Redox-dependent Ligand Switching in a Sensory Heme-binding GAF Domain of the Cyanobacterium Nostoc sp. PCC7120*

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The genome of the cyanobacterium Nostoc sp. PCC7120 carries three genes (all4978, all7016, and alr7522) encoding putative heme-binding GAF (cGMP-specific phosphodiesterases, adenylyl cyclases, and EhIA) proteins that were annotated as transcriptional regulators. They are composed of an N-terminal cofactor domain and a C-terminal helix-turn-helix motif. All4978 showed the highest affinity for protoheme binding. The heme binding capability of All7016 was moderate, and Alr7522 did not bind heme at all. The “as isolated” form of All4978, identified by Soret band (\(\lambda_{\text{max}} = 427\) nm), was assigned by electronic absorption, EPR, and resonance Raman spectroscopy as a hexacoordinated low spin FeIII heme with a distal cysteine ligand (absorption of \(\delta\)-band around 360 nm). The protoheme cofactor is noncovalently incorporated. Reduction of the heme could be accomplished by chemically using sodium dithionite and electro spectrochemically; this latter method yielded remarkably low midpoint potentials of about -445 and -453 mV (following Soret and \(\alpha\)-band absorption changes, respectively). The reduced form of the heme (FeIII state) binds both NO and CO. Cysteine coordination of the as isolated FeIII protein is unambiguous, but interestingly, the reduced heme instead displays spectral features indicative of histidine coordination. Cys-His ligand switches have been reported as putative signaling mechanisms in other heme-binding proteins; however, these novel cyanobacterial proteins are the first where such a ligand-switch mechanism has been observed in a GAF domain. DNA binding of the helix-turn-helix domain was investigated using a DNA sequence motif from its own promoter region. Formation of a protein-DNA complex preferentially formed in ferric state of the protein.

Hemes, in particular protoheme, are ubiquitous and essential protein cofactors for many biological reaction pathways (1–4). Nature has evolved quite variable protein folds competent for heme incorporation, in a covalent or noncovalent manner. The most apparent one from mankind’s point of view is the globin fold. However, many other protein domains bind hemes, modifying the chemical activity of the heme cofactor by the surrounding protein, thereby rendering it a redox-active component (5) or a gas sensor (6), for example. For these two functionalities, the heme-binding proteins (or protein domains) act as regulatory or signaling systems, allowing the organism to accommodate to changing environmental conditions.

A relatively large group of heme binding-proteins have a PAS (Per, period circadian protein, Arnt, aryl hydrocarbon receptor nuclear translocator protein, Sim, single-minded protein) domain. Others, such as nitrophorins (7), adopt the lipocalin structure. Only a few proteins have been described as binding...
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the heme cofactor in a GAF (cGMP-specific phosphodiesterases, adenylyl cyclase, and EIIA) domain (8, 9). The lipocalin motif is noticeable for its eight-stranded β-barrel structure, whereas PAS and GAF domains, despite a low sequence homology, share a similar structural topology of a five-stranded antiparallel β-sheet arrangement connected by surrounding α-helices, forming the ligand binding pocket (8, 10).

There are relatively few examples of proteins that contain a heme-binding GAF domain as follows: MA4561 from Methanosarcina acetivorans (11) and DosS (sometimes designated DevS) and DosT from Mycobacterium tuberculosis, which have been proposed as redox and gas sensors (5, 9). DosS and DosT are both tandem GAF domain proteins, where in both proteins, the N-terminal GAF domain, termed GAF DosS and GAF DosT, respectively, bind a heme (5, 9, 12, 13). Signaling in these proteins is accomplished through a histidine kinase, located down-stream of the second GAF domain. Both proteins coordinate the heme iron via a histidine residue (9, 10). However, although GAF DosS and GAF DosT share 75% amino acid sequence identity (14), each was assigned a different sensory function; both proteins are capable of binding gas molecules such as CO and NO in the ferrous oxidation state; however, only GAF DosT forms a stable complex with O2, whereas GAF DosS instead is oxidized to form the ferric complex (5). The binding of CO and NO to GAF DosS has little effect on the histidine kinase activity, but the change of the iron oxidation state does affect its activity remarkably, suggesting GAF DosS represents a redox sensory domain (15). In contrast, the ferrous oxidation state of GAF DosT is very stable, with the histidine kinase activity instead modulated by the concentration of the coordinating gas molecule, indicating a gas sensory function of GAF DosT (5). A similar GAF domain has been recently described for the histidine kinase MA4561 from M. acetivorans, termed GAF heme. Its heme iron is also likely coordinated by a histidine residue (11). However, in contrast to the β-type heme of GAF DosS and GAF DosT, the heme in MA4561 is covalently attached via a Cys side chain. The function of this domain is still under investigation; however, there is evidence that the GAF heme from M. acetivorans might be involved in (di)methyl sulfide metabolism.

A recent survey of cyanobacterial genomes (16) identified PAS domains assigned as potential heme-binding domains. GAF domains with a corresponding heme-binding function have so far not been reported in cyanobacteria. GAF domains, with tetrapyrrrole ligand-binding capacity, are most prominent in canonical photochromes, red-/far red-sensing photoreceptors present in both plants and bacteria (17), and in the related cyanobacteriochromes (16). In these chromoproteins, a cysteine residue in the GAF domain facilitates covalent binding of the chromophore, an open-chain tetrapyrrrole (bilin) derivative, to the protein scaffold, which undergoes light-driven photonicomer conversion between a resting and a signaling state.

Here, we report three genes (all4978, all7016 and alr7522) from Nostoc sp. PCC7120 all of which encode GAF domain proteins that show signatures for heme-binding sites. Annotation of the genome identifies them as transcriptional regulators based on a C-terminally located helix-turn-helix (HTH) domain of the LuxR type (Fig. 1) (18). The gene products were heterologously expressed, purified to homogeneity, and spectrally characterized. The highest loading of the heme was found for All4978. Moderate loading was found for All7016, and Alr7522 did not bind heme at all. In contrast to the heme-binding GAF domains from M. acetivorans and M. tuberculosis described above, which carry a histidine kinase as a signaling domain, the three GAF domain proteins from Nostoc carry an HTH motif at their C-terminal end. These proteins from Nostoc represent the first examples where a heme-binding GAF domain is combined, putatively in a regulatory fashion, with an HTH structural motif.

