Crystal Structures of the CO- and NO-Bound DosS GAF-A Domain and Implications for DosS Signaling in *Mycobacterium tuberculosis*

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Abstract

DosS is a sensor in *Mycobacterium tuberculosis* that differentially responds to O₂, NO, and CO, as well as to changes in the redox state of the prosthetic heme iron atom. The ferrous protein and its Fe(II)-NO and Fe(II)-CO complexes undergo autophosphorylation and subsequently transfer the phosphate group to DosR, a nuclear factor, to activate it. In contrast, autophosphorylation is negligible with the ferric protein and the Fe(II)-O₂ complex. To clarify the basis for this differential response to gases, we have determined the crystal structures of the NO- and CO-complexes of the DosS GAF-A domain, which contains the heme to which the gases bind. Comparison of these crystal structures with those reported for the phosphorylation-inactive ferric GAF-A domain suggest that the GAF-A domain is in a dynamic equilibrium between active and inactive states, and that the position of Glu87 in the heme cavity, which depends on the which gas is bound, acts as a modulator of the equilibrium, and therefore of catalytic activity.

Keywords

Mycobacterium tuberculosis; DosS; gas sensor; hemoprotein; conformational selection; x-ray crystallography

1. Introduction

Mycobacterium tuberculosis (*Mtb*) is a rod-shaped bacterium that causes most cases of tuberculosis in humans and results in over 1.7 million deaths each year [1]. The high level of *Mtb* in developing nations and the emergence of multi-drug resistant strains pose a serious threat to public health [2]. This threat is exacerbated by an environment in which industrial antibiotic development is not a priority due to a low return on investment [3]. Infection initially occurs in alveolar macrophages, in which the bacteria can reside in a non-replicating dormant state for prolonged periods [4], only to reemerge at a later time in immune-compromised individuals. The entry into dormancy allows the mycobacteria to evade the killing action of most commonly used antimicrobials [5]. Slightly elevated nitric oxide (NO) and carbon monoxide (CO) levels, as well as low O₂ concentrations in *Mtb*-engulfing granulomas, alter the expression of a 48-gene dormancy regulon (Dos) [1,3,4,6,7]. Two *Mtb* gas-sensing kinases, DosS and DosT, are important in turning on these genes and the consequent transition into dormancy [8,9]. DosS and DosT have four domains: a heme containing GAF-A domain, an adjacent GAF-B domain of unknown function, a histidine kinase domain, and finally an ATP binding domain. Both DosS and DosT undergo autophosphorylation at a conserved histidine in the kinase domain. This phosphate group is then transferred to an aspartate residue of DosR, the Dos regulon transcription factor. The heme groups in the GAF-A domains of both DosS and DosT have a histidine as the proximal iron ligand [10,11] and the kinase domain is catalytically active when the iron is reduced from the Fe(III) to the Fe(II) state, and more so when CO or NO is bound to the ferrous iron. Oxidation of the heme iron to the ferric state results in almost complete loss of activity. Although DosS and DosT are highly homologous (62% identity), UV-vis and EPR spectroscopy have shown that reduced ferrous DosS is prone to autooxidation to the ferric state in the presence of oxygen, whereas under the same conditions ferrous DosT forms a dioxygen complex that is stable for days [12,13]. The crystal structures of the ferric and ferrous DosS and DosT GAF-A domains have been reported [14,15]. However, they provide only limited insight

into the structural accommodations caused by the binding of ligands to the iron atom. Here we investigate the molecular mechanism of gas signaling mediated by the DosS GAF-A domain by determining the structures of the ferrous NO- and CO-bound proteins, the first DosS structures with a physiologically relevant gas ligand bound to the iron atom.

