Structural Analysis of a Nitrogenase Iron Protein from
*Methanosarcina acetivorans*: Implications for CO₂ Capture by a
Surface-Exposed [Fe₄S₄] Cluster

Lee A. Rettberg,a Wonchull Kang,a Martin T. Stiebritz,a Caleb J. Hiller,a,b Chi Chung Lee,a Jasper Liedtke,a Markus W. Ribbe,a,b Yilin Hu,a

aDepartment of Molecular Biology and Biochemistry, University of California, Irvine, California, USA
bDepartment of Chemistry, University of California, Irvine, California, USA

ABSTRACT Nitrogenase iron (Fe) proteins reduce CO₂ to CO and/or hydrocarbons under ambient conditions. Here, we report a 2.4-Å crystal structure of the Fe protein from *Methanosarcina acetivorans* (MaNifH), which is generated in the presence of a reductant, dithionite, and an alternative CO₂ source, bicarbonate. Structural analysis of this methanogen Fe protein species suggests that CO₂ is possibly captured in an unactivated, linear conformation near the [Fe₄S₄] cluster of MaNifH by a conserved arginine (Arg) pair in a concerted and, possibly, asymmetric manner. Density functional theory calculations and mutational analyses provide further support for the capture of CO₂ on MaNifH while suggesting a possible role of Arg in the initial coordination of CO₂ via hydrogen bonding and electrostatic interactions. These results provide a useful framework for further mechanistic investigations of CO₂ activation by a surface-exposed [Fe₄S₄] cluster, which may facilitate future development of FeS catalysts for ambient conversion of CO₂ into valuable chemical commodities.

IMPORTANCE This work reports the crystal structure of a previously uncharacterized Fe protein from a methanogenic organism, which provides important insights into the structural properties of the less-characterized, yet highly interesting archaeal nitrogenase enzymes. Moreover, the structure-derived implications for CO₂ capture by a surface-exposed [Fe₄S₄] cluster point to the possibility of developing novel strategies for CO₂ sequestration while providing the initial insights into the unique mechanism of FeS-based CO₂ activation.

KEYWORDS CO₂ capture, FeS cluster, iron protein, methanogen, nitrogenase
hydrocarbons under ambient conditions in the presence of Eu
-DTPA, further illustrating the unique reactivity of the [Fe4S4] cluster toward CO2 (16, 17). Together, these observations point to the nitrogenase Fe protein as a simple model system for mechanistic investigations of FeS-based CO2 activation and reduction.

Of the two Fe protein species that have been investigated for their reactivity toward CO2, MaNifH is particularly interesting given its ability to convert CO2 to CO and hydrocarbons. Despite its archaeal origin, MaNifH shares a sequence identity of 59% and a sequence homology of 72% with AvNifH. Like AvNifH, MaNifH is a homodimer of 60 kDa, and it contains an [Fe4S4] cluster that can adopt three oxidation states upon redox treatments: (i) the oxidized state ([Fe4S4]2+), which is generated upon treatment by indigodisulfonate; (ii) the reduced state ([Fe4S4]1−), which is generated upon treatment by dithionite (DT); and (iii) the “superreduced,” all-ferrous state ([Fe4S4]0), which is generated upon treatment by Eu
-DTPA (17). There are differences, however, in the electronic properties of MaNifH and AvNifH, which are reflected by a stronger S = 3/2 contribution to the electron paramagnetic resonance (EPR) spectrum of the reduced MaNifH and a decreased intensity of the parallel mode, g = 16.4 signal in the EPR spectrum of the superreduced MaNifH (17). These differences, along with the lower reduction potential of the [Fe4S4]1−/2+ pair of MaNifH (E0 = −395 mV) than that of AvNifH ([Fe4S4]1+/2+; E0 = −301 mV) (16), may contribute to the difference in the reactivities of MaNifH and AvNifH toward CO2. The redox dependence of this reaction is further illustrated by a substantially decreased CO2-reducing activity of both MaNifH and AvNifH in the presence of dithionite, a weaker reductant than Eu
-DTPA, which renders the clusters of these Fe proteins in the catalytically inefficient [Fe4S4]1− state (10, 16).

