The *In Vivo* Potential-Regulated Protective Protein of Nitrogenase in *Azotobacter vinelandii* Supports Aerobic Bioelectrochemical Dinitrogen Reduction *In Vitro*

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ABSTRACT: Nitrogenase, the only enzyme known to be able to reduce dinitrogen (N_2) to ammonia (NH_3), is irreversibly damaged upon exposure to molecular oxygen (O_2). Several microbes, however, are able to grow aerobically and diazotrophically (fixing N_2 to grow) while containing functional nitrogenase. The obligate aerobic diazotroph, *Azotobacter vinelandii*, employs a multitude of protective mechanisms to preserve nitrogenase activity, including a "conformational switch" protein (FeSII, or "Shethna") that reversibly locks nitrogenase into a multi-component protective complex upon exposure to low concentrations of O_2. We demonstrate *in vitro* that nitrogenase can be oxidatively damaged under anoxic conditions and that the aforementioned conformational switch can protect nitrogenase from such damage, confirming that the conformational change in the protecting protein can be achieved solely by regulating the potential of its [2Fe-2S] cluster. We further demonstrate that this protective complex preserves nitrogenase activity upon exposure to air. Finally, this protective FeSII protein was incorporated into an O_2-tolerant bioelectrosynthetic cell whereby NH_3 was produced using air as a substrate, marking a significant step forward in overcoming the crippling limitation of nitrogenase's sensitivity towards O_2.

INTRODUCTION

Ammonia (NH_3) is an important chemical commodity, with the majority of NH_3 being produced by the Haber-Bosch process at the expense of >1 % global energy.[1,2] This process requires high temperatures (500 ºC) and pressures (20 MPa) to produce NH_3 from molecular hydrogen (H_2) and nitrogen (N_2), resulting in the production of 3 % of global CO_2.[3] Nitrogenase is the only enzyme known to reduce N_2 to NH_3 where nitrogenase-harboring microbes are able to grow diazotrophically, using N_2 as their sole N source.[4,5] Being able to produce NH_3 at ambient pressure and temperature, nitrogenase presents an exciting alternative to this renowned process although it is irreversibly inhibited by low concentrations of molecular oxygen (O_2).

Some nitrogenase-containing microbes (such as *Azotobacter*) are obligate aerobes and N_2 fixation occurs under aerobic conditions despite this intricate protein being highly sensitive to irreversible damage by O_2; on the contrary, O_2 can even stimulate nitrogenase expression and N_2 fixation.[6] While oxidative phosphorylation, high respiration and an external mucus offer a degree of protection to nitrogenase from elevated O_2 concentrations, *Azotobacter vinelandii* also employs a "conformational switch" protective protein which reversibly locks the nitrogenase component proteins into a complex that can tolerate low O_2 concentrations for short periods of time.[7-9] Despite a number of investigations, little is known about the apparent redox-triggered protective mechanism of this conformational protein and the protection of nitrogenase from air (~21 % O_2) *in vitro* has not been studied.

The nitrogenase enzyme complex consists of an ATP-hydrolyzing reductive protein (Fe protein) and a substrate-reducing protein (MoFe protein, Figure 1).[4] The Fe protein is a homodimer of approximately ~66 kDa that contains a bridging [4Fe-4S] iron-sulfur cluster, where each monomer also contains an ATP binding site. The catalytic MoFe protein is a ~220 kDa dimer of heterodimers, where each heterodimer contains a bridging electron transfer [8Fe-7S] cluster (P cluster) and a catalytic cofactor, [7Fe-9S-Mo-C-homocitrate], commonly referred to as the FeMoco. The Fe and MoFe proteins undergo repeated transient associations where individual binding events result in the ATP-hydrolysis-coupled transfer of a single electron from the Fe protein to the P cluster of the MoFe protein (and ultimately to the FeMoco), where the eventual accumulation of $8e^-$ results in the reduction of N_2 and the reductive elimination of H_2 (Equation 1).[10,11]

$$16MgATP + 8e^- + 8H^+ + N_2 \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i$$

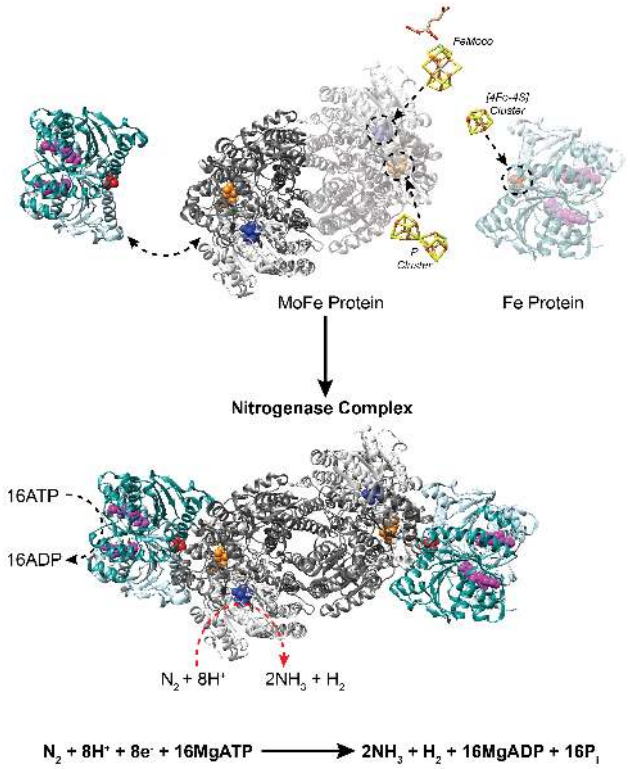


Figure 1. Structure and turnover of the Fe (cyan) and MoFe (gray) proteins of nitrogenase (PDB accession code: 4WZA). The [4Fe-4S] cluster is shown in red, the P cluster is shown in orange, the FeMoco is shown in blue and the magenta spheres represent bound nucleotides (ATP/ADP and homologues). For the molecular models of the cofactors, Fe is shown in orange, S is shown in yellow, Mo is shown in cyan and C is shown in light brown. The homocitrate is shown above the FeMoco.

(Equation 1)

The "conformational switch" protein of *A. vinelandii* (commonly referred to as the FeSII or "Shethna" protein) is a ~13 kDa [2Fe-2S] ferredoxin that contains a [2Fe-2S] cluster, whose nitrogenase-protective properties have been largely investigated *in vivo*.[7,8,12] FeSII has also been recombinantly-expressed in *Escherichia coli* and its O_2-responsive protective capabilities have been demonstrated for the non-physiological side reaction of nitrogenase *in vitro*, namely acetylene (C_2H_2) reduction to ethylene (C_2H_4) following exposure to low concentrations of O_2 (~2 %).[7,13] Previous research has determined that the FeSII protein forms a ~26 kDa homodimer, which after undergoing a conformational change to its "open" state (Figure 2), is thought to bind to the Fe and MoFe proteins (forming a catalytically-inactive tri-protein complex) where the Fe and MoFe proteins are protected from O_2; docking models have predicted the formation of the FeSII:Fe:MoFe complexes based on electrostatic interactions.[13,14] Upon reduction and the absence of O_2, the protective complex dissociates, the FeSII protein returns to a "closed" conformation and nitrogenase activity is restored. The FeSII protein is largely thought to be a redox-regulated switch where the oxidation state of the [2Fe-2S] cluster is proposed to expose or conceal the electrostatic Fe/MoFe-binding faces that bind the Fe and MoFe proteins; however, existent research has only investigated the effect of O_2 on the conformational switch and computational

