Distal Hydrogen-bonding Interactions in Ligand Sensing and Signaling by Mycobacterium tuberculosis DosS*

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Mycobacterium tuberculosis DosS is critical for the induction of M. tuberculosis dormancy genes in response to nitric oxide (NO), carbon monoxide (CO), or hypoxia. These environmental stimuli, which are sensed by the DosS heme group, result in autophosphorylation of a DosS His residue, followed by photontransfer to an Asp residue of the response regulator DosR. To clarify the mechanism of gaseous ligand recognition and signaling, we investigated the hydrogen-bonding interactions of the iron-bound CO and NO ligands by site-directed mutagenesis of Glu-87 and His-89. Autophosphorylation assays and molecular dynamics simulations suggest that Glu-87 has an important role in ligand recognition, whereas His-89 is essential for signal transduction to the kinase domain, a process for which Arg-204 is important. Mutation of Glu-87 to Ala or Gly rendered the protein constitutively active as a kinase, but with lower autophosphorylation activity than the wild-type in the Fe(II) and the Fe(II)-CO states, whereas the E87D mutant had little kinase activity except for the Fe(II)-NO complex. The H89R mutant exhibited attenuated autophosphorylation activity, although the H89A and R204A mutants were inactive as kinases, emphasizing the importance of these residues in communication to the kinase core. Resonance Raman spectroscopy of the wild-type and H89A mutant indicates the mutation does not alter the heme coordination number, spin state, or porphyrin deformation state, but it suggests that interdomain interactions are disrupted by the mutation. Overall, these results confirm the importance of the distal hydrogen-bonding network in ligand recognition and communication to the kinase domain and reveal the sensitivity of the system to subtle differences in the binding of gaseous ligands.

Tuberculosis, an airborne disease caused by Mycobacterium tuberculosis, is believed to have infected nearly 1 out of 3 people worldwide (1). Like any infection, tuberculosis leads to a host immune response resulting in recruitment of lymphocytes and macrophages (2). This process is associated with high levels of NO and CO produced by inducible nitric-oxide synthase and hemoxgenase-1 (HO-1), respectively, as part of the defense mechanism of macrophages (3–5). The ability of M. tuberculosis to successfully survive within the host for years in a clinically undetectable dormant state known as non-replicating persistence (NRP), in which it is resistant to most of the currently available treatments, makes it of paramount importance to elucidate this defense strategy (6). The mechanism of NRP is not fully understood; however, hypoxia (7, 8), high CO and NO levels (5, 9), nutrient deprivation (10), and the pH of the microenvironment (11) are among the factors leading to NRP. It has been shown that NRP can initiate changes in energy metabolism and cellular signaling pathways that lead to M. tuberculosis growth arrest. Decreased cellular activity is believed to be the main reason for the long term treatment protocols required to eradicate the bacteria. Moreover, in recent years, multiple drug-resistant and extremely drug-resistant strains of M. tuberculosis have emerged (12). Thus, the primary goal of new drug discovery is to design novel molecules to overcome drug resistance and to shorten the period of treatment.

Dormancy survival proteins DosS, also known as DevS, and DosT play an important role in this context. DosS and DosT are histidine kinase proteins with 61% sequence identity that, after intramolecular phosphorylation of one of their His residues, transfer the phosphate group to Asp-45 of the response regulator protein DosR. This induces the onset of 48 dormancy-related genes (13). DosT is constitutively expressed both in the presence and absence of O2, although DosS is induced by hypoxia as well as by CO and NO, two key players in the host defense mechanism. DosS is also induced by ascorbate and menaquinone under aerobic conditions (14). DosS and DosT have been shown to play an important role in the transition from the replicating state to NRP (15). Recently, Gautam et al. (16) showed that DosS has a critical role in survival of M. tuberculosis in C3HeB/FeJ mice, whereas deletion of DosR and DosT gave rise to a growth curve comparable with that of wild-type M. tuberculosis. Kumar et al. (17) demonstrated the importance of DosS by showing that induction of M. tuberculosis dormancy by CO depended primarily on sensing by DosS. These studies suggest that inhibition of DosS would block M. tuberculosis

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3 The abbreviations used are: NRP, non-replicating persistence; RMSF, root mean squared fluctuation.
from entering into the NRP state and hence that DosS is a potential target for drug design.

