A nitric oxide–binding heterodimeric cytochrome c complex from the anammox bacterium Kuenenia stuttgartiensisis binds to hydrazine synthase

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Anaerobic ammonium oxidation (anammox) is a microbial process responsible for significant nitrogen loss from the oceans and other ecosystems. The redox reactions at the heart of anammox are catalyzed by large multiheme enzyme complexes that rely on small cytochrome c proteins for electron shuttling. Among the most highly abundant of these cytochromes is a c-type cytochrome called NaxLS, which has distinctive biochemical and spectroscopic properties. Here, we present the 1.7 Å resolution crystal structure of this complex from the anammox organism Kuenenia stuttgartiensisis (KsNaxLS). The structure reveals that the heme irons in each subunit exhibit a rare His/Cys ligation, which, as we show by substitution, causes the observed unusual spectral properties. Unlike its individual subunits, the KsNaxLS complex binds nitric oxide (NO) only at the distal heme side, forming 6cNO adducts. This is likely due to steric immobilization of the proximal heme-binding motifs upon complex formation, a finding that may be of functional relevance, because NO is an intermediate in the central anammox metabolism. Pulldown experiments with K. stuttgartiensis cell-free extract showed that the KsNaxLS complex binds specifically to one of the central anammox enzyme complexes, hydrazine synthase, which uses NO as one of its substrates. It is therefore possible that the KsNaxLS complex plays a role in binding the volatile NO to retain it in the cell for transfer to hydrazine synthase. Alternatively, we propose that KsNaxLS may shuttle electrons to this enzyme complex.

Anaerobic ammonium oxidation (anammox) is a major process in the biogeochemical nitrogen cycle, and it is estimated to be responsible for 50–70% of the yearly loss of fixed nitrogen from the ocean to the atmosphere as N₂ (1, 2). In this process, chemolithoautotrophic bacteria convert ammonium (NH₄⁺) and nitrite (NO₂⁻) into N₂. Based on genetic, physiological, and biochemical information, it was proposed that the anammox process is carried out in three steps (Equations 1–3) involving NO and the unusual intermediate hydrazine (N₂H₄) (3, 4).

\[
\text{NO}_2^- + 2H^+ + e^- \rightarrow NO + H_2O \quad (\text{Eq. 1})
\]

\[
\text{NO} + NH_4^+ + 2H^+ + 3e^- \rightarrow N_2H_4 + H_2O \quad (\text{Eq. 2})
\]

\[
N_2H_4 \rightarrow N_2 + 4H^+ + 4e^- \quad (\text{Eq. 3})
\]

The first step is catalyzed by a nitrite reductase (Nir), the second step by hydrazine synthase (HZS) (5), and the final step by hydrazine dehydrogenase (HDH) (6). These enzymes are localized in a membrane-bound compartment called the anammoxosome (7). In the current model of the anammox process, the four electrons released in the final reaction are transferred to the menaquinone pool inside the anammoxosome membrane to set up a proton-motive quinol cycle (3, 8). The resulting proton gradient is finally utilized by an F₄₃FF₄-type ATP synthase to generate ATP. Electrons are proposed to be shuttled between soluble and membrane enzyme complexes by small c-type cytochromes (3, 9). Indeed, the genome of the anammox model organism Kuenenia stuttgartiensis encodes 65 c-type cytochromes that are predicted to be located inside the anammoxosome (10). Many of these are members of the low-molecular-weight class I and class II cytochrome c proteins (11), which could perform the proposed functions in electron transfer.

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The abbreviations used are: anammox, anaerobic ammonium oxidation; NO, nitric oxide; Nir, nitrite reductase; HZS, hydrazine synthase; HDH, hydrazine dehydrogenase; CO, carbon monoxide; AUC-SE, analytical ultracentrifugation sedimentation velocity; SEC-MALS, size-exclusion chromatography with multicycle static light scattering; SAXS, small-angle X-ray scattering; Ti(III)citrate, titanium (III) citrate; Ni-IDA, nickel(II) iminodiacetic acid; RMSD, root-mean-square deviation; 6cNO, 6-coordinate NO complex; 6cNO, 6-coordinate NO complex; KPi, potassium phosphate; PDB, Protein Data Bank; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; SHE, standard hydrogen electrode; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

The atomic coordinates and structure factors (codes 6R6M, 6R6N, and 6R6O) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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The NaxLS complex is the first example of a complex subunit possessing a rare His/Cys ligation (12). Moreover, the resonance (EPR) data, it was postulated that the heme in each of these, as well as on the sequence and electron paramagnetic resonance (EPR) analysis, suggests a function in NO binding of highly-reactive and volatile intermediates such as NO and hydrazine to protect the cell and/or to keep them from diffusing out. However, which of these small c-type cytochromes performs these electron transfer reactions and whether some of them perform other crucial tasks in the central anammox metabolism are unknown. Such other functions might include the binding of highly-reactive and volatile intermediates such as NO and hydrazine to protect the cell and/or to keep them from diffusing out.

Among the most abundant small cytochrome c proteins in anammox organisms are NaxL (a member of class IIb) and NaxS (class I). In the anammox model organism K. stuttgartiensis, the genes encoding NaxL and NaxS (kusta0087 and kusta0088, respectively) show the highest transcription levels of all monoheme c-type cytochromes (3) (Table S1) encoded in the genome. Previously, the homologues of these proteins from Jettinia caeni were purified as a heterodimeric complex (JcNaxLS) and were partially spectroscopically characterized (12). The UV-visible spectrum of JcNaxLS shows a blue shift of the Soret and α,β absorption peaks upon reduction, whereas other cytochrome c proteins typically show a red shift. Based on this, as well as on the sequence and electron paramagnetic resonance (EPR) data, it was postulated that the heme in each subunit possesses a rare His/Cys ligation (12). Moreover, the NaxLS complex is the first example of a complex between a class I and a class IIb c-type cytochrome.

Here, we describe the crystal structure of the NaxLS complex from K. stuttgartiensis (KsNaxLS). We provide a full analysis of the UV-visible spectroscopic properties of KsNaxLS, including the molecular origins of these unusual spectral shifts. We also demonstrate that KsNaxLS binds NO and CO. Importantly, we also show that the KsNaxLS complex interacts specifically with the hydrazine synthase complex, suggesting a function in NO and/or electron transport.