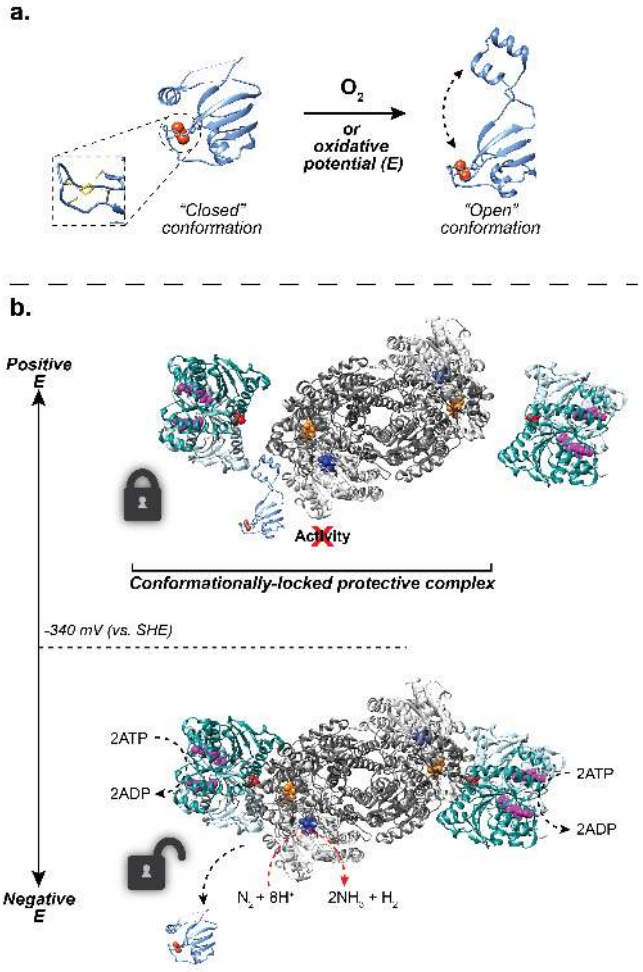


Figure 2. (a) Crystal structure of the FeSII protein in its "closed" (left) and "open" (right) conformational states (PDB accession code: 5FFI). The [2Fe-2S] cluster is shown in orange. Inset: The [2Fe-2S] cluster is shown bound to 4x cysteine residues of the FeSII protein, where Fe is shown in orange and S is shown in yellow. (b) Conformationally-locked MoFe/Fe/FeSII protein complex *vs.* the reduction potential of the FeSII protein, as determined in this study. For illustrational purposes, the FeSII protein is shown as a monomer. The color-code adopted for the MoFe and Fe proteins (and their cofactors) are the same as in Figure 1.

modeling has suggested that O_2-binding to the [2Fe-2S] cluster would require significant structural distortion (which the FeSII partially satisfies).[13,15] Redox titration experiments coupled with electron paramagnetic resonance (EPR) spectroscopy have determined the reduction potential of the [2Fe-2S] cluster of FeSII, which was calculated to be -262 mV *vs.* the Standard Hydrogen Electrode[16] (SHE). As the redox state of the [2Fe-2S] cluster is thought to control the conformational change of the FeSII protein (which presumably binds the nitrogenase components into an inactive complex), based on this we considered that the redox potential alone inside of *A. vinelandii* could act as regulatory machinery, even under O_2 concentrations that would otherwise not affect nitrogenase. Nevertheless, formation of the FeSII/MoFe/Fe protective complex under anoxic oxidative conditions (as induced by an electrochemical change only) would confirm the redox-switching nature of this protective protein, providing further insight into the internal electrochemical reactions of *A. vinelandii*.

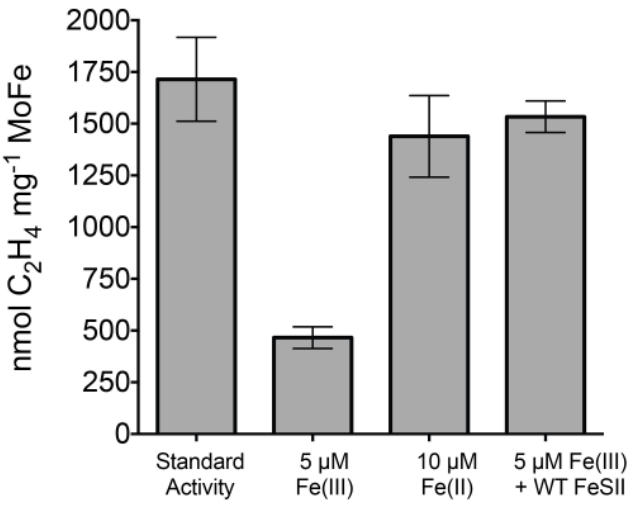


Figure 3. Inhibition of nitrogenase C_2H_2 reduction activity by Fe(III) or Fe(II) ($K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$) in the absence and presence of WT FeSII protein. Error bars represent one standard deviation ($n = 3$).

Enzymatic fuel cells (EFCs) are galvanic devices that employ enzymatic biocatalysts to facilitate the oxidation and reduction of substrates at the anode and/or cathode of a fuel cell.[17,18] The most common example of an EFC configuration is a glucose/O_2 EFC, where glucose oxidation to gluconolactone supplies electrons to an external circuit before reducing molecular oxygen (O_2) to water (H_2O) at the biocathode. Typically, EFCs configurations do not seek to produce valuable chemicals alongside electrical energy. We recently reported on the ability of electrochemically-reduced methylviologen ($MV^{\bullet+}$, N,N'-dimethyl-4,4'-bipyridinium) to support the bioelectrocatalytic reduction of N_2 to NH_3 in a H_2-fuelled EFC, where NH_3 was produced alongside electrical energy, however, the EFC required ATP and a strict anaerobic environment was necessary to preserve nitrogenase activity (ATP was continually regenerated by the use of creatine kinase and creatine phosphate).[19]

Here we investigated the FeSII protein of *A. vinelandii*, where the wild-type and 3x mutant FeSII proteins retained their ability to protect nitrogenase under 2 % O_2 for the non-physiological C_2H_2 reduction side reaction. Additionally, the wild-type (WT) FeSII protein was used to protect nitrogenase after exposure to air (21 % O_2) for C_2H_2 reduction as well as for N_2 reduction to NH_3. We also confirm that the FeSII protein does not require O_2 to initiate conformational protection, where Fe(III) (as potassium ferricyanide, $K_3[Fe(CN)_6]$) was found to inhibit nitrogenase activity by oxidative damage and the FeSII protein could protect such damage. Finally, the bioelectrochemical reduction of N_2 from air to NH_3 was demonstrated under continuous aerobic operation and $^1H/^{15}N$ NMR spectroscopy was used to confirm the production of $^{15}NH_3$ from $^{15}N_2$ "air" (79 % $^{15}N_2$/21 % O_2).