2. Materials and methods

2.1. Cloning of DosS GAF-A domain

We designed a construct analogous to the pHIS-Parallel [16] construct used for previous DosS GAF-A domain crystal structure determinations [15]. The GAF-A domain consisting of nucleotides encoding amino acids 63-210 followed by a stop codon was synthesized by Genewiz, Inc. (Berkeley, CA) and was provided in a pUC57 plasmid. The insert, containing a 5' histidine tag followed by a spacer, TEV cleavage site, and the GAF-A domain, was flanked by *NdeI* and *EcoRI* restriction sites. The resulting protein product was identical to that previously reported except for the loss of a Glu prior to amino acid 63, resulting in four additional amino acids (Met-Ser-Asp-Pro) rather than five (Met-Ser-Glu-Asp-Pro) preceding Asp63. The insert was amplified with pFU Ultra II (Agilent Technologies) using a standard protocol employing forward (5'-ATG TTC ATA TGT CGT ACT ACC ATC ACC AT -3') and reverse primers (5'- TTG GAA TTC TCA CTT AGC CTG CTG GTA G -3'), then cut with *NdeI/EcoRI*, and ligated into similarly cut pET-22b(+) (Life Technologies), to give a plasmid hereafter referred to as pDG22b. The plasmid integrity was confirmed by DNA sequencing.

2.2. DosS GAF-A domain expression and purification

Fresh colonies of BL21-Gold (DE3) cells harboring the pDG22b plasmid were grown overnight on Luria-Bertani broth (LB) agar plates supplemented with 100 mg/ml ampicillin. Colonies were resuspended in ~5 ml of LB broth, inoculated into 100 ml of LB supplemented with 100 µg/ml ampicillin, and

allowed to grow at 37 °C for 4 h. One liter flasks were inoculated with 10 ml of this starter culture and incubated at 220 RPM and 37 °C. When the culture reached O.D₆₀₀ ~ 1.0, the temperature was turned down to 18 °C, followed by addition of 0.2 mM isopropyl β-D-1-thiogalactopyranoside and 200 μM δ-aminolevulinic acid (final concentrations). Shaking was continued at 220 RPM and the culture was harvested 38 h later, yielding a dark red cell pellet. The cells from 6 liters of culture were lysed in 50 mM Kpi, pH 7.5, that included 100 mM NaCl, 20 mM imidazole, 0.5 mg/ml lysozyme, 20 μg DNase, 10 mM MgCl₂, 50 mM phenylmethylsulfonylfluoride, and 2 tablets of EDTA-free protease inhibitors (Roche). The cells were lysed by sonication and spun down at 20,000 RPM for 40 min. The soluble fraction was passed over a Ni-NTA column equilibrated in 50 mM Kpi, pH 7.4, containing 300 mM NaCl and 20 mM imidazole. The column was washed with equilibration buffer before DosS was eluted with a gradient from 20-250 mM imidazole. Pooled fractions were dialyzed into TEV protease buffer (50 mM Tris pH 8, 0.5 mM EDTA, 1 mM DTT) and were cleaved with AcTEV™ (Life Technologies) for 3 days at 4 °C. The cleaved DosS GAF-A domain was passed through a Ni-NTA column, concentrated and loaded onto a 26/60 Superdex S200 gel filtration column (GE Health Sciences) equilibrated in 20 mM Tris pH 7.5. The enzyme, which ran as a dimer at approximately 36 kDa, was concentrated to 1 mM for crystallography.

2.3. DosS GAF-A domain crystallography

Crystals were grown under similar conditions as previously reported (15-18% PEG 4000, 100 mM Tris pH 7, 200 mM calcium acetate) except that a mixture of 1.5 μl protein and 0.5 μl well solution was used instead of a 1:1 ratio [15]. The crystals were then transferred to a cryo solution consisting of 20% PEG4K, 175 mM calcium acetate, 10% glycerol and 10% (wt/vol) sucrose. They were then transferred in 10 μl of cryo solution to an Eppendorf placed inside an airtight Wheaton glass vial followed by purging with nitrogen. The vials were moved to an oxygen free anaerobic chamber where the crystals were slowly diluted with 400 μl CO- or NO-purged cryo buffer containing 20 mM sodium dithionite

(added after purging). The vials were resealed and removed from the glove box in preparation for freezing. Upon breaking the seal, crystals were transferred to a depression-well plate and frozen in liquid nitrogen as fast as possible (typically less than 1 min).

