NHase peptide sample in 10 mM NEM buffer, pH 8.0 of Fe-VCTLCSCY. Top panel: The top two traces show the parallel-mode EPR spectra (10 K, 80 mW, 9.37 GHz) of a sample of Fe-VCTLCSCY prepared anaerobically (top) and after 3 h exposure to air (bottom). The bottom two traces show the corresponding perpendicular-mode spectra (10 K, 10 mW, 9.63 GHz) of the sample exposed to air for 3 h, and the spectrum of the sample after further exposure to air for a total of 20 h, respectively. Bottom panel: The main trace shows the entire spectrum of Fe-VCTLCSCY exposed to air for 20 h, and the inset shows the g ~ 2 region in detail.
Upon additional exposure of Fe-NHase peptide complex to air, for 20 h, the high-spin signal persisted, retaining 95% of its intensity, and an additional signal with high amplitude was observed in the \( g \sim 2 \) region of the spectrum. This latter signal was characterized by an axial \( g_\perp \) resonance at \( g = 2.04 \) and two distinct apparently \( g_\parallel \) resonances at \( g = 2.20 \) and 2.16, respectively. These \( g \)-values lie somewhere between those of the (LFe)OTT nitrile hydratase model in dichloromethane (2.06, 2.03, 2.02) and a nitrile-bound model, [LFe-NCMe]\(^+\) (2.27, 2.18, 1.98), described by Grapperhaus and coworkers.\(^96\) Despite the very high amplitude of this signal, assigned to a low-spin Fe(III) center, its contribution to the total spin density was only about 8%. This is therefore consistent with retention of ~95% of the high-spin Fe(III) signal upon the appearance of the low-spin signal.

### 2.4. Discussion

The rationale for study of these Co- and Fe-NHase peptide complexes was to obtain information on the maturation of the active site of NHases. For NHase enzymes, maturation occurs \textit{in vivo} and involves the co-expression of an activator (\( \varepsilon \)) protein, which makes it difficult to study this process in detail. As all NHases contain a highly homologous amino acid sequence within the \( \alpha \)-subunit (\( \text{VCX}^1\text{LCSX}^2\text{-X}^1\text{-T(Co)/S(Fe)} \) and \( \text{X}^2\text{-Y(Co)/T(Fe)} \) that forms the metal binding site,\(^38\) an eight amino acid peptide sequence was synthesized based on \( \text{PtNHase} \). The addition of Co(II) to this NHase peptide under anaerobic conditions resulted in a green solution indicating a Co(II)-peptide binding interaction. Exposure to air results in a color change from green to light brown and finally deep brown suggesting oxidation of the Co(II) ion to Co(III). MS indicates
that the equitorial Cys residues also oxidizes as the oxygen atoms were added to the peptide. These data suggest the formation of the sulfinic and sulfenic acid ligands, identical to the PtNHase active site (Figure 24). The anerobic addition of Fe(II) to the NHse peptide results in a colorless solution that turns orange upon exposure to air. This color change also suggests the oxidation of Fe(II) to Fe(III) with the likely oxidation of the equitorial Cys residues. Interestingly, the oxidized Fe-NHase peptide complex does exhibit a green color with an absorbance around 700 nm, as is typically observed in Fe-type NHases.¹⁰¹

EPR spectra were obtained for both the Co- and Fe-NHase peptide complexes in their anerobic and aerobic states. In the case of the Co-NHase peptide complex, the Co(II) exists in both low-spin and high-spin states in essentially equal proportions, indicating two distinct chemical environments, consistent with MCD data. Upon air oxidation, the EPR signals were rapidly extinguished and no changes in the proportions of high- and low-spin Co(II) could therefore be assessed. These data are consistent with the formation of a low-spin Co(III) center which is \( S = 0 \), and therefore EPR silent. In the case of iron, a more detailed progression could be observed. The initial Fe(II) signal, exhibiting unusually small zero-field splitting, is slowly diminished upon oxidation and is replaced by high-spin signals, presumably due to Fe(III). Only after prolonged exposure to air is an additional signal, due to low-spin Fe(III), observed that, even after 20 h exposure, still accounts for < 10% of the Fe(III) in the sample.

The low-spin Fe(III) signals, likely two that differ in \( g \| \), are consistent with previously studied model compounds as they exhibit \( g \)-values within the range \( 2.3 > g > 1.97 \) and also resemble the oxidized form of Fe-type NHases.¹⁰² ¹⁰³ The high-spin Fe(III)
signals, though, are worthy of comment. A sharp resonance at \( g' = 6 \) is not unexpected for high-spin \( S = \frac{5}{2} \) Fe(III). However, the other resonances very convincingly appear to define a rhombic species with \( g' \)-values that are most straightforwardly attributed to an \( S = \frac{3}{2} \) system rather than \( S = \frac{5}{2} \). While \( S = \frac{5}{2} \) cannot be absolutely ruled out, it would seem an exceedingly unlikely coincidence that the very specific set of spin Hamiltonian parameters, including strains and temperature-dependent relaxation parameters, that would be required to describe this hypothetical \( S = \frac{5}{2} \) spin system would yield an EPR signal that is so readily and completely described by a simple set of \( S = \frac{3}{2} \) parameters.

The EPR data, then, very strongly suggest the presence of an \( S = \frac{3}{2} \) spin system and chemical sources for this must, then, be considered. First, Co(II), which is \( S = \frac{3}{2} \) in the high-spin state, is a rare metal in the general environment and is therefore highly unlikely to be present as a contaminant. Second, intermediate-spin \( S = \frac{3}{2} \) Fe(III) is extremely rare so, while theoretically a possibility, would appear unlikely on the grounds of probability. A third option is the binding of some paramagnetic system to iron that generates an \( S = \frac{3}{2} \) system, the most likely of which would appear to be nitric oxide to form an \{FeNO7\} system. The question then becomes the source of nitric oxide.

2.5. Conclusion

An eight residue peptide (VCTLCSCY) making up the active site ligands for \( PtNHase \) was designed and synthesized and shown to bind both Co(II) and Fe(II) ions forming metallopeptide complexes that mimic the active site of NHses based on electronic absorption, LCMS, MCD, and EPR data. Previously reported data for NHase enzymes, primarily Co-type NHases, suggest metal binding occurs \textit{via} a “self-subunit”
swapping mechanism in vivo where an apo-αε2 complex binds a Co(II) ion and the α-subunit is then inserted in an apo-αεβ2 complex by swapping α-subunits to form a Co(II)-bound-αεβ2 protein. These data suggest that the first step in NHase active site maturation is the coordination of Co(II) to the NHase active site. This first step is consistent with MALDI spectroscopic studies on the apo-α-subunit of Rhodococcus rhodechrous J1, which indicated that the equatorial Cys residues were modified after the Co(II) was inserted into the active site. Therefore, the availability of metal ion in the active site appears to be crucial for the post translational oxidation process. The next step involves the oxidation of the Cys-sulfur atoms, which in NHases has been proposed to occur through a step wise process where the Cys-sulfinic acid forms first followed by oxidation of the second Cys to a sulfenic acid. Combination of these data with the NHase peptide complex data reported herein, allows a mechanism for the metal ion maturation process in NHases (Figure 35). After that addition of divalent metal ion, equatorial Cys oxidation occurs with the active site metal ion remaining a high-spin divalent state. EPR data suggest that the active site metal ion then oxidizes to become trivalent but still remains in a high-spin state followed by the spin state change to low-spin. Upon continued exposure to air, the active site then forms the bis-sulfinic acid form and the metal ions remains trivalent and low-spin, similar to NHase enzymes which form the double sulfinated form with complete loss of enzymatic activity under aerobic conditions. Therefore, the metallo-NHase peptide complexes reported herein provide new insight onto the metal ion maturation process for NHases enzymes.
Figure 35. Sequential maturation of the NHase active site
CHAPTER 3

Catalytic Role of the Axial Cysteine Residue in Nitrile Hydratases

3.1. Introduction

Nitrile hydratases (NHases, EC 4.2.1.84) are intracellular metalloenzymes that catalyze the hydration of nitriles to their corresponding amides under ambient temperature and physiological pH.$^{84,106}$ They are important biocatalysts in the industrial production of commodity chemicals due to their ability to hydrate a broad range of nitriles under ambient conditions.$^{11b,107}$ NHases are $\alpha_2\beta_2$ heterotetramers $^4$ and the successful expression of soluble, active enzyme is dependent on the co-expression of an activator protein.$^{35}$ Their active site contains either a low-spin Fe(III) or Co(III) ion in a distorted octahedral geometry that is electron rich with two amides and three cysteine residues and a sixth axial ligand that is typically a water molecule (Figure 36).$^{37a,38-39,53,68}$ However, low-spin Fe(III) and Co(III) ions are classically thought to be exchange inert and such a negative coordination environment with an exchange inert metal ion would intuitively suggest that the Fe(III) and Co(III) ions that occupy the active sites of NHase enzymes would not be a good Lewis acid nor would they have the ability to bind nitriles and release product making them poor hydration catalysts. So how can NHase enzymes catalyze the hydration of nitriles given the highly unusual coordination environment of the active site metal ion?
Based on a significant number of model studies, DFT calculations, and enzyme crystallographic data, several hypotheses have been provided.\textsuperscript{108} First, in the enzymatically active form of all NHase enzymes, the equatorial cysteine residues are post-translationally modified to cysteine-sulfinic acid (Cys–SO\textsubscript{2}H) and cysteine-sulfenic acid (Cys–SOH).\textsuperscript{109} Oxidation of the equatorial cysteine residues is essential for catalysis and results in the removal of some of the negative charge from the M(III) center.\textsuperscript{108b} Second, DFT calculations revealed that the backbone amide ligands have significant imidate character, which provides only partial negative charge to the trivalent metal ion.\textsuperscript{48} Finally, like heme systems,\textsuperscript{65-66, 110} it has been proposed that the axial thiolate group in NHase enzymes "push" electrons, assisting the active site trivalent metal ion to bind and activate nitriles.\textsuperscript{37a, 71, 111} Model complex studies have shown that an axial
thiolate residue increases the ligand exchange rate since substituting the trans thiolate with a nitrogen ligand decreased the exchange rate by three-orders of magnitude.\textsuperscript{37a} Interestingly, all theoretical calculations assume that the axial Cys ligand is a thiolate, making it a strong $\sigma$-donor ligand and a good $\pi$-donor.\textsuperscript{37a, 48, 69} However, in NHase the thiolate character of the axial Cys ligand is likely modulated through a hydrogen bonding interaction with a strictly conserved $\alpha$Ser or $\alpha$Thr residue, which likely influences the axial Cys sulfur ligands $\pi$-donating ability and nucleophilicity. Combination of these post-translational modifications and hydrogen bonding interactions likely offset some of the anionic character of the NHase active site providing a synergistic effect, which specifically tunes the Lewis acidity of the active site trivalent metal ion.\textsuperscript{109a, b}

