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Salette Martinez
Loyola University Chicago

Xinhang Yang
Marquette University

Brian Bennett
Marquette University, brian.bennett@marquette.edu

Richard C. Holz
Marquette University, richard.holz@marquette.edu

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Salette Martinez

*Department of Chemistry, Marquette University,
Milwaukee, WI*

*Department of Chemistry and Biochemistry,
Loyola University Chicago,
Chicago, IL*

Xinhang Yang

*Department of Chemistry, Marquette University,
Milwaukee, WI*

Brian Bennett

*Department of Chemistry and Biochemistry,
Loyola University Chicago,
Chicago, IL*

Richard C. Holz

*Department of Physics, Marquette University,
Milwaukee, WI*

Abstract: Nitrile hydratase (NHase), an industrially important enzyme that catalyzes the hydration of nitriles to their corresponding amides, has only been characterized from prokaryotic microbes. The putative NHase from the eukaryotic unicellular choanoflagellate organism *Monosiga brevicollis* (*MbNHase*) was heterologously expressed in *Escherichia coli*. The resulting enzyme expressed as a single polypeptide with fused α - and β -subunits linked by a seventeen-histidine region. Size-exclusion chromatography indicated that *MbNHase* exists primarily as an $(\alpha\beta)_2$ homodimer in solution, analogous to the $\alpha_2\beta_2$ homotetramer architecture observed for prokaryotic NHases. The NHase enzyme contained its full complement of Co(III) and was fully functional without the co-expression of an activator protein or *E. coli* GroES/EL molecular chaperones. The homology model of *MbNHase* was developed identifying Cys400, Cys403, and Cys405 as active site ligands. The results presented here provide the first experimental data for a mature and active eukaryotic NHase with fused subunits. Since this new member of the NHase family is expressed from a single gene without the requirement of an activator protein, it represents an alternative biocatalyst for industrial syntheses of important amide compounds.

Abbreviations

- NHase, nitrile hydratase;
- ORF, open reading frame;
- ICP-MS, inductively-coupled plasma mass spectrometry;
- IMAC, immobilized metal affinity chromatography

Keywords: Nitrile hydratase, Enzyme kinetics, Cobalt, Hydrolysis

1. Introduction

Nitrile hydratases (NHases, EC 4.2.1.84) catalyze the hydration of nitriles to their corresponding amides.^{1,2} Since the currently employed industrial conditions used to hydrate nitriles to amides (either acid or base hydration), are often incompatible with the sensitive structures of many industrially and synthetically relevant compounds, NHases have attracted substantial interest as biocatalysts in preparative organic chemistry. For example, acrylonitrile and adiponitrile are used in the production of polyacrylamide and nylon-66, respectively, the latter of which is one of the most important industrial polyamides.³ Nitriles are also often used as synthetic starting materials since they add an extra carbon atom to an alkyl chain. Since nitriles are synthesized by plants, fungi, bacteria, algae, insects and sponges, there are several biochemical pathways for nitrile degradation.⁴ Enzymes involved in nitrile degradation pathways represent chemo-selective biocatalysts capable of hydrating nitriles under physiological reaction conditions.^{4,5}

X-ray crystallographic studies on bacterially-encoded NHases have revealed that they are $\alpha_2\beta_2$ heterotetramers with an active site in each α subunit consisting of three cysteine residues, two backbone amide nitrogen's, a water molecule, and either an Fe(III) ion (Fe-type) or a Co(III) ion (Co-type).² Two of the active site cysteine residues are post-translationally modified to cysteine-sulfinic acid ($-\text{SO}_2\text{H}$) and cysteine-sulfenic acid ($-\text{SOH}$), respectively, yielding an unusual metal coordination geometry, termed a "claw-setting". Oxidation of the equatorial Cys residues is critical for catalysis.⁶ Although the structures of Fe- and Co-type NHases are very similar, Fe-type NHases only bind Fe(III) and Co-type NHases only bind Co(III). This specificity is thought to be regulated by one of several open reading frames (ORFs), that encodes for an activator protein that has been identified just downstream from the structural α - and β -subunit genes in NHases.^{7,8,9} Even though Co- and Fe-type NHase enzymes share high sequence identity, their respective activator proteins are different in size (~ 14 kDa for Co-type vs. ~ 47 kDa for Fe-type NHase) and share little or no sequence identity.^{10,11} The prevailing dogma is that both Co- and Fe-type NHase enzymes require the co-expression of an activator protein to be soluble and fully active.^{7,8,9} The exception is the Fe-type NHase from *Comamonas testosteroni* Ni1 (CtNHase), which was shown to be functionally expressed without the co-expression of an activator protein.¹²

Until recently, NHases have only been found in prokaryotes; however, a novel gene that contains putative NHase α - and β -subunits was reported in several unicellular eukaryotic organisms such as *Monosiga brevicollis*, *Thecamonas trahens*, *Sphaeroforma arctica*, *Stephanoeca diplocostata*, *Salpingoeca rosetta*, *Emiliana huxleyi* and *Aureococcus anophagefferens*.^{13,14} Unlike prokaryotic NHases, the putative eukaryotic NHase ORF contains fused α - and β -subunits bridged by an insert that codes for multiple but variable numbers of His residues (Fig. 1). For example, the *M. brevicollis* gene encodes (His)₁₇, while the gene from *S. rosetta* encodes only (His)₂.^{13,14} The C-terminus of the eukaryotic holo-protein contains the strictly conserved metal-binding motif (CTLCSCY) found in the α -subunit of Co-type NHases and the homologous cobalt-containing thiocyanate hydrolase.¹⁵ Interestingly, no gene encoding an activator protein was identified.

