Cytochrome c nitrite reductase from the bacterium Geobacter lovleyi represents a new NrfA subclass

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Cytochrome c nitrite reductase (NrfA) catalyzes the reduction of nitrite to ammonium in the dissimilatory nitrate reduction to ammonium (DNRA) pathway, a process that competes with denitrification, conserves nitrogen, and minimizes nutrient loss in soils. The environmental bacterium Geobacter lovleyi has recently been recognized as a key driver of DNRA in nature, but its enzymatic pathway is still uncharacterized. To address this limitation, here we overexpressed, purified, and characterized G. lovleyi NrfA. We observed that the enzyme crystallizes as a dimer but remains monomeric in solution. Importantly, its crystal structure at 2.55-Å resolution revealed the presence of an arginine residue in the region otherwise occupied by calcium in canonical NrfA enzymes. The presence of EDTA did not affect the activity of G. lovleyi NrfA, and site-directed mutagenesis of this arginine reduced enzymatic activity to <3% of the WT levels. Phylogenetic analysis revealed four separate emergences of Arg-containing NrfA enzymes. Thus, the Ca2+-independent, Arg-containing NrfA from G. lovleyi represents a new subclass of cytochrome c nitrite reductase. Most genera from the exclusive clades of Arg-containing NrfA proteins are also represented in clades containing Ca2+-dependent enzymes, suggesting convergent evolution.

The global nitrogen cycle is an indispensable biogeochemical process, as nitrogen is a fundamental component of every living organism (1). One of its critical pathways is the dissimilatory nitrate reduction to ammonium (DNRA), a respiratory process that involves the reduction of nitrate to nitrite followed by the conversion of nitrite to ammonium (2). In contrast to denitrification, the DNRA process does not release nitrogen as gaseous compounds (e.g. N2 and the greenhouse gas N2O), thereby conserving nitrogen in the ecosystem and minimizing nutrient loss (3–5). The second step in the DNRA pathway, the conversion of nitrite to ammonium, is a six-electron, eight-proton process catalyzed by cytochrome c nitrite reductase, also known as NrfA (6, 7). In all available crystal structures to date (8–13), the enzyme is a dimer and contains five heme c cofactors per monomer with a Ca2+ ion located at the distal side of the heme-1 active site. Although the exact role of the calcium ion is uncertain, it is proposed to be involved in proton delivery (14) and structural stability (13), and/or to contribute to a positive electrostatic potential that is essential for substrate delivery to the active site (13, 14).

Geobacter lovleyi has emerged as a model representative of environmental bacteria that drives DNRA in nature (15). This organism has been shown to dominate a microbial community in an enrichment culture grown under limiting nitrate (16–18), a condition that enhances DNRA over denitrification. The switch from nitrate to nitrite as the electron acceptor in the enrichment culture did not change the dominant ribotype, suggesting that the DNRA pathway of G. lovleyi can efficiently use both substrates (17). This physiology was unexpected, given that Geobacter species are generally known to be reducers of iron oxide minerals (15).

In this paper, we report a NrfA subclass from G. lovleyi SZ. The isolated enzyme shares 31% or lower identity to the previously crystallized NrfAs. In contrast to the monomer-dimer equilibrium displayed by its homolog from Escherichia coli (19), the G. lovleyi NrfA remains a monomer in solution, enabling homogeneous samples for future mechanistic investigations. Notably, the crystal structure of G. lovleyi NrfA does not contain a calcium ion near the heme-1 active site, as in canonical NrfA enzymes. Instead, the enzyme contains an arginine in the region occupied by calcium in other crystallized homologs. Phylogenetic analysis and structure comparison provide insights into the function of the calcium ion in Ca2+-dependent NrfA homologs and adaptive responses of some environmental DNRA bacteria for Ca2+-independent nitrite reduction.

Results

G. lovleyi NrfA was heterologously overexpressed in S. oneidensis

We explored several strategies to overexpress G. lovleyi NrfA, ultimately selecting S. oneidensis MR-1 as the most efficient heterologous host. S. oneidensis expresses c-type heme proteins under both aerobic and anaerobic conditions (20), and strategies for overexpressing these proteins are well developed (21). Purification by Strep-tag II affinity chromatography followed by size-exclusion chromatography yielded 6–8 mg of highly purified WT NrfA per liter of culture, as indicated by denaturing gel electrophoresis (Fig. S1). The mass of the parent
New subclass of NrfA isolated from Geobacter lovleyi

Table 1
Data from dynamic light scattering

| Concentration (µM) | R_H (nm) | Polydispersity index (%) |
|-------------------|----------|--------------------------|
| 24                | 3.4      | 11.9                     |
| 52                | 3.6      | 10.9                     |
| 105               | 3.4      | 11.9                     |
| 165               | 3.4      | 11.9                     |
| 175               | 3.4      | 12.5                     |
| 266               | 3.3      | 13.6                     |
| 327               | 3.1      | 4.3                      |