**Results**

**KsNaxLS is a heterodimer**

KsNaxLS was purified from K. stuttgartiensis as a stable heterodimer (Fig. 1A). Moreover, the recombinantly expressed components KsNaxL and KsNaxS could be reconstituted into a heterodimer in vitro (Fig. 1B). The sedimentation coefficient distribution c(S) of the native KsNaxLS complex obtained by analytical ultracentrifugation sedimentation velocity (AUC-SV) resulted in a single symmetric peak at 2.7 S (Fig. S1). Using the calculated molecular mass of the KsNaxLS heterodimer (24,397 Da), the maximum possible sedimentation coefficient (i.e., the sedimentation coefficient of a perfectly spherical molecule of the same molecular mass) can be calculated as $S_{\text{max}} \approx 0.00361 \times M^{2/3}$. The ratio of this value and the measured sedimentation coefficient corrected to water at 20 °C ($s_{w,20}$) and $S_{\text{max}}$ indicates a protein complex with a globular shape ($S_{\text{max}}/s_{w,20} = 1.11$) (13). The sedimentation coefficient distribution c(S) of the recombinantly produced individual components of the KsNaxLS complex also resulted in single symmetric peaks at 1.8 S and 1.7 S for KsNaxL and KsNaxS, respectively (Fig. S1). The comparison of the measured $s_{w,20}$ values and the maximum possible sedimentation coefficients indicate that the components are globular and monomeric in solution ($S_{\text{max}}/s_{w,20}$ of 1.17 and 1.14 for KsNaxL and KsNaxS, respectively). AUC-SV of the reconstituted KsNaxLS complex from its overexpressed subunits (both with C-terminal His$_{10}$-tags) resulted in a sedimentation coefficient of 2.8 S ($s_{w,20}$ of 2.78 S), which is also consistent with a heterodimeric complex of the NaxL and NaxS components with a $S_{\text{max}}/s_{w,20}$ ratio of 1.16.

AUC-SE of the native KsNaxLS complex resulted in a molecular mass of 23.9 kDa, which also corresponds well to the calculated value for the heterodimer (24.4 kDa) (Fig. S2). An analysis of the native KsNaxLS complex and its reconstitution from heterologously expressed components by size-exclusion chromatography coupled with multianalyte light scattering (SEC-MALS) is shown in Fig. 1B. The size-exclusion chromatograms of KsNaxLS and its component KsNaxL all showed single peaks at elution volumes of 11.9 and 13.1 ml, respectively. MALS analysis resulted in 20.4 and 13.4 kDa for the KsNaxLS complex and KsNaxL, respectively. When the KsNaxL and KsNaxS components were mixed in a 2:1 molar ratio, they could be reconstituted into a stable complex, with the excess of KsNaxL remaining in a monomeric state. MALS analysis of the reconstituted complex resulted in a molecular mass of 22.8 kDa.

**NaxLS binds NO and interacts with hydrazine synthase**

Figure 1. Oligomeric state of KsNaxLS. A, 12% Tris-Tricine SDS-PAGE (S1) analysis of the natively purified KsNaxLS complex and its heterologously expressed components (with C-terminal His$_{10}$-tags). M, molecular mass markers (in kDa). L-His$_{10}$, recombinant His-tagged KsNaxL; S-His$_{10}$, recombinant His-tagged KsNaxS; LS (native), as-isolated KsNaxLS complex. B, SEC-MALS analysis of the KsNaxLS complex reconstitution from its individual components. Solid curves show SEC elution profiles measured as 280 nm; dots indicate the molecular mass in Da as determined by MALS. Green, recombinant His-tagged KsNaxL; blue, recombinant His-tagged KsNaxS; black, as-isolated KsNaxLS complex; and red, 2:1 mixture of recombinant His-tagged KsNaxL and NaxS. The red curve shows both the reconstituted KsNaxLS complex at 11.9 ml and the remaining KsNaxL at 13.1 ml.
Structures of WT and mutants of the KsNaxLS complex observed in the crystal (see below) (Fig. S3).

The 1.7 Å resolution X-ray structure of the WT, natively purified KsNaxLS complex, contains a heterodimer consisting of one KsNaxL and one KsNaxS subunit in the asymmetric unit. The molecular shape of the complex is somewhat elongated and resembles a pointing hand (Fig. 2), where the KsNaxL subunit forms the pointing finger(s) and KsNaxS the rest of the hand. The KsNaxL subunit forms a four-helix bundle, which is a typical characteristic of class II c-type cytochromes. KsNaxS possesses a class I cytochrome c-fold consisting of six α-helices. The contact surface area between the two subunits is 2120 Å² (out of the total surface area of the complex of 10,950 Å²); it is formed by the regions Asp-82 to Tyr-92 and Arg-115 to Thr-129 of KsNaxL, as well as Glu-29 to Gly-61 and Val-119 to Lys-126 of KsNaxS.

The c-type heme moieties of KsNaxL and KsNaxS are 19 Å apart in the complex (using the edge–to–edge distance (15)), and both are hexa-coordinated, with histidines serving as the proximal ligands (i.e. located on the side of the CXXC heme-binding motif), although cysteine residues, rarely acting as heme ligands in other c-type cytochromes, serve as the distal ligands (i.e. on the opposite side, Fig. 3, A and B). The distances between the heme iron and histidine Nε atoms are 2.0 Å in both KsNaxL and KsNaxS, and the heme–iron–to–cysteine–sulfur distances are 2.4 Å in both subunits. As expected for a class II c-type cytochrome, the heme-binding motif (120CRNCH124) in KsNaxL is located near the C terminus of the protein sequence, whereas the distal cysteine (Cys-32) ligand originates from the N-terminal part. The opposite is the case in KsNaxS where the heme-binding motif (36CYYCH39) is located close to the N terminus and the distal cysteine ligand (Cys-101) is located close to the C terminus, as is the case in other class I c-type cytochromes. The distal environment of the heme in KsNaxL is amphipathic, with several hydrophilic residues such as Gln-28, Ser-73, and Asn-7 at the side of the B and C pyrrole rings. Together with a solvent molecule, these residues form a hydrogen-bonding network. Interestingly, the Gln-28 Ne atom is within hydrogen-bonding distance (3.1 Å) of the iron-coordinating Sy atom of Cys-32. The opposite side (above the A and B pyrrole rings) contains hydrophobic residues such as Met-35, Trp-36, and Val-70. The environment at the proximal face of the NaxL heme is mainly hydrophobic, containing residues such as Leu-133, Val-134, and the terminal Pro-135 residue (Fig. 3A). The heme environment in KsNaxS is distinctly hydrophobic, being made up of several aromatic amino acids (such as Tyr-43, Phe-71, Phe-76, Phe-81, and Tyr-104) as well as other hydrophobic residues, e.g. Met-35, Val-73, and Ile-94 at both distal and proximal sides. Interestingly, the iron-coordinating Sy of the distal Cys-101 is within hydrogen-bonding distance (3.1 Å) of the Ne atom of His-93 (Fig. 3B).