The significantly decreased activity of Fe protein in a dithionite-driven reaction could prove advantageous for capturing CO2 in an early stage of CO2 reduction. Here, we report a 2.4-Å crystal structure of MaNifH that was generated in the presence of dithionite and an alternative CO2 source, bicarbonate. Structural analysis of this previously uncharacterized Fe protein from the methanogen nitrogenase family suggests that CO2 is possibly captured in an unactivated, linear conformation on the dithionite-reduced MaNifH; moreover, it reveals the initial coordination of CO2 by a conserved, surface-exposed arginine (Arg) pair in a concerted yet asymmetric manner, which could assist in trapping CO2 near the [Fe4S4] cluster via hydrogen bonding and electrostatic interactions. These results provide a useful framework for further exploration of the mechanism of CO2 activation by Fe proteins, which may enable future development of FeS catalysts for recycling the greenhouse gas CO2 into valuable chemical commodities.

RESULTS

Structural analysis of the dithionite-reduced MaNifH. Consistent with the presence of its [Fe4S4] cluster in the +1 oxidation state, MaNifH crystallized in the presence of dithionite had a characteristic brown color. The ∼2.4-Å structure of the dithionite-reduced MaNifH (PDB ID 6NZJ) adopts the same overall conformation as all Fe protein structures reported to date (9, 18–20), with each of its subunits folded as a single α/β-type domain and its [Fe4S4] cluster situated in a surface cavity between the two subunits (Fig. 1A and B). A closer examination of the region surrounding the active site of MaNifH (Fig. 1C) reveals the ligation of the [Fe4S4] cluster by four Cys residues: two from subunit A (Cys95A, Cys130A) and two from subunit B (Cys95B, Cys130B). Interestingly, the electron density omit map (Fo − Fc) of the active site of MaNifH (Fig. 1C, green mesh; also see Fig. S1 in the supplemental material) indicates the presence of additional electron density that lies immediately next to the crystallographic symmetry axis, seemingly held by two pairs of conserved Arg residues (R98A and R98B)—one from each of the two adjacent MaNifH subunit dimers.

Modeling the extra electron density in the structure of the dithionite-reduced MaNifH. Given that the additional electron density may originate from the small molecules in the protein preparations or crystallographic solutions, we then considered possible candidates and modeled water (see Fig. S2A in the supplemental material),
carbonate (Fig. S2B), glycerol (Fig. S2C), and CO₂ (Fig. S2D), respectively, into this density. Water is an unlikely contributor to this density, as modeling of one water molecule in the asymmetric unit and another in its symmetry mate results in substantial “leftover” electron density in the Fₒ – Fc omit map (Fig. S2A, green mesh). Carbonate and glycerol, on the other hand, could be modeled as two molecules—each at 50% occupancy—at the crystallographic symmetry axis with reasonable R factor values (see Table S1 in the supplemental material). Similarly, CO₂ could be modeled with reasonable R factor values at the crystallographic symmetry axis; only in this case, two molecules of CO₂—each at 100% occupancy—could be assigned to the asymmetric unit and its symmetry mate, respectively (Table S1). It should be noted that the modeling of two CO₂ moieties results in some negative electron density; however, the overall crystallographic statistics are reasonable to support this model (Table S1) despite the difficulty to conclusively assign this ligand near the crystallographic symmetry axis.

