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Mycobacterium tuberculosis WhiB1 is an essential DNA-binding protein with a nitric oxide sensitive iron-sulphur cluster

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Abstract

Mycobacterium tuberculosis is a major pathogen that has the ability to establish, and emerge from, a persistent state. Wbl family proteins are associated with developmental processes in actinomycetes, and *M. tuberculosis* has seven such proteins. Here it is shown that the *M. tuberculosis* H37Rv *whiB1* gene is essential. The WhiB1 protein possesses a $[4Fe\text{-}4S]^{2+}$ cluster that is stable in air but reacts rapidly with eight equivalents of nitric oxide to yield two dinuclear dinitrosyl-iron thiol complexes. The [4Fe-4S] form of WhiB1 did not bind *whiB1* promoter DNA, but the reduced and oxidized apo-WhiB1, and nitric oxide-treated holo-WhiB1 did bind to DNA. *Mycobacterium smegmatis* RNA polymerase induced transcription of *whiB1 in vitro*; however in the presence of apo-WhiB1 transcription was severely inhibited, irrespective of the presence or absence of the CRP protein Rv3676, which is known to activate *whiB1* expression. Footprinting suggested that autorepression of *whiB1* is achieved by apo-WhiB1 binding at a region that overlaps the core promoter elements. A model incorporating regulation of *whiB1* expression in response to nitric oxide and cAMP is discussed with implications for sensing two important signals in establishing *M. tuberculosis* infections.

Keywords

actinomycetes; iron-sulphur cluster; nitric oxide; TB; Wbl protein

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AUTHOR CONTRIBUTION

Laura Smith, Melanie Stapleton, Gavin Fullstone, Jason Crack, Salvatore Adinolfi, Debbie Hunt and Evelyn Harvey contributed to the experimental design, carried out the experiments, analyzed the data, and helped with the drafting of the paper. Andrew Thomson, Nick Le Brun, Roger Buxton and Jeffrey Green initiated the project, contributed to the experimental design, data analysis and preparation of the paper.

INTRODUCTION

Approximately one third of the world's population is infected by *Mycobacterium tuberculosis* [1]. Most infected individuals are essentially asymptomatic, a state characterized by the presence of *M. tuberculosis* in a persistent non-replicating state. However, 1 in 10 infected individuals will become ill with active tuberculosis during their lifetimes, contributing to ~1.8 million deaths in 2008 [1]. Thus, prevention of reactivation tuberculosis is a major healthcare challenge and understanding the reprogramming of gene expression that facilitates entry into, and emergence from, the persistent state is an important research goal [2,3].

The *M. tuberculosis* cyclic AMP receptor protein Rv3676 (CRP^{Mt}) is a global regulator that is required for virulence [4]. Amongst the genes regulated by Rv3676 (CRP^{Mt}) are *rpfA* and *whiB1* [4,5]. The *rpfA* gene encodes a protein that is involved in reviving dormant bacteria [6], and *whiB1* encodes a member of a family of proteins (Wbl) that are involved in developmental processes in actinomycetes [7]. Wbl proteins possess four conserved cysteine residues that bind a redox-sensitive iron-sulphur cluster [7-9]. It is assumed that the iron-sulphur cluster will prove essential in controlling Wbl protein function. This assumption is supported by experiments showing that all four conserved cysteine residues are essential for WhiD function in *S. coelicolor* [8]. Recombinant expression and aerobic isolation of all seven Wbl proteins of *M. tuberculosis* resulted in proteins partially occupied by [2Fe-2S] clusters, but after anaerobic reconstitution these proteins contained [4Fe-4S] clusters [9]. The presence of a predicted helix-turn-helix, combined with phenotypic studies of mutants, led to the suggestion that Wbl proteins are DNA-binding transcription factors [7, and references therein]. Consistent with this suggestion, *M. tuberculosis* WhiB3 interacts with promoter DNA of genes known to be differentially regulated in a *whiB3* mutant, and with the essential sigma factor SigA [10-12]. It has also been suggested that Wbl proteins, including *M. tuberculosis* WhiB1, function as protein disulphide reductases [13], although WhiD from *Streptomyces coelicolor* was shown to lack such activity [14].

The *M. tuberculosis* WhiB3 protein contributes to virulence [10] and *whiB3* expression is induced in mouse lungs and macrophages [15]. The WhiB3 [4Fe-4S] cluster is slowly degraded in the presence of O_2 resulting in total cluster loss, in contrast to the rapid reaction with O_2 of the *Escherichia coli* O_2-sensing transcription factor FNR which proceeds by cluster conversion from the [4Fe-4S] to the [2Fe-2S] form [16-18]. The WhiB3 and FNR [4Fe-4S] clusters also reacted with nitric oxide (NO) to form dinitrosyl-iron thiol complexes (DNIC) [16, 19]. Thus, it was suggested that WhiB3 acts as a sensor of the physiologically significant gases O_2 and NO to control expression of genes involved in intermediary metabolism.