RESULTS

FeSII is a Redox-Regulated Conformational Switch. Previous studies of FeSII and its protection of nitrogenase C_2H_2-reduction activity have demonstrated that low concentrations of O_2 (up to 2 % O_2) can irreversibly inhibit C_2H_2 activity and that the addition of native (purified from *A. vinelandii*) or recombinantly-expressed FeSII protein (such as that expressed in *E. coli*) can prevent inhibition, preserving nitrogenase activity.[8,13,20] It is largely assumed that the presence of O_2 results in the oxidation of the [2Fe-2S] cluster of the FeSII protein, which triggers a conformational change that allows the Fe and MoFe proteins to form a tri-protein protective complex with the FeSII protein. The most recent investigation into this conformational protection mechanism indicates that the *lid helix* of the FeSII protein is involved in electrostatically-binding the Fe and MoFe proteins (where HADDOCK was used to generate a binding model from the known structure of the FeSII and the inability of some mutant FeSII proteins to protect nitrogenase), which is exposed for Fe and MoFe binding upon oxidation by O_2.[13] We initially investigated the anoxic inhibition of nitrogenase by oxidative damage (where it is expected that the intricate FeS clusters of the Fe and/or MoFe proteins disassemble upon over-oxidation) by incubation of the Fe and MoFe proteins with Fe(III) in the absence and presence of the wild-type FeSII protein (Figure 3). At this stage, nitrogenase activity was evaluated by following the nonphysiological reduction of C_2H_2 to C_2H_4. Following incubation of the Fe and MoFe proteins in an excess of Fe(III) for 10 minutes at 30 ºC, an approximate 3x fold decrease in activity was observed. Incubation in Fe(III) in the presence of the FeSII protein, however, did not result in a significant decrease in activity. Additionally, incubation of the Fe and MoFe proteins in Fe(II) and in the absence of the FeSII protein did not significantly affect the activity of nitrogenase, confirming that inhibition was afforded by oxidative damage and that the protective FeSII-Fe-MoFe complex can be formed under anoxic oxidative conditions. Further, this confirms that the conformation state of the FeSII protein can be controlled solely by potential, which has implications that the FeSII protein does not only serve as a protective protein against O_2 *in vivo*, but that it may also serve as a nitrogenase regulator under oxidative potentials.

Spectroelectrochemistry of the FeSII Protein. We next sought to evaluate the oxidation and reduction of the FeSII protein using an electrochemical setup, using MV as a redox mediator. To date, the reduction potential of the FeSII protein has been investigated using EPR spectroscopy, whereby the potential of the solution was control by the addition of oxidizing or reducing agents and was measured with a 3-electrode electrochemical setup.[16] Recently, UV/visible spectroscopy was also used to interrogate the oxidation state of the [2Fe-2S] cluster of the FeSII protein where dithionite (DT) was used to reduce the [2Fe-2S] cluster and O_2 was used to oxidize the cluster, which was repeated and is reported within the Supporting Information (Supporting Information, Figure S1a).[13] We utilized a spectroelectrochemical setup to observe the oxidation and reduction of the [2Fe-2S] cluster by UV/visible spectroscopy while placing the protein under electrochemical control in anoxic conditions (Figure 4a). A Pt-mesh working electrode was placed in a thin-layer quartz cell and an undersized Pt-wire counter electrode was used alongside a saturated calomel (SCE) reference electrode to afford bulk electrolytic conditions.[21]

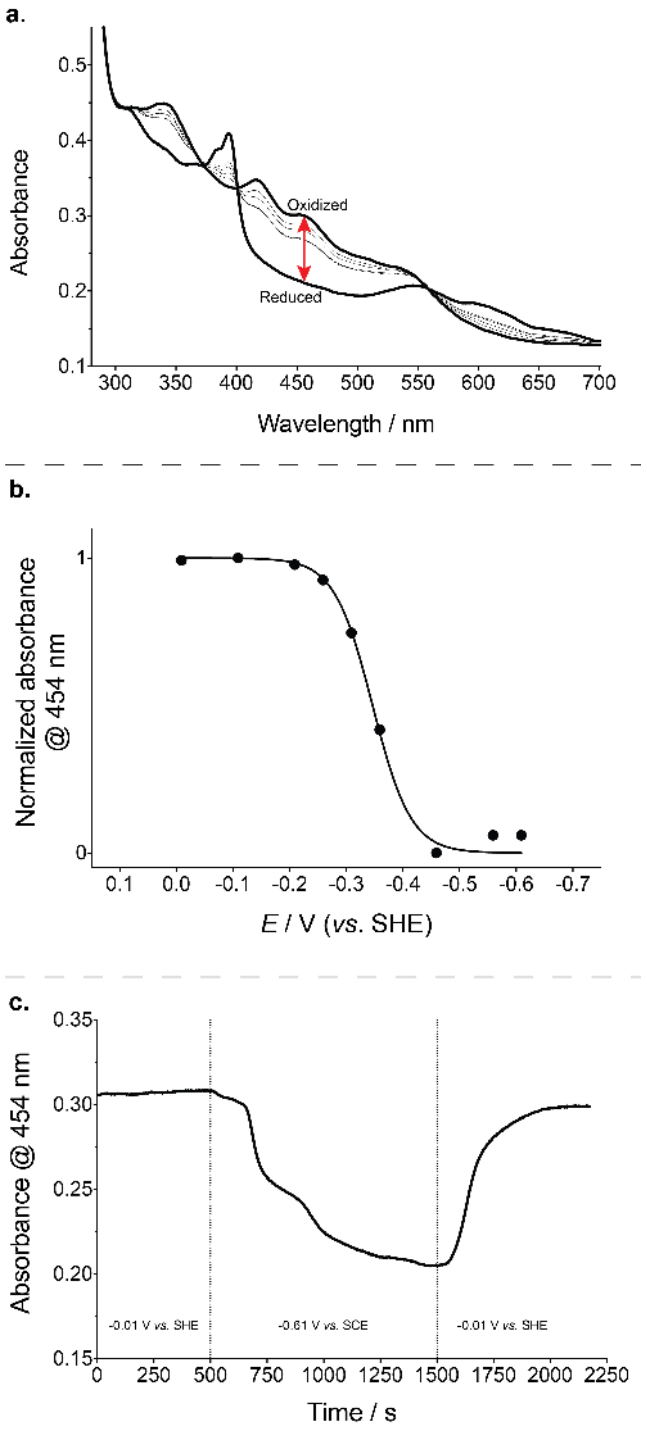


Figure 4. (a) UV/Visible absorption spectrum of the FeSII protein (80 μM) in the presence of MV (80 μM) in a thin-layer (2 mm) quartz anoxic spectroelectrochemical cell, where a Pt mesh working electrode was placed directly in the light path of the spectrophotometer. (b) Representative spectroelectrochemical determination of the reduction potential of the FeSII protein as mediated by MV at different applied potentials. (c) Spectroelectrochemical response of the FeSII protein upon switching the applied potential between oxidizing and reducing to the FeSII protein.

