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A Cobalt-Containing Eukaryotic Nitrile Hydratase

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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* (*Mb*NHase) 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 *Mb*NHase 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 *Mb*NHase 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 (− SO_2H) and cysteine-sulfenic acid (− 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 (*Ct*NHase), 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*, *Emiliania 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.

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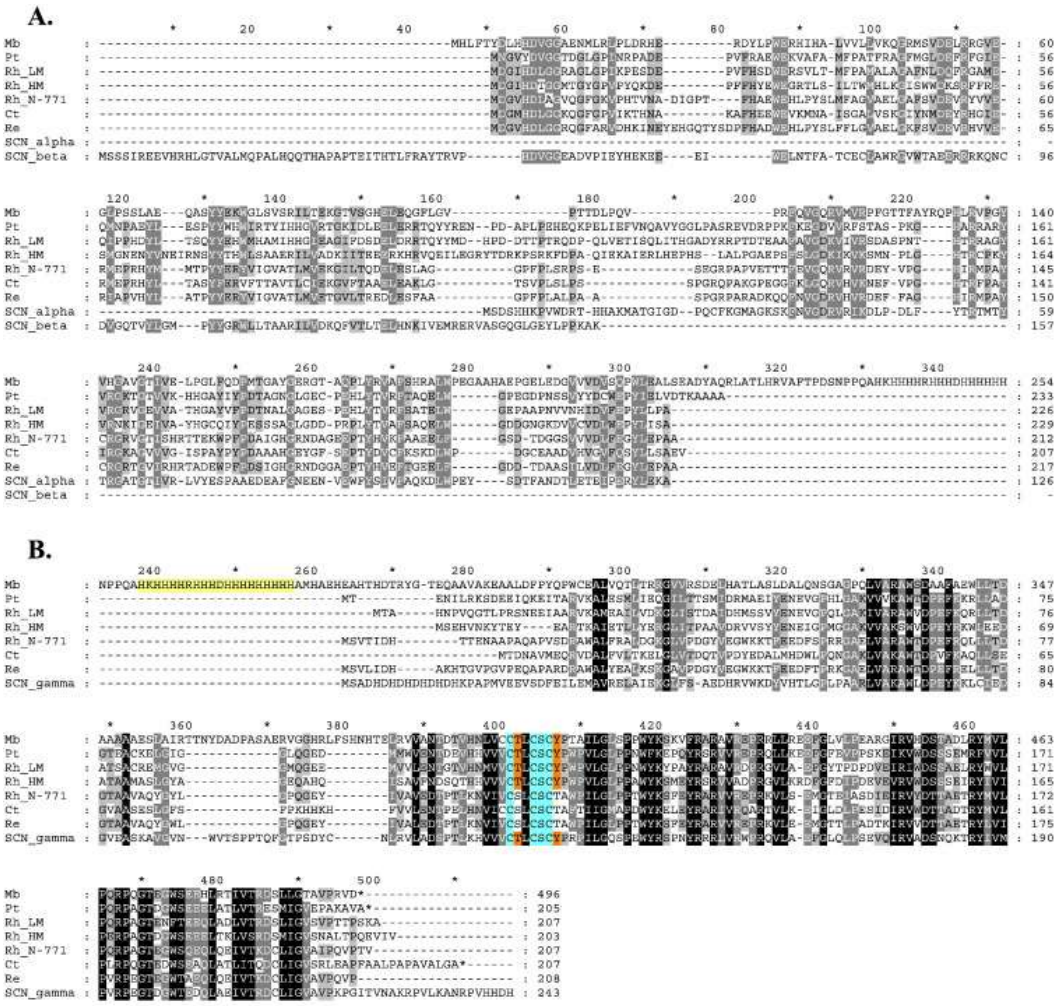

Fig. 1. Alignment of the amino acid sequence of the eukaryotic NHase from *M. brevicollis* and prokaryotic nitrile hydratases. A) Amino acid alignment of the N-terminus residues of the eukaryotic NHase from *M. brevicollis* and prokaryotic NHase β-subunits. B) Amino acid alignment of the C-terminus residues 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*

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

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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 $CoCl_2$ 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 NaH_2PO_4 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 *Mb*NHase 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 NaH_2PO_4 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 *Mb*NHase. Size-exclusion chromatography was used to remove remaining impurities and to assess the quaternary structure of *Mb*NHase (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

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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 *Mb*NHase homodimer was 111,207 g/mol with an extinction coefficient of 143,700 cm^{-1} M^{-1}. The molecular weight is in good agreement with SDS-PAGE data.