DFT calculations of the affinity of CO₂ to the dithionite-reduced MaNifH. To seek support for the assignment of CO₂ as the extra electron density in the crystal structure of MaNifH, we then used density functional theory (DFT) calculations to analyze the CO₂ affinity of the [Fe₄S₄]¹⁺ cluster in MaNifH. Consistent with our previous findings for both AvNifH-bound and synthetic [Fe₄S₄] clusters (10, 16), CO₂ does not interact well with the [Fe₄S₄]¹⁺ cluster of MaNifH and tends to dissociate from the cluster during the course of structural optimization; however, the two highly conserved Arg residues in MaNifH (R98⁴, R98⁸) form a cage-like configuration around the CO₂ molecule that assists in trapping it in close proximity to the cluster (see Movie S1 in the supplemental material). Interestingly, the location of the CO₂ moiety in the DFT-optimized model is in good agreement with half of the electron density pattern in the structure of MaNifH, except for a slight reorientation of CO₂ (Fig. 2). In comparison, DFT optimization reveals protonation of carbonate by R98⁸, followed by coordination of the resulting bicarbonate in a position parallel to the upper surface of the [Fe₄S₄] cluster, which is rather distinct from the perpendicular position modeled for carbonate in the crystal structure of MaNifH (see Fig. S3 in the supplemental material). This observation is important, as it provides theoretical support for the assignment of CO₂ as a potential ligand in the structure of the dithionite-reduced MaNifH protein. The fact that the MaNifH crystals were generated at a bicarbonate concentration in the same order of magnitude as that used to generate a CO₂-bound conformation of CO dehydrogenase (21) provides further support for the assignment of CO₂ in the MaNifH structure. In this scenario, the CO₂ moiety has its C atom placed at a distance of ~4 Å from the nearest Fe atom (Fe-3) of the [Fe₄S₄] cluster, with the NH₂⁺ groups of R98⁴ and R98⁸ assuming the “distal” and “proximal” positions, respectively, to Fe-3 (Fig. 2). This observation suggests a possible role of the conserved Arg pair in capturing CO₂ via hydrogen
bonding and/or electrostatic interactions, as well as a potentially asymmetric functionality of the two Arg residues in this process.

Examining the role of the conserved Arg pair of MaNiFH in CO₂ capture. To test the proposed role of conserved Arg residues in CO₂ capture, we performed site-directed mutagenic analysis and mutated R98 of MaNiFH to either a His or a Gly. Both R98H and R98G MaNiFH variants display the same S₁/₂ EPR signal as the wild-type protein, which is indicative of an unperturbed [Fe₄S₄] center in the +1 oxidation state (Fig. 3A). However, the R98H variant of MaNiFH retains ~80% CO₂-reducing activity, whereas the R98G variant loses ~85% of this activity (Fig. 3B), consistent with the preservation (i.e., the R→H mutation) or elimination (i.e., the R→G mutation) of the hydrogen bonding ability at the position of R98. The somewhat decreased activity of the R98H variant could be explained by a shorter side chain of His and, consequently, a reduced efficiency of this residue in hydrogen bonding/proton donation than Arg. The slight defect of His in proton donation would also account for a shift of the product profile of the R98H variant (hydrocarbon/CO ratio of 1.9) from hydrocarbon formation to CO formation compared to that of the wild-type MaNiFH (hydrocarbon/CO ratio of 2.7), as formation of CO requires fewer protons than that of hydrocarbons.

FIG 2 Crystal (A) and DFT-optimized (B) structures of MaNiFH with the extra electron density modeled as CO₂ and (C) an overlay of the two structures. The conserved pair of Arg residues assume “proximal” (R98B) and “distal” (R98A) positions, respectively, to the CO₂ moiety and the Fe-3 atom of the cluster (A and B), and CO₂ occupies a highly similar position in the crystal structure and the DFT model (C). The [Fe₄S₄] cluster and CO₂ moiety are shown in ball-and-stick presentation and colored as follows: Fe, orange; S, yellow; C, gray; O, red. The Cys ligands and the conserved Arg residues are shown as sticks.