Because cysteine as a distal heme ligand is relatively rare, we made several mutants for structural and spectroscopic investigations by replacing one or both distal cysteines with either methionine or glycine. Of these, mutants KsNaxL C32G/NaxS C101M formed crystals, and we determined their X-ray crystal structures to 2.0 and 1.9 Å resolution, respectively, to assess the mutations’ impact on the overall structure and heme environment. For the most part, there are no major structural differences between WT and mutant KsNaxLS complexes, and the cysteine–to–methionine mutations resulted in the methionine coordinating the heme iron as expected. Only the mutation of Cys-32 to methionine in KsNaxL caused some larger-scale structural effects: Gln-28 moving outward compared with the situation in the WT. This in turn allows residues 33–38 on the opposite helix to move slightly inward, with the side chain of Met-35 adopting a more stretched-out conformation than in the WT protein. Other than that, the mutations cause local changes only. In the

NaxLS binds NO and interacts with hydrazine synthase

Figure 2. Crystal structure of KsNaxLS. A, cartoon representation of the KsNaxLS complex. KsNaxL and KsNaxS are colored in green and blue, respectively. The hemes are shown a stick models. The interactions between the two subunits are predominantly polar, with 23 hydrogen bonds and five salt bridges. B, stereo figure showing a representative portion of the simulated annealing 2mFo−DFc composite omit map (beige surface, contoured at 1.0 σ) overlaid onto the heme-binding site of KsNaxL.
KsNaxL C32G/NaxS WT crystal structure, a water molecule acts as a sixth heme ligand, which is within hydrogen-bonding distance from Gln-28 (Fig. S4). Moreover, upon mutation of Cys-101 to methionine in KsNaxS, His-93 rotates away causing the formation of a hydrogen bond with one of the propionate groups of the heme (Fig. 4B).

**UV-visible spectroscopy of WT and mutant proteins**

The heterologously expressed KsNaxLS complex and its components were reddish brown. In all proteins (from which His-tags were removed), the Soret band in the as-isolated state was found to be located at 419–420 nm (Fig. 5, A–C). Upon reduction with 0.5 mM Ti(III)citrate, the Soret band showed a clear blue shift and the typical α- and β-bands appeared at 553 nm (551 nm for KsNaxL and KsNaxS) and 523 nm (521 nm for KsNaxL and 522 nm for KsNaxS), respectively (Fig. 5, A–C).

Interestingly, when Ti(III)-reduced KsNaxLS and its constituents were re-oxidized by sparging the sample with 1–2 ml of air, the Soret band further blue-shifted to 412 nm with simultaneous disappearance of α- and β-bands. Re-oxidation with 0.5 mM potassium ferricyanide, however, resulted in a red shift of the Soret bands back to 420 nm for KsNaxLS and its constituent KsNaxL and to 419 nm for KsNaxS (Fig. 6, A–C).

UV-visible spectra of different distal-cysteine–to–methionine mutants (with His-tags) in their as-isolated state as well as after reduction with 0.5 mM Ti(III)citrate are shown in Fig. 5, D–F. The Soret bands of the as-isolated states of all these mutants were located around 413–415 nm. Upon reduction with Ti(III)citrate, a red shift of the Soret band to 417–418 nm was observed in these mutants, with concomitant appearance of α- and β-bands at around 553 and 523 nm (Fig. 5, D–F). The double methionine mutant KsNaxL C32M/NaxS C101M was apparently isolated in a partially reduced state given the presence of weak α- and β-bands (Fig. 5D). The Soret peaks shifted back to around 408–414 nm upon re-oxidation by sparging with air (except for the mutant KsNaxL WT/NaxS C101M, which has a Soret maximum at 416 nm in the re-oxidized state, Fig. 6, D–F). Moreover, after full reduction the re-oxidation by air of this mutant took more than 1 h, and it resulted in a noticeable decrease in intensity of the α- and β-bands.

Although the mutant KsNaxL WT/NaxS C101G (Fig. S5) appeared to be in a completely oxidized state after purification, re-oxidation after full reduction yielded still partially reduced species as evident from minor α- and β-bands (in this case the spectra were measured immediately after sparging with air). Conversely, the single mutant KsNaxL C32G/NaxS WT (Fig. S5), which was isolated in its oxidized state, was immediately fully re-oxidized by air starting from the completely reduced state.

**Redox potential of KsNaxLS**

Spectropotentiometric measurements with KsNaxLS in an optically transparent thin-layer electrochemical cell resulted in irreversible reactions at the working electrode. Ti(III)-reduced KsNaxLS was therefore titrated with ferricyanide under anoxic conditions while monitoring the redox potential and UV-visi-
ble spectrum. The absorption at 417 nm showed a single, sharp transition during titration between $-300$ and $+50$ mV versus SHE, from which a redox potential of $-175$ mV versus SHE could be extracted by fitting with the Nernst equation assuming a 2-electron transition (Fig. 7). Nearly identical results were obtained at absorption wavelengths of 552 and 454 nm. Redox potentials much lower than $-300$ mV or much higher than $+50$ mV versus SHE could not be reached without using amounts of reducing/oxidizing agents that resulted in protein aggregation.

KsNaxLS binds NO and CO

The KsNaxLS complex and its components were tested for their ability to bind several small molecule ligands such as nitric oxide (NO), carbon monoxide (CO), hydroxylamine (NH$_2$OH), hydrazine (N$_2$H$_4$), nitrite (NO$_2$), azide (N$_3$), and cyanide (CN$^-$. Of these ligands, NO and CO were found to coordinate to the $c$-type hemes in the KsNaxLS complex as judged by UV-visible spectroscopy. When non-His–tagged, reduced KsNaxLS was incubated with NO, the Soret intensity only decreased slightly, pointing to an effect of the presence of the His-tag on the NO-binding properties of the protein (Fig. 8B). The individual recombinantly-produced components of the complex displayed much larger spectral changes upon NO exposure. Upon incubation with NO, Ti(III)-reduced KsNaxL showed a prominent shift of the Soret band to $395$ nm and formation of a shoulder at $416$ nm indicating coordination of the nitrosyl ligand to the heme iron (Fig. 8C). In reduced KsNaxS, the Soret band shifted to $415$ nm with formation of a shoulder at $396$ nm (Fig. 8D). Titration of reduced KsNaxLS complex with NO, monitored by UV-visible spectroscopy, indicated a dissociation constant ($K_d$) for NO of $12$ μM (Fig. 8E).

