Purification of the key enzyme complexes of the anammox pathway from DEMON sludge

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Abstract
Anaerobic Ammonium Oxidation ("anammox") is a bacterial process in which nitrite and ammonium are converted into nitrogen gas and water, yielding energy for the cell. Anammox is an important branch of the global biological nitrogen cycle, being responsible for up to 50% of the yearly nitrogen removal from the oceans. Strikingly, the anammox process uniquely relies on the extremely reactive and toxic compound hydrazine as a free intermediate. Given its global importance and biochemical novelty, there is considerable interest in the enzymes at the heart of the anammox pathway. Unfortunately, obtaining these enzymes in sufficiently large amounts for biochemical and structural studies is problematic, given the slow growth of pure culture anammox bacteria when high cell densities are required. However, the anammox process is being applied in wastewater treatment to remove nitrogenous waste in processes like DEamMONification (DEMON). In plants using such processes, which rely on a combination of aerobic ammonia-oxidizers and anammox organisms, kilogram amounts of anammox bacteria-containing sludge are readily available. Here, we report a protein isolation protocol starting from anammox cells present in DEMON sludge from a wastewater treatment plant that readily yields pure preparations of key anammox proteins in the tens of milligrams, including hydrazine synthase (HZS) and hydrazine dehydrogenase (HDH), as well as hydroxylamine oxidoreductase (HAO). HDH and HAO were active and of sufficient quality for biochemical studies and for HAO, the crystal structure could be determined. The method presented here provides a viable way to obtain materials for the study of proteins not only from the central anammox metabolism but also for the study of other exciting aspects of anammox bacteria, such as for example, their unusual ladderane lipids.

KEYWORDS
Anammox, enzyme complexes, protein purification

1 | INTRODUCTION

Nitrogen is an essential element for all life on Earth, but although molecular nitrogen (N₂) is the most abundant gas in our atmosphere, it is unavailable to most biological systems due to its low chemical reactivity in this form. For nitrogen to be incorporated into biological molecules, N₂ must first be converted to ammonium (NH₄⁺). This process, known as nitrogen fixation, is the first step in the biological nitrogen cycle, a set of metabolic reactions driven primarily by microorganisms that take the element through its various oxidation states.
which range from $-\text{III}$ to $+\text{V}$. Although most of these processes, such as nitrification, denitrification and ammonification are well known and understood, our picture of the biological nitrogen cycle was in fact incomplete until the discovery of anaerobic ammonium oxidation (anammox) in the 1990s. This process generates energy for the cell by combining ammonium with nitrite ($\text{NO}_2^-$), producing nitrogen gas and water via the highly unusual intermediate hydrazine. Moreover, anammox is estimated to be responsible for up to 50% of the total $\text{N}_2$ release from the oceans. Given its global importance and the highly unusual biochemistry involved, the anammox process has been the subject of much scientific interest.

In our current understanding of the anammox process, nitrite is first converted to nitric oxide ($\text{NO}$) in a one-electron reduction by a nitrite reductase. NO is then combined with $\text{NH}_3$ in a three-electron reduction reaction by the unique hydrazine synthase (HZS) enzyme complex, resulting in the formation of hydrazine ($\text{N}_2\text{H}_4$). This reaction likely proceeds via the formation of hydroxylamine ($\text{NH}_2\text{OH}$) which is channeled between two subunits of the HZS complex. The hydrazine, in turn, is oxidized to $\text{N}_2$ by hydrazine dehydrogenase (HDH) resulting in the release of three electrons. These various redox reactions drive an electron transport chain used to generate energy for the cell. Another key enzyme found in anammox bacteria is hydroxylamine oxidoreductase (HAO), which likely serves to convert any hydroxylamine escaping from the HZS complex back into NO. However, while considerable progress in elucidating the details of the anammox process had been made in recent years, many important questions remain.

The first anammox bacterium to be discovered, Candidatus Brocadia anammoxidans, was found in a waste water treatment plant (WWTP). Since then, various genera of anammox bacteria have been reported to inhabit virtually all possible habitats such as the soil, various marine ecosystems, freshwater systems, hydrothermal springs and low temperature zones. Nevertheless, anammox bacteria have posed considerable challenges in their cultivation, requiring sophisticated equipment such as sequencing batch reactors (SBRs), upflow-anoxic sludge blanket (UASB) reactors, non-woven biomass carriers and rotating biological contactors. All of these methods are based on the settling of bacterial aggregates embedded in biofilms containing extracellular polymeric substances (EPS). These EPS are comprised of various components such as proteins, nucleic acids, charged and neutral carbohydrates and humic acids. The resulting biomass formed gels at low temperatures, making it highly viscous and glutinous, complicating the purification of proteins from it for further biochemical studies.

