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ORIGINAL PAPER

The Fe-type nitrile hydratase from *Rhodococcus equi* TG328-2 forms an alpha-activator protein complex

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Abstract

Abstract An Fe-type nitrile hydratase $\alpha(\varepsilon)$ protein complex from *Rhodococcus equi* TG328-2 (*Re*NHase) was discovered and shown by MALDI-TOF to form a 1:1 complex. As isolated, the $\alpha(\varepsilon)$ protein complex exhibited no detectable NHase activity even in the presence of iron. The addition of the *Re*NHase β-subunit and Fe(II) to the *Re*NHase apo-α(ε) complex, provided an enzyme with a k_{cat} value of 0.7 ± 0.1 s^{-1} using acrylonitrile as the substrate, indicating that the β-subunit is important for the reconstitution of NHase activity. The addition of the reducing agent TCEP enhanced the activity by more than 50% (k_{cat} of 1.7 ± 0.2 s^{-1}). As the (ε) protein was previously shown to bind and hydrolyze GTP, the addition of GTP to the as-purified $\alpha(\varepsilon)$ complex provided a k_{cat} value of 1.1 ± 0.2 s^{-1}, in the presence of Fe(II) and β-subunit. The addition of TCEP to this combination further enhanced the activity (k_{cat} of 2.1 ± 0.3 s^{-1}). Apo α-subunit was expressed in purified and added to the (ε) protein and β-subunits plus Fe(II) and TCEP resulting in a k_{cat} value of 0.7 ± 0.2 s^{-1} suggesting an $\alpha(\varepsilon)$ complex can form in vitro. The addition of GTP to this sample increased the observed rate of nitrile hydration by ~ 30%, while TCEP free samples exhibited no activity. Taken together, these data provide insight into the role of the (ε) protein and the newly discovered $\alpha(\varepsilon)$ complex in NHase metallocenter assembly.

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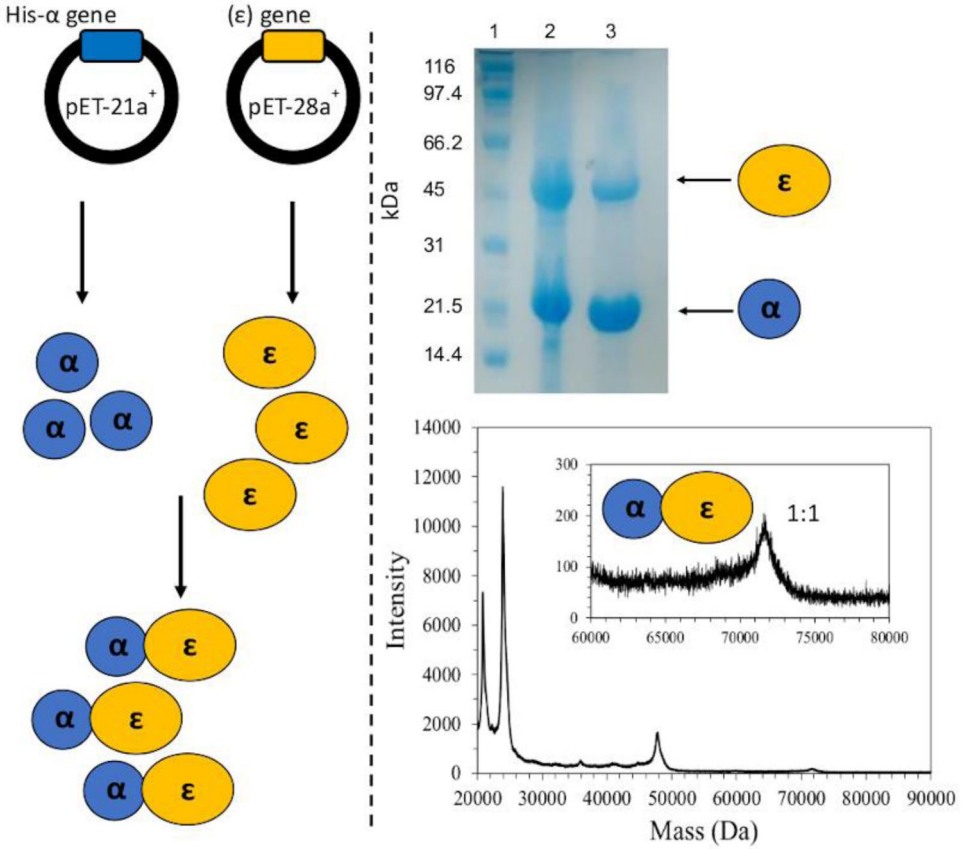
Graphic abstract



Keywords Nitrile hydratase · Iron · Hydration · Iron trafficking · Enzyme kinetics

Introduction

Nitrile hydratases (NHases, EC 4.2.1.84) are metalloenzymes that catalyze the hydration of nitriles to their corresponding higher value amides under mild conditions (room temperature and physiological pH) [1, 2]. X-ray crystallographic studies indicate that they are $\alpha_2\beta_2$ heterotetramers with an active site consisting of three cysteine residues, two amide nitrogens, a water molecule, and either a low-spin, non-heme Fe(III) ion (Fe-type) or a low-spin, non-corrin Co(III) ion (Co-type) [3, 4]. Two of the active site cysteine residues are post-translationally modified to cysteine-sulfinic acid ($-SO_2H$) and cysteine-sulfenic acid (–SOH) yielding an unusual metal coordination geometry, termed a "claw-setting." The protonation states of the active site equatorial sulfenic and sulfinic acids were suggested to be Cys-SOH and Cys-SO_2^- based on sulfur K-edge XAS and geometry-optimized DFT calculations [5]; oxidation of the equatorial Cys residues is essential for catalysis [6]. While the X-ray crystal structures of Fe- and Co-type NHases are very similar, Fe-type NHases are specific for Fe(III) while Co-type NHases are specific for Co(III) [3].