DosS contains two adjacent GAF domains, GAF-A and GAF-B. GAF-A has a heme prosthetic group, although the function of the GAF-B domain, which has no heme, remains unclear. DosS also has a kinase core with an ATP binding domain and a histidine phosphate-accepting domain that upon autophosphorylation initiates the signaling cascade (18). The crystal structure of the DosS GAF-A domain identifies His-149 as the proximal iron ligand and Tyr-171 as a highly conserved residue close to the heme (Fig. 1) (19). UV-visible and resonance Raman spectroscopy clearly demonstrate that DosS binds reversibly to O2, CO, and NO (20). In the presence of O2, DosS exhibits little autophosphorylation activity, whereas deoxy DosS and its ferrous CO and NO complexes show strong, time-dependent catalytic activity. The Y171F mutant loses its ability to discriminate between O2, CO, and NO and has little kinase activity with all three ligands; however, the deoxy Y171F mutant retains an activity similar to that of wild-type deoxy DosS (21). The importance of Tyr-171 is supported by the crystal structure, which shows a hydrogen-bonding network extending from a water molecule coordinated to ferric heme center through the side chains of Tyr-171, Glu-87, and His-89 (19). Reduction of the heme iron causes dissociation of the heme-bound water molecule and alteration of the hydrogen-bonding network with repositioning of Glu-87 and His-89 (19). To understand the ability of DosS to differentially sense diatomic gases, we investigated the role of the distal hydrogen-bonding network by site-directed mutagenesis of Glu-87 and His-89 combined with autophosphorylation activity assays, UV-visible and resonance Raman spectroscopy, and molecular dynamics simulations. The effect of Arg-204 was also examined based on the molecular dynamics results.

Materials and Methods

Site-directed Mutagenesis and Cloning of the DosS Distal Mutants—The full-length doss gene containing a C-terminal His, tag and engineered Ndel and BamHI restriction sites, previously constructed by amplification from M. tuberculosis H37Rv genomic DNA into a pET23a+ vector, was used as the template for mutagenesis (20). Site-directed mutagenesis was carried out using the QuikChange Lightning site-directed mutagenesis kit (Agilent, Santa Clara, CA, catalogue no. 210518) according to the prescribed protocol. The plasmid was then digested using Ndel and BamHI restriction enzymes (New England Biolabs, Ipswich, MA) and subcloned into a fresh pET23a+ vector. The resulting plasmid was verified using DNA sequencing. The E87A, E87D, E87G, H89A, H89R, and R204A mutants were prepared by this method.

Expression of the Full-length and Mutant DosS Proteins—Full-length DosS and its mutants were expressed and purified following the previously reported procedure with only minor modifications (20). The full-length doss construct in a pET23a+ vector was transformed into pT-GroE-transformed BL21(DE3) gold competent cells. A single colony was picked from an LB/AMP/chloramphenicol plate and grown overnight in LB medium containing ampicillin (100 μg/ml) and chloramphenicol (50 μg/ml). Approximately 20 ml of the overnight culture was transferred to 1.5 liters of LB culture medium containing ampicillin (100 μg/ml) and chloramphenicol (50 μg/ml). The cells were grown to A600 = 1.5 at 37 °C and 230 rpm, after which 1 mM 5-aminolevulinic acid was added. Protein expression was induced at 18 °C with isopropyl 1-thio-β-D-galactopyranoside at a final concentration of 0.2 mM. The culture was allowed to grow for a further 48 h, after which the cells were harvested by centrifugation at 5000 rpm at 4 °C.

Protein Purification—The cells were lysed in 50 mM phosphate buffer (pH 7.5), containing 250 mM NaCl, 10% glycerol, 1% Triton X-100, 0.5 mg/ml lysozyme, 0.1 mM phenylmethysulfonyl fluoride (PMSF), and one complete EDTA-free protease inhibitor mixture tablet (Sigma and Roche Applied Science catalog no.11873580001). The cell suspension was stirred at room temperature for 30 min, after which the cell membranes were disrupted by repeated sonication cycles at 50% (8 × 45-s cycles with 2 min cooling in-between) using a Branson model 450 Sonifier (VWR Scientific, Visalia, CA). The resulting mixture was centrifuged at 20,000 rpm for 60 min at 4 °C.

The supernatant was applied to a 5-ml HisTrap nickel affinity column (GE Healthcare) followed by washing with 20 column volumes of 50 mM phosphate buffer (pH 7.5), containing 10% glycerol and 300 mM NaCl. The column was further washed with the same buffer containing 10, 20, 40, and 80 mM imidazole (5 column volumes each) before the protein was eluted with a 200 mM final concentration of imidazole. The protein was dialyzed overnight in a dialysis buffer (50 mM Tris-HCl (pH 7.5), containing 5% glycerol and 1 mM EDTA). The protein was then purified using a DEAE-Sepharose column using a gradient elution from 0 to 400 mM NaCl in 50 mM Tris buffer (pH 7.5), containing 5% glycerol and 1 mM EDTA. The fractions contain-
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ing the protein were pooled, concentrated using an Amicon filter (30-kDa MWCO), aliquoted, and stored at -80 °C for future use.