When WT KsNaxLS as isolated from Kuenenia biomass was exposed to NO, the Soret band increased in intensity and showed a blue shift from $420$ to $417$ nm (Fig. S6A). With the recombinantly-produced His-tagged KsNaxLS protein, exposure to NO resulted in a much smaller increase in intensity of the Soret peak, although its position showed a clear
Figure 5. Spectroscopy of KsNaxLS. A–C, UV-visible spectra of recombinantly produced proteins after removal of the His-tag. A, natively purified, WT KsNaxLS, as well as recombinant, His-tagged; B, KsNaxL; C, KsNaxS; D–F, UV-visible spectra of His-tagged mutants. D, KsNaxL C32M/NaxS C101M; E, KsNaxL C32M/NaxS; and F, KsNaxL/NaxS C101M. Spectra of as-isolated and Ti(III) citrate-reduced samples are shown in black and red, respectively.
NaxLS binds NO and interacts with hydrazine synthase

Figure 6. Reoxidation after reduction. A–C, spectra of KsNaxLS and its constituents, reoxidized with air (red lines) or potassium ferricyanide (black lines) after reduction with Ti(III)citrate. Reoxidation by air results in a blue shift, whereas with ferricyanide the Soret band returns to the same position as in the as-isolated state. D–F, spectra of KsNaxLS distal Cys-to-Met mutants reoxidized with air after reduction with Ti(III)citrate.
blue shift from 419 to 417 nm (Fig. S6B). Thus, in both the reduced and as-isolated states, a difference in behavior toward NO was observed between His-tagged and non-His–tagged protein. At present, it is not clear what causes this difference in behavior.

Titration of as-isolated KsNaxLS complex with NO followed by UV-visible spectroscopy did not allow the $K_d$ value to be determined, as no saturation of any spectral feature could be reached using final NO concentrations up to 1 mM. This points to a low affinity with a $K_d$ in the millimolar range or higher in the as-isolated state.

Upon exposure to CO, the Soret bands of the reduced states of the KsNaxLS complex and its components sharpened and increased in their intensities (Fig. S7). The Soret band shifted to 417 nm–415 nm for the recombinant KsNaxLS complex, as well as its subunits. Moreover, in all cases two broad bands around 540 and 570 nm appeared, replacing the $\alpha$- and $\beta$-bands.

**KsNaxLS binds to hydrazine synthase**

The KsNaxLS complex (with a C-terminal His-tag on KsNaxS), individual KsNaxL and KsNaxS, as well as Kustc0563, another highly-expressed small cytochrome $c$ from *Kuenenia*, were used as “bait” proteins in a pulldown assay. To this end, they were bound to Ni-IDA beads, which were then incubated with a cell-free extract of *K. stuttgartiensis* after elution, SDS-PAGE (Fig. 9) followed by mass spectrometric analyses of the co-eluted proteins bands showed that only the KsNaxLS complex was able to pull down all three subunits of the HZS complex. In addition, a protein band at around 200 kDa appeared in all lanes when lysate was used. Peptide mass fingerprinting showed that this band belongs to KsHDH (6, 16). However, KsHDH was most likely bound nonspecifically, as it was also bound to the Ni-IDA beads without any bait protein. No co-eluting bands were observed when the single components KsNaxL or KsNaxS or the reference cytochrome $c$ Kustc0563 was used as bait. Similar results were obtained when immobi-

**Discussion and conclusions**

**KsNaxLS provides the first crystal structure of a complex between class I and II c-type cytochromes**

The crystal structure of KsNaxLS at 1.7 Å resolution provides the first molecular model of a complex between class I and class II c-type cytochromes. Moreover, based on its hexa-coordinated heme iron along with its four-helix bundle fold, NaxL can be assigned to subclass Ib of monoheme c-type cytochromes (11). The structure of KsNaxLS presented here is the first high-resolution structure of a member of this subclass. A molecular model, partially validated by NMR spectroscopy (17), exists of the His/Met-ligated cytochrome $c$-556 from *Rhodopseudomonas palustris*, another class Ib cytochrome. Its four-helix bundle fold shows an RMSD of 3.0 Å for 97 Ca-atoms with that of KsNaxL.

The purification of KsNaxLS from *K. stuttgartiensis* biomass as a stable heterodimeric complex, which was also the case for its orthologue from *J. caeni* (12), indicates that this oligomeric state is indeed physiologically relevant. Moreover, we show here that the recombinantly produced individual components KsNaxL and KsNaxS readily assemble into the heterodimer in vitro. Biophysical characterization in solution by AUC, SAXS, and SEC-MALS further corroborates that KsNaxLS exists as a heterodimer in solution. The crystal structure of the KsNaxLS complex shows that the interaction surface between the subunits constitutes a large fraction (~24%) of the total surface area. This strongly suggests that the heterodimeric assembly found in the crystal structure indeed represents the oligomeric state in solution. Our high-resolution structure shows that the c-type hemes in both KsNaxL and KsNaxS are coordinated by proximal histidine and distal cysteine ligands. This His/Cys heme ligation is relatively rare in nature with only a few examples known so far (18–24).

**The KsNaxLS complex possesses unusual UV-visible spectral properties**

In this study, we show that the mutation of the distal cysteines to either methionine or glycine in either one or both subunits of the KsNaxLS complex leads to blue-shifted Soret bands (413–415 nm in the as-isolated state) with respect to the WT complex (420 nm). Moreover, in all the mutants, a red shift to 417 nm takes place upon reduction, as opposed to the blue shift in WT KsNaxLS. These observations demonstrate that the unusual position of the Soret band and its blue shift upon reduction indeed originate from the His/Cys ligation in the KsNaxLS complex. The spectral features of the KsNaxLS complex and its components are reminiscent of those of cytochrome $c$ in which the axial Met had been replaced by Cys, suggesting a thiolate-Fe(III) ligation in both subunits of the KsNaxLS complex that is lost upon reduction (25). This was also pointed out for JcNaxLS (12). The crystal structure shows that in both subunits, a hydrogen bond is formed between the coordinating cysteine and another side chain (Gln-28 in KsNaxL and His-93 in KsNaxS). These would likely weaken the thiolate-Fe bond by reducing the $\sigma$-donor capability of the cys-
NaxLS binds NO and interacts with hydrazine synthase

A, KsNaxLS without His-tag

B, KsNaxLS with C-terminal His-tag

C, KsNaxL

D, KsNaxS

E

[Prot]_{total} = 11 \mu M

[Prot]_{total} = 4.5 \mu M
Figure 8. NO binding to reduced KsNaxLS and its components. A, effect of NO on the UV-visible spectra of recombinant, reduced KsNaxLS after removal of the His-tag; B, His-tagged KsNaxLS; C, KsNaxL (His-tag removed); and D, KsNaxS (His-tag removed). Reduced and NO-incubated reduced states are shown in blue and black, respectively. E, determination of binding constant of reduced KsNaxLS for NO, by titration with NO, and observation of the spectral change at 553 nm. Two series of measurements are shown, using protein concentrations of 11 and 4.5 μM protein (black and gray data points, respectively). The solid lines show the fits to each series using the average Kd of 12 μM.

Figure 9. SDS-PAGE analysis of pulldown experiments. Upper panel shows a Coomassie-stained gel, and the lower panel shows a heme-stained gel. The arrowheads indicate protein bands belonging to the three subunits of the potential interaction partner K. stuttgartiensis hydrazine synthase (HzsA, HzsB, and HzsC). The HzsB subunit does not contain a heme group and therefore does not show up in the heme-stained gel. Lane 63 indicates C-terminally 6×His-tagged Kustc0563 used as a control.