The successful cultivation of planktonic cells in a membrane bioreactor (MBR) solved this problem: MBR-derived material could easily be used for protein purification and enabled several detailed biochemical and structural studies of anammox proteins. However, enrichment of anammox bacteria in laboratory bioreactors is still extremely laborious and takes long periods of time (180-280 days to enrich up to ~80% in SBRs and ~350-400 days to enrich up to 95% in MBRs) when high cell densities are required to obtain large amounts (tens of grams) of biomass. In addition, significant feeding and monitoring capacities are needed to run such culture systems. These combined factors limit the supply of anammox cell material, seriously hindering anammox research. Despite recent progress in the elucidation of several central steps in the anammox pathway, numerous questions regarding the biochemistry of anammox bacteria remain, such as the roles of several membrane-bound complexes and many of the abundant c-type cytochromes, or the biosynthetic mechanism of their unique “ladderane” lipids. Therefore, it is necessary to find effective and fast ways to obtain anammox bacterial cell material that can readily be used for biochemical studies.

Recently, the anammox process has been applied increasingly in wastewater treatment, since it provides an alternative to conventional nitrogen removal systems that is both economical and environmentally friendly (reviewed by Kartal et al). Many species of anammox bacteria have been discovered in WWTPs, including Ca. Brocadia carolinensis, Ca. B. sinica, Ca. B. fulgida, Ca. Scalindua brodae and Ca. S. wagneri. Anammox-based wastewater treatment processes combine nitrification and anammox. In the SHARON-ANAMMox system, the nitrification process takes place in one reactor, and the anammox process in another. In other systems, the nitrification and anammox processes take place in the same reactor. This is the case for the CANON, SNAP and DEMON systems. In the DEMON process, from which the material for this study was obtained, a pH-controlled aeration system ensures that enough oxygen is present for nitrifying bacteria to convert about half of the incoming ammonia to nitrite. Anammox bacteria living in the same reactor then convert this nitrite and the remaining ammonia into nitrogen gas and water. The biomass in such a reactor consists of reddish granules and brown flocules. The flocules and granules from a DEMON plant in Strass, Austria have been separated and a phylogenetic analysis based on 16S rRNA sequencing was performed. This showed that the red granules were mainly inhabited by anammox bacteria closely related to Ca. Brocadia anammoxidans, while the bacterial population in the flocules was more diverse.

In this study, we investigate the presence of anammox bacteria and the bacterial population diversity in the granules from a full-scale DEMON plant in Baden-Württemberg, Germany. We selected a full-scale plant because removal of even several kilograms of biomass from the >1000 m³ total reactor volume has no significant effects on plant operation. Importantly, we purify central enzymes of the anammox pathway, such as Ca. fulgida hydroxylamine dehydrogenase (BH-DHD), hydrazine synthase (BH-HZS) and hydroxylamine oxidoreductase (BH-HAO). We show that these proteins are enzymatically active and that one of them yields crystals amenable to structure solution. The granular material was well behaved during purification, in contrast to the material obtained from an SBR culture of Kuenenia stuttgartiensis described earlier. These investigations demonstrate the possibility of using granular anammox material from WWTPs for biochemical and structural studies. Moreover, so far, most research on anammox biochemistry has been focused on the model organisms Kuenenia stuttgartiensis and Jettenia caeni, while other genera have remained largely unexplored biochemically. As many other anammox species also inhabit full-scale wastewater treatment reactors, this study investigates the possibility of accessing them in a convenient and cost-effective manner.
2 | MATERIALS AND METHODS

2.1 | Safety considerations

Although the DEMON process is typically used as a final step in wastewater purification, that is, after several other purification steps have been performed and the water is already largely purified, the material used in these experiments was obtained from a wastewater treatment plant processing waste from residential areas. Therefore, hepatitis A/B vaccinations were offered to all personnel working with the material, after consultations with the laboratory safety officers and a physician specializing in occupational safety. Depending on location and other relevant factors, other or additional vaccinations should be considered when these or similar experiments are to be reproduced using material from other wastewater treatment facilities.