**UV-visible Spectroscopy**—The UV-visible spectra of wild-type DosS and its mutants were obtained on an Agilent Hewlett-Packard 8453 diode-array spectrophotometer. The spectrophotometer was blanked with Tris-HCl buffer (pH 7.5) pretreated with Chelex 100 resin containing 50 mM KCl and 5 mM MgCl₂. The ferric protein was purified with argon and transferred to the glovebox. A few microliters of 100 mM sodium dithionite solution (50 mM) to argon-purged centrifuged relative to indene and CCl₄ and are accurate to ±1 cm⁻¹. CCl₄ was also used to check the polarization conditions. The integrity of the resonance Raman samples was confirmed by direct monitoring of their UV-visible spectra in the Raman capillaries before and after laser illumination.

**Autophosphorylation Assays**—An aliquot of wild-type or mutant DosS in 50 mM Tris-HCl buffer (pH 7.5) was placed inside the glovebox along with sodium dithionite and ATP (Sigma). The proteins were then reduced with excess dithionite (∼10–15 mM) followed by its removal using a Sephadex column (PD-10, GE Healthcare) equilibrated with Tris-HCl buffer (pH 7.5) pretreated with Chelex 100 resin containing 50 mM KCl and 5 mM MgCl₂. The UV-visible spectrum of the resulting protein was measured inside the glovebox to verify the concentration of the Fe(II) protein at the appropriate λmax, using $\epsilon = 130$ mm⁻¹ cm⁻¹. A 40-μl aliquot of the reduced protein was placed into each of four different septum-sealed microcentrifuge tubes. The reactions were initiated by addition of 5 μl of assay buffer, 100 μM final concentration of diethylammonium (Z)-1-(N,N-diethylamino)diazen-1-ium-1,2-diolate, or 5 μl of a saturated solution of CO or O₂. Each tube containing 45 μl of reaction mixture was taken out of the glovebox along with 20 μl of a 2 mM solution of cold ATP (Sigma). A 5-μl aliquot of [γ-³²P]ATP (PerkinElmer Life Sciences, 6000 Ci/mmol, 10 mCi/ml) was added to 20 μl of 2 mM unlabeled ATP solution (Sigma). The phosphorylation reaction was initiated by addition of 5 μl of the above ATP solution to each microcentrifuge tube. All the reaction mixtures (50 μl) contained a final concentration of 5 μM DosS protein, 100 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM MgCl₂, and a final ATP concentration of 200 μM (10 μCi). Aliquots (10 μl) removed at 3-, 6-, 9-, and 12-min time points were mixed with 5 μl of stop buffer (SDS loading buffer containing 10% 2-mercaptoethanol). These samples were loaded onto a 15% polyacrylamide gel (Criterion, Bio-Rad) and run at 150 V for 1 h. The gel was then vacuum-dried at 80 °C for 1 h and subsequently exposed in a PhosphorImager cassette (Molecular Dynamics) for 20 h. The levels of phosphorylated protein were imaged on a Storm 840 PhosphorImager (Amersham Biosciences) and quantified using ImageJ (National Institutes of Health). Phosphorylation of the wild-type Fe(II)-CO-bound protein stopped at the 20-min time point was used as a control for quantification purposes. The loading consistency was confirmed using Coomassie staining.

**Molecular Dynamics Studies**—Starting x-ray crystal coordinates for the wild-type, E87A, E87G mutants of the DosS-GAF-A domain were taken from Protein Data Bank codes 2W3E, 2Y79, and 2Y8H, respectively. The Visual Molecular Dynamics (22) 2.10 psfgen and solvate plugins were used to add hydrogen atoms and bulk solvent (8 Å cushion in the x, y, and z directions from the protein surface) and to generate a PSF topology file for simulation. The input force fields included the CHARMM36 (23) protein force field (top_all36_pro.rtf) for protein atoms, the accompanying water, ion force field for water (toppar_water_ions.str), and heme force field for the DosS heme moiety (toppar_all36_prot_heme.str). Active site crystallographic waters within 4 Å of the heme iron and active site residues 171, 87, and 89 were also included. The iron to nitrogen bond between the heme iron and the coordinating
histidine was added by referencing the PHEM patch available in the heme force field parameters. Charge on the system was neutralized with sodium ions using the Visual Molecular Dynamics Autoionize plugin.

All simulations were carried out on the shared UCSF cluster using the NAMD 2.10 molecular dynamics software package (24). Simulations were carried out at a constant temperature and pressure with rigid bonds set to “ALL,” and periodic boundary conditions. System charges were calculated using the Particle Mesh Ewald method with PMEGridSpacing = 1.0. Additional force field parameters included cutoff distance = 12 Å, switchdist = 10 Å, and pairlistdist = 14 Å. Initial equilibrations of surrounding water were carried out by holding protein atoms fixed for 4000 steps for 1-fs per step. Subsequently, energy minimization was carried out for 2 ns using 1-fs time steps. Simulations for later analysis were then carried out for not less than 40 ns using 2-fs time steps.