Here it is shown that the *M. tuberculosis* H37Rv *whiB1* gene is essential, and that the WhiB1 protein possesses a $[4Fe\text{-}4S]^{2+}$ cluster that reacts rapidly with 8NO molecules per cluster. Furthermore, apo-WhiB1 and NO-treated holo-WhiB1 (but not holo-WhiB1) bind at the *whiB1* promoter (P*whiB1*) to repress transcription. Therefore, it is concluded that the WhiB1 [4Fe-4S] cluster is a NO sensor, and that exposure to NO converts holo-WhiB1 from a non-DNA-binding form to a DNA-binding form capable of regulating transcription.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids are listed in Table 1. *Escherichia coli* cultures were grown in Luria-Bertani (LB) medium [20] in a 1:5 volume/flask ratio at 37°C with shaking at 250 rpm. For isolation of WhiB1, the expression plasmid (pGS2164) was selected for by

addition of kanamycin (50 μg ml^{-1}). DNA was manipulated using standard methodologies [20]. The *whiB1* open reading frame was amplified by PCR using primers MS40 (5′-TTTTTTGAATTCGATTGGCGCCACAAGGCGGT-3′) and MS41 (5′-TTTTTTCTCGAGTCAGACCCCGGTACGGGCTT-3′) containing engineered EcoRI and XhoI sites respectively. The amplified DNA was ligated into the corresponding sites of pET28a. The authenticity of the resulting plasmid (pGS2164) was confirmed by DNA sequencing and encoded a His_6-WhiB1 fusion protein.

Mycobacterium tuberculosis cultures (100 ml) were grown in 1 litre polycarbonate culture bottles (Techmate) in a Bellco roll-in incubator (2 rpm) at 37°C in Dubos broth containing 0.05% (v/v) Tween 80 supplemented with 0.2% (v/v) glycerol and 4% Dubos medium albumin. Middlebrook 7H11 agar was used for growth on plates. Where required, kanamycin was added at a final concentration of 25 μg ml^{-1}, hygromycin at 50 μg ml^{-1}, and gentamycin at 15 μg ml^{-1}.

Creation of a *M. tuberculosis* H37Rv conditional *whiB1* mutant

Approximately 1.5 kb of DNA sequence from each side of the *whiB1* gene (Rv3219) was amplified from *M. tuberculosis* genomic DNA using *PfuUltra* (Stratagene) with the following primer pairs: for the 5′ side Myc642 (5′-GCGGATCCCGAACAGGCACAGCATCA-3′) and Myc643 (5′-GCGGATCCCGCCAATCCATTAGTCGT-3′) (bp 3594224-3595722), and for the 3′ side Myc644 (5′-CGGCGGCCGCGGTCTGACGACTCAGTTCT-3′) and Myc645 (5′-CGGCGGCCGCGACGGTGTCCTGGTGTGC-3′) (bp 3595961-3597391). The 5′ fragments were cloned into the BamHI site and 3′ fragments into the NotI site of the suicide gene delivery vector p2NIL, which is capable of replication within *E. coli* but lacks a mycobacterial origin of replication [21]. A hygromycin cassette was cloned into the KpnI site of p2NIL to replace the *whiB1* gene. A PacI fragment from pGOAL17 containing the *lacZ* and *sacB* genes was inserted to give the completed suicide delivery vector p*whiB1*-KO (Table 1). This vector contains the *sacB* gene from *Bacillus subtilis* that causes lethality when expressed in *M. tuberculosis* making it an effective counter-selectable marker, and a *lacZ* gene. The virulent *M. tuberculosis* strain H37Rv was transformed with p*whiB1*-KO and selection was made for potential single crossovers as kanamycin-and hygromycin-resistant colonies expressing the *lacZ* gene. The veracity of the single crossover event was demonstrated by PCR with the following primers internal to the hygromycin cassette and external to the regions of homology: Myc152 (in Hyg cassette, 5′-CGTTAGAACGCGGCTAC-3′) and Myc1786 (5′-GCCATTGACGATGTGCTG-3′); Myc153 (in Hyg cassette, 5′-GGTCAGCGAACCAATCA-3′) and Myc1789 (5′-GATTACCTGATGTGGGTTCGC-3′); Myc1786 and Myc1787 (5′-GCACCGATTACAGACCAGT-3′); and Myc1789 and Myc1788 (5′-GCAACGCCCGCACGAAAGCC-3′). These colonies were streaked onto plates containing hygromycin to allow for double crossovers to occur and then serially diluted onto plates containing hygromycin, sucrose and X-Gal. No colonies with a double crossover phenotype (Hyg^R, $sucrose^S$ and white on X-Gal) were obtained at this stage suggesting that the *whiB1* deletion was lethal to the bacteria. Accordingly, a single-cross strain was transformed with a complementing plasmid (pDMH1) carrying the *whiB1* gene plus 293 bp 5′-flanking and 14 bp 3′-flanking DNA cloned into the EcoRI site of pKP203 (gentamycin-resistant), an integrase-negative derivative of the integrating vector pMV306. This was prepared by PCR of genomic DNA using the primers Myc1777 (5′-GCGAATTC-GCAAGAAAGCGGATCTGAGC-3′) and Myc1778 (5′-GCGAATTC-AGAACTGAGTCGTCAGACC-3′). Plasmid pDMH1 was co-transformed into the *whiB1* single crossover strain along with plasmid pBS-int carrying the integrase gene necessary to achieve integration of the plasmid into the chromosome. The pBS-int plasmid lacks a

