

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

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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*) that 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, an 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.

INTRODUCTION

Iron-sulfur (FeS) proteins utilize a wide array of FeS clusters to play key roles that range from electron transfer and catalysis to structural and regulatory functions in biological systems (1-7). A homodimer carrying a subunit-bridging $[\text{Fe}_4\text{S}_4]$ cluster at the protein surface, the iron (Fe) protein of nitrogenase is best known for its function as an obligate electron donor for its catalytic partner during substrate turnover (8, 9). Recently, the Fe protein from a diazotrophic microbe, *Azotobacter vinelandii* (designated *AvNifH*) was shown to act as a reductase on its own and catalyze the ambient reduction of CO_2 to CO via redox changes of its $[\text{Fe}_4\text{S}_4]$ cluster (10). Interestingly, while the cluster of *AvNifH* is believed to cycle between the $[\text{Fe}_4\text{S}_4]^{1+}$ (reduced) and $[\text{Fe}_4\text{S}_4]^{2+}$ (oxidized) states (11-15) for its function as an electron donor in nitrogenase catalysis, catalytic turnover of CO_2 by *AvNifH* on its own was observed when a strong reductant, europium(II) diethylenetriaminepentaacetic acid (Eu^{II} -DTPA; $E^0 = -1.14$ V at pH 8.0), poised its cluster in the all-ferrous, $[\text{Fe}_4\text{S}_4]^0$ state under *in vitro* conditions (10). Perhaps more interestingly, the Fe protein from a methanogenic microorganism, *Methanosarcina acetivorans* (designated *MaNifH*), was capable of reducing CO_2 past CO into hydrocarbons under ambient conditions in the presence of Eu^{II} -DTPA, further illustrating the unique reactivity of the $[\text{Fe}_4\text{S}_4]$ cluster toward CO_2 (16, 17). Together, these observations point to the nitrogenase Fe protein as a simple model system for mechanistic investigations of FeS-based CO_2 activation and reduction.

Of the two Fe protein species that have been investigated for their reactivity toward CO_2 , *MaNifH* is particularly interesting given its ability to convert CO_2 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 a $[\text{Fe}_4\text{S}_4]$ cluster that can adopt three oxidation states upon redox treatments: (i) the oxidized state ($[\text{Fe}_4\text{S}_4]^{2+}$),

which is generated upon treatment by indigodisulfonate; (ii) the reduced state ($[\text{Fe}_4\text{S}_4]^{1+}$), which is generated upon treatment by dithionite; and (iii) the ‘super-reduced’, all-ferrous state ($[\text{Fe}_4\text{S}_4]^0$), which is generated upon treatment by Eu^{II} -DTPA (17). There are differences, however, in the electronic properties of *Ma*NifH and *Av*NifH, which are reflected by a stronger $S = 3/2$ contribution to the EPR spectrum of the reduced *Ma*NifH and a decreased intensity of the parallel mode, $g = 16.4$ signal in the EPR spectrum of the super-reduced *Ma*NifH (17). These differences, along with the lower reduction potential of the $[\text{Fe}_4\text{S}_4]^{1+/2+}$ pair of *Ma*NifH ($E^0 = -395$ mV) than that of *Av*NifH ($[\text{Fe}_4\text{S}_4]^{1+/2+}$: $E^0 = -301$ mV) (16), may contribute to the difference in the reactivity of *Ma*NifH and *Av*NifH toward CO_2 . The redox dependence of this reaction is further illustrated by a substantially decreased CO_2 -reducing activity of both *Ma*NifH and *Av*NifH in the presence of dithionite, a weaker reductant than Eu^{II} -DTPA that renders the clusters of these Fe proteins in the catalytically inefficient $[\text{Fe}_4\text{S}_4]^{1+}$ state (10, 16).

The significantly decreased activity of Fe protein in a dithionite-driven reaction could prove advantageous for capturing CO_2 in an early stage of CO_2 reduction. Here, we report a 2.4 Å crystal structure of *Ma*NifH that is generated in the presence of dithionite and an alternative CO_2 source, bicarbonate. Structural analysis of this previously uncharacterized Fe protein from the methanogen nitrogenase family suggests that CO_2 is possibly captured in an unactivated, linear conformation on the dithionite-reduced *Ma*NifH; moreover, it reveals the initial coordination of CO_2 by a conserved, surface-exposed arginine (Arg) pair in a concerted yet asymmetric manner, which could assist in trapping CO_2 near the $[\text{Fe}_4\text{S}_4]$ cluster via hydrogen bonding and electrostatic interactions. These results provide a useful framework for further exploration of the mechanism of CO_2 activation by Fe proteins, which may enable future development of FeS catalysts for recycling the greenhouse gas CO_2 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: 6NZY) adopts the same overall conformation as all Fe protein structures reported to date (9, 18-20), with each of its subunits fold as a single α/β -type domain and its [Fe₄S₄] cluster situated in a surface cavity between the two subunits (Figure 1A, B). A closer examination of the region surrounding the active site of *MaNifH* (Figure 1C) reveals the ligation of the [Fe₄S₄] cluster by four Cys residues, two from subunit A (Cys95^A, Cys130^A) and two from subunit B (Cys95^B, Cys130^B). Interestingly, the electron density omit map (Fo-Fc) of the active site of *MaNifH* (Figure 1C, *green mesh*; also see Figure S1) 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 (R98^A and R98^B)—one from each of the two adjacent *MaNifH* subunit dimers.