Several open reading frames (ORFs) have been identified just downstream from the structural α- and β-subunit genes in both Co- and Fe-type NHases, and one of these genes has been proposed to function as an activator (ε) protein [7–9]. Current data indicate that both Co- and Fe-type NHase enzymes typically require the co-expression of an (ε) protein to be fully metallated, post-translationally modified, and functional [7–9]. While Co- and Fe-type NHases have high α- and β-subunit sequence similarity, their respective (ε) proteins differ in size and share little to no sequence identity, suggesting that their mechanism of metallocenter assembly is different [10–12]. Co-type NHase (ε) proteins are small (~ 15 kDa) and have significant sequence identity with the NHase β-subunit [13, 14]. The Co-type (ε) protein from *Rhodococcus rhodochrous* J1 was shown to form an $\alpha(\varepsilon)_2$ complex, which was proposed to bind Co(II) and insert it into apo-$\alpha_2\beta_2$ NHase via a "self-subunit swapping" mechanism [12]. The Co-type (ε) protein was also proposed

to facilitate oxidation of two active site Cys-residues. On the other hand, Fe-type NHase (ε) proteins are large (~45 kDa) and contain a highly conserved cysteine-rich (CXCC) motif that is a known metal binding site in other metallochaperones such as COX17 (copper) and the Hyp (nickel) proteins [6, 15]. While Fe-type NHase (ε) proteins may form an $\alpha(\varepsilon)_x$ complex and insert Fe(II) into the α-subunit, similar to Co-type (ε) proteins, no data exists to support this hypothesis.

Recently, an Fe-type NHase (ε) protein was expressed and characterized revealing that it is a member of the COG0523 subfamily of G3E P-loop GTPases and that GTPase activity is regulated by metal binding [16, 17]. Since the role of the Fe-type (ε) protein in NHase metallocenter assembly is directly related to NHase structure and function, investigating this process will identify aspects of metallocenter assembly that are essential for catalysis. Herein we describe the formation of an $\alpha(\varepsilon)$ protein complex for the Fe-type (ε) protein from *Rhodococcus equi* TG328-2 (*Re*NHase TG328-2). Kinetic data reveal that the addition of GTP and the *Re*NHase TG328-2 β-subunit under reducing conditions, results in NHase activity establishing the involvement of both the Fe-type (ε) protein and GTP in the maturation of Fe-type NHase enzymes.

Materials and methods

Materials

Guanosine triphosphate (GTP), isopropyl-β-D-1-thiogalactopyranoside (IPTG), tris(2-carboxyethyl)phosphine (TCEP), *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid (HEPES), 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride (Tris–HCl), and acrylamide were purchased from Sigma-Aldrich or Fisher scientific as the highest purity available. NEB and BL21 (DE3) competent *E. coli* cells were obtained from Agilent Technologies and plasmid preparation kits were purchased from Promega. All other reagents were purchased commercially and were the highest purity available.

Plasmid construction

The plasmid expressing the Fe-type nitrile hydratase from *Rhodococcus equi* TG328-2 (*Re*NHase TG328-2) was kindly provided by Professor Uwe Bornscheuer [18]. The original plasmid had the NHase α, β, and activator genes in tandem. Therefore, separate pET-28a$^+$ plasmids containing only the His_6-tagged *Re*NHase TG328-2 α-subunit and the His_6-tagged *Re*NHase TG328-2 β-subunit genes were synthesized by Genscript, while the pMCSG9 plasmid containing the His_6-MBP-*Re*NHase TG328-2 (ε) protein with a Tobacco Etch Virus (TEV) protease cleavage site between MBP and the *Re*NHase TG328-2 (ε) protein, as previously described, was used to express the *Re*NHase TG328-2 (ε) protein [16]. All plasmid sequences were confirmed using automated DNA sequencing at the Functional Biosciences DNA sequencing facility.