Recently, a theoretical study suggested that the axial thiolate ligand can form a disulfide bond with the sulfenic acid cyclic intermediate.\textsuperscript{37f, 85} However, no experimental data has been reported to support disulfide bond formation in the transition-state and its essence in the attack of the $\alpha$Cys-OH cyclic intermediate by a water molecule. One way to investigate the catalytic role of the axial Cys ligand is to mutate it to an alanine residue, which was attempted in the high-molecular mass Co-type NHase from \textit{Rhodococcus rhodochrous} J1 but no detectable enzyme expression was observed.\textsuperscript{87} Herein, the axial thiolate ligand in the Co-type NHase from \textit{Pseudonocardia thermophila} JCM 3095 (PrNHase) was mutated to an Ala residue in both the absence and presence of the activator protein. Surprisingly, the PrNHase $\alpha$C108A mutant exhibited detectable enzymatic activity with little to no cobalt present and X-ray crystallographic data provide evidence for the oxidation states of the equatorial Cys residues, providing insight in the active site maturation process. These data also establish for the first time that while
transient disulfide bond formation in the transition-state may be energetically favorable, it is not required for catalytic turnover.

3.2. Materials and Methods

3.2.1. Materials

All chemicals and reagents used were of the highest purity available and were used as purchased without further purification.

3.2.2. Methods

3.2.2.1. Primer design, mutagenesis, expression and purification.

Primers were designed using the PtnHase gene sequence and ApE software. The oligonucleotides were obtained from Integrated DNA Technologies Inc. and the forward primer sequence was 5'CCACGTCGTCGTCGCTCTC-3' while the reverse primer was completely complementary to the forward primer with the modified bases in bold, lower-case letters. Agilent’s QuickChange Lightning kit was used for mutagenesis. A modified QuickChange protocol was used for polymerase chain reaction (PCR) by amplifying the forward and reverse primers separately. Following an initial denaturation at 95 °C for 50 s, 3 PCR cycles were carried out and each cycle comprised incubation at 95 °C for 50 s, followed by incubation at 50 °C for 50 s, and a 15 min. incubation period at 68 °C. The process was completed with a 7 min. incubation period at 68 °C. The amplified gene sequence was then used in the second PCR with forward and reverse
primers mixed and the same PCR procedure except 16 cycles were used instead of three. The resultant mutant genes were inserted into pET28a+ plasmids. Gene sequencing was performed at Functional Biosciences to confirm successful mutagenesis. The recombinant gene was co-transformed with the gene for the PtNHase activator protein into BL21(DE3) *E. coli* cells. Both PtNHase wildtype and mutant enzymes were expressed and purified as previously described. The PtNHase αC108A mutant was expressed in the absence (αC108A-Act) and presence (αC108A+Act) of activator protein with Co(II) supplementation at induction. On the other hand, PtNHase was expressed in the absence of both Co(II) and activator, in the presence of Co(II) only, and in the presence of activator only. All purified PtNHase enzymes were stored in 50 mM HEPES buffer at pH 7.0.

3.2.2.2. UV-Vis spectroscopy and kinetic assay.

The protein concentration was determined at $A_{280}$ on a Shimadzu UV-2450 PC spectrophotometer equipped with a TCC temperature controller in a 1 cm quartz cuvette in 50 mM HEPES buffer pH 7.5. UV-Vis spectra were obtained at pH 7.5 in 50 mM HEPES buffer, and 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 $\Delta \varepsilon_{225} = 2.9 \text{ mM}^{-1} \text{ cm}^{-1}$. Assays were performed in triplicate (Table 5). The reaction was initiated by adding the enzyme to the reaction mixture (1 mL) containing various concentrations of 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. Kinetic data analysis was done using OriginPro 9.0 (OriginLab, Northampton, MA).

3.2.2.3. Metal analysis.

Protein samples for PtNHase wildtype and mutant enzymes were denatured using 8 M urea. A solution of 20% nitric acid and 10% hydrochloric acid was added to the samples to a final volume of 10 mL and incubated for 30 min at room temperature to ensure complete acid digestion. An identical mixture of nitric acid and hydrochloric acid was used as a blank. The samples were analyzed using inductively coupled plasma mass spectrometry (ICP-MS) at the Water Quality Center in the College of Engineering at Marquette University (Milwaukee, WI, USA) (Table 5).

3.2.2.4. Crystallization and Data Collection.

Crystallization of PtNHase was performed following the previously reported protocol with slight modifications in the precipitant concentration of 1.2 M sodium citrate tribasic instead of 1.4 M and 0.1 M HEPES, pH 7.5. Diffraction quality crystals grew within two weeks and belonged to the space group P321 with one copy of heterodimer in the asymmetric unit. For X-ray data collection, these crystals were frozen
following the protocol previously used. The diffraction data for each crystal were collected at the Advanced Photon Source (Argonne National Laboratory, Argonne, IL) Structural Biology Center (SBC) 19ID. The crystal diffraction images were recorded using a 4×4 tiled 300×300 mm² sensitive area CCD detector from Area Detector Systems Corp. (ADSC) at a wavelength of 1.0 Å. Each dataset was indexed, integrated and scaled using HKL2000. Data collection, processing and refinement statistics are given in Table 6.

3.2.2.5. Phasing, model building, and refinement.

Phasing was carried out with molecular replacement using the program Phaser from the CCP4 software suite. The structure of PtNHase (PDB code 1IRE), was used as the starting search model absent any water molecules. Rigid body refinement was followed by restrained refinement with Refmac5 and further manual model inspection and adjustments with Coot. When refinement converged, solvent molecules were added over several rounds. The \( R_{\text{free}} \) and the \( R_{\text{factor}} \) values for PtNHase αC108A+Act, αC108A−Act, WT PtNHase expressed with activator but no Co(II) supplementation (PtNHase−Co), and WT PtNHase expressed in the absence of activator protein and Co(II) supplementation (PtNHase−Co/Act) were 0.26/0.30, 0.13/0.16, 0.17/0.20, and 0.20/0.24 of the final model respectively (Table 6).
3.3. Results and Discussion

Mutation of the axial thiolate ligand in the Co-type NHase from *Rhodococcus rhodocrous* J1 was reported to affect protein expression as no enzyme was obtained from either the supernatant or inclusion bodies. In their study, the corresponding NHase activator protein was not co-expressed with the axial Cys mutant, which has been shown to be required for the functional expression of NHase enzymes. The lack of co-expression with the activator protein may in fact be the reason that no expression was observed. To test this hypothesis, the *PtNHase* αC108A mutant was over-expressed in the absence (αC108A-Act) and presence (αC108A+Act) of the *PtNHase* activator protein with Co(II) supplementation at the time of induction. Both mutant enzymes expressed as soluble proteins and were purified via IMAC and size exclusion chromatography. SDS-PAGE analysis revealed that both αC108A mutants were >95% pure (Figure 37). These data indicate, at least for *PtNHase*, that soluble enzyme can be obtained when expressed in the absence of the activator protein.
Table 5. Summary of the kinetic constants and the Co\textsuperscript{3+} ion content of each PtNHase protein.

<table>
<thead>
<tr>
<th>PtNHase</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>Co content/αβ dimer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PtNHase</td>
<td>1,790 ± 50</td>
<td>3.0 ± 0.4</td>
<td>1.7</td>
</tr>
<tr>
<td>PtNHase\textsuperscript{−Co}</td>
<td>ND\textsuperscript{a}</td>
<td>ND\textsuperscript{a}</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>PtNHase\textsuperscript{−Co/Act}</td>
<td>ND\textsuperscript{a}</td>
<td>ND\textsuperscript{a}</td>
<td>ND\textsuperscript{a}</td>
</tr>
<tr>
<td>αC108A\textsuperscript{−Act}</td>
<td>ND\textsuperscript{a}</td>
<td>ND\textsuperscript{a}</td>
<td>ND\textsuperscript{a}</td>
</tr>
<tr>
<td>αC108A\textsuperscript{+Act}</td>
<td>3.8 ± 0.8</td>
<td>7.0 ± 2.0</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>αC108A\textsuperscript{+Act} from crystals</td>
<td>3.8 ± 0.8</td>
<td>ND\textsuperscript{b}</td>
<td>ND\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}None Detected.
\textsuperscript{b}Not Determined

Interestingly, the αC108A\textsuperscript{−Act} mutant was colorless in 50 mM HEPES buffer at pH 7.5 even at concentrations exceeding 500 µM (Figure 37B) and contained no detectable metal ions based on ICPMS analysis (Table 5). On the other hand, the αC108A\textsuperscript{+Act} was light green at concentrations >500 µM (Figure 37B) but contained <0.1 Co(III) ions per αβ dimer, based on ICPMS analysis (Table 5). A UV-Vis spectrum was obtained for the αC108A\textsuperscript{+Act} mutant and exhibited $\lambda_{max}$ values at ~450, ~630, and >750 nm (Figure 38). Comparison to WT PtNHase, which exhibits $\lambda_{max}$ values at 440 and 550 nm suggests that the ligand-to-metal charge transfer (LMCT) bands observed for WT PtNHase are red-shifted for the αC108A\textsuperscript{+Act} mutant (Figure 38)\textsuperscript{116}. The observed red-shift in the αC108A\textsuperscript{+Act} mutant LMCT bands, clearly reflect that the altered electron density around
the Co(III) ion and the observed bands likely result from equatorial Cys and backbone amide LMCT. Given the low Co(III) content, the lack of an axial Cys ligand reduces the ability of the active site to bind cobalt due to the elimination of the electron donating ability of the axial thiolate. These data also confirm previous studies that indicate co-expression of PtNHase with the PtNHase activator protein is essential for metal insertion.23a, 26, 51, 75

![SDS-PAGE gel](image)

**Figure 37.** SDS-PAGE gel A1) PtNHase\(^{+\text{Act}}\), A2, A3) PtNHase \(\alpha\text{C108A}^{+\text{Act}}\); 1B) Purified PtNHase in 50 mM HEPES buffer at room temperature; A) 0.54 mM PtNHase\(^{+\text{Act}}\), B) 0.19 mM PtNHase \(\alpha\text{C108A}^{+\text{Act}}\), and C) 0.62 mM PtNHase \(\alpha\text{C108A}^{+\text{Act}}\).