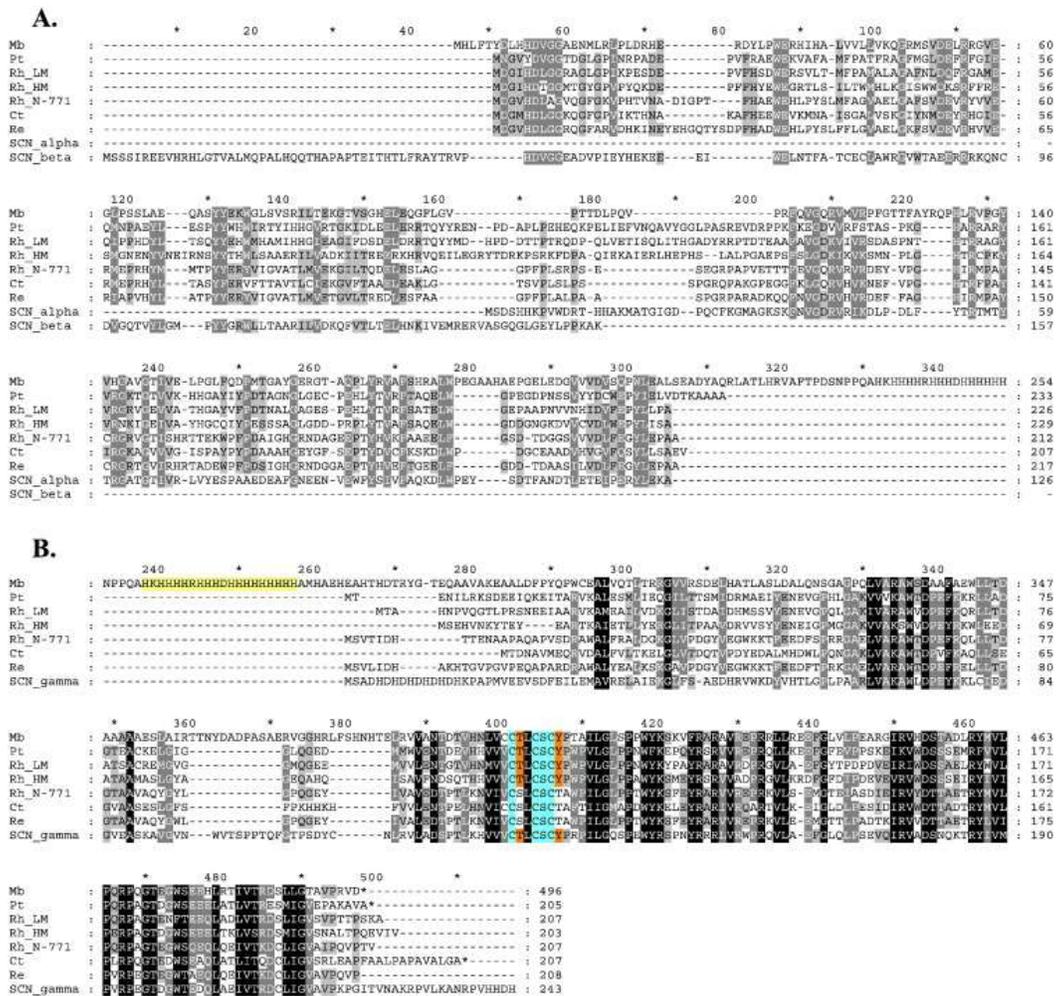


Fig. 1. Alignment of the amino acid sequence of the eukaryotic NHase from *M. brevicollis* and prokaryotic nitrile hydratases. A) Amino acid alignment of the N-terminus residues of the eukaryotic NHase from *M. brevicollis* and prokaryotic NHase β -subunits. B) Amino acid alignment of the C-terminus residues of the eukaryotic NHase from *M. brevicollis* and prokaryotic NHase α -subunits. Conserved residues among *Mb*NHase and prokaryotic NHases are highlighted in black and gray. The metal-binding motif is highlighted in cyan and active site Co-type NHase residues are in orange. The histidine-rich region is highlighted in light yellow. *Mb*NHase from *M. brevicollis*; Co-NHases: *Pt*NHase (*Pseudonocardia thermophila* JCM 3095), *Rh_LM*, (low-molecular weight, *Rhodococcus rhodochrous* J1), *Rh_HM* (high-molecular weight, *Rhodococcus rhodochrous* J1); Iron-NHases: *Rh_N-771* (*Rhodococcus* sp. N-771), *Ct* (*Comamonas testosteroni* Ni1), *Re* (*Rhodococcus equi* TG328-2); SCN_alpha, SCN_beta, and SCN_gamma, cobalt thiocyanate hydrolase (SCNase) from *Thiobacillus thioparus* THI115.

As no eukaryotic NHase enzyme has yet been described, we set out to determine whether the putative NHase gene from *M. brevicollis*

does indeed code for a functional NHase. The gene encoding the fused α - and β -subunits of the hypothetical NHase from *M. brevicollis* was heterologously expressed in *E. coli*. The resulting enzyme contained its full complement of Co(III) and was a fully functional NHase, all without the co-expression of an activator protein or *E. coli* GroES/EL molecular chaperones. This is the first report of a eukaryotic NHase and because this new member of the NHase family is expressed from a single gene without the requirement of an activator protein, it represents an alternative biocatalyst for industrial syntheses of important amide compounds.

2. Materials and methods

2.1. Materials

Acrylonitrile, Tris-HCl, and HEPES were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other materials were purchased at the highest quality available.