Table 2
Crystallographic statistics

| Parameter                  | Value(s) for G. lovleyi NrfA-1 |
|----------------------------|--------------------------------|
| No. of unique reflections | 122,238                        |
| No. of atoms               | 23,376                         |
| Protein (n)                | 21,161                         |
| Heme (n)                   | 1290                           |
| Sulfate (n)                | 30                             |
| Wilson B-factor (Å²)       | 34.2                           |
| R-factor                  | 0.19/0.24                      |
| R_mixed                   | 0.11/0.77                      |
| CC½¼                      | 0.36                           |

The crystal structure of G. lovleyi NrfA revealed key structural differences

Although G. lovleyi NrfA is a monomer in solution, it crystalized as a dimer, consistent with other structurally characterized NrfA homologs. Alignment of the G. lovleyi NrfA crystal structure with that from Sulfovirgopirillum deleyianum (PDB entry 1QDB), its closest structurally characterized homolog (Fig. S5), revealed a Cα root mean square deviation (RMSD) of 2.67 Å (ProMOL, Schrödinger, Inc.). The crystal, which resolved at 2.55 Å, belongs to the primitive monoclinic space group P2₁2₁2₁ with unit cell a = 110.9, b = 144.6, and c = 234.9 Å (Table 2). The dimer interface is dominated by three α-helices from each monomer, and it is primarily stabilized by a strong electrostatic contribution from two salt bridges (Fig. 1A) as well as H-bonding interactions. The structure revealed four bis-His coordinated hemes as well as a Lys-ligated heme (heme-1) that constitutes the active site. The hemes from this structure aligned well with the hemes from S. deleyianum NrfA (Fig. 1B).

The substrate binding site at the distal side of the active-site heme-1 is occupied by sulfate (efforts to obtain high-quality crystals with nitrite bound were unsuccessful), which is stabilized by the active-site residues Tyr-221, His-278, and Arg-122, as well as a water molecule (Fig. 2A). These active-site residues are strictly conserved in all NrfA enzymes characterized to date.

A key structural difference between G. lovleyi NrfA and the previously crystallized homologs can be found at the active-site region. Specifically, no calcium was found near the heme-1 active site in G. lovleyi NrfA. Instead, the active site contains an arginine residue (Arg-277) in the region that would otherwise be occupied by a calcium ion in other NrfA homologs (Fig. 2). Arg-277 forms a salt bridge with Glu-263 as well as H-bonds to both the side chain and amide oxygen atoms of the active-site tyrosine (Tyr-221). In addition, there is an n-cation interaction between Arg-277 and Tyr-221. In the Ca²⁺-containing homologs, the amide oxygen of this same tyrosine is ligated to the calcium (Fig. 2B). In contrast to the presence of two well-ordered

ion deduced from MS (electrospray ionization) was 56,196 Da, consistent with the mass of the polypeptide chain plus the five heme cofactors but no calcium ion. The metal content was further confirmed by inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Table S1). The UV-Vis and EPR spectra (Fig. S2 and S3) are consistent with previously characterized NrfA enzymes (10, 22–24).

Dynamic light scattering indicated that G. lovleyi NrfA remains a monomer in solution

Characterization of G. lovleyi NrfA by size-exclusion chromatography suggested that the enzyme is a monomer in solu-
H$_2$O molecules coordinated to the calcium ion in the Ca$^{2+}$-containing homologs, the presence of a water molecule in the vicinity of Arg-277 in *G. lovleyi* NrfA could not be definitively ascertained (i.e. only two out of six monomers revealed a well-ordered water molecule H-bonded to Arg-277). Interestingly, the positive and negative charges at both the Ca$^{2+}$ site and the Arg site are more or less balanced. To achieve this, the calcium is coordinated by Glu-216, its two H$_2$O ligands are H-bonded to Asp-267 (Fig. 2B), and its lysine ligand is H-bonded to Asp-400 (not shown). In *G. lovleyi* NrfA, however, charge balance is achieved by Arg-277 forming a salt bridge to Glu-263 (Fig. 2A). It is important to note that except for *Desulfovibrio desulfuricans*, all NrfA enzymes crystallographically characterized thus far, including *G. lovleyi* NrfA, were purified and crystallized without the addition of calcium to any of the buffers (8–13). In addition, our attempts to crystallize *G. lovleyi* NrfA in the presence of calcium yielded poorly diffracting crystals.

