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Diversity and evolution of nitric oxide reduction

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Summary:

Nitrogen is an essential element for life, with the availability of fixed nitrogen limiting productivity in many ecosystems. The return of oxidized nitrogen species to the atmospheric N_2 pool is predominately catalyzed by microbial denitrification ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$)[1]. Incomplete denitrification can produce N_2O as a terminal product, leading to an increase in atmospheric N_2O, a potent greenhouse and ozone depleting gas[2]. The production of N_2O is catalyzed by nitric oxide reductase (NOR) members of the heme-copper oxidoreductase (HCO) superfamily[3]. Here we propose that a number of uncharacterized HCO families perform nitric oxide reduction and demonstrate that an enzyme from *Rhodothermus marinus*, belonging to one of these families does perform nitric oxide reduction. These families have novel active-site structures and several have conserved proton channels, suggesting that they might be able to couple nitric oxide reduction to energy conservation. They also exhibit broad phylogenetic and environmental distributions, expanding the diversity of microbes that can perform denitrification. Phylogenetic analyses of the HCO superfamily demonstrate that nitric oxide reductases evolved multiple times independently from oxygen reductases, suggesting that complete denitrification evolved after aerobic respiration.

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Introduction

The HCO superfamily is extremely diverse, with members playing crucial roles in both aerobic (oxygen reductases) and anaerobic respiration (nitric oxide reductases). The superfamily currently consists of at least three oxygen reductase families (A, B and C) and three NOR families (cNOR, qNOR, and qCu_ANOR)[4]. The oxygen reductases catalyze the reduction of O_2 to water ($O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$) and share a conserved reaction mechanism[5] involving active-site metals, heme-Fe and Cu_B, as well as a unique redox-active cross-linked tyrosine-histidine cofactor that is essential[6] (**Figure 1**). The free energy of the reaction is converted to a transmembrane proton electrochemical gradient by two different mechanisms: (i) Taking the protons and electrons used in the chemistry from opposite sides of the membrane; and (ii) Pumping protons across the membrane, with the different oxygen reductase families exhibiting differential proton pumping stochiometries (n=4 for the A-family, and n=2 for the B and between C-families)[7–9]. Both the chemical and pumped protons are taken up from the electrochemically negative side of the membrane (bacterial cytoplasm) via proton-conducting channels that are comprised by conserved polar residues and internal water molecules. The oxygen reductases also vary in their secondary subunits that function as redox relays from electron donors to the active-site, with the A and B-families utilizing a Cu_A-containing subunit[10–12] and the C-family contain one or more cytochrome c subunits[13] (**Figure 1**).

The nitric oxide reductases (NORs) catalyze the reduction of nitric oxide to nitrous oxide ($2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O$). The NORs do not contain the cross-linked active-site tyrosine that is found in the O_2 reductases and, furthermore, contain a non-heme Fe_B in

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place of the Cu_B at their active sites[14,15]. The cNOR and qNOR families are closely related to the C-family oxygen reductases. In accordance with this relationship, the cNORs have a secondary cytochrome c-containing subunit, while in the qNORs the two subunits corresponding to those in the cNORs have been fused to a single subunit that lacks the heme c binding motif[15,16]. The qNORs accept electrons from quinols and not from cytochrome c. Importantly, the cNORs and qNORs each lack proton-conducting channels from the cytoplasm[14,17]. Consequently, these enzymes are not proton pumps and the chemical protons used to generate H_2O at the active site are taken from the same side of the membrane as are the electrons. Hence, the reaction does not generate any transmembrane charge separation and no proton motive force. In this work, we show using extensive phylogenomic analysis of publicly available sequence datasets that there are six new families of nitric oxide reductases, several of which are likely to generate proton motive force. This has important consequences for the efficiency of energy conservation associated with denitrification[18]. We have also isolated and biochemically characterized a member of one of the new families, eNOR verifying that it is a nitric oxide reductase.

Identification of seven new families of nitric oxide reductases using phylogenomics

Phylogenomic analyses of genomic and metagenomic data have identified at least seven new families belonging to the HCO superfamily (**Figure 2**). All of these families are missing the active-site tyrosine, suggesting that these putative enzymes do not catalyze O_2 reduction. Furthermore, their active-sites exhibit structural features never seen before within the superfamily (**Figure 1**). One of these families, closely related to qNOR has

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been proposed to be a nitric oxide dismutase contributing to oxygen production in *Methylomirabilis oxyfera*[19]. A second of these families is closely related to the cNOR but its function has not been easy to deduce. The remaining five families are closely related to the B-family of oxygen reductases (**Figure 2**). They all encode for homologs of Cu_A-containing secondary subunits, consistent with this evolutionary relationship (**Figures 1**). Based on modeled active-site structures and genomic context (the presence of associated denitrification enzymes in the genome), we propose that proteins within each of these five families perform nitric oxide reduction (**Figure 1**) and have named these eNOR, bNOR, sNOR, gNOR and nNOR. In the current work, we have isolated a member of the eNOR family and confirmed that, as proposed, this protein is an NO reductase.

Characterization of eNOR from *Rhodothermus marinus*

Rhodothermus marinus DSM 4252, a thermophilic member of the Bacteroidetes phylum has been classified as a strict aerobe[20], but its genome encodes a periplasmic nitrate reductase (NapA), two nitrite reductases (NirS and NirK), and a nitrous oxide reductase (NosZ), suggesting that it may also be capable of denitrification (**Extended Data Figure 1**). Although denitrification was not observed by a culture of *R. marinus* DSM 4252 under strictly anaerobic conditions, under microaerobic conditions isotopically labeled $^{15}NO_3^-$ was converted to $^{30}N_2$ (**Extended Data Figure 2**) Hence, *R. marinus* DSM 4252 is capable of complete aerobic denitrification ($NO_3^- \rightarrow N_2$). Blockage of the nitrous oxide reductase with acetylene results in the accumulation of N_2O (**Figure 3**), indicating that N_2O is an intermediate in the pathway. Although no genes encoding known NORs are in the genome, *R. marinus* DSM 4252 does encode for a member of the

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eNOR family (**Extended Data Figure 1**). This protein was detergent-solubilized from membranes of *R. marinus* DSM 4252, purified and characterized. The following can be deduced about the eNOR family of enzymes.

