A Heme-based Redox Sensor in the Methanogenic Archaeon Methanosarcina acetivorans*

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**Background:** Multidomain sensory kinases are involved in numerous receptive processes in all kingdoms of life.

**Results:** The multidomain sensory kinase MA4561 covalently binds a redox-active heme cofactor, which triggers kinase activity.

**Conclusion:** Covalently bound heme is utilized to detect redox changes.

**Significance:** Learning how Archaea perceive environmental stimuli will enhance our understanding of the evolution of prokaryotic signal transduction.

Based on a bioinformatics study, the protein MA4561 from the methanogenic archaon Methanosarcina acetivorans was originally predicted to be a multidomain phytochrome-like photosensory kinase possibly binding open-chain tetrapyrroles. Although we were able to show that recombantly produced and purified protein does not bind any known phytochrome chromophores, UV-visible spectroscopy revealed the presence of a heme tetrapyrrole cofactor. In contrast to many other known cytoplasmic heme-containing proteins, the heme was covalently attached via one vinyl side chain to cysteine 656 in the second GAF domain. This GAF domain by itself is sufficient for covalent attachment. Resonance Raman and magnetic circular dichroism data support a model of a six-coordinate heme species with additional features of a five-coordination structure. The heme cofactor is redox-active and able to coordinate various ligands like imidazole, dimethyl sulfide, and carbon monoxide depending on the redox state. Interestingly, the redox state of the heme cofactor has a substantial influence on autophosphorylation activity. Although reduced protein does not auto-phosphorylate, oxidized protein gives a strong autophosphorylation signal independent from bound external ligands. Based on its genomic localization, MA4561 is most likely a sensor kinase of a two-component system effecting regulation of the Mts system, a set of three homologous corrinoid/methyltransferase fusion protein isoforms involved in methyl sulfide metabolism. Consistent with this prediction, an M. acetivorans mutant devoid of MA4561 constitutively synthesized MtsF. On the basis of our results, we postulate a heme-based redox/dimethyl sulfide sensory function of MA4561 and propose to designate it MsmS (methyl sulfide methyltransferase-associated sensor).

Heme-based sensors are a diverse group of sensor proteins that are able to sense small gaseous ligands like oxygen (O2), carbon monoxide (CO), and NO and changes of the cellular redox state. So far, several heme-binding sensor domains have been characterized (for a review, see Ref. 1). Among them, a variety of globin-coupled sensors with a globin fold as the heme-binding site have been identified (2). Other examples are the heme NO/oxygen-binding proteins (3), which have similarity to eukaryotic soluble guanylate cyclases (4); the SCHIC (sensor containing heme instead of cobalamin) domain in AppA (5); and the CO-sensing transcriptional activator CooA from Rhodospirillum rubrum (6). Additionally, heme-containing PAS domains (7), like those in FixL (8), EcDox (9), and AxpDEA1 (10), and GAF domains, like those in DevS (DosS) and DosT (11, 12), have been reported. In virtually all cases, the proteins contain non-covalently bound b-type heme. Only two examples of covalently bound heme in sensor proteins have been described in the literature. DcrA from Desulfovibrio vulgaris contains a c-type heme in a periplasmic cytochrome c-like fold (13–15), and the protein GSU0303 from Geobacter sulfurreducens was shown to contain a periplasmic heme-binding PAS domain. In contrast to the sensory input, which is determined

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**The abbreviations used are:** PAS, Per (Drosophila period clock protein)-Arnt (vertebrate aryl hydrocarbon receptor nuclear translocator)-Sim (Drosophila single-minded protein); DMS, dimethyl sulfide; Fe(III), Fe(III)-protoporphyrin IX complex; Fe(III) heme complex, or hemin; Fe(II), Fe(II)-protopor-porphyrin IX complex or Fe(II) heme complex; GAF, domain conserved in cyclic GMP-specific and -stimulated phosphodiesterases, adenylate cyclases, and E. coli formate hydrogen-lyase transcriptional activator; H_ATPase, histidine kinase-like ATPase; Im, imidazole; RR, resonance Raman; THT, tetrahydrothiophene; MCD, magnetic circular dichroism; MsmS, methyl sulfide methyltransferase-associated sensor; HemAT, heme-based aerotactic transducer; EW, elution and washing; CAPS, N-cyclohexyl-3-aminopropanesulfonic acid; aa, amino acids; 6c-LS, six-coordinate low spin; Mb, myoglobin; cyt, cytochrome; T, tesla(s); PHY, phytocrome.
by the chemical nature of heme, the physiological output is as variable as the heme-binding domains themselves. HemATs function as regulators of aerotaxis (2), whereas other globin-coupled sensors regulate concentrations of second messengers (16). EcDos and ArPDEA1 are phosphodiesterases (10, 17), and CooA is a direct transcriptional activator (6), whereas AppA is an antirepressor for a transcriptional regulator (18). FixL, DevS, and DosT are sensor histidine kinases of two-component systems (8, 19). Recently, a globin-coupled histidine kinase also was identified (20). The only known heme-based sensor from Archaea is the aerotaxis transducer Hs-HemAT from *Halobacterium salinarum* (2).

Within the archaeal kingdom, one-component systems are more widely distributed than two-component systems (21), and indeed genes encoding two-component systems are only found in some euryarchaeal genomes and neither in the Crenarchaeota nor in the Nanoarchaeota (22). Therefore, two-component systems most likely evolved in bacteria and radiated into Archaea via horizontal gene transfer (21, 23). Typical bacterial two-component systems consist of a sensor histidine kinase and a corresponding response regulator (24). The sensor kinases are often multidomain proteins with a conserved kinase output domain fused to a variety of sensor domains that detect certain signals. The response regulators have a conserved input domain and non-conserved output domains (25). Upon signal recognition by the sensor kinase, a conserved histidine residue is autophosphorylated, and the phosphoryl group subsequently is transferred to a conserved aspartate residue in the response regulator input domain, resulting in activation of the output domain.

Based on sequence analyses and phylogenetic studies, the protein MA4561 from the methanogenic archaeon *Methanosaeta acetivorans* was predicted to be a phychochrome-like protein (26). Another study searching for two-component systems in Archaea showed that MA4561 contains a histidine kinase-like ATPase (H_ATPase) domain but seems to lack the H-box containing the conserved histidine residue that functions as the phosphoacceptor during autophosphorylation (22).

In this study, we investigated the function of MA4561 as well as different truncated variants thereof *in vitro* and *in vivo*. We show that MA4561 harbors a covalently bound heme cofactor in its second GAF domain. To our knowledge, this is the first report of a covalently attached heme cofactor in a cytoplasmic sensor protein. Autophosphorylation of the kinase domain is shown to be strongly dependent on the heme redox state. As deletion of MA4561 led to deregulated synthesis of a dimethyl sulfide-dependent methyltransferase, we argue for a redox/dimethyl sulfide signaling function for MA4561.