Using CAVER software (32), we identified a tunnel connecting the putative substrate and product channels (Fig. 3). The substrate channel in *G. lovleyi* NrfA is in a location similar to that in the *S. deleyianum* NrfA protein (8), and it is highly cationic because of the presence of several positively charged amino acid residues in the vicinity, i.e. Lys-124, Lys-225, Arg-277, and Arg-122. Whereas Arg-122 is strictly conserved in all NrfA proteins and Arg-277 is strictly conserved in Arg-containing NrfA proteins, Lys-124 and Lys-225 are highly conserved in Arg-containing but not in Ca$^{2+}$-containing NrfA homologs. Conversely, the product channel in *G. lovleyi* NrfA diverges from that of *S. deleyianum*. In *S. deleyianum*, the product channel hovers above heme-4 while in *G. lovleyi* it is near heme-3. Furthermore, the *G. lovleyi* NrfA product channel is surrounded with negatively charged carboxylates from heme-1 and heme-3 as well as from amino acid residues Glu-101 and Asp-114. These residues guide the exit of the positively charged ammonium ion.

**New subclass of NrfA isolated from Geobacter lovleyi**
**G. lovleyi NrfA displayed a kinetic behavior similar to that of previously characterized homologs**

In NrfA assays, dithionite-reduced methyl viologen was used as an artificial electron donor to drive the enzymatic reduction of nitrite. The disappearance of the intense blue color from the reduced methyl viologen was monitored to measure the activity of the enzyme. Despite lacking the calcium ion, *G. lovleyi* NrfA demonstrates kinetic behavior similar to that of previously characterized homologs. It displays zero-order kinetics with respect to the concentration of methyl viologen, first-order kinetics with respect to the concentration of the enzyme, and hyperbolic dependence of the rate with respect to the concentration of nitrite (Fig. 4). The $K_M$ and the $k_{cat}$ values obtained from the Michaelis-Menten curve were $27 \pm 2 \, \mu M$ NO$_2^-$ and $1,291 \pm 34 \, \mu mol$ NO$_2^-$ min$^{-1}$ mg$^{-1}$ enzyme, respectively. The *G. lovleyi* NrfA $K_M$ is similar to that reported for NrfA proteins purified from *E. coli* (10) and *S. oneidensis* (11). Whereas the $k_{cat}$ calculated here is higher than that reported for some other NrfA enzymes (10, 11, 23, 25, 33–36), it is well known that these values are heavily dependent on the exact reaction conditions (34).

Notably, *G. lovleyi* NrfA is unaffected by the absence of added calcium or the presence of 1 mM EDTA in the reaction buffer (Fig. S7). This is in contrast to the catalytic behavior displayed by its homolog from *S. deleyianum*, where the removal of its calcium by EDTA treatment reduced the activity of the enzyme by 50% (33). This further highlights that *G. lovleyi* NrfA catalyzes nitrite reduction in a Ca$^{2+}$-independent manner. To test the importance of the arginine residue located in the region otherwise occupied by calcium in other NrfA homologs, we generated enzyme variants carrying conservative (R277K and R277Q) and radical (R277A) substitutions. Each of the enzyme variants contains all five hemes, as deduced by MS (56,107 Da for R277A and 56,164 Da for R277K and R277Q). The rates obtained for these variants were significantly lower than that of the WT enzyme (Fig. 4, inset). These data suggest that the arginine residue in *G. lovleyi* NrfA is critical to catalysis and plays a role similar to that of the calcium ion in other homologs.