mycobacterial origin of replication and is therefore lost from the bacterium. Using this complemented strain for selection of double crossover events resulted in colonies with the required null phenotype ($Gent^R$, Hyg^R, $sucrose^S$ and white on X-Gal) of which after testing by PCR as above, seven out of eight colonies were genuine double crossovers. This deletion removes all but the initial 11 5′ DNA bases and 3 3′ bases of the *whiB1* gene. Subsequently the complementing plasmid in the *whiB1* null mutant was switched [22] using pKP186 (kanamycin-resistant) plasmids with 1192 bp DNA 5′ to *whiB1* (pDMH2, using primers Myc1779 (5′-GCGAATTCGCCGCGACCTGCTGGCGCAC-3′) and Myc1778) and 1192 bp DNA 5′ and 535 bp DNA 3′ to the *whiB1* gene (pDMH3, using primers Myc1779 and Myc1785 (5′-GCGAATTCCACGATGCGTTGTCGATGTC-3′) to give faster growing colonies (see *Results*).

Quantification of *whiB1* expression by qRT-PCR

RNA isolated as previously described [4] was used to prepare cDNA using a QuantiTect reverse transcription kit (Qiagen). Real-time quantitative PCR on this cDNA was carried out on the ABI Prism 7700 Sequence Detection system using the Fast SYBR Green Master Mix (Applied Biosystems). The primers were designed using the Primer Express software (Applied Biosystems). The forward *whiB1* primer was Myc947 (5′-TTCTTCCCGGTAGGAAACAGTG-3′), the reverse primer was Myc948 (5′-ATTACAGACCAGTTTCGCGTCA-3′). The forward primer from *sigA*, the normalizer gene, was Myc1790 (5′-TCGGTTCGCGCCTACCT-3′), the reverse primer Myc1791 (5′-GGCTAGCTCGACCTCTTCCT-3′).

Overproduction and purification of WhiB1

For isolation of recombinant WhiB1, cultures of the *E. coli* expression strain (JRG6050) were grown at 37°C in Lennox broth [20] to an OD of ~ 0.6, when IPTG (120 μg ml^{-1}) was added and the cultures were incubated at 25°C for a further 2 h. Cells were lysed after resuspension in 20 mM sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl by two passages through a French pressure cell at 37 MPa. The lysate was cleared by centrifugation and the resulting cell-free extract was applied to a nickel-charged Hi-Trap chelating column (GE Healthcare). The recombinant His_6-WhiB1 protein was eluted using a linear imidazole gradient (0-500 mM in 20 ml). WhiB1 containing fractions were either used immediately or stored at −20°C after addition of 1 mM DTT.

Reconstitution of WhiB1

WhiB1 was reconstituted under anaerobic conditions, in a Mark3 anaerobic workstation (DW Scientific). Protein samples were typically incubated with 25 mM Tris buffer (pH 7.4) L-cysteine (1 mM), DTT (2.5 mM) and a 10-fold molar excess of ammonium ferrous sulphate and an aliquot of NifS (225 nM final concentration) as described by Crack *et al.* [23]. Reactions were incubated at room temperature for up to 16 h before purification of the reconstituted protein by chromatography on a 1 ml HiTrap heparin column [24].

Total amino acid analysis, iron and reactive thiol assays

Amino acid analysis was carried out by Alta Bioscience (University of Birmingham, UK) by complete acid hydrolysis for 24 h at 110°C of aliquots of WhiB1 that had been previously assayed for protein concentration using the BioRad protein reagent with bovine serum albumin as the standard. The results of the analyses were used to determine that the BioRad assay overestimated the amount of protein present in the samples and a correction factor of 0.86 was required to obtain an accurate measurement of protein concentration.

Aliquots of reconstituted WhiB1 were used to determine their iron content. Samples were boiled in 1% (w/v) trichloroacetic acid for 5 min. The clarified supernatants were retained and mixed with saturated sodium acetate, bathophenanthroline sulphonic acid (0.3%) and sodium ascorbate (1.8%). After centrifugation the absorbance at 595 nm was measured. The amount of iron present was estimated by comparison to a standard curve prepared using an iron standard solution (BDH).

Reactive thiols were measured according to Thelander [25] using ~4 mM apo-WhiB1 protein in each reaction. Where indicated the apo-WhiB1 protein was denatured by pre-treatment with SDS to a final concentration of 1% (w/v).

