DosT and DevS are oxygen-switched kinases in *Mycobacterium tuberculosis*

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Abstract

Exposure of *Mycobacterium tuberculosis* to hypoxia is known to alter the expression of many genes, including ones thought to be involved in latency, via the transcription factor DevR (also called DosR). Two sensory kinases, DosT and DevS (also called DosS), control the activity of DevR. We show that, like DevS, DosT contains a heme cofactor within an N-terminal GAF domain. For full-length DosT and DevS, we determined the ligand-binding parameters and the rates of ATP reaction with the liganded and unliganded states. In both proteins, the heme state was coupled to the kinase such that the unliganded, CO-bound, and NO-bound forms were active, but the O₂-bound form was inactive. Oxygen-bound DosT was unusually inert to oxidation to the ferric state (half life in air >60 h). Though the kinase activity of DosT was unaffected by NO, this ligand bound 5000 times more avidly than O₂ to DosT (K_d [NO] ~5 nM versus K_d [O₂] = 26 μ M). These results demonstrate direct and specific O₂ sensing by proteins in *M. tuberculosis* and identify for the first time a signal ligand for a sensory kinase from this organism. They also explain why exposure of *M. tuberculosis* to NO donors under aerobic conditions can give results identical to hypoxia, i.e., NO saturates DosT, preventing O₂ binding and yielding an active kinase.

Keywords: FixL; GAF domain; heme-based sensor; histidine-protein kinase; host-microbe interactions; oxygen sensor; sensor kinase; response regulator; signal transduction

Supplemental material: see www.proteinscience.org

Oxygen is an especially important signal molecule to living organisms, and heme-based sensor proteins play a central role in sensing this ligand (Gilles-Gonzalez and Gonzalez 2005). Pathogenic and symbiotic bacteria

should logically possess O₂ sensors to guide their survival, since the availability of O₂ often changes dramatically from the primary site of attachment to the location in the host where the bacteria can best persist. For example, in the obligate aerobe *Mycobacterium tuberculosis*, infection begins with entry into the host via the richly aerated lungs; a macrophage then engulfs this bacterium and confines it into a phagosome, or ultimately a granuloma (Wayne and Sohaskey 2001; Kohler et al. 2002; Schnappinger et al. 2003). While information on the O₂ concentration in tuberculous human lesions is limited, there is clear evidence that O₂ is depleted from blocked human cavities to a sufficient degree that conditions corresponding to those seen in the Wayne-in vitro model of *M. tuberculosis* nonreplicative persistence are attained (Haapanen et al. 1959; Wayne and Hayes 1996; Wayne and Sohaskey 2001). The transcription-factor DevR governs nearly all genetic responses of *M. tuberculosis* to O₂

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Abbreviations: DosT, *M. tuberculosis* sensor kinase encoded by the *Rv2027c* gene; DevR, *M. tuberculosis* response regulator encoded by *Rv3133c* and also called DosR; DevS, *M. tuberculosis* sensor kinase encoded by *Rv3132c* and also called DosS; BjFixL, *Bradyrhizobium japonicum* FixL; RmFixL, *Sinorhizobium meliloti* FixL; GAF, regulatory domain originally named for its association with cGMP-regulated cyclic nucleotide phosphodiesterases, adenylate cyclases, and the bacterial transcriptional regulator FhlA; PAS, signal-detection domain originally named for its association with the Per, ARNT, and Sim proteins; deoxy, Fe^{II} form; oxy, Fe^{II}O₂ form; carbonmonoxy, Fe^{II}CO form; nitrosyl, Fe^{II}NO form.

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limitation (Dasgupta et al. 2000; Sherman et al. 2001; Saini et al. 2002, 2004a,b; Florkczyk et al. 2003; Parish et al. 2003; Park et al. 2003; Timm et al. 2003; Malhotra et al. 2004; Muttucumaru et al. 2004; Roberts et al. 2004; Talaat et al. 2004; Voskuil et al. 2004; Bagchi et al. 2005; Matsoso et al. 2005; Sohaskey 2005; Sharma et al. 2006; Singh et al. 2006; Reed et al. 2007). Given that about two billion people worldwide are infected with *M. tuberculosis*, it is urgently important to understand processes that are thought to assist its survival in its human host (Dye et al. 1999). Among these are the adaptive responses of this pathogen to hypoxia (Wayne and Hayes 1996; Wayne and Sohaskey 2001).

Rhizobia also experience a large drop in the O_2 concentration as they move from their initial site of attachment on a root hair to the site of chronic persistence in a newly formed symbiotic root nodule (Fischer 1994; Dixon and Kahn 2004; Brencic and Winans 2005). In these bacteria, in vitro hypoxia initiates gene-expression cascades similar to those induced during symbiosis with leguminous plants (Fischer 1994; Dixon and Kahn 2004; Brencic and Winans 2005). This leads to the expression of nitrogen-fixation genes as well as genes essential for surviving O_2 deprivation, such as those encoding alternative terminal oxidases for respiration in low O_2 (Fischer 1994; Dixon and Kahn 2004; Brencic and Winans 2005).

The biochemical mechanisms of hypoxic induction are well described for *Sinorhizobium meliloti* and *Bradyrhizobium japonicum*. In both cases, a classical prokaryotic two-component regulatory system made up of the FixL and FixJ proteins governs a direct response to O_2 (David et al. 1988; Sciotti et al. 2003). In FixL, a protein-histidine kinase activity is coupled to a neighboring heme-binding domain, such that O_2 switches off the kinase and hypoxia switches it on (Gilles-Gonzalez et al. 1991; Gilles-Gonzalez and Gonzalez 2005). Under hypoxic conditions, ferrous FixL has no O_2 bound to its heme and is called deoxy-FixL. Deoxy-FixL catalyzes the transfer of a γ -phosphoryl group from ATP to FixJ, a response-regulating transcription factor that functions as FixL's regulatory partner (Gilles-Gonzalez and Gonzalez 1993; Tuckerman et al. 2002; Dunham et al. 2003). The phosphorylation of FixJ causes it to dimerize and bind to target sites in DNA (Da Re et al. 1999). An intermediate of the phosphoryl-transfer reaction is a FixL that becomes transiently phosphorylated. Binding of O_2 to the heme is known to inhibit formation of the phospho-FixL intermediate (Sousa et al. 2005).