**EXPERIMENTAL PROCEDURES**

**Construction of Expression Plasmids**—The *M. acetivorans* C2A *maa4561* coding region and shorter variants were PCR-amplified from genomic DNA and cloned according to standard procedures. Details can be found in the supplementary Experimental Procedures and supplementary Tables S1 and S2.

**Overexpression and Purification of His<sub>S</sub>-tagged MA4561 Variants**—Recombinant full-length MA4561 and all variants fused to a C-terminal His<sub>S</sub> tag were produced using a T7/lac promoter-driven expression system (Novagen) in *Escherichia coli* strain BL21(DE3). Cells were grown in LB medium containing 100 μg/ml ampicillin at 37 °C to an *A*<sub>578</sub> of 0.5. Protein production was induced by the addition of 0.1 mM isopropyl β-thiogalactoside (full-length MA4561-His<sub>S</sub> and variants sGAF2-His<sub>S</sub>, PAS2-His<sub>S</sub>, GAF1-His<sub>S</sub>, and PAS1-His<sub>S</sub>) or 0.25 mM isopropyl β-thiogalactoside (variants sGAF2-His<sub>S</sub> and sGAF2C656A-His<sub>S</sub>), and cells were further incubated for 18–20 h at 17 °C. The cell pellet was either frozen and stored at −20 °C or directly resuspended in elution and washing (EW) buffer (50 mM Tris/HCl, pH 7.0, 300 mM NaCl, 10% glycerol, 0.05% Tween 20, 1 mM dithiothreitol, 0.25 mM 4-(2-aminoethyl)benzenesulfonyl fluoride; in the case of variants sGAF2-His<sub>S</sub> and sGAF2C656A-His<sub>S</sub>, no glycerol and Tween 20 were added) (6 ml of buffer/g of cell weight) and disrupted by sonication (Branson sonifier 250) or by acceleration disruption (Constant Systems Ltd. cell disruption system) at 40 k.p.s.i. (variants sGAF2-His<sub>S</sub> and sGAF2C656A-His<sub>S</sub>). Cell debris was removed by centrifugation (90 min at 28,000 × *g* at 4 °C), and the supernatant was either directly loaded onto a Co<sub>2</sub>-ligand affinity chromatography column (BD Talon<sup>®</sup>), which had been preequilibrated with EW buffer, or incubated with 100 μM hemin (in DMSO) for 1 h at 4 °C prior to this step. Unwanted proteins were washed off with 10 column volumes of EW buffer containing 10 mM imidazole (Im). Elution of His<sub>S</sub>-tagged proteins was performed with EW buffer containing 150 mM Im. Alternatively, the column was washed extensively with a minimum of 20 column volumes of EW buffer (without Im) after loading the protein (incubated with hemin), and all residual proteins were washed of the column with a total of 3 column volumes of EW buffer containing 200 mM EDTA. Fractions containing the desired protein were combined and dialyzed against an appropriate volume of dialysis buffer (50 mM Tris/HCl, pH 7.0, 100 mM NaCl; 50 mM sodium phosphate, pH 7.0, 100 mM NaCl; or 50 mM CAPS, pH 10.0, 100 mM NaCl) at 4 °C overnight. Proteins were concentrated if necessary using Vivaspin concentrator devices (molecular weight cutoff, 10,000; Sartorius Stedim Biotech). If higher purity was desired, the protein was subjected to gel permeation chromatography on a Superdex 200 10/300GL column (GE Healthcare). Fractions containing the desired protein were combined and again concentrated to an appropriate concentration (for calculated molecular masses and extinction coefficients, see supplementary Table S3).

**Overexpression and Purification of StrepII-tagged MA4561 Variants**—Recombinant full-length MA4561 and variant sGAF2 fused to a C-terminal StrepII tag were produced using a tet promoter-driven expression system (IBA) in *E. coli* strain BL21(DE3). Cell growth and purification were performed as described for the His<sub>S</sub>-tagged protein with the exception that protein production was induced with 200 ng/ml anhydrotretracycline, and the buffer for the purification procedure was Buffer W (100 mM Tris/HCl, pH 8.0, 300 mM NaCl, 1 mM EDTA, 10% glycerol, 0.05% Tween 20, 0.25 mM 4-[(2-aminoethyl)benzenesulfonyl fluoride; in the case of variant sGAF2, no glycerol and Tween 20 were added). For the affinity chromatography, a Strep-Tactin chromatography column (IBA) was preequilibrated with Buffer W was used. Unwanted proteins were washed off with 10 column volumes of Buffer W. Elution of StrepII-tagged protein was performed with a mix of 50 mM Tris and 1 mM imidazole. Fractions containing the desired protein were combined and dialyzed against an appropriate volume of dialysis buffer (50 mM Tris/HCl, pH 7.0, 100 mM NaCl; 50 mM sodium phosphate, pH 7.0, 100 mM NaCl; or 50 mM CATPS, pH 10.0, 100 mM NaCl) at 4 °C overnight. Eluted proteins were concentrated if necessary using Vivaspin concentrator devices (molecular weight cutoff, 10,000; Sartorius Stedim Biotech).
proteins was performed with Buffer E (Buffer W containing 2.5 mM desthiobiotin). Fractions containing the desired protein were combined, dialyzed, and concentrated as described for His₆-tagged proteins (for calculated molecular masses and extinction coefficients, see supplemental Table S3).

Overexpression and Purification of MA4561 Full-length Protein from M. acetivorans—MA4561-His₆ was produced in M. acetivorans strain WW73 (27) after liposome-mediated transformation (28) with ptet4561. Cells were grown under strictly anaerobic conditions at 37 °C to an A₅₇₈ of 1.5 in high salt medium (29) containing 125 mM methanol as the sole energy source. 2 μg/ml puromycin was added for selection of the pac gene encoding puromycin acetyltransferase (30), and 100 μg/ml tetracycline was added for induction of gene expression controlled by the hybrid mcrBtetO1 promoter (27). Cells were harvested by centrifugation, washed three times with anaerobic buffer (50 mM Pipes, pH 6.8, 400 mM NaCl, 13 mM KCl, 54 mM MgCl₂, 2 mM CaCl₂, 2.8 mM cysteine HCl, 0.4 mM Na₂S, 4 μM resazurin) and stored at −80 °C until use. Purification of homologously produced MA4561-His₆ was performed as described for the His₆-tagged protein produced in E. coli.

Heme Staining Procedure—Covalently bound heme in proteins can be detected with enhanced chemiluminescence (ECL) reagents due to peroxidase activity of the heme group (31). Proteins were separated by SDS-PAGE using sample buffer without β-mercaptoethanol and subsequently electroblotted onto a polyvinylidene fluoride (PVDF) (Roth) membrane. The membrane was covered with Luminata Forte Western blot horseradish peroxidase (HRP) substrate (Millipore) (about 1 ml for a 7-cm-sized membrane) and incubated for 1 min. Signals were detected using a charge-coupled device imaging system (Multi Image II, Alpha Innotech).