Kinetic characterization of the \(\alpha\text{C108A}^{-\text{Act}}\) and \(\alpha\text{C108A}^{+\text{Act}}\) mutant enzymes were conducted in 50 mM HEPES buffer at pH 7.0 using acrylonitrile as the substrate (Table 5). Not surprisingly, no activity was detected for the \(\alpha\text{C108A}^{-\text{Act}}\) under any conditions examined. However, unexpectedly, the PtNHase \(\alpha\text{C108A}^{+\text{Act}}\) mutant was catalytically competent and exhibited a \(k_{\text{cat}}\) value of 3.8 ± 0.8 s\(^{-1}\) with a corresponding \(K_m\) of 7.0 ± 2.0 µM (Table 5). The observed \(k_{\text{cat}}\) value is highly reproducible across multiple purifications and \(V_{\text{max}}\) is dependent on enzyme and substrate concentrations. The observed \(k_{\text{cat}}\) value for the \(\alpha\text{C108A}^{+\text{Act}}\) mutant is ~0.2% of that observed for WT
PtNHase and is consistent with the low level of Co(III) ions (<0.1) bound to the active site. The observation of catalytic activity for an NHase where the axial thiolate ligand is eliminated firmly establishes that the formation of a disulfide bond between the axial thiolate ligand and the equatorial sulfenic acid thiolate ligand in the transition-state, as proposed theoretically, is not essential for catalytic turnover.

**Figure 38.** UV-Vis spectra of PtNHase in 50 mM HEPES pH 7.5 (dashed) PtNHase\(^{+\text{Act}}\) and (solid) PtNHase \(\alpha C108A^{+\text{Act}}\) mutant at 25 °C.

Confirmation of the active site structures of the \(\alpha C108A^{-\text{Act}}\) and \(\alpha C108A^{+\text{Act}}\) mutant enzymes was obtained by X-ray crystallography. Quality colorless crystals were obtained for both the \(\alpha C108A^{-\text{Act}}\) and \(\alpha C108A^{+\text{Act}}\) mutant enzymes, unlike WT PtNHase crystals which are yellow-brown. The lack of any detectable color is consistent with the little or no Co(III) in their active sites. The crystals for the \(\alpha C108A^{-\text{Act}}\) and \(\alpha C108A^{+\text{Act}}\)
mutant enzymes diffracted to 1.28 Å and 1.39 Å, respectively, and the resulting overall structures were nearly identical to WT PtNHase, with only slight differences in the helices near the active site, with RMSD of 0.007Å for αC108A^-Act and 0.020Å αC108A^+Act. For αC108A^-Act, the mutation of the axial Cys residue is clearly supported by lack of electron density (Figure 39). No electron density was observed for a Co(II) in the active site, the uncoordinated αC111 was found to be in two conformations refined with 50% occupancies for both. Similar to the previously reported apo-PtNHase structure 21, one of the αC108A^-Act mutant αC111 confirmations formed a disulfide bond between the two equatorial cysteine residues (αC111 and αC113) with a bond distance of 2.1 Å (Figure 39A). In the second αC111 confirmation, the sulfur atoms of αC111 and αC113 are 3.8 Å apart, a distance too far for a disulfide bond. Despite the lack of post-translational modification of the equatorial cysteine residues in the αC108A^-Act mutant structure and the formation of a disulfide bond between αC111 and αC113, two conserved arginine residues from the β-subunit are within hydrogen bonding distances of αC111 and αC113 (3.1 to 3.8 Å) (Table 6, Figure 40A). βR175 exhibits two conformations and can only hydrogen bond to the sulfur atom of αC111 in one of the conformations (Figure 40A).
Figure 39. The active site crystal structure of A) αC108A<sup>+</sup>_Act, B) αC108A<sup>−</sup>_Act, and C) the X-ray crystal structure was obtained at 1.8 Å (PDB:1IRE from literature) generated using PyMOL™2.3.2 by Schrodinger, LLC. (distance is in Å).
Figure 40. The crystal structure showing the hydrogen bonding interaction of the active site residues with some of the second sphere amino acid residues of PtNHase: A) αC108A^{+Act}, B) αC108A^{-Act} and C) the X-ray crystal structure was obtained at 1.8 Å (PDB:1IRE) from literature generated using PyMOL™2.3.2 by Schrodinger, LLC. (distance is in Å).
Table 6. Statistics for the X-ray crystal structures of the PtNHase αC108A+Act, αC108A−Act, PtNHase−Co and PtNHase−Co/Act.

<table>
<thead>
<tr>
<th></th>
<th>αC108A+Act</th>
<th>αC108A−Act</th>
<th>PtNHase−Act</th>
<th>PtNHase−Co/Act</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data Processing</strong></td>
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<tr>
<td>Space group</td>
<td>P 3 2 1</td>
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<td>P 3 2 1</td>
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<tr>
<td>Cell dimension</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>α, β, γ (deg)</td>
<td>65.8, 65.8, 185.3</td>
<td>65.8, 65.8, 185.9</td>
<td>66.1, 66.1, 185.9</td>
<td>65.9, 65.9, 186.3</td>
</tr>
<tr>
<td>a, b, c (Å)</td>
<td>90.0, 90.0, 120.0</td>
<td>90.0, 90.0, 120.0</td>
<td>90.0, 90.0, 120.0</td>
<td>90.0, 90.0, 120.0</td>
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a The values for the highest-resolution bin are in parentheses
b R_{merge} = Σ|I_{obs} - I_{avg}|/ΣI_{avg}
c Precision-indicating merging R
d Pearson correlation coefficient of two “half” data sets
éR_{work} = Σ|F_{obs} - F_{calc}|/ΣF_{obs}
Five percent of the reflection data were selected at random as a test set, and only these data were used to calculate R_{free}
e Root-mean square deviation
Table 7. Bond distance between the cysteine residues at PtNHase active site.

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Interestingly, both equatorial αCys residues are properly oxidized to cysteine-sulfenic acid (αCys-SOH) and cysteine-sulfenic acid (αCys-SO₂H) acids with occupancies of 100% in the αC108A⁺Act structure (Figure 39B). Mutation of the axial αCys residue is clear and unexpectedly, no electron density is observed for a Co(III) ion in the active site. The lack of the axial αCys108 residue eliminates disulfide formation bond between the axial thiolate ligand and the equatorial sulfenic acid thiolate ligand in the transition-state. Therefore, such a transient disulfide bond is not essential for catalytic turnover. The sulfur atoms of the equatorial αC111 and αC113 residues are 3.8 Å apart, a distance too far for disulfide bond formation (Figure 40A). Similar to WT PtNHase (Figure 40C), the Cys-SO₂H and Cys-SOH oxygen atoms form hydrogen bonds with the strictly conserved βR52 and βR175 (Figure 40B). Hydrogen bond distances between the catalytic nucleophile Cys-SOH oxygen atoms and βR52 are on average 0.25 Å shorter than those observed in WT PtNHase but are longer by 0.23-0.54 Å for the Cys-SO₂H oxygen atoms (Table 7, Figure 40B and C). Proper oxidation of the two equatorial cysteine residues with no evidence for an active site metal ion prompted the question of

| αCSD111(SG) | αCO113(SG)A | 3.8 |
| αCSD111(SG) | αCys108(SG) | 3.7 |
| αCO113(SG)A | αCys108(SG) | 3.5 |
| αCO113(SG)B | αCys108(SG) | 2.8 |
| αCys113(SG)A | βArg52(NE) | 4.4 |
| αCys113(SG)B | βArg52(NE) | 6.2 |
| αCys113(SG)A | βArg52(NH2) | 3.4 |
| αCys113(SG)B | βArg52(NH2) | 5.2 |
| αCys113(SG)A | βArg52(NH2) | 6.1 |
| αCys113(SG)B | βArg52(NH2) | 4.4 |
| αCys113(SG)A | βArg52(NH2) | 5.3 |
| αCys113(SG)B | βArg52(NH2) | 3.5 |
| αSer112(OG) | βTyr68(OH) | 2.7 |

In contrast to WT PtNHase (Figure 40C), the Cys-SO₂H and Cys-SOH oxygen atoms form hydrogen bonds with the strictly conserved βR52 and βR175 (Figure 40B). Hydrogen bond distances between the catalytic nucleophile Cys-SOH oxygen atoms and βR52 are on average 0.25 Å shorter than those observed in WT PtNHase but are longer by 0.23-0.54 Å for the Cys-SO₂H oxygen atoms (Table 7, Figure 40B and C). Proper oxidation of the two equatorial cysteine residues with no evidence for an active site metal ion prompted the question of
whether the αCys-SOH can function as a nucleophile and hydrate nitriles in the absence of a metal ion. Therefore, crystals of the αC108A\textsuperscript{+Act} mutant were dissolved and assayed in 50 mM HEPES buffer at pH 7.0 and 25 °C using acrylonitrile as the substrate. Surprisingly, catalytic activity was observed with an identical $k_{cat}$ value of 3.8 ± 0.8 s\textsuperscript{-1} as the as purified αC108A\textsuperscript{+Act} mutant (Table 5). While trace amounts of Co(III) ions in the crystals cannot be ruled out, it is possible that the observed hydration activity is due to the substrate accessing the active site near the nucleophilic Cys-SOH moiety without binding to a metal ion, resulting in nucleophilic attack on the nitrile carbon.