Electron paramagnetic resonance (EPR) analysis

WhiB1 samples were transferred to EPR tubes and, where indicated, a 27-fold molar excess of proline NONOate was added for the indicated times before the samples were frozen in liquid nitrogen. EPR spectra were recorded with a Bruker EMX spectrometer equipped with a TE-102 microwave cavity and an ESR-900 helium flow cryostat (Oxford Instruments). Spectra were recorded at both 20 K and 77 K. No $[3Fe\text{-}4S]^{1+}$ signals were detected in any of the nitrosylated samples (not shown). The microwave power and frequency were 2 mW and 9.68 GHz, respectively, and the field modulation amplitude was 1 mT. Spectra were normalized to the same receiver gain. Spin intensities of paramagnetic samples were estimated by double integration of EPR spectra using 1 mM Cu(II), 10 mM EDTA as the standard.

Optical spectroscopy

Scanning spectrometry was carried out using a Cary 50 Bio UV-Vis spectrophotometer using Hellma 10 mm cuvettes with a screw-top lid to maintain anaerobic conditions. Oxygen or NO was injected into the cuvette as air saturated buffer or anaerobic solution of proline NONOate through the Hellma silicone seal in the lid using a Hamilton syringe. The concentration of NO released by aliquots of proline NONOate was measured using a NO electrode (ISO-NO™ Mark II; World Precision Instruments, Stevenage, UK) calibrated using dilutions of a saturated solution of NO gas in H_2O (1.91 mM at 20°C).

Protein disulphide reductase activity

The protein disulphide reductase activity of WhiB1 was measured as described by Holmgren [26] with modifications described by Crack *et al.* [14], except that WhiB1 was used in place of WhiD.

Electrophoretic mobility shift assays (EMSA), DNase I footprints and *in vitro* transcription reactions

The EMSAs were done using the indicated promoter DNA as described by Stapleton *et al.* [5] except that His_6-WhiB1 was used in place of CRP^{Mt}. Apo-WhiB1, reconstituted holo-WhiB1 or holo-WhiB1 treated with a 20-fold molar excess of proline NONOate was used as indicated. Radiolabelled DNA (~2 nM) was incubated with 0-50 μM His_6-WhiB1 in the presence of 40 mM Tris pH 8.0, 1 mM EDTA, 114 mM NaCl, 1 mM DTT, 10 mM $MgCl_2$, 0.25 mg ml^{-1} BSA and 1 μg calf thymus DNA, for 5 min on ice. Where indicated apo-WhiB1 was oxidized by treatment with 5 mM diamide before analysis. The resulting complexes were then separated on 6% polyacrylamide gels buffered with 0.5X TBE (45 mM Tris-borate, 1 mM EDTA). The sizes of the DNA fragments used were: *whiB1*, −289 to −4, obtained from pGS2060; and *rpfA*, −531 to −349; *3616c*, −1119 to−948, and −217 to −16; and *ahpC*, −127 to +1 (all obtained by PCR with genomic DNA as the template). Numbering is relative to the ATG start codons. For the competition assays 100-fold excess unlabelled

whiB1 or *rpfA* DNA was included in the EMSA reactions with radiolabelled *whiB1* as the target.

For DNase I footprinting, radiolabelled P*whiB1* DNA (~60 ng) was incubated with 20 μM apoWhiB1 and 2.5-20 μM CRP^{Mt} in the presence of 40 mM Tris pH 8.0, 75 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 5 % (v/v) glycerol, 1 mM DTT and 250 mg ml^{-1} BSA. The complexes were then digested with 1 unit of DNase I for 15-60 sec at 25°C. Reactions were stopped by the addition of 50 mM EDTA, followed by phenol/chloroform extraction. The DNA was ethanol-precipitated and resuspended in loading buffer (80% v/v formamide, 0.1% w/v SDS, 10% v/v glycerol, 8 mM EDTA, 0.1% w/v bromophenol blue, 0.1% w/v xylene cyanol) for electrophoretic fractionation on 6% polyacrylamide-urea gels and autoradiographic analysis. Maxam and Gilbert G tracks of the DNA fragments were used to provide a calibration [27].

In vitro transcription reactions were as described by Stapleton *et al.* [5] except that apo-WhiB1 was included as indicated. Apo-WhiB1 protein preparations lacked significant deoxyribonuclease and ribonuclease activities.