Figure 10. NO binding to reduced KsNaxLS and its components. A, effect of NO on the UV-visible spectra of recombinant, reduced KsNaxLS after removal of the His-tag; B, His-tagged KsNaxLS; C, KsNaxL (His-tag removed); and D, KsNaxS (His-tag removed). Reduced and NO-incubated reduced states are shown in blue and black, respectively. E, determination of binding constant of reduced KsNaxLS for NO, by titration with NO, and observation of the spectral change at 553 nm. Two series of measurements are shown, using protein concentrations of 11 and 4.5 μM protein (black and gray data points, respectively). The solid lines show the fits to each series using the average Kd of 12 μM.

**NaxLS binds NO and interacts with hydrazine synthase**

Crystallization experiments with NO- or CO-bound KsNaxLS or subunits thereof were unsuccessful, and incubation of crystals with either of these gases did not result in complex formation. However, our spectroscopic data provide detailed information on CO and NO binding. The UV-visible spectroscopic features of CO-bound KsNaxLS, its components, and its mutants (Soret band around 415–417 nm with increased intensity and flattening of α- and β-bands) are similar to the ferrous six-coordinated CO adducts of other cytochromes (29). This implies that the distal cysteine ligand is flexible enough to move out, enabling distal CO binding.

Upon NO incubation, the as-isolated state of KsNaxL shows a broadening of the Soret band around 395–400 nm. This has been associated in several studies with the formation of a five-coordinated NO (5cNO) adduct in which NO coordinates at the proximal side as the only axial ligand. The additional Soret peak at ~416 nm is suggestive of a six-coordinated NO (6cNO) adduct, where NO binds at the distal side as the sixth axial ligand (30–33). Thus, in the as-isolated state, the spectra of the NO-bound individual components combine features typical for 5cNO and 6cNO complexes and may represent a mixture of the two. Strikingly, however, the spectrum of the as-isolated KsNaxLS complex incubated with NO only shows features typical for a 6cNO adduct, i.e. an increase in its intensity and a shift of the Soret peak from 420 to 417 nm, whereas none of the features indicative of a 5cNO adduct appear. Thus, in the as-isolated state, complex formation appears to result in the exclusive production of 6cNO adducts.

In the reduced state, where the affinity for NO is much higher, the situation is somewhat different. Although the KsNaxL subunit in the reduced state also shows both 5cNO- and 6cNO spectral features upon incubation with NO, the isolated KsNaxS subunit displays a slight decrease in intensity as well as a blue-shift of the Soret band, features typical for a 6cNO adduct formation by reduced cytochromes c. When the reduced KsNaxLS complex is treated with NO, the Soret band decreases in intensity and shifts to 415 nm, again indicating the formation of a 6cNO adduct, but a small shoulder appears around 395 nm. This could be explained by the formation of a small amount of 5cNO complex, whereas most of the NO-bound protein forms a 6cNO adduct. The data thus suggest that in the reduced state, too, the formation of the complex favors the formation of 6cNO adducts. This may be explained by comparing the structure of the KsNaxLS complex with the
NaxLS binds NO and interacts with hydrazine synthase

Figure 10. Proximity of heme-binding motifs in the complex. The heme-binding motif on helix α4 of KsNaxL (120CRNCH124) is in contact with the heme-binding motif on helix α2 of KsNaxS 58CYCH58, as well as with the loop between α2 and α3.

structures of the individual components: the secondary structure elements that contain the proximal histidines are on the surface of the molecules in both individual monomers, but face each other in the complex (Fig. 10). The end of helix α4 containing the heme-binding motif of KsNaxL is in contact with helix α2, which contains the heme-binding motif of KsNaxS. Moreover, although the loop between α2 and α3 in KsNaxS is very rich in conserved glycine residues (Fig. S8), and thus likely confers considerable flexibility to the loop containing the proximal histidine in the monomeric state, in the complex it is in close contact with helix α4 of KsNaxL. The KsNaxLS complex thus appears specifically geared toward the exclusive formation of 6cNO complexes.

Binding to hydrazine synthase: possible functions of the KsNaxLS complex

Two possible roles come to mind for a protein such as KsNaxLS that binds specifically to HZS: nitric oxide and electron transfer. As one of the substrates of HZS, nitric oxide is a precious yet volatile and highly-reactive commodity for the cell. Thus, particularly under routine growth conditions where nitrite is limiting (3) and therefore NO is scarce, a means of retaining NO in the cell and delivering it to HZS would be beneficial. This would require a protein that is highly abundant, capable of NO binding, and interacts with the HZS complex. Indeed, based on transcriptionic data (Table S1) (3) and its ready purification from anammox biomass as reported in this study, it is evident that KsNaxLS is highly expressed in Kuenenia. Moreover, as reported by Ukita et al. (12), the JcNaxLS complex represents ~10% of the total molar protein content in Jettenia cells. Interestingly, however, a recent study (34) reports that when K. stuttgartiensis is grown on NO rather than on nitrite the transcription levels of the genes encoding KsNaxL and KsNaxS are reduced 20- and 10-fold, respectively. Additionally, the current study suggests that KsNaxLS predominantly forms a 6cNO adduct upon NO binding, similar to other NO-shuttling proteins. Our binding studies showed that in the reduced state KsNaxLS has a dissociation constant for NO of ~12 μM. The concentration of NO in anammox cells is not known. Rathnayake et al. (35) have reported NO concentrations of up to 2.7 μM in active anammox granules. However, the intracellular NO concentration is unlikely to be much higher than the low micromolar range, given the Kd of 2.5 μM for hydrazine dehydrogenase reported by Maalcke et al. (6). However, given the large amount of the KsNaxLS complex in the cell, it remains possible that NO binding by KsNaxLS assists in retaining the volatile NO inside the cell.

Alternatively or additionally, the NaxLS complex could function as an electron shuttle between HZS and an electron donor. Earlier, Ukita et al. (12) had noted that excess sodium dithionite was not able to effect complete reduction of JcNaxLS (which we also observed for KsNaxLS) and suggested that the JcNaxLS complex likely has an unusually low redox potential, given the low redox potential of ~660 mV versus SHE of dithionite (36). Because the reaction catalyzed by the HZS complex has a much higher midpoint potential (E’0 = +60 mV) (9), for HZS to accept electrons from a binding partner at such an extremely low redox potential would appear to be “wasteful” from a bioenergetic point of view. However, our redox titrations show that, unexpectedly, KsNaxLS has a redox transition at a much higher potential of only ~175 mV versus SHE (although additional redox transitions at a much lower redox potential cannot be excluded). This makes electron transfer from KsNaxLS to HZS much more economical in terms of energy conservation. Thus, given the properties of the KsNaxLS complex presented here, at least two possible functions for KsNaxLS present themselves, which are not mutually exclusive: NO retention/transfer to HZS and electron transfer to HZS. In vivo research on anammox organisms or in vitro reconstitution of the central anammox pathway could help settle this issue.