2.2. Plasmid construction

Protein sequences for the α - and β -subunit genes of the putative MbNHase were obtained from ORF 37534 (UniProt ID A9V2C1.1) of *M. brevicollis*. The predicted gene was synthesized by Integrated DNA Technologies, Inc. with optimized *E. coli* codon usage (SI Fig. 1) and cloned into the pIDT-SMART kanamycin resistant vector with *NdeI* and *HindIII* restriction sites. The gene was subsequently digested and ligated into the ampicillin resistant pET21a⁺ (EMD Biosciences) expression vector to create the plasmid pSMMa β . The sequence was confirmed using automated DNA sequencing at the University of Chicago Cancer Research Center DNA sequencing facility.

2.3. Expression of recombinant MbNHase in the absence of an activator protein

The hypothetical MbNHase gene was expressed alone, without co-expressing a Co-type activator protein or the *E. coli* chaperones GroES/EL, in the following manner. The pSMMa β plasmid was freshly transformed into *E. coli* BL21(DE3) competent cells (Agilent

Technologies) and a single colony was used to inoculate a 50 mL LB-Miller culture containing 50 µg/mL of carbenicillin with shaking overnight at 37 °C. This culture (~ 7 mL) was used to inoculate a 1 L culture containing 100 µg/mL of ampicillin and the cells were grown at 37 °C until the OD_{600 nm} reached 0.8–1.0. The culture was cooled on ice to 20 °C, induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) in the presence of 0.25 mM CoCl₂ and expressed at 20 °C for 16 h. Cells were harvested by centrifugation at 6370 × *g* for 10 min at 4 °C in a Beckman Coulter Avanti JA-10 rotor. Cell pellets were resuspended in buffer A (50 mM NaH₂PO₄ pH 8.0, 500 mM NaCl, and 10 mM imidazole with 5% glycerol) at a ratio of 5 mL per gram of cells, then sonicated for 4 min (30 s on 45 s off) at 21 W using a Misonix sonicator 3000. The crude extract was obtained after centrifugation in a JA-20 rotor at 31,000 × *g* and 4 °C for 20 min. The sample was subjected to another round of centrifugation before purification to remove particulate matter.

2.4. Purification of recombinant MbNHase

Crude extracts of MbNHase were loaded onto a 5 mL Ni(II)-nitrilotriacetic acid (NTA) Superflow Cartridge (Qiagen) for immobilized metal affinity chromatography (IMAC) using an ÄKTA FPLC P-960 column. The column was washed with four column volumes (CVs) of buffer A (50 mM NaH₂PO₄ pH 8.0, 500 mM NaCl, and 35 mM imidazole) followed by washes with 50 mM HEPES pH 8.0, 500 mM NaCl containing 60 mM and 110 mM imidazole, respectively. The protein was eluted with a linear gradient (0–100%) of buffer B (50 mM HEPES pH 8.0, 500 mM NaCl with 500 mM imidazole) over 20 CVs at a flow rate of 1 mL/min. Active protein fractions were pooled and concentrated with an Amicon Ultra-15 10,000 MWCO centrifugal filter unit (Millipore) resulting in ~ 10 mg/L of culture containing soluble MbNHase. Size-exclusion chromatography was used to remove remaining impurities and to assess the quaternary structure of MbNHase (Fig. SI-2). A 16/60 Superdex 200 prep grade (GE Healthcare) column was calibrated from 12.4 to 200 kDa using the MWGF200 gel filtration calibration kit (Sigma). Purified protein samples were analyzed by SDS-PAGE with a 12.5% polyacrylamide SPRINT NEXT GEL™ (Amresco). Gels were stained with Gel Code Blue (Thermo-Fisher Scientific). Protein concentration of purified protein

was determined using either a Coomassie (Bradford) Protein Assay Kit (Pierce) or by measuring the absorbance at 280 nm on a Shimadzu UV-2450 spectrophotometer. Theoretical molecular weights and protein extinction coefficients were calculated with the ExPASy compute pI/Mw tool.¹⁶ The molecular weight for the *MbNHase* homodimer was 111,207 g/mol with an extinction coefficient of $143,700 \text{ cm}^{-1} \text{ M}^{-1}$. The molecular weight is in good agreement with SDS-PAGE data.

2.5. Kinetic analysis of *MbNHase*

The enzymatic activity of *MbNHase* towards acrylonitrile (acrylamide; $\Delta\epsilon_{225} = 2.9 \text{ mM}^{-1} \text{ cm}^{-1}$) and benzonitrile (benzamide; $\Delta\epsilon_{242} = 5.5 \text{ mM}^{-1} \text{ cm}^{-1}$), was measured using a Shimadzu UV-2450 spectrophotometer. A 1 mL reaction consisted of 50 mM Tris-HCl buffer pH 7.0 at 25 °C and various concentrations of acrylonitrile or benzonitrile.¹⁷ Data analysis was performed using OriginPro 9.0 (OriginLab, Northampton, MA). The kinetic constants V_{max} and K_{m} (were calculated by fitting the data to the Michaelis and Menten equation. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the production of 1 μmol of the amide per minute at 25 °C.

2.6. Metal analysis

The metal content of purified protein was determined by inductively-coupled plasma mass spectrometry (ICP-MS). Purified *MbNHase* (1 mg) expressed in the presence and absence of added CoCl_2 . For comparison purposes, the Co-type NHase from *Pseudonocardia thermophila* JCM 3095 (*PtNHase*) (1 mg) was expressed and purified as previously described,¹⁸ and examined along with a control of buffer containing no protein. All protein samples were digested with concentrated nitric acid (0.863 mL) and heated at 70 °C for 1 h, allowed to cool to room temperature and then diluted to final concentration of 5% nitric acid. Samples were submitted for analysis at the Water Quality Center in the College of Engineering at Marquette University (Milwaukee, WI, USA).

