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Accelerated Publications

Shewanella oneidensis MR-1 H-NOX Regulation of a Histidine Kinase by Nitric Oxide†

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ABSTRACT: Nitric oxide (NO) signaling in animals controls processes such as smooth muscle relaxation and neurotransmission by activation of soluble guanylate cyclase (sGC). Prokaryotic homologues of the sGC heme domain, called H-NOX domains, have been identified and are generally found in a predicted operon in conjunction with a histidine kinase. Here, we show that an H-NOX protein (SO2144) from *Shewanella oneidensis* directly interacts with the sensor histidine kinase (SO2145), binds NO in a 5-coordinate complex similar to mammalian sGC, and in that form inhibits the activity of a histidine kinase (SO2145). We also describe the first account of NO formation by *S. oneidensis* under anaerobic growth conditions derived from nitrate and nitrite. These observations suggest that the *S. oneidensis* H-NOX and histidine kinase pair function as part of a novel two-component signaling pathway that is responsive to NO formation from higher nitrogen oxides used as electron acceptors when oxygen is low and thereby functioning as an environmental sensor.

A new family of prokaryotic proteins with high sequence identity (15−40%) to the heme domain of the mammalian soluble guanylate cyclase (sGC[1]) has recently been discovered (*1*, *2*). The heme domain of sGC is a specific receptor for nitric oxide [reviewed in ref (*3*)]. Several proteins in this family, termed H-NOX (for heme-nitric oxide/oxygen binding) proteins, have since been described in terms of ligand binding, structural characteristics, and spectroscopic properties (*2*, *4*−*7*). In prokaryotes they are present in predicted operons, and the downstream presence of histidine kinases is suggestive of a signaling role (Figure 1). However, a physiological role for these proteins has not been elucidated. In facultative aerobes like *Shewanella oneidensis*, the H-NOX is most often found within a predicted operon and upstream of a predicted histidine kinase suggesting that they might act in concert as a sensor−kinase pair similar to that seen with two-component signaling systems. In two component signaling, the sensor typically responds to some environmental change leading to an alteration of the cognate kinase activity. However, most two component signaling systems are so-named because they involve a sensor−kinase protein and a response regulator functioning together to bring

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[1] Abbreviations: sGC, soluble guanylate cyclase; H-NOX, heme-nitric oxide/oxygen; ODQ, 1*H*-[1,2,4]oxadiazole[4,3-*a*]quinoxalin-1-one; Ni-NTA, nickel nitriloacetic acid; NtrB, nitrogen regulator histidine kinase; NtrC, nitrogen regulator transcriptional activator; SO2144, *S. oneidensis* H-NOX; SO2145, *S. oneidensis* histidine kinase.

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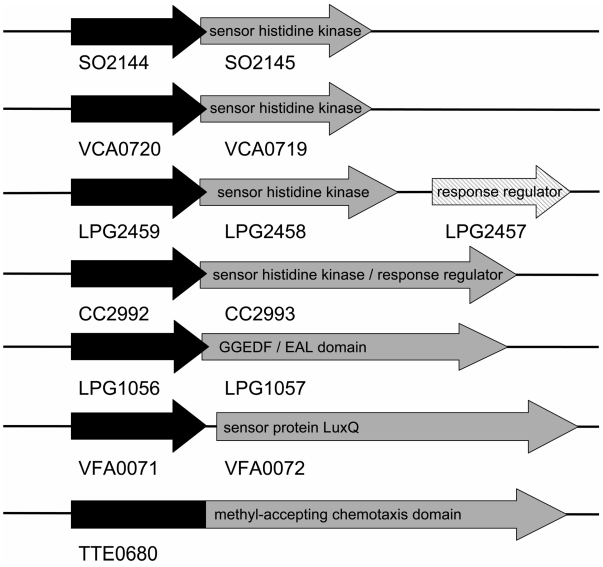