FixL has been noted to discriminate against unwanted ligands at the "switching" rather than the "binding" step (Gilles-Gonzalez and Gonzalez 2005). This stands in contrast to a sensor such as the mammalian soluble guanylyl cyclase, which specifically binds NO and rejects the false signal O_2 by using a heme pocket that simply excludes the latter. Oxygen binds less avidly than NO and

CO to FixL, yet O_2 specifically regulates FixL. Therefore FixL achieves its rejection of false signals such as NO, not by failing to bind those ligands, but by ignoring any bound ligand that is not a true signal.

Several lines of evidence indicate that, like the rhizobial FixJ, the DevR transcription factor of *M. tuberculosis* is controlled by one or more sensory protein-histidine kinases that directly detect O_2 . Genetic experiments suggest that DevR is important for survival of the pathogen during hypoxia in vitro (Sherman et al. 2001; Florkczyk et al. 2003; Parish et al. 2003; Park et al. 2003; Timm et al. 2003; Malhotra et al. 2004; Roberts et al. 2004; Saini et al. 2004a; Talaat et al. 2004; Voskuil et al. 2004; Sharma et al. 2006; Reed et al. 2007). Two closely related protein-histidine kinases, DosT and DevS (62.5% sequence identity) are thought to activate DevR, and the DevS protein is known to contain heme (Roberts et al. 2004; Saini et al. 2004a,b; Sardiwal et al. 2005; Ioanoviciu et al. 2007). The domain organization of DevS is reminiscent of that in FixL, with one notable difference being that FixL binds its heme within a PAS rather than a GAF domain (Fig. 1) (Gong et al. 1998). Thus far, only versions of DosT and DevS lacking heme, either because of deletion of their N-terminal region or treatment with denaturants, have been shown to phosphorylate themselves or DevR in vitro (Roberts et al. 2004; Saini et al. 2004a,b). Does the DosT protein contain heme, and if so, does the heme-GAF domain in DosT or DevS direct a response to gaseous ligands? In the current work, we isolate full-length soluble DosT and DevS, each with its full complement of heme, and examine switching in these sensors.

Results

DosT is soluble and contains one heme prosthetic group in its first GAF domain

Expression of the *dosT* gene in modest levels in *Escherichia coli*, and purification of the corresponding protein by relatively gentle methods, showed that the full-length protein is soluble in its native form. A distinctive red color was evident in the harvested cell pellet and became more intense in the cleared lysate and with every subsequent purification step. The purified DosT protein was recovered in the oxy state and had a UV/SoRET absorption ratio ($A_{280nm}/A_{415nm} = R_z$) of 0.6 (Fig. 1A). A pyridine hemochromogen assay of the purified protein's heme content found 1.1 heme b per DosT subunit (Appleby 1980). The site of heme binding in DevS was reported to be the first GAF domain (Sardiwal et al. 2005). Heme is similarly situated in DosT, as evidenced from the heme protein absorption of DosT₁₋₂₀₈ (Fig. 1A, inset).

Figure 1. A heme prosthetic group in full-length DosT and DosT_{1–208}. (A) Absorption spectrum of full-length ferrous DosT in air (red), and under anaerobic conditions (blue). This protein was found, by a pyridine hemochromogen assay, to contain a single heme per monomer. The *inset* shows the electronic absorption spectrum of DosT_{1–208}, i.e., the isolated N-terminal GAF domain of DosT, also in air (red) and under anaerobic conditions (blue). (B) Absorption spectrum of full-length ferrous DevS in air (red), and under anaerobic conditions (blue). The *inset* shows the electronic absorption spectrum of DevS_{1–210}, i.e., the N-terminal GAF domain, also in air (red) and under anaerobic conditions (blue). All proteins were in 50 mM Tris-HCl (pH 8.0) at 23°C. (C) Schematic representation of the DosT domain organization and its comparison to DevS and BjFixL. In *M. tuberculosis* DosT and DevS, a protein-histidine kinase region (HisKA plus HATPase_c regions) is preceded by two tandem GAF domains, and the heme resides in the first GAF domain. In BjFixL, a protein-histidine kinase region is preceded by two tandem PAS domains, and the heme resides in the second PAS domain. The domain boundaries and organizations in this figure were obtained with the SMART home server.

The absorption maxima for ferrous DosT in the deoxy state, or bound to O₂, CO, or NO, resemble those of FixL (Table 1). The spectra of deoxy-DosT indicate a penta-coordinate high-spin heme iron (Fig. 1A; Table 1). Previously, a DosT_{380–573} fragment consisting of only

the C-terminal kinase region was purified as a solubilized protein initially obtained from inclusion bodies (Saini et al. 2004b). This protein showed no sign of heme binding because it lacked the first GAF domain. A full-length S-tagged DosT has also been reported (Roberts et al. 2004). In that case, the solubilization of the protein from inclusion bodies by detergents and high pH had removed the heme, yielding the apoprotein (Roberts et al. 2004). Our discovery of heme in DosT is consistent with this protein's postulated involvement in the hypoxia/latency response of *M. tuberculosis* and with the strong resemblance of DosT to DevS (Fig. 1B; Roberts et al. 2004; Saini et al. 2004b; Sardiwal et al. 2005).

Full-length DevS with stoichiometric heme can be obtained

Modest overexpression of the *devS* gene in *E. coli* also yielded, for the first time, a soluble full-length holo-DevS, although this protein had been predicted to contain transmembrane regions (Fig. 1B; Saini et al. 2004a). The heme content of the full-length DevS, based on a pyridine hemochromogen assay, was 1.0 heme per subunit, in agreement with previous reports of heme binding by the DevS N-terminal GAF domain (Sardiwal et al. 2005; Ioanoviciu et al. 2007). Thus far, spectroscopic studies of DevS have examined only the first GAF domain, due to the reported great challenge of recovering the full-length heme-bound form. For example, despite measures such as the coexpression of a chaperone in *devS*-expressing *E. coli* strains and supplementation of the cultures with hemin to assist folding, elevated overproduction of DevS yielded a species that weakly bound heme ($K_d \sim 3 \mu\text{M}$) (Ioanoviciu et al. 2007). Our success with a slow rate of expression suggests that this might be needed for correct folding of the holo-DevS in *E. coli*.