Heme Titration Experiments—Variant sGAF2-His₆ purified without externally added heme was used to perform heme titration experiments in vitro. Increasing amounts of hemin (stock solution 4.6 mM in DMSO) were added to a 10 μM concentration of the protein, incubated for 1 h at room temperature, and subjected to the heme staining procedure.

Acidified Butanone Extraction—Acidified butanone extractions were carried out as described before (32). In detail, an aqueous protein solution was titrated with 1 mM HCl to give a pH of 1.5–2.0. An equivalent volume of ice-cold 2-butanol was added, and the solution was mixed gently. After cooling on ice, an upper organic layer and a lower aqueous layer were distinguishable. Covalently bound heme stayed in the aqueous layer, whereas non-covalently bound heme was extracted to the organic layer.

Western Blot Analysis—Detection of His₆-tagged protein was performed using the HisTag Antibody HRP Conjugate kit (Novagen) according to the manufacturer’s instructions. Extracts of M. acetivorans strains were electrophoretically separated, transferred to nitrocellulose membranes, and probed with polyclonal antiserum specifically cross-reacting with MtsF (but not with MtsD or MtsH) as described (62).

Optical Absorption Spectra—Optical absorption spectra were recorded on an 8453 UV-visible spectrophotometer (Agilent Technologies) at room temperature. For anaerobic conditions, rubber-sealed cuvettes were used. To obtain the Fe(II) complex, the protein was reduced by adding some crystals of sodium dithionite before sealing the cuvette. The solution was sparged with N₂ gas for at least 30 min to make it completely anaerobic. For preparation of the Fe(II)-CO complex, this solution then was flushed with CO gas for 30 min. To obtain complexes with Im or dimethyl sulfide (DMS), anaerobic stock solutions thereof were used to add specific concentrations to anaerobically prepared Fe(III) or Fe(II) complexes in rubber-sealed cuvettes.

Pyridine Hemochrome Assays—Pyridine hemochrome spectra were obtained according to modified, published protocols (33). Specifically, 500 μl of a 10 μM protein solution were mixed with 62.5 μl of pyridine and 62.5 μl of 0.5 M NaOH. Then an excess of crystalline sodium dithionite was added to the solution and mixed, and a spectrum of the reduced pyridine hemochromogen was recorded.

Resonance Raman Spectroscopy—Resonance Raman (RR) spectra were acquired using a WITec α300AR Raman microscope (Ulm, Germany). The excitation wavelength used was 532 nm from a frequency-doubled neodymium-doped yttrium aluminum garnet laser (CrystaLaser, Reno, NV). The exciting laser radiation was coupled into a Zeiss microscope through a wavelength-specific single mode optical fiber with a diameter of 50 μm. The incident laser beam was collimated via an achromatic lens and passed through a holographic band pass filter before being focused into the sample through a Nikon Plan Fluor (20×/0.45 numerical aperture) dry objective. Raman back-scattered light was collected through a microscopic objective and passed through a holographic edge filter onto multimode fiber (50-μm diameter) and into a 300-mm-focal length monochromator incorporating an 1800 g/mm grating blazed at 500 nm. Detection of Raman spectra was provided by a back-illuminated deep depletion charge-coupled device camera operating at −60 °C. The laser power at the sample point was adjusted to 3 milliwatts. Raman shifts were calibrated with cyclohexane. The accuracy of the peak positions of well defined Raman bands was ±1 cm⁻¹. The protein concentrations for the RR experiments were 500 μM in 50 mM sodium phosphate, pH 7.0, 100 mM NaCl.

Magnetic Circular Dichroism—Magnetic circular dichroism (MCD) spectra were measured on a Jasco J815 spectropolarimeter fitted with a Jasco MCD-1B magnet at a magnetic field strength of 1.41 T at 4 °C using a 0.2-cm-path length quartz cuvette. Jasco software was used for data acquisition and manipulation as reported previously (34). Because the molar absorptivity of the pyridine hemochromogen of the heme in variant sGAF2-His₆ (without Im) could not be determined, concentrations of the sGAF2 samples were tentatively determined based on an estimated value of ε = 115 mm⁻¹ cm⁻¹ (the value for ferric cytochrome b₅₃ for the Soret absorption peak (415 nm) of the Im complex of ferric sGAF2 at pH 7.0. UV-visible absorption spectra were recorded before and after each MCD measurement to track sample integrity. sGAF2 protein samples were studied in 50 mM sodium phosphate buffer containing 100 mM NaCl (NaP₄) at pH 7.0 and in 50 mM CAPS buffer at pH 10.0. The Im and tetrahydrothiophene (THT) adducts were prepared by adding 2 and 14 mM Im and THT to the protein, respectively. The ferrous species of sGAF2 was pre-
pared from ferric protein in the presence or absence of exogenous ligands (Im and THT) by flushing a rubber-sealed cuvette with nitrogen gas for about 10–15 min followed by the addition of a small amount of solid sodium dithionite.

**Kinase Assays**—Autophosphorylation assay was carried out in a reaction mixture containing 50 mM sodium phosphate, pH 8, 5 mM MgCl₂, 0.2 mM ATP, 20 μM MA4561-StrepII full-length protein, and 0.16 nmol of [γ-³²P]ATP (0.0185 MBq) at room temperature. Assays were performed in rubber-sealed cuvettes. The reaction was terminated after different time points by the addition of an equal volume of methanol. The reaction products were purified by Bio-Gel P-60 column (200–400 mesh) eluted with water. Phosphorylation assays were displayed by 2-D gel electrophoresis as described in Fig. 1 (A).

**Redox Potential of Variant sGAF2-StrepII**—Electrochemical experiments were performed in an electrochemical cell with an optical path length of 0.125 mm. As a working electrode, a gold mesh (82 mesh/inch; wire diameter, 0.06 mm; 65% transmission; Advent Materials) was used. Glassy carbon and Ag/AgCl (1 M KCl) electrodes were used as auxiliary and reference electrodes, respectively. The potential was controlled by a potentiostat (EG&G 273 A). UV-visible spectral changes were followed with a diode array spectrophotometer (HP8451, Hewlett-Packard) with a 320-nm cutoff filter. The following redox mediators were used (values of midpoint potentials are given in parentheses): 1,2-naphthoquinone (+145 mV), 1,4-benzoquinone (+78 mV), phenazine ethosulfate (+55 mV), methylene blue (+13 mV), duroquinone (+5 mV), 5,5,7-indigo trisulfonate (−35 mV), phenazine (−125 mV), 2-hydroxy-1,4-naphthoquinone (−145 mV), and anthraquinone 1,5-disulfonate (−170 mV).

An sGAF2-StrepII solution at pH 7.0 containing the mediator dyes was injected into the electrochemical cell, and a potential of −465 mV was applied for 1 min to completely reduce residual oxygen in the cell. Then the potential was reset to +440 mV to produce the Fe(III) complex of MA4561. The potential was reduced by 10 mV steps, and the reaction was equilibrated for 15 min. After equilibration, a spectrum was recorded, and the potential was again reduced. The redox reaction of sGAF2-StrepII was followed by the absorbance change in the Soret band at 428 nm.