Initially, the UV/visible absorption spectrum of oxidized (as prepared) FeSII protein was recorded from 280-700 nm in the presence of oxidized MV (MV^{2+}). Following the application of a MV^{2+}-reducing potential at the working electrode the [2Fe-2S] cluster of FeSII was reduced *via* mediated electron transfer from $MV^{\bullet+}$, resulting in a change in its absorption spectrum. Additionally, the reduction of MV^{2+} to $MV^{\bullet+}$ was observed by the appearance of a sharp peak ~380 nm and broad peaks between 550-700 nm (Supporting Information, Figure S1b). In order to determine the reduction potential of the [2Fe-2S] cluster and therefore the FeSII protein, the absorbance of the [2Fe-2S] cluster at 454 nm was recorded at different applied potentials, where the oxidation state of the $MV^{2+}/MV^{\bullet+}$ couple did not significantly absorb (Figure 4b). The reduction potential of FeSII was determined to be -340 ± 6 mV *vs.* SHE assuming a $1e^-$ transfer between the $[2Fe\text{-}2S]^{2+/1+}$ couple; the reduction potential of FeSII was previously-reported to be -262 mV *vs.* SHE by EPR.[16] Finally, Figure 4c demonstrates the reversibility of the bulk spectroelectrochemical method for FeSII oxidation and reduction, where the potential of the working electrode was stepped between an oxidizing potential (-0.01 V *vs.* SHE) and a reducing potential (-0.61 V *vs.* SHE) while the absorbance of the [2Fe-2S] cluster was followed at 454 nm.

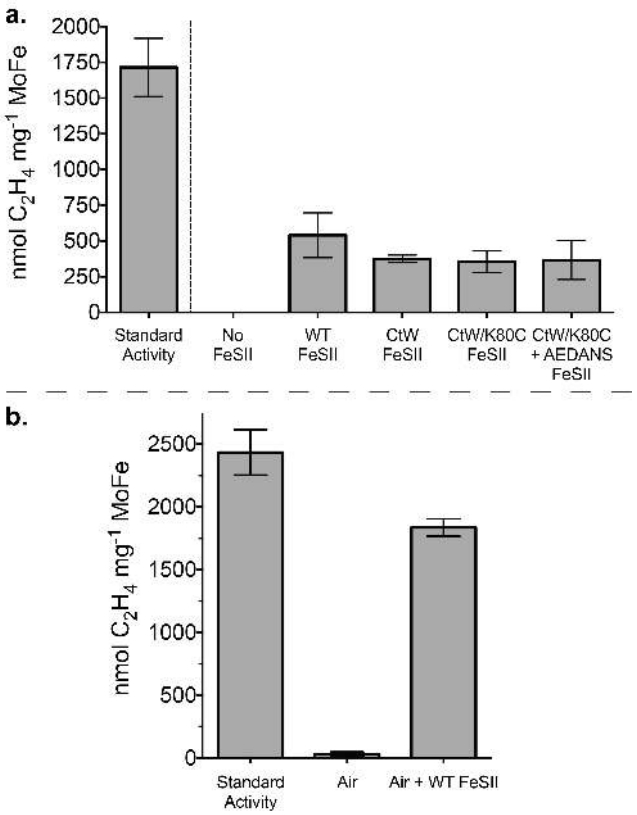


Figure 5. (a) Protection of nitrogenase C_2H_2 reduction activity upon exposure to 2 % O_2 for 10 minutes in the presence of wild-type (WT) FeSII protein, FeSII protein with a C-terminal additive tryptophan mutation (CtW), CtW FeSII with a K80C mutation (CtW/K80C) and CtW/K80C labeled with an IAEDANS fluorophore (CtW/K80C + AEDANS). (b) Protection of nitrogenase C_2H_2 reduction activity upon exposure to air for 1 minute in the absence and presence of WT FeSII protein. Error bars represent one standard deviation (n = 3).

Further, the oxidation and reduction of an alternative [2Fe-2S] ferredoxin (petF from *Synechococcus sp.*, recombinantly expressed in *E. coli*) was investigated by UV/visible spectroscopy using the spectroelectrochemical method reported above (Figures S2a and S2b, Supporting Information), where the

reduction potential was found to be similar to that of FeSII (-365 ± 11 mV *vs.* SHE).

FeSII Preserves Nitrogenase C_2H_2 Reduction Activity Following Exposure to Air. As stated above, previous research surrounding the use of FeSII to protect nitrogenase has solely investigated the nonphysiological reduction of C_2H_2 by nitrogenase by exposure to sub-atmospheric concentrations of O_2 (~2 % O_2). In order to create alternative sustainable NH_3-producing technologies to the Haber-Bosch process,[1] nitrogenase turnover of N_2 from air (*ca.* 79 % N_2 and 21 % O_2) is favorable and advantageous. Alternatively, anoxic N_2 gas must be supplied to enable NH_3 production. Thus, we next sought to investigate whether C_2H_2 reduction by nitrogenase could take place following exposure of the Fe and MoFe proteins to air, exposing nitrogenase to a < 10x fold increase in O_2 concentration.

The ability of FeSII to protect the Fe and MoFe proteins was first affirmed by adopting a previously-reported assay method by which the Fe and MoFe proteins were exposed to 2 % O_2 for up to 20 minutes, with slight modifications (Figure 5a).[13] The Fe and MoFe proteins were combined in an assay vial (containing MgATP, creatine phosphate/creatine phosphokinase to regenerate ATP and remove ADP, and 9:1 Ar:C_2H_2) in the absence and presence of wild-type FeSII, O_2 was injected to a final concentration of 2 % and the resulting assays were shaken at 30 ºC for 10 minutes. The presence of the Fe, MoFe and FeSII proteins was previously demonstrated to be necessary to afford protection from O_2, where no protection was observed in the absence of one of these three components and the Fe protein was demonstrated to be affected by O_2 to a greater extent than the MoFe protein.[13] Evaluation of residual C_2H_2-reduction activity by the quantification of C_2H_4 demonstrated that the FeSII protein could retain ~33 % C_2H_2 reduction activity under these conditions, confirming the successful expression and purification of active FeSII protein. C_2H_2 reduction activity was not observed under these conditions when Fe and MoFe were subjected to 2 % O_2 treatment in the absence of FeSII. In addition to FeSII, the above-mentioned alternative petF [2Fe-2S] ferredoxin was investigated where petF was not expected to offer the same conformational change-dependent protection as FeSII; petF did not significantly protect nitrogenase C_2H_2 activity under these conditions (data not shown).