1) The purified eNOR protein catalyzes the conversion of NO to N_2O (at 25°C, k_{cat} = 0.68 $\pm$ 0.21 NO.s^{-1} (n = 4) (**Figure 3**) but is unable to catalyze oxygen reduction **(Extended Data Figure 3).**

2) The enzyme contains a modified heme *a* that is present in both the low spin and high spin heme sites that are present in all HCOs (**Figure 3 and Extended Data Figures 3 and 4**). Although not identified as such, an eNOR appears to have been previously isolated from the aerobic denitrifier *Magnetospirillum magnetotacticum* MS-1[21,22]. It was unable to catalyze O_2 reduction, however NO was not tested as a substrate. The UV-Vis spectra of the *M. magnetotacticum* eNOR[21] is identical to the *R. marinus* eNOR, suggesting that the same modified heme *a* may be a general feature of all eNORs. Many eNOR operons, including that in *R. marinus* DSM 4252, contain a CtaA homolog, an O_2-dependent enzyme that converts heme O to heme A, consistent with the observation that eNOR is expressed under microaerobic conditions[23]. A mass spectrum of the hemes extracted from eNOR suggest that this heme is A_s, a previously isolated A-type heme with a different side chain from the cytochrome oxidase in *Sulfolobus acidocaldarius*[24].

3) Some members of the eNOR family (but not from *R. marinus* DSM 4252) have replaced one of the low-spin heme histidine ligands with a lysine, a modification that likely alters the redox midpoint potential of the heme[25] . This could be a modification due to the presence of a modified heme *a* at this site.

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4) The glutamate that ligates the active-site Fe_B in the cNOR/qNOR families is replaced by a glutamine in the eNOR enzymes (**Figure 1**). Hence the ligation of the active-site metal is distinct in the eNORs.

5) The eNORs contain a conserved set of polar residues homologous to those that define the proton-conducting K^B channel in the B-family O_2 reductases[26] extending from the cytoplasmic surface to the active site (**Figure 1**). This suggests that the protons consumed at the active site of the eNORs may originate from the cytoplasm, resulting in generation of a transmembrane voltage and energy conservation during catalytic turnover.

Active site features of novel nitric oxide reductase families

Our phylogenomic analysis allows the previously isolated qCu_ANOR from *Bacillus azotoformans*[27,28] to be identified as a member of the bNOR family. The bNOR enzymes contain an asparagine near the active site Fe_B, and also have conserved polar residues that suggests a proton-conducting channel (**Figure 1).** In addition to the experimental evidence that both eNOR and bNOR enzymes are NO reductases, there is reasonable evidence that the other newly identified families also perform nitric oxide reduction. The sNOR family has the same active-site structure as the bNOR family, strongly suggesting that it also performs nitric oxide reduction. Since the sNOR and bNOR families are independent clades, this provides an example of convergent evolution within the HCO superfamily (**Figures 1 and 2**). Another example of convergent evolution is the nNOR family which has the same conserved active-site residues as the cNOR and qNOR families (**Figure 1**), but is very distantly related to them. Note that in

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the nNOR proteins, one of the canonical histidine ligands to the low-spin heme is replaced by a methionine, which likely lowers its redox potential. This is similar to the lysine substitution found in some eNORs. Finally, the putative active-site residues in the gNOR proteins are unique in that one of the canonical histidine ligands to the non-heme metal (presumable Fe_B) is replaced by an aspartate. Although no gNOR enzyme has been characterized, a bioinorganic mimic of the gNOR active-site was shown to have nitric oxide reduction capability[29], suggesting that the gNORs are also likely to be functional NORs. The gNORs are also unique in that the cupredoxin fold in the secondary subunit lacks the residues that define the Cu_A redox center. Conserved residues that could bind quinol have been identified in the gNORs, suggesting that these enzymes are quinol oxidases rather than cytochrome c oxidases, similar to the qNORs.

One implication of the current work is that there is considerably more flexibility in the metal ligation at the active sites in the NO reductases than in the O_2 reductases. This may reflect the importance of the cross-linked histidine-tyrosine in preventing the release of ROS as well as the ability of O_2 reductases to function as proton pumps[30]. The newly identified families of NO reductases provide an opportunity to determine whether the chemistry of NO reduction is uniquely served by the non-heme Fe_B at the active sites, in the same way that Cu_B appears to be an absolute requirement of the proton pumping O_2 reductases.

Conserved proton channels and energetic efficiency of new denitrification pathways

Although both denitrification and aerobic respiration are highly exergonic processes, most of the enzymes in the denitrification pathway are not coupled to energy

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conservation, making denitrification significantly less efficient than aerobic respiration[31]. All of the HCO O_2 reductases contain conserved proton channels used to deliver protons from the cytoplasm to the active-site for chemistry and to pump protons across the membrane. These processes generate the proton motive force. In contrast, the cNORs do not have conserved proton channels from the cytoplasm[14,16] and instead receive their protons from the periplasm, which makes them incapable of conserving energy. It has been suggested that qNORs can uptake protons from the periplasm[15] but, no conserved proton channel can be identified in qNORs, suggesting that a majority of them are also unlikely to be capable of generating proton motive force. It is very significant that both the eNOR and bNOR families have conserved residues that closely resemble those found in the proton-conducting channel within the B-family of oxygen reductases[26] (**Supplementary Table 1, Extended Data Figure 5**). Similarly, the sNOR family also has conserved residues that suggest that these enzymes also contain a proton-conducting channel that could provide protons to the active site from the cytoplasm. Interestingly, the nNOR family, which has the same active site as cNOR and qNOR, has a conserved proton channel, suggesting that it can translocate protons across the membrane. (**Supplementary Table 1, Extended Data Figure 5**). These channels would allow the eNOR, bNOR, sNOR, and nNOR families to conserve energy via charge separation, and potentially by proton pumping. Recently it has been demonstrated that the qCu_ANOR from *Bacillus azotoformans*[27], which we have identified as a member of the bNOR family can generate a proton motive force[28]. This may also be true for the other NORs with putative proton-conducting channels. This has significance for the catalytic mechanism of the NORs since a proposed catalytic mechanism for the NORs contains no step that is

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sufficiently exothermic to drive charges across the membrane in the presence of a transmembrane votage.