2.2 | Collection and immediate processing of DEMON sludge

Biomass was removed from a deammonification (DEMON) plant in Baden-Württemberg, Germany, which has a total reactor volume of 1100 m³, and a maximum capacity of 480 m³/d, reducing the nitrogen (measured as ammonium) in the intake from ~1000 to <150 mg/L. The material was obtained as brown granules in a clear, colorless and odorless liquid. Sludge was collected below a hydrocylcone to enrich the granules which settled in minutes, after which the remaining supernatant was decanted. The remaining liquid was removed by pressing through a cotton cloth. 17 kg (wet-weight) of granules were used in this study. In order to prepare the sludge, it was frozen, ground in liquid nitrogen and kept at −20 °C until further processing. The material was then split into 100 ml portions, which were each subjected to sonication immediately after adding 100 μL of a 100 mM stock solution of phenylmethylsulfonyl fluoride in isopropanol. Sonication was performed with a Branson W-450 sonicor (G. Heinemann, Schwäbisch Gmünd, Germany) set to 50% amplitude, 0.5 s bursts with a total time of 10 min per portion. After sonication, the material was clarified by ultracentrifugation for 1 hr in a 45Ti rotor at 160000g, at 4 °C.

2.3 | DNA extraction and phylogenetic analysis

In order to determine the microbial diversity in the sample, genomic DNA was extracted from the DEMON granules using the PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, California). Fragments of genes encoding metabolic proteins and 16S rRNA were amplified from this material by PCR with Phusion High-Fidelity DNA Polymerase (New England Biolabs, Schwalbach, Germany), using degenerate primers for the α subunit (hzsA526F/1829R),[49] hydroxylamine oxidoreductase (haof1/haor3),[50] and 16S rRNA gene fragments from Planctomycetes (Pha46F/1392R).[51,52] Sequences and conditions are tabulated in the supplemental information (Tables S1 and S2, respectively). Any amplified fragments were investigated by agarose gel electrophoresis, after which they were cloned with the Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Carlsbad, California).

Plasmids were isolated using Wizard Plus SV Minipreps (Promega, Madison, Wisconsin) and checked for inserts of the expected size using EcoRI digestion followed by agarose gel electrophoresis. Positive clones were sequenced using the SP6 and M13 forward primers by GATC Biotech AG (Konstanz, Germany). SEQUATOR (http://sequator.com/index.html) was used to align raw sequencing results, whereas multiple DNA and protein sequences were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).[53] Phylogenetic trees were constructed with the Phylogeny.fr server (http://www.phylogeny.fr/).[54] Based on multiple sequence alignment results obtained using MUSCLE,[55] refinement of the alignments in Gblocks,[56] and maximum-likelihood tree reconstruction with PhyML,[57] MEGA 6.0[58] was used to prepare phylogenetic tree figures.

2.4 | Cell lysis

Approximately 200 g of DEMON granules kept at −20 °C were mixed with 600 ml buffer A (20 mM Tris-HCl pH 8.0) and the material was dispersed by stirring at 8 °C. Then, the mixture was homogenized in ~50 ml portions in a 100 ml Potter tube equipped with a strong stirring motor in order to rotate the pestle at 1000-1400 rpm. The dark brown material was centrifuged for 20 min (4 °C, 8000g) and the pellet mixed with another 600 ml of buffer A. For each 100 ml of material, two Complete protease inhibitor cocktail tablets were added (Roche Applied Science, Mannheim, Germany), after which lysozyme and DNase I were added to concentrations of 0.25 and 0.05 mg/ml, respectively. The material was then split into 100 ml portions, which were each subjected to sonication immediately after adding 100 μL of a 100 mM stock solution of phenylmethylsulfonyl fluoride in isopropanol. Sonication was performed with a Branson W-450 sonicator (G. Heinemann, Schwäbisch Gmünd, Germany) set to 50% amplitude, 0.5 s bursts with a total time of 10 min per portion. After sonication, the material was clarified by ultracentrifugation for 1 hr in a 45Ti rotor at 160000g, at 4 °C.