Inertness of DosT to oxidation

An unusual characteristic of the DosT protein was the extraordinary stability of the ferrous state to O₂ exposure, with the oxidation in air at 37°C being too slow to measure accurately (Fig. 2). Based on multiple-linear-regression analysis of whole spectra, <6% oxidation to the met form (Fe^{III}) was detectable after 16 h of monitoring. We estimate that the half-life of ferrous DosT in air exceeds 60 h. For comparison, note that the half-life of ferrous sperm whale myoglobin in air is about 11 h, and that of ferrous FixL is only 15 min (Quillin et al. 1993; Gonzalez et al. 1998). Addition of electron shuttlers such as methyl viologen (5 μM) accelerated the reaction to about 17% oxidation after 16 h in air (Fig. 2). DosT was also relatively stable toward oxidizing reagents, such as ferricyanide, that are routinely used in

Table 1. Absorption maxima of ferrous-DosT and DevS Species at pH 8.0 and 25°C

	Deoxy, Fe ^{II}		Oxy, Fe ^{II} O ₂			Carbonmonoxy, Fe ^{II} CO			Nitrosyl, Fe ^{II} NO		
	Soret, γ	Q-Band (α/β)	γ	β	α	γ	β	α	γ	β	α
DosT	430	554	415	542	578	422	542	568	420	546	576
DevS	430	558	416	542	578	422	542	568	420	546	575
BjFixL ^a	437	556	419	545	562	427	548	560			

^aGilles-Gonzalez et al. (1994).

stoichiometric amounts with heme proteins to generate the ferric forms instantaneously. Reaction with even a four-fold excess of ferricyanide took several minutes. The strong resistance of DosT to oxidation rules out any possibility that this protein could serve as a sensor of redox potential. It may be that this inertness indicates a built-in protection in DosT against oxidative and nitrosative attack by the phagosomes of macrophages (Kohler et al. 2002; Schnappinger et al. 2003). Compared with DosT, the autooxidation rate of DevS in air was at least 10-fold faster, with the half-life of oxy-DevS being 4 h at 37°C (Table 2; Supplemental Fig. S1). Consequently, the slow oxidation of DosT cannot be simply explained by the association of its heme with a GAF domain. It will be interesting and valuable to understand the basis of the stability of the DosT heme against oxidation, since oxidation is an important process in all O₂-binding heme proteins and is currently one of the problems complicating the development of heme-based blood substitutes.

Equilibrium and kinetic parameters for ligand binding to DosT at 25°C

From direct titrations of the sensors with ligand, we found O₂ to bind without cooperativity (Hill coefficient $n = 1.0$) and with an equilibrium dissociation constant (K_d) of 26 μM for DosT and 3.0 μM for DevS (Fig. 3A,B; Table 2). Oxygen affinities in this range are common in heme-based sensor proteins and no doubt reflect the O₂ concentrations at which hypoxic adaptation becomes necessary for various cell types (Gilles-Gonzalez and Gonzalez 2005). The association-rate constant for binding of O₂ to DosT was 0.79 $\mu\text{M}^{-1}\text{s}^{-1}$, about fivefold higher than for BjFixL, but around 20-fold lower than for sperm whale myoglobin (Fig. 3C; Table 2). This fit a general pattern of the ligand-binding properties of DosT being intermediate between those of heme-PAS sensors and those of myoglobins. For DevS, the O₂ on-rate constant ($k_{\text{on}} = 8.8 \mu\text{M}^{-1}\text{s}^{-1}$) resembled that of sperm whale myoglobin and was 11-fold higher than for DosT (Fig. 3D; Table 2). In contrast to the association rate constants, the values for the O₂ off-rate constants were similar for the two *M. tuberculosis* sensors (DosT $k_{\text{off}} \sim 20 \text{ s}^{-1}$ and DevS $k_{\text{off}} \sim 12 \text{ s}^{-1}$) (Table 2; Supplemental Fig. S2).

Carbon monoxide also bound to DosT without cooperativity (Hill coefficient $n = 1.0$); the K_d value measured by titration with CO was 0.94 μM (Fig. 4A; Table 2). For DevS, the CO affinity was too high to be measured directly, and the K_d value for CO binding was estimated to be about 36 nM from the on- and off-rate constants (Table 2). Whereas DevS was myoglobin-like in its rate of association with CO ($k_{\text{on}} = 1.8 \mu\text{M}^{-1}\text{s}^{-1}$), for DosT the CO on-rate constant was about 40-fold lower ($k_{\text{on}} = 0.050 \mu\text{M}^{-1}\text{s}^{-1}$) and intermediate between those of BjFixL and sperm whale myoglobin (Table 2; Supplemental Fig. S2). The directly measured CO off-rate constant for both DosT and DevS was 0.060 s^{-1} and typical of CO-binding heme proteins (Table 2; Supplemental Fig. S2).

The K_d values for binding of NO were found to be about 5 nM for DosT and 10 nM for DevS by competition of NO against CO (Fig. 4B,C). These values are 1000- to 2000-fold lower than for sperm whale myoglobin (Table 2) (Olson and Phillips 1997). Since NO affinities have been reported for very few heme proteins, one cannot say how the values for DevS and DosT compare with “typical” heme proteins. Similarly, the actual NO concentrations within granulomas are unknown.

Figure 2. Inertness of DosT to oxidation. Absorption spectra of DosT in the presence of 5 μM of methylviologen at time zero (gray curve) and after 16 h (dashed-black curve). Also shown is the spectrum of the ferric form produced by oxidation with ferricyanide (solid-black curve). The inset shows an expansion of the Q-band region of the spectrum. Spectra were recorded in 50 mM Tris-HCl (pH 8.0), at 37°C.