RESULTS

The *M. tuberculosis whiB1* gene is essential

Attempts to create a *M. tuberculosis whiB1* deletion mutant were unsuccessful. Although single crossovers were obtained it was not possible to select for double crossover events. Therefore, a conditional mutant was created by including a wild-type copy of the *whiB1* gene on a plasmid (pDMH1) together with 293 bp DNA upstream of the *whiB1* open reading frame. When pDMH1 was present, double crossovers that disrupted the chromosomal *whiB1* gene were isolated. When the vector (pKP203) was present, double crossover events were not be selected. Therefore, it was concluded that under the conditions used here the *whiB1* gene is essential for *M. tuberculosis* H37Rv. However, even in the presence of plasmid-encoded WhiB1 (pDMH1), the conditional *whiB1* mutant grew slowly, forming small colonies on 7H11 medium. This growth defect was accounted for by the low level of expression of *whiB1* from pDMH1 in the *whiB1* mutant, which was only ~20% of that obtained from the chromosomal copy of the wild-type strain, as indicated by qRT-PCR. Complementation by a plasmid containing an extra 899 bp upstream of *whiB1* (pDMH2) resulted in an improved growth rate, the formation of normal sized colonies, and an increase in expression of *whiB1* (equivalent to ~65% of the wild type). A plasmid that in addition also contained an extra 521 bp downstream of *whiB1* (pDMH3) resulted in a further increase in *whiB1* expression (equivalent to ~130% of wild type). These observations suggest the presence of positive regulatory elements located upstream of −293 bp in the *whiB1* promoter, and also up to 521 bp downstream of the *whiB1* gene, or that the local structure of the chromatin for the chromosome and plasmid are sufficiently different to facilitate enhanced expression of chromosomal *whiB1* compared to plasmid-borne *whiB1*. The *whiB1* gene has its own promoter [5,29], and the gene downstream of *whiB1* (Rv3220c) has the opposite orientation. Hence, it is unlikely that polar effects could account for either, the apparent essentiality of *whiB1* or the observation that DNA downstream of *whiB1* is needed for full complementation of *whiB1* expression. It should be noted that *whiB1* has previously been reported as a non-essential gene based on transposon site hybridization (TraSH) screening [30], but there are several possible reasons for false negative results, including transposon insertion in non-essential regions either in or adjacent to essential genes resulting in TraSH probes that are still able to hybridize. This could be a particular problem for small genes such as *whiB1*.

WhiB1 contains an O_2-insensitive $[4Fe\text{-}4S]^{2+}$ cluster

The essential nature of *M. tuberculosis whiB1* causes difficulty in assigning function. Several members of the Wbl family of proteins, including WhiB1, have been shown to possess iron-sulphur clusters [9]. Although the properties of these iron-sulphur clusters are relatively poorly characterized, it is assumed that they are important for Wbl protein function. Therefore, the *M. tuberculosis* H37Rv WhiB1 protein was overproduced as a soluble His_6-tagged protein in *E. coli* BL21(λDE3) transformed with pGS2164. On the affinity column a dark brown band was formed, which eluted as pale yellow-brown fractions, with UV-visible spectra typical of a [4Fe-4S] cluster (not shown). Upon longer term storage under aerobic conditions the preparations lost their colour, and in the absence of reducing agents the WhiB1 protein was prone to aggregation. Analysis by non-reducing SDS-PAGE showed the presence of different species with molecular masses and N-terminal amino acid sequences consistent with the presence of monomeric, dimeric and trimeric WhiB1 containing inter- and intra-molecular disulphide bonds (not shown). The presence of disulphide bonds in apo-WhiB1 was confirmed by measuring the amount of reactive thiol using 5,5′-dithio-bis-nitrobenzoic acid (DTNB) in the absence of reducing agents under native conditions and under denaturing conditions (after treatment with 1% SDS). This revealed the presence of two (1.88±0.27, n=9) reactive thiols in the native soluble apo-protein, implying the presence, on average, of one disulphide per apo-WhiB1. The number of reactive thiols per apo-WhiB1 decreased to 1.15±0.15 (n=9) after treatment with SDS, implying that unfolding the protein promoted additional disulphide bond formation (on average 1.5 per monomer). This propensity to form disulphide bonds accounts for the pattern of WhiB1 migration on non-reducing SDS-PAGE.

Reconstitution of the WhiB1 iron-sulphur cluster under anaerobic conditions was accompanied by an increase in absorbance around 420 nm (Figure 1A). Total amino acid analysis confirmed the authenticity of the isolated protein and allowed accurate estimation of protein content such that after removing excess reagents from reconstitution mixtures by chromatography on heparin Sepharose, the iron content of the reconstituted protein was measured as 3.57±0.06 Fe atoms per WhiB1 protein (n=3). In this form the protein was stable for several days if kept under anaerobic conditions and exhibited no tendency to aggregate, unlike apo-WhiB1 (see above). The reconstituted WhiB1 absorbance band at 420 nm was bleached by the addition of the reductant dithionite (Figure 1B). Exposure of the reconstituted protein to O_2, by addition of air-saturated buffer equivalent to a final concentration of 110 μM O_2 for up to 2 h, only slightly decreased absorbance at 420 nm (Figure 1C). Thus, it appears that the WhiB1 [4Fe-4S] cluster is more stable in the presence of O_2 than those of other Wbl proteins (WhiD and WhiB3), which degrade significantly when exposed to air [14,16]. The anaerobic CD spectrum of WhiB1 had positive features at 427 nm and 509 nm (Figure 1D) that were similar to those of WhiD [14], but different from those of the O_2 sensor FNR [23], indicating that the environment of the O_2-insensitive WhiB1 iron-sulphur cluster is different from that of the O_2-sensitive FNR iron-sulphur cluster.