Distribution and environmental relevance of new NOR families

The new HCO NOR families have broad phylogenetic and environmental distributions (**Table 1, Supplementary Tables 2, 3**). The eNOR, gNOR, and nNOR families are found in both Bacteria and Archaea, whereas the sNOR family is found in a number of bacterial phyla. To date, the bNOR family has only been found only in the *Bacillales* order of Firmicutes (**Supplementary Table 2**). Phylogenetic analysis of metagenomic data shows that the majority of eNOR, sNOR, gNOR, and nNOR enzymes are from uncharacterized organisms, suggesting that many more organisms are capable of nitric oxide reduction. Furthermore, the new HCO NOR families are found in a wide variety of environments (**Table 1, Supplementary Figure 2**), suggesting that they play roles in many ecosystems. The eNOR family is very common in nature, having a broad distribution similar to the cNOR and qNOR families. Recently, the eNOR from zetaproteobacteria was implicated in denitrification coupled to iron oxidation, occurring in iron-rich microbial mats in hydrothermal vents[32]. Interestingly, in these environments, the various reactions in the denitrification pathway occur in different microorganisms, providing an additional level of flexibility.

Many organisms encode NORs from multiple families (e.g., *Methylomirabilis oxyfera* has a qNOR, sNOR and gNOR, and *Bacillus azotoformans* has a qNOR, sNOR, and bNOR). This suggests that selection for different enzymatic properties (NO affinity, enzyme kinetics, energy conservation, or sensitivity to inhibitors) or the concentration of

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O_2 may be important factors in determining their distribution, similar to the HCO oxygen reductase families[7]. Analysis of the presence of denitrification genes (nitrite reductases, nitric oxide reductases, and nitrous oxide reductases) within sequenced genomes indicates that many more organisms are capable of complete denitrification than previously realized. Our current understanding of the diversity of organisms capable of performing denitrification is far from complete.

Our evolutionary analysis shows that nitric oxide reductases have evolved many times independently from oxygen reductases (**Figure 2**). The current data show that NORs have originated from both the B and C-families of oxygen reductases, enzymes that are adapted to low O_2 environments. These oxygen reductases can reduce NO at high concentrations *in vitro*[33] so it is not surprising that small evolutionary modifications would lead to enzymes capable of NO reduction at the lower NO concentrations produced during denitrification. The fact that NO reductases are derived from oxygen reductases strongly suggests that complete denitrification evolved after aerobic respiration. This places important constraints on the nitrogen cycle before the rise of oxygen.

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Table 1. Environmental distribution of the HCO NOR families. Distribution of NOR families in sequenced genomes versus environmental datasets. The newly discovered NOR families account for approximately 2/3 of currently known diversity and 1/2 of the abundance of NORs in Nature.

	NCBI-Genomes	IMG-metagenomes	GTDB-genomes
A-family	20290	102368	45135
B-family	1238	4683	2021
C-family	13976	23015	14981
qNOR	4388	7680	3458
cNOR	2801	4824	2594
eNOR	68	2709	547
sNOR	95	872	344
bNOR	51	12	200
nNOR	6	289	32
gNOR	10	913	156
NOD	8	539	108
N2O	25	597	n.a.

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Figure 1. Comparison of HCO active sites. a) Active-site and proton channel properties of the five characterized HCO families (A-family, B-family, C-family, cNOR, and qNOR). The oxygen reductases all have an active-site composed of high-spin heme, a redox-active cross-linked tyrosine cofactor, and a copper (Cu_B) ligated by three histidines. The A-family has two conserved proton channels, whereas the B and C-families only have one. The active-sites of the nitric oxide reductases are composed of a high-spin heme and an iron (Fe_B) that is ligated by three histidines and a glutamate. Notably they are missing the tyrosine cofactor. The cNOR and qNOR are also missing conserved proton channels, making them non-electrogenic. b) Sequence alignment of the active-sites of the newly discovered HCO families that are related to the B-family. c) Predicted active-sites and proton channels for the new HCO families. The eNOR, bNOR, sNOR, and nNOR families contain completely conserved proton channels.

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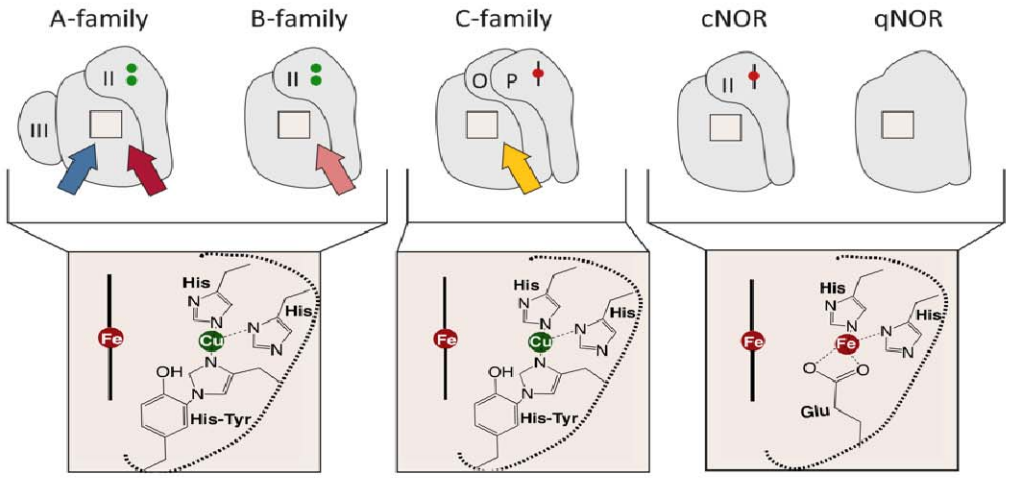
A