Diffraction data for the CO- and NO-bound DosS GAF-A domain proteins were collected at the Advanced Light Source (Lawrence Berkeley National Laboratory), beamline 8.3.1. For the CO-bound DosS GAF-A, 4 s exposures were collected in 0.8° oscillations from $\Phi = 48^\circ$ to $\Phi = 152^\circ$ with a crystal to detector distance of 250 mm. The NO-bound data were collected using 1° oscillations from $\phi = 79^\circ$ to $\phi = 184^\circ$ and 1 s exposures. Data were integrated and scaled in XDS [17]. Intensities were converted to structure factors using Truncate from the CCP4 suite [18] and 5% of the data was flagged for an R_{free} test set. The unit cells (**Table 1**) differed enough from previously published structures that molecular replacement was necessary for phasing using pdb file 2W3D and Phaser [19]. Refinement was performed in the Phenix package [20] with a strategy that included individual coordinates, isotropic B-factors for individual atoms as well as TLS groups as outlined in **Table 1**.

3. Results

3.1. CO-Bound DosS GAF-A Domain Crystal Structure

The DosS GAF-A domain crystallizes as a dimer (**Fig. 1A**) with two monomers of the DosS GAF-A domain in the asymmetric orthorhombic space group (**Table 1**). The dimer interface involves the hydrophobic regions of the N and C-terminal helices. This dimer is probably also present in solution, as the GAF-A domain purifies at an approximate molecular weight of 30 kDa on a Superdex 200 column (see methods). The PDBe PISA (v.1.51) webserver also predicts the dimer to exist in solution with a buried surface area of $4120 \text{ \AA}^2$. Each monomer in the dimer is related by a non-crystallographic symmetry operator that rotates around the z-axis parallel to helices H1 and H6 (see **Fig. 1B**). There is an overall $0.46 \text{ \AA}$ RMSD between the two monomers.

Although speculative, one possible interpretation of the formation of the GAF-A dimer in solution and in the crystal is that the GAF-A and GAF-B domains form such an internal dimer interface in the full-length protein.

The general topology of the CO-bound crystal structure (chain B) is depicted in **Fig. 1B**. The GAF-A domain has four beta strands ($\beta 2$, $\beta 1$, $\beta 5$ and $\beta 4$) that provide the majority of the binding pocket residues. N-terminal and C-terminal helices (H1 and H6) are paired in hydrophobic contacts with each other and the active site β -strands. A heme-b moiety with a histidine as the proximal iron ligand forms the floor of the binding pocket. In **Fig. 2B**, monomer B of the CO bound structure is shown aligned at the heme against the reduced Fe(II) crystal structure (PDB ID: 2W3F). While the $\beta 4$ - $\beta 5$ loop in chain B was modeled fully, in chain A the $\beta 4$ - $\beta 5$ loop residues 163-165 could not be modeled due to poor electron density. The overall structure is shown to pivot almost as a unit counterclockwise about helix H3. This creates an offset in the H1 and H6 helices as well as the beta sheets running along the binding pocket. This rotation is particularly evident when viewed as color coded RMSD values as in **Fig. 2B**, where red shows deviations greater than 1.5Å. The greatest deviations occur at the N-terminus of helix H1 and the C-terminus of helix H6, and in the $\beta 4$ - $\beta 5$ and $\beta 1$ - $\beta 2$ loops.

The CO ligand binds with an N-Fe-C angle of $\sim 176^\circ$ in molecule A and 178° in molecule B (**Table 2**), close to the theoretical value of 180° for heme-iron CO binding. In **Fig. 3A** the DosS GAF-A heme-CO (4YNR) complex is shown with the CO ligand inside positive omit-map density scaled to 3-sigma. The Fe-C atom distances are also close to the ideal value of 1.9 Å. In **Fig. 4A**, the CO ligand is depicted surrounded by hydrophobic pocket residues, including Ile121, which has an alternate conformation. In **Fig. 5B**, the Fe(II)-CO and unligated Fe(II) crystal structures are aligned, showing key residues and water molecules in the active site. The Tyr171 residue at 2.7 Å is within hydrogen-bonding distance to the CO oxygen atom. The water molecules present in the reduced structure (blue) have vacated in the CO bound structure (yellow). The counter clockwise rotation

causes key active site residues to shift position. Phe98, Tyr95, Tyr171, His89, Glu87, Ile121, Ile125 and Pro115 also shift counter clockwise. However, the Glu87-His89 salt bridge present in the reduced structure is maintained upon binding CO. The translation of the H4 helix causes Ile125 and especially Ile121 to penetrate deeper into the active site. This entry of Ile121 into the active site in the CO bound structure increases the hydrophobic environment and causes loss of active site water that is present in the unligated Fe(II) crystal structure.