Since the $Pt$NHase αC108A\textsuperscript{+Act} mutant enzyme contains properly oxidized equatorial αCys residues while the αC108A\textsuperscript{+Act} mutant enzyme does not, these data beg the question of the role of the metal ion and the activator protein in the post-translational modification of the equatorial cysteine residues. To address this question, WT $Pt$NHase was expressed under three different conditions, which were with activator but no Co(II) supplementation ($Pt$NHase\textsuperscript{Co}), no activator but with Co(II) supplementation ($Pt$NHase\textsuperscript{−Act}), and with neither activator or Co(II) supplementation ($Pt$NHase\textsuperscript{Co/Act}) and compared to the αC108A\textsuperscript{−Act} and αC108A\textsuperscript{+Act} mutant structures. Purified $Pt$NHase\textsuperscript{Co}, $Pt$NHase\textsuperscript{−Act}, and $Pt$NHase\textsuperscript{Co/Act} were colorless and contained no detectable cobalt (Table 5). Kinetic analysis of each $Pt$NHase expression variant in 50 mM HEPES buffer at pH 7.0 and 25 °C using acrylonitrile as the substrate indicated that these enzymes did not exhibit detectable catalytic activity. These data suggest that like αC108A\textsuperscript{−Act}, no post-translational modification of the equatorial αCys residues occurs without both activator and Co(II) present.
Figure 41. The active site crystal structure of PtNHase A) PtNHase\(^{\text{Co/Act}}\) with 2Fo-Fc electron density map contoured at 1.0\(\sigma\), B) An overlap of the PtNHase\(^{\text{Co/Act}}\) and PtNHase\(^{\text{Co}}\) (PDB:1UGQ from the literature, C) PtNHase\(^{\text{Act}}\) with 2Fo-Fc electron density map contoured at 1.0\(\sigma\), and D) an overlap of the PtNHase\(^{\text{Act}}\) and PtNHase\(^{\text{Co}}\), generated using PyMOL\textsuperscript{TM}2.3.2 by Schrodinger, LLC. (distance is in Å).
Figure 42. The crystal structure showing the hydrogen bonding interaction of the active site residues with some of the second sphere amino acid residues of PtNHase A) PtNHase\textsuperscript{Co/Act} with B) An overlap of the PtNHase\textsuperscript{Co/Act} and PtNHase\textsuperscript{Co} (PDB:1UGQ from the literature, C) PtNHase\textsuperscript{Act}, and D) An overlap of the PtNHase\textsuperscript{Act} and PtNHase\textsuperscript{Co}, generated using PyMOL\textsuperscript{TM}2.3.2 by Schrodinger, LLC. (distance is in Å).

Confirmation of the lack of post-translational modification for PtNHase\textsuperscript{Co}, PtNHase\textsuperscript{Act}, and PtNHase\textsuperscript{Co/Act} was provided by X-ray crystallography. X-ray quality colorless crystals were obtained for both PtNHase\textsuperscript{Co} and PtNHase\textsuperscript{Co/Act} and diffracted to 1.39 and 1.49 Å, respectively (Table 6). The resulting overall structures were nearly identical to WT PtNHase, with only slight differences in RMSD of 0.029 Å for PtNHase\textsuperscript{Co} and 0.026 Å for PtNHase\textsuperscript{Co/Act} (Table 6). The structure for PtNHase\textsuperscript{Act} was previously reported by Wakagi and co-workers and was used for comparison purposes (Figure 41 and Figure 42 B&D).\textsuperscript{21} For PtNHase\textsuperscript{Co} and PtNHase\textsuperscript{Co/Act}, the αC113 sulfur atoms
reside in two different conformations with occupancies of (A) ~30 and (B) ~70% (Figure 41A and C). The distances between the axial cysteine αC108 residue sulfur atom and the αC113 sulfur atom was 2.8 and 3.6 Å for $Pt\text{NHase}^{-\text{Co/Ac}}$ and 2.8 and 3.5 Å for the $Pt\text{NHase}^{-\text{Co}}$ (Figure 41A and C; Table 7). For comparison purposes, WT $Pt\text{NHase}$ exhibits a 3.1 Å distance between the sulfur atoms of αCys108 and αCys113-SOH, while the distance is 2.0 Å for $Pt\text{NHase}^{-\text{Co}}$. The observed αC108-αC113 sulfur-sulfur distances for $Pt\text{NHase}^{-\text{Co/Act}}$ and $Pt\text{NHase}^{-\text{Act}}$ are longer than typical disulfide bonds, but fall within the Vander Waals radii for two sulfur atoms, which is ~3.6 Å. These data indicate that in both $Pt\text{NHase}^{-\text{Col/Act}}$ and $Pt\text{NHase}^{-\text{Act}}$, like $Pt\text{NHase}^{-\text{Co}}$, a disulfide bond exists for conformation (A) but not for confirmation (B). It should be noted that the distances between sulfur atoms of αC111 and αC108 for $Pt\text{NHase}^{-\text{Col/Act}}$, $Pt\text{NHase}^{-\text{Act}}$, and WT $Pt\text{NHase}$ are 3.6 Å, 3.6 Å, and 3.3 Å respectively, indicating that no disulfide bond is present. Combination of these data indicate that co-expression of activator along with Co(II) supplementation upon induction are required for both metal ion insertion and post-translational modification of the equatorial Cys ligands.
3.4. Conclusions

The data reported herein on the PtNHase alanine mutant indicate that disulfide bond formation in the transition-state, as proposed theoretically, is not essential for catalysis. That the activator protein and the metal ion are required for posttranslational modification of the equatorial cysteine is clear. These data support the proposal that the activator protein assists with metal and the post-translational modification of the active site equatorial Cys residues. The possible weak disulfide bonds observed for apo-PtNHase enzymes are likely the result of a lack of divalent metal ions and activator, which inhibits metal ion insertion into the apo-enzyme in vitro. 118
CHAPTER 4
Determinations of the Protonation States of Catalytically Important Residues in a Transition-State Inhibitor Complex of a Co-Type Nitrile Hydratase via Neutron Diffraction

4.1. Introduction

Nitriles are commonly found in biological systems as cyanoglycosides or cyanolipids, but many plants also produce nitriles including ricin and β-cyanoalanine for self-defense purposes.\(^5,^{119}\) Nitrile hydratases (NHases, EC 4.2.1.84) are members of the nitrile degradation pathway and catalyze the hydration of nitriles to amides under ambient conditions and physiological pH.\(^{108b,120}\) NHases can also hydrate a wide range of synthetic nitrile substrates, which has resulted in their intense biotechnological exploitation as biocatalysts in preparative organic chemistry and in several industrial applications, most notably in the production of acrylamide and nicotinamide.\(^{121}\) A key advantage of NHases is their stereoselectivity, which is of particular importance in the pharmaceutical arena.\(^{122}\) NHases have also proven useful in the bioremediation of chemical and wastewater runoff, specifically for the hydration of nitrile-based pesticides such as bromoxynil, and are thus becoming increasingly recognized as a truly new type of “Green” chemistry.\(^{123}\)

Despite the biological, industrial, and bioremediation importance of NHase enzymes, many details of their reaction mechanism and metallocenter assembly remain poorly defined. Knowledge of their chemistry, together with advances in protein
screening and genetic engineering, will also provide insight into hitherto unconsidered catalytic reactions and exploitable chemistries, and impetus for development of a new area of catalytic chemistry through biomimetic and protein engineering. The successful determination of the protonation states of the active site amino acid residues will benefit society by facilitating a more intelligent design and manufacture of nitrile based chiral pharmaceuticals and industrially important specialty chemicals such as acrylamide and nicotinamide.¹²⁴

Figure 43. The X-ray crystal structure of PtNHase active site obtained at 1.8 Å (PDB:1IRE from literature generated using PyMOL™2.3.2 by Schrodinger, LLC. NHases are metalloenzymes that contain either a low-spin (\(S = \frac{1}{2}\)) non-heme Fe(III) (Fe-type) or a low-spin (\(S = 0\)) non-corrin Co(III) (Co-type) ion in their active site.¹⁰⁸b X-ray crystallographic studies on NHases reveal that they are \(\alpha_2\beta_2\) heterotetramers with an active site metal ion coordinated by three cysteine residues, two
amide nitrogen atoms, and a water or hydroxyl moiety (Figure 43).\textsuperscript{108,125} Two of the active site cysteine residues are post-translationally modified to cysteine-sulfinic acid (–\text{SO}_2\text{H}) and cysteine-sulfenic acid (–SOH), respectively, yielding a coordination geometry termed a “claw-setting”.\textsuperscript{38} Biologically, cobalt has otherwise been confirmed only in vitamin B12 and bound within a corrin-type ring, so NHases represent a rare case of a biologically important, non-corrin ring Co(III) containing metalloenzyme.\textsuperscript{108b} The protonation states of the active site equatorial sulfenic and sulfinic acids were suggested to be Cys-SOH and Cys-SO\textsubscript{2}\textsuperscript{−} based on sulfur K-edge XAS and geometry-optimized DFT calculations,\textsuperscript{109b} oxidation of the equatorial Cys residues is essential for catalysis.\textsuperscript{109a} The catalytic relevance of these moieties is established, but information on their mechanistic roles and the catalytic significance of their protonation states is only just starting to evolve.
Figure 44. The structure of PtNHase complexed with inhibitors A) Stereo view of PtNHase bound by BuBA after soaking in a cryoprotectant containing BuBA and B) Stereo view of PtNHase bound to BuBA after co-crystallization with BuBA (PDB: 4OB1).
The X-ray crystal structures of the PtNHase-1-butaneboronic acid (BuBA) complex formed by either co-crystallization (1.5 Å) or soaking (1.6 Å) and the PtNHase-phenylboronic acid (PBA) structure via co-crystallization (1.3 Å) have been reported (Figure 44). These X-ray crystal structures revealed the oxygen atom of the sulfenic acid ligand (αCys113-OH) is covalently bound to the boron atom of BuBA with a bond distance of 1.6 Å (Figure 44). This covalent bond, which had not been observed previously, is the result of nucleophilic attack of the sulfenic acid oxygen atom on the empty $p_z$ orbital of the boron atom and the subsequent loss of a boronic acid oxygen atom. These two structures represent a “snapshot” of two potential intermediate states in nitrile hydration depicting nucleophilic attack by the sulfenic acid ligand and the initial stage of the product-release step. The p$K_a$ of CysSO$_2^-$ is 6 to 7 and given that an Arg residue forms a hydrogen bond (Figure 43) to CysSO$_2^-$ and Arg has a p$K_a$ of 12 to 14, these two species are expected to be charge separated at the pH of maximal activity, which is 7.5. The p$K_a$ of SO$_2^-$ is 10 to 11 suggesting that SO$_2^-$ is protonated at pH 7.5. The structural characterization of both Fe-type and Co-type NHase enzymes has provided some insight into how the molecular structure controls enzyme function; however, details regarding the protonation states of active site residues and a metal coordinated water molecule, that have been shown to be essential for catalysis, remain unclear.