FIGURE 1: Genomic organization of H-NOX proteins. H-NOX operon organization is shown for *Shewanella oneidensis* (SO2144, SO2145), *Vibrio cholerae* El Tor (VCA0720, VCA0719), *Legionella pneumophilia* Philadelphia (LPG 2459, LPG2458, LPG2457), *Caulobacter crescentus* (CC2992, CC2993), and *Vibrio fischeri* (VFA0071, VFA0072). The H-NOX gene can be followed by a predicted cytoplasmic sensor histidine kinase, sometimes with a response regulator (LPG genes). *Legionella pneumophilia* Philadelphia also contains an additional H-NOX operon (LPG1056, LPG1057) with the H-NOX upstream of a predicted a diguanylate cyclase gene. CC2993 contains a receiver domain. VFA0071/VFA0072 is unique in that the hybrid kinase that follows the H-NOX gene contains an HPT domain as well as a receiver domain. TTE0680 illustrates the gene from *Thermoanaerobacter tengcongensis,* and is a fusion of an H-NOX domain to a methyl accepting chemotaxis protein. Overlap in arrows indicates overlapping reading frames. Operon predictions were accessed at MicrobesOnline (www.microbesonline.org) from the VIMSS database (*23*).

about a response (*8*). Regulation by cytoplasmic sensor H-NOXs and histidine kinases represents a smaller subset of this kind of signaling (*9*). A prototypic example of a sensor system involving a cytoplasmic kinase is NtrB that is involved in the regulation of nitrogen metabolism (*10*, *11*). Histidine kinases are central to bacterial "two-component" signal transduction and typically function to translate environmental information into specific adaptive responses within the cell (*12*, *13*). Histidine kinase-mediated responses are diverse and include regulation of chemotaxis, pathogenesis, and metabolism (*14*−*16*). Many of these kinases are regulated through specific protein−protein interactions in which auto-kinase activity is enhanced or inhibited in response to partner binding (*16*, *17*).

In this report we show that the H-NOX (SO2144) from *S. oneidensis* has similar NO binding properties to the NO sensor sGC, specifically it is stable in the ferrous oxidation state and binds NO and CO but not O_2. This ligand discrimination against O_2 is crucial to sGC, as it must sense low levels of NO in the presence of a much higher concentration of O_2. We also report that SO2145 is a soluble histidine kinase that is autophosphorylated in an ATP-dependent reaction. We also found that the NO-bound form of the H-NOX SO2144 inhibits the autophosphorylation histidine kinase SO2145. The products of respiratory denitrification by *S. oneidensis* have been subject of controversy over the past 20 years (*18*−*20*). Much of the past studies have centered on NH_3, N_2O, and N_2 formation, while NO production has not been directly addressed. Here we show for the first time that this organism is indeed capable of generating NO under anaerobic growth when supplied with NO_3^- and NO_2^-. Overall the results suggest that *Shewanella* may sense changes in the aerobic/anaerobic environment by detecting NO via the H-NOX SO2144. The NO-bound form of SO2144 would then inhibit the histidine kinase SO2145, affecting a signaling cascade that leads to a yet to be determined response in *Shewanella*.

EXPERIMENTAL PROCEDURES

Materials. The pET20b expression vector, *Escherichia coli* Tuner(DE3)pLysS and DH5α competent cells were purchased from Novagen. *S. oneidensis* (strain MR-1) genomic DNA was from ATCC. Restriction enzymes were obtained from New England Biolabs. IPTG was from Promega. The Expand High Fidelity PCR kit and the Rapid Ligation Kit were from Roche. Plasmid purification kits, gel extraction kits, Ni-NTA superflow resin, and Ni-NTA magnetic beads were purchased from Qiagen. Pefabloc protease inhibitor was obtained from Pentafarm. Benchmark protein ladder and precast 10−20% tris-glycine gels were from Invitrogen. DEA/NO was supplied by Cayman Chemicals. CO (99.5%) was from Airgas. SDS, Coomassie Blue R-250, and Bradford protein dye were from Bio-Rad. PCR primers were synthesized by Elim Biopharmaceuticals Inc. Radionucleotides were obtained from MP Biopharmaceuticals. The Western blot kit was purchased from Pierce. All other chemicals were supplied by Sigma.