Experimental Procedures

Cloning and Protein Preparation—DNA encoding full-length proteins and the GAF domains of All4978, All7016, and Alr7522 were PCR-amplified from genomic DNA of Nostoc sp. PCC7120 and cloned into PET vectors, thereby furnishing the recombinant proteins with His tags allowing for facile affinity purification. Details can be found in supplemental Table S1. Besides the wild-type proteins, for GAF All4978 the following site-directed mutations were generated: Y41F/Y41G, C92S/C92G, H95A/H95G, H97A/H97G, H99A, and C138S (Table 1 and supplemental Table S1). For the expression of all proteins, transformed Escherichia coli BL21 cells (DE3) were grown in LB medium at 37 °C and 200 rpm to OD600 nm = 0.8, at which time the cells were induced by addition of 1 mM isopropyl β-D-thiogalactopyranoside. Growth was continued for 14 h at 18 °C. For all further experiments, wild-type and mutated GAF All4978 showing the highest loading with heme were employed.

To improve the heme/protein ratio, hemin (Sigma) was added in some experiments before induction (10 mg/ml in 0.1 M NaOH per 1 liter of culture) (12). Wild-type and mutated proteins were purified as described (19). Harvested cells were lysed, and the supernatant after centrifugation was loaded onto an IMAC column. After elution from the affinity column, protein solutions were concentrated for further studies. The purity of the proteins was confirmed by SDS-PAGE (4–12% BisTris Gel, Novex).

Electronic Absorption Spectroscopy—Absorption spectra of protein solutions (~5 μM in phosphate buffer, see above) were recorded at room temperature in 1-cm quartz cuvettes (UV-2401 spectrophotometer, Shimadzu). For ferrous compounds, a gas-tight cuvette was used that was purged thoroughly with N2 or CO prior to the addition of reductants.

Butanone Extraction (20)—800 μl of 2-butane and 200 μl of 1 M HCl were added to a sample of 2 ml of GAF All4978. The mixture was gently vortexed and kept for several minutes. Non-covalently bound heme was found in the upper organic phase, whereas covalently bound heme remains in the lower aqueous phase.

Pyridine Hemochrome Assay—The pyridine hemochrome assay was carried out as described (21). In detail, 0.5 ml of protein solution was mixed with 0.5 ml of 200 mM NaOH, 40% (v/v)
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pyridine, and 3 μl of 0.1 m of K3Fe(CN)6. Upon measurement of its UV-visible spectrum (600–400 nm), several crystals of Na2S2O4 (2–5 mg) were added, and the UV-visible spectrum was recorded again.

Spectroelectrochemical titrations were carried out using an SEC-C spectroelectrochemical cell (1-mm light path) equipped with a platinum gauze working electrode and a platinum counter-electrode (ALS Co., Ltd., Japan) at room temperature. A silver/AgCl reference electrode (E = −205 mV versus SHE) was attached (BASi, Inc.). Protein samples were rendered essentially O2-free through dialysis (nominal molecular mass was attached (BASi, Inc.). Protein samples were rendered

with palladium catalysts. Reduction was performed by the addition of 5 mM ascorbic acid, 5 mM glutathione (GSH), 2 mM flavine dinucleotide (FAD), or 100 μM nicotinamide adenine dinucleotide (NADH) (5).

Magnetic Circular Dichroism Spectroscopy (MCD)—MCD measurements were performed at room temperature using a JASCO (Model J-715) spectropolarimeter equipped with a 1.4 tesla permanent magnet (Olis) in quartz cuvettes with 1 cm path length. Four spectra were accumulated between 300 and 700 nm for each sample with the longitudinal magnetic field in direction of, or opposite to the light beam (±B). The respective baseline spectra were subtracted before further processing. Because the observed spectra are a combination of the CD and the MCD signals (∆Aobs (±B) = ∆Acd ± ∆A MCD), pure MCD spectra were calculated as shown in Equation 1,

\[ \Delta A_{MCD} = \frac{\Delta A_{obs}(+B) - \Delta A_{obs}(-B)}{2} \]  

Resonance Raman Spectroscopy—Samples of ~50 μM protein were generally used. Anaerobic samples were prepared in an anaerobic chamber (N2/H2 (98:2) atmosphere) furnished with palladium catalysts. Reduction was performed by the addition of Na2S2O4 and was followed spectrophotometrically. Samples were transferred to quartz tubes (3.8 mm diameter) connected to a valve. CO was added by flushing of the septum-closed sample with CO gas. NO was added by the addition of diethylammonium 2-(N,N-diethylamino)-diazenolate-2-oxide (Enzo Life Science) in 2–3-fold excess. After freezing of the solution in liquid N2, the tubes were sealed under vacuum by glass melting.

Resonance Raman (RR) spectra were recorded with a scanning double monochromator. The excitation line at 406.7 nm was provided by a coherent K-2 Kr+ ion laser, and the sample was rotated throughout the measurement to minimize radiation damage. For measurements in frozen solution, samples of ~50 μM were filled into 3-mm quartz tubes and kept in a quartz Dewar filled with liquid N2 during the measurement.

FTIR Spectroscopy—The degassed protein solution was saturated with CO before being reduced under controlled conditions with Na2S2O4 in an anaerobic chamber. Samples were transferred under anaerobic conditions to a 50-μl gas-tight transmission cell (pathlength, 50 μm) equipped with CaF2 windows with 2 cm resolution. FTIR spectra were recorded on a Bruker IFS 66v/S FTIR spectrometer equipped with an MCT photo-conductive detector and a KB beam splitter. The temperature was set to 25 °C with a thermostat (RML, LAUDA).