Hydrogen atoms/ions comprise half of the atoms in a protein molecule and are of chemical and structural importance. Determining the positions of hydrogen atoms in proteins reveals: hydrogen bonding interactions, the protonation states of catalytically important amino acid residues, and the location and geometry of the water molecules.
In X-ray crystallography, it is difficult to locate the positions of the hydrogen atoms/ions, therefore, a combination of X-ray crystallography and neutron diffraction studies are ideal. Neutron diffraction can directly reveal the positions of hydrogen atoms, the replacement of hydrogen atoms with deuterium helps to improve their visibility because it has a positive scattering length. The intensity of the neutron source is weak compared to X-ray sources, which requires large crystals and longer exposure times for neutron diffraction experiments. To address these issues, exchangeable hydrogen atoms are typically replaced with deuterium, perdeuterated enzymes are prepared, and large crystals are required.

The catalytic relevance of αCys\textsuperscript{113}-OH and its protonation states over the course of the reaction profile is critically important but remains elusive. Several NHases have produced crystals that diffract well, and X-ray crystal structures have been reported with relatively high resolutions (~1.2 Å). However, attempts to measure data at ultrahigh resolution, needed for assessing the protonation states, revealed significant radiation damage at and around the metal site, including both post-translationally modified cysteine residues. Therefore, combined X-ray and neutron diffraction crystallography is necessary in order to identify the protonation states of these catalytically critical residues and thus shed new light on the catalytic mechanism of NHase enzymes. High quality, large crystals (0.7 mm x 0.8 mm x ~1 mm; P3\textsubscript{2}21, unit cell: α=β=90°, γ=120°, a=b=65.7 Å, c=185.6 Å) can be reliably grown for the Co-type nitrile hydratase from \textit{Pseudonocardia thermophila} JCM 3095 (PtNHase) in both H\textsubscript{2}O and D\textsubscript{2}O buffers in the absence and presence of boronic acids. Moreover, large crystals of perdeuterated PtNHase have also been successfully produced in collaboration with the deuteration lab
at Oak Ridge National Laboratory (ORNL). Neutron diffraction data collected thus far, in collaboration with Dr. Dean Myles at ORNL, suggest a neutron structure for PtNHase in the absence and presence of boronic acid inhibitors is forthcoming.

4.2. Materials and Methods

4.2.1. Materials

All reagents were purchased commercially and were the highest purity available.

4.2.2. Methods

4.2.2.1. Hydrogen/deuterium exchange of PtNHase.

Plasmid prep, co-transformation, growth, expression and purification of PtNHase were performed as previously described.\textsuperscript{112b} The peak fractions were collected and concentrated followed by buffer exchange into 50 mM HEPES D\textsubscript{2}O buffer at pD 7.0 using an Amicon Ultra-15 centrifugal filter device. SDS-PAGE analysis was performed to determine protein purity and kinetic analysis was carried out previously reported described.\textsuperscript{112b}
4.2.2.2. **Expression of PtNHase in H\textsubscript{2}O and D\textsubscript{2}O minimal media.**

Two different minimal media solutions were prepared:

- Holme minimal media-containing EDTA
- Studier minimal media

**Table 8.** Base components of minimal media solutions.

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium chloride dihydrate</td>
<td>0.5</td>
</tr>
<tr>
<td>Cobalt(II)chloride</td>
<td>0.1</td>
</tr>
<tr>
<td>Cupric sulfate pentahydrate</td>
<td>0.15</td>
</tr>
<tr>
<td>Ferric chloride hexahydrate</td>
<td>16.7</td>
</tr>
<tr>
<td>Manganese(II)sulfate monohydrate</td>
<td>0.1</td>
</tr>
<tr>
<td>EDTA disodium dihydrate</td>
<td>22.3</td>
</tr>
<tr>
<td>Zinc sulfate heptahydrate</td>
<td>0.2</td>
</tr>
</tbody>
</table>

To prepare minimal media, (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} (0.686g), KH\textsubscript{2}PO\textsubscript{4} (0.156 g), Na\textsubscript{2}HPO\textsubscript{4}.H\textsubscript{2}O (0.648 g), (NH\textsubscript{4})\textsubscript{2}-H-citrate (0.049 g) and glycerol (1.0 g) were added to 80 mL of NANO-pure water and dissolved with stirring. After complete dissolution, 0.025 g of MgSO\textsubscript{4}.7H\textsubscript{2}O was added, followed by 100 μL of 100 mg/mL CARB and 100 μL of KAN (50 mg/mL). Finally, 100 μL of the base minimal media solution was added (Table 8). The pH of the media was adjusted to 7.0 and was sterile filtered using acrodisc 0.2 mm syringe filters and stored in tightly closed clean dry falcon tubes. The 50 mL of
D₂O media (had the same components as the hydrogenated media except for D₂O instead of H₂O) used in this experiment was provided by the Bio-Deuteration Lab at ORNL.

4.2.2.3. Synthesis of perdeuterated PtNHase.

The PtNHase β-α-His₆ plasmid and the PtNHase activator plasmid were cotransformed into BL21(DE3) competent cells using the High Efficiency Transformation Protocol (C2984). Screening was performed using the Holme and Studier media to find the most suitable conditions for expression of PtNHase. A single colony was obtained and introduced into 40 mL of each media type with 40 μL of KAN (50 mg/mL) and CARB (100 mg/mL). The culture was incubated overnight at 30 ºC and 150 rpm. Unfortunately, there was no observed cell growth. The temperature was then increased to 37ºC with 225 rpm shaking and after 25 hrs an OD₆₀₀ of 1.25 and 1.51 was obtained in the Holme and Studier media, respectively. The cells obtained from the Holme media were grown in D₂O minimal media. This was done progressively by increasing the amount of D₂O Holme minimal media from 25 % v/v up to 100 % (Table 9). The culture volume was 5 mL for each set-up with 5 μL of KAN (50 mg/L) and CARB (100 mg/L).
4.2.2.4. Glycerol stock preparation.

A glycerol stock was prepared from the final 100% D₂O Minimal media culture. Aliquots of 1.4 mL of the cell culture were transferred into conical tubes containing 0.7 mL of a 50% glycerol buffer that had previously been autoclaved and mixed by inverting the tube several times followed by flash-freezing. This stock was then stored at -80ºC.

4.2.2.5. Small-scale growth, expression and purification of PtNHase in D₂O Holme minimal media.

Table 9. Summary of adapting cells to D₂O minimal media.

<table>
<thead>
<tr>
<th>D₂O media % Volume</th>
<th>Starting OD₆₀₀</th>
<th>Final OD₆₀₀</th>
<th>Temp. (ºC)</th>
<th>RPM</th>
<th>Time (Hrs.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25%</td>
<td>0.10</td>
<td>2.5</td>
<td>37</td>
<td>225</td>
<td>23</td>
</tr>
<tr>
<td>50%</td>
<td>0.10</td>
<td>1.7</td>
<td>37</td>
<td>225</td>
<td>24</td>
</tr>
<tr>
<td>75%</td>
<td>0.18</td>
<td>1.5</td>
<td>37</td>
<td>225</td>
<td>15</td>
</tr>
<tr>
<td>100%</td>
<td>0.11</td>
<td>1.0</td>
<td>37</td>
<td>225</td>
<td>15</td>
</tr>
<tr>
<td>100%</td>
<td>0.27</td>
<td>1.5</td>
<td>37</td>
<td>225</td>
<td>22</td>
</tr>
<tr>
<td>100%</td>
<td>0.21</td>
<td>1.3</td>
<td>37</td>
<td>225</td>
<td>24</td>
</tr>
<tr>
<td>100%</td>
<td>0.33</td>
<td>1.5</td>
<td>37</td>
<td>225</td>
<td>26</td>
</tr>
</tbody>
</table>

D₂O minimal media was supplied by the Bio-Deuteration Lab-ORNL (Table 10). 50 μL of KAN (50 mg/L) and CARB (100 mg/L) were added to the media. A starter culture was prepared in 4 mL of D₂O media using the previously prepared PtNHase
glycerol stock in D$_2$O, which was incubated for 49 hours at 225 rpm and 37 °C, providing an OD$_{600}$ of 0.541. This starter culture was added to 45 mL of D$_2$O Holme minimal media that had an initial OD$_{600}$ of 0.104 and was incubated for 12 hours with 225 rpm and 37 °C, resulting in an OD$_{600}$ of 1.185. The culture was allowed to cool to 11 °C after which the culture was induced with 0.1 mM IPTG and supplemented with 250 mL of a 1 M cobalt chloride solution. After induction, the culture was incubated at 20 °C and 225 rpm for 18 hours. The cells were harvested by centrifugation at 6000 rpm at 4 °C for 20 min. The cells were resuspended at 2 mL per gram in buffer 3A, pH 7.5 (50 mM NaH$_2$PO$_4$, 500 mM NaCl and 10 mM Imidazole) containing 5% v/v of glycerol. The protein was purified using the same protocol as previously reported,$^{112b}$ except that D$_2$O HEPES buffer (50mM) was used for buffer exchange. Kinetic analysis was carried out as previously described.$^{112b}$
Table 10. Components of D\textsubscript{2}O Holme minimal media.