To measure the potentials accurately for the reduction and oxidation, well-designed mediator mixtures were added (concentration, 0.1 mM) to a 0.4 mM concentration of the protein. For the reduction, a mixture of 1,2-naphthoquinone, phenazine ethosulfate, 1,4-benzoquinone, duroquinone, and 2-hydroxy-1,4-naphthoquinone was used. The oxidation was measured starting from the reduced state (−400 mV) to +300 mV with the following redox mediators: 1,2-naphthoquinone, phenazine ethosulfate, 1,4-benzoquinone, duroquinone, methylene blue, 5,5,7-indigo trisulfonate, phenazine, and anthraquinone 1,5-disulfonate. The obtained data points were fitted to the Nernst equation.

**Knock-out and Complementation**—The gene encoding MA4561 was deleted in-frame from the M. acetivorans chromosome using pMP44del4561 and a markerless mutagenesis procedure (35). Plasmid pMP44del4561 was constructed by cloning (via Sacl and SpeI) of a 910-bp fragment (PCR-amplified using primers 19 and 20) into pMP44 (35), which resulted in pMP44del4561up. Inserting into this plasmid (via Sphi and SpeI) a 1003-bp fragment (PCR-amplified using primers 21 and 22) corresponding to the downstream region of ma4561 gave pMP44del4561. The genotype of M. acetivorans D4561 (deleted for ma4561) was verified by DNA hybridization (Southern) using an internal fragment of ma4561 (PCR-amplified using primers 23 and 24; supplemental Table S1) as probe. M. acetivorans D4561 was complemented using ptet4561.

**RESULTS**

The Putative Sensor Kinase MA4561—In silico analysis of MA4561 revealed the presence of two alternating N-terminal PAS and GAF domains fused to a C-terminal H_ATPase domain (Figs. 1 and 2A). A previous bioinformatics characterization of both GAF domains predicted the protein to be a phytochrome-like photoreceptor mainly because of the presence of a conserved cysteine residue in the first GAF domain (26). We overproduced and purified different variants of MA4561 (Fig. 1) to identify possible cofactors and characterized their biochemical properties.

**Heterologously Purified MA4561 Contains a Heme Cofactor in the Second GAF Domain**—Purified full-length MA4561–His₆ contained heme as indicated by a typical heme absorption spectrum with a Soret band at 415 nm and a visible band at 532 nm with a shoulder at about 565 nm (Fig. 2B). The protein was not fully saturated with heme as the Soret band was very low in comparison with the protein absorption at 280 nm. Addition of hemin to the clarified lysate as described under “Experimental Procedures” led to a significant increase in Soret band absorption (Fig. 2C). Purified variant GAF2-His₆ (aa 1–751) showed the same absorption properties as the full-length protein where the heme content also increased by addition of external hemin (data not shown). Variants PAS2–His₆ (aa 1–580), GAF1–His₆ (aa 1–457), and PAS1–His₆ (aa 1–290) were also purified but
did not show any heme absorption properties even when hemin was added to the clarified lysate (Fig. 2, B and C; only variant PAS2 is shown). Variant sGAF2-His6 (aa 580–804) was purified with very little heme bound but showed an intense Soret band at 414 nm and a visible band at 533 nm with a shoulder at about 564 nm when external hemin was added (Fig. 2, B and C).

The protein-heme complex of purified full-length MA4561-His6 could not be further oxidized by the addition of potassium ferricyanide, indicating the existence of an Fe(III) complex (data not shown). The spectrum of an Fe(II) complex was obtained by reduction with dithionite and had a Soret band at 429 nm and -bands at 562 and 528 nm, respectively.

Upon exposure of this Fe(II) complex to CO gas, Fe(II)-CO complex formation was observed with a Soret band at 424 nm and visible bands at 532 and 560 nm (Fig. 3A). The Fe(II) complex was easily oxidized by aeration with oxygen. As the autoxidation rate in the presence of dithionite is very high, the Fe(II) complex (with excess dithionite) was subjected to gel permeation chromatography on a NAP5 column (GE Healthcare) under aerobic conditions to remove free dithionite. Under these conditions, the Fe(II) complex either binds the oxygen from air to form an Fe(II)-O2 complex (hemoglobin served as a control; data not shown) or is oxidized to form an Fe(III) complex. No stable Fe(II)-O2 complex was detected under this condition. Furthermore, aeration of the Fe(II)-CO complex with O2 did not alter the spectrum (data not shown). The spectra of the Fe(III) and the Fe(II) complexes were stable over a pH range from 6.0 to 10.0 (data not shown). All obtained heme complexes (Fe(II), Fe(II)-CO, and Fe(III)) of the heme binding variants (i.e. full-length MA4561-His6, GAF2-His6, and sGAF2-His6) have optical absorption properties indicative of six-coordinate complexes with only slight differences (Fig. 3).

To test whether any other cofactors bind to other domains of MA4561, we used variant PAS2-His6, which only lacks the heme-binding second GAF domain and the H_ATPase domain, to perform binding assays with the putative phytochrome chromophores biliverdin IXα and phycocyanobilin as well as blue light receptor chromophores FAD and FMN but did not obtain any binding (data not shown). Thus, we ruled out the former prediction of MA4561 being a phytochrome-like protein.

Homologously Produced MA4561 Also Binds Heme—To exclude artifacts resulting from heterologous protein production in E. coli and to test whether heme is the native cofactor to MA4561, we produced full-length MA4561-His6 in M. acetivorans. The purification was identical to that of the protein derived from E. coli except that no external hemin was added to the lysate. The His6-tagged protein was detectable in Western blots (Fig. 4A) and showed the same absorption properties as that purified from E. coli (Fig. 4B).

Imidazole Influences the Coordination Structure of the Heme Cofactor—As mentioned above, all His6-tagged variants showed optical absorption properties indicative of six-coordinate complexes. In many heme proteins, a histidine residue serves at least as one amino acid to coordinate the heme central iron. Standard purification protocols for native His6-tagged proteins use Im to elute the tagged proteins from the metal affinity chromatography column. Because Im resembles the side chain of histidine residues and has the potential to be coor-
to form the Fe(II) complex, the Soret band shifted to 432 nm, and a peak occurred in the visible region with a maximum at 556 nm and shoulders on both sides at around 528 and 574 nm, respectively. Additionally, a small peak at 623 nm was obtained (Fig. 5A). Aeration with CO led to an Fe(II)-CO complex with the same absorption properties as for the Im-purified variant (compare Figs. 5A and 3B, dashed lines). Upon addition of Im to the Fe(III) complex as well as to the Fe(II) complex, optical absorption spectra changed drastically. The obtained complexes exactly matched the “Fe(III)” and “Fe(II)” complexes when purification was performed with Im (compare Figs. 5B and 3B), indicating formation of Fe(II)-Im and Fe(III)-Im complexes. Both complexes could be converted into each other by reducing the Fe(III)-Im complex with dithionite or oxidizing the Fe(II)-Im complex with potassium ferricyanide or O₂. Addition of CO to the Fe(II)-Im complex produced the above described Fe(II)-CO complex (e.g. Figs. 3B and 5A). To completely rule out effects of the tag and purification system used, we constructed, produced, and purified variants MA4561-StrepII (full-length) and sGAF2-StrepII (aa 580–804). All optical absorption properties for full-length MA4561-StrepII (data not shown) and sGAF2-StrepII (Fig. 5, C and D) were identical to those of the His₆-tagged variants when purified without Im.