EPR Spectroscopy—X-band EPR measurements were performed at 6.25 K using a Bruker E500 spectrometer, equipped with an Oxford Instruments ESR 935 cryostat and ITC4 temperature controller. Experimental parameters were as follows: \( v_{mw} = 9.65 \text{ GHz} \); \( P_{mw} = 0.2-20 \text{ milliwatts} \); modulation amplitude = 1 millitesla, and modulation frequency 100 kHz. Spectra were fitted as a single S = ½ species with rhombic g-tensor, consistent with low spin Fe(II) iron signals. Spectral simulations were performed numerically using the EasySpin package (24) in MATLAB. An isotropic line width of 3 millilitesa was used.

Homology Modeling—Homology modeling was performed with the SWISS-MODEL server (25–27) using the PDB files of DosS and DosT (2W3G and 2VZW) as templates. Refinement was performed with the Swiss-PDBViewer version 4.1 (28) using the GROMOS96 (29) implementation for local energy minimizations. Modeling results were evaluated by WhatCheck (30) and Procheck (31).

Electrophoretic Mobility Shift Analysis—The digoxigenin gel shift kit (2nd Generation, Roche Applied Science) was used for all DNA binding tests. For DNA binding tests, a 25-bp double-stranded oligonucleotide from the all4978 promoter region (forward, 5′-GCTGGTATTAGCATAGAAGTAATTG-3′, and reverse, 5′-CAATTACTTCTATGCTAATACGAG-3′) was synthesized (Metabion International AG) and labeled at both ends with digoxigenin. Full-length All4978 protein (N-terminally His-tagged) was purified as described previously. For control experiments, protein EL222 together with its DNA target (65) were used under identical conditions as described for All4978. The GAF domain of All4978 alone (no binding capacity due to removed HtH motif) was used under the same experimental conditions as described for the full-length protein. 15 nM DNA probes were incubated with different concentrations of the purified protein for 30 min at room temperature, in 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5% glycerol. The mixtures were then loaded onto a 10% TBE gel (Invitrogen) at 4 °C in 0.5× TBE buffer (89 mM Tris-HCl, pH 8.0, 89 mM borate, and 2 mM EDTA). For the control experiment using unlabeled DNA and in the experiments described above, 150 nM unlabeled DNA was added. Gel running, transfer, and imaging were done as described by the manufacturer; gels were scanned with an LAS-4000 imager (Fuji film). When reducing conditions were required, fresh dithionite solution (5 mM final concentration) was added to all of the buffers and reaction mixtures.
**Results**

**Structural, Absorbance, and Magnetic Properties of “As Isolated” GAFAll4978**

GAFAll4978 encodes a protein of 224 amino acids that, according to protein domain prediction programs, is composed of two domains, an N-terminal GAF part (aa 17–147) and a C-terminally located HtH motif (aa 163–216). Most experiments on GAFAll4978 were performed with a construct (GAFAll4978) containing the GAF domain and short N- and C-terminal extensions (positions 1–170) plus an N-terminal His6 tag. All7016 (aa 255 as full-length protein) contained the same structural elements as follows: an N-terminal GAF domain (aa 19–146) and a C-terminal HtH motif (aa 178–230). Expression products covering aa 4–168 and aa 1–173 were used as GAFAll7016 and GAFAlr7522 in this study, respectively.

The full-length protein and also the GAF domain expressed separately form dimers, as determined by gel filtration chromatography irrespective of the oxidation state (data not shown).

A survey for other heme-binding GAF domains identified DosS from *M. tuberculosis* as the structurally closest neighbor in current literature (also see under “Homology Modeling”). Despite the structural homology of these two proteins, a sequence alignment revealed only a moderate similarity between the two proteins (Fig. 1). In DosS, the proximal ligand to the heme iron was identified as His-149 (15). For the corresponding region of GAFAll4978, sequence alignment revealed an interesting pattern of three alternate histidine residues at positions 95–99 (HDHGH), preceded by a cysteine at position 92 (in DosS, the corresponding position is occupied by Pro-146). To determine whether a histidine residue serves also as proximal ligand to the heme in GAFAll4978, each of the three histidines was individually mutated.

Upon IMAC purification of the heterologously expressed protein in standard LB medium, the red-colored GAFAll4978 exhibits an absorbance spectrum typical of a heme protein (Fig. 2). Spectra of full-length protein and the separately expressed GAF domain are qualitatively identical; quantitatively they differ by an increased pigment-to-protein ratio ($A_{427}/A_{280}$) of the latter as a consequence of its shorter protein chain. Recombinant expression of full-length All4978 or GAFAll4978 in the presence of 5-aminolevulinic acid (1 mM) or hemin (5 \(\mu\)M) resulted in only a minor (~10%) increase of the pigment-to-protein ratio. Similarly, the addition of heme to the purified protein did not significantly increase the absorbance in the range of 400–600 nm.

**Figure 1.** A, amino acid sequence alignment between GAF domains: GAFAll4978, GAFAll7016, GAFAlr7522, and GAFDosS. Secondary structure elements were derived from the x-ray structure of GAFDosS (PDB code 2W3G). The proximal heme ligand His-149 of GAFDosS and the potential proximal heme ligands for GAFAll4978, Cys-92 and His-95, are shown by stars. B, amino acid sequence alignment between helix-turn-helix motifs: HtHAll4978, HtHAll7016, HtHAlr7522, and HtHNarL. Secondary structure elements were derived from the x-ray structure of HtHNarL (PDB code 1JE8). The C-terminally HtH domain of All4978 is highly similar to NarL of *E. coli* (PDB code 1JE8), nitrate reductase, an important enzyme to nitrogen metabolism. In NarL, the regulation proceeds via phosphorylation of a Rec domain (N-terminal, aa 10–124), followed by structural changes of the C-terminal HtH domain. Multiple alignment was done using the T-coffee software and visualized using ESPript.