B

```
A-family_1M56   PVLYQHILWFFGHPEVYIIVLPAFGIVSHVIATFAK--GYLPMVYAMVAIGVLGFVVWAHHMYT
B-family_3S8F   PLVARTLFWWTGHPIVYFWLLPAYAIIYTILPKQAG--SDPMARLAFLLFLLLSTPVGFHHQFA
eNOR            AAWYRQMYWIIGHGSQQINLAAMITVWYFLTHVVGG--SEKLSRTAFILYLFFINMGAAHHLLA
bNOR            VMVARTLFWAFGHTAVNIWYLTAVSAWYVIVPKIIG--SDMLTRVVIIALVIMNITGGFHHQII
sNOR            PLLSKNLIYAFGHIFANSIIYMGVIAVYEIFPKYTG--VYGNFLIAWNASTLFTMIIYPHHLLM
gNOR            ALLYKNVYWWGLDLIADGLVLIYVAGSWYLLAMLLT--MQHIARAALFVELVVSWFVWSHHLLS
nNOR            PWPFNVAFWLFAHNLMEAMGIMALAAVYALVPLYTR--SPGMGVLAVGLYTLSAIPAFGHHLYT
C-family_3MK7   GATDAMVQWWYGHNAVGFFLTAGFLGIMYYFVPKQA--SYRLSIVHFWALITVYIWAGPHHLHY
cNOR_3O0R       LTRDKFYWWWVVHLWVEGVWELIMGAILAFVLVKIT--IEKWLYVIIAMALISGIIGTGHHYFW
qNOR_3AYF       FTMADFWRWWIIHLWVEGIFEVFAVVVIGFLLVQLR--TVRALYFQFTILLGSGVIGIGHHYYY
                --------------Helix VI--------------  ---------Helix VII--------
```

C

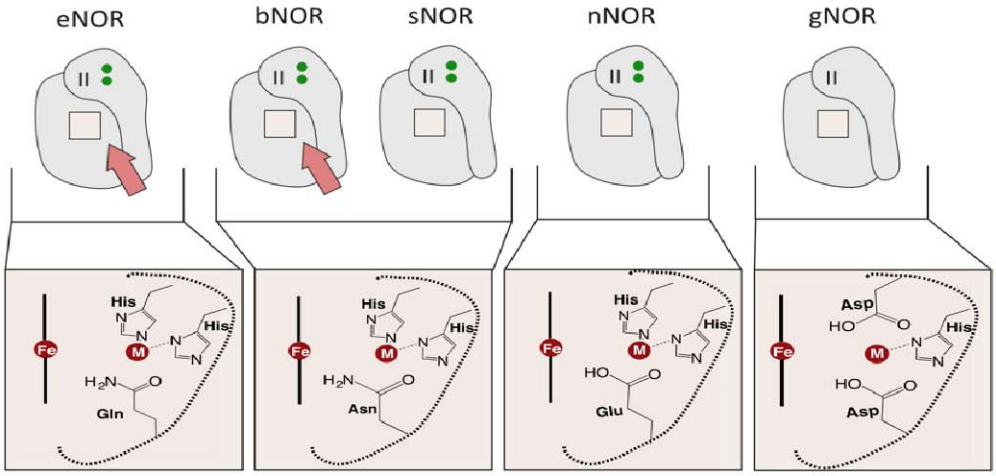


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Figure 2. Evolution of nitric oxide reductases. Phylogenetic tree of the NORs related to the B-family oxygen reductases. All of the new NOR families are derived from oxygen reductase ancestors. Nodes on the tree marked with stars indicate unique indels that validate the branching topology. B-family oxygen reductases are in shades of blue, whereas nitric oxide reductases are in shades of red.