Dimethyl Sulfide Binds to the Heme Cofactor—Because MA4561 is encoded on the chromosome in the vicinity of a system involved in metabolism of methyl sulfides (as discussed later), we tested whether DMS can bind to the heme cofactor of MA4561. DMS is a typical thioether heme ligand resembling the side chain of methionine residues. Therefore, a coordination by the heme iron is feasible. Addition of DMS led to either Fe(III)-DMS or Fe(II)-DMS complexes, which could be converted into each other by reduction and oxidation as already observed for the Im complexes (Fig. 5G). The Fe(III)-DMS and Fe(II)-DMS complexes were nearly indistinguishable from the Fe(III)-Im and Fe(II)-Im complexes with only slightly shifted Soret bands. The Fe(III)-DMS Soret maximum was shifted to a longer wavelength by about 2–3 nm in comparison with the

FIGURE 3. Absorption spectra of imidazole-purified His₆-tagged MA4561 variants. UV-visible spectra of full-length MA4561-His₆ (A) and variant sGAF2-His₆ (B) purified with imidazole as “Fe(III)” (solid line), “Fe(II)” (dotted line), and Fe(II)-CO (dashed line) complexes are shown. Maxima of the Soret bands and of the ω- and β-bands of the “Fe(II)” complex are indicated in the spectrum. The visible region in each panel is enlarged by the factor indicated in the spectrum. For explanation of the quotation marks, see “Results.” AU, absorbance units.

FIGURE 4. Western blot analysis and UV-visible spectra of homologously purified MA4561. A, full-length MA4561-His₆ purified from E. coli (lane 1) and from M. acetivorans (lane 2) was immunologically detected by the C-terminal His₆ tag. Migration positions of standard proteins with known relative molecular weights (M, × 1000) are given on the left. B, UV-visible spectra of full-length MA4561-His₆ purified from M. acetivorans. The absorption properties are the same as for the imidazole-purified protein from E. coli. Fe(III)-Im (solid line), Fe(II)-Im (dashed line), and Fe(II)-CO (dotted line) complexes are shown. The visible region is enlarged by the factor indicated in the spectrum. AU, absorbance units.
Fe(III)-Im complex, whereas the Fe(II)-DMS Soret maximum was shifted to a shorter wavelength by about 2–3 nm compared with the Fe(II)-Im complex. Furthermore, the Fe(II)-CO complex was converted to an Fe(II)-DMS complex upon addition of DMS and vice versa. Although imidazole was easily displaced by CO and DMS, the opposite ligand exchanges were not possible (indicative of significantly higher binding affinities to the heme for DMS and CO).

FIGURE 5. Absorption spectra of sGAF2 variants. UV-visible spectra of EDTA-purified variant sGAF2-His6 (A), variant sGAF2-StrepII (C), and EDTA-purified variant sGAF2C656A-His6 (E) as Fe(III) (solid line), Fe(II) (dotted line), and Fe(II)-CO (dashed line) complexes as well as EDTA-purified variant sGAF2-His6 (B) and variant sGAF2-StrepII (D) supplemented with imidazole as Fe(III)-Im (solid line) and Fe(II)-Im (dashed line) complexes are shown. Pyridine hemochrome spectra (F) of variant sGAF2-StrepII (solid line) and EDTA-purified variant sGAF2C656A-His6, (dashed line) are shown. Variant sGAF2-StrepII supplemented with DMS (G) as Fe(III)-DMS (solid line) and Fe(II)-DMS (dashed line) complexes is shown. Effects of different pH values on absorption properties of variant sGAF2-StrepII (H) as Fe(III) (solid line) and Fe(II) (dotted line) at pH 7.0 and Fe(III) (long dashed line) and Fe(II) (short dashed line) at pH 10.0 are shown. Maxima of the Soret bands and the visible region in the reduced protein (i.e. Fe(II) (A, C, E, and H), Fe(II)-Im (B and D), and Fe(II)-DMS (G)) as well as \( \alpha \)-band maxima of the pyridine hemochromes (F) are given in the spectrum. The visible region in each panel is enlarged by the factor indicated in the spectrum. AU, absorbance units.
**Different pH Values Influence the Coordination Structure of the Heme Cofactor**—Variant sGAF2-StrepII was used to test the pH stability of the optical absorption spectra. The Fe(III) complex showed no significant changes in the spectrum in buffers with pH 5.4, 7.0, and 10.0. The very broad Soret peak at 429 nm and the weakly resolved visible region with a maximum at 557 nm with shoulders on each side was detectable at pH 7.0 and 5.4. In contrast, the Soret peak at pH 10.0 was sharper and shifted to 425 nm, and in the visible region, two clearly resolved peaks were detectable with the β-band at 527 nm and the α-band at 557 nm. In all cases, an additional peak at around 625 nm was observed (Fig. 5H; only pH 7.0 and 10.0 are shown).

**Heme Is Covalently Bound to MA4561 at a 1:1 Stoichiometry**—Pyridine hemochromogen assays were initially performed to determine the protein to heme ratio. Interestingly, the α-band of full-length MA4561 (His₆- and StrepII-tagged variants) as well as variant GAF2-His₆ and variant sGAF2 (His₆- and StrepII-tagged variants) had a maximum at 553 nm (Fig. 5F; only variant sGAF2-StrepII is shown). Typically, the pyridine hemochromogen of heme b shows an α-band maximum at 556 nm, whereas the α-band maximum of heme c pyridine hemochromogen is 550 nm (36–38). The shift from 556 nm in heme b to 550 nm in heme c is due to saturation of both heme vinyl groups when covalently attached to two cysteine residues in a CXXCH motif of the protein. It was reported that in Cytochrome c and AXCH variants of a c-type cytochrome the α-band maximum is 553 nm due to the saturation of only one heme vinyl group, whereas the other stays unsaturated (38–40). Because the pyridine hemochromogen of MA4561 displays exactly this absorbance, we assume that the heme group in MA4561 is also bound covalently to the protein via one thioether linkage to a cysteine residue.