3.2. DosS GAF-A NO-Bound Crystal Structure

The Fe(II)-NO bound GAF-A domain is similar in structure to that of the CO bound structure. As in the case of the CO-bound structure, residues 161-162 of the β 4- β 5 loop are missing from Chain A due to poor electron density. The dimer topology is analogous to that of the CO-bound crystal structure with an all-atom RMSD of 0.36 Å between the two monomers. In **Fig. 2C**, monomer B of the Fe(II)-NO crystal structure is shown aligned at the heme against the reduced Fe(II) bound structure. The counterclockwise rotation around helix H3 is analogous to that described above for CO bound DosS, although the degree of movement in all regions is noticeably reduced in both monomers. The H4 helix and Ile121 do not move as far into the active site as in the CO-bound structure, as seen when comparing **Fig. 5B** and **Fig. 5C**. In **Fig. 4B**, NO bends towards the Y171 ligand while in **Figure 4C** it bends away from it. In both cases the NO oxygen atom is within hydrogen bonding distance of the Tyr171 hydroxyl (2.5 Å and 2.9 Å, respectively). NO has one electron in its π^* antibonding orbital giving maximal overlap with an empty iron 3d orbital at an Fe-N-O angle of $\sim 140^\circ$ [22] and Fe-N distance of 1.7 Å (**Table 3** and **Fig. 4C**). The experimental Fe-N-O angles for chain A and B are about 153° and 155° , respectively. Deviations from ideal geometry are common in enzymes where active site amino acids interact with the distal atom of the gaseous ligand. Myoglobin, in particular, takes values from 109° - 160° when binding NO [23]. In comparison to the CO-bound structure, the NO ligand tilt allows two water molecules into the active site. The two water molecules above the Tyr171 side-chain are actually alternate conformations of the

same water entity. A hydrogen bond network is present between the active site water molecules and the Tyr171 OH group, the NO oxygen atom, and the heme propionate oxygen atom. The presence of these water molecules most likely prevents Ile121 from further entering the active site, in contrast to its behavior in the CO bound crystal structure.

These structures cannot be compared to that of the DosS GAF-A Fe(II)-O₂ complex because autooxidation of this complex to the ferric state has prevented determination of its structure. Our earlier crystal structure of the DosT GAF-A domain contained two protein monomers in the unit cell, one in the unligated ferrous state and the other with a molecule of oxygen bound to the iron atom [14]. However, this structure is not suitable for comparison because (a) the critical hydrogen-bonding residues Glu87 and His89 of DosS are replaced in DosT by Gly85 and Arg89, (b) the bound oxygen molecule in DosT has an unusually long Fe-O bond distance (2.60 Å versus 2.37 Å for that in the *Escherichia coli* EcDos PAS domain) and thus may be abnormally ligated [24], and (c) the activations of DosS and DosT may not be mediated by identical mechanisms.

The structure of the DosS GAF-A Fe(III)-CN complex was determined by Cho *et al.* [15], who used it as a generic model for diatomic ligand coordination. The Fe(III)-CN complex of P450_{cam} was used by others as a surrogate for the Fe(II)-O₂ complex of that protein [25] and was found to mimic the O₂ iron geometry and the structural changes found in the authentic P450_{cam} Fe(II)-O₂ complex [26,27]. The crystal structure of the DosS GAF-A Fe(III)-CN complex is aligned in **Fig. 6** with the structures of the Fe(II)-CO and Fe(II)-NO complexes. This alignment shows that the crystal structures are largely similar with the greatest difference in the $\beta 1$ - $\beta 2$ loop, where the CN⁻ bound structure moves the furthest of the three (maximum of 3.9 Å) relative to the ferrous, unliganded structure. Glu87 in the Fe(III)-CN complex flips upward to approximately the same position as in the Fe(II)-CO and Fe(II)-NO structures.