2.5 | Protein purification

The protocol for protein purification described here was modified from that described earlier,[59] adding a final size exclusion chromatography step. The clarified lysate was first submitted to a Q Sepharose column (50 ml, GE Healthcare, Uppsala, Sweden) (measured as ammonium) in the intake from ~1000 to <150 mg/L. The material was obtained as brown granules in a clear, colorless and odorless liquid. Sludge was collected below a hydrocylcone to enrich the granules which settled in minutes, after which the remaining supernatant was decanted. The remaining liquid was removed by pressing through a cotton cloth. 17 kg (wet-weight) of granules were obtained from 50 L of DEMON sludge. The material was shock-frozen in liquid nitrogen and kept at −20 °C until further processing.

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Superose 6 (10/300 GL) column (GE Healthcare, Uppsala, Sweden). The purified proteins were concentrated by ultrafiltration to an $A_{280} \text{ cm}^{-1} \text{ cm}^{-1}$ of ~20 for both HDH and HAO, and ~45 for HZS, flash-frozen in small aliquots in liquid nitrogen and stored at ~80 °C. The purity of the final preparations was checked using 15% SDS-PAGE, where the gels were first stained with a heme-sensitive method, followed by Coomassie staining.

### 2.6 Protein identification by MALDI mass spectrometry

To confirm protein identities, slices containing the relevant bands were excised from 15% SDS-PAGE gels and digested with trypsin. The resulting fragments were analyzed by MALDI-TOF on an Axima TOF (Shimadzu Biotech, Duisburg, Germany), with α-cyano-4-hydroxycinnamic acid as the matrix. Tryptic peptides were identified using the MASCOT software (Matrix Science Inc., Massachusetts). Total mass analysis was performed by electrospray ionization time-of-flight (ESI-TOF) mass spectrometry on a maXis spectrometer (Bruker Daltonik GmbH, Bremen, Germany).

### 2.7 UV-Vis Spectroscopy

Proteins were diluted in 20 mM potassium phosphate buffer, pH 7.0, after which spectra were measured in 200 μl quartz micro-cuvettes (1 cm path length; Hellma Analytics, Müllheim, Germany) at 25 °C using a Jasco V-650 spectrophotometer (Jasco GmbH, Gross-Umstadt, Germany) at 0.5 nm bandwidth. Spectra were first collected in the as-isolated state of the proteins, after which a few crystals of sodium dithionite were added to collect spectra of the reduced states. The effects of substrates on the spectra were investigated by adding 10 mM of either hydroxylamine hydrochloride or hydrazine sulphate (pH set to 7.0 in each case). Spectra were processed using the Jasco software and plotted using OriginLab 8.0.

### 2.8 Activity assays

The activities of $Bf$HAO and $Bf$HDH were assayed as was described for the *K. stuttgartiensis* enzymes, with minimal modifications. In each case, the reduction of bovine heart cytochrome c (cyt c; Sigma-Aldrich, Schnelldorf, Germany) was measured by following the increase in absorption at 550 nm ($\Delta\varepsilon = 19,600 \text{ M}^{-1} \text{ cm}^{-1}$). One milliliter reaction mixtures were prepared, containing 50 μM cyt c and 1 μg of the respective enzymes.

**FIGURE 1** Biodiversity of DEMON granules. Genbank accession numbers are shown on the right. A, Diversity of anammox organisms as investigated by amplification of hydrazine synthase alpha (HZSa) genes. A single consensus HZSa sequence was obtained from the DEMON sample (red dot) which showed 99.7% sequence identity to the HZSa from *B. fulgida*. B, Diversity of AOB as investigated by amplification of hydroxylamine oxidoreductase (HAO) genes. The observed clones cluster with *Nitrosomonas europaea* or *N. eutropha*.
enzyme in 20 mM potassium phosphate, pH 7.0. Measurements were performed in polystyrene cuvettes (Sarstedt AG, Nümbrecht, Germany), recording the absorption at 550 nm using a Jasco V-650 spectrophotometer (Jasco GmbH, Gross-Umstadt, Germany) at 35 °C. After following the absorption for 1 min, the reaction was started by adding the appropriate amount of substrate (hydroxylamine hydrochloride, pH set to 7.0 for HAO and hydrazine sulfate, pH 7.0 for HDH). To investigate the influence of hydroxylamine on HDH activity, 1 mM hydroxylamine was added to the mixture before starting the reaction. Initial reaction rates were extracted by fitting with GraphPad Prism (GraphPad Software Inc., La Jolla, California) using the Michaelis-Menten equation.

