Analyzing the function of the insert region found between the α and β-subunits in the eukaryotic nitrile hydratase from Monosiga brevicollis

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ABSTRACT

The functional roles of the (His) $_{17}$ region and an insert region in the eukaryotic nitrile hydratase (NHase, EC 4.2.1.84) from Monosiga brevicollis (MbNHase), were examined. Two deletion mutants, MbNHase $^{238\ 257}$ and MbNHase $^{219\ 272}$, were prepared in which the (His) $_{17}$ sequence and the entire insert region were removed. Each of these MbNHase enzymes provided an $_{2\ 2}$ heterotetramer, identical to that observed for prokaryotic NHases and contains their full complement of cobalt ions. Deletion of the (His) $_{17}$ motif provides an MbNHase enzyme that is 55% as active as the WT enzyme when expressed in the absence of the Co-type activator ( ) protein from Pseudonocardia thermophila JCM 3095 (PtNHase^{act}) but 28% more active when expressed in the presence of PtNHase^{act}. MbNHase $^{219\ 272}$ exhibits 55% and 89% of WT activity, respectively, when expressed in the absence or presence of PtNHase^{act}. Proteolytic cleavage of MbNHase provides an $_{2\ 2}$ heterotetramer that is modestly more active compared to WT MbNHase (k_{cat} = 163 ± 4 vs 131 ± 3 s ^{1}). Combination of these data establish that neither the (His) $_{17}$ nor the insert region are required for metallocentre assembly and maturation, suggesting that Co-type eukaryotic NHases utilize a different mechanism for metal ion incorporation and post-translational activation compared to prokaryotic NHases.

1. Introduction

Nitrile hydratases (NHases, EC 4.2.1.84) are metalloenzymes that contain either a non-heme Fe(III) ion (Fe-type) or a non-corrin Co(III) ion (Co-type) in their active site [1,2]. NHases catalyze the hydration of nitriles to their corresponding higher value amides under mild conditions (room temperature and physiological pH) and have attracted substantial interest as biocatalysts in preparative organic chemistry and bioremediation processes [3 8]. NHases have historically been only found in prokaryotes; however, multiple eukaryotic organisms were shown to contain genes that potentially encode NHase enzymes [9,10]. We recently cloned and over-expressed the candidate gene from the eukaryotic organism Monosiga brevicollis in E. coli and characterized a fully functional Co-type NHase gene product, with fused - and -subunits linked by a (His) $_{17}$ containing region (MbNHase) (Fig. 1) [11]. Size-exclusion chromatography indicated that MbNHase is an ( )$_2$ homodimer in solution, analogous to the $_{2\ 2}$ heterotetrameric architecture of prokaryotic NHases, of which numerous X-ray crystal structures exist [1,2,12,13].

Several open reading frames (ORFs) have been identified just downstream from the structural - and -subunit genes in prokaryotic NHases, and one of these genes has been proposed to function as an activator ( ) protein [14 16]. The prevailing dogma is that both Co- and Fe-type NHase enzymes require the co-expression of an ( ) protein to be fully metallated, post-translationally modified, and fully functional [14 16]. No such ( ) protein has been identified for eukaryotic NHases, such as MbNHase [9,10]. It is tempting to speculate that the insert region within MbNHase, which contains a (His) $_{17}$ motif, plays the role of the ( ) protein. Histidine rich regions are present in some cobalamin (vitamin B12) biosynthetic pathway proteins, e.g., the chelatase CbiX enzyme from Bacillus megaterium and CobW from Pseudomonas denitrificans [17]. Histidine rich regions are also found in accessory proteins involved in metallocentre assembly of nickel hydrogenases and ureases,

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

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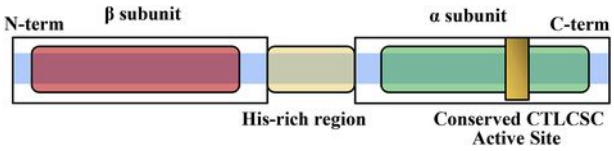

Fig. 1. Scheme showing the arrangement of the NHase - and -subunits in eukaryotes.

such as HypB from Bradyrhizobium japonicum and Rhizobium leguminosarum, SlyD from Escherichia coli and Helicobacter pylori, UreE from Klebsiella aerogenes, and Hpn and Hpn-like proteins from Helicobacter pylori [18].

To investigate metallocentre assembly in MbNHase, we created three different altered MbNHase enzymes. First, we obtained a proteolytically cleaved MbNHase enzyme in which the - and -subunits were separated providing an enzyme that structurally mimics prokaryotic NHase enzymes. Second, a mutant MbNHase enzyme, in which the entire (His) $_{17}$ motif was removed (MbNHase $^{238\ 257}$), was constructed and expressed in the presence and absence of the prototypical prokaryotic Co-type ( ) activator protein from Pseudonocardia thermophila JCM 3095 (PtNHase^{act}). Finally, a mutant was constructed in which the entire insert region was deleted (MbNHase $^{219\ 272}$), leaving only the classical prokaryotic NHase - and -subunit analogs; this mutant was also expressed in the presence and absence of PtNHase^{act}. Functional interrogation of these species provides important insight into the role of the insert region in MbNHase and how eukaryotic NHase enzymes are metallated and post-translationally modiWed.