Table 2. Equilibrium and kinetic parameters for DosT and DevS at pH 8.0 and 25°C

Protein	O ₂				CO			NO	ATP
	<i>k</i> _{on} (μM ^{−1} s ^{−1})	<i>k</i> _{off} (s ^{−1})	<i>K</i> _d (μM)	<i>k</i> _{ox} ^a (h ^{−1})	<i>k</i> _{on} (μM ^{−1} s ^{−1})	<i>k</i> _{off} (s ^{−1})	<i>K</i> _d (μM)	<i>K</i> _d (μM)	<i>K</i> _M (μM)
DosT	0.79	20 ^b	26	<0.01	0.05	0.06	0.94	0.005	39
DevS	8.8	12.5	3.0	0.17	1.8	0.06	0.036 ^c	0.020	73
BjFixL ^d	0.14	20	140	2.7	0.0050	0.045	9		47
SW Mb ^e	14	16	1.2	0.060 ^f	0.50	0.019	0.037	0.0000045 ^g	

^aRate of oxidation to the Fe^{III} form: measured for the ferrous form in air at 37°C, except for BjFixL, which was at 25°C.

^bCalculated from *k*_{on} and *K*_d values.

^cCalculated from *k*_{on} and *k*_{off} values.

^dGilles-Gonzalez et al. (1994, 1995). Sperm whale myoglobin data.

^eRohlfs et al. (1990).

^fBrantley et al. (1993).

^gOlson and Phillips (1997).

Divalent-metal requirement of the DosT or DevS reaction with ATP

Protein-histidine kinases require divalent cations for their activity, and the DosT and DevS proteins are typical in that regard, with Mg²⁺ being usually preferred for catalysis, being most effective at physiological concentrations, or both (Fig. 5). An apparent inhibition by Ca²⁺ concentrations above the micromolar level, initially noted for the DosT kinase fragment, appeared to occur for the full-length proteins. For both DosT and DevS, this effect of Ca²⁺ disappeared when a physiological background of Mg²⁺ was supplied (Fig. 5). Therefore, although Ca²⁺ is linked to NO production and macrophage activation (lysosome-phagosome fusion), it is unlikely that an inhibition by Ca²⁺ is physiologically relevant for DosT or DevS.

Oxygen switches off reaction of DosT or DevS with ATP

We investigated whether ligands such as O₂, CO, and NO could regulate the DosT or DevS autophosphorylation with ATP. We chose to examine this reaction because heme-ligand regulation of protein-histidine kinases such as FixL is known to be qualitatively the same for the autophosphorylation as for the turnover reaction, i.e., ligands that do not influence autophosphorylation also fail to affect turnover (Dunham et al. 2003). On the other hand, the protein substrate FixJ was noted to enhance the rates of the FixL kinase reactions (Sousa et al. 2005). Consequently, rates of autophosphorylation, although qualitatively useful, should not be taken to represent the rates of protein-substrate turnover (*k*_{cat}) by the kinases. Measurements of *k*_{cat} require protein substrate that can be quantitatively phosphorylated, and preparations of DevR are being optimized to this end.

The autophosphorylation reactions in Figure 6 demonstrate clearly and for the first time that the *M. tuberculosis* DosT and DevS proteins are heme-based sensors, and that switching is accomplished at physiologically plausible

concentrations of one specific ligand: O₂. The *K*_M values for the autophosphorylation reactions of deoxy-DosT and deoxy-DevS with respect to ATP were 39 and 73 μM, respectively, in a range like that seen for FixL proteins (Table 2; Supplemental Fig. S3). Deoxy-DosT reacted with 500 μM ATP/Mg²⁺ at an initial rate of 3.0% min^{−1} (Fig. 6A). This was about six times faster than a previously reported phosphorylation of the DosT_{380–573} kinase fragment (Saini et al. 2004b). The deoxy-DosT autophosphorylation was also about six times faster than that of FixL (Tuckerman et al. 2002; Dunham et al. 2003). Oxy-DosT had an activity of only 0.062% min^{−1}, i.e., about 50 times lower than that of the deoxy-protein (Fig. 6A,D). For deoxy-DevS, the initial rate of autophosphorylation was 0.25% min^{−1}, a rate close to that reported for FixL, although slower than that of DosT (Fig. 6B; Gilles-Gonzalez and Gonzalez 1993; Tuckerman et al. 2002; Dunham et al. 2003). Binding of O₂ slowed the autophosphorylation rate of DevS to 0.041% min^{−1}: an “inhibited” rate similar to that measured for oxy-DosT (0.062% min^{−1}) (Fig. 6; Supplemental Figs. S4, S5).

For either DosT or DevS, full autophosphorylation activity was restored simply by removing O₂ from the oxy form. Under our assay conditions, the phosphorylated sensors (either DosT with a phosphohistidine at H392 or DevS with a phosphohistidine at H395) were relatively stable to hydrolysis, as verified by inspecting the reaction products for free radiolabeled phosphate on polyethyleneimine thin-layer chromatographic plates (Supplemental Fig. S6). Such verifications show that our kinase preparations contained no phosphatases, spurious or intrinsic. Examination of ferric DevS species showed them to be inactive (Supplemental Fig. S5).