Construction of Expression Plasmids. SO2144 H-NOX and SO2145 histidine kinase open reading frames were amplified from *S. oneidensis* genomic DNA using Expand High Fidelity PCR (Roche). The upstream and downstream primers for SO2144 were 5′-GTC CCA TAT GAA AGG AAT TAT TTT TAA TGT G-3′ and 5′-CAC TGC GGC CGC TCA GTC ATT TTG TAA CTC G-3′. The downstream primer 5′-CAC TGC GGC CGC GTC ATT TTG TAA CTC GAT G-3′ was used for the SO2144 construct with a C-terminal hexa-histidine tag. The upstream and downstream primers for SO2145 were 5′-GTC CCA TAT GAC TGA CAG CGA AAA TCC CTA T-3′ and 5′-CAC TGC GGC CGC CTA GGT TAT CGA GCT AGA AG-3′. The downstream primer 5′-CAC TGC GGC CGC GGT TAT CGA GCT AGA AGT C-3′ was used for the SO2145 construct with C-terminal hexa-histidine tag. Upstream primers for both open reading frames contained *Nde*I restriction sites, and downstream primers contained *Not*I sites. PCR fragments were purified using 1% agarose and isolated using a gel extraction kit. The PCR fragments were digested and ligated into a pET20b expression using a Rapid Ligation Kit. The ligation reactions were then transformed into and propagated in DH5α cells. All constructs were sequenced by Elim Biopharmaceutics Inc.

Expression Cloning. Plasmids pET20b/SO2144 or pET20b/SO2145 were transformed into *E. coli* Tuner (DE3)pLysS cells. Protein expression was carried out as previously described (*21*).

Protein Purification. Non-His-tagged proteins were purified as described previously (*2*). His-tagged SO2144 and SO2145 constructs were purified as above with the following changes. A 10 mL Ni-NTA column was substituted for the

Toyopearl Q 650M anion exchange column. Cell pellets from three 1 L cultures containing either His-tagged SO2144 or SO2145 were thawed on ice and resuspended in 100 mL of buffer A (50 mM NaH_2PO4, 20 mM imidazole, 300 mM NaCl, 5% glycerol). Pefabloc protease inhibitor was added to 1 mM and β-mercaptoethanol to 5 mM. Resuspended cells were lysed by sonication as described (*2*). Supernatant was collected by ultracentrifugation at 42,000 rpm and applied to a 10 mL Ni-NTA Superflow (Qiagen) column at 0.5 mL/min using a Biologic LP (Bio-Rad). The column was washed with 20−30 column volumes of buffer A, followed by a linear gradient of buffer B (50 mM NaH_2PO_4, 250 mM imidazole, 300 mM NaCl, 1 mM β-mercaptoethanol) over 10−15 column volumes. Fractions containing the appropriate protein were collected and purity was assessed by SDS−PAGE as greater than 95%. Protein identity was confirmed by N-terminal sequencing. All protein concentrations were determined in triplicate by the Bradford method.

UV/Vis Spectroscopy. Spectra were collected on a Varian Cary 300 Bio UV/vis spectrometer as previously described (*2*). SO2144-Fe^{2+} was prepared by the addition of 10 mM $Na_2S_2O_4$ for 30 min at 25 °C and desalted using a PD10 column (Amersham) equilibrated with deoxygenated buffer C (50 mM DEA, 150 mM NaCl, 5% glycerol, pH 8.0). The sample was placed in a septum-sealed anaerobic cuvette, and spectra were recorded against a baseline of buffer C from 200 to 700 nm. SO2144-Fe^{2+}−NO was prepared anaerobically with the addition of 10-fold excess DEA/NO to SO2144-Fe^{2+} and incubation at 25 °C for ∼3 half-lives (45 min). SO2144-Fe^{2+}−NO was desalted, and spectra were recorded in a septum-sealed anaerobic cuvette against a baseline of buffer C from 200 to 700 nm. SO2144-Fe^{2+}−CO was prepared by placing SO2144-Fe^{2+} in an anaerobic Reacti-Vial and flushing the headspace with CO gas for 10 min. Protein was transferred to a septum-sealed anaerobic cuvette, and spectra were obtained against a blank of buffer C from 200 to 700 nm.