Results

**UV-visible Spectral Analysis of Wild-type DosS and Its Mutants**—The Fe(III)-DosS native protein exhibited a Soret absorbance at 407 nm and Q-bands at 500 and 630 nm in the visible region, in accordance with the previously reported spectral values for this protein (25). Reduction of the protein to the Fe(II) state with dithionite caused a red shift of the Soret maximum to 430 nm, with a Q-band at 560 nm. The Fe(II)-CO form of DosS had a strong and sharp absorbance maximum at 422 nm, with a Q-band at 560 nm. The NO-bound protein of DosS had a strong and sharp absorbance maximum at 422 nm, and Q-bands at 500 and 630 nm in the visible region, in accordance with the previously reported spectral values for this protein (25). The E87G mutant had a Soret band at 425 nm (Fig. 2), corresponding to the presence of O2 with a Soret maximum at 421 nm (27). The Fe(II)-O2 form shows a Soret band at 425 nm (Fig. 2, inset). The E87G mutant had a Soret band at 425 nm (Fig. 2, inset). The similarity of the Fe(II)-O2 spectra for the wild-type DosS and the E87G mutant to that of the wild-type protein suggests that the distal mutations do not significantly alter the heme binding pocket in DosS. The UV-visible data clearly suggest that some mutations increased the sensitivity of the heme-oxo complex to autoxidation, but precise autoxidation rates were not measured.

**Resonance Raman of the H89A Mutant**—To better evaluate possible changes at the heme center that might contribute to signaling, we compared by resonance Raman spectroscopy the wild-type and the H89A variant, which has the most differentiated catalytic behavior (see below). High frequency resonance Raman spectra of the ferric and ferrous H89A variant protein obtained with a 413-nm excitation are virtually superimposable to that of wild-type DosS (Fig. 3A), supporting conserved porphyrin geometries, iron spin states, and coordination numbers in both the oxidized and reduced proteins. Specifically, the ferric proteins exhibit a 6-coordinate high spin/low spin mixture assigned to the presence of an aqua/hydroxy distal ligand, although the ferrous proteins are pure 5-coordinate high spin species bound to the proximal histidine, His-149. Low frequency resonance Raman spectra of the reduced proteins obtained with a 442-nm excitation further support the lack of rearrangement of the heme binding pocket in the H89A variant, which exhibits an intense band at 214 cm⁻¹ assigned to an Fe–His stretching vibration, ν(Fe–N_His), and peripheral propionate and vinyl C–C–C deformation modes at 364 and 408 cm⁻¹, as observed previously in wild-type DosS (Fig. 3B).

The resonance Raman spectra of the O2 complexes also show remarkable conservation between the wild-type and H89A proteins. In both wild-type and variant proteins, the Fe–O2 stretching mode, ν(Fe–O2), is observed at 564 cm⁻¹ and shifts to 541 cm⁻¹ with 16O2 (Fig. 4A). Previous studies have shown that the ν(Fe–O2) mode is conserved in full-length DosS and in the truncated GAF-A domain (28). However, different conformers were observed when the CO and NO adducts were compared (28). Specifically, resonance Raman spectra of the wild-type GAF-A domain identified two CO complex conformers as follows: one with a ν(CO) at 1936 cm⁻¹ that represents a carbonyl ligand hydrogen-bonded to Tyr-171, and one with a ν(CO) at 1971 cm⁻¹ that represents a carbonyl ligand free of hydrogen bond interactions. Only the latter conformer was observed in the full-length wild-type protein and in the construct that included both the GAF-A and GAF-B domains (28). These differences between full-length and GAF-A proteins were interpreted in terms of restricted conformational flexibility of the heme distal pocket through interdomain interactions between the GAF-A and GAF-B domains (28). Remarkably, the resonance Raman spectra of the CO complex of full-length H89A shows the same two conformers previously seen only in the truncated GAF-A wild-type construct (Fig. 4B), suggesting that the H89A mutation might disrupt interdomain interactions in DosS and the control it provides on carbonyl conformers at the heme distal pocket.

The resonance Raman characterization of the nitrosyl complexes is less discriminative than for the CO complexes because the ν(NO) frequencies approach those of strong porphyrin vibrations and lead to Fermi coupling between ν(NO) and porphyrin vibrations. Despite these spectral perturbations, 15N16O–15N18O difference spectra of full-length H89A more
closely match those obtained with the truncated GAF-A protein than with wild-type DosS (Fig. 4C). As described previously (28), positive Raman bands at 1638 cm\(^{-1}\) are assigned to \(\nu(\text{NO})\) modes from nitrosyl conformers free of hydrogen bond interactions, although distinctive negative bands near 1530 cm\(^{-1}\) are assigned to \(\nu(15\text{N}18\text{O})\) modes from a nitrosyl engaged in hydrogen bond interaction(s) within the heme distal pocket.