2.5. Kinetic analysis of MbNHase

The enzymatic activity of *Mb*NHase towards acrylonitrile (acrylamide; $\Delta\varepsilon_{225}$ = 2.9 mM^{-1} cm^{-1}) and benzonitrile (benzamide; $\Delta\varepsilon_{242}$ = 5.5 mM^{-1} 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 *Mb*NHase (1 mg) expressed in the presence and absence of added $CoCl_2$. For comparison purposes, the Co-type NHase from *Pseudonocardia thermophila* JCM 3095 (*Pt*NHase) (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 *Mb*NHase and *Pt*NHase were obtained at 25 °C in a 1 cm quartz cuvette in 50 mM HEPES buffer containing 300 mM NaCl, pH 8.0, for *Mb*NHase or pH 7.5 for *Pt*NHase.

2.8. Homology model of the MbNHase

A homology model of the *Mb*NHase was developed using the X-ray crystal structure of *Pt*NHase (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 *Mb*NHase.[21,22] Comparison of the energy minimized *Mb*NHase α- and β-subunit homology model to the X-ray crystal structure of *Pt*NHase, using the MatchMaker in UCSF Chimera, reveals that the *Mb*NHase α- and β-subunit homology model displays the typical dimerization and catalytic domains of *Pt*NHase with a Needleman-Wunsch[23] score of 285 and an average RMSD of 1.129 Å over the aligned 354 Cα 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 *Mb*NHase 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).

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3.2. Characteristics of purified MbNHase

*Mb*NHase 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 *Mb*NHase homodimer (111.2 kDa) that was not fully denatured under the conditions used. In contrast to prokaryotic NHases, *Mb*NHase 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 *Mb*NHase 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 *Mb*NHase expressed in the presence of 0.25 mM $CoCl_2$ is capable of hydrating acrylonitrile, the standard substrate for NHase kinetic analysis, with a k_{cat} of 130 ± 3 s^{-1} and a K_m of 78 ± 3 mM, providing a catalytic efficiency k_{cat}/K_m of 1.7 s^{-1} mM^{-1}. Since the turnover rate is in line with other reported Co-type NHase enzymes (k_{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] *Mb*NHase is also capable of hydrating benzonitrile with k_{cat} of 33 ± 1 s^{-1} and a K_m = 18 ± 1 mM, providing a catalytic efficiency k_{cat}/K_m of 1.8 s^{-1} mM^{-1}. These data suggest that *Mb*NHase exhibits a higher affinity for aromatic nitrile substrates

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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 *Mb*NHase expressed in the absence of an activator protein contained its full complement of Co(III). After purification, *Mb*NHase expressed in the presence of 0.25 mM $CoCl_2$ exhibits the characteristic amber color of a Co-type NHase enzymes (Fig. 2B, inset).[2] ICP-MS data indicate that *Mb*NHase expressed in the presence of 0.25 mM $CoCl_2$ contained ~ 1.8 equivalents of cobalt per homodimer while *Mb*NHase expressed in the absence of $CoCl_2$ contained 1.6 cobalt ions per homodimer. The UV–Vis spectrum of *Mb*NHase expressed in the presence and absence of cobalt shows the characteristic S → Fe(III) ligand-to-metal-charge-transfer (LMCT) band at ~ 320 nm (ε = 19,392 M^{-1} cm^{-1}) (Fig. 2B).[2] These data show that *Mb*NHase 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 *Mb*NHase 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 *Ct*NHase.[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 *Mb*NHase 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 *Mb*NHase 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

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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 *Mb*NHase, 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 *Mb*NHase, a homology model was developed using the X-ray crystal structure of the prototypical prokaryotic Co-type NHase from *Pseudonocardia thermophila* JCM 3095 (*Pt*NHase; PDB code: 1IRE) as a template (Fig. 3A). Only the α- and β-subunits of *Mb*NHase were modeled as no suitable template structure exists for the entire *Mb*NHase peptide. The α- and β-subunit model of *Mb*NHase suggests that it is, in general, structurally similar to other Co-type NHase enzymes (Fig. 3A). The α-subunit of *Mb*NHase 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 *Pt*NHase. The Co(III) ion of *Mb*NHase 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 *Mb*NHase 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

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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.