Expression of recombinant ReNHase TG328-2 α, β and (ε) proteins

The pMCSG9 plasmid containing the His_6-MBP-TEV-*Re*NHase TG328-2 (ε) gene was transformed into BL21 magic cells for the soluble expression of the *Re*NHase TG328-2 (ε) protein. Similarly, individual expression systems for the *Re*NHase TG328-2 α- and β-subunits were prepared by transforming the pET-21a$^+$ plasmids containing the His_6-*Re*NHase TG328-2 α-subunit gene and the His_6-*Re*NHase TG328-2 β-subunit gene into BL21 (DE3) cells. A single colony of each transformation was typically used to inoculate separate 50 mL flasks of LB Miller culture containing 50 μg/mL of kanamycin and allowed to grow at 37 °C with constant shaking overnight. These cultures were used to inoculate 6 L of LB Miller culture containing kanamycin (50 μg/mL) and ampicillin (100 μg/mL). Cells were allowed to grow at 37 °C with constant shaking until an optical density of ~0.8–1.0 at 600 nm was reached. In each case, the culture was cooled to 18 °C and induced with 0.1 mM Isopropyl-β-D-1- thiogalactopyranoside (IPTG) and shaken for an additional 16 h at 18 °C. Cells were pelleted by centrifugation at 5000×*g* for 10 min and resuspended in 50 mM phosphate buffer at pH 7.5 (500 mM NaCl, 50 mM imidazole, and 10% glycerol) for *Re*NHase TG328-2 α- and β-proteins and 50 mM Tris buffer at pH 7.4 (200 mM NaCl) for the *Re*NHase TG328-2 (ε) protein. All cell paste was stored at − 80 °C until needed.

Purification of recombinant ReNHase TG328-2 (ε) protein

Cells containing the His_6-MBP-TEV-*Re*NHase TG328-2 (ε) plasmid were lysed by ultrasonication (Misonix Sonicator 3000) in 30 s increments for 4 min at 21 W. Cell lysate was separated from cell debris by centrifugation for 40 min. at 10,000×*g*. The supernatant was loaded onto two, pre-equilibrated 5 mL MBP-Trap columns (GE Healthcare) and washed with 20 column volumes of 20 mM Tris–HCl buffer at pH 7.5 containing 200 mM NaCl. *Re*NHase TG328-2 (ε) protein was eluted with a 10 mM maltose gradient in 20 mM Tris–HCl buffer at pH 7.5 containing 200 mM NaCl. Fractions containing *Re*NHase TG328-2 (ε)-MBP-TEV-His_6 were pooled and the buffer exchanged into 50 mM Tris–HCl buffer at pH 7.5 containing 10% glycerol (0.5 mg/mL). His_6-TEV protease (total protein: TEV (w/w) = 50:1) and 10 mM tris(2-carboxyethyl)phosphine (TCEP) was added

to the protein sample and stirred gently for ~ 36 h at 4 °C. The sample containing cleaved *Re*NHase TG328-2 (ε) protein was concentrated to 5 mL and loaded onto two 5 mL MBP-Trap columns (GE Healthcare) connected to an ÄKTA Prime Plus FPLC system that was previously equilibrated with 20 mM Tris–HCl buffer at pH 7.5 containing 10% glycerol and 5 mM TCEP. The cleaved *Re*NHase TG328-2 (ε) protein was collected in the flow through and concentrated. The protein was then buffer exchanged into 50 mM sodium phosphate buffer at pH 7.5 containing 10% glycerol and 5 mM TCEP to a total volume of 5 mL and loaded onto a pre-packed 5 mL immobilized-metal affinity chromatography (IMAC) nickel-nitrilotriacetic acid (Ni–NTA) column (QIAGEN) connected to an ÄKTA Prime Plus FPLC system that was previously equilibrated with 50 mM sodium phosphate buffer at pH 7.5 containing 10% glycerol, 5 mM TCEP. The *Re*NHase TG328-2 (ε) protein was collected in the flow through and shown to be pure via SDS-PAGE (12.5%).

EDTA treatment of recombinant ReNHase TG328-2 (ε) protein

Purified *Re*NHase TG328-2 (ε) protein was treated with 10 mM EDTA in 50 mM HEPES buffer, pH 7.5 containing 20 mM TCEP and incubated overnight under nitrogen at 4 ˚C. The EDTA and TCEP were removed by dialysis against Chelexed 50 mM HEPES buffer, pH 7.5. The metal-free *Re*NHase TG328-2 (ε) protein was concentrated and the protein concentration determined using a Bradford assay. Samples were frozen at − 80 °C until needed.

Purification of the ReNHase TG328-2 α- and β-proteins

Cells were lysed by ultra-sonication using a microtip (30 s on and 45 s off) over 8 min at 21 W. The supernatant was separated from the cell debris by centrifugation at 4 °C for 40 min. at 10,000×*g*. Based on SDS-PAGE (12.5%) analysis, both the α- and β-proteins expressed as inclusion bodies. Therefore, the pellet proteins were re-suspended in 4 M urea and centrifuged at 4 ˚C and 10,000×*g* for 40 min. The supernatant was dialyzed at 4 °C against 50 mM HEPES buffer, pH 7.5 containing 2 M urea using a 10 kDa cutoff dialysis bag. Successive dialysis steps were undertaken at 4 °C against 50 mM HEPES buffer, pH 7.5 containing 1 M, 0.5 M urea and no urea, respectively, with the dialysis buffer exchanged at least three times every 3 h.

The solubilized α- and β-proteins were loaded individually onto pre-equilibrated 5 mL IMAC-Ni–NTA columns. The columns were washed with 10 column volumes of 50 mM sodium phosphate buffer at pH 7.5 (300 mM NaCl, and 10 mM imidazole). Both the His_6-tagged α- and β-proteins were eluted with a linear gradient (0–100%) of a high imidazole content buffer (50 mM NaH_2PO_4 pH 7.5, 300 mM NaCl, 10% glycerol, 200 mM imidazole) at a flow rate of 1 mL/min. The fractions containing protein were concentrated and shown to be soluble and pure by SDS-PAGE (12.5%). Samples were frozen at − 80 °C until needed.