Modeling the extra electron density in the structure of 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 (Figure S2A), carbonate (Figure S2B), glycerol (Figure S2C) and CO₂ (Figure 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 (Figure 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 (Table S1). 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 symmetric 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 is 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 *Ma*NifH. To seek support for the assignment of CO₂ as the extra electron density in the crystal structure of *Ma*NifH, we then used density functional theory (DFT) calculations to analyze the CO₂ affinity of the [Fe₄S₄]¹⁺ cluster in *Ma*NifH. Consistent with our previous findings for both *Av*NifH-bound and synthetic [Fe₄S₄] clusters (10, 16), CO₂ does not interact well with the [Fe₄S₄]¹⁺ cluster of *Ma*NifH and tends to dissociate from the cluster during the course of structural optimization; however, the two highly conserved Arg residues in *Ma*NifH (R98^A, R98^B) form a cage-like configuration around the CO₂ molecule that assists in trapping it in close proximity to the cluster (Video S1). 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 *Ma*NifH except for a slight reorientation of CO₂ (Figure 2). In comparison, DFT optimization reveals protonation of carbonate by R98^B, 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 *Ma*NifH (Figure S3). 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 *Ma*NifH protein. The fact that the *Ma*NifH 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 *Ma*NifH structure. In this scenario,

the CO₂ moiety has its C atom placed at a distance of ~4 Å to the nearest Fe atom (Fe3) of the [Fe₄S₄] cluster, with the NH₂⁺ groups of R98^A and R98^B assuming the ‘distal’ and ‘proximal’ positions, respectively, to Fe3 (Figure 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 *Ma*NifH 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 *Ma*NifH to either a His or a Gly. Both R98H and R98G *Ma*NifH variants display the same $S = 1/2$ EPR signal as the wildtype protein, which is indicative of an unperturbed [Fe₄S₄] center in the +1 oxidation state (Figure 3A). However, the R98H variant of *Ma*NifH retains ~80% CO₂-reducing activity, whereas the R98G variant loses ~85% of this activity (Figure 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 (hydrocarbons/CO ratio: 1.9) from hydrocarbon formation to CO formation as compared to that of the wildtype *Ma*NifH (hydrocarbons/CO ratio: 2.7), as formation of CO requires fewer protons than that of hydrocarbons.

Proposal of a plausible mechanism for the initial capture of CO₂ by *Ma*NifH. To obtain further insights into the mechanism of CO₂ capture by nitrogenase Fe proteins, we compared our dithionite-reduced *Ma*NifH structure (PDB ID: 6NZY) that is potentially bound with CO₂ with a previously reported, dithionite-reduced *Av*NifH structure (PDB ID: 1G5P) that is free of CO₂ (19).

Consistent with a high degree of sequence homology between *Ma*NifH and *Av*NifH, the subunits A and B in *Ma*NifH show C α -deviations of only 0.599 and 0.616 Å, respectively, relative to those in *Av*NifH; yet, the two subunit chains in *Ma*NifH are more similar to each other in terms of secondary structural elements, particularly with respect to the structurally less conserved α -helical regions (Figures S4, S5). More strikingly, compared to their counterparts in *Av*NifH, there is a notable movement of the two subunits of *Ma*NifH 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 (Figure S6; Video S2). A top-view comparison between the two structures further reveals a ‘linearization’ of helices C^A and C^B in *Ma*NifH relative to those in *Av*NifH, which is accompanied by a substantial swing of the Arg pair, R98^A and R98^B (located at the tips of helices C^A and C^B), toward the center of the surface cavity (Figure S6; Video S3). Such a movement of the conserved Arg pair could reflect a concerted action of the ‘distal’ R98^A and the ‘proximal’ R98^B in the initial capture of CO₂ in an unactivated, linear conformation near the Fe₃ atom of the [Fe₄S₄] cluster (*see* Figure 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 *Av*NifH (10) led to the proposal of binding of an activated CO₂ moiety via coordination of C to Fe₃ of the cluster and coordination of O to the guanidinium group of the ‘proximal’ R100^B (corresponding to the ‘proximal’ R98^B in *Ma*NifH), with the latter potentially donating protons for the subsequent C-O bond cleavage.

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]) as a pair of Lewis acid/base to facilitate scission of a C-O bond (21-24). In the absence of such a heterometal-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 C to the nearest Fe³⁺ 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 (Fe³⁺) 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 of 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 into useful chemical commodities.

MATERIALS AND METHODS

Protein Purification and Crystallization. All protein purification steps were carried out anaerobically using Schlenk techniques. His-tagged *MaNifH* was purified by immobilized metal affinity as described elsewhere (17, 28). Reagents for protein crystallization were purchased from Hampton Research and were thoroughly de-aerated 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 N₂ for data collection.

MaNifH was crystallized at room temperature by microbatch method under a layer of Al's oil (Hampton Research). The purified *MaNifH* protein was desalted on a G-25 fine column equilibrated with buffer M (10 mM EPPS (pH 8.0), 100 mM NaCl, 10% (v/v) 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 μ L protein solution and 3 μ L precipitant solution (2.3 M ammonium sulfate, 7% (w/v) 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 two weeks and were flash-frozen in liquid nitrogen for data collection.

Data Collection and Structural Determination. The diffraction data of *MaNifH* crystals were collected at 100 K on beamline 8.2.1 of Advanced Light Source using a wavelength of 0.9774 Å and an ADSC Q315r CCD detector. 501 images were recorded for *MaNifH* 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 CCP4 package (29). Molecular replacement was performed with *Phaser* in PHENIX (30) using the structure of the *Clostridium pasteurianum* NifH protein (PDB ID: 1CP2) (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 *MaNifH* 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 modelling are summarized in Table S1. The structure of DT-reduced *MaNifH* (PDB ID: 6NZY) was deposited in the Protein Data Bank (<https://www.wwpdb.org>) and will be released upon publication.