The WhiB1 iron-sulphur cluster is highly sensitive to NO

Nitric oxide is a key component of the host response to mycobacterial infection, and iron-sulphur clusters serve as sensors of NO in some bacterial regulatory proteins (e.g. NsrR and FNR) [31]. Therefore, reconstituted WhiB1 was titrated with the NO releasing compound proline NONOate under anaerobic conditions and the response of the iron-sulphur cluster was monitored by obtaining UV-visible spectra (Figure 2A). Before addition of NO, the extinction coefficient of the WhiB1 iron-sulphur cluster was estimated to be ~18,000 M^{-1} cm^{-1}, which is similar to reported values for other [4Fe-4S] containing proteins. Nitric oxide addition caused decreased absorbance at 420 nm and increased absorbance at ~355 nm, with

apparent isobestic points at 395 nm and 480 nm, indicative of formation of DNICs [19]. Under these conditions 8NO (7.5-8.2) molecules reacted per WhiB1 [4Fe-4S] cluster (Figure 2B), suggesting the formation of an unusual octa-nitrosylated iron-sulphur cluster. The spectral properties of the product and the observed stoichiometry of the reaction are consistent with the formation of two dinuclear DNICs. Accordingly, using the extinction coefficient (~8,500 M^{-1} cm^{-1}) determined by Cruz-Ramos *et al.* [19], the absorbance increase at 360 nm upon completion of the NO titration was equivalent to the formation of ~2.5 dinuclear DNICs per iron-sulphur cluster. Remarkably, unlike the reaction of WhiB1 with O_2, the spectral changes upon exposure to NO were extremely rapid. Using stopped flow to monitor absorbance changes at 364 nm upon mixing of a 92-fold molar excess of NO with holo-WhiB1 (7 μM iron-sulphur cluster) the reaction was complete within 30 sec of mixing; the kinetic details of the reaction of WhiB1 with NO will be reported elsewhere.

Reconstituted WhiB1 under anaerobic conditions was EPR silent, consistent with the presence of a diamagnetic $[4Fe\text{-}4S]^{2+}$ cluster (Figure 3). However, monomeric DNICs have characteristic EPR spectra [32]. After treatment with a 27-fold molar excess of proline NONOate under anaerobic conditions for 10 min an axial EPR spectrum (g = 2.048, 2.03, and 2.022) was detected, indicative of the presence of 6 μM monomeric DNIC arising from 17 μM iron-sulphur cluster (i.e. ~10% of total Fe was present as an EPR detectable monomeric DNIC). Because DTT was present in the reaction buffer, some of this intensity may have arisen from a DTT-coordinated DNIC species. The intensity of the 2.03 signal decreased to that equivalent to 2.8 μM monomeric DNIC (equivalent to 4% total Fe as monomeric DNIC) after further incubation. Taken together with the UV-visible spectra, to which both mononuclear and dinuclear DNICs contribute, the EPR data suggest that exposure of holo-WhiB1 to NO results in the formation of a mixture of EPR active monomeric and EPR silent DNICs, the latter presumed to be the dinuclear form, with the EPR-silent form as the major species. Similar observations were made with the *E. coli* FNR protein after exposure to NO [19].

Under anaerobic conditions the DNIC form of WhiB1 was stable for at least 2 h at 25°C in the presence of 1 mM DTT (Figure 4, spectrum 2). Introduction of air caused the absorbance at 360 nm to decrease by ~20% after 2 h (Figure 4, spectrum 3), and only after >24 h was the DNIC form degraded to apo-WhiB1 (Figure 4, spectrum 4).

WhiB1 is an NO-responsive DNA-binding protein

It has been suggested that Wbl proteins are transcription factors and/or general protein disulphide reductases (see above). Therefore, the protein disulphide reductase activity of apo-WhiB1, prepared by prolonged exposure to air, was tested in an insulin reduction assay [26]. Upon reduction of disulphide bonds the insulin-B chain precipitates, which can be monitored at 600 nm. For comparison *E. coli* thioredoxin was used as a *bone fide* general protein disulphide reductase. Although increased insulin reduction was observed as the concentration of apo-WhiB1 increased, the rates of reaction were slow compared to those obtained with a much lower concentration of thioredoxin and were similar to the rate obtained when both thioredoxin and WhiB1 were omitted from the reactions (Table 2). Garg *et al.* reported significant WhiB1 protein disulphide reductase activity using a similar assay, and later showed that WhiB1 specifically interacted with and was able to reduce disulphide bonds in the α(1,4)-glucan branching enzyme GlgB [13,33]. However, the data reported here indicate that *M. tuberculosis* WhiB1 is not a general protein disulphide reductase, but do not exclude the possibility that apo-WhiB1 could act as a specific protein disulphide reductase.