Nitric oxide and carbon monoxide fail to switch off DosT or DevS

In contrast to the strongly inhibited oxy-form of DosT, the fully saturated carbomonoxy and nitrosyl forms of the

Figure 3. Kinetic and equilibrium parameters for binding of O₂ to DosT and DevS (pH 8.0) and 25°C. (A) The equilibrium dissociation constant for binding of O₂ to DosT was determined by titrating the deoxy form (2 μM) with 1–1200 μM O₂. Changes in fractional saturation were calculated from multiple linear regression of whole spectra, taking the deoxy-DosT and O₂-saturated DosT spectra as the bases. The data were fit to a nonlinear Hill equation ($R^2 = 0.999$), from which a $K_d = 26 \mu\text{M}$ ($\pm 1 \mu\text{M}$) and Hill coefficient $n = 1.0$ were determined (GraphPad Prism software version 4.03). (B) The equilibrium dissociation constant for binding of O₂ to DevS was determined similarly by titrating the deoxy form (2 μM) with 0.8–256 μM O₂; the data analysis gave a $K_d = 3.0 \mu\text{M}$ ($\pm 0.5 \mu\text{M}$) and Hill coefficient $n = 1.0$. (C) The on-rate constant for binding of O₂ to deoxy-DosT was determined from examinations of O₂ rebinding to deoxy protein produced from laser-flash photolysis of the O₂-bound form. The disappearance of the deoxy form is shown during bimolecular rebinding of O₂ after the flash photolysis, as monitored at 435 nm. Each k_{obs} represents the average of five measurements of the rate of DosT (4 μM) association at a given concentration of O₂. A k_{on} value of $0.79 \mu\text{M}^{-1}\text{s}^{-1}$ was calculated from the slope of the plotted k_{obs} versus ligand concentration. The inset shows examples of the kinetic traces for binding of DosT to 256, 512, and 770 μM O₂ (black), along with the single-exponential fits that yielded the k_{obs} values (gray). (D) The on-rate constant for binding of O₂ to deoxy-DevS was determined similarly from examinations of O₂ rebinding after laser-flash photolysis of the oxy form and found to be $k_{on} = 8.8 \mu\text{M}^{-1}\text{s}^{-1}$. The inset shows examples of kinetic traces for binding of DevS to 64, 128, and 512 μM O₂ (black), along with the single-exponential fits that yielded the k_{obs} values (gray). All measurements were in 50 mM Tris-HCl, 50 mM KCl, 5% (v/v) ethylene glycol (pH 8.0) at 25°C.

proteins were completely active (Fig. 6; Supplemental Fig. S4). For DevS, the results were qualitatively similar and also showed inhibition for only the oxy-form (Fig. 6; Supplemental Fig. S5). Our results therefore show that DosT and DevS are designed to sense O₂ and to discriminate against regulation by CO or NO (Fig. 6; Supplemental Figs. S4, S5). Since both of these latter nonregulating ligands bind avidly to the proteins, the discrimination in signaling must clearly be effected at the “switching step,” as noted for FixL (Gilles-Gonzalez and Gonzalez 2005).

Our findings of O₂ regulation of DosT and DevS provide the first conclusive demonstration of any heme-based sensor in *M. tuberculosis* (Fig. 6). Previously, DevS was shown to have kinase activity and was independently shown to contain heme, but its kinase activity had not

been reported to respond to a heme ligand. The true test for the establishment of a direct sensor is not the occurrence of ligand binding and enzyme activity in one protein, but rather the demonstration that ligand binding can reversibly switch the enzymatic activity (Gilles-Gonzalez and Gonzalez 2005).

Discussion

To our knowledge, the discovery of O₂ switching of DosT and DevS represents the first identification of a signal ligand for a *M. tuberculosis* kinase (Figs. 6, 7). This work also provides the first evidence of a direct O₂ effect on a component of the *M. tuberculosis* proteome. DosT and DevS are heme-based O₂ sensors with K_d values of 26 and 3 μM, respectively, for binding of O₂ (Fig. 3A,B;

Figure 4. Equilibrium parameters for binding of CO and NO to DosT and DevS (pH 8.0) at 25°C. (A) The equilibrium dissociation constant for binding of CO to DosT was determined by titrating DosT (1 μM) with 0.3–940 μM CO. The changes in fractional saturation were calculated from multiple-linear regression of whole spectra, taking the spectra of deoxy-DosT and CO-saturated DosT as the bases. The data fit well to a nonlinear Hill equation ($R^2 = 0.998$), from which a $K_d = 0.94 \pm 0.04 \mu\text{M}$ and Hill coefficient $n = 1.0$ were determined (GraphPad Prism software version 4.03). (B) The equilibrium dissociation constant for binding of NO to DosT was determined by competitively titrating carbonmonoxy-DosT (equilibrated in 240 μM CO) with 0.50–9.0 μM NO. The changes in fractional saturation were calculated from multiple-linear regression of whole spectra, taking the spectra of carbonmonoxy-DosT and nitrosyl-DosT as the bases. The data analysis yielded a $K_d \sim 5 \text{ nM}$ for binding of NO to ferrous DosT. (C) The equilibrium dissociation constant for binding of NO to DevS was determined similarly by competitively titrating carbonmonoxy-DevS (equilibrated in 10 μM CO) with 0.50–28 μM NO. The data analysis yielded a $K_d \sim 10 \text{ nM}$ for binding of NO to ferrous DevS. All measurements were in 50 mM Tris-HCl, 50 mM KCl, 5% (v/v) ethylene glycol (pH 8.0) at 25°C.

Table 2). As such, these two proteins will be switched on at the various low O_2 concentrations that have been reported for the physiological observations of hypoxic responses by *M. tuberculosis*. In each case, the deoxy form is the active kinase, and conversion to the oxy form switches off the activity by preventing an initial auto-phosphorylation of the sensor with the γ -phosphoryl group from ATP (Fig. 7). This is entirely analogous to the way the deoxy-FixL kinase activity is switched off by O_2 (Gilles-Gonzalez and Gonzalez 1993). In contrast to

FixL, which is slightly inhibited by CO or NO, no regulation was detectable for DosT or DevS saturated with either of these ligands (Fig. 6; Tuckerman et al. 2002; Dunham et al. 2003).

Why feature two sensors for modifying a single-response regulator? One explanation is that the sensors might simply be redundant. Alternatively, the sensors could belong to different physiological states, with DosT being the DevR kinase most likely to survive oxidative assault from the host immune system. Yet another possibility is that the pathogen might require an O_2 dose response achievable only with two sensors of different affinities and kinetics (Table 2; Sousa et al. 2007).