**Phylogenetic analysis revealed a diverse evolutionary path of Arg-containing NrfA proteins**

We identified a wide taxonomic diversity of bacteria with NrfA proteins carrying the critical arginine residue (Arg-277) of the *G. lovleyi* NrfA (Fig. 5), suggesting a novel subclass of Arg-containing, Ca$^{2+}$-independent NrfA enzymes. To gain insights into the phylogenetic diversity of Arg-containing NrfA proteins, we constructed a phylogenetic tree from a refined alignment of 445 bacterial NrfA proteins. These analyses reconstructed the previously reported phylogenetic clustering of NrfA proteins in 18 distinct clades (designated A to R) (37), which groups the *Geobacter* NrfA proteins in clade I (Fig. 5). This clade is polyphyletic and includes NrfA proteins from...
genera (Desulfuromonas, Pelobacter, and Geobacter) in the two families (Geobacteraceae and Desulfuromonadaceae) described in the order Desulfuromonadales (15). The clade also includes strictly anaerobic genera from the orders Clostridiales (Desulfovibrio and Dethiobacter), Nitrospirales (Thermodesulfovibrio), Thermodesulfobacteriales (Thermodesulfobacterium), Desulfovibrionales (Desulfovibrio), and Syntrophobacteriales (Syntrophobacter) (Fig. 5). Despite its taxonomic diversity, the clade only contains NrfA proteins carrying the conserved face through heme-5 (11, 39). It is not uncommon, however, for NrfA proteins isolated from the periplasmic soluble fractions to elute as monomers by size-exclusion chromatography (25–28), as we observed for G. lovleyi NrfA. A DLS experiment on purified E. coli NrfA performed at various concentrations indicated, however, that this homolog exists in a monomer-dimer equilibrium, even at concentrations as low as 9 μM (19). This suggests that the enzyme is likely a monomer at dilute concentrations but dimerizes at higher concentrations. We performed similar DLS experiments on G. lovleyi NrfA, and our results indicate that the enzyme remains monomeric even at concentrations as high as 300 μM. This suggests that the G. lovleyi NrfA has a dimer dissociation constant, Kd, that is significantly higher than that measured for its homologs (11, 34, 40, 41). This is a significant finding, because in vitro mechanistic studies are often interpreted with the assumption that NrfA is a functional dimer based on the crystal structure (Fig. 1). The fact that G. lovleyi NrfA remains in the monomeric state even at very high protein concentrations suggests that the situation is more complex.

Although G. lovleyi NrfA-1 crystallizes as a homodimer and displays a fold and heme arrangement similar to those of previously crystallized homologs (8–13), it lacks the calcium ion near the heme-1 active site seen in the canonical NrfA enzymes. Instead, we identified a conserved arginine residue (Arg-277) that is critical for enzyme activity. The calcium ion associated with other NrfA proteins has been proposed to participate in the proper positioning of two strictly conserved active-site residues, histidine and tyrosine. Cunha et al. (13) demonstrated that the NrfA active site is architecturally similar to that of peroxidase classes II and III, which also contain a calcium ion in a similar location in the heme active site. The calcium ion in NrfA and both class II and class III peroxidases are structurally coupled to an active-site histidine. Release of the calcium ion in both Phanerochaete chrysosporium lignin and manganese peroxidases resulted in the histidine binding to the heme and deactivating the enzyme (42, 43). This led Cunha et al. to hypothesize that the calcium ion in NrfA plays a similar role and prevents the active-site histidine from binding to the heme-1 iron (13). This is achieved by coordination of the calcium to the side chain oxygen of the conserved glutamine residue that is contiguous to the histidine (Fig. 2B). The Arg-277 in G. lovleyi NrfA, which is extensively H-bonded to Tyr-221 and Glu-263 (Fig. 2A), is contiguous to the active-site histidine, His-278. This H-bonding network prevents the active-site histidine from binding to the heme-1 iron, similar to what was suggested for the Ca2+-binding NrfAs. The <3% activity of the R277K amino acid variant relative to the WT enzyme (Fig. 4) may be because of the inability of the lysine to form the same H-bonding network as the arginine. Additionally, the calcium

**Discussion**

G. lovleyi, first isolated from creek sediment samples cultivated under strict anaerobiosis using acetate-amended enrichment culture (38), can couple the oxidation of acetate to the reduction of U6+, Fe3+, Mn4+, trichloroethene, tetrachloroethene, and, most relevant to this paper, NO3− and NO2− (15). This reaction is most likely catalyzed by NrfA-1, the only one of two paralogs that is encoded in an operon with the putative membrane-bound reductase partner NrfH (15). We successfully expressed NrfA-1 in Shewanella oneidensis MR-1 and purified it in the mature form. The enzyme had the same heme content (Table S1), similar UV-Vis and EPR spectroscopic signatures (Figs. S2 and S3), and similar kinetics (Fig. 4) compared to those of previously characterized NrfA homologs.

G. lovleyi NrfA, like all NrfA enzymes crystallized thus far (8–13), has a homodimeric structure. This dimeric structure is often assumed to be functional in vivo, and it is quite possible that intermolecular electron transfer occurs at the dimer interface through heme-5 (11, 39). However, the second paralog branched separately, clustering together with other Geobacter NrfA proteins.