2.9 | Crystallization, data collection and structure determination

Protein samples were concentrated to an A_{280}^{1cm} of 20 for HAO and HDH, or 30 for HZS, in 25 mM HEPES/KOH, 25 mM KCl, pH 7.5. Crystallization conditions were screened in 96-well plate sitting drop setups with a Mosquito LCP crystallization robot (TTPlabtech, Cambridge, UK). All crystallization setups were kept at 20 °C. Small, dark red crystals grew within a few days from setups containing B/HAO using 1.0 M lithium chloride, 0.1 M Tris pH 8.5 and 10% w/v PEG 6000 as the reservoir solution. These crystals were cryoprotected in mother liquor supplemented with 25% (v/v) ethylene glycol and flash-cooled in liquid nitrogen. Diffraction data collected at the X10SA beamline at the Swiss Light Source (SLS) of the Paul-Scherrer-Institute (Villigen, Switzerland) at 100 K on a PILATUS 6 M detector (Dectris, Baden, Switzerland) which were processed using XDS to phase by molecular replacement with PHASER, using the structure of the HAO from _Kuenenia stuttgartiensis_ (KsHAO, PDB: 4N4J) as the search model. The structure was iteratively rebuilt using COOT and refined with REFMAC. Data collection and refinement statistics are given in supplemental Table S3. The final structure was validated using the MolProbity server (http://molprobity.biochem.duke.edu/) and

![FIGURE 2](image_url)

**FIGURE 2** A, Purification of anammox proteins from DEMON granules as described in the text. B, 4-20% Gradient Tris-glycine SDS-PAGE gel of the protein preparations. M: Molecular size marker, with the molecular masses indicated in kDa. α/β/γ: hydrazine synthase subunits. Since the β-subunit of hydrazine synthase does not contain heme, it does not show up with heme staining (right).
3 | RESULTS AND DISCUSSION

3.1 | Identity of anammox bacteria in DEMON granules

Since the first anammox-based WWTP in Rotterdam commenced operation in 2002, the number of such plants has grown to over 100 worldwide and the number is still increasing. Thus, material from such plants is becoming more and more accessible for research purposes. DEMON granules are usually dense, with an anaerobic core containing the anammox bacteria and a micro-aerobic outer layer in which the aerobic ammonium-oxidizing bacteria (AOB) reside. On the other hand, the smaller, low-density floccules also encountered in such reactors are less likely to possess an anaerobic core with anammox bacteria. Thus, the size and density of the granules are an important measure of the health of the anammox-based sludge and are good indicators for the suitability of the material for protein purification from anammox bacteria. The significant difference in the density between floccules and granules helps to separate them while suspended in aqueous solution.

With the method employed here, the only anammox species that could be detected in granular sludge obtained from the DEMON reactor sampled was *Brocadia fulgida* (Figures 1A and S1). Several other studies on the biodiversity of WWTPs also reported *Brocadia* as the dominant genus of anammox bacteria. Likewise, the AOB in the analyzed DEMON granules appears dominated by *Nitrosomonas europaea* and *N. eutropha* (Figure 1B). Although we did not determine the relative abundance of anammox bacteria and AOB, the multi-milligram yields of anammox proteins (see below) indicate that a considerable fraction of the bacterial population is made up by anammox organisms.

3.2 | Protein purification

To access the anammox cells, efficient destruction of the highly resilient EPS making up the bulk of the granules was essential. To this end, around 200 g of frozen granules were first subjected to an aggressive mechanical step in order to achieve homogenization. Lysozyme was added to assist lysis and DNase I was added to mitigate the viscosity caused by nucleic acids. A protease inhibitor cocktail was used to minimize proteolysis. The cells were then lysed by sonication in small portions. Using this protocol, we obtained a cleared lysate that did not form a gel at low temperatures, even in the absence of reducing- or chelating agents or at high salt concentrations. Possibly, lysate obtained from DEMON granules contains other polysaccharides or other potentially gel-forming materials than that obtained from *Kuenenia stuttgartiensis* cultures. This would mean that protein purification from DEMON biomass offers considerable advantages. After ultracentrifugation, the lysate was immediately loaded onto a strong anion exchange column (Q Sepharose). This step might be critical in removing most of the remaining EPS materials, which mainly consist of negatively charged polysaccharides which would bind strongly to the column and only elute at very high salt concentrations. Indeed, when a DEAE (a weak anion exchanger)
Michaelis-Menten kinetics of BfHAO and BfHDH compared with their homologues