**Kinase Activity of Wild-type DosS and Its Mutants**—The activities of the proteins were measured using an autophosphorylation assay in which the proteins, both in the deoxy and ligand bound states, were incubated with \([\gamma-32\text{P}]\text{ATP}\) for varying times to measure the extent and time dependence of DosS phosphorylation. Previous studies have shown that the Fe(II)-deoxy, Fe(II)-CO, and Fe(II)-NO DosS complexes have significant activity (25, 26, 29). The relative activities for each form of the protein were measured with respect to the corresponding activity of the Fe(II)-CO form of wild-type DosS at the 20-min time point determined simultaneously, as described under “Experimental Procedures.” More importantly, the wild-type and the mutant proteins remained ligand-bound during the entire time of the assay, as judged by UV-visible spectroscopy conducted under identical conditions. Furthermore, adding a saturated solution of an appropriate gas to the deoxy protein or purging the sealed Eppendorf tubes containing the deoxy protein with the gas led to the same results.

Wild-type DosS, as expected, was active in the Fe(II)-deoxy, Fe(II)-NO, and Fe(II)-CO forms and had little activity in the \(\text{O}_2\)-bound form; the general trend in the activity of the wild-
type protein was consistent with earlier reports (25, 26, 29). As we were interested in looking at the initial rate of the reaction, the autophosphorylation reactions were monitored at 3, 6, 9, and 12 min. As shown in Fig. 5A, the activities of the protein in all forms showed a time-dependent increase. Mutations of His-89 to arginine (H89R) and of Glu-87 to aspartic acid (E87D) were expected to preserve the active site hydrogen-bonding network, whereas the H89A and E87G mutants should disrupt this network (Fig. 1).

Distal Histidine Mutants (H89R and H89A)—The H89R mutant on binding of NO or CO was about 50% less active than the corresponding wild-type complexes (Table 1), although the deoxy and Fe(II)-O₂ forms had little activity (Fig. 5B). The H89A mutant, in which the hydrogen-bonding interactions should be completely disrupted, did not show significant autophosphorylation activity for any form of the protein (Fig. 5C and Table 1), demonstrating the importance of this network for protein activity.

Earlier, the important role of Tyr-171 in ligand recognition was demonstrated by the finding that in the Y171F mutant only the Fe(II)-deoxy protein exhibited autophosphorylation activity; all the other forms had little kinase activity (25). The present results argue that disrupting the hydrogen bonding of His-89 to Tyr-171 via Glu-87 interferes with communication of the signal to the kinase domain, as even the deoxy form without a ligand has little activity.

Distal Glutamate Mutants (E87G, E87D, and E87A)—Mutation of Glu-87 to Gly had a minimal effect on autophosphorylation of the Fe(II) deoxy and Fe(II)-NO states (Fig. 5E and Table 1). However, this mutation diminished the activity of the Fe(II)-CO complex. Interestingly, the Fe(II)-O₂ protein had much higher activity than the wild type, which had little activity. Although the E87D mutant was expected to retain its activity on ligand binding, as it can participate in hydrogen-bonding interactions, it was in fact active only with NO, although the Fe(II) and Fe(II)-CO forms had negligible activity, and the mixture of Fe(II)-O₂ and Fe(III) obtained after exposure to O₂ had no measurable activity (Fig. 5F and Table 1). Mutation of Glu-87 to Ala reduced the activity of the Fe(II) and Fe(II)-CO proteins by half compared with the wild type. The mixture of Fe(II)-O₂ and Fe(III) obtained on exposure to O₂ showed a
FIGURE 5. Representative autophosphorylation gel and quantification of the activity of wild-type (A), H89R (B), H89A (C), E87D (D), E87G (E), E87A (F), and R204A (G) mutants of DosS in the Fe(II) state (red) and after its exposure to CO (black), O2 (magenta), and NO (green). 1st lanes, wild-type bound to CO at 20 min; 2nd to 5th lanes, Fe(II); 6th to 9th lanes, exposure of Fe(II) to CO; 10th to 13 lanes, exposure of Fe(II) to O2; and 13th to 17th lanes, exposure of Fe(II) to NO at 3-, 6-, 9-, and 12-min time points, respectively.
modest increase in activity (15%) compared with the wild-type protein (9%), although it was lower than the E87G mutant (38%) (Fig. 5F and Table 1). However, it must be noted that E87G is the only mutant of this position to yield a stable oxy species (Fig. 2D), whereas the others appeared at least partially oxidized. Therefore, the apparent difference in the activities of the oxy states of these mutants may in part reflect oxidation of the E87A and E87D to an essentially inactive ferric state. Overall, the distal glutamic acid mutants recognized and responded to NO as well as the wild-type protein and retained approximately half the activity of the wild type in the Fe(II) deoxy and Fe(II)-CO states.