2. Materials and methods

2.1. Materials

Acrylonitrile, 2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris-HCl), and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) were obtained from Sigma-Aldrich. Oligonucleotides and genes were obtained from Integrated DNA Technologies, Inc. All other reagents were purchased commercially and were the highest purity available.

2.2. Expression and puri?cation of MbNHase

Protein sequences for the - and -subunit genes of the putative WT MbNHase were obtained from ORF 37534 (UniProt ID A9V2C1.1) of M. brevicollis and the predicted gene was synthesized by Integrated DNA Technologies, Inc. with optimized E. coli codon usage. This gene was cloned into the kanamycin resistant pET21a (EMD Biosciences) expression vector to create the plasmid pSMM , as previously reported [11].

A single colony was used to inoculate separate starter cultures of 50 mL LB Miller media containing the appropriate antibiotics (pET28a+: 50 mg/mL kanamycin) and allowed to grow at 37 °C with constant shaking overnight. These cultures were used to inoculate 6 L of LB Miller media containing the appropriate antibiotics and allowed to grow at 37 °C with constant shaking until an optical density of 0.8 1.0 at 600 nm was reached. The cultures were cooled on ice to 20 °C and induced with 0.1 mM isopropyl- -D-1- thiogalactopyranoside (IPTG), supplemented with 0.25 mM of $CoCl_2$, and shaken for an additional 16 h at 20 °C.

Cells were pelleted by centrifugation at 6370 × g for 10 min at 4 °C in a Beckman Coulter Avanti JA-10 rotor and resuspended in buZer A (50 mM sodium phosphate buZer, pH 7.5, containing 300 mM NaCl, 5% glycerol, and 10 mM imidazole) at a ratio of 5 mL per gram of cells. Cells were lysed by ultrasonication (Misonix Sonicator 3000) for 4 min (alternating 30s on and 45s oZ) at 21 W. Cell lysate was separated from cell debris by centrifugation in a JA-20 rotor at 31,000 × g at 4 °C for 20 min. Cell lysate was puriWed using immobilized metal aYnity chromatography (IMAC; 100 mg protein/5 mL column) on a GE ÄKTA Fast Protein Liquid Chromatography (FPLC) system at 4 °C. The column was washed with 50 mL of buZer A followed by 50 mL of buZer A containing additional imidazole (35 mM). The protein was eluted using a linear gradient (0 100%) of buZer B (50 mM NaH_2PO_4 pH 7.5, 300 mM NaCl, 10% glycerol, 525 mM imidazole) at a Xow rate of 1 mL/min resulting in MbNHase being eluted between 150 and 240 mM imidazole.

Fractions containing WT MbNHase were pooled and concentrated to 1 mL using an Amicon Ultra-15 (Millipore) and loaded onto a 16/60 Superdex 200 prep grade (GE Healthcare) polishing column using buZer C (50 mM HEPES and 300 mM NaCl at pH 8.0). Pure WT MbNHase was concentrated using an Amicon Ultra-15 (Millipore) and analyzed by SDS-PAGE with a 12.5% polyacrylamide SPRINT NEXT GEL (Amresco). Gels were stained with Gel Code Blue (Thermo-Fisher ScientiWc). The protein concentration of puriWed WT MbNHase was determined by measuring the absorbance at 280 nm on a Shimadzu UV-2450 spectrophotometer. The calculated molecular weight for the WT MbNHase homodimer is 111,207 g/mol with an extinction coefWcient of 143,700 $cm^{-1} M^{-1}$. The molecular weight is in good agreement with SDS-PAGE data [11].

2.3. Expression and puri?cation of MbNHase mutants

The genes encoding for the MbNHase - and -subunits, where the entire (His) $_{17}$ motif was removed (MbNHase $^{238\ 257}$), were synthesized by Integrated DNA Technologies, Inc. with optimized E. coli codon usage and cloned into the kanamycin resistant pET28a $^{+}$ (EMD Biosciences) plasmid. The - and -subunit genes were designed for co-expression by overlapping the TGA stop codon of the -subunit with the ATG start codon of the subunit (Figure SI-1), like the expression of PtNHase [16]. In addition, the - and -subunit genes where the entire insert region was deleted (MbNHase $^{219\ 272}$), were obtained by polymerase chain reaction (PCR) using the primers (Sense primer: CAACCATGGGTACCG AGCAGGCGGCGGTG; Anti-sense primer: CATAAGCTTTTAGTGGTGGTGGTGATGATGATCAACACGCGGCAG) for the -subunit and (Sense primer: ACG CATATG ATGCACCTGT TCA CCTACGACCTGCA; Anti-sense primer: AGTCCTCGAGTTACGCTTG CGGCGGGTTGCTA) for the -subunit. The -subunit gene was sub-cloned between NcoI and HindIII sites, while the -subunit gene was sub-cloned between NdeI and XhoI sites within the pCOLADuet-1 expression vector (Novagen). The sequences were conWrmed by Functional Biosciences. The plasmids containing the MbNHase $^{238\ 257}$ and MbNHase $^{219\ 272}$ deletion mutant genes were transformed into BL21 magic cells for the soluble expression of the MbNHase $^{238\ 257}$ and MbNHase $^{219\ 272}$ enzymes. Expression and puriWcation of these mutant enzymes were carried out in an identical manner to that described above for WT MbNHase [11].