2.7. Electronic absorption spectra

Electronic absorption spectra were recorded on a Shimadzu UV-2600 spectrophotometer equipped with a TCC-240A temperature controlled cell holder. Spectra of *MbNHase* and *PtNHase* were obtained at 25 °C in a 1 cm quartz cuvette in 50 mM HEPES buffer containing 300 mM NaCl, pH 8.0, for *MbNHase* or pH 7.5 for *PtNHase*.

2.8. Homology model of the *MbNHase*

A homology model of the *MbNHase* was developed using the X-ray crystal structure of *PtNHase* (PDB code:1IRE) as a template.^{19,20} Sequence analysis of the target and template α - and β -subunits only, revealed that these proteins are of similar length, with a sequence identity of 29%, and exhibit no significant sequence gaps. The YASARA homology-building program was used to construct the structural homology model of *MbNHase*.^{21,22} Comparison of the energy minimized *MbNHase* α - and β -subunit homology model to the X-ray crystal structure of *PtNHase*, using the MatchMaker in UCSF Chimera, reveals that the *MbNHase* α - and β -subunit homology model displays the typical dimerization and catalytic domains of *PtNHase* with a Needleman-Wunsch²³ score of 285 and an average RMSD of 1.129 Å over the aligned 354 Ca atom pairs.

3. Results and discussion

3.1. Sequence alignment of *MbNHase* with prokaryotic NHases

The predicted NHase polypeptide encoded by the *M. brevicollis* ORF 37534 contains 495 amino acids.¹⁴ The first ~ 240 AA of the N-terminus of this putative NHase align with the β -subunit of prokaryotic NHases (Fig. 1A), while the last ~ 240 AA of the C-terminus aligns with, and has high similarity (35–45%) with prokaryotic NHase α -subunits (Fig. 1A). The α - and β -subunit regions in *MbNHase* are connected by a histidine rich region containing seventeen histidine residues while the C-terminus region contains the signature NHase metal-binding domain of Co-type NHases (Fig. 1B).

3.2. Characteristics of purified MbNHase

MbNHase was expressed in *E. coli* without the co-expression of the *E. coli* molecular chaperones GroES/EL or an activator protein. The protein was purified by IMAC using the seventeen His residues naturally encoded in the insert between the α - and β -subunit regions as the tag, providing ~ 10 mg/L of soluble protein. SDS-PAGE analysis revealed a band with molecular weight of ~ 55 kDa (Fig. 2A), which is in good agreement with the theoretical molecular weight of 55.6 kDa. A faint second band is also observed at ~ 110 kDa, which is consistent with a small fraction of MbNHase homodimer (111.2 kDa) that was not fully denatured under the conditions used. In contrast to prokaryotic NHases, MbNHase is expressed as a fused protein where both the α - and β -subunits reside in a single polypeptide as predicted by Foerstner et al.¹⁴ Even though this is the first example of a fused eukaryotic NHase, the expression of a subunit-fused bacterial NHase from *Pseudomonas putida*²⁴ has also been reported, however, in this case the subunit-fusion was accomplished by engineering a proline-glycine dipeptide linker between the two subunit genes by PCR. Size-exclusion chromatography indicated that MbNHase exists primarily as an $(\alpha\beta)_2$ homodimer in solution, analogous to the $\alpha_2\beta_2$ homotetramer architecture observed for prokaryotic NHases, with a small fraction ($< 10\%$) present as a monomer (Fig. SI-2).

3.3. Kinetic characterization of MbNHase

Kinetic analysis revealed that MbNHase expressed in the presence of 0.25 mM CoCl₂ is capable of hydrating acrylonitrile, the standard substrate for NHase kinetic analysis, with a k_{cat} of $130 \pm 3 \text{ s}^{-1}$ and a K_{m} of $78 \pm 3 \text{ mM}$, providing a catalytic efficiency $k_{\text{cat}}/K_{\text{m}}$ of $1.7 \text{ s}^{-1} \text{ mM}^{-1}$. Since the turnover rate is in line with other reported Co-type NHase enzymes ($k_{\text{cat}} = 50$ to 1500 s^{-1}),^{2,18} it was hypothesized that the metal ion was properly inserted and that both active site Cys residues (Cys403 and Cys405) were properly oxidized in the absence of an activator protein, which is typically required for full NHase enzymatic activity.^{7,8,9} MbNHase is also capable of hydrating benzonitrile with k_{cat} of $33 \pm 1 \text{ s}^{-1}$ and a $K_{\text{m}} = 18 \pm 1 \text{ mM}$, providing a catalytic efficiency $k_{\text{cat}}/K_{\text{m}}$ of $1.8 \text{ s}^{-1} \text{ mM}^{-1}$. These data suggest that MbNHase exhibits a higher affinity for aromatic nitrile substrates

compared to aliphatic substrates, following the trend observed for most Co-type NHases.^{4,15}

3.4. Metal analysis and spectral characterization of *MbNHase*

A combination of UV-Vis spectroscopy and metal analysis was used to determine if *MbNHase* expressed in the absence of an activator protein contained its full complement of Co(III). After purification, *MbNHase* expressed in the presence of 0.25 mM CoCl₂ exhibits the characteristic amber color of a Co-type NHase enzymes (Fig. 2B, inset).² ICP-MS data indicate that *MbNHase* expressed in the presence of 0.25 mM CoCl₂ contained ~ 1.8 equivalents of cobalt per homodimer while *MbNHase* expressed in the absence of CoCl₂ contained 1.6 cobalt ions per homodimer. The UV-Vis spectrum of *MbNHase* expressed in the presence and absence of cobalt shows the characteristic S → Fe(III) ligand-to-metal-charge-transfer (LMCT) band at ~ 320 nm ($\epsilon = 19,392 \text{ M}^{-1} \text{ cm}^{-1}$) (Fig. 2B).² These data show that *MbNHase* contains its full complement of Co(III) and can thus be expressed in *E. coli* in the absence of a Co-type NHase activator protein or the *E. coli* molecular chaperones GroES/EL. Given the high iron content of LB media, these data also suggest that the *MbNHase* has high affinity for cobalt and can be classified as a Co-type NHase.