Histidine Kinase Autophosphorylation Assay. The kinase activity of SO2145 was assayed by the addition of 10 μCi of ATP [γ-^{32}P], 1 mM ATP, and 5 mM $MgCl_2$ to 5 μM SO2145 in buffer C at 25 °C. Aliquots were quenched at t = 0, 0.16, 0.5, 1, 3, 5, 10, 20, and 60 min with SDS−PAGE loading buffer. Samples were separated on 10−20% tris-glycine gels at 225 mV for 65 min. Gels were washed 3 × 15 min in 30% MeOH, 5% glycerol on an orbital shaker and dried overnight on a slab gel dryer (Hoeffer Scientific Instruments). The dried gels were exposed overnight (16−24 h) on a Kodak phosphorimaging plate. Data were collected on a Storm Phosphorimager (Amersham) at 100 μm resolution and quantified with the software package Imagequant (Amersham). Experiments were run in duplicate. The anti-His-tagged Western blot was carried out according to the manufacturers instructions.

H-NOX/Kinase Activity Assays. To determine the effect of the SO2144 H-NOX on SO2145 kinase activity, 1 μM SO2145 histidine kinase in buffer C was incubated with 0, 1, 5, 10, 25, 50, and 100 μM SO2144-Fe^{2+}−NO, SO2144-Fe^{2+}−CO, SO2144-Fe^{3+} or 100 μM SO2144-Fe^{2+} for 30 min at 25 °C. Reactions were initiated by addition of 10 μCi of ATP [γ-^{32}P], 500 μM ATP, and 2.5 mM $MgCl_2$. Assays were quenched at 30 min with SDS−PAGE loading buffer and analyzed by SDS−PAGE for 65 min at 225 mV. Gels were washed and dried and data was collected as described above. Each experiment was performed in duplicate or triplicate.

H-NOX−Kinase Interaction Assay. A 50 μL slurry of Ni-NTA magnetic beads (binding capacity 15 μg) was pre-equilibrated with 5 × 500 μL of buffer A. The Ni-NTA magnetic beads were first prebound with an excess (30 μg) amount of His-tagged SO2145 for 30 min at 25 °C in a 1.7 mL microcentrifuge tube. The SO2145-bound beads were then washed with 1 × 150 μL of buffer A and decanted. *E. coli* supernatant (centrifugation at 42,000 rpm) containing non-His-tagged SO2144 (5−10 mg/mL) was then added to the immobilized His-tagged SO2145 to a final volume of 300 μL and placed on a rotating shaker for 1 h at room temperature. The protein/bead mixture was then placed into the magnetized chamber and decanted. The beads were then washed with 3 × 300 μL of buffer A. Protein elution was carried out by the addition of 30 μL of buffer B and analyzed by SDS−PAGE and N-terminal sequencing.

Nitric Oxide Production in Shewanella oneidensis. S. oneidensis MR1 was grown under anaerobic conditions as follows. A single colony was grown aerobically in 5−10 mL of LB for 8−10 h at 30 °C. A 2 mL aliquot was then used to inoculate 150 mL of MR1 medium (*22*) containing 30 mM D,L-lactic acid as a carbon source. The culture was incubated aerobically at 30 °C until an OD_{600} of ∼0.5−0.8. The rate of cell growth did not change significantly over the course of the experiment. Cells were harvested by centrifugation and resuspended in MR1 medium containing 30 mM D,L-lactic acid to a final OD_{600} of ∼0.6. The resuspended culture was then split into 8 mL cultures in 17 mL Hungate anaerobic culture tubes and was made anaerobic by flushing with argon for 15 min. The following electron acceptors were added: 5 mM $NaNO_3^-$, 5 mM $NaNO_2^-$, or 5 mM $Na_2S_2O_3$. Colony forming units (CFUs) were assessed at various time points. NO production was determined using a Sievers nitric oxide analyzer NOA 280i. A headspace sample (100 μL) from each culture tube was withdrawn using a gastight Hamilton syringe, and NO was quantified by comparison to NO gas standards. Samples were analyzed in triplicate and standards in quadruplicate. MR1 medium saturated with NO gas was used to generate a calibration curve. All standards were generated in 17 mL Hungate anaerobic culture tubes containing 8 mL of MR1 media. The samples were flushed with NO gas that was purified by bubbling it through 50% w/v KOH for 20−30 min. A sample of the headspace (5 μL) was taken to make a 1:1000 dilution in a 5 mL anaerobic tube (Supelco). Different volumes of headspace (2.5, 5, 7.5, 10, 12.5, 15 μL) were taken from this tube and injected into the NOA. With each injection representing a known amount of NO, the calibration curve was then plotted based on moles of NO versus peak area generated.