**Figure 2.** Absorbance spectra of full-length GAFAll4978 and GAFAlr7522, as well as construct GAFAll4978, and the separately expressed GAF domain (GAFAll4978). The spectra show the absorbance range of 420–700 nm, with a peak at around 427 nm, characteristic for heme proteins. The inset shows the zoomed-in view of the absorbance peak, highlighting the difference in pigment-to-protein ratio between the full-length protein and the GAF domain.
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The electronic absorbance spectrum of the as isolated protein is characterized by sharp $\alpha$- and $\beta$-bands at 571 and 540 nm, respectively, indicative of a low spin (LS) Fe$^{11+}$ ($S = \frac{1}{2}$) (Fig. 2A and Table 1). A Soret band at 427 nm and a prominent $\delta$-band at 360 nm are also clearly visible, characteristic of thiolate heme ligands (32, 33). Commensurate EPR measurements on the as isolated protein identified a single EPR active species centered about $g \approx 2$ (Fig. 2B). The EPR signal displays fast magnetic relaxation as evidenced by the high microwave flux that can be used to measure the signal at cryogenic temperatures (<30 K) without significant power saturation ($P_0 > 2$ milliwatts at 25 K); as shown in Fig. 2B, the magnitude of the EPR signal is the same using two microwave fluxes, 0.2 and 2 milliwatts, after accounting for the expected microwave power dependence. The intensity of the signal shows a linear dependence with the reciprocal of the measurement temperature (Fig. 2B, inset). Both of these properties are consistent with assigning the EPR signal to a single $S = \frac{1}{2}$ species. No additional EPR signal was observed at low magnetic fields where high spin heme iron signals can be observed.

The line shape of the EPR signal is characteristic of a LS ferriheme. Such species typically display a rhombic EPR spectrum (three inflection points), with at least one turning point greater than $g = 2$ and one turning point less than $g = 2$. The relatively narrow width ($g$ spread) of the EPR signal is indicative of a thiolate-coordinated heme (P-type). The fitted $g$ values [2.37, 2.23, 1.92] are very similar to that seen for the heme cofactor of P450$_{cam}$ [2.41, 2.25, 1.91], which is coordinated by a thiolate residue and a water (for tabulated values see Walker (76)). Using these $g$ values, estimates for the ligand field parameters can be deduced as follows: the tetrahedral ($\Delta / \Lambda$) and the rhombic splitting ($|V| / |\Delta|$), where $V$ and $\Delta$ describe the energy level splittings of the occupied $t_{2g}$ orbitals ($d_{xz}$, $d_{yz}$, and $d_{xy}$), and $\Lambda$ indicates the spin-orbit coupling. Using the ”proper” axis system ($|V| / |\Delta| \leq \frac{3}{2}$) as defined by Taylor (34), these $g$ values yield $\Delta / \Lambda = -5.37$ and $|V| / |\Delta| = 0.40$, and constrain $g_2$ to lie in the plane of the heme ring. The strong similarity of these values to that of P450$_{cam}$ suggests the redox potential of GAF$_{All4978}$ and P450$_{cam}$ is likely to be similar.

The addition of strong ligands to ferriheme, such as imidazole, NO, and CN$^-$, did not change the absorbance spectrum suggesting that the distal site of the heme iron is either coordinated by a ligand residue (hexa-coordinated form) or its open coordination site is sterically shielded by the surrounding protein pocket. As indicated above, EPR data would suggest that if the ferric heme was hexa-coordinated, the 6th ligand would be water.

The as isolated protein ($\lambda_{\text{max}} = 427$ nm), characterized by EPR and by Raman as an Fe$^{1+}$ species, has been treated by K$_2$[Fe(CN)$_6$]$_{10}$, which caused a shift of the Soret band to 412 nm. Reduction of the as isolated protein by Na$_2$S$_2$O$_4$ led to an upshift of the 427-nm Soret band to 424 nm and a gain in intensity, concomitant with a concurrent change of the line shape of the $\alpha$- and $\beta$-bands (Fig. 3 and Table 1). Exposure of the sample to air by opening the cuvette yielded rapid re-oxidation of the heme cofactor once S$_2$O$_4^{2-}$ was consumed, again generating, in vivo and in vitro conditions; however, no spectral indications of incorporation of the bilin cofactor could be found.
with a slightly shifted absorbance, the short wavelength form ($\lambda_{\text{max}} = 413$ nm). This reduced, re-oxidized sample showed the same Raman spectrum as the as isolated protein (see below), confirming its Fe$^{II}$ state. The reduction experiment was performed over a pH range from 5.5 to 10 without any significant change of the spectral properties. When kept under reducing conditions, the addition of CO resulted in a significant change of the absorbance spectrum: the Soret band and the $\alpha$- and $\beta$-bands downshifted to 420, 568, and 539 nm, respectively, indicating that, in contrast to the ferriheme state, the reduced heme does bind CO and, as identified in a separate experiment, also NO (Fig. 3). Similar effects were observed for the addition of NO and CO to the reduced GAFAll7016 domain (data not shown) unambiguously demonstrating that these novel heme-GAF domains in their reduced form bind these diatomic gas molecules. Homology modeling (see below) suggested a functional involvement of tyrosine 41 in the gas binding capacity. We thus mutated this residue into phenylalanine and glycine and repeated the CO-binding experiment. Also, electrochemical reduction was performed with these mutants. Spectra of CO-loaded wild-type and Y41F/Y41G mutants are virtually identical, and also the reduction experiment yielded potentials as found for the wild-type protein, excluding a functional role of Tyr-41 in the gas binding capacity and the redox sensing of All4978.

**Chemical Reduction and Spectroelectrochemical Titration**—The chemical reduction was performed by adding a freshly prepared solution of sodium dithionite (Fig. 3). The effect of reduced FMN was investigated, too, because it has been reported to accelerate reduction of DosS (15). However, the presence or absence of FMN did not affect the kinetics of the reduction of GAFAll4978 with 1 mM Na$_2$S$_2$O$_4$. The reduction rate was not increased by the addition of ascorbic acid, GSH, FAD, or NADH (data not shown). Similar to reduction by sodium dithionite, the electrochemical titration led to a shift of the 427-nm Soret band and modification of the $\alpha$-band at 559 nm (Fig. 4). Fits of the electrochemical titration curves yielded inflections at $-449$ mV ($-445 \pm 2$ mV for the 424-nm band and $-453 \pm 2$ mV for the 559-nm band) versus SHE. The mean, $-449$ mV versus SHE, is a remarkably low reduction potential for a heme protein (35).