FIG 3 Spectroscopic and catalytic features of the wild-type and variant MaNiFH. (A) EPR spectra and (B) CO₂-reducing activities of wild-type and variant MaNiFH. EPR spectra were collected at 10 K. The wild-type and R98H and R98G variant MaNiFH are dimers of ~60 kDa and contain 3.7 ± 0.1, 3.9 ± 0.4, and 3.8 ± 0.2 nmol Fe per nmol protein, respectively. Like the wild-type MaNiFH, the R98H and R98G variants display the same [Fe₄S₄]⁺⁺ characteristic, S = 1/2 EPR signal in the dithionite-reduced state (A), yet they display disparate activities in CO₂ reduction (B). The hydrocarbon/CO ratios (calculated based on total nmol of reduced carbons) of the wild-type MaNiFH and R98H variant are 2.7 and 1.9, respectively, suggesting a shift from hydrocarbon formation to CO formation in the latter case.
Proposal of a plausible mechanism for the initial capture of CO₂ by MaNifH. To obtain further insights into the mechanism of CO₂ capture by nitrogenase Fe proteins, we compared our dithionite-reduced MaNifH structure (PDB ID 6NZJ) that is potentially bound with CO₂ with a previously reported, dithionite-reduced AvNifH structure (PDB ID 1G5P) that is free of CO₂ (19). Consistent with a high degree of sequence homology between MaNifH and AvNifH, the subunits A and B in MaNifH show Cα deviations of only 0.599 and 0.616 Å, respectively, relative to those in AvNifH, yet the two subunit chains in MaNifH are more similar to each other in terms of secondary structural elements, particularly with respect to the structurally less conserved α-helical regions (see Fig. S4 and S5 in the supplemental material). More strikingly, compared to their counterparts in AvNifH, there is a notable movement of the two subunits of MaNifH with respect to each other, which flattens the surface cavity and consequently “pushes” the [Fe₄S₄] cluster further toward the surface where a CO₂ molecule could be modeled (see Fig. S6 and Movie S2 in the supplemental material). A top-view comparison between the two structures further reveals a “linearization” of helices CA and CB in MaNifH relative to those in AvNifH, which is accompanied by a substantial swing of the Arg pair, R98A and R98B (located at the tips of helices CA and CB), toward the center of the surface cavity (see Fig. S6 and Movie S3 in the supplemental material). Such a movement of the conserved Arg pair could reflect a concerted action of the “distal” R98 and the “proximal” R98 in the initial capture of CO₂ in an unactivated, linear conformation near the Fe-3 atom of the [Fe₄S₄] cluster (Fig. 4). Further activation of CO₂ into a bent, carboxylate-like conformation may continue to employ an asymmetric mechanism. Previous DFT calculations of CO₂ activation by the catalytically competent, all-ferrous AvNifH (10) led to the proposal of binding of an activated CO₂ moiety via coordination of C with Fe-3 of the cluster and coordination of O with the guanidinium group of the “proximal” R100 (corresponding to the “proximal” R98 in MaNifH), with the latter potentially donating protons for the subsequent C-O bond cleavage.

FIG 4 Comparison of the CO₂-free (A) and CO₂-captured (B) conformations of Fe protein, showing concerted yet asymmetric movement of a pair of conserved Arg residues that potentially capture CO₂ near the [Fe₄S₄] cluster. The CO₂-free and CO₂-captured conformations are represented by the homologous AvNifH and MaNifH, respectively. The movement of the “proximal” Arg (R100 in AvNifH and the corresponding R98 in MaNifH) and the “distal” Arg (R100 in AvNifH and the corresponding R98 in MaNifH) is shown from two angles.
DISCUSSION

In light of a plausible asymmetric mechanism of CO₂ activation by Fe protein, it is interesting to consider the mechanism proposed for the Ni-dependent CO dehydrogenase in CO₂ activation, which involves the action of the Fe/Ni atoms of its heterometallic C-cluster ([NiFe₄S₄]²⁻) as a pair of Lewis acid/base to facilitate scission of a C-O bond (21–24). In the absence of such a hetermetal-based asymmetry, it is plausible that activation of CO₂ by the homometallic [Fe₄S₄] cluster would resort to a structure-based asymmetry that enables interactions between O and the guanidinium group of the proximal Arg, as well as binding of CO to the nearest Fe-3 atom. It is worth noting that the proposed asymmetric functionality of the conserved Arg pair in CO₂ activation is consistent with the previously established regulatory mechanism of nitrogenase activity through ADP-ribosylation of only one of these conserved Arg residues (25), whereas the structure-based suggestion of a single reactive Fe (Fe-3) site for CO₂ activation may have certain relevance to the unique Fe site that was identified by earlier Mössbauer studies of the all-ferrous Fe protein (14). While the functions of these asymmetric elements await further elucidation, the current study provides a useful framework for investigating the structural basis of Fe protein-based CO₂ capture and activation. Moreover, the strategy utilized by the Fe protein to trap CO₂ by a pair of surface-located arginines loosely resembles the approaches that employ nitrogen-based ligands, such as metal-organic frameworks (MOFs) with amine or amide groups (26) or protein amyloid fibers comprising lysines in stacked sheets (27), for CO₂ capture and sequestration. The fact that the arginine residues of the Fe protein trap CO₂ in the close proximity to a surface-exposed [Fe₄S₄] cluster for further processing may provide a conceptual basis for the future development of MOF- or protein-based FeS catalysts that couple the capture of CO₂ with the recycling of this greenhouse gas into useful chemical commodities.