| Organism                  | Brocadia fulgida | Jettienia caeni | Kuenenia stuttgartiensis | Brocadia anammoxidans |
|---------------------------|------------------|-----------------|--------------------------|-----------------------|
| HAO Kinetics              |                  |                 |                          |                       |
| Locus tag                 | Broful00019 (BfHAO) | KSU1_D0438 (JcHAO) | Kustc1061 (KsHAO)         | BuHAO                 |
| $V_{\max}^{\text{NH}_2\text{OH}}$ ($\mu$mol min$^{-1}$ mg$^{-1}$) | 1.71             | 9.6             | 4.8 ± 0.2                | 21                    |
| $K_{\text{cat}}^{\text{NH}_2\text{OH}}$ ($s^{-1}$) | 5.2              | 19              | 15                       | 64                    |
| $K_{\text{M}}^{\text{NH}_2\text{OH}}$ ($\mu$M) | 20               | 33              | 4.4 ± 0.9                | 26                    |
| $K_{\text{cat}}/K_{\text{M}}^{\text{NH}_2\text{OH}}$ ($s^{-1}$ $\mu$M$^{-1}$) | 0.3              | 0.58            | 3.4                      | 2.5                    |
| MW oligomer (kDa)         | 184              | 174a            | 184                      | 183                   |
| MW monomer (kDa)          | 61               | 58              | 61.5                     | 58                    |
| Oligomer                  | $\alpha_3$       | $\alpha_a$      | $\alpha_3$               | $\alpha_3$            |
| P460 absorption maximum (nm) | 468              | 468             | 468                      | 468                   |
| References                | This study       | 47              | 11                       | 76                    |

| HDH kinetics              |                  |                 |                          |                       |
| Locus tag                 | Broful01550-51 (BfHDH) | KSU1_B0738 (JcHDH A) | Kustc0694 (KsHDH)         | BuHDH                 |
| $V_{\max}^{\text{N}_2\text{H}_4}$ ($\mu$mol min$^{-1}$ mg$^{-1}$) | 0.51             | 6.2             | 11 ± 1.2                 | ND                    |
| $K_{\text{cat}}^{\text{N}_2\text{H}_4}$ ($s^{-1}$) | 1.7              | 13.4            | 36                       | ND                    |
| $K_{\text{M}}^{\text{N}_2\text{H}_4}$ ($\mu$M) | 7.6              | 5.5             | 10 ± 2.2                 | ND                    |
| $K_{\text{cat}}/K_{\text{M}}^{\text{N}_2\text{H}_4}$ ($s^{-1}$ $\mu$M$^{-1}$) | 0.22             | 2.4             | 3.5                      | ND                    |
| MW oligomer (kDa)         | 200.7 (~1600)    | 186a            | 201.5 (~1600)            | ND                    |
| MW monomer (kDa)          | 66.9             | 62              | 67.2                     | ND                    |
| Oligomer                  | $\alpha_3$ ($[\alpha_3]_a$) | $\alpha_a$        | $\alpha_3$ ($[\alpha_3]_a$) | ND                    |
| P460 absorption maximum (nm) | 473              | 472             | 473                      | 473                   |
| References                | This study       | 48              | 10                       |                       |

Note: Values for the J. caeni, K. stuttgartiensis, and B. anammoxidans enzymes are reproduced from the references given below each column. Abbreviation: ND, not determined.

*Previously, size-exclusion chromatography results suggested JcHAO$^{47}$ and JcHDH$^{48}$ to be dimers. The same method applied to KsHAO and BfHAO suggested these proteins to be dimeric, too. However, because several more accurate biophysical methods (AUC, crystallography and SEC-MALS) unambiguously show that these proteins are trimeric, we believe that JcHAO and JcHDH are most likely trimers in solution as well.