RESULTS

The protein SO2144 was initially identified from a BLAST search as a probable H-NOX protein, with ∼30% sequence identity to the heme domain of sGC. SO2144 is located in a predicted operon with a histidine kinase (SO2145) (*23*). To characterize the putative H-NOX SO2144 and to investigate whether SO2144 and SO2145 might participate in a two-component signaling pathway, SO2144 and SO2145

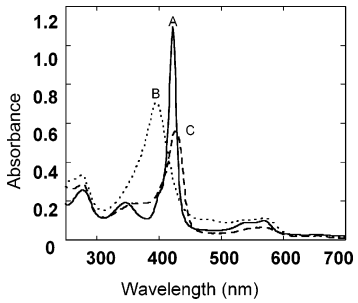

FIGURE 2: UV/vis spectra of H-NOX SO2144. A: The spectrum of the 6-coordinate, low-spin $Fe^{2+}-CO$ complex (solid line), with the Soret maximum at 424 nm. B: The spectrum of the 5-coordinate $Fe^{2+}-NO$ complex (dotted line), with the Soret maximum at 398 nm. C: The spectrum of the 5-coordinate, high-spin Fe^{2+} unligated species (dashed line), with the Soret maximum at 427 nm.

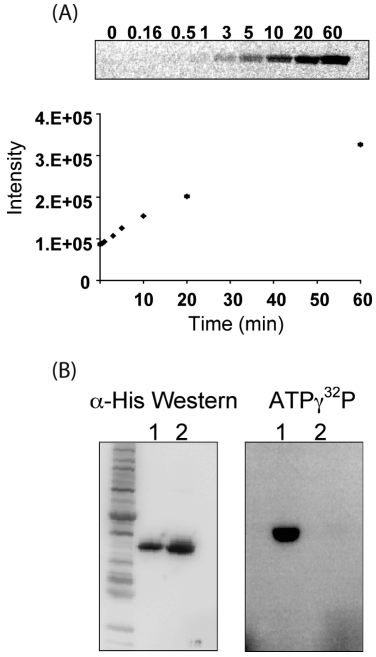

FIGURE 3: Autophosphorylation of SO2145. Kinase autophosphorylation assays were carried out with radiolabeled ATP as described in the Experimental Procedures, and aliquots were taken at 0, 0.16, 0.5, 1, 3, 5, 10, 20, and 60 min and analyzed by SDS−PAGE and autoradiography. Panel A: Autoradiograph of a kinase assay with a plot of the relative signal intensity ± standard error from two experiments. Panel B: Anti-His-tag Western blot of WT SO2145 kinase (lane 1) and the mutant H72A (lane 2) at the predicted site of autophosphorylation accompanied by an autoradiograph of WT (lane 1) and the mutant kinase (lane 2).

were cloned, expressed in *E. coli*, and purified to homogeneity and the identities confirmed by N-terminal sequencing.

UV/Vis Spectroscopy. As purified, the H-NOX SO2144 does indeed contain heme and forms stable complexes with NO and CO, displaying electronic spectra that are nearly identical to sGC (*24*) (Figure 2) including no measurable affinity for O_2.

Kinase Activity Assays. SO2145 was found to be constitutively active *in vitro*, exhibiting time-dependent autophosphorylation in the presence of ATP (Figure 3A). The rate of phosphorylation is slow and not unlike that observed with other *in vitro* histidine kinases, such as VanS involved in vancomycin resistance (*25*). We have not determined the stoichiometry of the phosphorylation nor have we studied the stability of the phosphorylated enzyme. The level of