MATERIALS AND METHODS

Protein purification and crystallization. All protein purification steps were carried out anaerobically using Schlenk techniques. His-tagged MoNiFH was purified by immobilized metal affinity as described elsewhere (17, 28). Reagents for protein crystallization were purchased from Hampton Research and were thoroughly deaerated by vacuum/Ar-fill cycling before use. All crystals were generated at room temperature in an anaerobic chamber (Coy Laboratory Products), coated with Parabar 10312 oil (Hampton Research) as a cryo-protectant, and flash-frozen in liquid nitrogen for data collection.

MoNiFH was crystallized at room temperature by a microbatch method under a layer of Al’s oil (Hampton Research). The purified MoNiFH protein was desalted on a G-25 fine column equilibrated with buffer M (10 mM EPPS [pH 8.0], 100 mM NaCl, 10% [vol/vol] glycerol, and 2 mM dithionite [DT]) and then concentrated to 10 mg/ml by Amicon Ultra-4 30-kDa centrifugal filter units. The crystals were grown by evaporating a mixture of 1 M protein solution and 3 M precipitant solution (2.3 M ammonium sulfate, 7% [wt/vol] polyethylene glycol 3350 [PEG 3350], 12 mM carbonate, and 2 mM DT) under Al’s oil. The protein solution was brown, indicating that the protein-bound cluster was present in the reduced, +1 state. Brown crystals grew after 2 weeks and were flash-frozen in liquid nitrogen for data collection.

Data collection and structural determination. The diffraction data of MoNiFH crystals were collected at 100 K on beamline 8.2.1 of Advanced Light Source using a wavelength of 0.977 Å and an ADSC Q315r charge-coupled device (CCD) detector. A total of 501 images were recorded for MoNiFH at a distance of 450 mm, with an oscillation angle of 0.25° and an exposure time of 0.25 s. The raw data were indexed and processed using iMosflm and Scala in the CCP4 package (29). Molecular replacement was performed with Phaser in PHENIX (30) using the structure of the Clostridium pasteurianum NiFH protein (PDB ID 1CFP) (19) as a search model. The initial model was further improved by cycles of manual building and refinement using Coot and PHENIX (30–32). At the end of the refinement cycle, water, carbonate, glycerol, or CO₂ was manually put into the model of MoNiFH and further refined for 3 cycles using PHENIX. The stereochemical quality of the final structures was evaluated by MolProbity (33). All molecular graphics were prepared using PyMol (34). Data collection and statistics for refinement and ligand modeling are summarized in Table S1.

Strain construction and activity analyses. Strains expressing R98H and R98G MoNiFH variants were constructed via site-directed mutagenesis of the wild-type Methanosarcina acetivorans nifH sequence carried on a pET14b vector (17), followed by transformation of the resultant plasmids into Escherichia coli strain BL21(DE3). The in vitro CO₂-reduction assays were carried out in 9.4-ml assay vials with crimped butyl rubber serum stoppers. Each assay contained, in a total volume of 1.0 ml, 500 mM Tris-HCl (pH 10.0), 0.5 mg Fe protein (wild-type or R98H or R98G variant MoNiFH), and 100 mM Eu³⁺-DTPA. In addition, the headspace of each assay contained 100% CO₂ (for reactions) or 100% Ar (for controls). The assays were assembled without protein and Eu³⁺-DTPA and repeatedly flushed and exchanged with CO₂, followed by equilibration for 30 min until pH stabilized at ~8.0. The reaction was initiated upon addition of MoNiFH,
followed immediately by addition of Eu³⁺-DTPA and incubation with continuous shaking at 30°C for 300 min until the reaction was complete. Following the quenching of each assay by 100μl of 30% trichloroacetic acid, the headspace sample was examined for the production of CO and hydrocarbons as described previously (16).