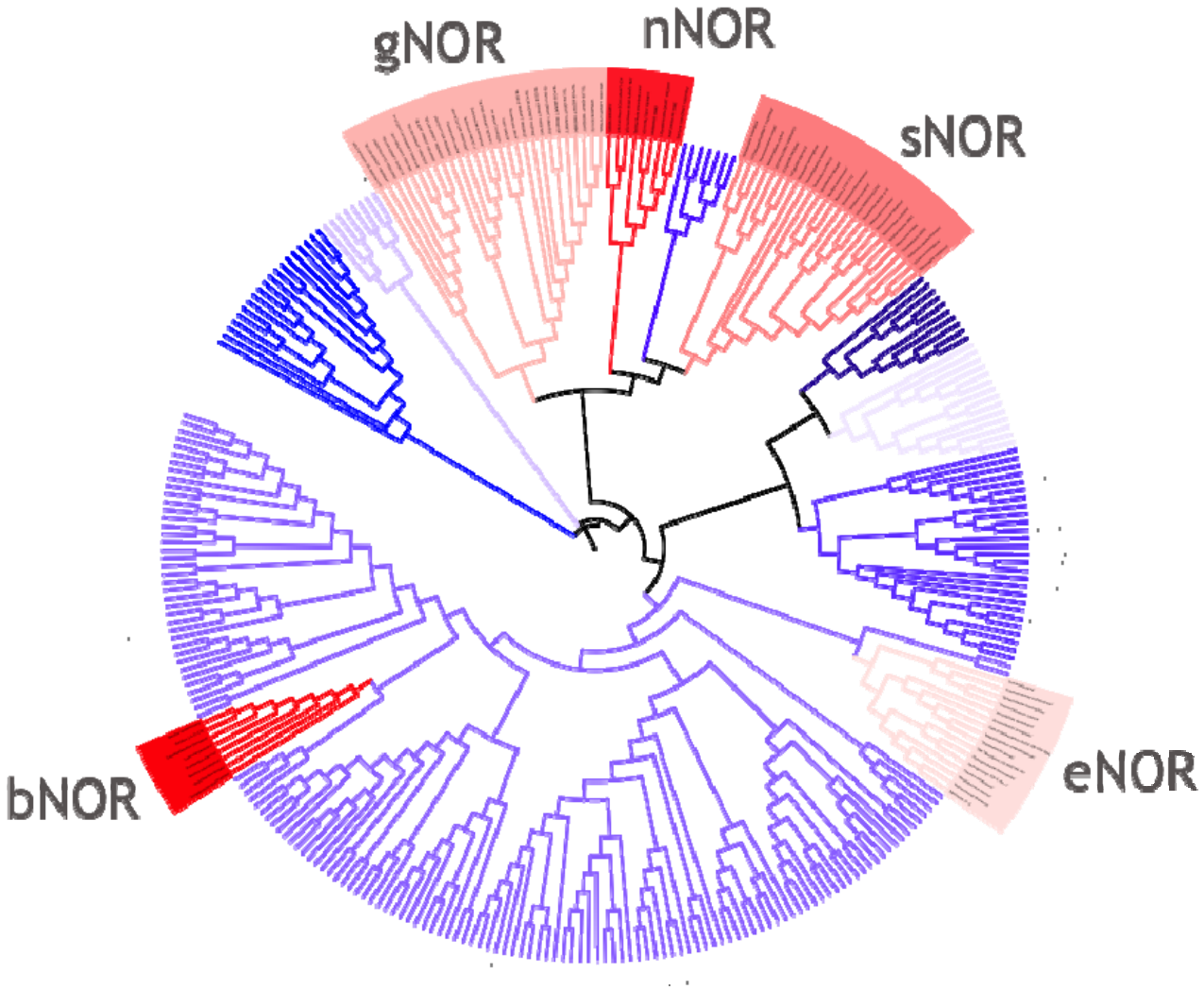

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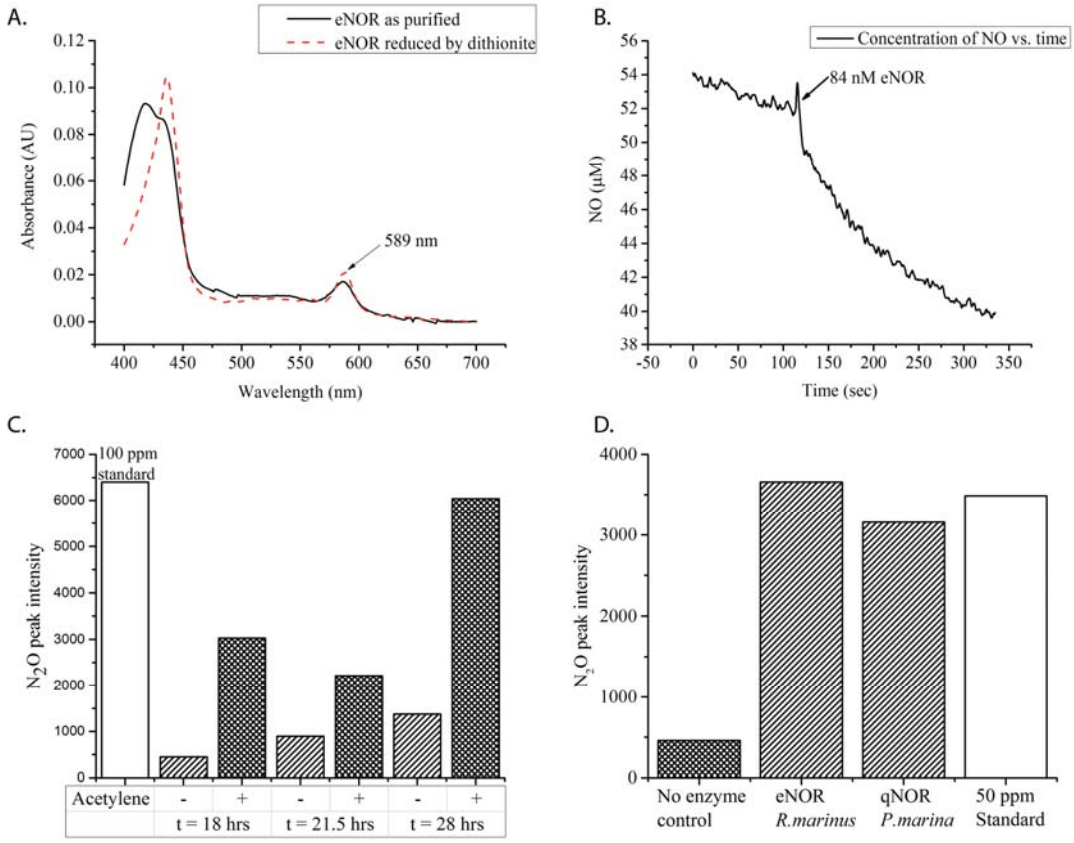
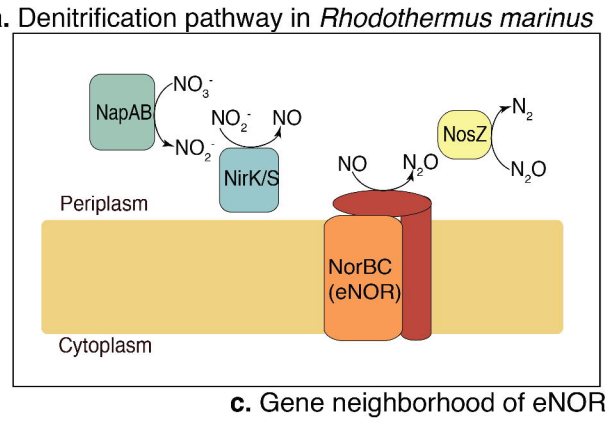
Figure 3. Biochemical Characterization of the eNOR from *Rhodothermus marinus*. a) UV-Vis spectra. b) NO and O_2 activities. c) GC data



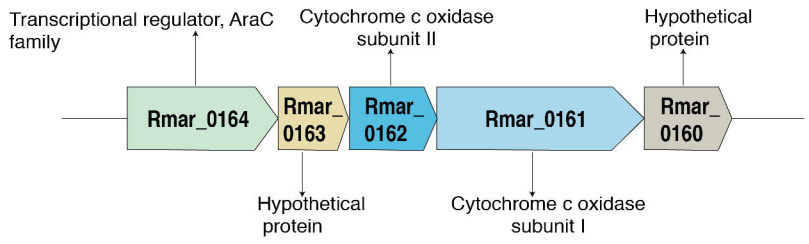
Extended data Figure 1

a. Denitrification pathway in *Rhodothermus marinus*



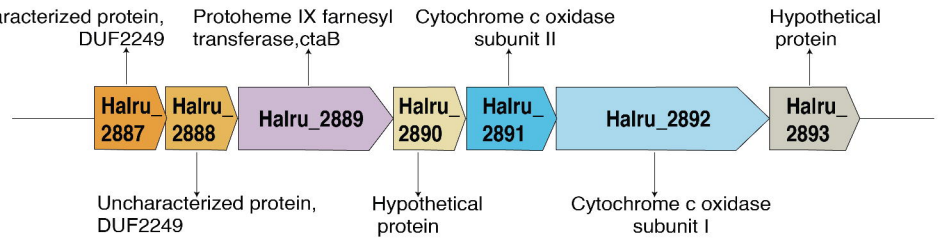
b. Gene neighborhood of eNOR in *Rhodothermus marinus*