Numerous workers have noted that exposure of *M. tuberculosis* to NO donors under aerobic conditions causes the DevR transcription factor, i.e., a known target of DosT and DevS activation, to induce a similar set of genes as during hypoxia (Kwon 1997; Nathan and Shiloh 2000; Shiloh and Nathan 2000; Chan et al. 2001; Voskuil et al. 2003; Chan and Flynn 2004; Sohaskey 2005; Schnappinger et al. 2006). This observation has, in some cases, been taken to imply that the hypoxic responses of *M. tuberculosis* are somehow mediated by NO. It is important not to interpret a failure to sense O_2 as sensing of NO. The results are more simply explained by NO's avid binding ($K_{d(\text{O}_2)}/K_{d(\text{NO})} \sim 5000$) to DosT, coupled with its incapacity to regulate the kinase. Even at the low (micromolar) levels of NO generated by the NO donors used in in vivo experiments, to achieve concentrations of dissolved O_2 sufficient to displace a significant fraction

Figure 5. Influence of divalent cations on the reactions of DosT and DevS with ATP. Autophosphorylations of deoxy-DosT or deoxy-DevS (4–5 μM protein in 0.5–1 mM ATP) and the indicated divalent cation(s) were carried out at pH 8.0 and 23°C. In the experiments examining the effect of calcium in a high-magnesium background, 0.5 mM MgCl_2 was added to DosT and 1.0 mM MgCl_2 to DevS. Reactions were in 50 mM Tris-HCl, 50 mM KCl, 5.0% (v/v) ethylene glycol (pH 8.0); they were begun by introducing the ATP ($[\gamma\text{-}^{32}\text{P}]$ -labeled, 0.21 Ci/mmol), and they were stopped (at 0.5, 1.0, 2.0, 4.0, 8.0, 10, and 14 min for DosT and 0.5, 1.0, 1.5, 3.0, 6.0, 12, 24, and 48 min for DevS) by mixing 10- μL aliquots of the reaction mixtures with one-third volume of a “stop buffer” containing 40 mM EDTA and 2% (w/v) sodium dodecyl sulfate. The products were electrophoresed, and levels of the phosphorylated protein in dried gels were quantified as described under Materials and Methods. Initial rates of reaction were obtained by fitting each rate curve to a single exponential and obtaining the rate at early times.

Figure 6. Specific sensing of O₂ by DosT and DevS. (A) Kinetics of the phosphorylation of unliganded ferrous DosT (deoxy, open circles), and the O₂- (closed circles), NO- (triangles), and CO-saturated forms (squares) at pH 8 and 23°C. Note the 20-fold expansion of the ordinate for the oxy-DosT data in the inset. For deoxy-DosT, the autophosphorylation rate was determined by fitting the data to a first-order single-exponential equation ($R^2 = 0.995$), and for oxy-DosT the rate was determined by fitting the data to a linear regression ($R^2 = 0.994$). (B) Kinetics of the phosphorylation of unliganded ferrous DevS (deoxy, open circles), and the O₂- (closed circles), NO- (triangles), and CO-saturated forms (squares) at pH 8 and 23°C. The rates were determined by fitting the data to a linear regression ($R^2 \geq 0.99$). (C) Autoradiographs of the gels showing the autophosphorylation time courses for the deoxy (anaerobic) and oxy (O₂) forms. (D) Quantification of the relative autophosphorylation activities of the liganded species, i.e., the percent activity for each ligand-saturated species compared with the same protein in the unliganded state. All autophosphorylations contained 30–40 pmol of protein and 0.5–1 mM ATP/MgCl₂ in 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 5.0% (v/v) ethylene glycol, (pH 8.0) at 23°C. Reactions were begun by introducing the ATP [γ -³²P]-labeled, 0.21 Ci/mmol, and stopped (at 0.5, 1.0, 2.0, 4.0, 8.0, 10, and 14 min for DosT and 0.5, 1.0, 1.5, 3.0, 6.0, 12, 24, and 48 min for DevS) by mixing 10- μ L aliquots of the reaction mixtures with one-third volume of a “stop buffer” containing 40 mM EDTA and 2% (w/v) sodium dodecyl sulfate. Levels of phosphorylated protein in gels dried after electrophoresis or low-molecular weight products on dried thin-layer chromatography plates were quantified as described under Materials and Methods.

of the NO and inhibit DosT, at least five atmospheres of pure O₂ would be required. Likewise, the ligation of CO to aerobic DosT or DevS will cause these sensors to behave as if hypoxically activated.

The results in Figure 6 show that O₂ is the true signal for DosT. *M. tuberculosis* resides mainly in the lungs. So, it is reasonable that the first indication of the formation of granulomas would be a fairly modest hypoxia. The

simplest way to detect hypoxia is by directly sensing O₂. We propose that the DosT and DevS proteins serve as triggers of the *M. tuberculosis* hypoxia/latency response by directly sensing O₂ (Fig. 7).

This study of DosT and DevS was inspired in great part by observations on FixL (Gilles-Gonzalez and Gonzalez 2005). Although the interaction of *M. tuberculosis* with humans is obviously not symbiotic, striking parallels

Figure 7. Schematic representation of the effect of O_2 on the DosT/DevS/DevR system. Under normoxic conditions, i.e., conditions of air saturation ($256 \mu M O_2$), both DosT and DevS will become saturated with O_2 , and their kinase activity will be switched off (top). Under hypoxic conditions, DosT and DevS will exist predominantly in the deoxy state and be active kinases (bottom). Catalysis of phosphoryl transfer from ATP to DevR, by either DosT or DevS, will trigger a hypoxia response: a cascade of gene expression enhancing the production (or activity) of some proteins while decreasing the production (or activity) of others. The hypoxia response is correlated with the latency of *M. tuberculosis*.