2.4. Expression and puri?cation of WT MbNHase and the mutants in the absence or presence of PtNHase^{act}

The gene encoding the Co-type activator ( ) protein from Pseudonocardia thermophila JCM 3095 (PtNHase^{act}) was synthesized by Integrated DNA Technologies, Inc. with optimized E. coli codon usage and cloned into the kanamycin resistant pET21a^{+} (EMD Biosciences) expression vector to create the pSPT ^{act} plasmid. The pSPT^{act} plasmid was co-expressed with the previously reported pSMM plasmid encoding recombinant MbNHase [11] that had been freshly transformed into BL21(DE3) (Stratagene) competent cells. In addition, the pSPT^{act} plasmid was co-expressed with the plasmids containing the MbNHase $^{238\ 257}$ and MbNHase $^{219\ 272}$ deletion mutant genes in BL21 magic cells. Expression and puriWcation of WT MbNHase,

MbNHase $^{238\,257}$ and MbNHase $^{219\,272}$ co-expressed with PtNHase^{act} was carried out in an identical manner to that described above for WT MbNHase [11].

2.5. Kinetic analysis

The enzymatic activity of WT MbNHase and each variant was determined using acrylonitrile as the substrate (acrylamide; $_{225}$=2.9 mM ^{1}cm ^{1}). The rate of nitrile hydration was determined by continuously monitoring the formation of acrylamide at 225 nm using a Shimadzu UV-2450 spectrophotometer equipped with a TCC-240A temperature controlled cell holder [19]. A typical 1 mL reaction consisted of 50 mM Tris-HCl buZer pH 7.0 at 25 °C and various concentrations of acrylonitrile (0 450 mM). One unit (U) of MbNHase activity is deWned as the formation of 1 mol of acrylamide per minute. To obtain the kinetic parameters, V_{max} and K_m, the initial velocities from at least three independent measurements were Wtted to the Michaelis-Menten equation using OriginPro 9.0 (OriginLab, Northampton, MA). Kinetic data for each MbNHase variant were determined more than three times for multiple puriWcations, all of which provided consistent results.

2.6. Metal analysis

The metal content of WT MbNHase and each variant, expressed in the presence and absence of $CoCl_2$, was determined by inductively-coupled plasma mass spectrometry (ICP-MS). For comparison purposes, the Co-type NHase from Pseudonocardia thermophila JCM 3095 (PtNHase) was expressed and puriWed as previously described [20], and the metal content determined along with a buZer control that contained no protein. All protein samples were pretreated with 1 M urea and digested with concentrated nitric acid (0.863 mL) followed by heating at 70 °C for 1 h, allowed to cool to room temperature, and then diluted to a Wnal 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 and electron paramagnetic resonance spectra

Electronic absorption spectra were recorded on a Shimadzu UV-2450 spectrophotometer equipped with a TCC-240A temperature-controlled cell holder. Spectra for WT MbNHase and each variant as well as PtNHase were obtained at 25 °C in a 1 cm quartz cuvette in 50 mM HEPES buZer containing 300 mM NaCl, pH 8.0. X-band EPR spectra for WT MbNHase were recorded at 4 K, 0.1 mW in 50 mM HEPES buZer containing 300 mM NaCl, pH 8.0 on a Bruker EMX TDU/L-upgraded spectrometer equipped with an ER4112 SHQ (9.47 GHz) resonator, a Cold-Edge/Bruker RDK-408 4K Stinger recirculating cryocooler, an Oxford Instruments ESR900 helium Xow cryostat, and Oxford Instruments Mercury iTC temperature controller.

3. Results and discussion

3.1. Proteolytic cleavage of WT MbNHase

Active, mature MbNHase was obtained when expressed in the absence of an ( ) protein or the E. coli GroES/EL molecular chaperones [11] [13], whereas prokaryotic NHase activation appears to absolutely require an activator protein [14 16]. These Wndings beg the question: how is MbNHase functionally expressed? We hypothesized that the insert region, which contains a His $_{17}$ section (Fig. 1), plays a key role in metallocentre assembly and therefore, created three altered MbNHase enzymes targeting this insert region.