***Rhodothermus marinus* DSM 4252: NC_013501**



c. Gene neighborhood of eNOR in other organisms include genes for heme *a* synthesis

Halovivax ruber XH-70, DSM 18193: Halru_Contig_35



d. Sequence alignment of eNOR - Subunit I showing conserved amino acid residues according to *R.marinus* eNOR numbering

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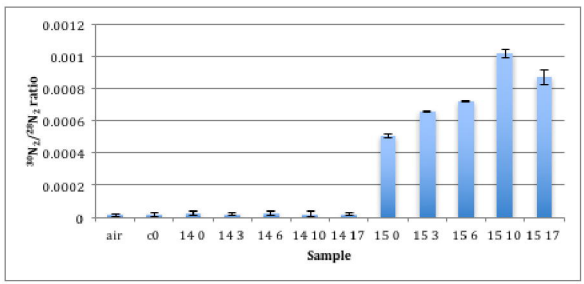
Extended Data Figure 2: Rhodothermus marinus does perform complete denitrification. a. R.marinus converted $^{15}NO_3^-$ to $^{30}N_2$. b. Growth curve of *R. marinus*, measured using OD_{600nm} and NO_3^- utilization, measured by calorimetry. c. *R. marinus* growth under denitrifying and non-denitrifying conditions. d. Phenotypic differences of *R. marinus* cultures, under denitrifying and non-denitrifying conditions.

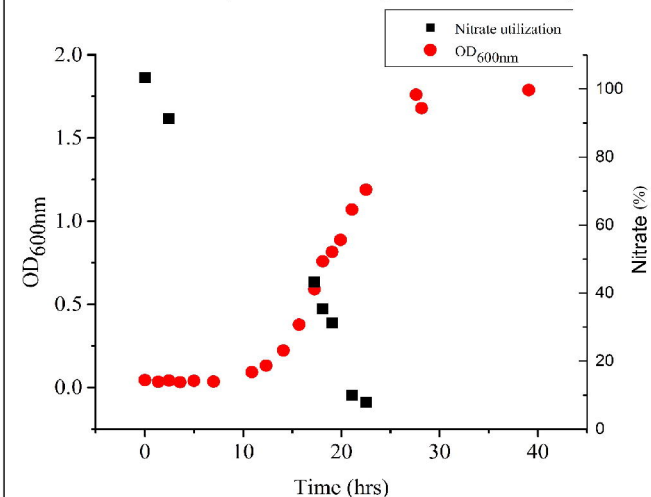
a.

Conversion of $^{15}NO_3^-$ to $^{15}N_2$ in pure cultures of *Rhodothermus marinus*

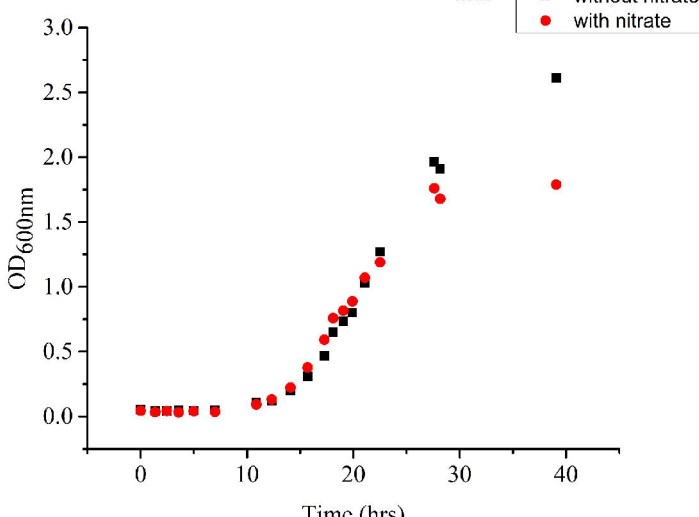


b.

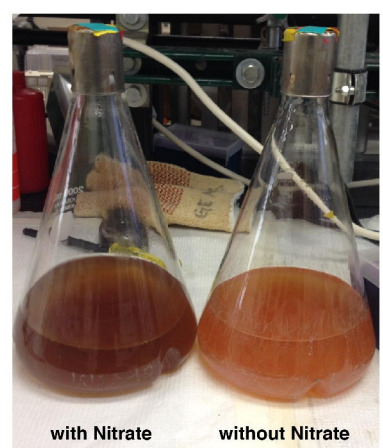
Nitrate consumption evaluated by a colorimetric assay



c. **Growth of *Rhodothermus marinus* observed at $OD_{600\ nm}$**

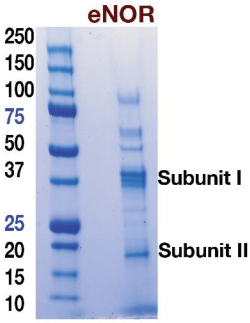


d. **Phenotypic differences between cultures of *Rhodothermus marinus* grown with and without nitrate**



Extended Data Figure 3: Characteristics of eNOR from *Rhodothermus marinus* a. SDS-PAGE gel electrophoresis of eNOR. b. Mass spectrometric identification of eNOR. c,d. Absence of O_2 reduction by *R. marinus* eNOR, in comparison to robust O_2 reduction by *T. thermophilus* ba_3-type oxygen reductase. e. UV-visible spectrum of eNOR f. Pyridine hemochrome-spectra of extracted hemes from eNOR showing a peak which is atypical of hemes *a, b or c*.

a. Electrophoresis of eNOR on a SDS-PAGE gel



Estimated Molecular Weight (MW)

Subunit I: 62.5
Subunit II: 20.5

b.