Experimental procedures

Protein expression and purification

The native KsNaxLS complex was purified from K. stuttgartiensis cultured as suspended cells in a membrane bioreactor (37, 38). Cells were collected by centrifugation at 8000 rpm for 15 min. The pellet was resuspended in 20 ml of 20 mM potassium phosphate buffer (KPi), pH 7.0. Cells were disrupted by
NaxLS binds NO and interacts with hydrazine synthase

passing them through a French press three times at 1200 p.s.i., and the lysate was incubated with 1% (w/v) sodium deoxycholate for 1 h under mild stirring. Soluble proteins were separated from debris by ultracentrifugation for 1 h at 150,000 × g (Discovery 100 centrifuge with a T-1270 rotor; Sorvall, Newtown, CT). FPLC was performed with an AKTA purifier (GE Healthcare, Uppsala, Sweden). A 30-ml Q-Sepharose XL column (GE Healthcare, Uppsala, Sweden) was equilibrated with 20 mM Tris-HCl buffer, pH 8.0. Cell extract was loaded at 2 ml/min. Nonbinding proteins were washed off with Tris-HCl buffer until the Æ280 signal was stable. KsNaxLS was eluted isocratically at 200 mM NaCl in 20 mM Tris-HCl buffer, pH 8.0 (flow rate 2 ml/min, collected at 2 ml/fraction). Fractions were pooled, desalted, and concentrated. The resulting sample was loaded onto a 10-ml ceramic hydroxyapatite packed column (Bio-Rad), which had been previously equilibrated with 20 mM KPi buffer, pH 7.0. KsNaxLS was eluted isocratically with 500 mM KPi buffer, pH 7.0 (flow rate 2 ml/min, collected at 2 ml/fraction). Pooled fractions were desalted and concentrated using Vivaspin 20 spin filters. For heterologous expression of the KsNaxLS complex and its individual components, the kusta0087 (KsNaxL) and kusta0088 (KsNaxS) genes were cloned either as bicistronic operon or individually into a pUC19-derived vector (39); the primers and vectors used are given in Tables S2 and S3. The native N-terminal signal peptides (Fig. S8) were replaced by the strong signal peptide from the small tetraheme cytochrome c (STC, SO2727) obtained from the expression host Shewanella oneidensis MR-1 (40) (in case of the bicistronic operon, this was done only for the upstream gene kusta0087). The pUC19-derived vectors containing the inserts were transformed into S. oneidensis MR-1 ΔendA (41) by electroporation at 0.55 kV, 25 microfarads, 200 ohms using a Gene Pulser II (Bio-Rad). Large-scale purification, 2-liter batches (6–10 liters total) of LB medium were supplemented with 50 μg/ml kanamycin and inoculated with 1% (v/v) of S. oneidensis MR-1 overnight culture. Initially, large-scale cultures were grown in 5-liter Erlenmeyer flasks (without baffles) at 30 °C and 100 rpm for 5–6 h until an OD600 of 0.6–0.8, upon which the temperature was lowered to 20 °C and the cultures were shaken at 60 rpm for a further 60–70 h. The cells were harvested by centrifugation at 6000 rpm, 4 °C for 10 min in a FibreliteTM F9 – 6 × 1000 LEX rotor (Thermo Fisher Scientific, Darmstadt, Germany). The cell pellets were either directly used for purification or frozen in liquid nitrogen and stored at −80 °C. The expressed proteins were purified using immobilized metal ion-affinity chromatography using nickel-nitrilotriacetic acid–agarose (Qiagen, Hilden, Germany) followed by size-exclusion chromatography using a Superdex 75 (10/300 GL) column connected to an Äkta Purifier FPLC system (GE Healthcare, Uppsala, Sweden). For spectroscopic, electrophotometric, and NO-binding studies of WT proteins, the hexahistidine tags were removed by incubation with tobacco etch virus protease overnight prior to the size-exclusion step.

For reference purposes, another typical monoheme c-type cytochrome from K. stuttgartiensis, Kustc0563, was cloned, expressed, and purified with a C-terminal hexahistidine tag in the same way as described above for the individual components of KsNaxLS. Protein purity was assessed by 15% SDS-PAGE analysis. The identity of the proteins was confirmed by MALDI-MS peptide mass fingerprinting, and total molecular masses were determined by ESI-MS.

**Protein crystallization**

Initial precipitant screenings with the native KsNaxLS complex and its mutants (KsNaxL C32G/NaxS WT and KsNaxL C32M/NaxS C101M) were performed using the sitting drop vapor-diffusion method. 100 nl of concentrated proteins were screened against 100-ml precipitant solutions from commercial crystallization screens pre-pipetted into 96-well sitting drop trays (Greiner XTL low-profile, Greiner Bio One, Frickenhausen, Germany) using a Mosquito crystallization robot (TTP Labtech Ltd., Melbourne, UK) at 20 °C. For larger-scale hanging drop vapor-diffusion crystallization setups, 1 μl of KsNaxLS (WT complex or mutants) protein stock (A1cm = 6 – 7 in 25 mM HEPES/KOH, pH 7.5, 25 mM KCl) was mixed with 1 μl of precipitant solution containing 20–25% (w/v) PEG 3000 and 0.1 M sodium citrate, pH 5.5. The resulting drops were equilibrated against 800-μl reservoir solution in 24-well Linbro plates. All the crystals from WT and mutant complexes were cryo-protected by short soaking in reservoir solution supplemented with 20% (v/v) ethylene glycol, followed by flash-cooling in liquid nitrogen.

**Diffraction data collection and crystal structure determination**

High-resolution native data and high-redundancy single-wavelength anomalous diffraction data were collected at beamline X10SA at the Swiss Light Source (SLS) of the Paul-Scherrer-Institute (Villigen, Switzerland). Diffraction data sets were collected using the rotation method with oscillation ranges of 0.1° (fine-slicing) while the crystals were kept at 100 K. Diffraction images were recorded using a PILATUS 6M detector (Dectris, Baden, Switzerland). Diffraction data were processed using XDS (42), and data statistics are given in Table 1. Experimental phases using data from single-wavelength anomalous diffraction experiments were obtained using AutoSHARP 2.0 (Global Phasing Ltd, Cambridge, UK). Density modification was performed using DM (43). Structural models were iteratively built using COOT (44) and refined using REFMAC (45). Model statistics are given in Table 2. Structure validation was performed using the validation tools in COOT and with MolProbity (http://molprobity.biochem.duke.edu/)6 (52). Figures were prepared using PyMOL (Schrödinger LLC).