**EPR spectroscopy analyses.** The EPR samples were prepared in a Vacuum Atmospheres glove box and flash-frozen in liquid nitrogen prior to analysis. The DT-reduced samples contained 2 mM DT, 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 10% (vol/vol) glycerol. EPR spectra were recorded by an ESP 300 Ez spectrophotometer (Bruker) interfaced with an ESR-9002 liquid-helium continuous-flow cryostat (Oxford Instruments) using a microwave power of 50 mW, a gain of 5 × 10⁶, a modulation frequency of 100 kHz, and a modulation amplitude of 5 G. Five scans were recorded for each EPR sample at a temperature of 10 K and a microwave frequency of 9.62 GHz.

**Density functional theory calculations.** The mechanism of CO₂, carbonate, and bicarbonate coordination was studied with the DFT programs in the Turbomole package, version 7.0 (35). Atomistic models of the [Fe₄S₄] cluster and its immediate protein environment were generated from the structure of MoNifH (PDB ID 6NZJ [this work]) in the DT-reduced, [Fe₄S₄]¹⁺ state. The models were selected as described previously (10) and contained the [Fe₄S₄] cluster and C95A, C95B, C130A, C130B, R98A, R98B, F133A, F133B, and the main-chain atoms of the residues A96A, A96B, A97A, A97B, G97A, G97B, G131A, G131B, G132A, and G132B of MoNifH to account for all interactions of the cluster with the protein backbone. N termini were saturated with acetyl groups according to the crystallographic atom positions. Hydrogen atoms were added to the model with Open Babel (36), assuming protonation of the Arg residues. During structural optimizations, the atoms of the cluster, the side-chain atoms of the cluster-coordinating Cys residues (including Cγ), the side-chain atoms of the Arg residues (starting from Cγ), the benzene groups of the Phe residues, and all hydrogen atoms were allowed to spatially relax. All other atoms were kept structurally frozen. The models were treated as open-shell systems in the unrestricted Kohn-Sham framework. Solvent effects were treated implicitly by the conductor-like solvent screening model (COSMO) (37), assuming a dielectric constant of ε = 40. The structures were optimized with the TPSS (Tao-Perdew-Staroverov-Scuseria) functional (38). A def2-TZVP basis set (39, 40) was used for the [Fe₄S₄] cluster, the side-chain atoms of the Cys residues (including Cγ atoms), the atoms of the guanidinium groups, and the cluster-bound CO₂, carbonate, and bicarbonate moieties. A def2-SVP basis set was assigned to all remaining atoms to accelerate the calculations. Computational time was further reduced by utilizing the resolution-of-the-identity approximation (41, 42). Antiferromagnetic coupling in the Fe₄S₄ cluster was accounted for by the broken symmetry approach (43–45).

**Data availability.** The structure of DT-reduced MoNifH (PDB ID 6NZJ) has been deposited in the Protein Data Bank (https://www.wwpdb.org) and will be released upon publication.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at [https://doi.org/10.1128/mBio.01497-19](https://doi.org/10.1128/mBio.01497-19).

**FIG S1**, JPG file, 0.8 MB.

**FIG S2**, JPG file, 2.3 MB.

**FIG S3**, JPG file, 1.4 MB.

**FIG S4**, JPG file, 0.8 MB.

**FIG S5**, JPG file, 2.8 MB.

**FIG S6**, JPG file, 2.3 MB.

**TABLE S1**, PDF file, 0.1 MB.

**MOVIE S1**, MPG file, 7.8 MB.

**MOVIE S2**, MPG file, 7.9 MB.

**MOVIE S3**, MPG file, 6.6 MB.

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