All Fe- and Co-type NHase enzymes reported to date require the co-expression of an activator protein for metal ion insertion and the post-translational oxidation of the two equatorial active site cysteine residues.^{11,25} The only exception is *CtNHase*.¹² For *M. brevicollis*, no ORF encoding for an NHase activator or accessory protein, which is usually found downstream of the prokaryotic NHase genes, has been reported.^{13,14} Based on the binding ability of *MbNHase* during purification to the Ni-NTA column material, even though no His-tag was engineered, it is clear that the histidine-rich region found in the insert between the β - and α -subunits of *MbNHase* is capable of tight, but reversible binding of divalent transition metal ions. It is tempting, then, to speculate that the histidine-rich insert plays a role similar to that of the activator protein in metal acquisition and/or insertion. Histidine rich regions are present in some proteins of the cobalamin (vitamin B12) biosynthetic pathway, e.g., the chelatase CbiX enzyme

from *Bacillus megaterium* and CobW from *Pseudomonas denitrificans*.²⁶ Histidine rich regions are also found in metallochaperone and accessory proteins involved in metallocenter assembly of nickel hydrogenases and ureases, such as HypB from *Bradyrhizobium japonicum* and *Rhizobium leguminosarum*, SlyD from *Escherichia coli* and *Helicobacter pylori*, UreE from *Klebsiella aerogenes*, Hpn and Hpn-like proteins from *Helicobacter pylori*.²⁷ Based on the similarity of the histidine rich region with accessory proteins involved in the metallocenter assembly of cobalamin, hydrogenases, and ureases, it is possible that the histidine rich insert that links the β - and α -subunits of *MbNHase*, and those found in other eukaryotic NHases, play a role in cobalt binding and possible insertion of the cobalt ion into the active site.

3.5. Homology model of *MbNHase*

Since no three-dimensional X-ray crystal structure exists for *MbNHase*, a homology model was developed using the X-ray crystal structure of the prototypical prokaryotic Co-type NHase from *Pseudonocardia thermophila* JCM 3095 (*PtNHase*; PDB code: 1IRE) as a template (Fig. 3A). Only the α - and β -subunits of *MbNHase* were modeled as no suitable template structure exists for the entire *MbNHase* peptide. The α - and β -subunit model of *MbNHase* suggests that it is, in general, structurally similar to other Co-type NHase enzymes (Fig. 3A). The α -subunit of *MbNHase* is embedded in the center of the β -subunit, while the N-terminus of the α -subunit forms a helix that is embedded among three helices from the β -subunit. The model reveals that within 4.0 Å of the Co(III) binding site, all of the amino acid residues are identical to those in *PtNHase*. The Co(III) ion of *MbNHase* is five coordinate and bound by C400, C403, and C405 as well as two backbone amide nitrogen's, where C403 and C405 are both modeled as oxidized cysteine-sulfinic and cysteine-sulfenic acids, respectively (Fig. 3B). The oxidation of the equatorial Cys residues is expected since *MbNHase* exhibits nitrile hydratase activity and oxidation of the equatorial Cys residues is required for catalytic activity.⁶ Two amide nitrogen atoms and the oxidized sulfur atoms are roughly in the same plane as the metal ion. The sulfur atom from C400 forms a coordination bond roughly perpendicular to this plane, which results in a slightly distorted square-based pyramidal geometry. On

the opposite side of C400, no sixth ligand such as water was modeled but is often observed for Co-type NHase enzymes.² The sulfinic and sulfenic acid ligands are within hydrogen bonding distance of two strictly conserved and catalytically important β -Arg56 and β -Arg167 residues found in Co-type NHase enzymes. These Arg residues are conserved in all bacterial NHases and play a role in the stabilization of the "claw-setting" structure through the hydrogen bonding.