To further strengthen the assumption that heme is covalently bound to MA4561, the protein was subjected to a specific heme staining procedure. Variant sGAF2-His₆ (with Im) purified with heme in the clarified lysate contains covalently bound heme (Fig. 6A, lane 1). In contrast, this variant purified without hemin contains only a little heme (Fig. 6A, lane 2). The covalent attachment of the heme to the protein was additionally verified with other methods, such as MALDI-TOF-MS and denaturing HPLC (data not shown), as well as acidified butanone extraction (Fig. 6B). In addition, the covalently bound heme was verified for the full-length MA4561-StrepII protein (Fig. 6C, lane 3).

As variant sGAF2-His₆ contained very little bound heme when directly purified from *E. coli*, it was used in titration experiments with hemin. 10 μM protein was titrated with increasing amounts of hemin and stained for protein as well as for covalent heme (Fig. 6A). The heme signal increased with increasing amounts of heme up to a hemin concentration of 5 μM (Fig. 6A, lane 5). Upon the addition of 7.5 μM hemin, an additional signal in the heme stain became visible that is due to saturation of both heme vinyl groups when covalently bound to two cysteine residues in a CXXCH motif of the protein.

**Heme Is Covalently Bound to Cysteine 656**—The pyridine hemochrome spectra shown in Fig. 5F pointed toward a cys-
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FIGURE 7. Resonance Raman analyses of sGAF2 variants. The RR spectra of wild type (a) and C656A mutant (b) of the oxidized (A) and reduced (B) forms in the high wave number region are shown. The excitation wavelength was 532 nm. The sample concentration was 500 μM in 50 mM sodium phosphate, pH 7.0, 100 mM NaCl. a.u., absorbance units.

The Raman bands of the ferric form of wild type sGAF2–His₆ at 1377, 1506, 1573, and 1645 cm⁻¹ (Fig. 7A, a) are assigned to ν₄, ν₃, ν₂, and ν₅ respectively, and their wave numbers indicate that the heme adopts a six-coordinate low spin (6c-LS) state. Similar results were observed for the sGAF2C656A–His₆ variant (Fig. 7A, b), implying that the mutation of Cys656 to alanine has no significant effect on the heme coordination structure and the spin state. The ν₄, ν₃, ν₂, and ν₅ bands of the Fe(II) complex of wild type sGAF2–His₆ (Fig. 7B, a) are observed at 1360/1369, 1498, 1592, and 1631 cm⁻¹, respectively, suggesting a 6c-LS type of heme. Besides, ν₁ is located at 1360 and 1369 cm⁻¹, suggesting the presence of two conformations in which the heme adopts a 6c-LS state. The mutation of Cys656 perturbs the populations of the 1360 and 1369 cm⁻¹ fractions. The population of the 1360 cm⁻¹ species is much higher than that of 1369 cm⁻¹ (Fig. 7B, b). Also, the intensity of the Raman bands at 1555 and 1607 cm⁻¹ is significantly enhanced than that in the wild type protein, indicating that the interactions of the heme vinyl groups with the surroundings are altered upon mutation of cysteine residue 656.

Axial ligation of the heme cofactor (MCD)—MCD spectra of ferric and ferrous states of sGAF2–His₆ (without Im) are shown in Fig. 8 where analogous states of horse heart myoglobin (Mb), HRP, and cytochrome b₅ (cyt b₅) are overlaid for comparison. At pH 7.0, ferric sGAF2 exhibits an MCD spectrum (Fig. 8A, solid line) that is similar to that of ferric HRP (dashed line) and ferric Mb (dotted line), five-coordinate and water-ligated six-coordinate heme proteins, respectively, in the visible region (450–700 nm). However, a closer comparison of the trough intensities at ~640 nm indicates that the spectrum of ferric sGAF2 is more similar to that of HRP than Mb. In the Soret region (350–450 nm), an asymmetric MCD spectrum having a prominent peak at ~404 nm (intensity, ~7.5 M⁻¹ cm⁻¹ T⁻¹)
and two troughs at \( \sim 370 \) and \( \sim 430 \) nm (\( < 5 \text{ M}^{-1} \text{ cm}^{-1} \text{ T}^{-1} \)) does not resemble either a weak negative feature (a trough at \( \sim 420 \) nm; \( \sim 4 \text{ M}^{-1} \text{ cm}^{-1} \text{ T}^{-1} \)) of HRP or a broadly symmetric derivative-shaped spectral feature (\( \sim 15 \text{ M}^{-1} \text{ cm}^{-1} \text{ T}^{-1} \)) of Mb. The MCD spectrum of ferric sGAF2 at pH 10.0 (gray line) is clearly different from that at pH 7.0 in that one of the visible region troughs (\( \sim 550 \) nm) is shifted to \( \sim 575 \) nm, and a Soret region feature becomes derivative-shaped with considerably enhanced intensity (\( +34/-40 \text{ M}^{-1} \text{ cm}^{-1} \text{ T}^{-1} \)). The resultant spectral features are indicative of an increased 6c-LS species.

In the ferrous state, sGAF2 (Fig. 8B, solid line) has MCD spectral features that are broadly similar to those of ferrous Mb (which contains a five-coordinate heme; Fig. 8B, dotted line) including quite intense Soret peaks (\( \sim 125 \text{ M}^{-1} \text{ cm}^{-1} \text{ T}^{-1} \)) except for a more enhanced and derivative-like shape in the visible region around 555 nm for sGAF2 than for Mb. At pH 10.0, this derivative-shaped spectral line becomes much more intense. Concomitantly, the Soret peak intensity diminishes by \( \sim 50\% \) (Fig. 8B, gray line). These MCD spectral changes are indicative of an increase in the fraction of a ferrous 6c-LS heme such as bisamine (or bis-His)-ligated ferrous heme (see below).

Fig. 8C compares ferric Im (solid line) and ferric THT (a cyclic thioether) adducts (dashed line) of sGAF2 with ferric cyt \( b_5 \) (dotted line). These three heme adducts display similar MCD spectral patterns, namely relatively intense derivative-shaped Soret spectra and mainly positive (450–560 nm) and negative (560–700 nm) features with a prominent trough at 570–580 nm. The MCD spectral similarity for the ferric Im complex of sGAF2 and ferric cyt \( b_5 \) (which contains a bis-His-ligated heme) suggests identical ligation modes for the two heme proteins. Because bisamine (or bis-His)-ligated and bisthioether-ligated ferric hemes have been shown to have similar MCD spectra (46), mono-Im (or His)/monothioether-bound ferric heme also is expected to share this similarity. In Fig. 8D, MCD spectra of ferrous Im and ferrous THT adducts are compared with the spectrum of ferrous cyt \( b_5 \). As seen in their ferric state (Fig. 8C), the MCD spectral patterns of these three ferrous complexes show common patterns. In the visible region, extremely intense derivative-shaped features around \( \sim 555 \) nm are notable. In the Soret region, the MCD spectra of the THT-bound sGAF2 and cyt \( b_5 \) are similar to each other in pattern (except for some intensity difference), whereas the Im complex of sGAF2 shows a somewhat different pattern with a noticeably intense trough at \( \sim 425 \) nm. A possible cause of this difference could be incomplete formation of the Im complex; however, an increase of Im concentration (2–50 \text{ mM}) does not change the outcome.