Strain Construction and Activity Analyses. Strains expressing R98H and R98G *MaNifH* variants were constructed via site-directed mutagenesis of the wildtype *Methanosarcina acetivorans nifH* sequence carried on a pET14b vector (17), followed by transformation of the resultant plasmids into the *E. 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 (wildtype, R98H or R98G *MaNifH*), and 100 mM Eu^{II}-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 Eu^{II}-DTPA, 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 *MaNifH*, followed immediately by addition of Eu^{II}-DTPA and incubation with continuous shaking at 30°C for 300 min until the reaction was complete. Following the quenching of each assay by 100 µL 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 N₂ prior to analysis. The DT-reduced samples contained 2

mM DT, 50 mM Tris-HCl (pH 8.0), 500 mM NaCl and 10% (v/v) 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 5×10^4 , 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 *Ma*NifH (PDB ID: 6NZY; this work) in the DT-reduced, [Fe₄S₄]¹⁺ state.

The models were selected as described previously (10) and contained the [Fe₄S₄] cluster and C95^A, C95^B, C130^A, C130^B, R98^A, R98^B, F133^A, F133^B and, additionally, the main chain atoms of the residues A96^A, A96^B, A97^A, G97^B, G131^A, G131^B, G132^A and G132^B of *Ma*NifH 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 C α), the side-chain atoms of the Arg residues (starting from C γ), 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 $\epsilon=40$. The structures were optimized with the TPSS functional (18). A def2-TZVP basis set (39, 40) was used for the [Fe₄S₄] cluster, the side chain atoms of the Cys residues (including

C α 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 FeS cluster was accounted for by the broken symmetry approach (43-45).

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FIGURES

Figure 1

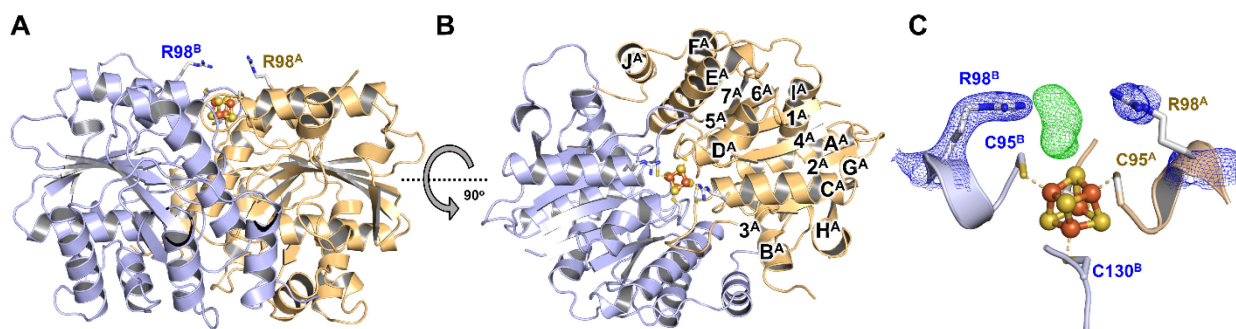


FIG 1 Side (A) and top (B) views of the 2.4 Å crystal structure of *MaNifH*. The subunits are shown as ribbons (subunit A, light orange; subunit B, light blue). The α -helices (A^A-J^A) and β -sheets (1^A-7^A) of subunit A are indicated. The [Fe₄S₄] cluster is shown in ball-and-stick presentation (Fe, orange; S, yellow). (C) The electron density (2Fo-Fc) of the active site of *MaNifH* was contoured at 1.5 σ level for the conserved Arg pair (blue meshes), and the omit map (Fo-Fc) of the additional electron density (green mesh) that is unaccounted for in the structure was contoured at 3.0 σ level. The four Cys ligands (C95^A, C130^A, C95^B, C130^B) and the conserved Arg pair (R98^A, R98^B) are shown as sticks.

Figure 2

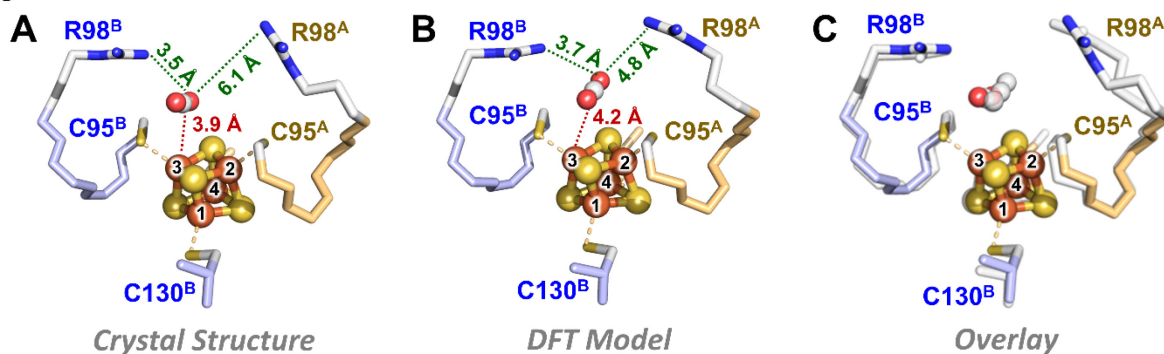


FIG 2 Crystal (A) and DFT-optimized (B) structures of *MaNifH* with the extra electron density modeled as CO₂, and (C) an overlay of the two structures. The conserved pair of Arg residues assume ‘proximal’ (R98^B) and ‘distal’ (R98^A) positions, respectively, to the CO₂ moiety and the Fe³⁺ atom of the cluster (A, 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.