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration in 50mL (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium sulfate</td>
<td>350</td>
</tr>
<tr>
<td>Sodium phosphate dibasic</td>
<td>263</td>
</tr>
<tr>
<td>Potassium phosphate monobasic</td>
<td>80</td>
</tr>
<tr>
<td>Ammonium citrate dibasic</td>
<td>25</td>
</tr>
<tr>
<td>Glycerol</td>
<td>250</td>
</tr>
<tr>
<td>Magnesium sulfate hexahydrate</td>
<td>10</td>
</tr>
<tr>
<td>Calcium chloride dihydrate</td>
<td>0.025</td>
</tr>
<tr>
<td>Cobalt (II) chloride</td>
<td>0.005</td>
</tr>
<tr>
<td>Cupric sulfate pentahydrate</td>
<td>0.008</td>
</tr>
<tr>
<td>Ferric chloride hexahydrate</td>
<td>0.835</td>
</tr>
<tr>
<td>Magnesium (II) sulfate monohydrate</td>
<td>0.006</td>
</tr>
<tr>
<td>EDTA disodium dihydrate</td>
<td>1.0</td>
</tr>
<tr>
<td>Zinc sulfate heptahydrate</td>
<td>0.009</td>
</tr>
</tbody>
</table>

4.2.2.6. Preparation of Enfors media.

An 800 mL solution of Enfors media was prepared combining 53.0 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 37 mM Na\textsubscript{2}HPO\textsubscript{4}, 11.8 mM KH\textsubscript{2}PO\textsubscript{4} and 2.2 mM ammonium citrate dibasic. The salts (5.50 g (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 4.20 g Na\textsubscript{2}HPO\textsubscript{4}, 1.28 g KH\textsubscript{2}PO\textsubscript{4} and 0.40 g ammonium citrate dibasic) used for the preparation of the media were dissolved in 100 mL of D\textsubscript{2}O by stirring. This solution was evaporated to dryness using a Rotary evaporator by increasing the temperature stepwise from 20 °C to 50 °C at 150 rpm. This process was repeated
The dry residue obtained after evaporation was dissolved in 700 mL of D$_2$O after which 0.4 g MgSO$_4$.7D$_2$O, 0.4 mL of minimal media solution (Table 8), 4.0 g of glycerol, 40 mg of Kan and 80 mg of Carb were added to the mixture in a stepwise manner with stirring. D$_2$O was added to an end volume of 800 mL volume. The solution was then sterile filtered and stored in a dry clean bottle. Two feed-stocks were used during the fermentation process; feed-1 (10 \% NaOD (50 mL)) and feed-2 (10 \% glycerol-15 g, 0.2 \% MgSO$_4$.7H$_2$O-1.5 mL).

4.2.2.7. Preparation of the starter and fermenter feed culture.

The cells from the previously D$_2$O adapted glycerol stock were used to prepare a starter culture. The cells were introduced into 3 mL of the 75 \% D$_2$O Enfors media and this was progressively increased to 100\% D$_2$O Enfors media. This was achieved by centrifugation of the cells and resuspending them in 100\% D$_2$O Enfors media. These cells were then added to 4 mL of Enfors media and after 24 hours, an OD$_{600}$ of 1.6 was obtained. The culture was then centrifuged at 500 rpm, 25 \°C for 10 min and resuspended in 4 mL of Enfors media. Approximately 3.33 mL of the resuspended culture was added to 50 mL of Enfors media giving an initial OD$_{600}$ of 0.08. The culture was left overnight at 250 rpm and 30 \°C, resulting in an OD$_{600}$ of 0.7. To this culture, 50 mL of Enfors media was added providing an OD$_{600}$ of 0.35. The temperature was increased to 37 \°C at 250 rpm and after 7 hrs. an OD$_{600}$ 2.32 was reached. A 50 mL starter culture in Enfors media was inoculated for 24 hours with a starting OD$_{600}$ of 0.08 at 30 \°C at 250 rpm and regrown as indicated in Table 11.
Table 11. Tracking fermentor seed culture.

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>OD&lt;sub&gt;600&lt;/sub&gt;</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.08</td>
<td>30 ºC</td>
</tr>
<tr>
<td>13</td>
<td>0.7</td>
<td>30 ºC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>diluted to 100 mL total; temp</td>
</tr>
<tr>
<td>13</td>
<td>0.35</td>
<td>increased to 37 ºC</td>
</tr>
<tr>
<td>17</td>
<td>1.1</td>
<td>37 ºC</td>
</tr>
<tr>
<td>24</td>
<td>2.32</td>
<td>37 ºC</td>
</tr>
</tbody>
</table>

4.2.2.8. Culture fermentation.

The fermenter was cleaned and dried and the detachable parts were cleaned and autoclaved before being reassembled and calibrated as described in the manual. An 800 mL solution of Enfors media at pH of 7.0 was added to the fermenter. The dissolved oxygen (DO) probe, pH probe and temperature probe were calibrated as instructed in the manual. Nitrogen was blown into the media and through all the pumps to drive out any dissolved oxygen and also to drive out any water vapor in the system. Dry air was then introduced into the media until a 100% dissolved oxygen level was reached. The level of dissolved oxygen was monitored so that a concentration of 100% was maintained. This set-up was left for 7 hours before introducing the starter culture. The initial OD<sub>600</sub> was 0.29, DO 100%, pH 7.0, agitation 200 rpm and temperature of 35 ºC. The DO decreased to 30% within 4 hrs and was maintained at this level throughout the process. Once the
DO level was stabilized at 30%, the temperature was reduced to 25°C and the agitation increased to 650 rpm. After ~12 hrs, the agitation level was decreased to ~420 rpm and maintained for the remainder of the growth while the pH was slightly lowered and maintained at pH 6.9. Feed-2 started after 13 hrs. and was linearly increased while feed-1 was introduced after 8 hrs. After 20 hrs. of growth, an OD$_{600}$ of 10.3 was reached. At this time, the temperature was lowered to 20 ºC and the culture was induced with 200 µL of 1 M IPTG and supplemented with 500 µL of 1 M CoCl$_2$. After 32 hrs., an additional 100 µL of 1 M IPTG and 300 µL of 1 M CoCl$_2$ were added to the culture. Approximately 150 mL of feed-2 and 29 mL of feed-1 were consumed after 46 hrs. providing a final culture OD$_{600}$ of 14.2.

4.2.2.9. Cell harvesting, protein purification, crystallization and crystal analyses.

Cells were harvested by centrifugation at 6,370 g, 4 ºC, for 20 min providing 36 g of cells, which were resuspended at 2 mL/g in buffer 3A (50 mM NaH$_2$PO$_4$, pH 7.5, 500 mM NaCl, 10 mM Imidazole). This solution was stored at -80 ºC. All procedures done from this point were carried out on ice unless otherwise indicated. Approximately 20 mL of cell paste stored at -80 ºC was thawed on ice and the cells lysed using a 21 W Misonex sonicator 3000 for 12 min (30 s on and 45 s off). The cell debris was removed by centrifugation twice at 31,000g, 4 ºC, for 25 min., the purified perdeuterated PrNHase was used to determine the conditions for purification and crystal growth. The purity of PrNHase was analyzed using a 12.5% SDS-PAGE gel.
D$_2$O crystallization buffer was prepared with sodium citrate concentrations from 0.8 to 1.6 M and pD ranging from 7.5 to 8.2. A hanging drop diffusion method was used for crystal screening. A drop of 2 μL volume was used with a final protein concentration of 10 mg/mL and a reservoir volume of 30 μL and kept at ~4 °C. All the wells had precipitate after one week and no crystals were formed. A second crystal screen was set-up using the sitting drop diffusion method. In this case, the D$_2$O crystallization buffer (1.4 M sodium citrate and 0.1 M HEPES and pD 7.9) was used. The well volume was 20 μL containing 10 mg/mL of protein with a crystallization buffer volume of 800 μL, Some of the wells co-crystallization with 10 mM inhibitor-Phenyl boronic acid (PBA) or Butane boronic acid (BuBA) was used. Crystals appeared after one week and analyzed on a home source X-ray diffractometer to assess X-ray diffraction quality.

With the successful crystallization of perdeuterated PtNHase, the remaining cell paste was used to purify additional perdeuterated PtNHase that was used to set up crystal trays using the sitting drop diffusion method. The well volume for this set-up was 200 μL with a protein concentration of 10 mg/mL and a reservoir volume of 40 mL containing the crystallization buffer (1.4 M sodium citrate, pH 7.9 and 0.1 M HEPES). Some of the wells were set-up containing 2 μL of 0.1 M of the competitive inhibitor Phenyl boronic acid (PBA) or Butane boronic acid (BuBA) at ~4 °C. After 8 weeks, 1.6 mm x 0.9 mm x 0.5 mm crystals were obtained, which were mounted in quartz capillaries at room temperature for analysis on MaNDi at ORNL.