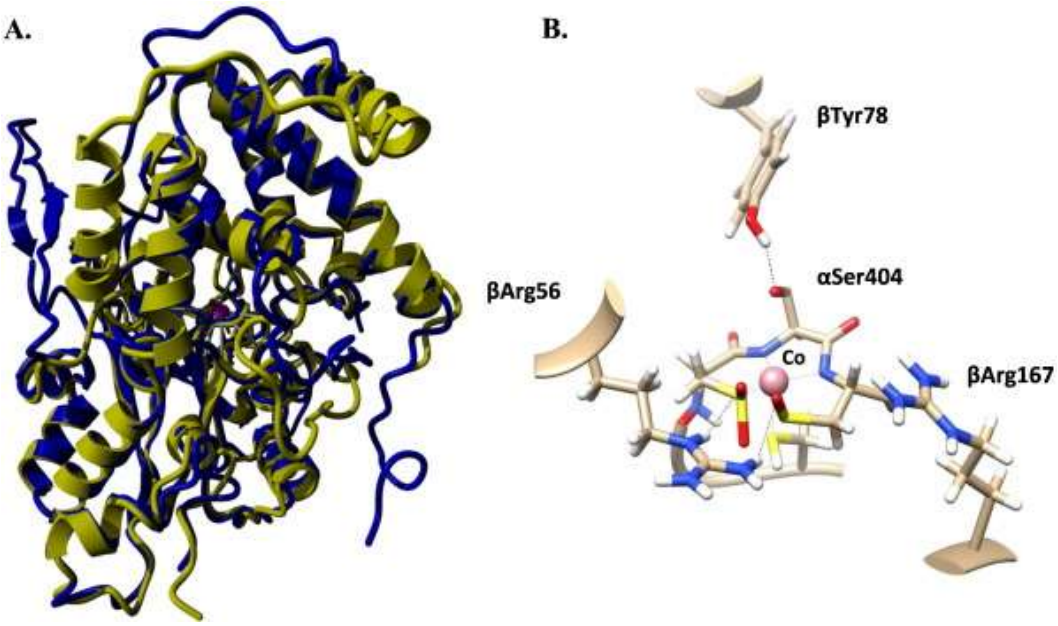

Fig. 3. Homology model of *Mb*NHase. A) The *Mb*NHase homology model for the α- and β-subunits only are highlighted in blue is overlaid with the X-ray crystal of *Pt*NHase (PDB code:1IRE) which is in green. B) The active site of *Mb*NHase derived from the homology model showing the classical "claw-setting"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