Figure 3

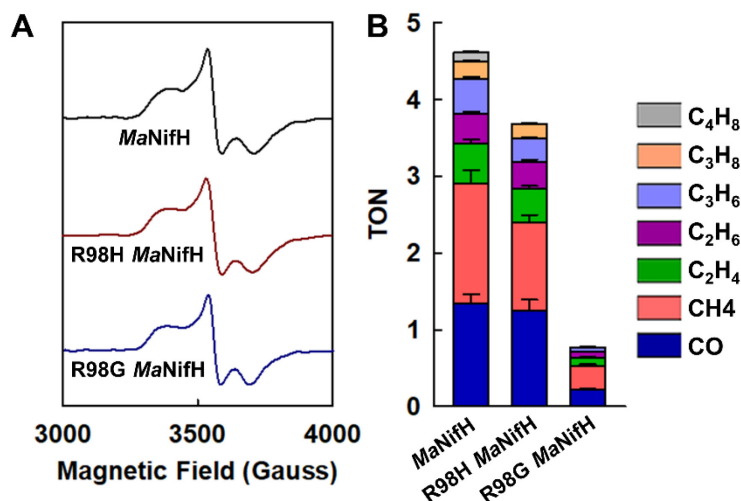


FIG 3 Spectroscopic and catalytic features of the wildtype and variant *MaNifH*. **(A)** EPR spectra and **(B)** CO_2 -reducing activities of wildtype and variant *MaNifH*. EPR spectra were collected at 10 K. The wildtype, R98H and R98G *MaNifH* are dimers of ~60 kDa and contain 3.7 ± 0.1 , 3.9 ± 0.4 and 3.8 ± 0.2 nmol Fe per nmol protein, respectively. Like the wildtype *MaNifH*, the R98H and R98G variants display the same $[Fe_4S_4]^+$ -characteristic, $S=1/2$ EPR signal in the dithionite-reduced state (A); yet they display disparate activities in CO_2 reduction (B). The hydrocarbons/CO ratios (calculated based on total nmols of reduced carbons) of the wildtype *MaNifH* and R98H variant are 2.7 and 1.9, respectively, suggesting a shift from hydrocarbon-formation to CO-formation in the latter case.

Figure 4

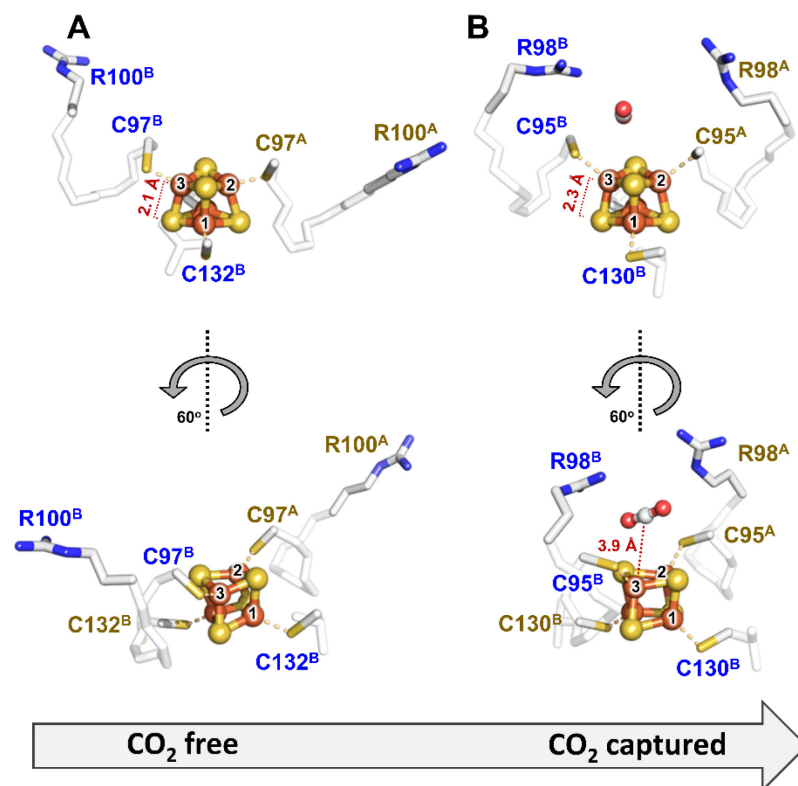


FIG 4 Comparison of the CO₂-free (A) and CO₂-captured (B) conformations of Fe protein, showing concerted yet asymmetric movement of a pair of conserved Arg residues that potentially capture CO₂ near the [Fe₄S₄] cluster. The CO₂-free and CO₂-captured conformations are represented by the homologous *Av*NifH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

SUPPORTING TABLE

Table S1. Data collection, refinement and ligand model statistics of *MaNifH* (PDB ID 6NZJ)

Space group	<i>P6₅22</i>				
<i>Cell dimensions</i>					
a, b, c (Å)	96.16, 96.16, 320.24				
α, β, γ (°)	90.00, 90.00, 120.00				
Wavelength (Å)	0.97741				
Number of reflections measured	419,306 (35,917)				
Number of unique reflections	35,959 (3,432)				
Resolution (Å)	83.3 – 2.4 (2.5–2.4) ^a				
R _{meas} (%)	11.1 (78.1)				
CC _{1/2}	0.999 (0.810)				
Mean I/σ(I)	5.4 (1.0)				
Completeness (%)	100.0 (99.9)				
Multiplicity	11.9 (11.2)				
Resolution (Å)	83.3 – 2.4				
No. of reflections	35,369				
R _{work} / R _{free} ^b (%)	18.31 / 21.80				
<i>Number of atoms (non-H)</i>					
Overall	4,147				
Protein	3,952				
Ligand	23				
Solvent	172				
<i>Average B factors (Å²)</i>					
Overall	49.01				
Protein	48.95				
Ligand	42.46				
Water	51.34				
<i>Ramachandran plot statistics</i>					
Favored (%)	96.49				
Allowed (%)	3.51				
<i>R.M.S. deviations</i>					
Bond lengths (Å)	0.008				
Bond angles (°)	0.90				
<i>Statistics for the plausible ligands</i>					
	R _{work} (%) ^c	R _{free} (%) ^c	Occupancy of the ligand ^d	Average B factor of the ligand (Å ²)	RSCC ^e
No ligand	18.31	21.80	-	-	-
CO ₂	18.27	21.78	1.00	50.69	0.89
Glycerol	18.22	21.78	0.46	41.82	0.95
Carbonate	18.23	21.84	0.51	45.27	0.95