Purification of an ReNHase TG328-2 α(ε)$_x$ complex

Cells were lysed by ultra-sonication using a microtip (30 s on and 45 s off) over 4 min at 21 W. Cell lysate was separated from cell debris through centrifugation at 4 °C for 40 min. at 10,000×*g*. Protein was purified from the re-suspended pellet using 4 M urea as the target protein was found with the cell debris. The resulting solution was centrifuged at 4 for 40 min. at 10,000×*g*. The supernatant was dialyzed using a 10 kDa cutoff dialysis bag at 4 °C. The first dialysis step was done in 50 mM HEPES buffer at pH 7.5 containing 2 M urea. The second and third steps were done with 1 M and 0.5 M urea in 50 mM HEPES buffer at pH 7.5, respectively. The supernatant was dialyzed an additional three times in urea free 50 mM HEPES buffer at pH 7.5. The sample to buffer ratio was 100: 1 at each dialysis step and the dialysis buffer was changed every 16 h.

Dialyzed supernatant was loaded onto a pre-equilibrated 5 mL IMAC-Ni–NTA column. The column was washed with 10 column volumes of 50 mM sodium phosphate buffer at pH 7.5 (300 mM NaCl, 40 mM butyric acid, and 10 mM imidazole). His_6-tagged protein was eluted using 10 column volumes of 50 mM sodium phosphate buffer at pH 7.5 (300 mM NaCl, 40 mM butyric acid, and 200 mM imidazole). The eluted sample was concentrated and analyzed by SDS-PAGE (12.5%).

Another sample was purified under denaturing conditions by loading solubilized protein onto a pre-packed 5 mL IMAC-Ni–NTA column that had been equilibrated with 4 M urea and 50 mM HEPES buffer at pH 7.5. Purification was performed using an ÄKTA Prime Plus FPLC system. The column was washed with 10 column volumes of 50 mM HEPES buffer at pH 7.5 containing 4 M urea. The urea concentration was gradually decreased to 0% by increasing the percentage of urea free 50 mM HEPES buffer at pH 7.5 over 5 column volumes. The column was then washed with 5 column volumes of 50 mM HEPES buffer at pH 7.5. His_6-tagged protein was eluted using 50 mM sodium phosphate buffer at pH 7.5 containing 300 mM NaCl, 40 mM butyric acid, and 200 mM imidazole. Fractions were collected and analyzed by SDS-PAGE (12.5%).

MALDI TOF analysis of the ReNHase TG328-2 α(ε)$_x$ complex

Matrix-assisted laser desorption/ionization time of flight (MALDI TOF) mass spectroscopy (MS) was utilized to

examine the *Re*NHase TG328-2 $\alpha(\varepsilon)_x$ complex in 50 mM HEPES, pH 7.5 (1 mg/mL). The matrix consisted of α-cyano-4-hydroxycinnamic acid (α-CHCA) dissolved in a minimal amount of acetone, which was applied to the MALDI target (0.5 µL). Once dried, 0.5 µL of a 1 mg/mL *Re*NHase TG328-2 $\alpha(\varepsilon)_x$ complex protein solution was applied along with a calibrant spot containing albumin followed by another 0.5 µL of α-CHCA matrix solution. Samples were dried under air and then MALDI TOF MS spectra were recorded on a Voyager-DE™ PRO BioSpectrometer (EVISA).

In vitro *activation of ReNHase TG328-2*

The purified *Re*NHase TG328-2 α-subunit in 50 mM HEPES buffer, pH 7.5 was mixed with Fe(II), Fe(II) + activator, or Fe(II) + activator + GTP in the absence and presence of TCEP anaerobically (Table 1). Each of the previous combinations was also mixed with purified β-subunit (Table 1). Samples prepared in the presence of TCEP were incubated for ~ 36 h at 4 °C, after which TCEP, GTP, and unbound Fe(II) was removed by buffer exchange using a 10 kDa Amicon ultra centrifugal filtration unit. The enzymatic activity of each combination towards acrylonitrile (acrylamide; $\Delta\varepsilon_{225} = 2.9\ mM^{-1}\ cm^{-1}$) was measured using a Shimadzu UV-2450 spectrophotometer. A 1 mL reaction consisted of 50 mM HEPES buffer, pH 7.5 at 25 °C, 80 mM acrylonitrile and 1 µM *Re*NHase TG328-2 α-subunit. 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.

Metal content

The metal content of each purified protein was determined by inductively-coupled plasma mass spectrometry (ICP-MS). Each protein (1 mg) was incubated overnight in 8 M urea followed by digestion with a mixture of 2% HNO_3 and 0.5% HCl. After 24 h, the digested protein samples were filtered using 0.2 µM syringe filters and submitted, along with a control of buffer containing no protein, for analysis at the Water Quality Center in the College of Engineering at Marquette University (Milwaukee, WI, USA).

Table 1 ICP-MS metal analysis data for the apo-α-subunit and apo-$\alpha(\varepsilon)_x$ complex

Protein	Protein: Fe mole ratio
apo-α-subunit	1: 0.06
apo-α(ε) complex	1: 0.12

Electronic absorption spectra

All electronic absorption spectra were recorded on a Shimadzu UV-2600 spectrophotometer equipped with a TCC-240A temperature-controlled cell holder. Spectra were obtained at 25 °C in a 1 cm quartz cuvette in 50 mM HEPES buffer, pH 7.5.