Model Structure of sGAF2 Variant—To structurally support the spectroscopic data, the sGAF2 domain (aa 608–768) was modeled using the PHYRE2 server (47). Based on six template sequences, which were all GAF domains or GAF domain-like structures, a model of the second GAF domain of MA4561 was generated (Fig. 9). The modeled structure was also used to predict putative ligand-binding sites using 3DLigandSite (48). Best hits were obtained for heme molecules. One of the heme vinyl

![Figure 8. MCD analyses of variant sGAF2-His6.](image-url)
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FIGURE 9. Structural model of the heme-binding pocket of MA4561. The second GAF domain (aa 608–768) of MA4561 was modeled using the PHYRE2 server (47), and the modeled structure was used to predict putative ligands binding to this structure using 3DLigandSite (48). The heme cofactor with highest priority is shown in the middle as sticks with the central iron atom as a sphere, and the protein structure is shown as a ribbon (interesting aa are shown as sticks). The cysteine residue (Cys656) that is the covalent heme-binding site is located close to one of the two heme vinyl groups. Two histidine residues (His702 and His704) and a methionine residue (Met653) are located at the proximal heme site. Two tyrosine residues (Tyr632 and Tyr665), a methionine residue (Met648), and a histidine residue (His646) are located at the distal heme site.

FIGURE 10. Autophosphorylation activities of full-length MA4561-StrepII. The Fe(III), Fe(II), Fe(II)-CO, Fe(III)-DMS, and Fe(II)-DMS complexes were incubated with radioactively labeled ATP, and the reaction was stopped after 1-, 5-, 15-, and 30-min incubation time, subjected to SDS-PAGE, transferred to a PVDF membrane, and detected by autoradiography (a). The same membrane was stained with Ponceau S for proteins (b). Only the Fe(III) and the Fe(III)-DMS complexes showed autophosphorylation activity increasing over time.

Groups is in direct vicinity to the cysteine 656 residue, which is the covalent attachment site for the heme. Putative axial ligands like histidine residues or methionine residues are located in the vicinity of the heme iron and strengthen the conclusion of the MCD data.

Autophosphorylation of MA4561 Is Dependent on the Heme Redox State but Not on External Ligands—As MA4561 contains an H_\text{ATPase} domain at its C terminus, we tested whether the protein is autophosphorylated using radioactively labeled ATP. Kinase assays of all heme complexes of full-length MA4561-StrepII were performed (Fig. 10). Although the Fe(II) complex showed increasing autophosphorylation over time, the Fe(II) and Fe(II)-CO complexes were not autophosphorylated (Fig. 10). Binding of DMS had no influence on the kinase activity with the Fe(II)-DMS complex showing autophosphorylation activity and the Fe(II)-DMS complex not showing autophosphorylation activity (Fig. 10). The same was true for 1m complexes (data not shown).

Redox Potential of MA4561—To gain insight into the oxidation state of MA4561 within \textit{M. acetivorans}, the redox potential of recombinant variant sGAF2-StrepII was determined using redox titration experiments. These measurements obtained midpoint potentials of −95 ± 10 and −75 ± 10 mV for the oxidation and reduction reactions, respectively (Fig. 11). Full-length MA4561-His\textsubscript{6} (with Im) showed comparable midpoint potentials of −91 ± 10 and −85 ± 10 mV for the oxidation and reduction reactions, respectively (data not shown).

MA4561 Regulates the Synthesis of MtsF, a Corrinoid/Methyltransferase Fusion Protein—Because we suspected MA4561 to play a role in signal transduction for regulating the vicinally encoded MtsH and potentially MtsD and MtsF, three highly similar corrinoid/methyltransferase fusion proteins involved in DMS metabolism (62), a markerless in-frame deletion of the \textit{ma4561} coding region was constructed. The strain (D4561) grew indistinguishably from the wild type under all conditions tested (growth on methanol, trimethylamine, and acetate; data not shown). To test the influence of MA4561 on the synthesis of the corrinoid/methyltransferase fusion proteins, Western blot analyses were performed with the only available antiserum specific for the MtsF protein. MtsF was not detectable in Western blots when wild type cells were grown on methanol (Fig. 12, lane 1) (62). Addition of 5 mM DMS to the growth medium resulted in a strong MtsF signal (Fig. 12, lane 2). Interestingly, in strain D4561 carrying the “empty” complementation vector pMR08, this DMS-dependent regulation of MtsF was abol-
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The axial coordination of the heme iron in the protein is not significantly affected by a mutation of the cysteine residue to an alanine residue as shown by UV-visible (Fig. 5E) and RR spectra (Fig. 7). Although the heme in the Fe(II) complex seems to be found in a mixture of two slightly different six-coordinate states (see below), one of those is more dominant in the cysteine to alanine mutation. The in vivo relevance of this covalent attachment is thus unclear and requires further characterization. Differences in biophysical properties other than spectral properties might be influenced by the covalent binding.

MA4561 Contains Heme in a Large, Accessible Binding Pocket—Extensive UV-visible spectral analyses of differentially tagged heme binding variants of MA4561 unraveled an influence of the purification system used on the heme cofactor. When Im was used for the purification of the His$_{6c}$-tagged variants, the UV-visible spectral properties differed from those obtained with StrepII-tagged variants and Im-free His$_{6c}$-tagged variants. The tag itself had no influence on the optical absorption properties as the spectra of the StrepII-tagged variants were identical to those of Im-free His$_{6c}$-tagged variants. With addition of Im to the Im-free variants (independent from the tag used), the spectra obtained perfectly matched the spectra of the Im-purified protein. As a consequence, we suggest a heme pocket architecture that is accessible even to larger molecules like Im. This assumption is strengthened by the fact that also DMS (or alternatively the even bulkier THT) can coordinate the heme.

Coordination Structure of the Heme Cofactor Is Heterogeneous—For more insight into the coordination structure of the heme, we performed extensive UV-visible spectral analyses combined with RR and MCD measurements. The UV-visible spectra of the Fe(III) complex as purified from E. coli without Im with a His$_{6c}$ tag as well as with a StrepII tag showed a Soret band at about 407 nm and weak bands in the visible region of about 505 and 630 nm at pH 7.0. These absorption properties are indicative of a six-coordinate high spin heme as found in HemAT (52). The absorption properties clearly differ from the 6c-LS heme found in the direct oxygen sensor EcDos from E. coli (53, 54), recombinant barley hemoglobin (55), CO sensor CooA from R. rubrum (6, 56), and globin-coupled histidine kinase A/GcHK from Anaeromyxobacter sp. Fw109-5 (20) and from the five-coordinate high spin heme found in YddV (57) and BfFixL from Bradyrhizobium japonicum (58).

In the RR measurements, a 6c-LS heme was observed, whereas MCD data suggest a high spin state and a five-coordinate structure rather than a six-coordinate structure. Resonance conditions used in the RR experiments most likely would not allow detection of a five-coordinate high spin heme species. Therefore, a clear assignment of the coordination structure cannot be made, suggesting a mixture of different coordination states.