The DNA-binding ability of WhiB1 was tested using electrophoretic mobility shift assays (EMSA) with the *whiB1* promoter (P*whiB1*) as the target. Apo-WhiB1 (0.08±0.04 Fe atoms

per monomer) in the presence of the reducing agent DTT bound P*whiB1* (Figure 5A, lanes 2-5). The smeared appearance of the EMSAs at lower protein concentrations suggests that the complex is relatively unstable and dissociates during electrophoresis (Figure 5A, lane 3). DNA-binding by WhiB1 was inhibited by the presence of the [4Fe-4S] cluster (Figure 5A, lanes 6-9), but was restored after treatment of holo-WhiB1 with NO (Figure 5B). Furthermore, DNA-binding was abolished following reconstitution of an iron-sulphur cluster into NO-treated WhiB1 (not shown). In the absence of additional reducing agent (DTT) NO-treated WhiB1 formed a second retarded species of lower mobility (Figure 5B, lane 5), which was absent following the addition of DTT (Figure 5B, lane 10). A similar species was also present when apo-WhiB1 was analyzed (Figure 5A, lane 5). Oxidation of apo-WhiB1 by treatment with 5 mM diamide increased the amount of the slow migrating DTT-sensitive complex (Figure 5C, compare lanes 2 and 5). These data, and the relative stability of the DNIC-WhiB1 (Figures 3 and 4), suggests that the aerobic EMSAs shown in Figure 5B contained mostly dinuclear DNIC-WhiB1 mixed with some reduced and oxidized apo-WhiB1. These results are consistent with the assignments of the slower migrating complex as that formed between P*whiB1* and oxidized (disulphide) WhiB1, and the faster migrating complex as that formed between P*whiB1* and reduced (dithiol) apo-WhiB1 and/or DNIC-WhiB1. Specificity of the DNA-binding was demonstrated by the inability of apo-WhiB1 to bind *rpfA*, *ahpC* and Rv*3616c* promoter DNA (not shown). Specificity of DNA-binding was further demonstrated by competition experiments in which a 100-fold excess of unlabelled P*whiB1* DNA inhibited binding of both oxidized (Figure 5C, compare lanes 2 and 3) and reduced (Figure 5D, compare lanes 2 and 3) apo-WhiB1 at labelled P*whiB1*, whereas 100-fold excess unlabelled *rpfA* promoter DNA did not (Figure 5C, compare lanes 2 and 4; Figure 5D, compare lanes 2 and 4). Thus, it was concluded that DNA-binding by WhiB1 is complex and is affected by the oxidation state of the apo-protein and the chemical form ([4Fe-4S] or DNIC) of its cofactor.

DNase I footprinting of apo-WhiB1 at P*whiB1*

Footprinting of apo-WhiB1 at P*whiB1* revealed a distinctive DNase I cleavage pattern characterized by a protected region (W1) located at −42 to −3 relative to the transcript start (Figure 6). The footprint also exhibited several hypersensitive sites, suggesting that apo-WhiB1 causes significant distortion of P*whiB1* (Figure 6). W1 was located downstream of the activating Rv3676 binding site (CRP1, centred at −58.5, [5] and overlapped the repressing Rv3676 binding site (CRP2, centred at −37.5) and the −10 element of P*whiB1*.

Apo-WhiB1 represses *whiB1* transcription *in vitro*

Previous work has shown that *whiB1* expression is activated by intermediate concentrations (2.5 μM) of the *M. tuberculosis* cAMP receptor protein (Rv3676), but that higher concentrations (20 μM) result in repression of *whiB1* expression [5]. *In vitro* transcription reactions confirmed this pattern of regulation (Figure 7, compare lanes 1, 5 and 9). In the absence of Rv3676, apo-WhiB1 significantly inhibited *whiB1* transcription at concentrations as low as 2 μM (Figure 7, lanes 1-4). In the presence of activating concentrations of Rv3676 (Figure 7, lanes 5-8), or in the presence of repressing concentrations of Rv3676 (Figure 7, lanes 9-12), apo-WhiB1 still repressed *whiB1* transcription. It was therefore concluded that apo-WhiB1 acts as an inhibitor of *whiB1* expression, and that this inhibition is more severe than that mediated by high concentrations of Rv3676 and silences any Rv3676-dependent activation of *whiB1* expression.

Discussion

Nitric oxide reacts with proteins that contain iron-sulphur clusters, non-heam iron, heam and thiols [34], and the consequent toxic effects are exploited by host immune systems in the

response to pathogenic bacteria [35], providing the selection pressure for such bacteria to evolve mechanisms for NO detoxification [36]. To mount an effective defence to counter the effects of NO, bacteria must reprogram gene expression. Several NO-sensing regulators are known, including: the non-haem iron protein NorR; the haem-containing DosS-R and DosT-R two-component systems; and the iron-sulphur proteins FNR, IRP1, NsrR, SoxR, and WhiB3 [19,31,37-44]. Reaction of NO with the [4Fe-4S] cluster of the O_2 sensor FNR yields a DNIC form that is incapable of DNA binding [19]. Similarly, NO nitrosylates the [2Fe-2S] cluster of NsrR to inactivate the protein [39]. In contrast, reaction of the SoxR [2Fe-2S] cluster with NO to form a protein bound DNIC activates the protein [40]. The [4Fe-4S] cluster of WhiB3 reacts with NO to form DNIC species, but it is the cluster-free oxidized form that binds DNA with high affinity [12]. IRP1 is a post-transcriptional regulator of iron homeostasis in higher organisms [41]. Like WhiB3, the apo-form of IRP1 binds nucleic acid (in this case mRNA), and its [4Fe-4S] cluster reacts with NO to yield protein bound DNIC [41,42]. Exposure of IRP1 to NO activates mRNA binding, probably by promoting cluster disassembly [43,44]. Here it is shown that the WhiB1 iron-sulphur cluster reacts specifically and very rapidly with NO to convert the protein from a non-DNA-binding form to a DNA-binding form capable of transcription regulation *in vitro*. Expression of *whiB1* in response to NO *in vivo* has not been specifically tested, but the evidence obtained thus far suggests that the essential protein WhiB1 is likely to play a role in establishing *M. tuberculosis* infections by controlling gene expression in response to macrophage-generated or endogenous NO.