PuriWed WT MbNHase exhibits a single band at 55 kDa on SDS-PAGE (Fig.2) and a k_{cat} value of 131 ± 3 s ^{1}(K_m=83±10mM) in 50 mM Tris-HCl buZer pH 7.0 at 25 °C using acrylonitrile as the substrate, values that are indistinguishable from those reported previously (Table 1) [11]. Size-exclusion chromatography revealed that WT MbNHase exists primarily as an ( )$_2$ homodimer in solution, analogous to the $_2$ $_2$ heterotetramer architecture observed for prokaryotic NHases. However, storage of WT MbNHase at 4 °C in 50 mM Tris-HCl buZer, pH 7.0 for approximately two weeks, provided an MbNHase enzyme with a modestly increased k_{cat} value of 163 ± 4 s ^{1} but a similar

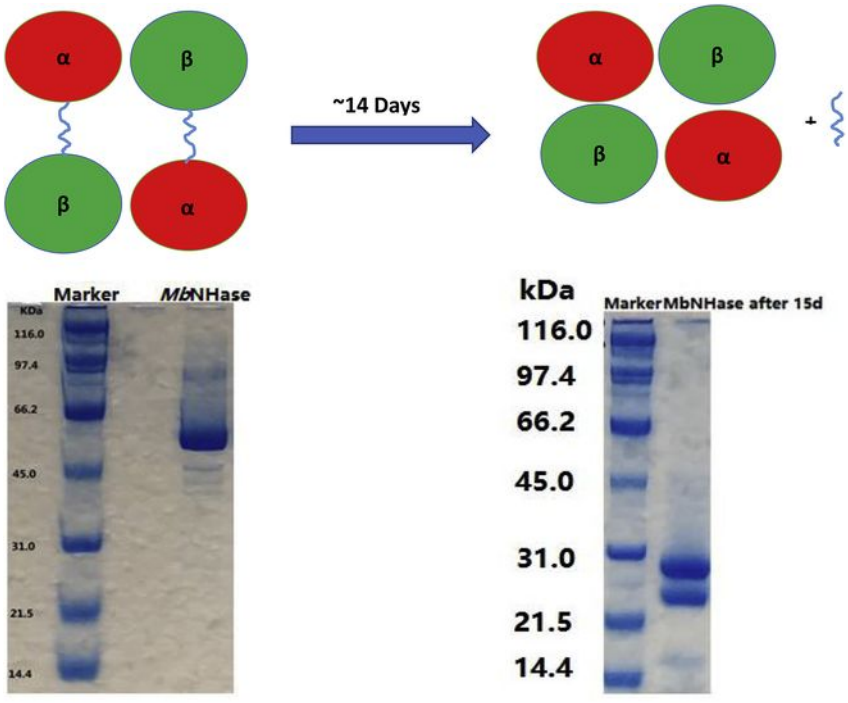

Fig. 2. The SDS-PAGE of (a) WT MbNHase and (b) the proteolytically cleaved MbNHase enzyme.

Table 1
Kinetic constants for the wild-type and mutant MbNHases.[a]

	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}mM^{-1}$)	Metal Content
Wild-type[b]	131 ± 3	83 ± 10	1.6	1.8 ± 0.1
Cleaved	163 ± 4	93 ± 15	1.8	1.9 ± 0.1
238-257	71 ± 4	104 ± 17	0.7	1.8 ± 0.1
238-257 + PtNHase[act]	166 ± 5	125 ± 17	1.3	2.3 ± 0.2
219-272	75 ± 8	73 ± 14	1.0	1.7 ± 0.1
219-272 + PtNHase[act]	117 ± 10	149 ± 23	0.8	1.7 ± 0.1

[a] Acrylonitrile was used as the substrate.
[b] Reference [11].

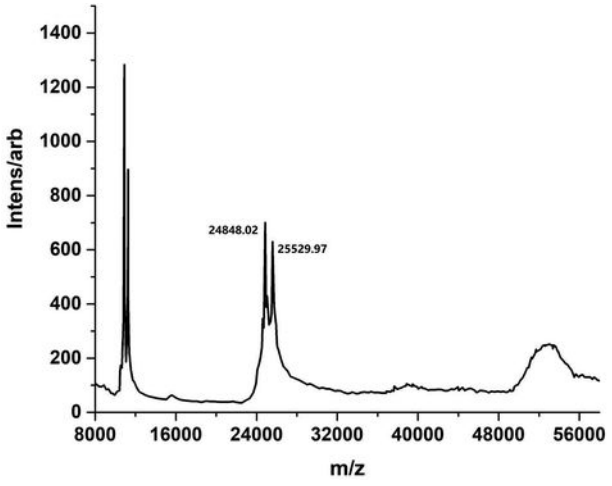

Fig. 3. MALDI-TOF Mass Spectra of proteolytically cleaved MbNHase revealing two peaks at 24.8 and 25.5 kDa corresponding to independent - and -subunits.