A sitting drop crystal screen was also set up with perdeuterated PtNHase and PtNHase in D$_2$O buffer with pD in the range 6.8 to 7.9 and sodium citrate concentrations of 1.4 and 1.2 M in 0.1 M HEPES. The 24 well plate used contained 10 mg/mL protein
and a reservoir volume of 800 μL. Crystals obtained from this screen were examined on beamlines 5BC sector 19BM of GM/CA@APS of the Advanced Photon Source (APS) using X-rays of 0.98 Å wavelength and Rayonix (formerly MAR-USA) 4×4 tiled CCD detector with a 300 mm² sensitive area. The perdeuterated protein crystals did not diffract to a high resolution and so, another screening was set using the H/D-exchanged PtNHase protein co-crystallized with PBA or BuBA. The resultant crystals were screened using a Rigaku HomeLab X-ray diffraction system, equipped with MicroMax-007 generator, Varimax-HF Optics, a Saturn-944+ CCD detector and Oxford Cryostream at room temperature. The largest crystals were mounted in quartz capillary tubes and shipped to ORNL for screening with neutron diffraction at IMAGINE and MaNDi. From the promising results of these screens a second attempt was made to synthesize perdeuterated protein at ORNL. The synthesis was successful, and a set of crystals screens were prepared to determine the best conditions. pD values between 6.9 and 7.1 were found to be most favorable so perdeuterated PtNHase was co-crystallized with BuBA and PBA. The crystals from this screening were analyzed via X-ray diffraction on a home source diffractometer. A large well plate of 300 μL volumes were then set-up to grow large crystals for neutron diffraction. A full X-ray data set has been collected for PBA and BuBA complexed perdeuterated crystals under cryo-conditions described previously on a Rigaku HomeLab X-ray diffraction system, equipped with MicroMax-007 generator, Varimax-HF Optics, a Saturn-944+ CCD detector and Oxford Cryostream.
4.3. Results and Discussion

The protonation states of active site residues in NHase enzymes have been proposed through a combination of EXAFS and theoretical studies, but no further experimental work has been done to support the previously reported information. Determination of the protonation states of active site residues is vital to understand the catalytic mechanism of nitrile hydratases. Current PtNHase crystals diffract well and the X-ray crystal structure has been solved to relatively high resolution (~1.2 Å). However, attempts to measure data at ultrahigh resolution, needed for assessing protonation states, revealed significant radiation damage at and around the metal site including both post-translationally modified cysteine residues. Therefore, combined X-ray and neutron diffraction crystallography is necessary to identify the protonation states of these catalytically critical residues and thus shed new light on the catalytic mechanism of NHase enzymes.

Neutrons interact directly with the nucleus whereas X-rays interact with the electron cloud around each atom. Therefore, the more electrons the more strongly these atoms contribute to the diffracted X-rays. On the other hand, the contribution to diffracted neutrons is dependent on specific isotopes so deuterium contributes more strongly the hydrogen, increasing signal-to-noise (S/N) by ~3-fold. Unfortunately, neutron diffraction crystallography requires large crystals (>1 mm³) which need to be grown in D₂O buffer. Protein deuteration is also hugely beneficial, typically providing an ~10-fold improvement in the S/N for neutron diffraction data. Given the difficulties in obtaining large crystals of perdeuterated proteins such as a large well-ordered crystal,
only sixty-seven neutron diffraction structures have been reported in the RCSB Protein Data Bank.

Given the mechanistic importance of determining the protonation states of active site residues in NHase enzymes and the fact that relatively large crystals of PtNHase that diffract to ~1.2 Å can be routinely grown, (0.7 mm x 0.8 mm x ~1 mm; space group: P3\(_2\)21, unit cell: \(\alpha=\beta=90^\circ, \gamma=120^\circ, a=b=65.7\) Å, \(c=185.6\) Å) in H\(_2\)O (Figure 43), neutron diffraction studies were undertaken. To determine the feasibility of solving a neutron structure of PtNHase, Dr. Dean Myles at ORNL obtained neutron diffraction data on PtNHase crystals grown in H\(_2\)O buffer using the High Flux Isotope Reactor (HFIR) IMAGINE-Quasi Laue-Single Diffractometer (Figure 45). Preliminary tests provided data to better than ~5 Å for these crystals, which was highly encouraging given the long unit cell edges (184 Å) and that IMAGINE is optimized for unit cell edges of ~100 Å (Figure 45b).\(^{130}\)

Based on these encouraging screening data, crystallization conditions utilizing D\(_2\)O buffer were optimized using large (>200 μl) sitting drops. Both micro and macro-seeding approaches were also utilized to increase crystal size. The optimized crystals grown in D\(_2\)O buffer resulted in significant improvement in signal-to-noise due to H/D exchange, which extended the resolution to better than ~2.9 Å on IMAGINE (Figure 44A and B). These encouraging data led to testing deuterated crystals on the MaNDi instrument, which is significantly more sensitive for proteins with larger cell dimensions. However, crystals grown for this experiment only diffracted to ~4 Å (data not shown). Examination of these test crystals by X-ray diffraction revealed a high level of mosaicity due to presence of small perfect crystals that are randomly misoriented resulting in a
significant amount of overlap of diffraction spots resulting in smearing. The poor-quality diffraction pattern was hypothesized to be due to protein packing problems within the crystals, which could have emerged from several factors such as i) impure protein used for crystallization due to presence of the traces of the activator protein, ii) dehydration of crystals during or after mounting in the capillaries due to little or no solvent around the crystals or poor sealing of the capillaries, iii) quick crystal formation, iv) longer cell dimensions, and/or v) poor packing of the WT enzyme.

Figure 45. Neutron diffraction on hydrogenated protein: a) Optical image of the PtNHase crystal used to obtain Laue diffraction data and b) Laue diffraction extends to about ~5A resolution for a crystal of PtNHase.
To improve the neutron diffraction resolution and to attempt to grow higher quality crystals of PtNHase, initially, perdeuteration of the enzyme was undertaken as it can increase the neutron diffraction S/N ratio by an additional ~10-fold. PtNHase was over-expressed in Enfors minimal media at pH of 7.0 minimal media expression was confirmed using SDS-PAGE (Figure 45). The perdeuterated PtNHase protein bands appear at the same positions as the WT hydrogenated enzyme. However, the protein yield was only 0.6 times that observed for WT PtNHase. The $k_{\text{cat}}$ value obtained for perdeuterated PtNHase was $1,200 \pm 100 \text{ s}^{-1}$, a value that is ~67% of that observed for WT PtNHase ($1,790 \pm 50 \text{ s}^{-1}$). The decreased activity observed for perdeuterated PtNHase is expected and due to the difference in bond strength of deuterium compared to hydrogen.

**Figure 46.** Neutron diffraction on H/D exchanged PtNHase: a) PtNHase crystals ((1x2) mm) and b) data frame of Laue diffraction extending to about 2.9 Å resolution.
which slows the rate of bond dissociation during proton transfer steps. With catalytically active perdeuterated PtNHase in hand, crystallization was attempted to obtain crystals large enough for neutron diffraction studies.

Crystallization conditions for the perdeuterated PtNHase enzyme were carried out in the absence and presence of the competitive inhibitors BuBA and PBA by varying pH values from 7.0 to 7.9 with two different salt concentrations (1.2 M and 1.4 M sodium citrate tribasic). This screening attempt was not successful as the protein precipitated in all hanging drops. A second trial was undertaken using 1.4 M sodium citrate, 0.1 M HEPES and pH 7.9, conditions that are identical to those used to crystalize hydrogenated PtNHase. Over the course of several weeks, high quality crystals were only obtained for the BuBA and PBA complexed PtNHase enzymes, which diffracted to ~2.0 Å on a home source X-ray diffractometer. With crystallization conditions established for the BuBA and PBA complexed PtNHase enzymes using the sitting drop method, crystallization trials were prepared in large wells (>200 µL) to obtain large crystals for neutron diffraction for both H/D-exchanged and perdeuterated PtNHase enzymes complexed with BuBA or PBA. Large crystals of ~0.8 mm by ~0.5 mm was obtained after approximately four weeks but after approximately eight weeks, these crystals had grown to ~1.6 mm x ~0.6 mm (Figure 46). As expected, crystal growth for the BuBA and PBA complexed perdeuterated and the H/D-exchanged protein was not the same as the crystals grown in D₂O.
**Figure 47.** SDS-PAGE analysis of the H₂O and D₂O PtNHase: 1) Hydrogenated PtNHase after Gel filtration, 2) Perdeuterated protein after gel filtration (grown and purified in our Lab), 3) Perdeuterated protein after gel filtration (grown and purified in ORNL), 4) Hydrogenated PtNHase after Gel filtration, 5) Perdeuterated protein after IMAC purification, and 6) Protein marker.
buffer and were larger compared to the perdeuterated crystals grown over the same time and under the same crystallization conditions. Neutron diffraction screening was done at ORNL on MaNDi to determine if these crystals were of high enough quality to obtain a full neutron diffraction data set. Surprisingly, the perdeuterated PtNHase-BuBA and PtNHase-PBA crystals screened on MaNDi diffracted to very low resolution (~6 Å) over 16 hours of exposure. Due to the poor diffraction of these crystals, a full data set was not obtained. Instead further analysis was done to determine the source of the poor crystal quality.

Several perdeuterated PtNHase-BuBA and PtNHase-PBA crystals were tested on a home source X-ray diffractometer, which revealed that smaller crystals (~0.2 mm$^3$) diffracted to ~1.9 Å with 0.5 mosaic spread compared to larger crystals (~0.8 mm$^3$) that diffracted only to ~3.0 Å with a mosaic spread of >1.0. These data indicate that the larger the crystals grew, the greater the mosaicity. While these data indicate that high
quality perdeuterated PtNHase-BuBA and PtNHase-PBA crystals could be obtained, new crystallization conditions were needed to obtain large crystals with low levels of mosaicity that would be amenable to neutron diffraction studies. As the crystal screening done thus far revealed crystal disorder, it was hypothesized that decreasing the rate of crystal growth to achieve better packing of the protein molecules during crystal formation would produce high quality crystals.