**Analytical ultracentrifugation**

AUC-SV was used to determine the oligomeric state of KsNaxLS in solution. For each sample, the buffer was exchanged to 25 mM KCl, 25 mM HEPES/KOH, pH 7.5, by repeated washings in 10-kDa MWCO Amicon tubes (Millipore, Schwabach, Germany). The samples were diluted to A1cm0.280 ~0.42. The measurement cells (Beckman Coulter Inc., Palo Alto, CA) were assembled according to the manufacturer’s instructions. The two sectors of the cells were filled with 400 μl

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of protein sample and 420 μl of buffer, respectively. Samples were sedimented at 30,000 rpm for 24 h at 20 °C in an An-60 Ti rotor in an XL-1 analytical ultracentrifuge (Beckman Coulter Inc.). Absorbance scans were recorded at 280 and 420 nm wavelength. The first 150 scans were processed using SEDFIT (46) to obtain the c(S) distributions, s20,w values (sedimentation coefficients corrected to pure water), and molecular masses assuming a fixed frictional ratio (f/fmin) of 1.2. Sednterp was used to calculate the density and viscosity of the buffer. A partial specific volume of 0.73 ml/g for the proteins was used to fit the data (13). AUC-SE measurements were performed to determine the molecular mass of the KsNaxLS complex and its components in solution. AUC-SE experiments were carried out in the same cells but with 200 μl of sample and 220 μl of buffer. Two angular velocities (20,000 and 30,000 rpm) were used to equilibrate the samples. Five photometric scans at 280 nm were recorded at each speed after 22 h of equilibration at 20 °C. AUC-SE data were processed using SEDPHAT (46).

**SEC-MALS**

Protein samples were separated on Superdex 75 gel-filtration columns (GE Healthcare, Uppsala, Sweden). Analyses were performed using a 1260 Infinity II HPLC system (Agilent Inc., Santa Clara, CA) equipped with a photodiode array detector SPD-M20A (Shimadzu, Duisburg, Germany). Static light-scattering analyses were performed in line using a DAWN HELEOS II multiangle scattered light photometer (Wyatt Technology, Santa Barbara, CA) combined with a refractive index detector Optilab T-rEX (Wyatt Technology, Santa Barbara, CA). The system was equilibrated with a buffer containing 50 mM HEPES, pH 7.5, and 150 mM KCl. A flow rate of 0.5 ml min⁻¹ was used. To calibrate the gel-filtration column, a standard (Bio-Rad) containing several proteins was used. 40 μl of each sample with concentrations up to 150–250 μM were injected using an autosampler. Data analysis was performed using the ASTRA 7.1.2 software (Wyatt Technology, Santa Barbara, CA), providing weight-averaged molar masses of the eluting species.

### Table 1
**Data collection and processing statistics**

|                      | KsNaxLS Fe-SAD | KsNaxLS native, PDB code 6R6M | KsNaxLS C32M/C101M, PDB code 6R6N | KsNaxLS C32G/WT, PDB code 6R6O |
|----------------------|----------------|-------------------------------|----------------------------------|---------------------------------|
| **Diffraction source** | SLS PXII       | SLS PXII                      | SLS PXII                         | SLS PXII                        |
| **Wavelength (Å)**    | 1.7433         | 1.000                         | 1.000                            | 1.000                           |
| **Temperature (K)**   | 100            | 100                           | 100                              | 100                             |
| **Detector**          | Pilatus 6 M    | Pilatus 6 M                   | Pilatus 6 M                      | Pilatus 6 M                     |
| **Rotation range per image (°)** | 0.1     | 0.15                          | 0.2                             | 0.2                             |
| **Total rotation range (°)** | 360     | 180                           | 180                             | 120                             |
| **Exposure time per image (s)** | 0.1     | 0.15                          | 0.2                             | 0.2                             |
| **Space group**       | P2,2,2         | P2,2,2                        | P2,2,2                           | P2,2,2                          |
| **α, β, γ (°)**       | 90, 90, 90     | 90, 90, 90                    | 90, 90, 90                       | 90, 90, 90                      |
| **Resolution range (Å)** | 40.0–2.3       | 40.0–1.7                      | 50.0–2.0                         | 50.0–1.9                        |
| **Total no. of reflections** | 454,148       | 184,876                       | 116,845                          | 182,263                         |
| **Completeness (%)**  | 100 (99.9)     | 98.8 (93.4)                   | 99.6 (99.4)                      | 99.6 (99.5)                     |
| **Redundancy**        | 19.8 (17.6)    | 6.3 (5.0)                     | 6.2 (6.4)                        | 8.5 (8.6)                       |
| **(f/σ(f))**          | 28.1 (5.6)     | 17.6 (2.2)                    | 13.7 (2.0)                       | 19.2 (3.0)                      |
| **Rmax (%)**          | 8.6 (56.2)     | 6.0 (61.0)                    | 12.3 (73.6)                      | 7.3 (96.0)                      |

### Table 2
**Refinement and model statistics**

|                      | KsNaxLS native, PDB code 6R6M | KsNaxLS C32M/C101M, PDB code 6R6N | KsNaxLS C32G/WT, PDB code 6R6O |
|----------------------|-------------------------------|----------------------------------|---------------------------------|
| **Resolution range (Å)** | 65.2–1.75                    | 65.7–2.0                         | 65.7–1.9                        |
| **Completeness (%)**  | 98.8                          | 99.5                             | 99.5                            |
| **α-Cutoff**          | None                          | None                             | None                            |
| **No. of reflections, working set** | 27949 | 17705                          | 20427                           |
| **No. of reflections, test set** | 1450          | 960                             | 1881                            |
| **Final R_pwr**       | 0.188                         | 0.200                            | 0.182                           |
| **Final R_exp**       | 0.210                         | 0.236                            | 0.206                           |
| **ML-based coordinate error (Å)** | 0.13  | 0.24                           | 0.20                           |
| **No. of non-H atoms** | 1632                         | 1626                            | 1620                            |
| **Protein**           | 86                            | 86                              | 86                              |
| **Heme groups**       | 192                           | 129                             | 173                             |
| **Water**             | 1910                          | 1841                            | 1879                            |
| **Average B factors (Å²)** | 0.010                  | 0.008                           | 0.007                           |
| **Angles (%)**        | 0.972                         | 1.063                            | 0.979                           |
| **Average B factors (Å²)** | 34.4                      | 41.2                            | 38.6                            |
| **Heme groups**       | 26.4                          | 34.7                            | 30.0                            |
| **Water**             | 40.0                          | 44.0                            | 45.5                            |
| **Ramachandran plot** | 98.5                          | 97.6                            | 98.5                            |
| **Most favored (%)**  | 1.5                           | 2.4                             | 1.5                             |
| **Allowed (%)**       | 0.0                           | 0.0                             | 0.0                             |

NaxLS binds NO and interacts with hydrazine synthase

[^1]: J. Biol. Chem. (2019) 294(45) 16712–16728
**NaxLS binds NO and interacts with hydrazine synthase**

Solution SAXS measurements were performed at the cSAXS beamline (X12SA) of the Swiss Light Source at the Paul Scherrer Institute, Villigen (Switzerland). KsNaxLS was concentrated to an $\Delta A_{280}$ of $-0.9$ in 25 mM HEPES/KOH, pH 7.5, 25 mM KCl. Scattering data were measured in a 1-mm diameter quartz capillary kept at 283 K. The X-ray photon energy was 11.2 keV, and 200 measurements of 0.5 s each were recorded over 10 positions along the length of the capillary, which was mounted at a distance of 2.133 m from a Pilatus 2M detector. Background measurements with buffer were performed using the identical capillaries, positions, and measurement protocol. Scattering data were used to a maximum momentum transfer of 0.25 Å$^{-1}$. Data analysis and three-dimensional reconstruction were performed using PRIMUS (47), GNOM (48), and GASBOR (49) from the ATSAS suite.