K_m value of 93 ± 15 mM, using acrylonitrile as the substrate (Table 1). SDS-PAGE analysis of aged MbNHase samples revealed two polypeptides of 24 and 26 kDa (Fig. 2), while size exclusion chromatography suggested an $_2$ $_2$ heterotetramer, identical to that observed for prokaryotic NHases. These data suggested that WT MbNHase is cleaved, likely by trace amounts of proteases. The observed increase in k_{cat} for the proteolytically cleaved MbNHase enzyme, represents a 20% increase in rate over WT MbNHase. The increased rate was very reproducible for batch-to-batch preparations, providing the calculated error of ±4 s^{-1}. The observed increase in activity is perhaps due to an easing of conformational stress induced by the insert region in and around the active site, although structural characterization will be required to conWrm this.

ConWrmation of an $_2$ $_2$ heterotetramer after proteolytic cleavage of WT MbNHase was obtained by MALDI-TOF mass spectroscopy (Fig. 3). Two masses were clearly observed at 24,848 Da and 25,530 Da, values that are consistent with SDS-PAGE estimates (Fig. 2) and correspond to the - and -subunits of MbNHase based on sequence comparison with the prototypical Co-type PtNHase enzyme (Fig. 4). The two MALDI-TOF MS peaks were of similar intensities, indicating a 1:1 : ratio. Interestingly, the observed molecular masses from MALDI-TOF MS suggests that a 5226 Da peptide is lost after proteolytic cleavage, comparable to the size of the complete insert region of WT MbNHase. Attempts to isolate the cleaved fragment by gel-Wltration were unsuccessful suggesting that the fragment is likely cleaved into small peptides by protease contaminants. Cleavage of MbNHase could be prevented by an additional gel-Wltration puriWcation step performed directly after IMAC; however, the addition of metal ion inhibitors such as EDTA or 1,10-phenanthroline had no effect on MbNHase cleavage. Addition of protease inhibitor cocktails, such as AEBSF, resulted in precipitation of MbNHase. Taken together, these data indicate that the single polypeptide of freshly isolated WT MbNHase, which contains fused - and -subunits linked by an insert region, is cleaved into separate - and -subunits upon ageing, likely by trace amounts of proteases, resulting in a modestly more active $_2$ $_2$ heterotetrameric form of MbNHase.

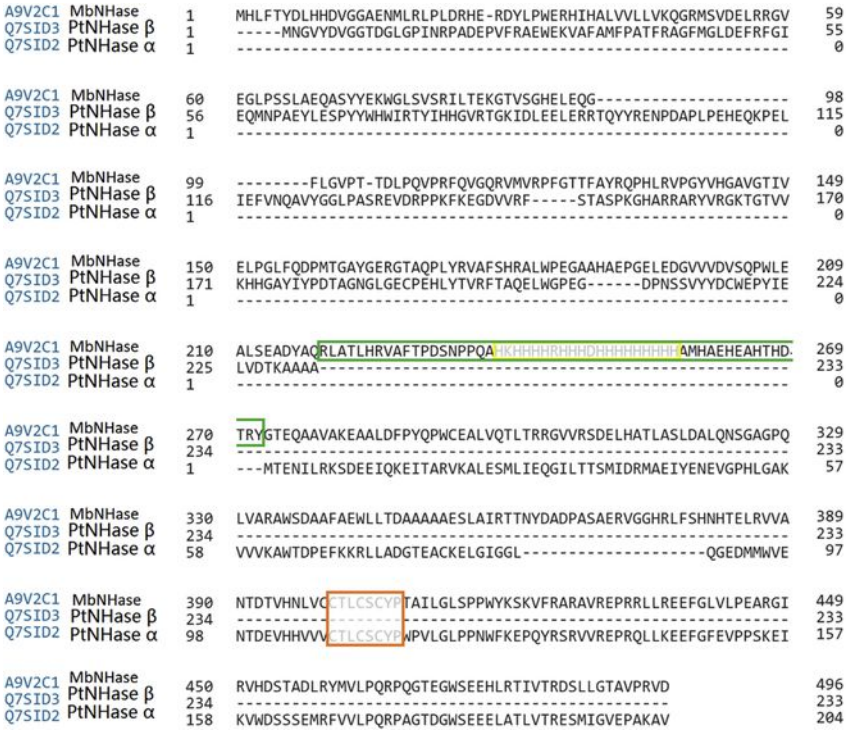

Fig. 4. Sequence alignment of MbNHase and PtNHase shows that the -subunits share 23% identity while the -subunits exhibit 32% identity (Orange: active site; green: insert region; yellow: histidine rich region). (For interpretation of the references to color in this Wgure legend, the reader is referred to the Web version of this article.)

A combination of UV Vis and EPR spectroscopy coupled with metal analyses was used to determine that the proteolytically cleaved MbNHase enzyme contained its full complement of Co(III). ICP-MS data indicated that the proteolytically cleaved MbNHase enzyme contained 1.9 ± 0.1 equivalents of cobalt per $_{2\ 2}$ heterotetramer, indistinguishable from WT MbNHase (Table 1) with no other metal ions detected above the background level of <10 ppb [11]. Proteolytically cleaved MbNHase exhibited the typical amber color of Co-type NHase enzymes in 50 mM HEPES buZer containing 300 mM NaCl, pH 8.0 (Fig. 5) [1], and the UV Vis spectrum reveals the characteristic S Co(III) ligand-to-metal-charge-transfer (LMCT) band at 315 nm (=2500 M ^{1}cm ^{1}) (Fig. 5), which is blue-shifted by 10 nm compared to WT MbNHase [11]. EPR spectra show no detectable Co(II) signals consistent with the presence of low-spin Co(III), which is diamagnetic (Figure SI-2). These data indicate that proteolytically cleaved MbNHase, retains its full complement of Co(III) and that neither the insert region nor the (His) $_{17}$ motif is required for catalysis in the proteolytically cleaved MbNHase enzyme.