Results and discussion

The prevailing dogma is that both Co- and Fe-type NHase enzymes require the co-expression of the α- and β-subunits with an (ε) protein to be fully metallated, post-translationally modified, and functional [19–21]. While (ε) proteins may indeed dictate the identity of the metal ion in Co-type NHases and participate in the controlled oxidation of the two equatorial Cys residues, no biochemical or structural data exist to support this hypothesis in Fe-type NHases. Recently, the Fe-type *Re*NHase (ε) protein was heterologously overexpressed and purified [16]. These data established, for the first time, that the *Re*NHase (ε) protein is a member of the COG0523 subfamily of G3E P-loop GTPases and that GTPase activity is regulated by metal binding. However, two significant questions remain unanswered: (i) does an $\alpha(\varepsilon)_x$ complex form for Fe-type NHases? and (ii) is GTP hydrolysis connected to iron insertion into the NHase α-subunit?

To determine if an Fe-type (ε) protein α-subunit complex can form, a plasmid that contained only the His_6-tagged α-subunit of *Re*NHase was prepared and co-expressed with untagged WT *Re*NHase (ε) protein. Expression of this plasmid produced inclusion bodies with proteins with MW's at ~ 23 kDa and ~ 46 kDa, corresponding to the NHase α-subunit and the (ε) protein, respectively (Fig. 1a; column 2). The NHase α-subunit and the (ε) protein were solubilized in 4 M urea followed by the step-wise removal of urea via dialysis and purification by IMAC [22]. The resulting, soluble NHase α-subunit and the (ε) proteins were shown to be pure via SDS-PAGE (Fig. 1a; column 3). Since only the α-protein is His-tagged but both proteins purify together on the IMAC column, these data suggest the formation of an $\alpha(\varepsilon)_x$ complex. To test this hypothesis, the solubilized $\alpha(\varepsilon)_x$ complex, in 4 M urea, was applied to an IMAC column. The (ε) protein, which does not contain a His_6-tag, was eluted in the wash buffer while the His_6-taged α-subunit remained bound to the column (Fig. 1b). As the WT *Re*NHase (ε) protein has no His_6-tag, it has no specific affinity towards the IMAC column when denatured and can only remain bound to the column due to complex formation with the NHase α-subunit in the absence of urea. Therefore, these data establish for the first time, that an Fe-type NHase α-subunit can bind to and form a complex with an Fe-type (ε) protein.

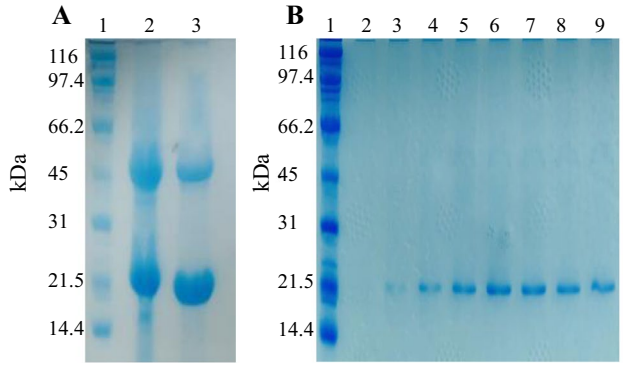


Fig. 1 SDS-PAGE analysis of **a** re-folded protein sample before and after Ni–NTA column purification (lane 1—protein marker, lane 2—re-folded protein before purification, lane 3—re-folded protein after the purification), **b** the eluted α-subunit protein fractions when purified under denaturation conditions (lane 1—protein marker, lane 2–9—eluted fractions at 200 mM imidazole concentration)

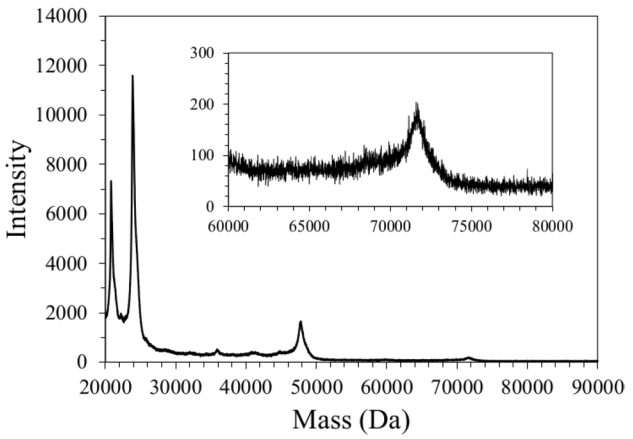


Fig. 2 MALDI TOF analysis of *Re* NHase α-subunit and activator protein (ε) complex

Table 2 Kinetic data for the $\alpha(\varepsilon)_x$ complex in the absence and presence of TCEP at 25 °C

Components	k_{cat} (s^{-1})	k_{cat} (s^{-1}) + TCEP
αε only	ND	ND
$\alpha\varepsilon + Fe^{2+}$	ND	ND
$\alpha\varepsilon + Fe^{2+} + GTP$	ND	ND
$\alpha\varepsilon + \beta + Fe^{2+}$	0.7 ± 0.1	1.7 ± 0.2
$\alpha\varepsilon + \beta + Fe^{2+} + GTP$	1.1 ± 0.2	2.1 ± 0.3