When the pH of the buffer was changed to pH 10.0, a mixture of a high spin and a low spin component was also detectable in MCD spectra, whereas the differences in UV-visible spectra were minor. This low spin component appears to be arising from a bis-His-type coordination structure as judged from the similarity to the Fe(III)-Im complex (as discussed below).
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Upon reduction of MA4561 with dithionite, an Fe(II) complex with a broad Soret band at about 432 nm and a visible band at about 557 nm with shoulders at about 528 and 574 nm is formed. An additional peak was observed at about 625 nm for the sGAF2 variants. These optical absorption properties cannot be clearly assigned to one coordination and spin state but rather look like a mixture of different states. With RR data, a mixture of two different 6c-LS states was observed, and the existence of two species was confirmed in the MCD measurements. At pH 10.0, one of the two states becomes predominant as judged from the MCD data and is similar to the bis-His-ligated Fe(II) form of cyt $b_5$.

The addition of Im or DMS (or rather THT for the MCD measurements) led to typical 6c-LS hemes for the Fe(III)-(Im/DMS/THT) as well as the Fe(II)-(Im/DMS/THT) (53, 54). The Im complexes were hardly distinguishable from the DMS/THT) complexes with only minor differences in UV-visible as well as MCD spectra. Similarities of the Im complexes to cyt $b_5$ led to the conclusion that a His/Im or in the case of DMS (THT) a His/DMS (THT) complex is formed that would resemble a bis-His (cyt $b_5$) or hardly distinguishable His/Met (many c-type cytochromes) ligation structure.

The structural model of sGAF2 generated supports the spectral data with putative axial ligands in proximity of the heme cofactor. Specifically, two histidine residues (His702 and His704) are located at the proximal side of the heme pocket that might be involved in coordinating the heme iron (Fig. 9). Initial mutational analyses of these residues suggest His702 as the proximal ligand. The distal side is occupied by two tyrosine residues (Tyr632 and Tyr665), one methionine residue (Met645), and one histidine residue (His646) that might be directly or indirectly involved in coordinating the heme iron depending on the redox state. Because ligands like CO, DMS, and Im are able to bind to the heme in MA4561, we envision the distal side of the heme pocket large enough to allow these molecules to enter and coordinate the heme iron. The above mentioned amino acids might be involved in stabilizing different ligation states of the heme without being a direct ligand to the heme iron. Alternatively, they might be exchanged as a direct ligand upon binding of external ligands as most data suggest six-coordinate states also in the absence of external ligands. Interestingly, the cysteine residue that binds the heme covalently is located near one of the heme vinyl groups, giving the modeled structure more confidence.

MA4561 Is a Redox Sensor—The heme in MA4561 is easily oxidized and reduced by different oxidizing and reducing agents independently of the coordination of the external ligands Im and DMS. Autophosphorylation is strongly dependent on the heme redox state, and activity was observed for the Fe(III), the Fe(III)-DMS, and the Fe(III)-Im complexes. In contrast, all reduced complexes had no autophosphorylation activity (i.e. Fe(II), Fe(II)-DMS, Fe(II)-Im, and Fe(II)-CO). As M. acetivorans is an obligate anaerobe (59), it is very unlikely that oxygen is the physiological signal leading to the oxidation of the heme iron and subsequently to an activation of the kinase.

Although it was shown that the organism can cope with microaerophilic conditions (60), we consider a change in redox state of the cell as a more realistic signal (see below).

The positive and negative ends of the redox potentials to which M. acetivorans is exposed may be defined by hydrogen at the negative end (with a redox potential of about −420 mV) and by the terminal electron acceptor of the methanogenic pathway, the heterodisulfide of coenzyme M and coenzyme B at the positive end (with a redox potential of about −140 mV) (61). The redox potential of the heme in MA4561 was determined to be about −80 to −90 mV. Under physiological conditions, the heme in MA4561 is therefore likely in the Fe(II) state, and consequently, the kinase activity is switched off. We envisage that MA4561 might be involved in redox sensing. Local accumulation of oxidized cofactors could lead to oxidation of the heme iron in MA4561 that in turn would switch on kinase activity. Alternatively, sulfur-containing metabolites like methyl sulfides could also be mediators for redox sensing either directly or indirectly (see below). Further analyses are warranted to substantiate this idea.

MA4561 Is Part of a System Regulating Methyl Sulfide Metabolism—MA4561 is encoded directly upstream of the transcriptional regulator MsrG (MA4560) (62). This regulator is the direct transcriptional activator of the protein MtsH (MA4558), which is one of three highly similar corrinoid/methyltransferase fusion protein isoforms required for methyl sulfide metabolism (62, 63). These enzymes are involved in DMS formation when grown with CO and are essential for growth with DMS as the sole energy source (64). There are two other Mts isoforms present in M. acetivorans. MtsD (MA0859) and MtsF (MA4384), which are also regulated individually by vically encoded transcriptional regulators. MtsD transcription is activated by MsrF (MA0862), and MtsF transcription is activated by MsrC (MA4383). MsrF is encoded directly downstream of the putative sensor kinase MA0863, which shares 68% identity and 84% similarity with MA4561. The regulator proteins all belong to the Msr family of regulators specific to Archaea (65). It can therefore be speculated that MsrF and MsrG, both encoded in putative transcriptional units with the respective kinases MA0863 and MA4561, might form two-component systems with these sensor kinases.

Interestingly, regulatory cross-talk among MtsD, MtsF, and MtsH has been demonstrated previously (63). Abolishing DMS-dependent regulation of synthesis of MtsF by removing MA4561 and restoring this regulation by complementation with its His$_6$-tagged variant demonstrates that (i) the tagged variant of the protein used for our in vitro studies is functional in vivo, (ii) MA4561 is directly or indirectly involved in regulation of MtsF (and presumably of the other Mts proteins as well), and (iii) MA4561 is a negative effector of MtsF synthesis. Based on these findings, we propose to name the protein MsmsS for methyl sulfide methyltransferase-associated sensor. Removing MsmsS leads to constitutive MtsF synthesis, which indicates constant MsrG (or MsrC)-dependent activation of mtsF expression.

Because autophosphorylation of MsmsS is dependent on the heme redox state rather than on ligand binding, the mechanism underlying this deregulation cannot easily be explained by CO-
or DMS-dependent phosphoryl transfer from the sensor kinase to the respective response regulator to activate it. Instead, binding of CO or DMS might keep MsmS in an unphosphorylated state as both gases are weak reductants. Thus, the presumed phosphoryl transfer could effect the inactivation of the response regulator. Although such a scenario is consistent with our observations, much of it is speculative. It is unknown whether the phenotype seen in D4561 is due to a direct interaction of DMS with MsmS, leading to a signal output other than kinase activity, or an indirect effect (e.g. accumulation of oxidized cofactors during growth on DMS). These questions will be addressed in the future.

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