3.2. Examination of the functional role of the (His)$_{17}$ insert

To investigate whether the (His) $_{17}$ region plays a role in metal ion insertion and/or the posttranslational modiWcation of the active site, the MbNHase $^{238\ 257}$ (His)$_{17}$ deletion mutant, was prepared. SDS-PAGE analysis revealed two bands at 25.5 and 28.5 KDa for MbNHase $^{238\ 257}$ while size-exclusion chromatography indicated that MbNHase $^{238\ 257}$ exists primarily as an $_{2\ 2}$ heterotetramer with a molecular weight of 108 kDa, much like prokaryotic Co-type NHases (Fig. 6). Kinetic analyses of the MbNHase $^{238\ 257}$ (His)$_{17}$ deletion mutant, expressed in the absence or presence of the prototypical Co-type activator ( ) protein, PtNHase^{act}, using acrylonitrile as the substrate, were performed in triplicate for multiple puriWcations in 50 mM Tris-HCl buZer, pH 7.0 at 25 °C providing k_{cat} values of 71 ± 4 s ^{1} and 166 ± 5 s ^{1} respectively. K_m values for the MbNHase $^{238\ 257}$ (His)$_{17}$ deletion mutant expressed in the absence or presence of the PtNHase^{act} were 104 ±17mM and 125 ± 17 mM, respectively (Table 1). The MbNHase $^{238\ 257}$ (His)$_{17}$ deletion mutant expressed in the absence or presence of PtNHase^{act} contained 1.8 ± 0.1 and 2.3 ± 0.2 equivalents of cobalt per $_{2\ 2}$ heterotetramer, respectively, with no other metal ions detected above the background level of <10 ppb. The MbNHase $^{238\ 257}$ (His)$_{17}$ deletion mutant expressed in either the ab-

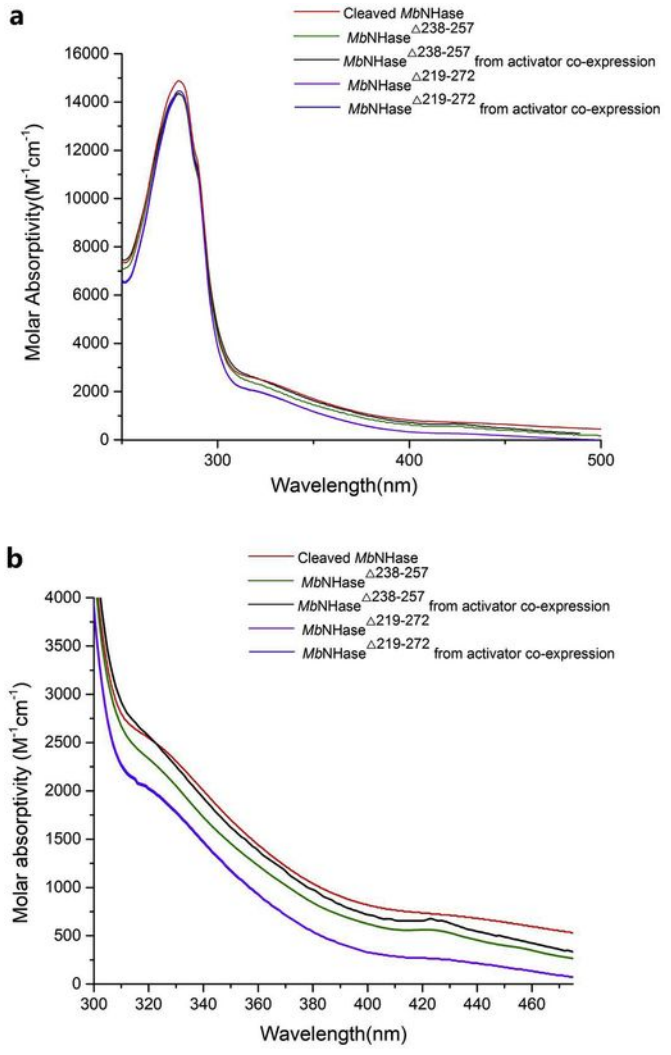

Fig. 5. UV Vis spectra of various MbNHase constructs between (a)280 and 500 nm; (b) 300 and 480 nm in 50 mM HEPES buZer containing 300 mM NaCl at pH 8.0. (b).