Protein identification by Mass spectrometry - eNOR

Subunit I - 24 % sequence coverage, MASCOT score: 354

```
  1  MAGLLLSGTY DAEGFRTCSV TGLRIHRDVE RYVKLFALTA VVALLIGGIS
 51  AIFVALTRWE VIGLADPPAF YKWLSIHAWN LLIFWMVFME VAILYIGGPL
101  VLGRPLPAPR LAGIGYFVML VAALMINHAI ATTEAPDSAP LLTSYAPLPS
151  PPLFYLGVIL FILGVIVAAL PFFITIWQEK QEYPNRSLPL VSFGAFITSI
201  IALEALLGGL ITYIPTFLWR IGVVEHIDAA WYRQMYWIIG HGSQQINLAA
251  MITVWYFLTH VVGGAEVVSE KLSRTAFILY LFFINMGAAH HLLADPGVHM
301  SWKHWNTSYA VYGAVLASMI HAFAIPAGLE AGRRKRGLGS QGLFGWLWSA
351  PWGNPVFSAT ALGIIMFGFI GGISGVVMGQ TQLNLTWHNT LGVPGHFHGT
401  VVLGTTLTFM ALVYFALPVL FNRDLAFKPL ARIQPYFYAV AMGLATIMMI
451  YLGVIYGVPR RHPSVMSFPG TDFSFAAASP LFAIFGVAAI LAIIAGASFL
501  LVAVGSLLFG KRVENPALVM PPLISGGSDD RPIHEYSMQG TFTLTLIFLA
551  VFAIVYAVNW YLLGRLWAIG
```