The phylogenetic analyses also provided evidence for the separate emergence of Arg-containing NrfA proteins within clades R, D, and N (Fig. 5), consistent with convergent evolution. These clades are hybrid, comprising both Arg-containing and Ca2+-binding NrfA proteins. Thus, in clade R, we identified Arg-containing NrfA proteins in the actinobacterial order Slackia and Cryptobacterium, within the family Eggerthellales, but the clade also includes a cluster of Egerthallales with canonical Ca2+-binding NrfA proteins. Clade D, on the other hand, only includes proteins from the order Alteromonadales (Shewanella and Ferrimonas), but the presence of the two NrfA subclasses splits the clade into two separate clusters. This clade provides examples of both the presence of canonical Ca2+-dependent NrfA proteins and the emergence of Arg-dependent NrfA proteins within the same species (e.g. Shewanella sedimentinis, Shewanella piezotolerans, and Ferrimonas balearica). The genus Shewanella is also represented in clade C, a phylogenetically distinct group of Ca2+-dependent enzymes represented by the NrfA protein from Shewanella oneidensis, for which a structure is available (11). Clade N is also hybrid, containing the two types of NrfA subclasses in two genera within the orders Clostridiales (Desulfitobacterium) and Thermoaerobacteriales (Carboxydothermus). Desulfotibacterium, for example, provides a clear example of NrfA diversification within the same genus, with most species containing the Ca2+-binding protein but one member (Desulfotibacterium dichlororescens) evolving the Arg-dependent protein (Fig. S9). Even within the same species (e.g. Ferrimonas balearica in clade D), there is the emergence of the Arg motif in phylogenetically distinct NrfA proteins.
ion may also be required for the proper positioning of an active-site Tyr residue. In Ca$^{2+}$-binding NrfA enzymes, the carbonyl oxygen of this tyrosine is ligated to calcium, presumably ensuring proper positioning. In *G. lovleyi* NrfA, Arg-277 is H-bonded to this tyrosine (Tyr-221) and ensures proper orientation.

**Figure 5.** Bayesian phylogenetic tree of NrfA proteins (*n* = 445) showing collapsed phylogenies for taxonomic orders represented in each of the 18 NrfA clades (designated A to R on the right), as reported by Welsh et al. [37]. The collapsed outgroup cluster (comprised of the genera *Dethiobacter*, *Geobacter*, *Pelobacter*, *Caldimicrobium*, *Thioalkalivibrio*, *Desulfurivibrio*, *Calditerrivibrio*, *Mucispirillum*, *Suterella*, and *Parasuterella*) is shown on top. The scale bar shows substitutions per site. Clade I, which contains only Arg-containing NrfA proteins, also includes the two NrfA paralogs of *G. lovleyi*: NrfA-1<sub>Glov</sub> and NrfA-2<sub>Glov</sub> (GLOV_RS01015). A tree showing the uncollapsed phylogenies is available in Fig. S9.
Another interesting feature in the Ca\(^{2+}\)-binding NrfA enzyme is the presence of a strictly conserved lysine residue whose amide oxygen is a ligand to calcium. The lysine ligand in a Ca\(^{2+}\)-binding NrfA may be involved in the delivery of nitrite to the active site through electrostatic interactions either by itself or in tandem with calcium. In *G. lovleyi* NrfA, no lysine is observed in the vicinity of Arg-277, suggesting that any role played by the lysine in a Ca\(^{2+}\)-dependent NrfA is presumably assumed by Arg-277. Previously, the calcium ion was believed to be involved in the delivery of catalytic protons through its two water ligands (14). In the asymmetric unit of the *G. lovleyi* crystal structure, however, only two of six monomers show an ordered water molecule H-bonded to Arg-277. This could mean that *G. lovleyi* NrfA has a different pathway for proton delivery, although given the resolution of the structure, it is impossible to make a definitive conclusion.

Interestingly, many Ca\(^{2+}\)-dependent proteins are known to have Ca\(^{2+}\)-independent homologs. Some examples include dysferlin (44), phosphatidylinositol-specific phospholipase C (45, 46), C-type lectins (47, 48), and protein kinase Ce (49). The obvious recurring theme in these Ca\(^{2+}\)-independent homologs is the appearance of either lysine or arginine as calcium replacement residues, presumably assuming the role of the calcium ion. For example, in the Ca\(^{2+}\)-independent homolog of phosphatidylinositol-specific phospholipase C, an arginine was found to be involved in lowering the pK\(_{a}\) of the inositol C2 hydroxyl group and in the activation of the phosphate group, similar to the function played by the calcium ion in the Ca\(^{2+}\)-dependent homolog (46).