ND none detected

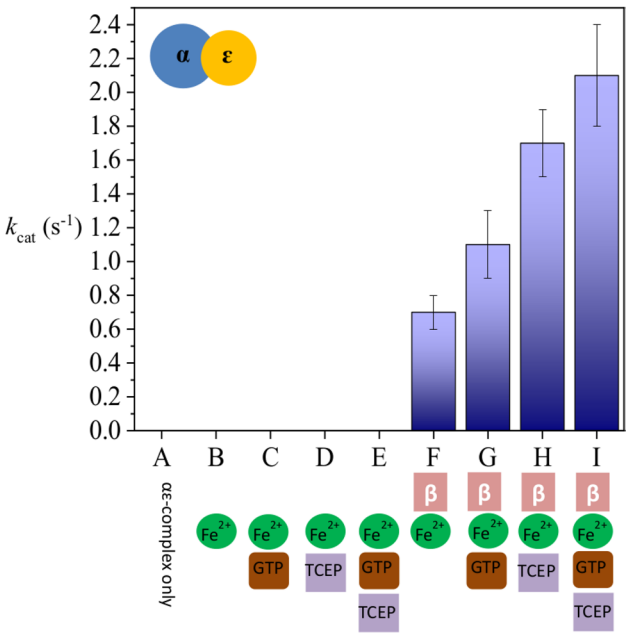


Fig. 3 Observed k_{cat} (s^{-1}) values of the α(ε) complex combined with Fe(II), β-subunit, GTP, or TCEP in 50 mM HEPES, pH 7.5 at 25 °C. All reactions were performed under nitrogen and incubated for 12 h

The Co-type (ε) protein from *Rhodococcus rhodochrous* J1 was shown to form an $\alpha(\varepsilon)_2$ complex, but Co-type NHase (ε) proteins are small (~ 15 kDa), less than half the size of Fe-type (ε) proteins (~ 47 kDa) [12–14]. To determine the molar ratio of the *Re*NHase $\alpha(\varepsilon)_x$ complex, MALDI-TOF MS data were obtained (Fig. 2). Masses were observed at 23,865 Da and 47,769 Da in a good agreement with the theoretical masses of the α-subunit (23,904 Da) and the (ε) protein (47,756 Da). A mass was also observed at 71,593 Da that is nearly identical to that mass expected for an α(ε) complex (71,660 Da). Therefore, these data suggest the existence of an α(ε) protein complex with a 1:1 mol ratio that can be dissociated into the α-subunit and the (ε) protein. Previous spectroscopic and isothermal titration calorimetry data revealed that the *Re*NHase (ε) protein is capable of binding one equivalent of Co(II) or Fe(II) [16]; however, metal analysis on the as-purified Fe-type NHase α(ε) complex indicated that the complex was essentially in the apo-form as it contained only 0.1 equivalent of iron (Table 1). Combination of these data with those for Co-type NHase (ε) proteins, which were shown to form an $\alpha(\varepsilon)_2$ complex that binds Co(II) and inserts it into apo-$\alpha_2\beta_2$ NHase via a "self-subunit swapping" mechanism [12], suggests that the Fe-type NHase (ε) protein can possibly play a similar role.

Having in hand the apo-form of an *Re*NHase α(ε) complex, provides the unique opportunity to investigate if the α(ε) complex can bind Fe(II) and if GTP hydrolysis plays a role in iron insertion into the NHase α-subunit. The as purified apo-α(ε) complex exhibits no detectable NHase activity under standard conditions (50 mM HEPES buffer at pH 7.5 and 25 °C) using 80 mM acrylonitrile as the substrate (Table 2; Fig. 3). As expected, apo-NHases

from *Pseudonocardia thermophila* (Co-type) and *Re*NHase (Fe-type) are inactive highlighting the importance of metal incorporation for NHase activity [23, 24]. Interestingly, purified α-subunit from the trimeric toyocamycin nitrile hydratase (TNHase) from *Streptomyces rimosus* exhibited ~ 0.3% of wild type activity, suggesting that the α-subunit alone is capable of hydrating nitriles [21]. For TNHase, the as-purified α-subunit contained cobalt which is likely why detectable activity is observed. The step-wise addition of Fe(II), the reducing agent TCEP and GTP to the apo-α(ε) complex followed by incubation under nitrogen for 12 h, produced no detectable NHase activity, suggesting that the *Re*NHase α-subunit on its own, unlike TNHase, is not capable of hydrating nitriles or iron was not incorporated into the active site.

Several residues in the β-subunit are known to be important for the NHase activity. For example, βArg56, which is strictly conserved, forms hydrogen bonds with both active site oxidized axial Cys ligands and, when replaced with lysine, NHase is nearly inactive [25, 26]. Similarly, a strictly conserved active site tyrosine (βTyr72), when mutated to Phe, significantly reduced the observed NHase activity [27]. We hypothesized that for Fe-type NHases, both the α and β subunits are required to reestablish catalytic activity. As no β-subunit from any NHase has been independently expressed and purified, a plasmid was constructed that expressed only the His_6-tagged *Re*NHase β-subunit. Similar to the α(ε) complex, the *Re*NHase β-subunit also formed inclusion bodies but was solubilized in 4 M urea followed by the step-wise removal of urea via dialysis and purification by IMAC (Figure S1) [22]. The as-purified β-subunit exhibited no detectable NHase activity, under standard assay conditions using acrylonitrile as the substrate.