4. Discussion

Full-length DosS is a four domain protein that must undergo some degree of conformational deformation to communicate the state of the GAF-A domain to the histidine kinase domain in order for histidine autophosphorylation to occur, an event that takes place nearly 200 amino acids away in the linear sequence. We report here the subtle changes that occur in the GAF-A crystal structure upon binding of NO and CO to the ferrous protein. Under anaerobic conditions, DosS has a robust autophosphorylation activity in both of these ligand-bound states, as well as in the ferrous state with no ligand coordinated to the heme iron atom [12, 28].

Only small structural shifts are required to accommodate the differences in the size and orientation of the iron ligands in DosS (and DosT). It is generally agreed that DosT is a gas and hypoxia sensor, whereas the role of DosS is less clear. In the ferrous state DosS, like DosT, responds to CO and NO, but its ready autooxidation in the presence of oxygen makes it unclear whether the very low autophosphorylation activity observed with oxygen is due to the ferrous dioxy complex or to the autooxidatively generated ferric state. In fact, DosS may have a dual function as both a gas and redox sensor [12].

Proteins in solution are flexible and constantly sample a range of conformations, among which there is a subset that enables autophosphorylation. Reducing the heme iron causes Glu87 to flip away from Tyr171, disrupting the cross-linked hydrogen-bonding network that is present in the ferric enzyme. Because the ferrous enzyme has robust autophosphorylation activity [12], it is likely that disruption of the interconnected hydrogen-bonding network is important for increased sampling of the catalytically productive conformations. From this perspective, anything that disrupts the hydrogen-bonding network might result in increased enzyme activity. Our molecular dynamics analysis of the ferric DosS GAF-A domain revealed a decreased flexibility, particularly in the H6 helix of the wild-type protein, compared to the E87G and E87A mutants [29]. The

observation that the E87G and E87A mutants have some autophosphorylation activity in the presence of oxygen may be a result of the increased flexibility modeled by MD simulations. The binding of ligands to the iron may cause a shift in the equilibrium towards conformations that favor optimal enzyme activity. Furthermore, the NO and CO ligands will stabilize the ferrous, catalytically active state, by inhibiting autoxidation [12]. CO binding has specifically been shown to slow down the oxidation from Fe(II) to Fe(III) with a K_{off} of 0.6 s^{-1} for CO vs. 12.5 s^{-1} for O_2 [30].

A low residual autophosphorylation activity of the ferric DosS protein is evidenced by the fact that the autophosphorylated protein slowly accumulates even in the presence of oxygen [12,28,30]. The equilibrium between enzymatically non-productive and productive conformations, albeit unfavorable, thus results in slow accumulation of the phosphorylated product. This requires that: (a) the rate of autooxidation in the presence of O_2 is on the same time scale as the rate at which DosS samples productive conformations, and (b) reoxidation of Fe(II) to Fe(III) is accompanied by a Glu87 flip back to its position in the oxidized conformation. The ferrous DosS GAF-A domain autoxidizes with a $t_{1/2} \sim 1.1 \text{ ms}^{-1}$ in the absence of chelators [30], a time that is at the minimum end of the time range for large scale protein conformational changes [31]. Evidence that the second condition is met is provided by a previously published DosS GAF-A crystal structure in which the protein had been reduced and subsequently reoxidized, (PDB ID:2W3G) [15]. In this instance both the reduced and oxidized states have been frozen in time within the same DosS GAF-A dimer (**Fig. 7**). Molecule A (grey) shows the reoxidized state in which Glu87 has flipped back towards Tyr171 to complete the H-bond network characteristic of the oxidized state. Molecule A is aligned against molecule B (aqua), in which autooxidation has not yet occurred, and in which Glu87 is flipped upwards. Subtle differences can also be seen in the H4 helix and $\beta 1$ - $\beta 2$ loops. The appearance of both oxidized and reduced conformations within a single dimer suggests that in solution the conformation characteristic of the oxidized enzyme is freely reversible. Therefore there is a competition between sampling molecular motions

that induce autophosphorylation and oxygen binding/autooxidation of the Fe(II) iron, with the presence of oxygen favoring oxygen binding and return to the less flexible ferric state.