^aValues in parentheses represent the highest resolution shell.

^bR_{work} was calculated using 95% of the data included in the refinement and R_{free} the 5% excluded data.

^cR_{work} and R_{free} were calculated by Phenix.Refine.

^dThe structure was refined by Phenix.Refine after the generation of each ligand.

^eReal-space correlation coefficient was provided by wwPDB Validation Server.

TABLE S1. Date collection, refinement and ligand model statistics of *MaNifH* (PDB ID 6NZJ). Shown are the values for the ligand-free model (upper) and the statistics for the models with the plausible ligands CO₂, glycerol and carbonate (lower).

SUPPORTING FIGURES

Figure S1

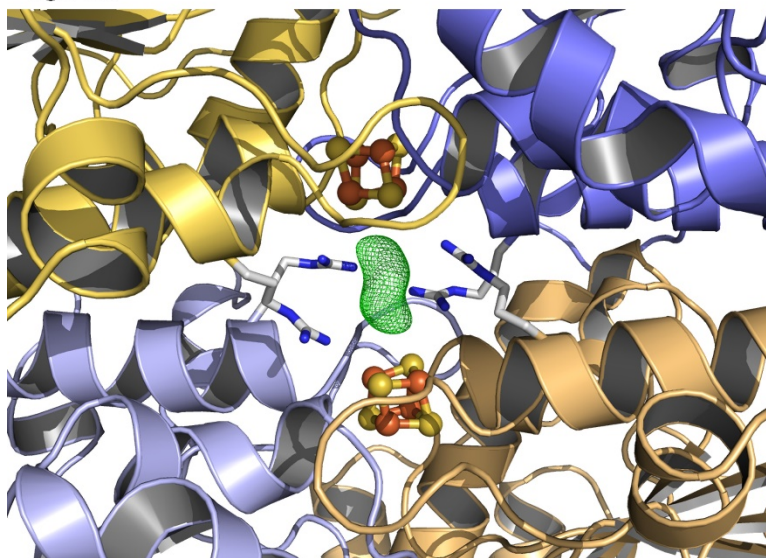


FIG S1 Structure of *MaNifH* without additional ligand. The subunits are shown as ribbons and colored light orange (subunit A) and light blue (subunit B) for the asymmetric unit, and yellow (subunit A) and dark blue (subunit B) for the symmetric mate. The Fo–Fc map (green mesh) is contoured at 3 σ . The side chains of R98 are shown as sticks. The [Fe₄S₄] clusters are shown in ball-and-stick presentation and colored as follows: Fe, orange; S, yellow.