Mutant FeSII proteins were next generated to investigate the redox-controlled conformational change of the FeSII protein by electrochemically-controlled Förster Resonance Energy Transfer (FRET). A single mutant of the FeSII protein was first generated to possess a C-terminal tryptophan residue (CtW, no other W residues are present on FeSII), where we hypothesized from evaluation of the crystal structure of FeSII that this W residue would be approximately located *behind* the [2Fe-2S] cluster in relation to the lid helix. A double mutant was next generated in which the CtW mutant possessed an additional mutation, K80C, where a cysteine mutation was hypothesized to provide a specific site to attach a FRET acceptor ((([(amino)ethyl]amino)naphthalene-1-sulfonic acid), AEDANS) where the W residue would act as its FRET donor.[22] While FRET was observed for the W-AEDANS FRET pair, control experiments designed to evaluate the extent of diffusion-mediated FRET indicated that the [2Fe-2S] cluster sufficiently quenched the FRET response (Figure S3, Supporting Information) and electrochemically-controlled

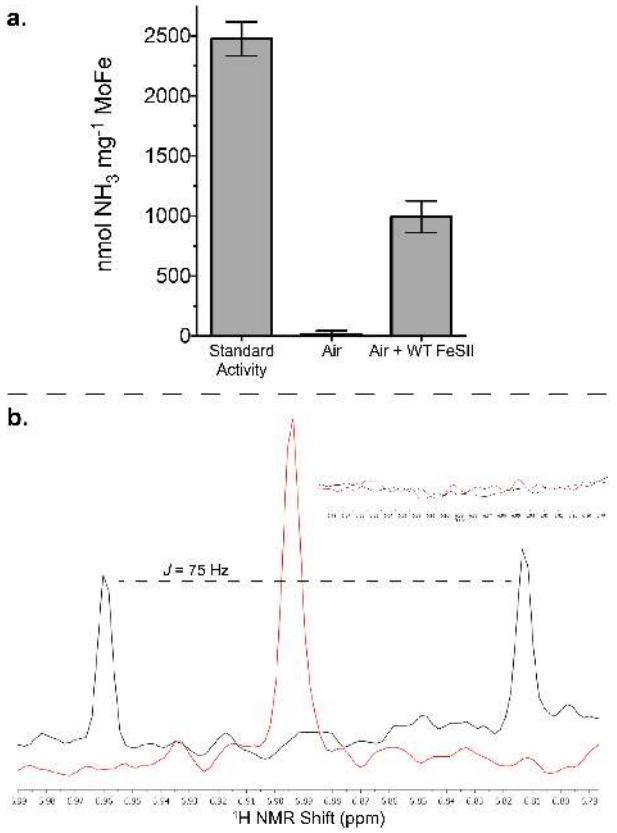
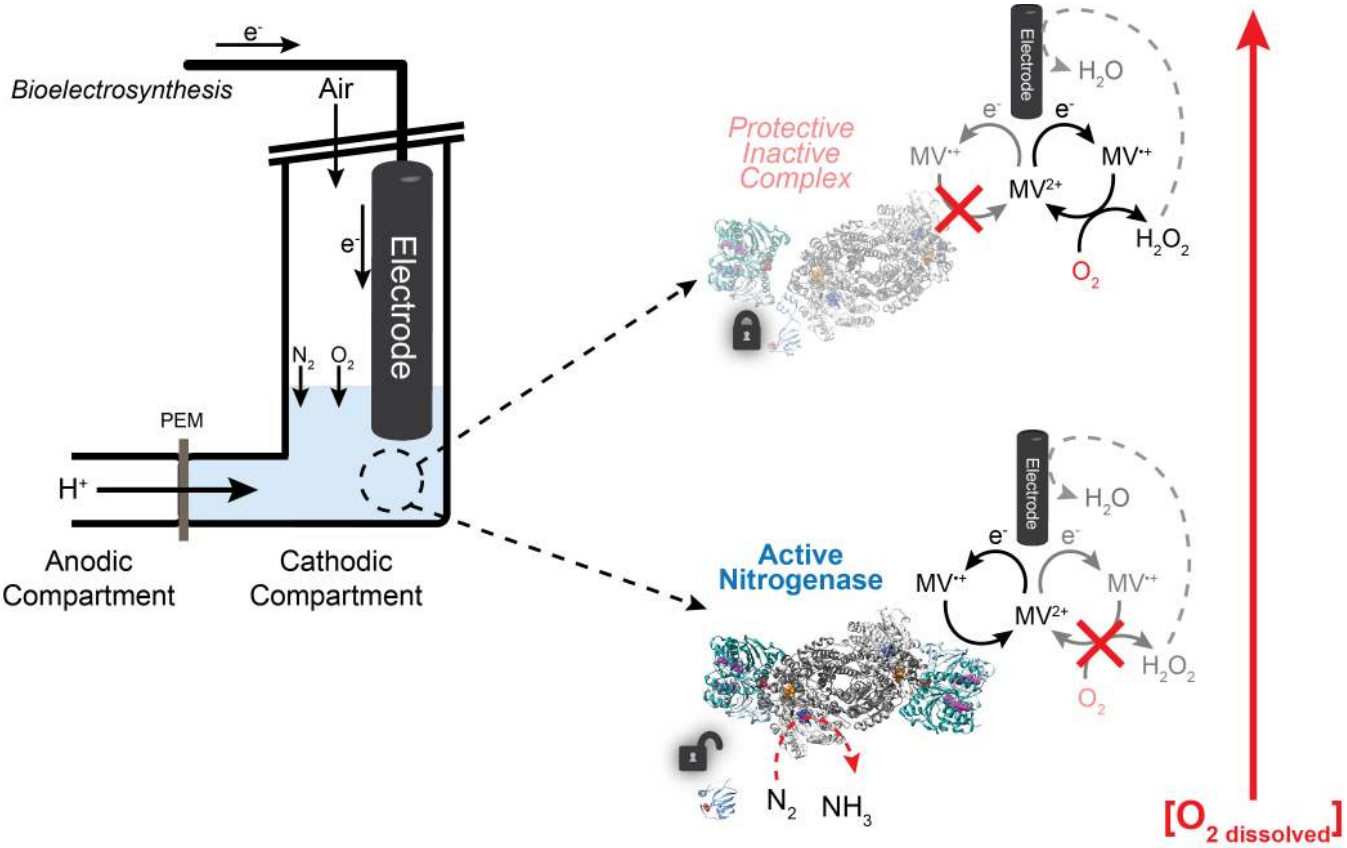


Figure 6. (a) Protection of nitrogenase N_2 reduction activity upon exposure to air for 1 minute in the presence of wild-type FeSII protein. (b) 1H NMR (500 MHz) spectra for NH_3 production by nitrogenase in the presence of FeSII following exposure to air for 1 minute, without (black) and with (red) ^{15}N decoupling. The inset NMR spectra were obtained in the absence of FeSII protection. Error bars represent one standard deviation (n = 3).

FRET was not evaluated further. Nevertheless, the CtW and CtW/K80C mutants were explored for their ability to protect Fe and MoFe from exposure to 2 % O_2, providing further information surrounding the electrostatic binding interactions between Fe, MoFe and FeSII (Figure 5a). The single (CtW), double (CtW/K80C) and AEDANS-labeled mutant (CtW/K80C + AEDANS) FeSII proteins all retained the ability to preserve C_2H_2-reduction activity upon exposure to 2 % O_2, with no observed significant decrease in protection.

After demonstrating the protection of Fe and MoFe by wild-type FeSII, we next evaluated the ability of FeSII to protect Fe and MoFe from exposure to air (*ca.* 21 % O_2). Due to this significant increase in the concentration of O_2, the (un)protected assays was exposed to air for only 1 minute (under stirring conditions) after which the headspace of the assay vial was refreshed with Ar and C_2H_2 before DT was injected to reduce the Fe, MoFe and FeSII proteins and facilitate C_2H_2 reduction. Remarkably, 75 % C_2H_2 reduction activity was preserved in the presence of the FeSII protein whereas greater than 98 % of activity was lost under unprotected conditions (in the absence of FeSII).