This view of ligand-assisted conformational selection, in which the inherent conformational equilibrium of the ferrous sensor is enhanced by binding of CO or NO, both of which disrupt the hydrogen bonding network and favor an upward swing of Glu87, is supported by the crystal structures reported here, as well as by earlier mutagenesis experiments, molecular dynamics simulations, and resonance Raman results [29]. However, an additional factor contributes to the promotion of DosS autophosphorylation. As discussed earlier, the crystal structure of the ferric DosS GAF-A domain with cyanide bound to the iron exhibits a conformation analogous, but not identical, to those of the ferrous CO- and NO-complexes (**Fig. 6**). Early microarray expression profiling showed that low levels of cyanide prevented induction of the dormancy regulon by NO and hypoxia [32]. This suggests indirectly that the ferric cyano complex has low autophosphorylation activity. We have directly measured the activity of DosS in the presence of cyanide by the protocol of Basudhar *et al.* [29] and shown it to have negligible autophosphorylation activity. The inactivity of the Fe(III)-CN complex, in view of the conformational similarities of the Fe(III)-CN with the Fe(II), Fe(II)-CO, Fe(II)-NO proteins, in all of which the hydrogen bond network is disrupted and Glu87 has flipped upwards, implies that a subtle difference enables autophosphorylation by the ferrous protein and the CO and NO complexes, but not the Fe(III)-CN complex. It is possible that the conformation of the Fe(III)-CN complex, in which the $\beta 1$ - $\beta 2$ loop is less able than the other two to “search” for the precise conformation that is required for catalysis to occur.

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Table 1. Data collection and refinement statistics

RMSD, root mean square deviation

Data set	DosS-CO PDB ID: 4YNR	DosS-NO PDB ID: 4YOF
Radiation source	ALS 8.3.1	ALS 8.3.1
Space group	P212121	P212121
Unit cell dimensions (a,b,c) (Å)	33.505, 79.247, 109.451	33.593, 78.847, 110.645
Resolution Range (Å)	30 – 1.92 (2.0033-1.9162)	37 - 1.90 (1.9755-1.8995)
Wavelength (Å)	1.115872	0.978550
Total observations	85,537	98,096
Unique reflections (highest shell)	22,480 (3,125)	23,788 (3,757)
Completeness (%) (highest shell)	96.9 (85.4)	99.1 (98.8)
CC(1/2) (highest shell)	99.9 (65.8)	99.9 (79.4)
R _{meas} (highest shell)	.062 (0.75)	.061 (0.62)
<I/σ> (highest shell)	17.8 (1.7)	16.7 (2.49)
Redundancy (highest shell)	3.8 (2.9)	4.1 (4.1)
B factor, Wilson plot (Å ²)	31.3	36.8
Reflections used in refinement	22,479	23,787
Resolution range (Å) used in refinement	19.01 – 1.92	37.14 – 1.90
No. of protein atoms fit	2,156	2,205
No. of heteroatoms fit	90	90
No. of waters fit	141	149
R _{work} %	18.5	18.6
R _{free} %	23.4	22.5
RMSD Bond length (Å)	.008	.008
RMSD Bond angle °	1.31	1.25
Standard Uncertainty Maximum Likelihood (Å)	0.21	0.21
Average B, heteroatoms	29.5	29.6
Average B, protein atoms	43.1	42.2

Table 2. RMSD values for structural elements.