The Arg-dependent homologs of the Ca\(^{2+}\)-dependent proteins could have emerged as an adaptive response to the scarcity of bioavailable calcium in their habitats. Microbial metabolic activities, including ammonification and precipitation of calcium with carbonates, reduce their bioavailability (50). This limitation exerts selective pressure to evolve proteins with arginine or lysine residues as a replacement for calcium. The strict conservation of arginine in the Ca\(^{2+}\)-independent subclass of NrfA enzymes points to a critical role for this residue in roles otherwise assumed by lysine or calcium in other enzymes. Consistent with this, replacing the active-site arginine with a lysine (R277K) reduced the nitrite reductase activity of the *G. lovleyi* NrfA to levels comparable with a radical substitution (R277A). This suggests a higher fitness conferred by this substitution relative to Lys (51, 52). This helps define diagnostic features of NrfA proteins of the Ca\(^{2+}\)-dependent and -independent subclasses. Welsh et al. (37) defined NrfA enzymes as pentaheme nitrite reductases containing a conserved KXQH or KXRH motif between the third and fourth heme-binding sites. Our phylogenetic analyses (Fig. 5) and amino acid sequence alignment (Fig. S8) identified KXQH as specific to Ca\(^{2+}\)-dependent enzymes. This motif has three conserved residues of the active site (Lys-279, Gln-281, and His-282 in *S. deleyianum*; Fig. 2B). These residues likely are critical not only in calcium ion coordination (Lys-279 and Gln-281) (8) but also for the maintenance of high substrate binding affinity (Gln-281) (53) and for the proper orientation of nitrite for N-O bond cleavage (His-282) (36).

We found, however, that the distinctive feature of Ca\(^{2+}\)-independent enzymes is the presence of an XXRH motif with the conserved active-site histidine (His-278 in *G. lovleyi*) and arginine residue (Arg-277 in *G. lovleyi*) that assumes the roles of the calcium ion in these enzymes. This motif evolved separately in 4 clades of NrfA proteins, branching clades R, D, and N into the Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent subclasses and establishing clade I as a phylogenetically diverse group of Arg-containing NrfA proteins represented by *G. lovleyi* NrfA (Fig. 5). The unifying feature of all the bacteria in clade I is that they are obligate anaerobes with metal, sulfur, and/or sulfate reductive metabolisms (15, 54). Some of these organisms use metals as electron acceptors for respiration in reactions that can be inhibited by calcium (55). Their oxidative metabolisms also generate carbon dioxide, which favors carbonate precipitation and calcium removal from their habitats (50). Thus, the emergence of Ca\(^{2+}\)-independent NrfA proteins provided these microorganisms a competitive advantage to respire nitrate and nitrite despite the limited bioavailability of the calcium ion.

**Experimental procedures**

**Protein expression and purification**

The plasmid used for the overexpression of the enzyme was synthesized by GenScript (Piscataway, NJ). Briefly, the native signal sequence (MRSKLLVPVAAALCGAATAC) at the N terminus of the WT *G. lovleyi* NrfA-1 (GLOV_RGS05135) paralog was replaced with pelB, and the Strep-tag II was added at the C terminus. The corresponding gene construct was codon optimized and inserted into pBAD202/D-TOPO (Invitrogen) downstream from the ribosome binding site by 11 non-coding base pairs. The resulting plasmid was then used for site-directed mutagenesis (Phusion DNA polymerase, New England Biolab) to generate the R277K, R277A, and R277Q NrfA variants using appropriate primers. The WT NrfA and its variant plasmids were then individually transformed into the overexpression host, *Shewanella oneidensis* MR-1 (21). Enzyme overexpression was performed by inoculating 10 ml of an overnight culture into a liter of Terrific broth supplemented with kanamycin to a final concentration of 50 \(\mu\)g L\(^{-1}\). The resulting culture was incubated at 30 °C with constant shaking at 160 rpm until an optical density of 0.6 was reached. The culture was then induced with arabinose to a final concentration of 0.02% and incubated for an additional 12 h before harvesting. The cells were harvested by centrifugation, resuspended in 50 ml of 100 mM Tris buffer at pH 7.5, flash-frozen in liquid nitrogen, and stored at −80 °C until further use.

Purification of the enzyme was accomplished by thawing the frozen 50-ml cell suspension and immediately adding 250 units of Benzonase (Millipore Sigma), 2 tablets of dissolved cOmplete™, Mini, EDTA-free protease inhibitor mixture (Roche), and 150 kU of rLysosome (Millipore Sigma). The cell lysate was then allowed to incubate for 4 h at room temperature with constant stirring, after which the suspension was stored at 4 °C overnight. The next day, the cell suspension was centrifuged at 47,000 relative centrifugal force for 30 min to separate the cell membrane from the lysate. The ionic strength of the lysate was increased by the addition of NaCl to a final concentration of...
**New subclass of NrfA isolated from Geobacter lovleyi**

150 mM, and the solution was passed through a 0.22-µm filter. The protein was then purified by affinity chromatography using a Strep-Tactin XT high-capacity column (IBA Lifesciences). Following protein binding, the column was washed with 12 column volumes of 100 mM Tris buffer containing 150 mM NaCl at pH 8.0. The bound enzyme was eluted using 50 mM biotin. The enzyme was further purified by size-exclusion chromatography (Enrich SEC 650, Bio-Rad) at a flow rate of 0.5 ml/min using 50 mM HEPES with 150 mM NaCl at pH 7.0. Fractions within 80% of the elution band were collected, and the purity was assessed by SDS-PAGE. The molecular weight of the enzyme was determined by MS.