3.3 | UV-Vis spectroscopy

Both BfHAO and BfHDH exhibited Soret peaks at 408 nm as well a broad absorption around 550 nm (Figure 3A,B), as was described earlier for the corresponding enzymes from Kuenenia stuttgartiensis, KsHAO and KsHDH. The $A_{408}/A_{280}$ ratio was 3.5 for both proteins. Upon reduction with dithionite, the Soret band shifted to 420 nm and $\alpha$- and $\beta$-band appeared at 554 and 524 nm for both BfHAO and
BfHDH (Figure 3A,B). An additional band at around 468 nm for BfHAO (Figure 3A) and at 473 nm for BfHDH (Figure 3B) also appeared, which is caused by the tyrosine-heme crosslink in the P₄₆₀ cofactor. BfHZS possessed UV-Vis spectroscopic features similar to those described for the homologous HZS from Kuenenia stuttgartiensis (KsHZS).[8] In the as-isolated state, the Soret band was located at

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**FIGURE 4** X-ray crystal structure of BfHAO. A, overall structure of the BfHAO trimer shown in two perpendicular orientations. B, the fold of BfHAO (red) is very similar to that of the K. stuttgartiensis homolog (green). C, Positions and orientations of the heme cofactors in the trimers of BfHAO (gray) and KsHAO (blue). The P₄₆₀ cofactors are shown in yellow. D, Closeup of the active site. In addition to the covalent bonds to the cysteines of the CXXCH binding motif that are typical for c-type hemes, the P₄₆₀ cofactor engages in two covalent bonds to Tyr₄₄⁹* from an adjacent subunit, causing strong ruffling of the porphyrin ring. Residues 258-261 were not visible in the electron density maps.
407 nm and a broad band was observed in the 550 nm region. The $A_{407}/A_{350}$ ratio was 1.5. Upon reduction with dithionite, the Soret band was red-shifted to 420 nm and the characteristic α- and β-bands appeared at 554 and 523 nm, respectively (Figure 3C). In all cases, dithionite reduction led to an increase of the Soret band intensity. $Bf$HZS, $Bf$HAO and $Bf$HDH were incubated with hydroxylamine and hydrazine in their as-isolated state. The effects of the substrates were similar to those described for KsHZS, KsHAO, and KsHDH, respectively.\cite{5,10,11} For instance, there was no effect of either hydroxylamine or hydrazine on the UV-Vis spectrum of $Bf$HZS, whereas hydroxylamine resulted in the partial reduction of $Bf$HAO (Figure 3A). Hydrazine also caused partial reduction of $Bf$HAO, but with less prominent spectroscopic effects than hydroxylamine (Figure 3A). For $Bf$HDH, hydroxylamine did not have any effect on the UV-Vis spectrum, whereas its substrate hydrazine caused partial reduction of the enzyme (Figure 3B).

### 3.4 Activity assays

The ability of $Bf$HDH to catalyze the four-electron oxidation of hydrazine to $N_2$ was demonstrated using excess bovine cytochrome $c$ as electron acceptor. The oxidation of hydrazine followed Michaelis-Menten kinetics with a $V_{max}$ of 0.51 μmol min$^{-1}$ mg$^{-1}$ protein and a $K_s$ of 7.6 μM for the substrate hydrazine. These data are in line with published kinetic data for HDH from other sources (Table 1). As expected, hydroxylamine inhibited the activity of $Bf$HDH as previously shown for KsHDH. Similar to KsHAO,\cite{11} $Bf$HAO also catalyzed the oxidation of hydroxylamine, when excess bovine cytochrome $c$ was used as an artificial redox partner. $Bf$HAO followed Michaelis-Menten kinetics with a $V_{max}$(NH$_2$OH) of 1.7 μmol min$^{-1}$ mg$^{-1}$ and a $K_s$(NH$_2$OH) of 20 μM, these values being similar to the published catalytic properties of other HAO proteins (Table 1). $Bf$HAO was also observed to oxidize hydrazine; albeit at a much lower rate (Michaelis-Menten parameters were not calculated). These results indicate that $Bf$HDH and $Bf$HAO purified from a full-scale deammonification reactor can be used for further biochemical and biophysical characterization.