Figure S2

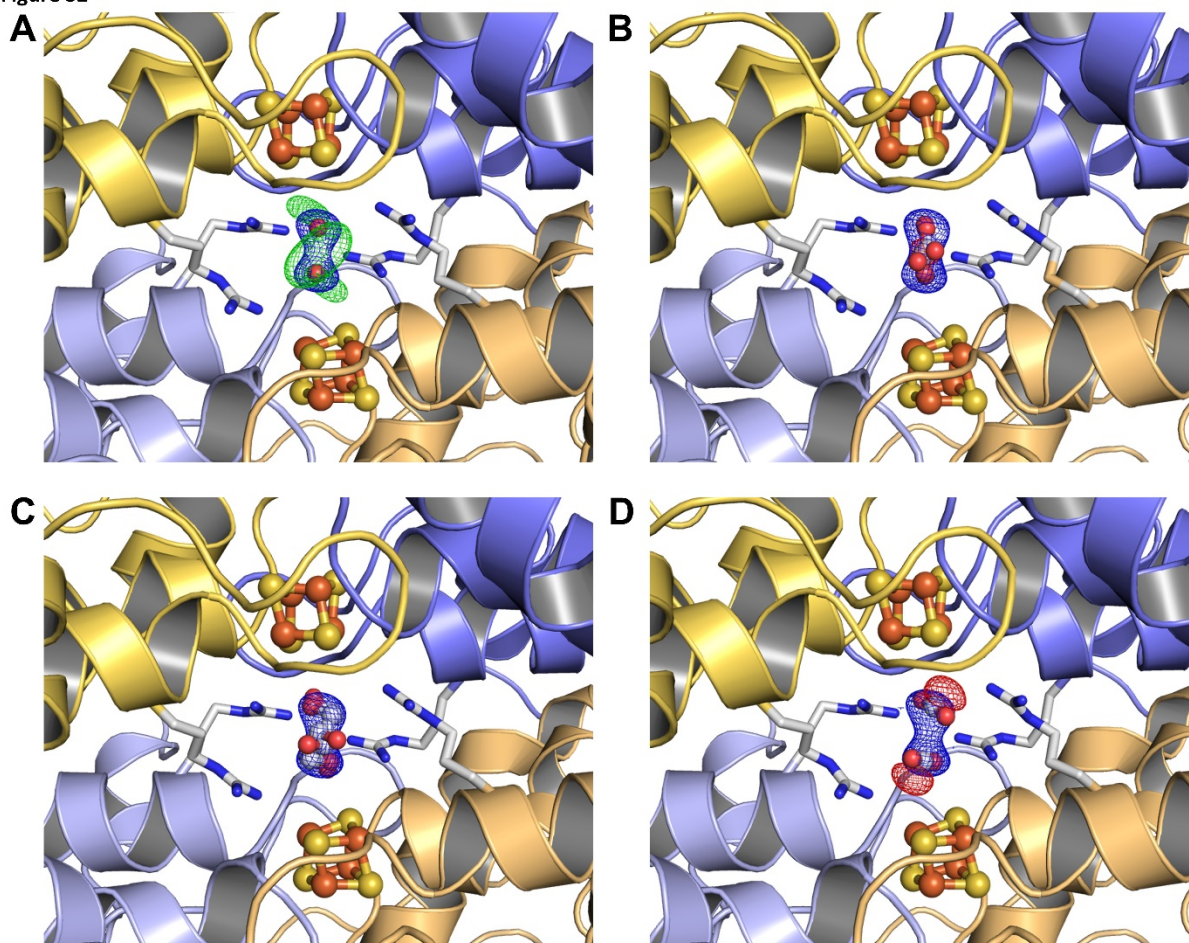


FIG S2 Structures of *MaNifH* modeled with two water molecules (**A**), two carbonate molecules (**B**), two glycerol molecules (**C**), and (**D**) two CO₂ moieties, respectively. The subunits are shown as ribbons and colored light orange (subunit A) and light blue (subunit B) for the asymmetric unit, and yellow (subunit A) and dark blue (subunit B) for the symmetric mate. The Fo–Fc map (red mesh) is contoured at -3σ , the Fo–Fc map (green mesh) is contoured at 3σ , and the 2Fo–Fc (blue mesh) map is contoured at 1.5σ . One water (A) or CO₂ (B) molecule was modeled in the asymmetric unit and the other in the symmetry mate, respectively. The two carbonate (B) or glycerol (C) molecules were placed on the crystallographic axis. The side chains of R98 are shown as sticks. The [Fe₄S₄] cluster and the water, carbonate and glycerol moieties are shown in ball-and-stick presentation and colored as follows: Fe, orange; S, yellow; C, gray; O, red.