### TABLE 1

| GAFAll4978 | As isolated | Reduced |
|------------|-------------|----------|
|            | $\delta$ | $\alpha$ | $\beta$ | Heme content$^a$ | Soret | $\alpha$ | $\beta$ |
| WT         | 360      | 427      | 570     | 541 | 1 | 424 | 559 | 538 |
| Fe$^{II}$-CO | 1       | 420      | 568     | 539 | 1 | 420 | 553 | 528 |
| Fe$^{II}$-NO | 1       | 420      | 553     | 528 | 1 | 420 | 553 | 528 |
| Y41G      | 360      | 426      | 571     | 540 | 0.51 | 425 | 558 | 529 |
| Y41F      | 360      | 426      | 571     | 541 | 0.75 | 425 | 558 | 529 |
| C92S      | 411      | 412      | 0.06    | 0.06 | 0.06 | 419 | 553 | 528 |
| H95G      | 360      | 426      | 567     | 541 | 0.46 | 425 | 557 | 530 |
| H95G      | 360      | 425      | 570     | 540 | 0.37 | 424 | 557 | 528 |
| D96A      | 423      | 417      | 536     | 531 | 0.12 | 422 | 556 | 528 |
| H97G      | 417      | 0.09     | 424 | 558 | 525 |
| H95A      | 417      | 0.09     | 424 | 558 | 525 |
| C138S     | 360      | 427      | 570     | 541 | 0.60 | 424 | 559 | 538 |
| Y41G      | 360      | 426      | 571     | 540 | 0.51 | 425 | 558 | 529 |
| Y41F      | 360      | 426      | 571     | 541 | 0.75 | 425 | 558 | 529 |
| C92G      | 411      | 412      | 0.06    | 0.06 | 0.06 | 419 | 553 | 528 |
| H95G      | 360      | 426      | 567     | 541 | 0.46 | 425 | 557 | 530 |
| H95G      | 360      | 425      | 570     | 540 | 0.37 | 424 | 557 | 528 |
| D96A      | 423      | 417      | 536     | 531 | 0.12 | 422 | 556 | 528 |
| H97G      | 417      | 0.09     | 424 | 558 | 525 |
| H95A      | 417      | 0.09     | 424 | 558 | 525 |
| C138S     | 360      | 427      | 570     | 541 | 0.60 | 424 | 559 | 538 |

$^a$ Ratio between the heme absorption (Soret band) of mutant and WT after normalizing to the protein absorption at 280 nm is shown.

$^b$ This mutation led to a very unstable heme binding.

**Chemical Reduction and Spectroelectrochemical Titration**—The chemical reduction was performed by adding a freshly prepared solution of sodium dithionite (Fig. 3). The effect of reduced FMN was investigated, too, because it has been reported to accelerate reduction of DosS (15). However, the presence or absence of FMN did not affect the kinetics of the reduction of GAFAll4978 with 1 mM Na$_2$S$_2$O$_4$. The reduction rate was not increased by the addition of ascorbic acid, GSH, FAD, or NADH (data not shown). Similar to reduction by sodium dithionite, the electrochemical titration led to a shift of the 427-nm Soret band and modification of the $\alpha$-band at 559 nm (Fig. 4). Fits of the electrochemical titration curves yielded inflections at $-449$ mV ($-445 \pm 2$ mV for the 424-nm band and $-453 \pm 2$ mV for the 559-nm band) versus SHE. The mean, $-449$ mV versus SHE, is a remarkably low reduction potential for a heme protein (35).

**RR and MCD Spectroscopy Reveal Low Spin State and Hexacoordination of the Ferric GAFAll4978**—RR spectra of frozen solutions were recorded with excitation into the blue edge of
the Soret band (406.7 nm). The RR spectrum of the as isolated GAFAll4978 recorded at 77 K is shown in Fig. 5, and the most relevant modes are summarized in Table 2. The observation of an intense so-called oxidation state marker transition, $\nu_4$ appearing at 1374 cm$^{-1}$ typical of ferriheme proteins (36, 37), provides further evidence that as isolated GAFAll4978 contains a ferriheme. Another important feature is the so-called spin-state marker transition, $\nu_3$ appearing at 1502 cm$^{-1}$, which is a fingerprint for hexa-coordinated low-spin (6cLS) heme (1500–1510 cm$^{-1}$) (38). The positions of other prominent core marker bands (e.g. $\nu_2$ and $\nu_{10}$) are also consistent with a 6cLS heme (39).

The $\nu_{C-H}$ modes characteristic of the heme vinyl substituents are well separated (1615 and 1627 cm$^{-1}$), indicating a rather different environment of the vinyl groups of rings A and B (36, 40).

MCD spectroscopy is another useful method for the exploration of iron coordination and spin-state in heme proteins (41–44). Room temperature spectra of the as isolated GAFAll4978 (Fig. 6) yielded characteristic signatures of ferric LS hemes that are less complex than those of the corresponding HS ($S = 5/2$) electron configuration (42). The ferriheme MCD spectrum of GAFAll4978 is similar to the LS spectrum of the His/Met-liganded cytochrome $c$ (45), and Cys-liganded cytochrome P450s, the latter assignment being in line with the EPR results described above (46–49).

**RR and MCD Spectroscopy Reveal Histidine Ligation of Ferrous GAFAll4978**—RR spectra of the heme iron in the diamagnetic LS form, GAFAll4978[Fe$^{II}$-CO] ($S = 0$), or in the paramagnetic HS form, HS GAFAll4978[Fe$^{II}$] ($S = 2$), provide information on the modified coordination environment induced by cofactor reduction. Reduced samples without (5c) and with an extra ligand (NO or CO) (6c) at the Fe$^{II}$ were measured under the same conditions, but in the absence of oxygen (Fig. 7 and Table 2). The most prominent core-size marker band of heme proteins in the high frequency region is the oxidation state marker $\nu_4$ that indicates the presence of Fe$^{III}$ (1370–1375 cm$^{-1}$) or Fe$^{II}$ (1350–1375 cm$^{-1}$) (36, 37, 50–52). For the unliganded species, the $\nu_4 = 1359$ cm$^{-1}$ mode clearly demonstrates that the iron is in its reduced state. A shoulder at 1375 cm$^{-1}$ may indicate the presence of a small fraction of ferric form. The above-mentioned spin-state marker $\nu_3$ changes with the spin state of iron but is independent of the oxidation state, i.e. 1460–1470 cm$^{-1}$ for 5cHS Fe$^{III}$ and 1490–1510 cm$^{-1}$ for 5cLS or 6cLS Fe$^{III}$ (38, 51, 52). Therefore, the spectral features seen at 1493 and 1501 cm$^{-1}$ indicate a LS complex for both sixth ligands, NO and CO. This is also consistent with the positions of $\nu_2$ and $\nu_{10}$.