Region ^a	4YNR aligned on		4YOF aligned on	
	Monomer A	Monomer B	Monomer A	Monomer B
Loop β 1- β 2	5.32 ^b	4.66	4.06	3.08
Loop β 4- β 5	4.20	2.57	2.47	1.18
N-term H1	5.34	5.62	4.60	4.78
C-term H6	4.68	5.14	4.15	4.52

^aLoop β 1- β 2 measured at GLN92; Loop β 4- β 5 measured at VAL161 (4YOF/A), VAL162 (4YNR/A), and ARG164 (4YOF/B and 4YNR/B); N-term H1 measured at ASP61 (monomer A) and ASP63 (monomer B); C-term H6 measured at ARG204 (4YOF/A) and ALA203 (others)

^bMonomers were aligned at the heme and the maximum rmsd of a given region in the other monomer was measured in Å

Table 3. Bond angles and bond lengths in the crystal structures of the DosS GAF-A domain Fe(II)-CO and Fe(II)-NO complexes

Crystal structure	N ϵ -Fe-C/N angle Å	Fe-C/N distance Å	N ϵ -Fe distance Å
DosS GAF-A-CO chain A	175.8	1.94	2.16
DosS GAF-A-CO chain B	178.1	1.84	2.08
DosS GAF-A-NO chain A	155.2	1.67	2.07
DosS GAF-A-NO chain B	153.7	1.71	2.08

Figure legends

Fig. 1. Structure of the ferrous, CO-bound DosS GAF-A domain: (A) Crystallographic dimer interface and (B) structure of chain B of the crystallographic dimer with the structural elements differentially colored. The pseudo non-crystallographic 2-fold axis in the dimer is parallel to the H1 and H6 helices and is located between the two monomer structures perpendicular to the plane of the figure. According to convention, in B the N-terminus is blue and the C-terminus red, with each structural element in a color that transitions between these two extremes. The proximal histidine iron ligand is not shown for clarity.

Fig. 2. Illustration of the changes undergone in conversion of the DosS GAF-A domain from: (A) the ferric to the ligand-free Fe(II) state; (B) the ligand-free Fe(II) to the Fe(II)-CO complex; and (C) the ligand-free Fe(II) to the Fe(II)-NO complex. The lower model in each pair is rotated relative to the upper model by 90° about a vertical axis through the iron atom. The structural elements are color coded to indicate RMSD changes, with red representing the largest changes and deep blue the lowest. The specific RMSD values are shown in **Table 2**. In (A), the N- and C-termini of the protein are labeled.

Fig. 3. The DosS GAF-A (A) heme-CO (4YNR) and (B) heme-NO (4YOF) complexes, with the CO and NO ligands, respectively, shown inside positive omit-map density scaled to 3-sigma. For clarity, the surrounding protein atoms have been omitted. This figure was generated with UCSF Chimera [21].

Fig. 4. DosS GAF-A domain active sites from the crystal structures of: (A) the Fe(II)-CO complex, (B) Chain A of the Fe(II)-NO complex, and (C) Chain B of the Fe(II)-NO complex.

Fig. 5. The position of key residues and water molecules in alignments of the unligated Fe(II) DosS GAF-A domain (purple) with: (A) the Fe(III) protein (gray); (B) the Fe(II)-CO complex (yellow); and (C) the Fe(II)-NO (magenta) complex. Water molecules are shown as free-floating spheres.

Fig. 6. Superposition of the crystal structures of the DosS GAF-A domain in the Fe(II)-CO (yellow), Fe(II)-NO (magenta, and Fe(III)-CN (grey) states. The alignment was done by superposition of the heme groups.

Fig. 7. Superposition of the two structures of the DosS GAF-A domain occurring in a single crystallographic dimer. Molecule A (grey), which has been reduced and then undergone autooxidation to the ferric state, has Glu87 flipped downwards toward Tyr171. In Molecule B (aqua), autooxidation has not yet occurred and Glu87 is flipped upwards. The relevant residues are labeled. The structures are taken from PDB files 2W3E and 2W3G [15].

Figure 1

DosS GAF-A Fe(II)-CO

A

B

Figure 2

Figure 3

Figure 4

A. Fe(II)-CO

B. Fe(II)-NO-Chain A

C. Fe(II)-NO-Chain B

Figure 5

Figure 6

DosS GAF-A NO-CO-CN Overlay

Figure 7

DosS GAF-A Re-oxidized Overlay