**Arg-204 Mutant**—His-89 is in a polar patch of residues within the active site (Fig. 1). It is located close to the junction of the GAF-A and GAF-B domains, so its position in the crystal structure of the isolated GAF-A domain may not accurately reflect its position in the full-length protein. Although not seen in the crystal structures (Fig. 1), molecular dynamics simulations (see below) pointed to a persistent hydrogen bond between His-89 and Arg-204. The interaction between His-89 and Arg-204 was therefore investigated by mutating Arg-204 to Ala, a non-polar residue. The UV-visible spectra of the R204A mutant, which were very similar to those of the other mutants (Fig. 2F), showed normal binding of both CO and NO and autodissociation with O₂. Nevertheless, the autophosphorylation activity of the R204A mutant, both in the absence and presence of ligands, was below the limit of detection for all the forms of the protein (Fig. 5G).

**Molecular Dynamics Simulations**—Molecular dynamics simulations have been used to investigate the *in vitro* behavior of the wild-type DosS GAF-A domain and its E87A and E87G mutants. Given the complexities and subtleties of inferring structural changes with different gases, we focused these studies on oxygen binding, as this is the situation in which both the E87A and E87G mutants display particularly unusual behavior.

The E87A and E87G mutants had higher activities on exposure to oxygen than the wild-type protein (Table 1). To interpret these results, we considered the impact of active site water molecules on the hydrogen-bonding network depicted in Fig. 1. The wild-type, E87A, and E87G proteins display distinct hydrogen-bonding patterns that are present at varying frequencies throughout the MD simulations. The most frequent possibilities are portrayed in 6. In Fig. 6A, two bridging waters complete the active site hydrogen-bonding network from Tyr-171 to Glu-87 and then to His-89. This is depicted graphically in 7, where **open circles** indicate the presence of a given bonding network at specific points in the simulation. In the wild-type protein, the Tyr-171–Glu-87 single water bridge (Fig. 7, **blue circles in top panel**) occurs 49.9% of the time, and the Glu-87–His-89 water bridge (Fig. 7 **green circles in top panel**) is present in 50.9% of the simulation, although they are both present 33.5% of the time. The wild-type enzyme may also form a network in which there is a direct hydrogen bond between Tyr-171 and Glu-8, and a salt bridge between Glu-87 and His-89 (Fig.

### TABLE 1
Quantification of autophosphorylation activity of wild-type DosS and its mutants after 12 min

| Protein        | Fe(II) | Fe(II)-CO | Fe(II)-O₂ | Fe(II)-NO |
|----------------|--------|-----------|-----------|-----------|
| WT-DosS        | 43 ± 8 | 60 ± 10   | 9 ± 3     | 70 ± 6    |
| E87G-DosS      | 36 ± 3 | 36 ± 2 (NS) | 38 ± 1 (NS) | 73 ± 1 |
| E87A-DosS      | 23 ± 7 | 30 ± 12 (NS) | 15 ± 5 (NS) | 70 ± 12 |
| E87D-DosS      | 19 ± 7 | 16 ± 5 (NS) | 4 ± 2 (NS) | 59 ± 2 (NS) |
| H89R-DosS      | 11 ± 1 | 32 ± 8 (NS) | 10 ± 2 (NS) | 38 ± 7 (NS) |
| H89A-DosS      | 10 ± 10 | 16 ± 12 (NS) | 5 ± 2 (NS) | 11 ± 5 (NS) |
| R204A-DosS     | ND     | ND        | ND        | ND        |

* p < 0.01.
* p < 0.0001.
* NS mean not significant, p > 0.05.
* ND means not detectable.
6C). This occurs over 25.5% of the simulation and can be seen graphically as black circles in Fig. 7. If we take the overlap of these two cases, the hydrogen-bonding network in the wild-type protein is maintained nearly 60% of the time.

The DosS-E87A mutant does not have the same possibilities for intra-protein hydrogen bonding as the wild type. Because of the non-polar substitution in the E87A mutant, waters are required to bridge the distance from Tyr-171 to His-89. Fig. 6B shows the most common scenario in which two waters bridge the gap. This occurs over 32% of the simulation (Fig. 7, green circles in lower panel). It is worth noting that this largely occurs in the first 13 ns of the 40-ns simulation and is almost never revisited. An alternative in which only one hydrogen-bonding water bridges Tyr-171 and His-89 occurs only 1% of the time (Fig. 7, blue circles in bottom panel). This probably reflects a low sampling of conformations in which Tyr-171 and His-89 are close enough to be bridged by a single water. It is clear from the last two lines in Fig. 7 that the hydrogen-bonding pattern is severely altered in the E87A mutant. In Fig. 6A the active site water network in the wild-type ferric enzyme is compared with that of the wild-type ferrous enzyme (Protein Data Bank code 2W3F) in which the Glu-87 residue has flipped upwards (Fig. 8B). In the latter case the local water network is obliterated, and this is the environment that oxygen encounters when binding to the Fe(II) enzyme. Consequently, the hydrogen-bonding pattern of the wild-type enzyme may function as a switch that must be disrupted to maintain inactivity on oxygen binding.