FeSII Preserves Nitrogenase N_2 Reduction Activity Following Exposure to Air. To the best of our knowledge, the ability of FeSII to preserve nitrogenase's N_2 reduction activity *in vitro* has not been demonstrated; evaluation of this possibil-



Scheme 1. Self-regulated aerobic N_2 reduction by nitrogenase in a bioelectrosynthetic cell. N_2 was introduced to the cell by the injection of air (*ca.* 79 % N_2 and 21 % O_2) over the headspace of the solution. To provide further evidence for the aerobic production of NH_3, $^{15}N_2$ "air" was prepared by mixing $^{15}N_2$ (79 %) and O_2 (21 %) in a syringe prior to injection to the bioelectrosynthetic cell.

ity is important for future aerobic N_2-reducing technologies. After establishing that FeSII could preserve C_2H_2-reduction activity following exposure to air, we evaluated remaining N_2 activity. Air was introduced into the nitrogenase assay vial containing MoFe, Fe and FeSII for 1 minute under stirring at room temperature, followed by evacuation, the addition of N_2 and DT. NH_3 was first quantified by the use of *ortho*-phthalaldehyde and fluorescence spectroscopy,[19,23,24] where the absence of FeSII resulted in a loss of almost all activity and the inclusion of FeSII preserved approximately 40 % of nitrogenase activity (Figure 6a).

Creatine produced from the regeneration of ATP in the assay solution (by the action of creatine phosphokinase on ADP and creatine phosphate) can also result in fluorescence when evaluating NH_3 by the use of *ortho*-phthalaldehyde, although the fluorescence response resulting from the detection of NH_3 is usually of significantly-increased magnitude that correction to H^+ turnover by nitrogenase (yielding creatine only) is typically adequate when quantifying NH_3 by this method (as was the case in this study). Nevertheless we turned to the use of NMR spectroscopy to provide further evidence for the production of $^{15}NH_3$ from $^{15}N_2$ following exposure to air, which corroborated the production of NH_3 by nitrogenase (Figure 6b). ^1H NMR spectra of $^{14}NH_4Cl$ and $^{15}NH_4Cl$ standards are presented within the Supporting Information (Figures S3a and S3b), where a diagnostic triplet (J = ~52 Hz) or doublet (J = ~72 Hz) was observed for the ^1H NMR spectra of $^{14}NH_4Cl$ and $^{15}NH_4Cl$, respectively.[25]

Aerobic Bioelectrosynthetic NH_3 Production from Air. Nitrogenase is notoriously sensitive to irreversible inhibition upon exposure to O_2, thus, the production of NH_3 from untreated air by nitrogenase is of high interest. We recently demonstrated the prospect of producing NH_3 from N_2 and while simultaneously producing electrical energy, however, there were 2 major drawbacks to the reported EFC: (i) the requirement of ATP for nitrogenase turnover, and (ii) it was necessary to operate the EFC under anaerobic conditions.[19] With these two limitations in mind, we investigated the employment of FeSII in an aerobic bioelectrosynthetic system where we hypothesized that N_2 would be reduced to NH_3 and FeSII preserved nitrogenase activity in the presence of O_2. We previously demonstrated the ability of MV to supply electrons to nitrogenase in a bioelectrosynthetic cell[19] and other researchers have demonstrated the ability of MV redox polymers to provide O_2-protection to another O_2-sensitive enzyme, hydrogenase.[26,27] The presence of H_2 enables hydrogenase to generate protective $MV^{\bullet+}$ equivalents by enzymatic turnover, however, oxidative enzymatic turnover of $MV^{\bullet+}$ by nitrogenase (such as MV-supported N_2 reduction) would theoretically result in the removal of MV-catalyzed protection from O_2. As shown in Scheme 1, this bio-inspired system is self-regulatory since nitrogenase turnover results in the oxidation of $MV^{\bullet+}$ to MV^{2+} and the concomitant oxidation of FeSII, whose conformational change locks the Fe and MoFe proteins of nitrogenase into an inactive tri-protein complex. During this time there is a shift in bias towards the reduction of MV^{2+}, maintaining a significantly-reducing environment for nitrogenase. We antic-

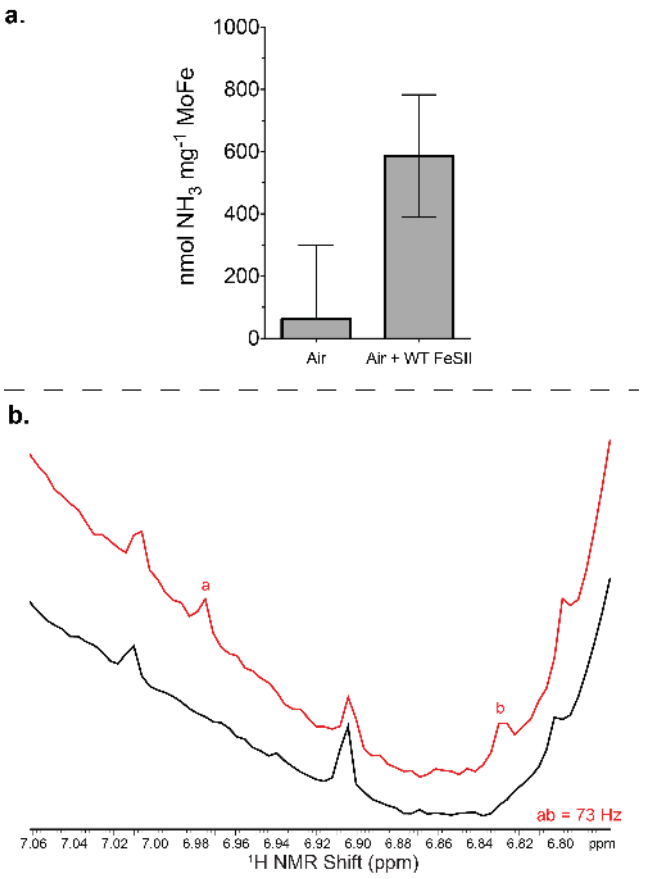


Figure 7. (a) Fluorimetric quantification of NH_3 produced by nitrogenase bioelectrosynthetic N_2 reduction from air in the absence and presence of FeSII. Experiments were corrected to H^+ turnover in the presence of FeSII under Ar containing to which O_2 was injected to an equivalent concentration of O_2 found in air (79 % Ar/21 % O_2). Error bars represent one standard deviation (n = 3). (b) ^1H-^{15}N NMR (500 MHz) of $^{15}NH_4^+$ produced by the bioelectrosynthetic reduction of $^{15}N_2$ "air" (79 % $^{15}N_2$/21 % O_2) in the presence of FeSII, with (black) or without (red) ^{15}N decoupling.

ipate that this could further protect nitrogenase from any irregular surges in [O_2], as O_2 would be reduced more efficiently under increased concentrations of $MV^{\bullet+}$. Additionally, momentary exposure of the solution to O_2 results in the formation of this protective complex, where the electroreduction of MV^{2+} to $MV^{\bullet+}$ is diverted to the removal of O_2 in the solution by its electrocatalytic reduction to hydrogen peroxide (H_2O_2), which is further reduced at the carbon electrode (Figures S5a and S5b, Supporting Information).