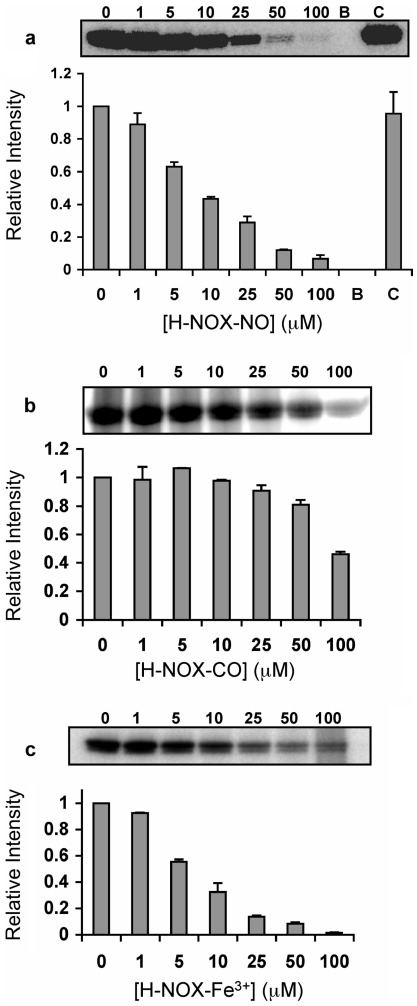

FIGURE 4: Effect of the SO2144 H-NOX on the kinase activity of SO2145. Kinase assays containing 1 μM SO2145 were carried out for 30 min with radiolabeled ATP in the presence of different H-NOX heme complexes as described in the Experimental Procedures, and samples were analyzed by SDS−PAGE and autoradiography. (a) The H-NOX $Fe^{2+}-NO$ complex inhibits kinase activity. Top: Autoradiograph of a kinase assay in the presence of increasing amounts of H-NOX $Fe^{2+}-NO$ complex. B is a blank lane; C is an assay carried out with 100 μM H-NOX−Fe^{2+}. Bottom: Plot of relative signal intensity ± standard error from three experiments. (b) The H-NOX $Fe^{2+}-CO$ complex has little effect on kinase activity. Top: Autoradiograph of a kinase assay in the presence of increasing amounts of H-NOX Fe^{2+}-CO complex. Bottom: Plot of relative signal intensity ± standard error from three experiments. (c) The H-NOX−Fe^{3+} species inhibits kinase activity. Top: Autoradiograph of a kinase assay in the presence of increasing amounts of H-NOX−Fe^{3+}. Bottom: Plot of relative signal intensity ± standard error from three experiments. The background in the scanned gels is different, and this was left unadjusted.

phosphorylation is dependent on a number of factors as outlined by Russo and Silhavy (*26*). A mutant, H72A, of SO2145 was constructed at the predicted site of phosphorylation, and no autophosphorylation was observed under the same reactions conditions (Figure 3B). The effect of ligation and oxidation state of the SO2144 H-NOX heme ($Fe^{2+}-NO$, $Fe^{2+}-CO$, Fe^{2+}, and Fe^{3+}) on the SO2145 kinase activity was also examined. Unligated SO2144-Fe^{2+} had no effect on kinase activity at any concentration tested (Figure 4a, lane C). However, the SO2144-$Fe^{2+}-NO$ complex inhibited autophosphorylation of the kinase in a concentration-dependent manner. At 5−10 μM, SO2144-$Fe^{2+}-NO$

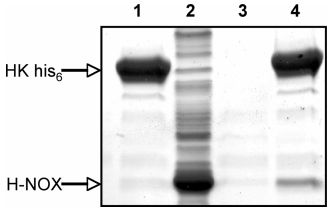

FIGURE 5: SO2144 H-NOX interacts with SO2145 kinase. Pull-down experiments were carried out using magnetic Ni-NTA beads, His-tagged kinase, and *E. coli* lysate containing overexpressed untagged HNOX as described in the Experimental Procedures. Lane 1: + His-tagged SO2145 kinase − SO2144 H-NOX. Lane 2: + SO2144 H-NOX loading control. Lane 3: + S02144 H-NOX − His-tagged SO2145 kinase. Lane 4: + SO2144 H-NOX + His-tagged SO2145 kinase.

gave 50% inhibition (Figure 4a). The SO2144-Fe^{2+}−CO complex also inhibited kinase activity; however, inhibition was not observed until the concentration of SO2144-Fe^{2+}−CO was 100-fold over that of the kinase (Figure 4b). The SO2144-Fe^{3+} species also inhibited kinase activity as effectively as the Fe^{2+}−NO complex (Figure 4c). However, the unligated H-NOX−Fe^{2+} species is very stable *in vitro* and quite difficult to oxidize (requiring more than 100-fold excess of common oxidizing agents such as $K_3Fe(CN)_6$ or ODQ for 30 min at 25 °C), suggesting that this oxidation state may not be relevant under physiological conditions. Due to the similar inhibition profiles between the ferrous nitrosyl H-NOX and the oxidized H-NOX on kinase activity, we investigated whether NO was oxidizing the heme and if kinase inhibition was due to the redox state of the iron. Spectral studies showed, however, that in the presence and absence of the kinase, with and without saturating levels of ATP, the SO2144-Fe^{2+}−NO complex is quite stable and does not oxidize throughout the course of the assay (data not shown). In addition, the kinase activity itself is unaffected by NO when present at 5−10 μM (identical concentrations as those used in the H-NOX reactions) (data not shown).