The effect of global conformational change on the wild-type, E87A, and E87G mutants was analyzed by investigating the root mean squared fluctuation (RMSF) over the course of the simulation. The RMSF of carbon atoms plotted for the E87A (blue) and E87G (orange) mutants are consistently higher than that of the wild-type (green) (Fig. 9A). In particular, the C-terminal helix composed of residues 183–204 shows the most consistent RMSF increases in the variants compared with the wild-type protein. This region of the GAF-A domain would be most likely to significantly affect both the positioning of the rest of the DosS protein and interdomain interactions. To aid in visualizing these fluctuations, an amalgamation of snapshots (every 100 frames) are overlaid in (B and C) for the wild-type and E87A mutants, with each shown in two orientations reflecting a 90° rotation about a vertical axis. The snapshots are color-coded chronologically, with the earliest in darkest blue and the latest in darkest red. The wild-type has considerably less movement than the E87A mutant.

Discussion

DosS is a heme-based sensor that detects changes in intracellular redox state and the presence or absence of gaseous signaling molecules. Understanding the effect of the binding of different gaseous ligands to DosS is important in terms of drug...
therapy, as the dormant state of *M. tuberculosis* is less susceptible to the currently available treatment regimens. A crystal structure of the GAF-A domain of wild-type DosS indicated that the carboxyl group of Glu-87 hydrogen-bonds with the hydroxyl of Tyr-171 and the imidazole of His-89, thus forming a hydrogen-bonding network that may be important in the function of the heme center (Fig. 1) (19). In this study, the role of the hydrogen-bonding network in the differential response to gaseous ligands was examined.

It is well established that both DosS and DosT are active as kinases in the Fe(II)-deoxy, Fe(II)-CO, and Fe(II)-NO forms and have little activity in the Fe(III) and Fe(II)-O₂ forms (20, 26). Although DosT forms a stable Fe(II)-O₂ complex, there is disagreement in the literature as to whether DosS is a gas or redox sensor. Parrish *et al.* (2), Ioanoviciu *et al.* (27), and Sousa *et al.* (29) observed stable formation of the Fe(II)-O₂ complex, although other groups have reported immediate oxidation of the Fe(II) to the Fe(III) heme on exposure to O₂ (19, 26, 27). In this study, the UV-visible spectrum of wild-type DosS was recorded in Tris buffer (pH 7.5). Exposure of the Fe(II) wild-type DosS to O₂ resulted in rapid formation of the Fe(III) state, as reported previously (19, 26). However, on pretreating the buffer with Chelex 100, the Fe(II)-O₂ complex was observed with O₂, emphasizing the importance of metal removal for formation of a stable Fe(II)-O₂ complex. All the experiments performed in this study utilized buffers pretreated with Chelex 100. Although not observed by us (27), Cho *et al.* (19) reported that exposure of the ferrous wild-type DosS GAF-A domain to O₂ resulted in its oxidation to the Fe(III) state even when the buffer was chelated. Clearly, autoxidation of the Fe(II)-O₂ state is highly sensitive to the protein and solution environment. The extent to which the mycobacteria respond to the DosS Fe(II)-O₂ complex versus the ferric protein is unclear, as both states suppress kinase activity, and we have previously shown that endogenous electron donor proteins can reduce ferric DosS to the ferrous state (27). This ambiguity does not occur with DosT, which gives a highly stable Fe(II)-O₂ complex.

DosT exhibits similar activity to DosS in the presence of gaseous ligands, but it contains Gly and Arg residues at the positions occupied in DosS by Glu-87 and His-89, respectively (Fig. 10). Thus, a priori, the E87G and H89R mutants should more closely approximate the environment of DosT in DosS. Furthermore, the DosS E87D mutation should help to preserve the hydrogen-bonding network, whereas the E87A and H89A mutations should disrupt this network. In fact, mutation of Glu-87 to Gly or Ala yielded a protein that formed a stable Fe(II)-O₂ complex or a mixture of the Fe(II)-O₂ and Fe(III) forms, respectively (Fig. 2, D and E), as reported previously (30). Although the E87D mutant also gave rise to a mixture of the Fe(II)-O₂ and Fe(III) forms (Fig. 2C), it exhibited negligible kinase activity (4%) in comparison with the E87G (38%) and E87A (15%) mutants (Table 1). Interestingly, replacement of His-89 by an Ala (H89A) or Arg (H89R) yielded proteins that more readily autoxidized to the ferric state (Fig. 2, A and B), emphasizing the importance of the hydrogen-bonding network in determining the oxidizability of the metal center.