To evaluate the ability of this bioelectrochemical setup to produce NH_3 from the aerobic reduction of N_2 from air, a reductive potential of -0.76 V *vs.* SHE was applied to the working electrode to supply a sufficient overpotential for the rapid electrochemical reduction of MV^{2+}, 1 mL of air was injected into the headspace of the bioelectrosynthetic vial and bioelectrosynthesis was conducted for ~4 hours. Initial NH_3 quantification by *ortho*-phthalaldehyde (corrected to H^+ turnover under Ar with an equivalent injection of 100 % O_2) demonstrated that aerobic N_2 fixation produced 586 ± 197 nmol NH_3 mg^{-1} MoFe protein in the presence of FeSII (Figure 7a). Interestingly, small quantities of NH_3 were produced in the absence of FeSII, suggesting that the electroreduction of MV^{2+} alone could offer a degree of anoxic protection. To provide further evidence for aerobic turnover an "artificial" air mixture (1 mL of 79 % $^{15}N_2$ and 21 % O_2, balance Ar) was reduced by bioelectrosynthesis and the presence of $^{15}NH_4^+$ was confirmed by ^1H NMR spectroscopy (± ^{15}N decoupling to confirm the presence of $^{15}NH_4^+$ when $^{15}N_2$ was used as the substrate, Figure 7b). Interestingly, the diagnostic peaks of $^{14}NH_4^+$ were also observed for the $^{15}N_2$ bioelectrosynthetic reaction and ^{14}N decoupling proved these protons to be coupled to ^{14}N (Figures S6a and S6b, Supporting Information), reiterating the importance of control experiments when attempting to reduce N_2 to NH_3. In this case, we hypothesize that $^{14}NH_4^+$ was introduced into the sample upon ultrafiltration of the samples prior to NMR spectroscopy.

DISCUSSION

It was recently demonstrated that the conformational change of the FeSII protein was responsible for its binding of the MoFe and Fe proteins, whereby this tri-protein complex results in the protection of nitrogenase activity under 2 % O_2.[13] All studies to date, however, have used O_2 to induce this conformational protection and have only postulated that the redox state of the [2Fe-2S] cluster is responsible for this conformational change. We have demonstrated that Fe(III) is able to significantly affect nitrogenase activity under anoxic conditions as a function of oxidative damage (Fe(II) did not affect the activity of nitrogenase) and that the FeSII protein is able to preserve nitrogenase activity, confirming that the redox state of the [2Fe-2S] is indeed responsible for conformational protection. This information also suggests that FeSII possesses a regulatory role within *A. vinelandii*, whereby the potential of the cell could also regulate nitrogenase activity.

Spectroelectrochemistry of the FeSII protein under electrochemical control was used to determine the reduction potential of the [2Fe-2S] cluster, which was determined to be -340 ± 6 mV *vs.* SHE, in close agreement with the previously reported reduction potentials as determined by EPR spectroscopy. Additionally, MV was found to both oxidize and reduce the [2Fe-2S] cluster of the FeSII protein as a function of the reaction quotient of the $MV^{2+}/MV^{\bullet+}$ redox couple.

To date, the ability of FeSII to protect nitrogenase from O_2 has been demonstrated under [O_2] ~2 %. Due to interest in alternative NH_3-production biotechnologies, we investigated the ability of FeSII to preserve nitrogenase activity upon exposure to air (*ca.* 21 % O_2). After exposing the MoFe and Fe proteins to air in the presence of FeSII, 75 % C_2H_2 reduction activity was retained, whereas the absence of the FeSII resulted in the loss of 98 % C_2H_2 reduction activity. We also demonstrated upon exposure to air in the presence of the FeSII protein that 40 % N_2 reduction activity is preserved; nitrogenase lost almost all N_2 reduction activity in the absence of the FeSII protein.

With the possibility of performing bioelectrosynthetic N_2 reduction under *pseudo*-aerobic conditions in mind, we explored the possibility of utilizing bioelectrosynthesis to drive MV-mediated N_2 reduction by nitrogenase (Scheme 1), where the FeSII protein was included to provide protection to nitrogenase while reducing N_2 from air. NMR spectroscopy confirmed the production of NH_4^+ from air when using $^{14}N_2$ or $^{15}N_2$ "air" as the N source. The presence of $^{14}NH_4^+$ contamination in the samples prepared using $^{15}N_2$ highlight the importance of proper control experiments to account for contaminants when detecting the production of NH_4^+.[28]

Future studies should investigate the tolerance of this proof-of-concept configuration to air as well as the apparent stability of this configuration in order to progress towards a sustainable aerobic N_2-reducing biotechnology. We recently reported on a H_2/N_2 EFC that could simultaneously produce electrical energy and NH_3, however, the EFC required strictly-anaerobic conditions and the input of ATP; the findings of this manuscript indicate that it may be possible to construct an O_2-tolerant NH_3-producing EFC in the near future.

MATERIALS AND METHODS

Growth of *A. vinelandii* and Purification of MoFe and Fe Proteins. A mutant strain of *A. vinelandii* (derived from strain DJ) was grown and both N-terminal poly(histidine)-tagged MoFe protein and wild-type Fe protein were purified as reported previously.[19] Briefly, *A. vinelandii* was grown in 18 L batches on modified Burk medium (containing 10 mM NH_4^+) and the cells were derepressed by centrifugation and resuspension into NH_4^+-free media to enhance nitrogenase production once an $OD_{600\ nm}$ of ~ 1.5 had been reached. Crude extracts containing both His-tagged MoFe and wild-type Fe were prepared in an anoxic tent (Coy Laboratory Products, MI, USA) under < 0.5 ppm O_2 by lysing the cells with osmotic shock in the presence of 2 mM DT. His-tagged MoFe was purified by FPLC by immobilized metal affinity chromatography (IMAC) over 2x HisTrap HP columns (GE Healthcare) connected in series, before being purified further by anion exchange chromatography (Q-Sepharose, GE Healthcare) over a linear gradient of NaCl (200 – 650 mM). Wild-type Fe protein was purified by anion exchange chromatography (Q-Sepharose) and size-exclusion chromatography (Sephacryl S-200, GE Healthcare). Protein concentrations were determined by the Biuret method, corrected to DT.

Recombinant Expression and Purification Wild-Type and Mutant FeSII Proteins. Wild-type and mutant FeSII proteins were produced by modification of a previously-published procedure.[13] The gene of *A. vinelandii* encoding the FeSII protein (AVIN_39700) was purchased as a synthetic gene from Integrated DNA Technologies, Inc. and cloned into a pre-linearized pET28a expression vector (Novagen) by Gibson assembly. Mutant FeSII proteins were generated using the QuikChange Lightning kit (Agilent) and primers were designed by the use of the associated software and provided by the DNA/Peptide Facility, part of the Health Sciences Core at the University of Utah. Sequencing of the final plasmids confirmed the successful incorporation of the desired mutations. *E. coli* BL21 (DE3) C43 cells (OverExpress™, Sigma, chemically-competent) were transformed with the corresponding vectors and grown in LB medium containing 50 μg mL^{-1} kanamycin, where protein expression was induced once the $OD_{600\ nm}$ had reached ~ 0.6 by the addition of isopropylthiogalactoside (IPTG, 0.2 mM) and $FeCl_3$ (50 μM). After 6 hours of induction at 30 ºC the cells were harvested by centrifugation, washed, and lysed in 50 mM acetate buffer (pH 5.2) by the use of a microfluidizer (3x passages @ ~18,000 psi). Following centrifugation to remove the cell debris, the red FeSII protein in the supernatant was purified by cation exchange chromatography (2x HiTrap SP columns, 5 mL, GE Healthcare, 0 – 100 mM NaCl) and size-exclusion chromatography (Sephacryl S-100, GE Healthcare) before being exchanged into MOPS buffer (pH 7.0, 50 mM) over an FPLC desalting column (HiPrep desalting column, 5 mL, GE Healthcare). Protein concentrations were determined by the μ-Bradford assay. A control [2Fe-2S] ferredoxin (petF from *Synechococcus sp.*) was obtained by the same procedure, although an N-terminal His-tag was introduced and the protein was purified by IMAC. ~50 mg of purified FeSII was routinely purified from ~2 L of *E. coli* grown in LB under the above conditions.