H-NOX−Kinase Interaction Assays. The interaction between SO2144 and SO2145 was examined using a Ni-NTA magnetic bead capture assay. Utilizing a hexa-His-tagged construct of SO2145, a specific protein−protein interaction between the H-NOX protein and histidine kinase was observed (Figure 5). The converse experiment using tagged H-NOX protein and non-tagged kinase gave identical results. N-terminal sequencing was used to confirm the identity of the associated proteins. The interaction was independent of ligation state of the H-NOX as both the NO bound and the ferrous unligated H-NOX were pulled down (data not shown). The strength of the interaction could be influenced by ligation state, but that was not observed here.

NO Formation by S. oneidensis. NO formation by *S. oneidensis* was investigated as described. NO formation occurred only under microaerobic/anaerobic conditions and showed an absolute dependence on either NO_3^- or NO_2^- (Figure 6). NO formation from NO_2^- was observed within minutes, whereas, in cultures supplemented with NO_3^-, there was a 15−30 min lag before NO was detected, suggesting that the reduction of NO_3^- to NO proceeds via NO_2^-. NO was not detected in the presence of sodium thiosulfate, an alternate electron acceptor, or in control samples without bacteria. NO formation was not observed using boiled bacterial cultures supplemented with NO_3^- or NO_2^- (data not shown). Although no change in pH was observed during the course of the experiments, the formation of NO upon acidification of nitrite cannot be ruled out.

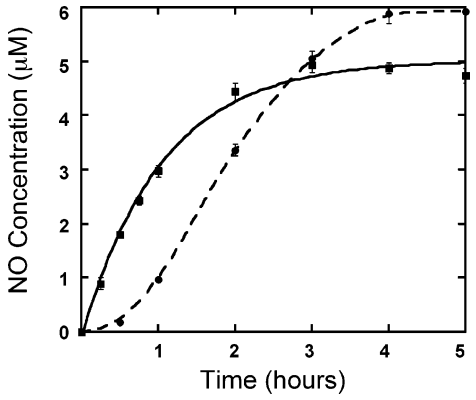

FIGURE 6: NO production by *S. oneidensis*. Bacteria were grown anaerobically in the presence of 5 mM nitrite (solid line) or 5 mM nitrate (dashed line), and headspace NO was measured with a chemiluminescence NO analyzer. *S. oneidensis* is capable of producing NO in the presence of either alternate electron acceptor.

DISCUSSION

Our previous studies with H-NOX domains have outlined key molecular details of ligand binding, especially with regard to discrimination against O_2 as a ligand (*5*, *7*, *27*). As predicted, SO2144 forms stable NO and CO complexes and can be oxidized to the ferric state. Further we show that the NO complex regulates the kinase activity of SO2145. These results, coupled with our finding that *S. oneidensis* synthesizes NO under microaerobic conditions, suggest that SO2144 binds NO and initiates a signal transduction pathway via the SO2145 histidine kinase, reminiscent of sGC function in eukaryotes. sGC contains an H-NOX domain in the N-terminus of the β-subunit. Once activated, sGC catalyzes the conversion of GTP to the secondary messenger cyclic GMP, which is involved in a signaling cascade that affects a variety of processes such as vasodilation and neurotransmission.