exist between the infections by these mycobacteria in humans and the formation of symbiotic root nodules by rhizobia in their leguminous hosts. In both cases, bacteria are switching to a hypoxic lifestyle inside of a eukaryotic cell. FixL proteins trigger cascades of bacterial gene expression in response to hypoxia that eventually lead to a state in which replication is virtually, if not literally stopped (Fischer 1994; Dixon and Kahn 2004; Brencic and Winans 2005). DosT has been proposed to play a similar role (Roberts et al. 2004; Saini et al. 2004b). The morphology of the nonreplicating cells bear some similarities, including an accumulation of cytoplasmic lipid droplets, as observed for *M. bovis BCG* and *Rhizobium etli* (Cevallos et al. 1996; Florczyk et al. 2003). In these respects, the latency of *M. tuberculosis* resembles a symbiosis. For example, leguminous plants typically initiate root nodule formation with symbiotic bacteria when their soil is nitrogen poor, i.e., when resources are scarce. Might a host immune response that would normally completely clear the mycobacteria instead, try to contain them into granulomas when some aspects of immune function are compromised? After controlling the bacteria into a granuloma, the relationship would confer protection to both microbe and host: to the microbe because persistence in a granuloma prevents the host from clearing the infection, and to the host because the granuloma prevents the massive tissue damage seen in full-blown clinical cases of tuberculosis. Others have also noted a potential for a sort of symbiosis between *M. tuberculosis* and their human host, though

arguing from a different set of observations. For example, it has been suggested that the strong T-cell response to an initial *M. tuberculosis* infection, with subsequent granuloma formation, is beneficial to the host for control of the infection and to the pathogen as a means to increase its efficacy of transmission once reactivated from its protracted latency (Flynn and Chan 2005). A latent *M. tuberculosis* infection is not a true symbiosis, of course, but would be more akin to a Faustian bargain struck during inopportune circumstances.

Materials and Methods

Genetic manipulations

The full-length *dosT* and *devS* genes, formerly called *Rv2027c* and *Rv3132c*, respectively, were amplified by PCR with *M. tuberculosis H37Rv* genomic DNA as the template. The oligonucleotide primers for the amplifications were designed to add a NdeI site before the 5' end and an HindIII site after the 3' end of the gene. Fragments encoding the first GAF domain of each protein and consisting of codons 1–208 for *dosT* and 1–210 for *devS* were similarly amplified. Template DNA was a gift from Dr. David Russell (Veterinary Medical Center, Cornell University), and primers were based on the *M. tuberculosis H37Rv* genome database (Cole et al. 1998). The amplified products were inserted as NdeI–HindIII fragments into a pUC19-derived *E. coli* vector. The resulting expression vectors conferred ampicillin resistance and maintained the relevant genes (full-length *dosT*, *dosT*_{1–208}, full-length *devS*, or *devS*_{1–210}) under *tac*-promoter regulation. Cloning of the correct fragments was confirmed by DNA sequencing (McDermott Center for Human

Growth and Development at the University of Texas Southwestern Medical Center).

Gene expression and protein purification

A 4-L culture of *E. coli* strain TGI harboring the *dosT*, *dosT*_{1–208}, *devS*-, or *devS*_{1–210}-bearing plasmid was grown overnight in a Bioflow 3000 fermentor at 37°C, 200–500 rpm, and 20% of atmospheric O₂. When the culture reached an OD_{600 nm} of about 0.5, expression of the recombinant protein was induced with 1 mM IPTG. The harvested cells were lysed by sonication, and the lysate was cleared by centrifugation at 70,000 rpm (Ti 70 rotor, Beckman). Since the cleared lysates of both *dosT*- or *devS*-expressing cells were red, later tracking of the proteins during their purification was done from their 415-nm absorption (Quadt UV/Vis Detector, Bio-Rad). To purify full-length DosT or DevS, the corresponding cleared lysate was brought to 30% saturated ammonium sulfate, and a red precipitate was recovered. This was redissolved in 10% saturated ammonium sulfate and desalted on a size-exclusion column (Sephadex G-25) pre-equilibrated with 50 mM Tris-HCl, 50 mM NaCl, 5% (v/v) glycerol, and 10 mM β-mercaptoethanol (pH 7.5). The protein mixture was chromatographed on an anion-exchange column (DEAE-Sephacel, Amersham) with thorough washing in 100 mM NaCl and elution from 200 mM NaCl in 50 mM Tris-HCl, 5% (v/v) glycerol, 10 mM β-mercaptoethanol (pH 7.5). The heme protein-containing fractions were further purified by gel filtration (Superdex S-200) on a column pre-equilibrated with 50 mM Tris-HCl, 50 mM NaCl, 5% (v/v) glycerol (pH 8.0). Approximately 25 mg of >95% pure DosT or DevS was recovered. The protein concentration was measured by the micro BCA protein assay (Pierce Biotechnology, Inc.), with BSA as the standard. The heme content of the purified proteins was quantified by a pyridine hemochromogen assay, with hemin as the standard (Appleby 1980).

The DosT_{1–208} and DevS_{1–210} truncations were purified by the same series of fractionation steps, with the following variations: A 30%–60% cut of saturated ammonium sulfate was initially used to recover the hemeprotein from the cleared *E. coli* lysate, and the anion-exchange fractionation used loading and elution buffers containing 75 mM NaCl and 150 mM NaCl, respectively, in 50 mM Tris-HCl, 5% (v/v) glycerol, 10 mM β-mercaptoethanol (pH 7.5).

Absorption spectra

All of the spectra were measured on a Cary 4000 UV-Visible Spectrophotometer (Varian) for proteins in 50 mM Tris-HCl, 50 mM KCl and 5% (v/v) ethylene glycol (pH 8.0), and 23°C, unless otherwise stated. Deoxy-DosT or DevS was prepared inside an anaerobic chamber (Coy Laboratory Products, Inc.) by adding dithionite and immediately removing this reducing agent with a desalting column (Sephadex G25). Oxy-DosT or DevS was prepared by mixing the deoxy proteins with O₂- or air-saturated buffer. Carbonmonoxy-DosT or DevS was prepared by mixing the proteins with CO-saturated buffer.