The addition of the *Re*NHase β-subunit and Fe(II) to the *Re*NHase apo-α(ε) complex followed by incubation under nitrogen for 12 h, provided detectable NHase activity ($k_{cat} = 0.7 \pm 0.1$ s^{-1}) (Table 2; Fig. 3). While this k_{cat} value is only ~ 0.1% of the value observed for WT *Re*NHase, it is highly reproducible across multiple purifications, is dependent on the α(ε) complex and β-subunit concentrations, incubation time, and the concentration of acrylonitrile. It is also greater than the k_{cat} value (0.44 ± 0.04 s^{-1}) reported for the TNHase α-subunit only enzyme [21]. The addition of TCEP to this combination more than doubled the observed k_{cat} value to 1.7 ± 0.2 s^{-1} (Table 2; Fig. 3). Reducing conditions likely enhance metal ion binding to the apo-α(ε) complex due to the reduction of disulfide bonds, one of which was proposed to occur between the axial cysteine thiolate sulfur and the sulfenic acid sulfur atom of the NHase active site and/or in the proposed CXCC metal binding site of Fe-type (ε) proteins [24]. These data indicate that apo-α(ε) complex, in combination with β-subunit and Fe(II) provides NHase activity.

The ability to reconstitute *Re*NHase activity by the addition of Fe(II) and β-subunit, under reducing conditions, to the α(ε) complex allows the question of whether GTP hydrolysis is connected to iron insertion into the NHase α-subunit, to be addressed. The addition of GTP to the as-purified α(ε) complex in 50 mM HEPES buffer at pH 7.5 and 25 °C using 80 mM acrylonitrile as the substrate, in the presence of Fe(II) and β-subunit, followed by incubation under nitrogen for 12 h., resulted in NHase activity (k_{cat} value of 1.1 ± 0.2 s^{-1}). The addition of GTP enhances the observed activity by ~ 35% vs. the observed k_{cat} value for the same combination without GTP. As reducing conditions have been shown to enhance the rate of acrylonitrile hydration, TCEP was added to this mixture, which indeed further enhanced the observed NHase activity ($k_{cat} = 2.1 \pm 0.3$ s^{-1}) (Table 2; Fig. 3). The addition of TCEP nearly doubled the observed k_{cat} value compared to the same combination of α(ε) complex, Fe(II), GTP, and β-subunit. These data indicate that combination of the *Re*NHase α(ε) complex with Fe(II), GTP, and β-subunit, in situ and under reducing conditions, provides detectable and reproducible NHase activity. Moreover, while GTP increases the observed NHase k_{cat} value, it is not essential.

In all of the recombination experiments described thus far, the as-purified α(ε) complex was used; however, an unanswered question is can purified α–subunit and (ε) protein be mixed to form, in situ, an α(ε) complex that can bind Fe(II) and give rise to NHase activity. To explore this question, the apo-forms of purified *Re*NHase (ε), α- and β-subunit proteins were purified and mixed in a stepwise fashion with Fe(II), GTP, and TCEP. In the absence of TCEP, all combinations of (ε) protein in 50 mM HEPES buffer at pH 7.5 and 25 °C incubated with α- and β-subunits and Fe(II) and/or GTP under nitrogen for 12 h., resulted in no detectable activity (Table 3; Fig. 4). The addition of TCEP to the α- and β-subunits plus Fe(II), with no (ε) protein present, surprisingly, provided NHase activity ($k_{cat} = 0.7 \pm 0.2$ s^{-1}). The observed activity is nearly identical to the activity observed when pre-formed α(ε) complex was combined with β-subunit and Fe(II). The observation of NHase activity without the addition of (ε) protein, suggests that the reduction of the active site Cys residues is

Table 3 Kinetic data for the α- and β-subunits in the absence and presence of TCEP at 25 °C

Mixed components	k_{cat} (s^{-1})	k_{cat} (s^{-1}) + TCEP
α + β + ε + GTP	ND	ND
α + β + Fe^{2+}	ND	0.7 ± 0.2
α + β + Fe^{2+} + ε	ND	0.8 ± 0.2
α + β + Fe^{2+} + ε + GTP	ND	1.0 ± 0.2