Upon addition of the strong ligands NO and CO, the oxidation state marker band shifted to 1374 and 1372 cm$^{-1}$, respec-
Values typical of ferroheme nitrosyls and carbonyls (53).

In contrast, the spectra are rather similar to each other and also to the unliganded sample that is consistent with the expected 6cLS situation. More importantly, the low frequency region of the Fe\textsuperscript{II}-CO spectrum can be used for the assignment of the \(\nu_{\text{Fe-CO}}\) stretch vibration by subtraction of the spectrum of the unliganded form. The anti-correlation of the C–O bond strength and the Fe–C bond strengths strongly depends on the ligand \textit{trans} to the CO. This is a result of the competition between CO and the axial ligand for the \(d_z^2\) acceptor orbital of iron. Thus, the pair of \(\nu_{\text{Fe-CO}}\) and \(\nu_{\text{C-O}}\) vibrational frequencies, the latter of which was obtained by FTIR spectroscopy (Fig. 7A, inset), can be used to assign the proximal ligand type. In Fig. 8, the position of \(\nu_{\text{Fe-CO}}\) falls within the anti-correlations of thiolate-liganded hemes, His-liganded heme proteins, and 5c-liganded hemes. Clearly, Cys is not the proximal ligand. However, GAF\textsubscript{All4978}[Fe\textsuperscript{II}-CO] is surprisingly close to the 5c line. A similar position was found for the case of Rev-erb\(\beta\) (54), which is His-liganded in the CO-bound form. To complete the Raman measurements, the ferrous-state sample was exposed to air, yielding the ferric state protein (\(\lambda_{\text{max}} = 413\) nm), again showing the same Raman spectrum as measured for the as isolated form of GAF\textsubscript{All4978}.

The MCD and the absorbance spectrum of the reduced/CO-bound system further supports a His-ligated type of CO heme. A number of other redox-dependent ligand-switch proteins, e.g. CooA, Rev-erb\(\beta\), and \textit{Drosophila} E75, show similar MCD peak positions (supplemental table in Ref. 54). We therefore conclude that in the reduced state the CO-coordinated heme is ligated by a histidine residue instead of the cysteine found in the oxidized state.

A ligand switch between a cysteine and a histidine residue, which is dependent on the oxidation state of the iron atom of heme, has been reported for a number of heme-binding proteins (Table 3) (54–60). All4978 displays an interesting sequence motif (\(^{\text{SS}}\)HDHG) in the region below the heme ring plane; any one of these three histidines might represent the putative switching partner. A close-by cysteine residue (Cys-92) might be the other ligand-switching partner. Accordingly, a number of variant proteins were prepared by site-directed mutation, and their heme binding capability was determined.

### TABLE 2

Core marker bands of GAF\textsubscript{All4978} in the ferric state as isolated and in the ferrous state without (5c) and with various distal ligands (6c)

| Mode | Ferric/cm\(^{-1}\), as isolated | Ferrous/cm\(^{-1}\) |
|------|---------------------------------|-------------------|
| \(\nu_\alpha\) | 1585 | 1580 |
| \(\nu_\beta\) | 1502 | 1493-1501 |
| \(\nu_\gamma\) | 1374 | 1359 |
| \(\nu_\delta\) | 674 | 677 |
| \(\nu_\mu\) | 1637 | 1619 |
| \(\nu_\mu\) | 747 | 754 |

FIGURE 6. MCD spectra of GAF\textsubscript{All4978} recorded at room temperature (\(B = 1.4\) tesla) in 20 mM KPi. The spectrum of the as isolated form is given as a dotted line. The ferrous form (gray) was generated in a nitrogen atmosphere upon addition of 2 mM Na\textsubscript{2}S\textsubscript{2}O\textsubscript{4}. The carbonyl liganded sample (black) was prepared in a CO atmosphere upon addition of 2 mM Na\textsubscript{2}S\textsubscript{2}O\textsubscript{4}.

FIGURE 7. RR spectroscopy (\(\lambda_{\text{ex}} = 406.7\) nm) of frozen samples (77 K) of ferroheme GAF\textsubscript{All4978} in 20 mM KPi, without ligand (5c, gray, in bold) and with NO (black, thin line) and CO (black, in bold) as sixth ligand. A, low frequency part. Top, difference spectrum (black) between GAF\textsubscript{All4978}[Fe\textsuperscript{II}] and GAF\textsubscript{All4978}[Fe\textsuperscript{II}-CO] is displayed to identify \(\nu_{\text{Fe-CO}}\). The resulting band at 514 cm\(^{-1}\) was fitted with a single Voigt function (gray inset). The inset shows the C=O band in the FTIR spectrum of the sample recorded at 25 °C. B, high frequency part.
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The suggested function of Cys-92 could be verified from C92S/C92G variants; both mutations led to nearly complete loss of heme incorporation (C92S showed ~10% heme content compared with the heme content of the WT protein, and C92G ~6%, respectively, see Table 1 (keep in mind that the heme loading of the WT protein itself is relatively low)). However, the assignment which of the three candidate histidine residues is serving as ligand in the FeII state is not straightforward. Single mutations of all three histidine residues led to a decrease in heme binding. For His-95 variants (H95A/H95G), binding was reduced to 46 and 37%, respectively. For both His-97 and His-99 variants, a more dramatic decrease was observed (Table 1) compared with the heme content of the WT protein, and C92G/H99A carried only 10% of the WT heme content. This result seems to support assigning the axial ligand switch (Fig. 1). Moreover, as GAFDosS does not undergo a Cys → His switch and thus does not carry a cysteine at the corresponding position, reliance of the sequence alignment alone is somewhat uncertain.

