

SUPPLEMENTAL INFORMATION

Characterization of anammox hydrazine dehydrogenase, a key N₂-producing enzyme in the global nitrogen cycle

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SUPPLEMENTAL FIGURE LEGEND

FIGURE S1. **Multiple protein sequence alignment of hydrazine dehydrogenase and other HAO-like octaheme proteins.** The Figure shows the alignment of hydrazine dehydrogenase kustc0694 from *Kuenenia stuttgartiensis* (KsHDH), its close homologs and paralogs from other anammox bacteria, hydroxylamine oxidase (KsHOX, kustc1061) from *K. stuttgartiensis*, and hydroxylamine oxidoreductase from *N. europaea* (Neuro_HAO). Predicted N-terminal signal sequences are printed in blue. These sequences are absent in proteins with known crystal structures (KsHOX, NeHAO). The CX₂CH heme binding motifs of the heme *c* molecules are highlighted red (white letters); primed numbers represent the histidines proximal ligands to the respective heme *c* molecules as deduced from the KsHOX and NeHAO crystal structures. Heme 3 in KsHDH and its homologs having an unusual CX₄CH binding motif is highlighted pink (white lettering). The tyrosine involved in the covalent binding to the catalytic heme 4 (P₄₆₀) in KsHOX (Tyr-491) and NeHAO is highlighted purple (white letters). The aspartate, histidine and tyrosine residues (Tyr-358 in NeHAO) near the catalytic site are highlighted blue (white letters). Note that the tyrosine is apparently conserved in all proteins. However, in KsHOX the tyrosine is moved away from the catalytic site by several Ångströms by a two-amino acid contraction and it is replaced at that position by a methionine (Met-323) (12). This same contraction is found in the HDH proteins. A 15-amino acid sequence in the C-terminal part, which is specific for KsHDH and its close homologs is printed in bold. Peptide sequences identified for KsHDH by MALDI-TOF analyses are underlined. Protein identifiers and abbreviations represent the following: kust, *K. stuttgartiensis*; KSU-1_HzoB (ZP_10100863) and KSU-1_HzoB (ZP_10098714), hydrazine dehydrogenase/ oxidase (Hzo) from anammox enrichment culture KSU-1; BROSI, anammox bacterium *Brocadia sinica* (PRJDB103); scal, HAOs from anammox bacterium *Scalindua profunda* (Taxon Object IDs 2017108002 and 2022004002 at JGI).