Determinations of equilibrium and kinetic parameters for binding of ligand

All measurements of kinetics used 2–5 μM of DosT in 50 mM Tris-HCl, 50 mM KCl, 5% (v/v) ethylene glycol (pH 8.0) at

25°C. Ligands were prepared in the same buffer. The measurements were done with a LKS-60 stopped-flow/flash photolysis spectrometer fitted with a Pi-star stopped-flow drive unit (Applied Photophysics Ltd.). For sample excitation, the LKS.60 spectrometer was coupled to a Quantel Brilliant B Nd:YAG laser with second-harmonic generation. Data acquisition was provided by an Agilent 54830B digital oscilloscope for fast measurements or a 12-bit ADC card within the instrument work station for slow measurements. To determine the rates of O₂ or CO association, a quartz cuvette was filled in an anaerobic chamber with deoxy-DosT or DevS, and an aliquot of a saturated O₂ or CO solution was added to bring the sample to the desired final concentration of ligand (60–1024 μM for O₂, and 30–480 μM for CO). The cuvette was immediately stoppered and brought to the LKS-60 for measurement. Rebinding of ligand after flash photolysis was followed from the change in the absorbance at 435 nm for O₂, or at 420 nm for CO. At least five kinetic traces were averaged at each ligand concentration. All of the association kinetics fit a single-exponential process, i.e., each with one *k*_{obs} value. The reported association rate constants were determined from the slope of *k*_{obs} (s^{−1}) versus ligand concentration (μM), determined by linear regression (*R*² ≥ 0.99). The entire determinations of the O₂ and CO on-rate constants were repeated at least three times, with several different protein samples.

Rates of CO dissociation from carbonmonoxy-DosT or DevS were followed at 423 nm after mixing a solution of ferrous protein equilibrated with a low CO concentration with a solution supplying a large excess of O₂ in a stopped-flow apparatus. For carbonmonoxy-DosT, equilibration was with 20 μM CO, and 256–1280 μM O₂ were added. For DevS, equilibration was with 0.5 μM CO, and 640 μM O₂ was added.

The rate of O₂ dissociation from oxy-DevS was followed at 423 nm after mixing a solution of ferrous DevS equilibrated with 40 μM O₂ with a solution supplying a large excess of sodium dithionite (1.25 mM), in a stopped-flow apparatus.

The equilibrium dissociation constant for binding of O₂ was directly measured by mixing deoxy-DosT or DevS with buffer (50 mM Tris-HCl, 50 mM KCl, 5% [v/v] glycerol [pH 8.0]) containing 0.80–1200 μM O₂. The basis spectra for the deoxy and oxy states were used to determine the saturation at varying O₂ concentrations by multiple linear combination of whole spectra. A plot of the saturation versus ligand concentration was fitted (*R*² > 0.99) to a nonlinear Hill Plot equation using GraphPad Prism software version 4.03. The equilibrium dissociation constant for binding of CO to deoxy-DosT was similarly determined by directly titrating the protein with buffer (50 mM Tris-HCl, 50 mM KCl, 5% [v/v] glycerol, [pH 8.0]) containing 0.30–940 μM CO. To estimate the equilibrium dissociation constant for binding of NO, ferrous DosT or DevS was equilibrated with CO and competitively titrated with NO. For DosT, equilibration was in 240 μM CO (in 50 mM Tris-HCl, 50 mM KCl, 5% [v/v] glycerol, [pH 8.0]) and the competitive titration was with 0.5–9.0 μM NO. For DevS, equilibration was in 10 μM CO in the same buffer, and the competitive titration was with 0.50–28 μM NO.

Autophosphorylation assays

Deoxy-DosT was prepared inside an anaerobic chamber by incubating the purified protein for more than 15 min with 10 mM dithiothreitol. Oxy-DosT was prepared by adding pure O₂ to the deoxy protein and maintaining a continuous atmosphere of pure O₂ during the phosphorylation reaction. Carbonmonoxy-DosT

was prepared by adding CO-saturated buffer to a final concentration of 100 μ M CO in the reaction mixture. Nitrosyl-DosT was prepared by adding NO-saturated buffer to a final NO concentration of 40 μ M NO. Deoxy-DevS was prepared by reducing this protein with dithionite inside an anaerobic chamber and promptly removing this reducing agent with a gel-filtration column (Sephadex-G25). Conversion to the oxy, carbonmonoxy, or nitrosyl forms was as described for DosT. Ferric DevS was prepared by exposing the protein to an equimolar level of ferricyanide and removing this oxidizing agent with a bio-spin column (Bio-Rad). Cyanomet-DevS was made by adding 20 mM KCN to ferric DevS.

All DosT and DevS species were verified before and after each reaction from the absorption spectra. Assays were done with 4–5 μ M DosT or DevS and 0.5–1.0 mM ATP/MgCl₂ (unlabeled ATP from Sigma and [γ -³²P]ATP from Amersham Pharmacia Biotech, specific activity 0.21 Ci/mmol, in 50 mM Tris-HCl, 50 mM KCl, 5.0% [v/v] ethylene glycol [pH 8.0]) unless otherwise specified. Reactions were begun by introducing the ATP; they were stopped at timed intervals (0.5, 1.0, 2.0, 4.0, 8.0, 10, and 14 min for DosT; 0.5, 1.0, 1.5, 3.0, 6.0, 12, 24, and 48 min for DevS) by mixing 10- μ L aliquots of the reaction mixtures with one-third volume of “stop buffer” (40 mM EDTA, 2% [w/v] sodium dodecyl sulfate, 0.40 M Tris-HCl, 50% [v/v] glycerol, and 2% [v/v] β -mercaptoethanol [pH 6.8]). The products were electrophoresed on 11% (w/v) polyacrylamide gels (Laemmli 1970). To verify the stability of the phosphorylations, aliquots of the stopped reaction mixture (1 μ L) were fractionated on polyethyleneimine-cellulose thin-layer chromatographic (TLC) plates developed with 0.75 M NaH₂PO₄ (pH 3.5). Levels of phosphorylated protein in the dried gels and of low-molecular weight species on the TLC plates were quantified with a PhosphorImager (Bio-Rad Personal Molecular Imager FX).

Electronic supplemental material

Figures showing autoxidation of DevS, determinations of O₂ off-rate from DevS and of the on- and off-rate constants for CO binding to DosT and DevS, determinations of K_M with respect to ATP, raw DosT autophosphorylations, raw DevS autophosphorylations, and verification of the low minimal free-phosphate production from the protein phosphorylations are presented as supplemental information. File name: Dev_DosT_Suppl_Figs.doc

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