The *S. oneidensis* H-NOX and kinase interact directly, forming a complex that is independent of the H-NOX ligation state. The *S. oneidensis* H-NOX has the same ligand binding properties as the sGC H-NOX domain, and the kinase activity of SO2145 is controlled in trans by the NO-complex of the SO2144 H-NOX. CO binding to the H-NOX domain inhibits kinase activity but to a much lesser extent than NO. This is also the case for sGC where NO is known to be the physiologically relevant activator (*24*). One possibility for the inhibitory effects on the kinase by the CO bound H-NOX might be that a small population of the ferrous CO complex is in an "activated" conformer and only when a large excess of the protein is present are the effects on the kinase detectable by our assay. This exchange between active and inactive conformers has been well documented with NtrC (*28*, *29*). Since the ferrous, unligated H-NOX has no effect on kinase activity *in vitro*, we assume that under growth conditions where the heme of SO2144 is likely to be ferrous and unligated, SO2145 is likely to be autophosphorylated and able to catalyze a phosphoryl transfer to an as yet unidentified cognate response regulator.

The effect on histidine kinase activity of the binding of a diatomic gas to a heme center is best exemplified by FixL,

a heme-containing histidine kinase expressed by *Rhizobium meliloti* in the root nodules of *Medicago sativa* (alfalfa) (*30*, *31*). Under anaerobic conditions, the FixL heme is unoccupied, and the protein is constitutively active, leading to autophosphorylation on a conserved histidine residue. This phosphoryl group is then directly transferred to a cognate response regulator FixJ, which in this form activates gene transcription for nitrogen fixation (*31*). However, under aerobic conditions, the FixL heme binds O_2, which negatively regulates kinase activity, leaving FixJ unphosphorylated and nitrogen fixation genes inactivated (*30*, *31*). *In vivo* FixL functions as an O_2 sensor, which requires the heme to be in the ferrous oxidation state. Yet, in nonphysiological experiments *in vitro*, FixL−Fe^{3+} in one case stimulated ATP turnover when assayed with FixJ and in another case inhibited turnover (*32*, *33*). Thus, the ferric induced inhibition of the histidine kinase SO2145 by the H-NOX SO2144 observed here may represent an analogous *in vitro* artifact. Given that oxidation of H-NOX SO2144 is difficult and slow, the functional *in vivo* oxidation state of the SO2144 heme is most likely to be ferrous.

The enzymatic machinery for NO synthesis in *Shewanella* is not clear. In eukaryotes, the NO that activates sGC through binding to the heme domain is synthesized by nitric oxide synthase (NOS) (*3*). NOS-like oxygenase domains have been found in some prokaryotes, and appear to be involved in an unusual nitration reaction but not in the formation of NO (*34*). Regardless, there is no predicted NOS-like oxygenase domain in *S. oneidensis*. Several bacteria have been shown to produce NO during the process of respiratory denitrification (*35*−*37*). *S. oneidensis* is a facultative anaerobe that is capable of utilizing a variety of alternative electron acceptors in the absence of oxygen, including NO_3^- and NO_2^-. NO_3^- and NO_2^- reduction to N_2O and NH_3 in *S. oneidensis* has been previously characterized (*18*−*20*); however, NO production by *S. oneidensis* has never been reported (*18*−*20*). Nitrite reduction in bacteria is typically catalyzed by copper-containing nitrite reductases (*38*) or by heme-containing cytochrome cd1 nitrite reductase (*38*, *39*) as part of a denitrifying pathway. *S. oneidensis* has a predicted NO_3^- reductase and a predicted ammonia-forming c552 nitrite reductase, but does not appear to have a homologue of the NO-forming NO_2^- reductase. Thus, as stated above, the enzymatic source of NO in *S. oneidensis* is not obvious.

The results reported here establish the first link between a member of the H-NOX family of heme-based sensors and a downstream signal transduction protein, histidine kinase (SO2145), and suggest a novel two-component signaling pathway that is regulated by NO. Why does *S. oneidensis* have an NO-responsive two-component signaling system? Facultative anaerobes require an alternate electron acceptor when O_2 levels fall below that necessary to sustain aerobic respiration. Our results lead us to propose that the NO derived from NO_3^- and NO_2^- reduction under oxygen-depleted growth serves as an early signal to the organism that O_2 is limiting, and that NO_3^- is available to respire. This NO signal would be transduced by the H-NOX/kinase pair, resulting in changes in the regulation of downstream transcriptional elements in response to the new growth conditions, leading to the expression of genes required for growth in the absence of oxygen. Further, it does appear that H-NOX domains are sensors that use a ferrous heme and the associated ligand binding properties to regulate diverse responses from bacteria to humans.

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