### 3.5 Structure of $Bf$HAO

As expected from the sequence similarity (~87%), the fold of $Bf$HAO is very similar to that of KsHAO (PDB id: 4N4J),\cite{11} and the two structures can be superimposed to an rmsd of 0.57 Å for 436 Cx atoms (Figure 4B). $Bf$HAO consists mainly of α-helices, with the central axis of the trimer being surrounded by three parallel helices from each subunit running parallel around a central cavity (Figure 4A). The active site heme 4 shows the typical binding pattern for a c-type heme to its associated C$^{2+}$XXC$^{2+}$H$^{2+}$ binding motif: the heme’s vinyl groups are covalently bound to Cys221 and Cys224, while the proximal side of the heme iron is coordinated by His225. However, as in KsHAO, the heme’s C4 and C5 atoms form covalent bonds with Tyr449 from an adjacent subunit, resulting in a striking degree of ruffling of the heme\cite{11,77-79} (Figure 4D) as is typical for octaheme c-type cytochromes catalyzing oxidative reactions, and which is believed to determine the direction of electron flow in these enzymes.\cite{6,9,11,80}

No density was observed for a distal ligand, although the presence of a small ligand such as a solvent molecule cannot be excluded at the current resolution. The other seven c-type hemes in the monomer all show bis-His ligation. Together, the 24 hemes present in the trimer form a ring-like structure that is very similar to that found in KsHAO (Figure 4C). Three regions, Gln314-Lys334, Gly258-His261 (containing the catalytic His/Asp pair\cite{11}), and part of the “N1”-domain found in KsHAO\cite{11} did not show electron density and were therefore not modeled. These regions surround the tunnel leading to the active site. Given the similarity between $Bf$HAO and KsHAO, it appears reasonable to assume that these regions occupy comparable positions in $Bf$HAO. As they are located on the surface of the protein, the Gln314-Lys334 and N1 regions could have been proteolytically cleaved. In case of the Gly258-His261 loop, however, this appears unlikely as it is buried inside the complex. Thus, it is more likely that the lack of electron density for this loop is caused by disorder. Indeed, in the phenylhydrazine complex of KsHAO the corresponding loop occupied another position than in the unliganded structure, illustrating the flexibility of this region in the Kuenenia homolog.\cite{11} Interestingly, the Gln314-Lys334 loop covers both the active site heme 4, as well as two other c-type hemes in the KsHAO structure. Possibly, flexibility of this loop allows access to the c-type heme binding sites for the enzymes involved in forming the bonds between the c-type hemes and their binding motifs.

### 4 CONCLUSION

Research into anammox biochemistry has been complicated by the fact that the key enzymes such as HZS, HAO and HDH contain multiple cofactors, often of a unique nature and/or covalently attached, and therefore cannot be expressed heterologously. Thus, these proteins have had to be painstakingly purified from slow-growing anaerobic cultures, and obtaining yields in the tens of mg range has only been possible because of the high expression levels of these proteins. Expanding these investigations to as yet unstudied anammox species and/or proteins that are not as highly abundant, such as for example, the various membrane complexes implicated in the anammox process, or those involved in the synthesis of the unique ladderane lipids found in anammox bacteria,\cite{35} would benefit greatly from a convenient supply of large amounts of biomass. Here we establish the usefulness of granular sludge from a deammonification (DEMON) reactor for the isolation of proteins with a view to studying the anammox pathway. We show that at the time of sampling, the dominating anammox species in the DEMON granules was *Brocadia fulgida*. We demonstrate that enzymes central to the anammox metabolism such as HZS, HAO and HDH can be purified in good yield: several mg were obtained from only 200 g of DEMON biomass, an amount of starting material...
that is insignificant compared to the virtually unlimited amounts readily available in full-scale DEMON plants. As demonstrated, the amounts of purified protein obtained are more than sufficient for biochemical investigations such as enzyme kinetics or binding studies. The amounts obtained are sufficient even for crystallographic studies, which typically require comparatively large amounts (several mg) of sample. Less abundant proteins than those investigated here would of course yield smaller amounts from the same quantity of starting material, but the current shift in structural biology towards cryo-electron microscopy, which requires much less sample, will ameliorate this. Indeed, *Brocadia fulgida* HDH purified from DEMON sludge using this protocol has already been used in a structural- and biochemical study of hydrazine oxidation by anammox organisms.

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**CONFLICT OF INTEREST**

The authors declare no conflict of interests.

**DATA AVAILABILITY STATEMENT**

Crystal structure coordinates and structure factor amplitudes have been deposited in the protein data bank (http://www.rcsb.org) under accession code 6T5E (DOI: 10.2210/pdb6T5E/pdb). All other data are available from the authors upon request.

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