Homology Modeling—Heme binding GAF domains are found in two histidine kinases, DosS and DosT, from M. tuberculosis. Both GAFDosS and GAFDosT have been structurally characterized by x-ray crystallography (PDB code 2W3G (15) and 2VZW (10), respectively). Sequence alignments between GAFAll4978 and GAFDosS or GAFDosT using T-Coffee (Fig. 1) (14) revealed high amino acid sequence homologies (69 and 68%, respectively). In addition, secondary structure predictions of GAFAll4978 using PSIPRED closely matched secondary structural elements of GAFDosS and GAFDosT (61). Homology models built with PDB code 2W3G as template resulted in higher quality parameters than models built with PDB code 2VZW. The former model based on the structure of GAFDosS (Fig. 9A) was investigated in more detail. To allow docking in the pocket, the heme had to be rotated by ~180° around the z axis, which passes through the iron and is perpendicular to the heme plane. After this rotation, the protein structures of GAFDosS and GAFAll4978 show a high degree of homology (root mean square deviation value of 0.57 Å for the backbone atoms calculated by Swiss-PdbViewer (27, 28)).

The 92CXXHDHGH motif lies in a loop region. In GAFDosS, His-149 had been identified as the proximal ligand to the heme group, and it is located in a loop region connecting the β3- and β4-strands (Pro-140 to Thr-154) (15). The sequence alignments (Fig. 1A) reveal that His-95 of GAFAll4978 corresponds to His-149 of GAFDosS. Thus, in the GAFAll4978 model, His-95 was inserted as the heme ligand of the iron in the reduced (FeII) state, as identified by the spectroscopic results discussed above. In addition, to accommodate the requirement that the iron in the oxidized (FeIII) state has a Cys coordination, the sequence alignment was modified by placing Cys-92 of GAFAll4978 in line with His-149 of GAFDosS, and the model building was repeated. The His-95- and the Cys-92-ligated forms are compared with each other in Fig. 9B, showing the two peptide chains in blue and yellow, respectively. Both proximal ligands are located in a highly flexible region of the structure that may support ligand switching. The model indicates that the conformational change may create a large rearrangement in the turn Ile-82-Gly-98, but nevertheless it preserves the overall structure of GAFAll4978.

DNA Binding—The presence of the C-terminally located HtH motif called for attempts identifying DNA binding capacity of All4978. A survey in the genome neighborhood did not yield any apparent interacting gene product or an operon structure. All4978 is followed by two genes encoding ribosomal RNA (16S and 23S) and is preceded by a gene encoding for a hypothetical protein. A gene for a hypothetical protein is also found on the opposite strand. Thus, due to the absence of genes that might potentially be functionally related and be regulated by All4978, we suggested autoregulation by binding to its own promotor. Similar experiments had been reported for other switchable DNA-binding proteins, e.g. EL222 from Erythrobacter litoralis (62). This protein serves as a blue light sensor that (preferentially in its lit state) binds to a short stretch of its own promotor. In fact, control experiments using EL222 (courtesy Dr. Kevin Gardner, City University of New York) as reference and even a hybrid protein composed of the heme-binding GAF domain of All4978 and the DNA-binding HtH domain of EL222 showed the expected binding to the EL222 target DNA (data not shown). Under the same conditions established for the control experiments, we performed binding studies with All4978, using a 25-bp stretch of DNA as target. Applying increasing amounts of All4978, we find clear evidence for a protein-DNA complex. Under the experimental conditions,
TABLE 3

Selected examples of ligand switching sensory heme proteins with ferric Fe-SCys and ferrous Fe-NHis coordination

| Protein      | Organism    | Domain fold | Motif     | Function         |
|--------------|-------------|-------------|-----------|------------------|
| GAF_A14978   | Nostoc sp. PCC7120 | CRP         | CPRHDHG    | DNA binding      |
| CooA (55)    | R. rubrum    | Nuclear receptor | CMH      | CO sensing, DNA binding |
| Rev-erbβ (54, 56) | Vertebrates | Nuclear receptor | HLVCP    | Nuclear receptor |
| E75 (54, 57) | Invertebrates | Nuclear receptor | CPX_A/HX11,CP | Transcription factor |
| HRI (58)     | Mammals      | PAS         | CP         | Kinase of eIF    |
| Npas2 (59)   | R. sphaeroides | HtH/PAS     | CH         | Transcriptional regulator |
| PpsR (60)    |              |             | HX146,Cl   | DNA binding      |

Discussion

GAF_A14978 represents a new heme-binding GAF domain protein; there are only few examples reported so far (9, 11), and none have yet been identified in cyanobacteria. As part of the genome-annotated transcription regulator All4978, it carries an HtH motif in its C-terminal domain. Although GAF_A14978 shares a high degree of structural homology with GAF_DosS and GAF_DosT, the heme iron coordination is entirely different with a Cys sulfur acting as the axial ligand of the iron in its ferric oxidation state. Like GAF_DosS, but unlike GAF_DosT (5), the protein is not able to bind O2 despite its facile oxidation within seconds. A similar experiment, as reported previously (5), gave additional proof that GAF_A14978 does not bind oxygen; addition of cyanide to the oxidized form yields an absorbance peak at 540 nm as reported for GAF_DosS (Fig. 5A). However, in contrast to GAF_DosS reduction of the heme cofactor of GAF_A14978 by S2O42− was not enhanced in the presence of cytosolic reducers like FMN (E° = −220 mV) and NADH (E° = −320 mV) (63), suggesting its heme cofactor has a very low reduction potential as compared with typical heme irons. Although no intracellular potential has been reported for Nostoc, one might assume a redox sensing by the proteins described here. Nostoc forms heterocysts capable of nitrogen fixation, only under very reducing conditions allowing nitrogenase activity. When the intracellular potential is increased, Nostoc cells stop nitrogen fixation, potentially concurrent with changing into the ferric state of the heme-binding proteins. Such conversion needs to be rapid in order to protect the oxygen-sensitive nitrogenase complex. The ineffectiveness of naturally occurring mediators was corroborated by the spectroelectrochemical titration measurements that yielded a midpoint potential for the hemes of −445 ± 2 and −453 ± 2 mV, respectively (Soret and α-band monitored). Interestingly, reduction with S2O42− (E° = −660 mV) (64) causes a ligand switch from Cys-92 to His-95. Such behavior representing an activation/de-activation switch is well established for a number of other heme sensory proteins with very distinct folds (Table 3) (3, 59, 65).