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proteins. The homology model suggests that the *Mb*NHase 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 *Mb*NHase align with those of *Pt*NHase 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 *Mb*NHase 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 *Mb*NHase (Fig. SI-1), a size-exclusion chromatography elution profile (Fig. SI-2), and an image of an SDS-PAGE for apo *Mb*NHase (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 *Mb*NHase 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
```

Biochimica et Biophysica Acta: Proteins and Proteomics, Vol 1865, No. 1 (January 2017): pg. 107-112. DOI.

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

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 *Mb*NHase 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 *Mb*NHase, respectively.

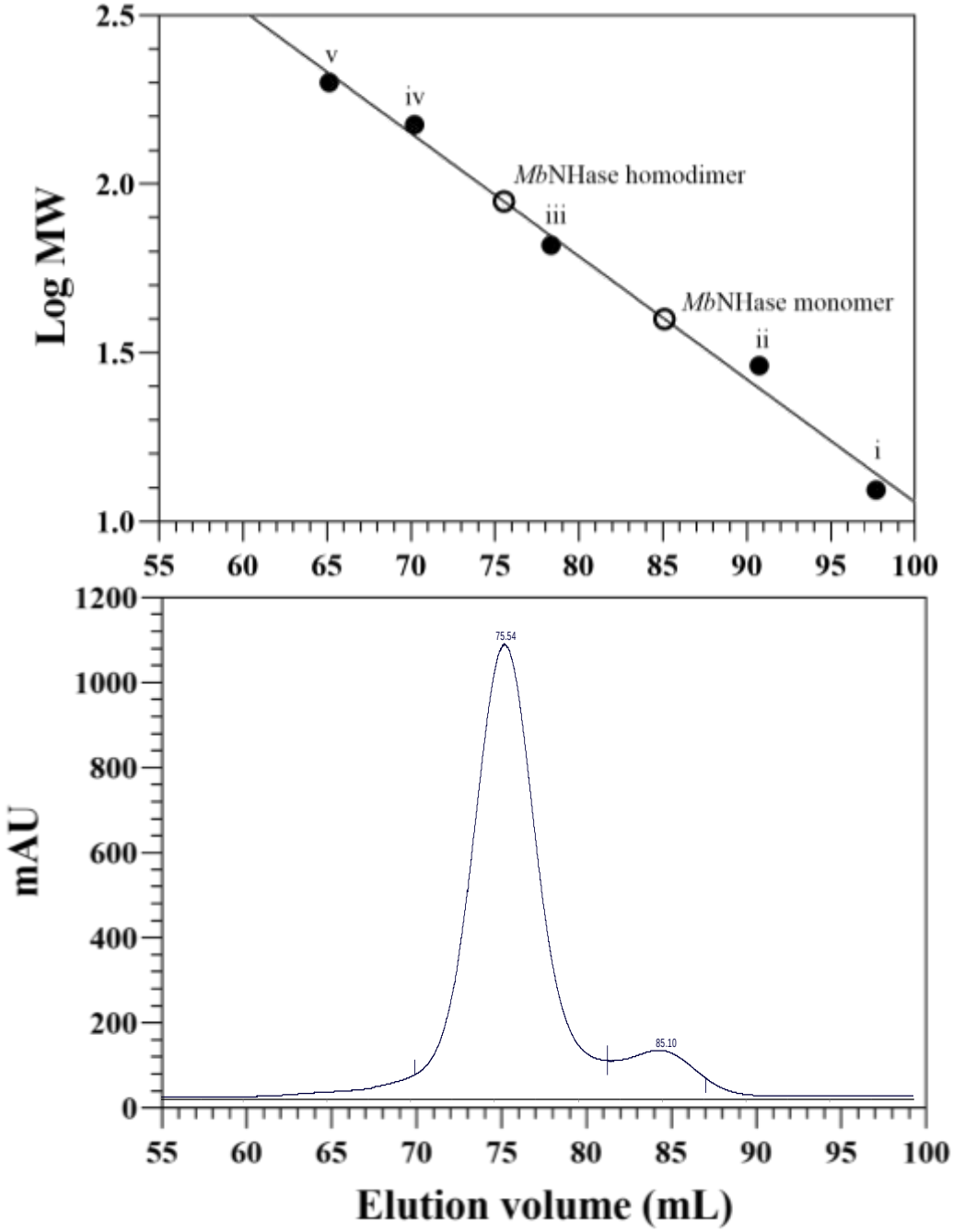
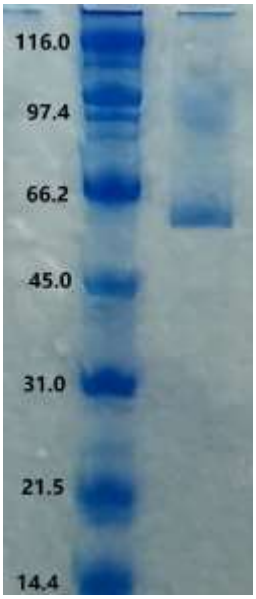


Figure SI-3. SDS-PAGE analysis of apo-*Mb*NHase: lane 1 molecular weight standards, lane 2 purified apo *Mb*NHase.



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5. Manuscript Title: A cobalt-containing eukaryotic nitrile hydratase

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Corresponding Author's Name: Richard C. Holz

5. Manuscript Title: A cobalt-containing eukaryotic nitrile hydratase

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

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5. Manuscript Title: A cobalt-containing eukaryotic nitrile hydratase

6. Manuscript Identifying Number (if you know it): BBAPAP_39824

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Dr. BENNETT has nothing to disclose.

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Please visit http://www.icmje.org/cgi-bin/feedback to provide feedback on your experience with completing this form.

ICMJE Form for Disclosure of Potential Conflicts of Interest

Instructions

The purpose of this form is to provide readers of your manuscript with information about your other interests that could influence how they receive and understand your work. The form is designed to be completed electronically and stored electronically. It contains programming that allows appropriate data display. Each author should submit a separate form and is responsible for the accuracy and completeness of the submitted information. The form is in six parts.

1. Identifying information.

2. The work under consideration for publication.

This section asks for information about the work that you have submitted for publication. The time frame for this reporting is that of the work itself, from the initial conception and planning to the present. The requested information is about resources that you received, either directly or indirectly (via your institution), to enable you to complete the work. Checking "No" means that you did the work without receiving any financial support from any third party -- that is, the work was supported by funds from the same institution that pays your salary and that institution did not receive third-party funds with which to pay you. If you or your institution received funds from a third party to support the work, such as a government granting agency, charitable foundation or commercial sponsor, check "Yes".

3. Relevant financial activities outside the submitted work.

This section asks about your financial relationships with entities in the bio-medical arena that could be perceived to influence, or that give the appearance of potentially influencing, what you wrote in the submitted work. You should disclose interactions with ANY entity that could be considered broadly relevant to the work. For example, if your article is about testing an epidermal growth factor receptor (EGFR) antagonist in lung cancer, you should report all associations with entities pursuing diagnostic or therapeutic strategies in cancer in general, not just in the area of EGFR or lung cancer.