Nitrogenase Activity Assays. Nitrogenase activity assays were performed as previously reported, with minor modifications.[19] As recently demonstrated,[13] maximal protection from O_2 is achieved at near-stoichiometric quantities of Fe, MoFe and FeSII. Thus, 9.6 mL crimped reaction vials (Wheaton) contained 1.35 nmol of the Fe, MoFe and FeSII proteins each. Each 1 mL assay was comprised of an ATP-regenerating system (6.7 mM $MgCl_2$, 30 mM creatine phosphate, 5 mM ATP, 0.2 mg mL^{-1} creatine phosphokinase and 1.3 mg mL^{-1} bovine serum albumin) in 100 mM MOPS buffer (pH 7.0). In order to test the ability of the FeSII protein to protect nitrogenase from exposure to O_2 or Fe(III), complete assays were prepared (in the absence of excess DT) and 2 % O_2 or Fe(III) was injected, followed by incubation at 30 ºC with shaking for 10 minutes. Following this incubation period, DT was injected to a final concentration of 20 mM to reduce residual O_2/Fe(III) and to initiate substrate turnover. Air-exposure assays were prepared as above, although air was added to a sealed vial equipped with a magnetic stirred bar for 1 minute at room temperature, where a needle open-to-air was used to pierce the septa of reaction vials that were under vacuum. Following this incubation period, the air was removed under vacuum and the headspace of the vial was filled with the substrate (100 % N_2 or 1:9 C_2H_2/Ar) and DT was added to reduce the proteins and initiate substrate turnover. Activity assays were performed for 10 minutes at 30 ºC and were terminated by the addition of 400 mM EDTA (300 μL). C_2H_4 was quantified by GC-FID equipped with a HP PLOT Q column and NH_3 was quantified by the fluorescent product of its reaction with *ortho*-phthalaldehyde.[19]

NMR Spectroscopy. ^1H and ^{15}N NMR spectroscopy was used to confirm the production of NH_3 by nitrogenase following exposure to various concentrations of O_2. For ^1H NMR spectroscopy, samples were first spun through a 10 kDa MWCO membrane to remove the proteins before the addition of HCl (final concentration ~1 M). ^1H ($\pm^{15}N$ decoupling) experiments were used to confirm the identity of $^{15}NH_4^+$ from bioelectrosynthetic experiments when the quantity of $^{15}NH_4^+$ was not sufficient for direct observation.

Spectroelectrochemistry of FeSII. A thin layer (d = 2 mm) gas-tight quartz cell was used for the spectroelectrochemical determination of the FeSII reduction potential, in which a thin Pt mesh working electrode was coupled with a small Pt wire counter electrode and a SCE reference electrode; potentials were applied *vs.* SCE and provided *vs.* SHE by $E_{SHE} = E_{SCE} + 241$ mV at 25 ºC.[29] FeSII (80 μM) and MV (80 μM) were mixed under anoxic conditions before connecting the cell to a potentiostat and recording UV/visible spectra. Each measurement (applied potential) required < 20 minutes to equilibrate.

Aerobic Bioelectrosynthesis. A custom-made glass H-cell was used to perform bioelectrocatalysis, where the working electrode was separated from the reference (SCE) and counter (Pt) electrodes with a Nafion 212 proton exchange membrane. The working electrode consisted of reticulated vitreous carbon foam cut into a cylindrical shape (0.9 cm diameter, 1.5 cm in height) where the top of the electrode was coated with paraffin wax and connected to a conductive wire. The working compartment of the H-cell had an approximate volume of 10 mL, to which a 3 mL assay solution was added along with a stirrer bar. The complete ATP-regenerating assay solution containing 2 mM MV^{2+} was first reduced for ~1 minute to afford partial MV^{2+} reduction before injection of the MoFe, Fe and FeSII proteins (1:1:1 stoichiometry, 12.2 nmol protein in total). Immediately following protein injection, 1 mL of air was added to the sealed cell (and vented to atmospheric pressure) and bioelectrosynthesis was conducted for ~4 hours. In addition to the use of *ortho*-phthalaldehyde, the production of NH_3 was confirmed by NMR whereby the injected "air" sample consisted 79 % $^{15}N_2$ and 21 % O_2 and ^1H NMR spectroscopy ($\pm\ ^{15}N$ decoupling) was used to determine the presence of $^{15}NH_4^+$.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website: Spectroelectrochemistry control experiments; spectroelectrochemistry of petF [2Fe-2S] ferredoxin; FRET of C_tW/K80C + AEDANS FeSII; ^1H NMR of $^{14}NH_4Cl$ and $^{15}NH_4Cl$; H_2O_2 electroreduction; ^1H-^{14}N (de)coupling NMR spectra.

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Author Contributions

The manuscript was written by R.D.M. and S.D.M. All authors have given approval to the final version of the manuscript.

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Notes

Electrochemical experiments were performed *vs*. a saturated calomel reference electrode (SCE) and reported *vs.* SHE by the following calculation: $E_{SHE} = E_{SCE} + 241$ mV.[29]

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ABBREVIATIONS

ATP, adenosine triphosphate; ADP, adenosine diphosphate; FeSII, iron-sulfur conformational protection protein; CtW, C-terminal tryptophan mutant; Fe protein, iron protein; MoFe protein, molybdenum-iron dependent protein; MV^{2+}, methylviologen (oxidized); $MV^{\bullet+}$, methylviologen (reduced); DT, sodium dithionite/hydrosulfite.

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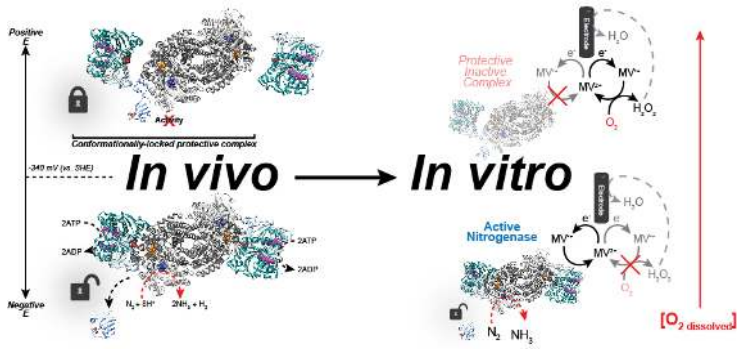
Positive E
Negative E
Conformationally-locked protective complex
In vivo
In vitro
Protective Inactive Complex
Active Nitrogenase
[O2 dissolved]