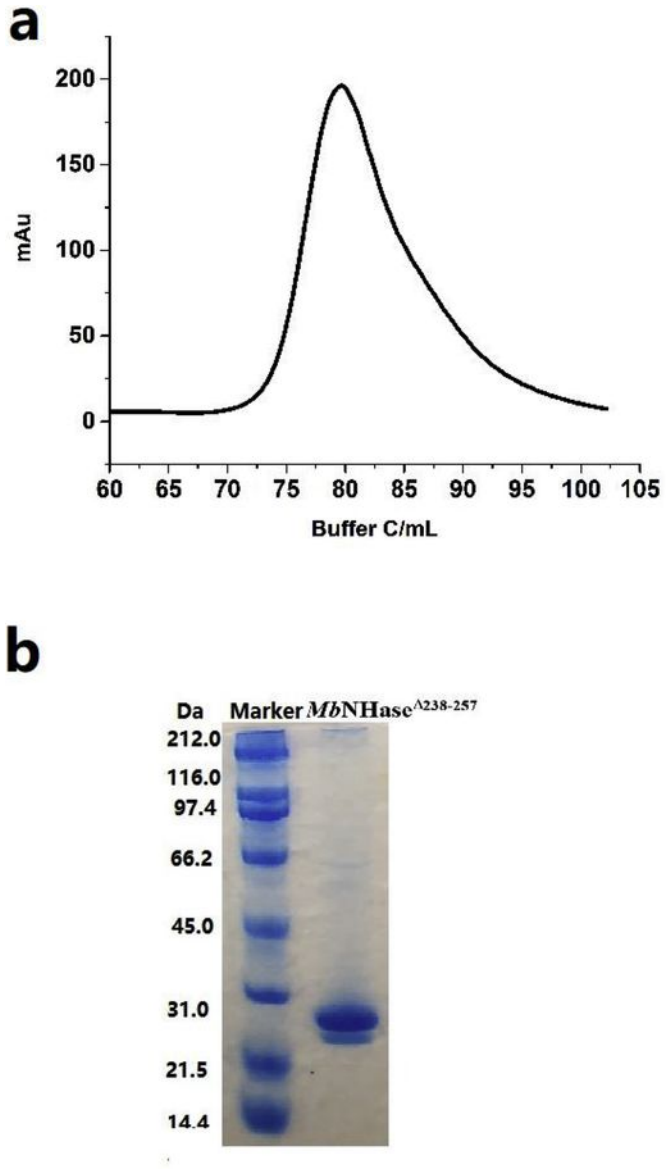

Fig. 6. PuriWcation of the MbNHase $^{238\ 268}$ mutant. a) size-exclusion column indicating a single peak corresponding to an $_{2\ 2}$ heterotetramer; b) SDS-PAGE gel page showing that the puriWed and -subunits are two independent proteins.

sence or presence of PtNHase^{act} in 50 mM HEPES buZer containing 300 mM NaCl, pH 8.0 exhibited the characteristic S Co(III) LMCT band at 320 nm (=2200 M ^{1}cm ^{1}) (Fig. 5), nearly identical to WT MbNHase [11]. While the $(His)_{17}$ region is not required for metal uptake or active site maturation, expression in the presence of the prototypical prokaryotic activator protein, PtNHase^{act}, does enhance the observed k_{cat} value by 60%, which is greater than would be expected from the slight increase (20%) in metal content. Therefore, PtNHase^{act} does assist in the activation of the MbNHase $^{238\ 257}$ $(His)_{17}$ deletion mutant but is not required for post-translational modiWcation of the active enzyme.

3.3. Investigation of the insert region of MbNHase

The MbNHase $^{219\ 272}$ insert region deletion mutation provides an enzyme with the entire insert region between the and regions of WT MbNHase removed (Fig. 1). Removal of this insert region essentially converts the eukaryotic MbNHase into a prokaryotic NHase analog, allowing the direct comparison of MbNHase $^{219\ 272}$ and PtNHase. The MbNHase $^{219\ 272}$ insert region deletion mutant expressed in the absence of PtNHase^{act} exhibited two bands on SDS-PAGE at 25.5 and 26.5 kDa. Size-exclusion chromatography indicated that MbNHase $^{219\ 272}$ exists primarily as an $_{2\ 2}$ heterotetramer with a molecular weight of 104 kDa, indistinguishable from proteolytically cleaved WT MbNHase and prokaryotic Co-type NHases such as PtNHase. Surprisingly, co-expression of MbNHase $^{219\ 272}$ in the presence of PtNHase^{act} yielded a single band at 52 kDa on SDS-PAGE which is consistent with an complex. However, it could also be due to the formation of an $(\ )_2$ complex as PtNHase^{act} is 14 kDa so an $(\ )_2$ complex would have a mass of 53.5 kDa. Interestingly, the Co-type ( ) protein from Rhodococcus rhodochrous J1 was shown to form an $(\ )_2$ complex, which was proposed to bind Co(II) and insert it into apo- $_{2\ 2}$ NHase via a self-subunit swapping mechanism [21]. The Co-type ( ) protein was also proposed to facilitate oxidation of two active site Cys-residues. Attempts to separate the 52 kDa proteins into their individual components were unsuccessful even in the presence of 8 M urea, 1 M dichlorodiphenyltrichloroethane (DDT) or SDS at 95 °C for 10 min (Fig. 7).