To test this hypothesis, crystal screens were prepared to investigate the correlation between crystal quality and both salt concentration and pH of the crystallization buffer. Varying salt concentration led to the discovery that decreasing the salt concentration contributed to slower rates of crystal growth and that PtNHase-BuBA and PtNHase-PBA crystals obtained under these conditions diffracted to higher resolutions compared to those grown at high salt concentration with similar pH. PtNHase-BuBA and PtNHase-PBA crystals were then screened to establish the optimal pH at which perdeuterated PtNHase would crystallize while minimizing precipitation. Decreasing the pH yielded crystals with very little enzyme precipitation and the perdeuterated PtNHase-BuBA and PtNHase-PBA crystals diffracted at higher resolution (~1.3 Å). From these data, crystal screens combining two salt concentration (1.2 M & 1.4 M sodium citrate tribasic) and various pH values between 6.9 and 7.5 were prepared for both perdeuterated and hydrogenated PtNHase-BuBA and PtNHase-PBA complexes. Good quality H/D exchanged PtNHase-BuBA and PtNHase-PBA crystals that diffracted at ~1.2 Å resolution at APS were obtained from lower pD values (7.3 to 6.9) and salt concentrations of 1.2 M sodium citrate tribasic and 0.1 M HEPES. Having successfully generated high quality H/D exchanged PtNHase-BuBA and PtNHase-PBA crystals from
the 24 well sitting drop trays that diffracted at higher resolution with X-ray in a 20 μL well, a similar set of conditions were used for crystallization screens using 200 μL wells to obtain large crystals (>1 mm³), which appeared after ~4 months (Figure 47 and Figure 48). Neutron diffraction screening done at ORNL for H/D BuBA and PtNHase-PBA crystals on IMAGINE (Figure 49) revealed that these crystals diffracted beyond ~3 Å using the limited band-pass (λ = 2.8-4.5 Å) mode. However, when indexed and integrated, the relatively large unit cell means that spatial overlap is clearly significant, which indicates that obtaining better than ~3 Å is unlikely. Ideally, ~2 Å data is required for collecting a full neutron data set. Therefore, the best PtNHase-BuBA crystal was used to collect a test data set on MaNDi at SNS, which is optimized for larger unit cells (Figure 50). While overall diffraction is similar to IMAGINE, the higher resolution reflections are clearly resolved in the time of flight dimension on MaNDi. With large crystals of H/D exchanged PtNHase-BuBA and PtNHase-PBA in hand, we were awarded 24 days of beamtime at ORNL on MaNDi for spring data collection. Unfortunately, ORNL experienced a radiation leak that shut the beamline down until June 2019. Even so, we were granted 24 days of beamtime on MaNDi set for late summer or early fall to collect complete data sets on H/D exchanged PtNHase-BuBA and PtNHase-PBA crystals.
Figure 49. H/D exchanged PrNHase crystals a) co-crystallized with BuBA at 4 ºC, pD 6.9, b) co-crystallized with BuBA at 4 ºC, pD 7.1, c) co-crystallized with PBA at 4 ºC, pD 6.9, and d) co-crystallized with PBA at 4 ºC, pD 7.1.
Figure 50. The H/D-exchanged PtNHase crystals in 1.2 M sodium citrate and 0.1 M HEPES co-crystallized with BuBA and PBA after 5 months.

Finally, perdeuterated crystal of PtNHase-BuBA and PtNHase-PBA have now been obtained and from the screening experiments on a home X-ray diffractometer, they diffracted to ~1.9 Å with a relatively low mosaicity of ~0.6. As these crystals can provide an ~10-fold enhance in neutron diffraction data, they will be screened at the beginning or the granted fall beamtime and if they diffract to higher resolution than the H/D exchanged crystals, will be used in place of the H/D crystals for complete data set collection.
Figure 51. Laue diffraction extends to better than 3 Å for crystals of PrNHase-BuBA grown in D$_2$O on IMAGINE. a) data frame and b) indexed and integrated snapshots.
Figure 52. Laue diffraction patterns extends to better than 3 Å for crystals of H/D-exchanged PtNHase grown in D₂O pD 7.1 test shots taken on MaNDi. a) 30 min exposure for BuBA and b) 15 hrs exposure test shot for PBA
In conclusion, large crystals (>1 mm³) have been obtained for the H/D exchanged $Pt\text{NHase-BuBA}$ and $Pt\text{NHase-PBA}$ complexes. These large crystals diffract to ~1.2 Å resolution at APS and were found to diffract neutrons to better than ~3 Å on MaNDi with low mosaicity. As such, 24 days of beamtime on MaNDi have been awarded to collect full neutron data sets, suggesting that a neutron structure for $Pt\text{NHase}$ complexed by the competitive inhibitors BuBA and PBA is forthcoming. While testing H/D exchanged $Pt\text{NHase-BuBA}$ and $Pt\text{NHase-PBA}$ crystals, expression conditions for perdeuteration of $Pt\text{NHase}$ were developed and perdeuterated $Pt\text{NHase}$ was obtained in good yield and successfully crystallized. As with H/D exchanged $Pt\text{NHase}$, high quality crystals that diffract to better than ~1.9 Å on an in-house X-ray diffractometer were only obtained for the $Pt\text{NHase-BuBA}$ and $Pt\text{NHase-PBA}$ complexes. The ability of these crystals to diffract neutrons will be tested and if they prove superior to the H/D $Pt\text{NHase-BuBA}$ and $Pt\text{NHase-PBA}$ crystals, will be used to collect full neutron diffraction data sets rather than the H/D crystals. Based on the data obtained thus far, a full neutron structure of $Pt\text{NHase}$ is forthcoming and would constitute only the 68th published neutron diffraction structure of a protein and provide the first experimental evidence for the protonation states of catalytically relevant residues in NHases. Future work will include screening additional conditions to obtain high quality, large crystals of perdeuterated $Pt\text{NHase}$ and perdeuterated $Pt\text{NHase}$ mutant enzyme in both the absence and presence of BuBA and PBA as well as the product of nitrile hydration.
4.4. Conclusion

The trials done on deuterated and per-deuterated NHases point out that PrNHase remains stable and have yielded large crystals for neutron diffraction studies. The H/D-exchanged inhibitor complexed crystals diffract well to higher resolutions compared to the protein not co-crystallized with the inhibitors. The trials have strongly indicated the need for obtaining large, good quality crystals in order to successfully obtain data on the protonation states of the catalytically important active site residues.
CHAPTER 5

CONCLUSIONS

The studies reported herein have provided important information in elucidating the active site maturation process and reaction mechanism of NHases. In chapters 2, a metallopeptide mimic was designed and revealed that the metal ion is inserted as a divalent ion in a high-spin state. It then oxidizes into a high-spin trivalent species and eventually converts into a low-spin trivalent ion as the active site equatorial Cys residues oxidize. It is the low-spin state that is found in a functional NHases. In chapter 3, mutation of the axial thiolate ligand provided insight that the strictly conserved axial cysteine residue is important for catalysis but not essential. However, its role in metal ion insertion has been strongly supported through metal analysis studies. The axial cysteine therefore ensures maturation of the active site by providing a conducive environment for metal ion oxidation. This role is shared with the activator protein, which aids in the metal ion insertion and oxidation processes of the equatorial cysteine residues. There are some other components that are required for active site maturation like the metal ion, in its absence the equatorial cysteine residues were not oxidized. From this study, it is evident that the nature of the axial ligand, the metal ion, and the activator protein work together to enable the post-translational modifications for a functional NHase protein.

In chapter 4, neutron diffraction studies have provided insight into how to obtain large (>1 mm$^3$), high quality crystals of PrNHase in both D$_2$O buffer and in its
perdeuterated form. It was discovered that PtNHase can be synthesized as a perdeuterated protein and yield crystals of both the BuBA and PBA complexes that diffract to ~1.2 Å resolution at the Synchrotron and ~1.9 Å at an in-house X-ray diffractometer. Neutron diffraction of large H/D-exchanged PtNHase-BuBA or PBA inhibitor complex crystals provided data to better than 3.0 Å. These data are very exciting as they indicate well-ordered crystals that should be amenable for the collection of a full neutron diffraction data set in the fall of 2019, which will further elucidate the protonation states of important active site residues, paving the way for a more detailed catalytic reaction mechanism.

Given the informative results obtained from these studies, there still exists a lot of gaps that need to be filled in order to establish a more conclusive reaction mechanism and the active site sequential maturation process for NHases. Some of the areas that still need to be investigated include: i) mutating the axial thiolate ligand in Fe-type NHases, which can be probed with using EPR, Resonance Raman spectroscopy, stopped flow, and MCD to elucidate the maturation process and trap intermediate species, ii) how to insert metal back into the apo-alanine mutant and determine the possible catalytic efficiency with a fully metallated enzyme; iii) developing the experimental parameters under which metal ions can be inserted into the apo-NHase enzymes, iv) to further probe the active site maturation process and optimize conditions that would enable a functional metallopeptide based on the NHase metal binding motif.
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intermediates and a proposed mechanism for self-subunit swapping maturation. 
*Biochemistry-Us 2010, 49 (44), 9638-48.*


# APPENDIX

## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>NHase</td>
<td>Nitrile Hydratase</td>
</tr>
<tr>
<td>Co</td>
<td>Cobalt</td>
</tr>
<tr>
<td>Fe</td>
<td>Iron</td>
</tr>
<tr>
<td>PtNHase</td>
<td><em>Pseudonocardia thermophila</em> JCM 3095NHase</td>
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<tr>
<td>CtNHase</td>
<td><em>Comamonas testosteroni</em> Ni1 NHase</td>
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<td>MbNHase</td>
<td><em>Monosiga Brevicollis</em> NHase</td>
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<td>ReNHase</td>
<td><em>Rodococcus equi</em>-TG328-2</td>
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<td>SCNase</td>
<td>Thiocyanate hydrolases</td>
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<tr>
<td>Act</td>
<td>Activator protein</td>
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<tr>
<td>L-NHase</td>
<td>Low molecular weight NHase</td>
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<tr>
<td>Cys-SO(H)</td>
<td>Cysteine-sulfenic acid</td>
</tr>
<tr>
<td>Cys-SOOH</td>
<td>Cysteine-sulfinic acid</td>
</tr>
<tr>
<td>PivCN</td>
<td>Pivalonitrile</td>
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<tr>
<td>M</td>
<td>Metal</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>LMCT</td>
<td>Ligand-to-metal charge transfer band</td>
</tr>
<tr>
<td>EPR</td>
<td>Electron paramagnetic resonance</td>
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<tr>
<td>DFT</td>
<td>Density functional theory</td>
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<tr>
<td>UV-Vis</td>
<td>Ultra Violate Visible absorption spectroscopy</td>
</tr>
<tr>
<td>XAS</td>
<td>K-edge X-ray absorption spectroscopy</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>ICP-EAS</td>
<td>Inductively coupled plasma atomic emission spectroscopy</td>
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<tr>
<td>IMAC</td>
<td>Immobilized metal affinity chromatography</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>BuBA</td>
<td>1-Butaneboronic acid</td>
</tr>
<tr>
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<td>Phenylboronic acid</td>
</tr>
<tr>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<tr>
<td>DTNB</td>
<td>5,5'-dithio-bis-(2-nitrobenzoic acid)</td>
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