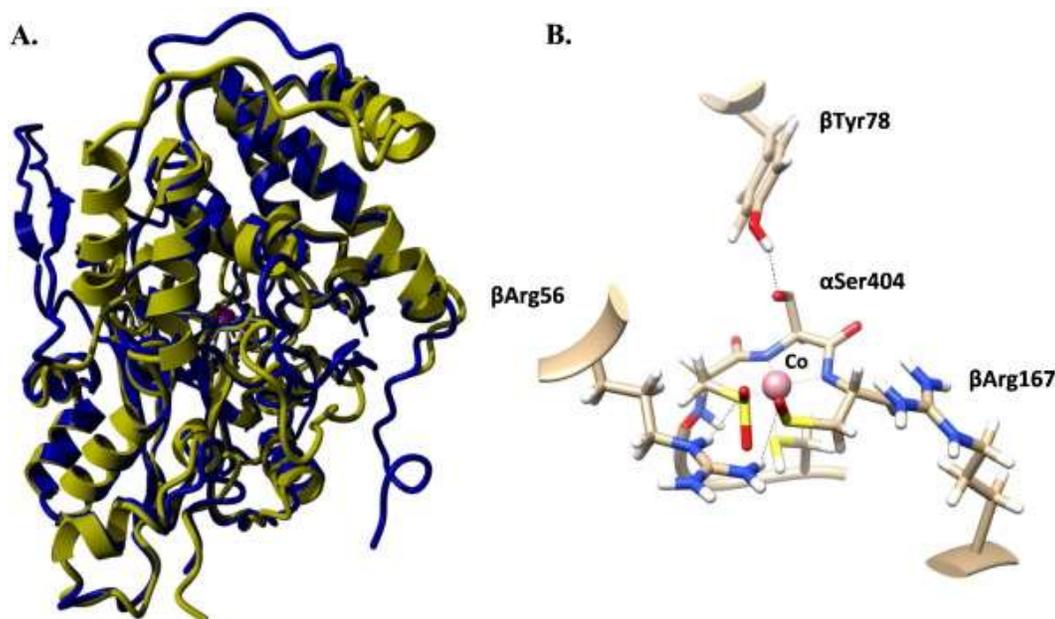


Fig. 3. Homology model of *Mb*NHase. A) The *Mb*NHase homology model for the α - and β -subunits only are highlighted in blue is overlaid with the X-ray crystal of *Pt*NHase (PDB code: 1IRE) which is in green. B) The active site of *Mb*NHase derived from the homology model showing the classical "claw-setting" of an NHase.

4. Conclusion

The construct presented here provides the first evidence of a functional subunit-fused eukaryotic NHase. We found that expression of *Mb*NHase does not require an NHase activator protein or the *E. coli* chaperone proteins GroEL/ES. These data are contrary to the accepted view regarding the strict requirement of co-expressing NHases with activator proteins for fully active enzymes. Based on UV-Vis and ICP-MS data, *Mb*NHase binds its full complement of Co(III) ions in its active site and the equatorial Cys residues are appropriately oxidized, which is a requirement for full enzymatic activity. Both metal ion insertion and Cys oxidation are functions previously ascribed to NHase activator

proteins. The homology model suggests that the *MbNHase* polypeptide encoding for the α - and β -subunits is capable of folding like a prototypical prokaryotic Co-type NHase. The position of the predicted active site residues of *MbNHase* align with those of *PtNHase* including the highly conserved β -Arg residues in prokaryotic Co-type NHases that form hydrogen bonds to the active site oxidized Cys residues.²⁸ Both of these β -Arg residues are catalytically important for both Fe- and Co-type prokaryotic NHase enzymes. Since *MbNHase* is a new member of the NHase family that is expressed from a single gene without the requirement of an activator protein, it represents an alternative biocatalyst for industrial syntheses of important amide compounds.

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Corresponding author at: Department of Chemistry, Marquette University, PO Box 1881, Milwaukee, WI 53201, United States.
Current address: Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824, United States.

Appendix A. Supplementary data

Appendix A. Supplementary data

Supplementary information includes DNA and amino acid sequences of *MbNHase* (Fig. SI-1), a size-exclusion chromatography elution profile (Fig. SI-2), and an image of an SDS-PAGE for apo *MbNHase* (Fig. SI-3). Supplementary data associated with this article can be found in the online version, at doi: <http://dx.doi.org/10.1016/j.bbapap.2016.09.013>.

Supplementary Material

Figure SI-1. The DNA sequence encoding the nitrile hydratase from *Monosiga brevicollis* was codon-optimized for over-expression in *E. coli* and synthesized by IDT (Iowa, USA). The codon-optimized gene and amino acid sequences of *MbNHase* are shown below.

```
1 ATG CAT CTG TTC ACG TAT GAT CTG CAC CAT GAC GTG GGC GGT GCG 45
1 Met His Leu Phe Thr Tyr Asp Leu His His Asp Val Gly Gly Ala 15

46 GAA AAC ATG CTT CGT CTG CCG CTG GAT CGT CAC GAA CGT GAT TAC 90
16 Glu Asn Met Leu Arg Leu Pro Leu Asp Arg His Glu Arg Asp Tyr 30

91 CTG CCG TGG GAA CGT CAT ATT CAC GCT TTG GTA GTG TTG CTG GTC 135
31 Leu Pro Trp Glu Arg His Ile His Ala Leu Val Val Leu Leu Val 45

136 AAA CAA GGC CGT ATG AGC GTT GAT GAG TTG CGT CGC GGG GTC GAA 180
46 Lys Gln Gly Arg Met Ser Val Asp Glu Leu Arg Arg Gly Val Glu 60

181 GGC CTG CCT TCC TCA TTA GCG GAA CAG GCG AGC TAT TAC GAA AAG 225
61 Gly Leu Pro Ser Ser Leu Ala Glu Gln Ala Ser Tyr Tyr Glu Lys 75

226 TGG GGG TTG TCG GTG TCA CGT ATT CTG ACG GAA AAA GGC ACG GTC 270
76 Trp Gly Leu Ser Val Ser Arg Ile Leu Thr Glu Lys Gly Thr Val 90

271 TCA GGT CAC GAA CTG GAG CAA GGG TTC TTA GGT GTT CCG ACG ACG 315
91 Ser Gly His Glu Leu Glu Gln Gly Phe Leu Gly Val Pro Thr Thr 105

316 GAT CTG CCG CAG GTG CCC CGC TTC CAG GTG GGC CAA CGT GTA ATG 360
106 Asp Leu Pro Gln Val Pro Arg Phe Gln Val Gly Gln Arg Val Met 120

361 GTG CGT CCG TTC GGG ACA ACG TTT GCA TAC CGC CAA CCC CAT CTG 405
121 Val Arg Pro Phe Gly Thr Thr Phe Ala Tyr Arg Gln Pro His Leu 135

406 CGT GTT CCG GGG TAT GTT CAT GGA GCC GTG GGC ACT ATC GTG GAA 450
136 Arg Val Pro Gly Tyr Val His Gly Ala Val Gly Thr Ile Val Glu 150

451 CTG CCT GGG CTG TTT CAG GAT CCT ATG ACC GGC GCT TAC GGG GAA 495
151 Leu Pro Gly Leu Phe Gln Asp Pro Met Thr Gly Ala Tyr Gly Glu 165
```