Kinetic analysis of the MbNHase $^{219\ 272}$ insert region deletion mutant expressed in the absence or presence of PtNHase^{act} in 50 mM Tris-HCl buZer, pH 7.0 at 25 °C provided k_{cat} values of 75 ± 8 s ^{1} and 117 ± 10 s ^{1}, respectively, using acrylonitrile as the substrate (Table 1). These kinetic data suggest that the MbNHase $^{219\ 272}$ insert region deletion mutant expressed in the presence of PtNHase^{act} is likely an complex; even so, the Co-type NHase ( ) proteins are known to have significant sequence identity with the NHase -subunit [22,23], so $(\ )_2$ complex might be expected to be catalytically competent, but not likely more active than an $_{2\ 2}$ heterotetramer such as was observed for the MbNHase $^{219\ 272}$ insert region deletion mutant expressed in the absence of PtNHase^{act}. The MbNHase $^{219\ 272}$ insert region deletion mutant expressed in the absence or presence of PtNHase^{act} is not particularly stable in 50 mM Tris-HCl buZer, pH 7.0 at 25 °C and loses >95% of its activity over the course of a few hours, indicating that the insert region plays a role in stabilizing the WT MbNHase enzyme. The MbNHase $^{219\ 272}$ insert region deletion mutant contained 1.7 ± 0.1 equivalents of cobalt per $_{2\ 2}$ heterotetramer, irrespective of the co-expression of PtNHase^{act}, a value nearly identical to that observed for WT MbNHase (Table 1). UV Vis spectra of the MbNHase $^{219\ 272}$ insert region deletion mutant expressed in the absence or presence of PtNHase^{act} in 50 mM HEPES buZer containing 300 mM NaCl, pH 8.0 were indistinguishable from each other and WT MbNHase (Fig. 5). Therefore, MbNHase $^{238\ 257}$ binds its full complement of Co(III) ions without the assistance of an ( ) protein, indicating that the insert region is also not required for metal ion binding or active site maturation.

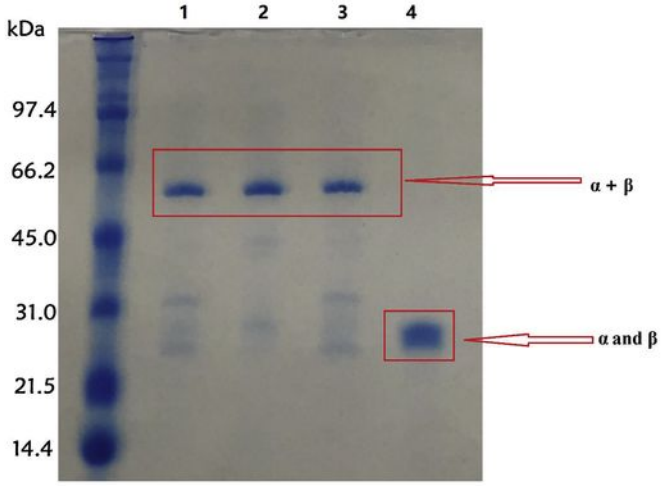

Fig. 7. SDS-PAGE gelof MbNHase $^{219\ 272}$. Column 1: MbNHase $^{219\ 272}$ from activator co-expression; Column 2: MbNHase $^{219\ 272}$ from PtNHase activator co-expression treated by 8 mM Urea; Column 3: MbNHase $^{219\ 272}$ from activator co-expression treated by 8M urea and 1M DDT; Column 4: MbNHase $^{219\ 272}$ without activator.

4. Conclusion

Characterization of the eukaryotic NHase from Monosiga brevicollis constructs described herein, conWrm that MbNHase does not require an NHase activator protein nor the E. coli chaperone proteins GroEL/ES for metallocentre assembly, including metal ion insertion and active site post-translational maturation. In addition, these data indicate that the $(His)_{17}$ region found within the insert that links the - and -subunits is not required for metal ion incorporation or active site maturation. The fact that the proteolytically cleaved WT MbNHase enzyme and the MbNHase $^{238\ 257}$ $(His)_{17}$ deletion mutant each exhibit a modest increase in activity compared to WT MbNHase, suggests that the insert region likely induces a structural strain on the active site that has a limiting effect on the catalytic rate of hydration. The lack of the need for either an intrinsic or an extrinsic activator polypeptide for MbNHase is in stark contrast to the absolute requirement for an activator for assembly and activation of the otherwise similar prokaryotic NHases. The pertinent and related outstanding questions are, How can the MbNHase metallocentre self-assemble and self-activate?" and, Why do the prokaryotic NHase metallocenters require activators for assembly and activation? The genetically engineered functional constructs of MbNHase described herein represent an important new tool with which to further address these important questions using structural and spectroscopic methods.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.abb.2018.08.013.

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