Figure S3

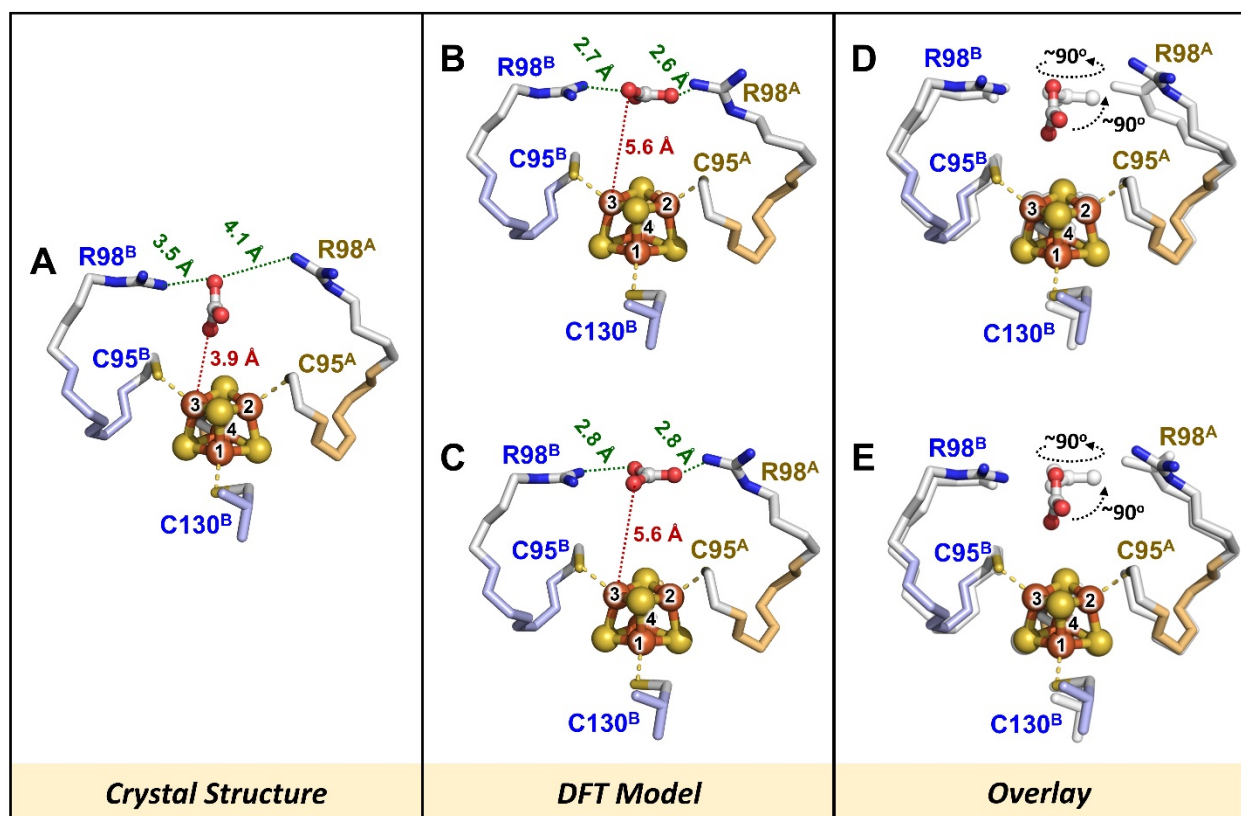


FIG S3 Crystal structure of *MaNifH* with the extra electron density modeled as carbonate (A) and DFT-optimized structure of *MaNifH* with carbonate (B) or bicarbonate (C) as the substrate. In the crystal structure, the carbonate moiety assumes a position perpendicular to the upper surface of the [Fe₄S₄] cluster (A). Based on DFT-based structural optimization, however, carbonate is first protonated by R98^B and the resulting bicarbonate assumes a position parallel to the upper surface of the [Fe₄S₄] cluster (B). The DFT-optimized structure calculated directly with bicarbonate (C) closely resembles that achieved with carbonate upon protonation (B). Overall, carbonate occupies a different position in the crystal structure than (bi)carbonate in the DFT models, which can be best visualized by the overlay of the carbonate-bound crystal structure with the (bi)carbonate-bound DFT models that originate either from the protonation of carbonate (D) or directly from bicarbonate (E). In both cases, the (bi)carbonate moiety swings ~90° from a position that is perpendicular to one that is parallel to the upper surface of the [Fe₄S₄] cluster. Furthermore, the (bi)carbonate moiety rotates ~90° counterclockwise in parallel to the upper surface of the cluster. The hydrogen atoms of bicarbonate are omitted from the figure, as they cannot be discerned by x-ray crystallography. The [Fe₄S₄] cluster and carbonate/bicarbonate moieties are shown in ball-and-stick presentations and colored as follows: Fe, orange; S, yellow; C, gray; O, red. The Cys ligands and the conserved Arg residues are shown as sticks.

Figure S4

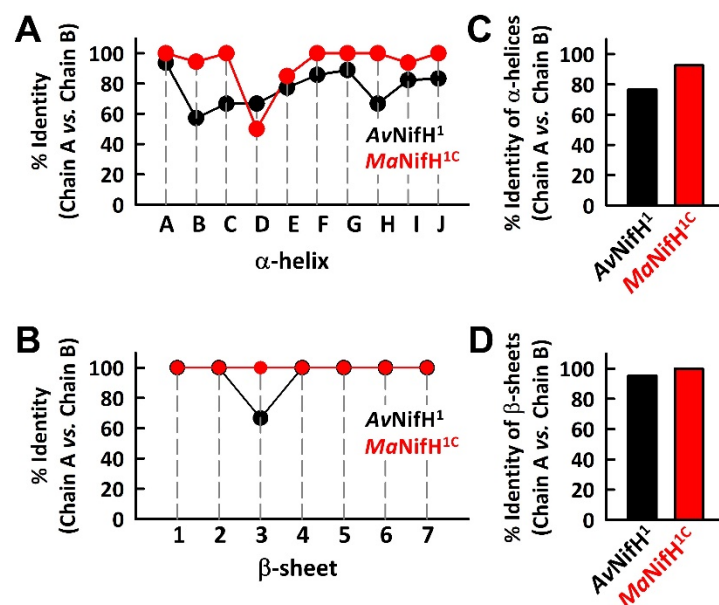


FIG S4 Identities between subunits A and B within *AvNifH* and *MaNifH*. Percentage identities between individual α -helices (**A**) and β -sheets (**B**) of subunits A and B within *AvNifH* (black) and *MaNifH* (red). Overall percentage identities between α -helices (**C**) and β -sheets (**D**) of subunits A and B within *AvNifH* (black) and *MaNifH* (red). Data from FIG S5 were used to calculate these percentages. C α -deviations of subunit A vs. subunit B: *AvNifH* (0.356 Å) and *MaNifH* (0.334 Å).