Report all sources of revenue paid (or promised to be paid) directly to you or your institution on your behalf over the 36 months prior to submission of the work. This should include all monies from sources with relevance to the submitted work, not just monies from the entity that sponsored the research. Please note that your interactions with the work's sponsor that are outside the submitted work should also be listed here. If there is any question, it is usually better to disclose a relationship than not to do so.

For grants you have received for work outside the submitted work, you should disclose support ONLY from entities that could be perceived to be affected financially by the published work, such as drug companies, or foundations supported by entities that could be perceived to have a financial stake in the outcome. Public funding sources, such as government agencies, charitable foundations or academic institutions, need not be disclosed. For example, if a government agency sponsored a study in which you have been involved and drugs were provided by a pharmaceutical company, you need only list the pharmaceutical company.

4. Intellectual Property.

This section asks about patents and copyrights, whether pending, issued, licensed and/or receiving royalties.

5. Relationships not covered above.

Use this section to report other relationships or activities that readers could perceive to have influenced, or that give the appearance of potentially influencing, what you wrote in the submitted work.

Definitions.

Entity: government agency, foundation, commercial sponsor, academic institution, etc.
Grant: A grant from an entity, generally [but not always] paid to your organization
Personal Fees: Monies paid to you for services rendered, generally honoraria, royalties, or fees for consulting , lectures, speakers bureaus, expert testimony, employment, or other affiliations
Non-Financial Support: Examples include drugs/equipment supplied by the entity, travel paid by the entity, writing assistance, administrative support, etc.
Other: Anything not covered under the previous three boxes
Pending: The patent has been filed but not issued
Issued: The patent has been issued by the agency
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

Section 3. Relevant financial activities outside the submitted work.

Place a check in the appropriate boxes in the table to indicate whether you have financial relationships (regardless of amount of compensation) with entities as described in the instructions. Use one line for each entity; add as many lines as you need by clicking the "Add +" box. You should report relationships that were **present during the 36 months prior to publication**.

Are there any relevant conflicts of interest? ☐ Yes ☑ No

Section 4. Intellectual Property -- Patents & Copyrights

Do you have any patents, whether planned, pending or issued, broadly relevant to the work? ☐ Yes ☑ No

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Section 5. Relationships not covered above

Are there other relationships or activities that readers could perceive to have influenced, or that give the appearance of potentially influencing, what you wrote in the submitted work?

☐ Yes, the following relationships/conditions/circumstances are present (explain below):

☑ No other relationships/conditions/circumstances that present a potential conflict of interest

At the time of manuscript acceptance, journals will ask authors to confirm and, if necessary, update their disclosure statements. On occasion, journals may ask authors to disclose further information about reported relationships.

Section 6. Disclosure Statement

Based on the above disclosures, this form will automatically generate a disclosure statement, which will appear in the box below.

Evaluation and Feedback

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