ND none detected

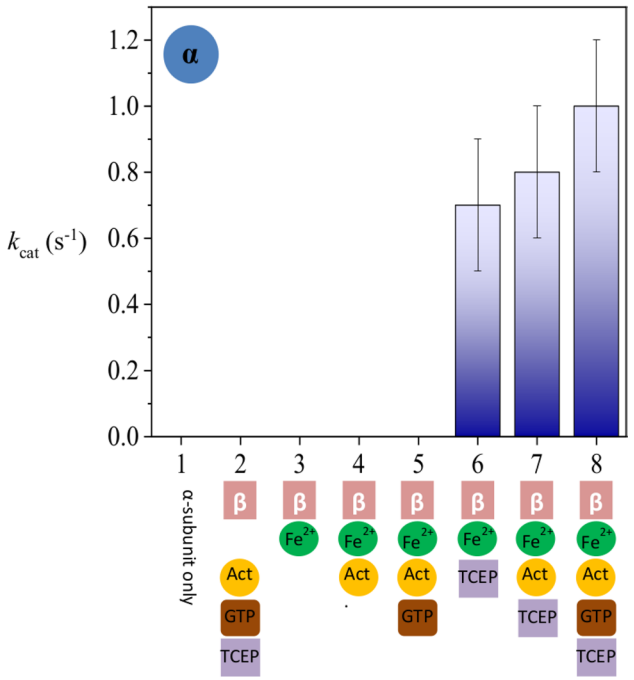


Fig. 4 Observed k_{cat} (s^{-1}) values of the α-subunit combined with (ε) protein, Fe(II), β-subunit, GTP, or TCEP in 50 mM HEPES, pH 7.5 at 25 °C. All reactions were performed under nitrogen and incubated for 12 h

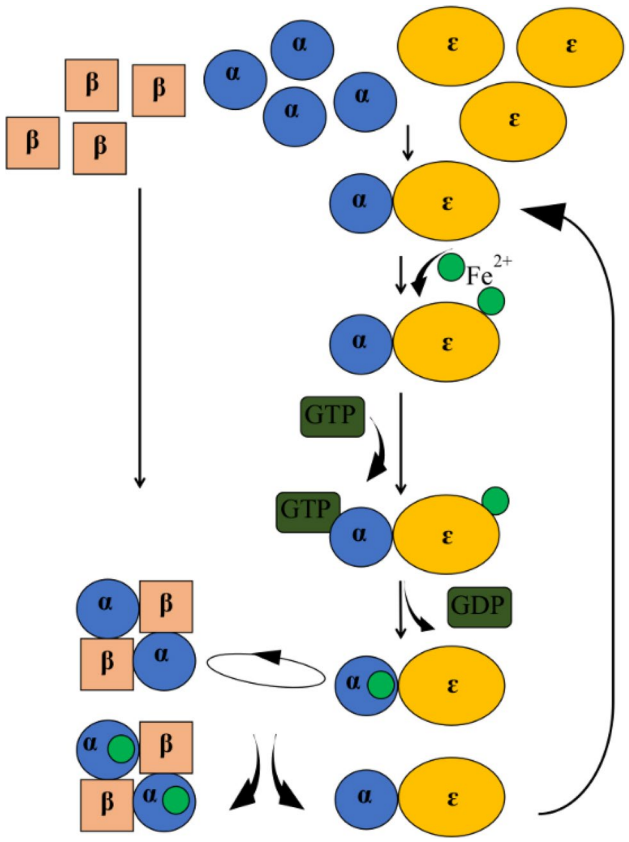


Fig. 5 A proposed model iron incorporation into the Fe-type NHase α-subunit and activation to $\alpha_2\beta_2$ heterotetramer

likely critical for Fe(II) incorporation, consistent with X-ray crystallographic data on apo-NHase enzymes [28]. Interesting, the addition of (ε) protein to this mixture did not change the observed activity with error of the experiment ($k_{cat} = 0.8 \pm 0.2\ s^{-1}$); however, the addition of both (ε) protein and GTP increased the observed rate of nitrile hydration by ~ 30% ($k_{cat} = 1.0 \pm 0.2\ s^{-1}$). These data suggest that in vitro activation of NHase likely requires the reduction of the active site Cys residues and, at least in vitro, activator is not required for NHase activity to be observed but when present, the combination of (ε) protein and GTP supports metal ion insertion into the α-subunit, which is required for NHase activity.

In conclusion, evidence is provided for the formation of the first known Fe-type α(ε) complex, which was shown to form a 1:1 mol ratio complex. This complex can be mixed in vitro with β-subunit and Fe(II) resulting in detectable NHase activity. The addition of GTP enhances the rate of acrylonitrile hydration indicating that GTP plays facilitating the incorporation of iron into the α-subunit, but is not essential. On the other hand, the addition of TCEP markedly enhances the rate of nitrile hydration, likely due to the reduction of either a disulfide bond in the NHase active site and/or in the proposed CXCC metal binding site of the (ε) protein. The independent expression and purification of *Re*NHase α- and β-subunits allowed purified α-subunit to be mixed with (ε) protein, in situ, to form the α(ε) complex, which could then be mixed with β-subunit, Fe(II), and/or GTP in the absence and presence of TCEP. These data indicate that NHase activity can be observed under reducing conditions in the presence of GTP. Comparison of these data with those reported for Co-type NHase (ε) proteins, which were proposed to insert Co(II) into apo-$\alpha_2\beta_2$ NHase via a "self-subunit swapping" mechanism [12], implies that a similar mechanism might be active for Fe-type NHases (Fig. 5). If so, an Fe-type α(ε) complex forms rather than the $\alpha(\varepsilon)_2$ complex observed for Co-type enzymes. This Fe-type α(ε) complex is proposed to bind Fe(II) under reducing conditions and insert iron into the NHase active site in the α-subunit. This metal loaded α-subunit can then "self-subunit swap" with apo α-subunit to form a metallated, NHase enzyme.

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Author contributions KPWL prepared expression plasmid, carried out protein expression, purification, enzymatic assays and prepared samples for metal analysis, and analyzed the results. RCH conceived of the idea and wrote the paper with KPWL and BB.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest with the contents of this article.

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