Yukl *et al.* (25) previously demonstrated a critical role for Tyr-171 by establishing that a Y171F mutation disabled kinase autophosphorylation in the presence of all gaseous ligands, while preserving intact the activity of the deoxy state. Tyr-171 is hydrophobic to Glu-87 (Fig. 1). Interestingly, the DosT-like DosS E87G mutant was active in the deoxy form as well as when CO or NO was bound. Most surprisingly, unlike wild-type DosS or DosT, it was also active on binding of O₂. The Fe(II)-O₂ complex of the E87G mutant was red-shifted by 4 nm compared with that of wild-type DosS (Fig. 2D), a difference that may be related to its autophosphorylation activity. Strikingly, both the Y171F and E87G DosS mutants exhibited a loss of ligand discrimination ability, even though their responses were orthogonal to each other. The crystal structure of the E87G GAF-A domain revealed the presence of water molecules at the active site (30). These water molecules, which replace the side chain of the normal Glu residue, can maintain the hydrogen-bonding network from heme to His-89, consistent with our
activity assay results. The E87A mutant, which exhibited a decreased activity in all but the Fe(II)-NO state (Fig. 5F), was shown in a previously published crystal structure of the E87A GAF-A domain to have no clear electron density for any water molecules in the active site (30).

The DosS H89R mutant had a kinase activity profile on binding to CO (active), NO (active), and O₂ (low activity) similar to that of wild-type DosS and DosT, even though the activity was reduced (Fig. 5, A and B, and Table 1). The inactivity of the H89R deoxy form may be due to the steric bulk of the Arg residue, which causes Glu-87 to flip to a conformation similar to that in the ferric protein. In contrast, the H89A mutation rendered the protein essentially inactive. Its inactivity suggested that hydrogen-bonding interactions of His-89 play a key role in signal transduction to the kinase domain. Furthermore, molecular dynamics studies indicated that Arg-204 can form a hydrogen bond to His-89 and suggested that mutation of Arg-204 might inactivate the protein. This was confirmed by our finding that the R204A mutant had no measurable kinase activity (Fig. 5G), a finding that provides strong support for the proposed role of this residue.

The results clearly show that the hydrogen-bonding network is important for differential recognition of the gas ligands. We previously showed that Tyr-171 is critical for ligand discrimination, but here we demonstrate that Glu-87 also plays an important role. The E87G and E87A mutants were active as kinases with all the gas ligands, although the E87D mutant had little activity except when NO was bound. The His-89 residue appears to be critical for signal transduction to the kinase domain, as the H89R mutant, which maintained some hydrogen bonding, was partially active, whereas the H89A mutant had no kinase activity above background levels. The disturbance of the distal hydrogen-bonding network in the H89A mutant is also apparent in the resonance Raman data of the H89A CO and NO complexes as they show multiple conformers previously observed with the truncated GAF-A only domain, but not when the GAF-B domain is present. From these data, it is tempting to suggest that GAF-A/GAF-B interdomain interactions play a role in stabilizing the hydrogen-bonding network at the heme distal pocket and the signal transduction to the kinase domain. Finally, the inference from molecular dynamic simulations that Arg-204 hydrogen-bonds to His-89 and that this interaction is important for signal transmission is supported by the finding that mutation of Arg-204 completely inactivates the full-length protein.

Indirect support for a critical role of the hydrogen-bonding network and an interdomain contact involving His-89 and Arg-204 in signal recognition and transmission is provided by the fact that the UV-visible and resonance Raman properties of the wild-type and H89A mutants are essentially identical, which precludes a role for changes in the iron spin state or porphyrin conformation in either these processes.

As shown by the crystal structures of the GAF-A domain of ferric and ferrous DosS (Fig. 8), the catalytically active ferrous protein has a different hydrogen-bonding network than the ferric protein. The partial kinase activity of the ferrous protein, compared with the CO- or NO-bound proteins, is likely to reflect a more mobile active site in the absence of an iron ligand that allows it to sample both the active and inactive conformations. In the Fe(II)-O₂ complex the terminal oxygen of the dioxygen ligand is a good hydrogen-bond acceptor and, like the water ligand of the ferric state, supports a hydrogen bond network that stabilizes the catalytically inactive state. In contrast, in the Fe(II)-CO and Fe(II)-NO complexes, the poorer hydrogen-bonding properties of the terminal oxygen of the CO and NO give rise to hydrogen-bonding patterns that more closely resemble that of the ferrous, ligand-free, catalytically active protein. In this conformation, Glu-87 swings upward (Fig. 8), and this shift of Glu-87 may be important for activation of the kinase domain.

**Author Contributions**—D. B. did all the analytical work on DosS and its mutants; Y. M. did the molecular dynamics simulations; E. T. Y. carried out resonance Raman spectroscopy; S. S. designed and verified the plasmids, and C. R. N. expressed the E87A DosS mutant. P. M. L. directed and funded the resonance Raman work, and P. R. O. M. conceived, funded, and directed the project. All the authors participated in writing the manuscript.

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