**Small-angle X-ray scattering**

All spectroscopic measurements of WT proteins were performed on heterologously-expressed proteins after removal of the hexahistidine tags unless otherwise stated, and for mutant proteins the His-tags were not removed. Spectra in the UV and visible range (200–700 nm, scan speed 400 nm/min) were recorded in 200-μl quartz micro-cuvettes with 1.0-cm path length (Hellma GmbH, Müllheim, Germany) at 0.5 nm bandwidth using JASCO V-650 or V-760 spectrophotometers (Jasco GmbH, Gross-Umstadt, Germany) at room temperature. The data were processed using the Jasco32 software. For qualitative spectra, protein samples were diluted in 25 mM KCl, 25 mM HEPES/KOH, pH 7.5. Ferrous protein samples were prepared in a nitrogen-filled glove box (Belle Technology Ltd., Weymouth, UK, <10 ppm O$_2$) using thoroughly degassed and argon-saturated buffers. Samples of the KsNaxLS complex and its mutants (A$_{280}^\text{cm}$ = 0.25–0.5) were reduced by adding freshly prepared 10 mM Ti(III)citrate (about 1 mM final concentration) in a buffer containing 25 mM KCl, 25 mM HEPES/KOH, pH 7.5. The reduced samples were transferred to stoppered quartz microcuvettes to measure the spectra outside of the glove box. Afterward, the cuvette was opened, and 10 ml of air supplied from a syringe was gently bubbled through the samples to obtain the spectra of the re-oxidized proteins.

**UV-visible spectroscopy**

Afterward, the cuvette was removed from the glove box, and an absorption spectrum was measured immediately using a JASCO V-650 spectrophotometer (Jasco GmbH, Gross-Umstadt, Germany) between 400 and 700 nm, with a 0.5-nm data interval and at a data acquisition rate of 400 nm/min. For each measurement, the NO concentration in the stock solution was determined using the Griess assay (50). Two data sets were measured, one at a KsNaxLS concentration of 4.5 μM and one at 11 μM, and the absorption change at 553 nm was used to determine binding. Each binding curve was fitted independently using the quadratic equation, and the weighted arithmetic mean of the fit results was calculated to arrive at an estimated $K_d$ of 12 μM.

**Redox titration**

To determine redox potentials, the protein was titrated with Ti(III)citrate or ferricyanide inside a nitrogen-filled glove box (Belle Technology Ltd., Weymouth, UK, <10 ppm O$_2$) using thoroughly degassed and argon-saturated buffers. 1.5 ml of protein solution (15 μM in 25 mM HEPES/KOH, pH 7.0, 100 mM KCl) was mixed with 5 μM each of the following redox mediators: potassium ferricyanide, 2,5-dimethyl-p-benzoquinone, 1,2-naphthoquinone; phenazine methosulfate, 1,4-naphthoquinone; phenazine ethosulfate, 5-hydroxy-1,4-naphthoquinone, 2-methyl-1,4-naphthoquinone, 2,5-dihydroxy-p-benzoquinone, 2-hydroxy-1,4-naphthoquinone; anthraquinone; sodium anthraquinone-2-sulfonate; benzyl viologen; and methyl viologen. The mixture was placed in a quartz cuvette (10 mm path length), and a small magnetic stirring bar was added. The cuvette was placed in a CV100 cuvette holder (Thorlabs, Dachau, Germany) equipped with collimating lenses (LA4647, Thorlabs, Dachau, Germany) and fiberoptic light guides that were fed out of the glove box through silicone-filled plugs. The cuvette holder was placed on a miniature magnetic stirrer, and light was supplied from a CLX500 xenon lamp (Zeiss, Oberkochen, Germany) through one of the fiberoptic cables, whereas the exiting light was fed through the other light guide into an AvaSpec ULS2048CL spectrometer (Avantes, Apeldoorn, The Netherlands). The redox potential was monitored using a 0.5-mm diameter platinum wire.
**NaxLS binds NO and interacts with hydrazine synthase**

and a 3.5-mm diameter Ag/AgCl, 4 m KCl reference electrode (Pine Research, Durham, NC) using a custom-built, high-input impedance recording millivoltmeter. The protein was tittered with 10- µl portions of ferricyanide or Ti(III) stock solution (10 mM on redox potential plateaus, 0.5 mM for Ti(III)citrate, and 1 mM for ferricyanide close to redox transitions) using a gas-tight Hamilton syringe. Absorption spectra were recorded when the potential difference had reached a stable value. The Nernst equation was used to fit the data, and the obtained values were corrected to reflect the potentials with respect to the SHE assuming an offset of 200 mV.

**Pulldown assay**

The KsNaxLS complex (with a C-terminal His-tag on KsNaxS), its individual subunits, and Kustc0563, another small soluble cytochrome c from *K. stuttgartiensis*, were used as bait by immobilizing them on Ni-IDA Profinity beads (Bio-Rad, Munich, Germany), which were pre-equilibrated with wash buffer (150 mM NaCl, 50 mM HEPES/NaOH, pH 7.5). For the preparation of a cell lysate from *K. stuttgartiensis* biomass, 180–200 mg of cell pellet was resuspended in 1 ml of lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl and 0.4% (v/v) Triton X-100). The cells were disrupted by sonication using a Branson 250 sonifier (G. Heinemann, Schwäbisch Gmünd, Germany) equipped with a sonication microtip at 50% amplitude, 0.5 s bursts, and a total of 20 pulses that was repeated three times. The lysate was incubated for 15 min while rotating in a cold room at 8 °C. Then, the lysate was cleared by centrifugation at 16,000 × g, 15–20 min at 4 °C. 50 µl of Ni-IDA beads loaded with the individual immobilized bait proteins were incubated with 100 µl of *K. stuttgartiensis* cell lysate in 2-ml Eppendorf tubes while rotating at 8 °C for 60 min. In parallel, another set of bait-loaded Ni-IDA beads (50 µl) was incubated with 100 µl of purified *K. stuttgartiensis* hydrazine synthase (KsHZS) protein solution (A

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