496 CGC GGT ACA GCT CAA CCG CTG TAC CGC GTG GCA TTC AGT CAT CGC 540
166 Arg Gly Thr Ala Gln Pro Leu Tyr Arg Val Ala Phe Ser His Arg 180

541 GCG CTG TGG CCG GAA GGG GCC GCG CAC GCC GAA CCT GGC GAA CTG 585
181 Ala Leu Trp Pro Glu Gly Ala Ala His Ala Glu Pro Gly Glu Leu 195

586 GAA GAT GGC GTA GTC GTG GAT GTC AGT CAG CCA TGG TTA GAG GCT 630
196 Glu Asp Gly Val Val Val Asp Val Ser Gln Pro Trp Leu Glu Ala 210

631 TTA TCT GAA GCT GAT TAC GCA CAG CGT CTG GCG ACG CTG CAT CGT 675
211 Leu Ser Glu Ala Asp Tyr Ala Gln Arg Leu Ala Thr Leu His Arg 225

676 GTG GCG TTT ACT CCT GAT AGC AAC CCG CCA CAA GCG CAT AAG CAC 720
226 Val Ala Phe Thr Pro Asp Ser Asn Pro Pro Gln Ala His Lys His 240

721 CAT CAT CAC CGT CAT CAT CAT GAC CAC CAT CAT CAT CAT CAT CAT 765
241 His His His Arg His His His Asp His His His His His His His 255

766 CAC CAC GCT ATG CAC GCC GAA CAT GAA GCT CAT ACC CAC GAT ACC 810
256 His His Ala Met His Ala Glu His Glu Ala His Thr His Asp Thr 270

811 CGT TAC GGC ACC GAA CAG GCC GCG GTA GCG AAG GAA GCG GCA TTG 855
271 Arg Tyr Gly Thr Glu Gln Ala Ala Val Ala Lys Glu Ala Ala Leu 285

856 GAT TTC CCG TAT CAG CCC TGG TGT GAA GCT CTG GTC CAG ACT CTT 900
286 Asp Phe Pro Tyr Gln Pro Trp Cys Glu Ala Leu Val Gln Thr Leu 300

901 ACC CGC CGC GGG GTC GTG CGC TCG GAC GAG CTG CAT GCT ACG CTG 945
301 Thr Arg Arg Gly Val Val Arg Ser Asp Glu Leu His Ala Thr Leu 315

946 GCA TCT CTG GAT GCA CTG CAA AAC TCA GGC GCA GGG CCT CAG CTG 990
316 Ala Ser Leu Asp Ala Leu Gln Asn Ser Gly Ala Gly Pro Gln Leu 330

991 GTG GCG CGC GCC TGG TCC GAT GCT GCT TTC GCG GAG TGG TTA CTG 1035
331 Val Ala Arg Ala Trp Ser Asp Ala Ala Phe Ala Glu Trp Leu Leu 345

1036 ACC GAT GCC GCT GCG GCG GCA GAG AGC TTA GCA ATC CGC ACC ACG 1080
346 Thr Asp Ala Ala Ala Ala Ala Glu Ser Leu Ala Ile Arg Thr Thr 360

1081 AAT TAC GAC GCG GAT CCG GCC TCT GCC GAG CGT GTG GGC GGC CAT 1125
361 Asn Tyr Asp Ala Asp Pro Ala Ser Ala Glu Arg Val Gly Gly His 375

1126 CGC CTG TTT TCG CAC AAT CAT ACG GAA TTA CGT GTC GTT GCG AAC 1170
376 Arg Leu Phe Ser His Asn His Thr Glu Leu Arg Val Val Ala Asn 390

1171 ACC GAC ACC GTA CAT AAC TTA GTA TGC TGC ACG CTG TGT TCT TGT 1215
391 Thr Asp Thr Val His Asn Leu Val Cys Cys Thr Leu Cys Ser Cys 405

1216 TAC CCG ACC GCG ATC TTA GGG CTT AGT CCA CCG TGG TAT AAG TCA 1260
406 Tyr Pro Thr Ala Ile Leu Gly Leu Ser Pro Pro Trp Tyr Lys Ser 420

1261 AAA GTG TTC CGC GCG CGT GCG GTC CGC GAA CCG CGT CGC TTG CTG 1305
421 Lys Val Phe Arg Ala Arg Ala Val Arg Glu Pro Arg Arg Leu Leu 435

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1306 CGT GAA GAA TTT GGC CTG GTG TTA CCG GAA GCG CGT GGT ATT CGT 1350
436 Arg Glu Glu Phe Gly Leu Val Leu Pro Glu Ala Arg Gly Ile Arg 450

1351 GTT CAT GAT AGC ACT GCA GAT CTG CGT TAC ATG GTT CTG CCC CAA 1395
451 Val His Asp Ser Thr Ala Asp Leu Arg Tyr Met Val Leu Pro Gln 465

1396 CGC CCG CAA GGC ACA GAA GGC TGG TCT GAG GAA CAT CTG CGT ACC 1440
466 Arg Pro Gln Gly Thr Glu Gly Trp Ser Glu Glu His Leu Arg Thr 480

1441 ATC GTT ACC CGC GAT AGC CTT CTC GGT ACC GCG GTG CCG CGC GTG 1485
481 Ile Val Thr Arg Asp Ser Leu Leu Gly Thr Ala Val Pro Arg Val 495

1486 GAT TAA 1491
496 Asp End 497

Figure SI-2. Gel filtration elution profile of *MbNHase* for the determination of the quaternary structure in solution and molecular mass. Marker proteins used for gel filtration: (i) cytochrome c, 12.4 kDa, (ii) carbonic anhydrase, 29 kDa, (iii) albumin 66 kDa, (iv) alcohol dehydrogenase, 150 kDa, and (v) beta-amylase, 200 kDa. Peaks at 75.54 mL and 85.10 mL correspond to dimer and monomer of *MbNHase*, respectively.