**Extinction coefficient**

Duplicate samples of the WT enzyme were submitted to the Proteomics Core Facility at the UC–Davis Genome Center (Davis, CA) for amino acid analysis. Immediately before sending the samples, the absorbance at 410 nm (λ_max) was determined. From the measured absorbance and the enzyme monomer concentration obtained by amino acid analysis, the extinction coefficient was calculated to be 547 mM⁻¹ min⁻¹. The monomeric concentration of samples used for the succeeding experiments was determined spectrophotometrically using this extinction coefficient.

**ICP-OES**

A 120-µl sample of 208 µM enzyme was digested in 45% nitric acid (≥99.999% purity based on trace metal) at 50 °C overnight. The ICP-OES sample was prepared by diluting the digested sample with deionized water to 5 ml. A solution for blank measurement was prepared in the same manner, except that sample buffer was used instead of the enzyme. Calcium and iron standards with concentrations between 0.1 and 2.0 ppm were prepared in 2% nitric acid. Metal analysis was performed using a Varian 710-ES Axial ICP-OES at a flow rate of 10 rpm. Quantitative analysis of calcium was monitored at 317.933, 393.366, and 396.847 nm, whereas iron was monitored at 234.350, 239.563, and 259.940 nm. Each measured value was an average of three replicates.

**DLS**

A stock solution of 300 µM NfrA was prepared, and the concentrated enzyme was centrifuged at 17,000 relative centrifugal force for 30 min to ensure the removal of any particulate matter. Samples with concentrations between 25 and 300 µM were then prepared by serial dilution, after which the exact concentrations of these samples were determined spectrophotometrically. DLS was performed using DynaPro Nanostar (Wyatt Technology) initially offset using the same buffer as that used for the enzyme. Each measurement was an average of 20 acquisitions with 15 s per acquisition and performed at 25 °C using a 658-nm wavelength. The enzyme’s theoretical monomer and dimer R_H values were calculated using DYNAMICS V7 (Wyatt Technology) given the monomeric molecular weight of 56,196 Da.

**Crystal structure determination**

The enzyme in HEPES buffer (pH 7.0) containing 150 mM NaCl and 30 mM ammonium sulfate was concentrated to 10 mg/ml. The protein was crystallized by sitting-drop vapor diffusion using the crystallization buffer (200 mM sodium malonate and 15% PEG 3350 at pH 6.5) with a protein:buffer ratio of 2:1 (v/v). The crystals were allowed to grow to full size for 2–3 weeks at 4 °C and cryoprotected using 20–30% glycerol prior to flash freezing in liquid N2. Diffraction data were collected at 1.72 Å (slightly shorter than the K edge of Fe at 1.74 Å) at the 21-ID-D beamline of the Advance Photon Source (APS) at Argonne National Laboratory. The data sets were indexed, integrated, and scaled using the HKL2000 suite (56). The initial trial to solve the phasing problem using molecular replacement failed, and the structure of G. lovleyi NfrA was eventually solved by single-wavelength anomalous dispersion using Fe anomalous signals in Phenix.AutoSol (57) at 2.7 Å. Nearly all the amino acid residues were built using Phenix.AutoBuild. This initial model was then used as a search template in molecular replacement to solve the structure at 2.55 Å in CCP4 (58) using the first half of the diffraction data with less radiation damage. Model building and refinement were conducted iteratively in Coot (59) and Phenix.refine to manually correct geometry and insert both the hemes and sulfate. In the final model (Table 2), one asymmetric unit contained three homodimers, and each monomer includes residues 35–480, five hemes, and one sulfate ion in the active site.