Figure S5

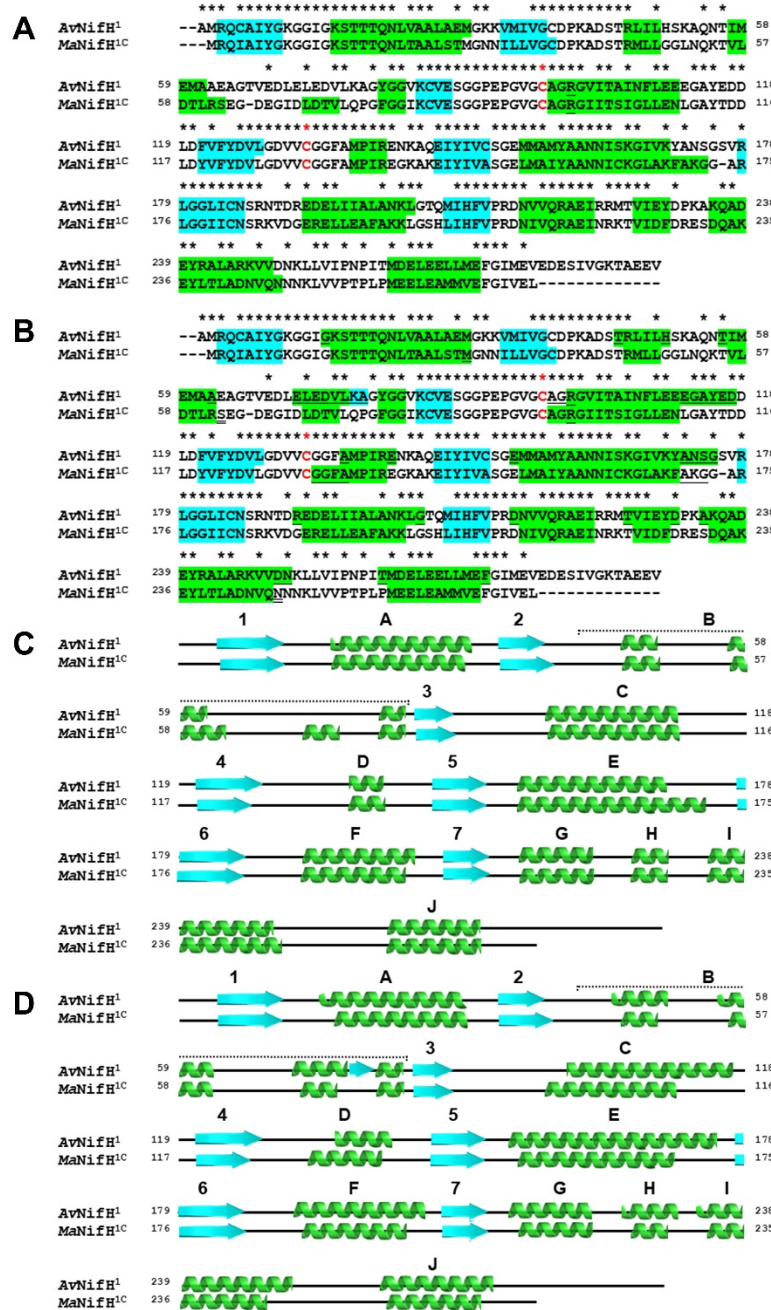


FIG S5 Subunits A and B of *AvNifH* and *MaNifH*. Comparisons between (A, B) primary sequences and (C, D) secondary structures of subunits A (A, C) and B (B, D) of *AvNifH* and *MaNifH*. The identical residues in the primary sequences are indicated with *, and the conserved Cys ligands for the [Fe₄S₄] cluster are shown in red (A, B). The α -helices are colored green, and the β -sheets are colored cyan (C, D); and the primary sequences corresponding to these structural elements are highlighted with the corresponding colors (A, B).

Figure S6

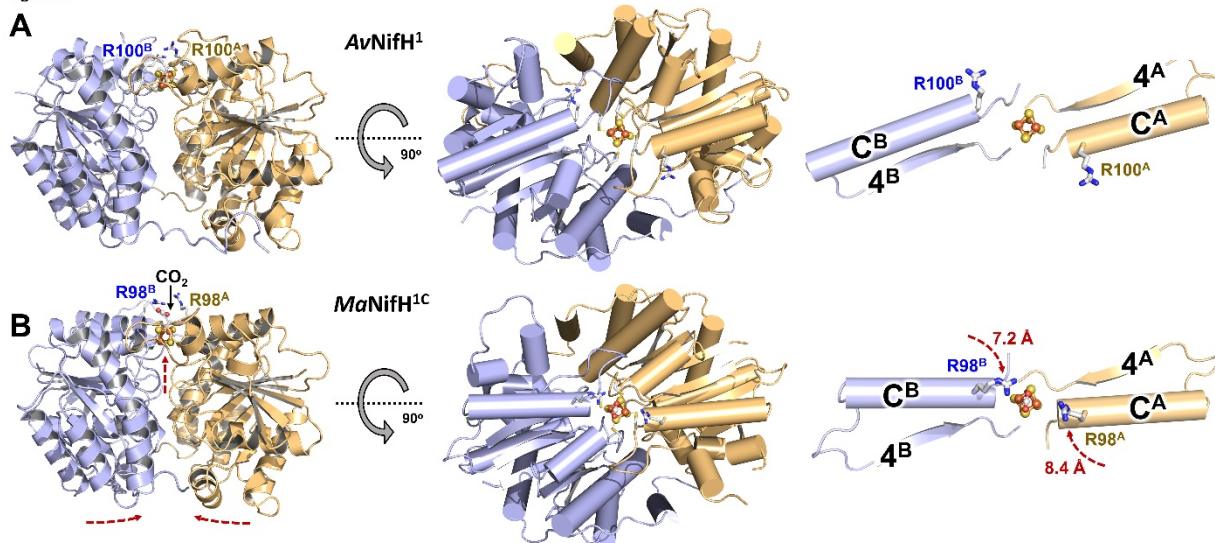


FIG S6 Structural comparison between *AvNifH* and *MaNifH*. Side (*left*) and top (*middle*) views of *AvNifH* (A) and *MaNifH*, with the alignments of helices C^B and C^A and the positions of the Arg pairs (located at the tips of helices C^B and C^A) highlighted (*right*). The movements of Arg residues in *MaNifH* (B, *right*) relative to those in *AvNifH* are indicated by dashed red arrows. Subunits are shown as ribbons (side view) or cylinders (top view) and colored light orange (subunit A) and light blue (subunit B), respectively. 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.

SUPPORTING VIDEOS (Captions)

Video S1. Course of DFT-based structural optimization (TPSS/def2-SVP/def2-TZVP) of the CO₂-captured conformation of *MaNifH* in the [Fe₄S₄]¹⁺ state (*S*=1/2). The CO₂ moiety in the DFT-optimized structure is rendered gray, and the CO₂ moiety in the crystal structure is colored as follows: C, gray; O, red.

Video S2. Conformational morphing of *AvNifH* into *MaNifH* (side view). Both structures are depicted and colored as described in the legend of FIG S1. The conformational morphing plugin of PyMol 2.1.1 (<https://pymol.org>) was used to generate the movie, with *AvNifH* as the start conformation and *MaNifH* as the end conformation. Refinement cycles: 3; number of output states: 100; interpolation method: RigiMOL).

Video S3. Conformational morphing of *AvNifH* into *MaNifH* (top view). The movie was generated as described in the legend of Video S2.