Subunit II - 60 % sequence coverage, MASCOT score: 2022

```
  1  MSSPLKPLEG NWWGIPANRY EKLWVQISVV WGLILFGWMV GWSFFGNQNQ
 51  HGPTYRIEPA TYRAKLAAYR EASTATEQGL RPAGEDVYIA AMRYGFDGLP
101  VILKAGKTYR LHLTSYDVQH GFSIRPEHAL SKQVTFQLLP GYEWVIPMRF
151  DEPGVYHVIC NEFCGIGHRT MHGIFIVEE
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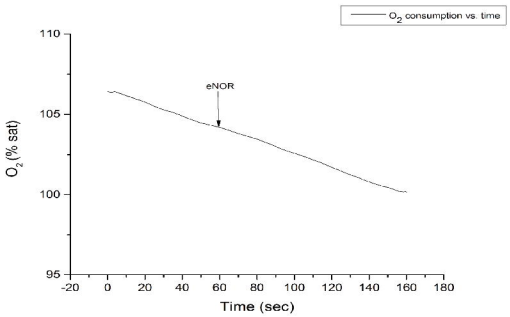
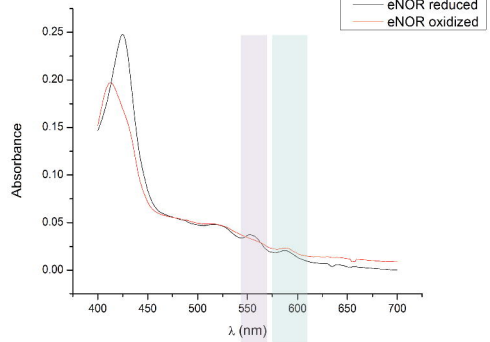
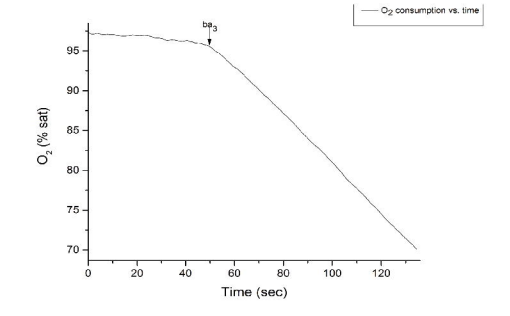
c. Absence of oxygen reduction activity by eNOR from *Rhodothermus marinus*



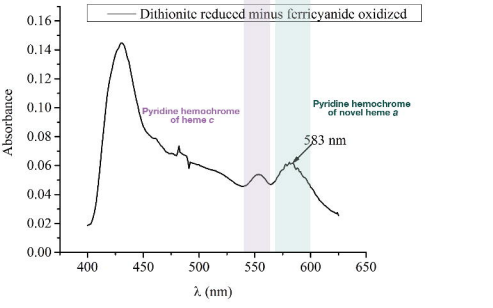
e. UV-visible spectrum of eNOR containing membrane fraction



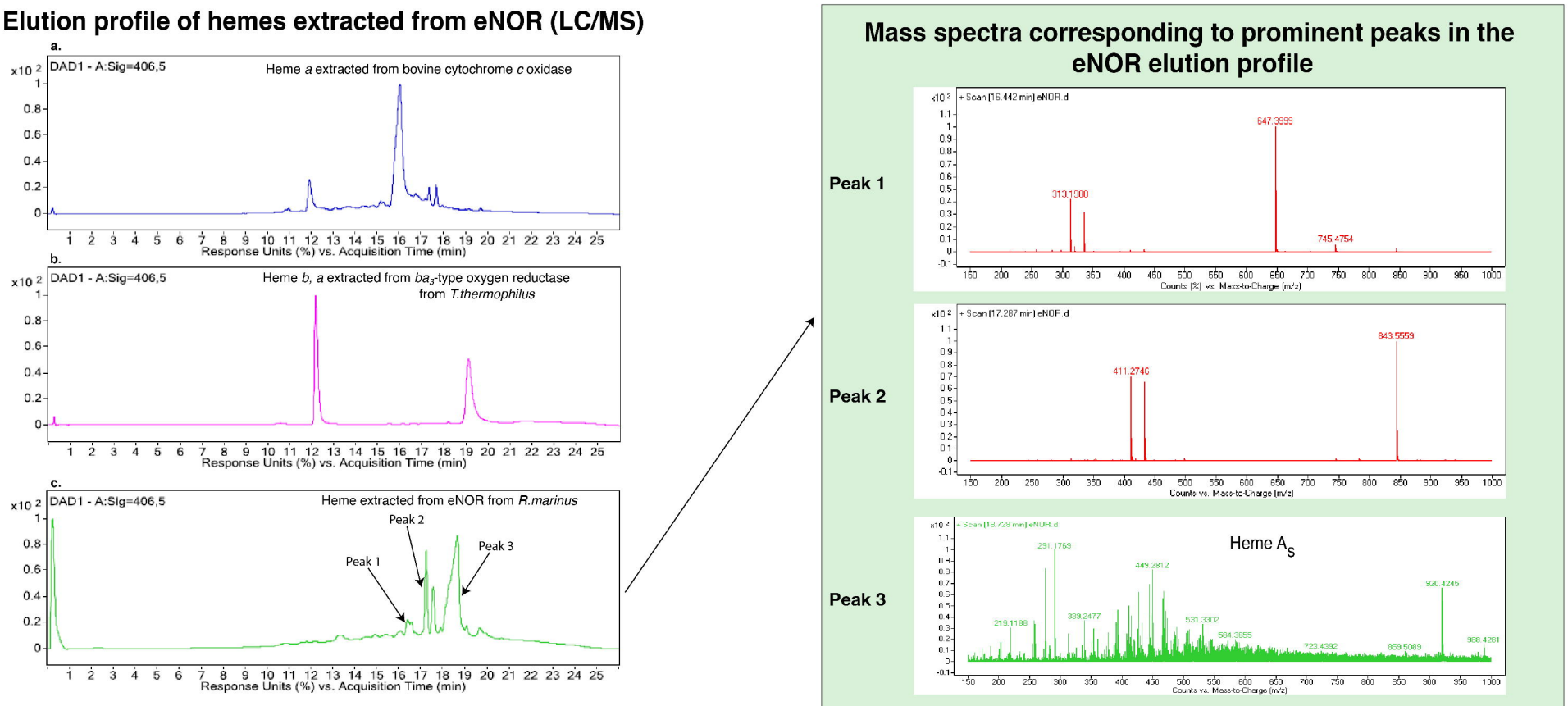
d. Oxygen reduction activity of cytochrome ba_3 oxygen reductase from *Thermus thermophilus*



f. Pyridine hemochrome spectra of eNOR

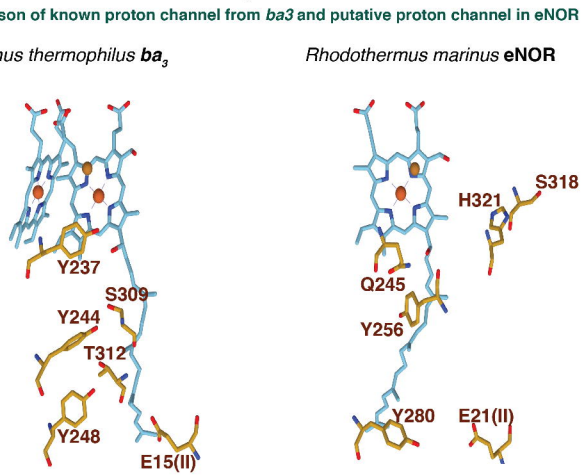
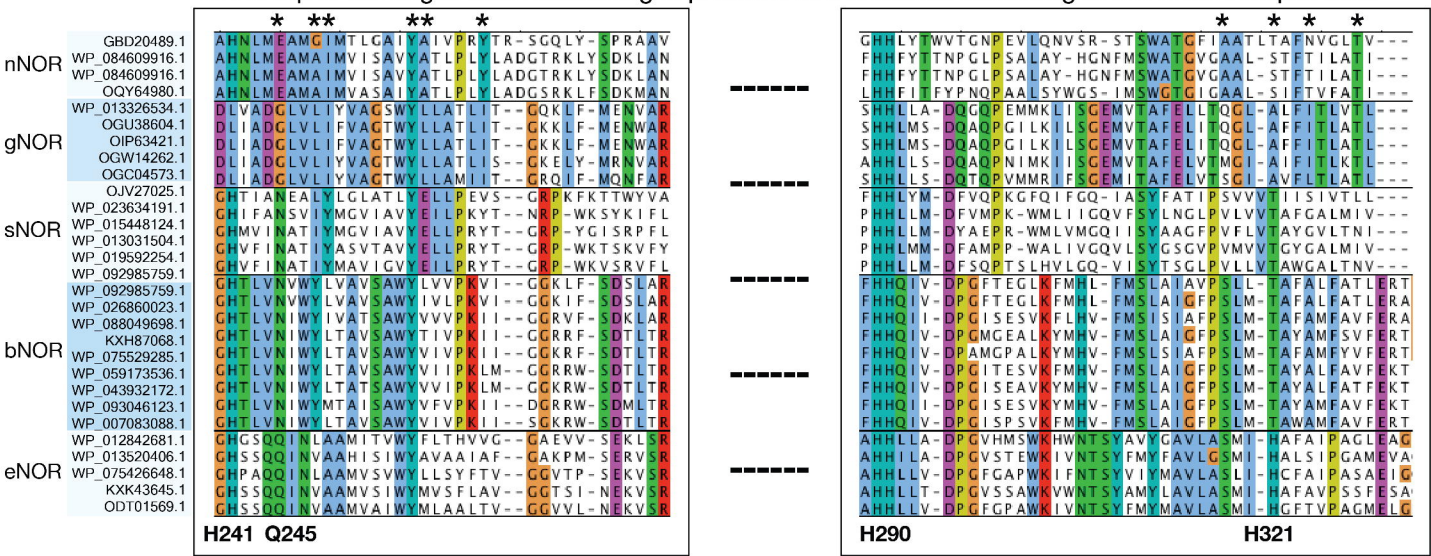


Extended Data Figure 4: Identification of hemes extracted from eNOR. Comparing the elution profile of extracted hemes from partially purified R.marinus eNOR to bovine cytochrome c oxidase (A-type, t=16 min) , T. thermophilus ba3-type oxygen reductase (b- and As-type hemes, t=12 min and t=19 min) reveals that the heme is most likely an As-type heme. Mass spectra of the peak at ~19 min from the eNOR hemes elutionprofile confirms that the heme is an As-type heme with a molecular weight of 920 Da.



Extended Data Figure 5: Proton channel in eNOR of Rhodothermus marinus. eNOR contains conserved residues in Helix VII, similar to the location of K-proton channel residues in *T. thermophilus* ba_3-type oxygen reductase.

a. Comparison of known proton channel from $\boldsymbol{ba_3}$ oxygen reductase and putative proton channel in eNOR



b. Conserved residues forming putative proton channels in eNOR, sNOR, bNOR and nNOR are marked with a ‘ * ’ at the top of the alignment. Numbering is provided at the bottom according to the eNOR sequences.