The high degree of structural homology between GAF_A14978 and GAF_DosS allowed building a structural model of GAF_A14978 for both its oxidized (Cys-92 liganded) and reduced (His-95 liganded) forms (Fig. 9). Verification of His-95 as the proximal ligand in the ferrous state remains uncertain to some extent; the sequence alignment gives preference to His-95, whereas the mutagenesis experiments would be more consistent with either His-97 or His-99. Ligand switching has also been reported for other heme-binding proteins where it was identified as activation/de-activation mechanism for sensor functions (Table 3). A similar mechanism might be envisaged here for GAF_A14978 which carries an HtH motif in its C-terminal domain.
Changing the coordinating ligand upon reduction is understood as a consequence of the loss of charge stabilization between CysS/H\textsubscript{11002} and the heme iron as shown in Reaction 1.

\[(\text{ppIX})\text{Fe}^{2+} + \text{Cys}^- + \text{H}^+ \rightarrow (\text{ppIX})\text{Fe}^- + \text{NHis} + \text{H}_2\text{Cys}\]

**REACTION 1**

One might assume that the conformational change induced by the ligand switch results in modulation of a catalytic or DNA binding domain associated with the sensory heme domain. An example for this process is found for CooA, a transcriptional activator. Here, a Cys-His switch was accomplished by CO binding, which then allows formation of the DNA complex. The ligand switch was examined by NMR and EPR spectroscopy (66).

Examples of other ligand-switching heme proteins are presented in Table 3. Supported by the mutagenesis experiments (Table 1), other Cys, His, and Met residues in the GAF\textsubscript{All4978} structure can be excluded as heme ligands. Among the folds for which sensory heme domains were found, PAS domains may be mentioned because they share a high degree of structural homology with GAF domains. A structural feature among many, but not all, heme sensory proteins is the presence of a Pro following the coordinating Cys (67). This CP motif is also present in GAF\textsubscript{All4978} (Table 3 and Fig. 1). Another common feature of ligand-switching heme sensors is that the coordinating His residue is mostly found at the positions of aa 1–3 away from the CP motif, but there are exceptions where the distance in sequence can be much larger (Table 3). An example is the nuclear receptor E75 (54, 57), where a relatively large distance between the CP motif and the putative His ligand is proposed.

A special case has been reported for the HtH transcription factor PpsR (a heme-binding and DNA-binding transcription factor) expressed in the photosynthetic bacterium *Rhodobacter sphaeroides*, which contains two PAS domains (60). In this case, the heme-binding Cys is located in the HtH domain, whereas the ligand-switching His residue (upon reduction) was identified as part of one of the PAS domains, suggesting that the heme binding pocket is formed as part of an interdomain surface. Here, the heme-coordinating Cys is followed by an Ile residue. In summary, the currently known ligand-switching heme sensory domains show very large fold diversity. GAF\textsubscript{All4978} and its paralogs in *Nostoc* extend this diversity and represent the first example of a ligand-switching heme-GAF domain. Apparently, the sensing mechanism among heme-binding GAF domains is accomplished in many different ways.

An important structural and functional aspect among the heme-sensing proteins is the nature of the proximal ligand, which also shows a very high degree of diversity involving water, Glu, His, and N-terminally Pro (68). The spectroscopic analysis for GAF\textsubscript{All4978} clearly demonstrates a 6cLS complex in case of the ferric form. However, the structural models do not provide evidence for a 6th side-chain ligand. In the case of GAF\textsubscript{DosS} and GAF\textsubscript{DosT}, a proximal Tyr plays a critical role for the sensory function (10, 15, 69). Similar as in these two proteins, Tyr-41 of GAF\textsubscript{All4978} is located in the model above the heme ring plane, yet at a relatively large distance to the iron (4.3 Å), making a bond formation between the iron and the phenolic oxygen fairly unlikely. In fact, the finding of similar results for the wild-type protein and its Y41F/Y41G mutants speaks against a direct involvement of Tyr-41 unlikely. Alternatively, a water molecule might be positioned in between serving as a ligand that is replaced by NO or CO in the reduced state. There exists no other protein residue candidate for this ligand. In contrast, P450s also exhibit 6cLS spectra in case of the substrate-unbound state, suggesting that the ligand field of Cys\textsuperscript{−} together with a proximal water ligand is strong enough to create the LS complex.
Cys-His Ligand Switching in Heme-binding GAF Proteins

state.7 Surprisingly, even diatomic ligands with high affinity for ferric hemes, i.e. NO or CN−, did not bind to the ferric (FeIII) form of GAFAll4978, suggesting that the distal pocket must be rather crowded. Such crowding could be explained by the presence of the Tyr-41 side chain reaching into the distal pocket.

The protein is found as a dimer, irrespective of its state of oxidation. This raises the question for the mechanism of DNA binding, as usually HTH motifs bind as dimers, which under the constitutionally dimeric composition determined here would make a regulation difficult to understand. However, one might consider the yet speculative oxidation state-dependent formation of a heterodimeric complex with another transcription factor in the presence of the DNA target.

Preliminary results point to binding to a sequence isolated from the promoter region of All4978, preferentially when the protein is in the ferric state. Further investigations on the precise binding site and the binding mechanism, e.g. homodimer to heterodimer change (see above), will require experiments extending the results reported here.

Overall, this study adds a new type of heme pocket to the very diverse members of heme sensory domains. GAFAll4978 and possibly GAF7016 represent the first example of a combination of a heme-binding GAF domain with an HTH motif, which are typical motifs involved in gene expression regulation.

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