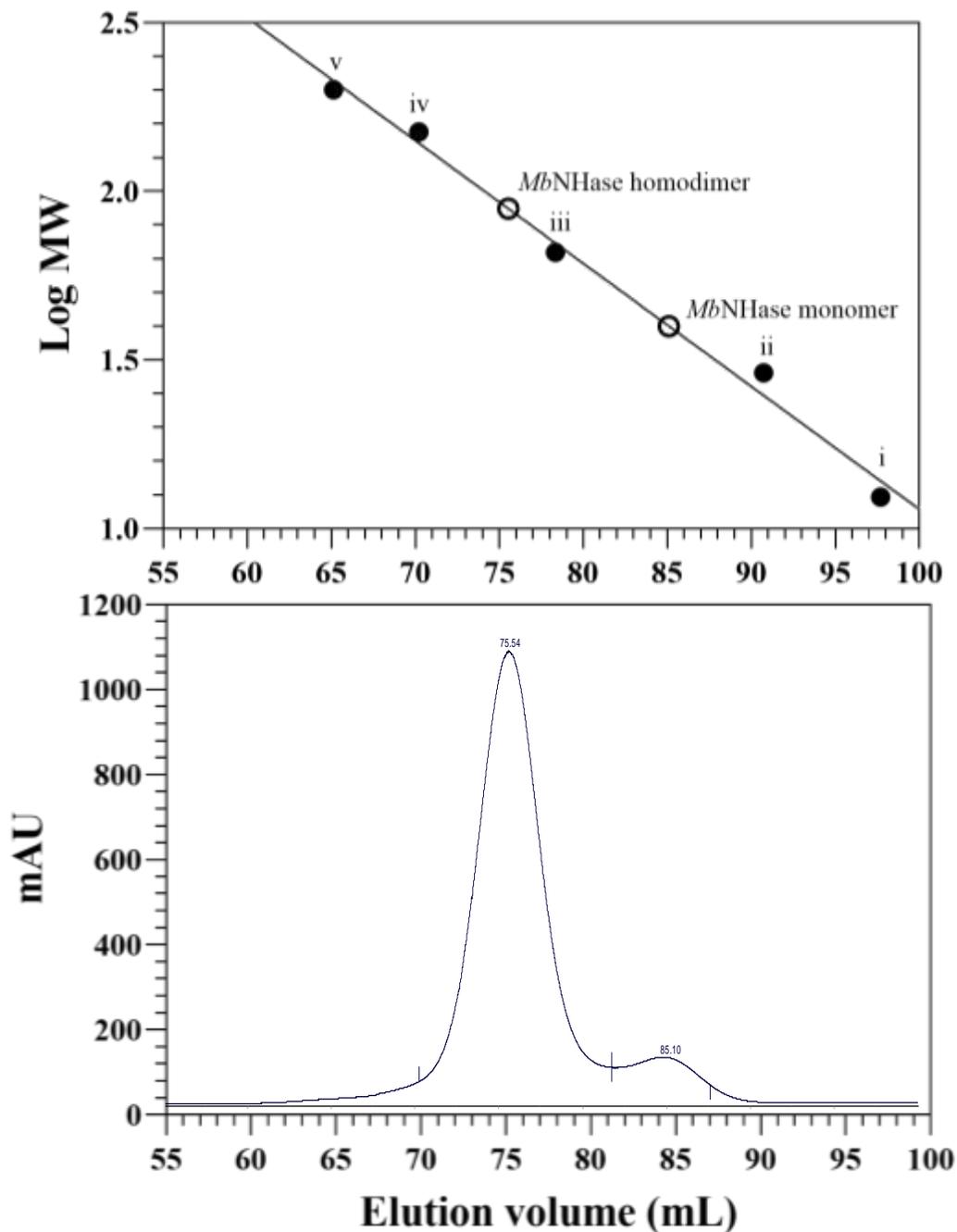
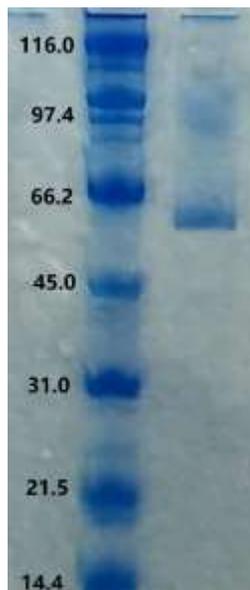


Figure SI-3. SDS-PAGE analysis of apo-*Mb*NHase: lane 1 molecular weight standards, lane 2 purified apo *Mb*NHase.



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5. Manuscript Title A cobalt-containing eukaryotic nitrile hydratase		
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Licensed: The patent has been licensed to an entity, whether earning royalties or not

Royalties: Funds are coming in to you or your institution due to your patent

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Section 1. Identifying Information

1. Given Name (First Name)
Richard

2. Surname (Last Name)
Holz

3. Date
03-October-2016

4. Are you the corresponding author? Yes No

5. Manuscript Title
A Cobalt-Containing Eukaryotic Nitrile Hydratase

6. Manuscript Identifying Number (if you know it)
BBAPAP39824

Section 2. The Work Under Consideration for Publication

Did you or your institution **at any time** receive payment or services from a third party (government, commercial, private foundation, etc.) for any aspect of the submitted work (including but not limited to grants, data monitoring board, study design, manuscript preparation, statistical analysis, etc.)?

Are there any relevant conflicts of interest? Yes No

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