**Activity assay**

The activity assay was performed as previously described (34), with minor modifications. Briefly, 50 mM HEPES buffer at pH 7.0 containing 0.8 mM methyl viologen was prepared (with or without 2 mM calcium chloride for R277A, R277Q, and R277K activity assays) and degassed under vacuum for 30 min. Deionized water and a solution of 250 mM EDTA were also degassed. Inside an anaerobic chamber (Coy Laboratory Products), solutions of 10 mM dithionite and 1 mM nitrite were prepared using degassed water and buffer, respectively. A 1 µM sample of enzyme was also prepared by diluting a concentrated sample with an appropriate volume of buffer, and the sample was incubated for at least 30 min inside the chamber. The exact concentration of the enzyme was then determined spectrophotometrically. A 3-ml reaction solution consisting of 0.8 mM methyl viologen (buffer), 0.04–0.06 mM dithionite, and 0.5–2.0 mM enzyme was prepared in a crimped-top modified cuvette. In assays where EDTA was required, EDTA was added to a final concentration of 1 mM. The desired amount of nitrite (10 µM to 1 mM) was then added to the side of the cuvette above the solution to avoid initiating catalysis. The cuvette was sealed with a rubber septum, tightly secured with a crimped aluminum lid, and brought outside the chamber for the kinetic analysis using a spectrophotometer initially baselined using the same buffer. Catalysis was initiated by quickly inverting the cuvette three times to introduce the nitrite previously added to the side of the cuvette. The absorbance at each time point was converted to the concentration of the methyl viologen using ε_600 = 13,700 M⁻¹ cm⁻¹, and the rate of decrease of the methyl viologen was
used to calculate the rate of the reaction. $K_M$ and $V_{\text{max}}$ values were determined by fitting the Michaelis-Menten curve using the Michaelis-Menten function in Origin [Origin(Pro), version 2019b, OriginLab Corporation, Northampton, MA, USA].

**Phylogenetic analysis**

Phylogenetic analysis was performed in the same manner as that previously described (37). Approximately 13,400 NrfA amino acid sequences contributed by Joel Klappenbach in FUNGENE (RRID:SCR_018749) were retrieved, excluding sequences from environmental samples as well as sequences from unknown species. The number of sequences was further trimmed to 13,200 by aligning the sequences and retaining only the ones that had the conserved CXXCK and CXXCH heme-binding motifs as well as the conserved His and Tyr active-site residues. The sequences were segregated into two groups: the arginine-bearing and the putative Ca$^{2+}$-bearing groups (Fig. S8). Each group was then individually aligned using MUSCLE (60) and clustered at 98% identity using UCLUST’s cluster_fast command (61). The two groups were then realigned against each other to generate a single multiple-sequence alignment consisting of 445 sequences. Bayesian phylogenetic analysis for the 445 sequences was performed in Beauti/Beast v1.10.4 (62) using the LG + G + I model, which was determined using MEGA v10.1 maximum likelihood model selection (63). For this model, a single chain of Markov chain Monte Carlo was run for 10,000,000 generations and sampled at every 10,000th generation. With a burn-in of the first 1,000,000 generations, effective sample size values of >200 were achieved for all statistical parameters. This burn-in was used in TreeAnnotator (Beast package module) to compute the final consensus tree. Postprocessing for the final presentation of the tree was performed using FigTree (Beast package module) and MEGA V10.1.

**Data availability**

The crystal structure presented in this paper was deposited in the Protein Data Bank (PDB entry 6V0A). The ICP-OES data, crystallographic statistics, Coomassie-stained gel and Western blotting, UV-Vis spectrum, EPR spectrum, data from size-exclusion chromatography, G. lovleyi and S. deleyianum NrfA protein alignment, G. lovleyi active-site difference density map, activity assay with and without EDTA, sequence alignment of NrfA proteins from select species, and the uncollapsed phylogenetic tree can be found in the supporting information. The alignment of all 445 sequences is uploaded separately as Dataset S1. The dynamic light scattering data, activity assay, collapsed phylogenetic tree, and all remaining data are contained within the paper.

**Acknowledgments**—We thank Prof. Cheryl Kerfeld and Dr. Bryan Ferlez (MSU-Plant Research Laboratory) for the use of their DLS instrument and for their technical expertise.

**Author contributions**—J. C., N. L., G. R., and E. L. H. conceptualization; J. C., S. L., Z. W., G. R., and J. H. data curation; J. C., S. L., Z. W., V. S. A., N. L., G. R., and J. H. formal analysis; J. C., S. L., Z. W., V. S. A., G. R., and J. H. investigation; J. C., S. L., V. S. A., N. L., G. R., J. H., and E. L. H. methodology; J. C. writing—original draft; J. C., S. L., N. L., G. R., J. H., and E. L. H. writing—review and editing; S. L., Z. W., V. S. A., and J. H. visualization; N. L., G. R., and E. L. H. resources; N. L., G. R., J. H., and E. L. H. supervision; N. L., G. R., and E. L. H. funding acquisition; N. L., G. R., and E. L. H. project administration.

**Funding and additional information**—This work was supported by the U.S. Department of Energy, Office of Science, Basic Energy Sciences, under award no. DE-SC0017952 (to G. R. and E. L. H.) and award no. DE-SC0018173 (to N. L.).

**Conflict of interest**—The authors declare no conflicts of interest in regards to this work.

**Abbreviations**—The abbreviations used are: DNRA, dissimilatory nitrate reduction to ammonium; ICP-OES, inductively coupled plasma-optical emission spectroscopy; DLS, dynamic light scattering; RMSD, root mean square deviations.

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