Iron-sulphur clusters are intrinsically sensitive to O_2 and thus the stability of the WhiB1 iron-sulphur cluster in air is worthy of comment. The highly conserved CXXCXXXXXC motif in Wbl proteins, which contains three of the four Cys ligands of the iron-sulphur cluster, is also present in the extremely sensitive O_2 sensor FNR (Figure 8) [17]. In the latter, the O_2-sensitivity of the iron-sulphur cluster is determined by the protein fold [17,18,45,46]. A systematic study of FNR in which the amino acid (Ser) located downstream of Cys 2 (labelled 2 in Figure 8) was replaced by all possible alternatives showed that Pro in this position enhanced the stability of the FNR iron-sulphur cluster in air [18]. The presence of Pro immediately adjacent to Cys 2 may go some way to account for the relative O_2-insensitivity of the iron-sulphur cluster of WhiB1 and other Wbl proteins. A second stabilizing substitution is replacement of the Leu residue located upstream of Cys 3 by His [45]. In WhiB1 and WhiD this position is occupied Glu; however a systematic study of the effects of amino acid replacements at this position has not been reported and so the effects of the presence of Glu rather than Leu at this position are unknown.

In spite of its insensitivity to O_2, the WhiB1 [4Fe-4S] cluster is remarkably sensitive to NO, reacting extremely rapidly to generate DNIC species. The CD spectrum of WhiB1 is different from that of the O_2 sensor FNR [23], indicating that the environment of the O_2-insensitive WhiB1 iron-sulphur cluster is different from that of the O_2-sensitive FNR iron-sulphur cluster. Further detailed biochemical analysis will be necessary to determine the molecular basis underpinning the different responses of these two gas sensors.

In contrast to previous reports [13], the data presented here indicate that WhiB1 is not a general protein disulphide reductase (Table 2). Nevertheless, the propensity of the apo-protein to form intra- and inter-molecular disulphide bonds suggests that such redox chemistry is possible, but might require an as yet unidentified reductase and specific target proteins for reduction [33]. WhiB1 interacts with the α(1,4)-glucan branching enzyme GlgB, which is essential for optimal growth of *M. tuberculosis* [30], and thus WhiB1 might be bifunctional, acting as both a gene regulator and a specific GlgB reductase.

The apo-WhiB1 footprint at P*whiB1* showed a 37 bp protected region (−40 to −3, relative to the transcript start) that contained related DNA sequences ($^{-32}$TGACA^{-28} and $^{-14}$TGACA^{-10}), but it would be premature to propose an apo-WhiB1 binding site sequence; further footprints and/or SELEX experiments are necessary. The footprint was characterized by hypersensitive sites spanning a region that was too large to be accounted for by simple binding of an apo-WhiB1 monomer, suggesting multiple apo-WhiB1-DNA interactions (Figure 6). Based on the location of the apo-WhiB1 footprint the simplest explanation for repression of *whiB1* transcription is that apo-WhiB1 blocks RNA polymerase (RNAP) binding. Alternatively, by analogy with WhiB3 [10], apo-WhiB1 could interact with the sigma factor, SigA, to repress *whiB1* transcription by inhibiting RNAP activity or by preventing RNAP from escaping the promoter.

It has recently been shown that upon infection of macrophages a mycobacterial-derived cAMP burst promotes bacterial survival by interfering with host signalling pathways [47]. Moreover, cAMP is important in *M. tuberculosis* gene regulation [4,5,48]. Dual regulation of *whiB1* expression by the cAMP-responsive regulator CRP^{Mt} (Rv3676) and the NO-responsive WhiB1 protein might provide a mechanism to integrate the transcriptional response to two important signals associated with infection (Figure 9). Whatever the detailed mechanism proves to be, the evidence thus far suggests that in the macrophage environment (high NO, high cAMP) transcription of the essential gene *whiB1* will be inhibited. This is consistent with entry into the persistent state. At low NO concentrations (immunocompromised state) holo-WhiB1 is formed, de-repressing *whiB1* transcription, consistent with emergence from the dormant state. The reason for the essentiality of *whiB1 in vitro* is not established, but the data suggest that either sufficient NO is generated during *in vitro* culture of *M. tuberculosis* to activate WhiB1, or that the [4Fe-4S] form regulates as yet unidentified essential gene(s), or that the interaction between WhiB1 and GlgB [30,33] is essential for normal growth. Recall that WhiB2 is also essential and binds DNA in the apo-form [49,50], and that the transcription factor IscR regulates distinct groups of genes in its [2Fe-2S] and apo-forms [51].

In conclusion, it is shown that *M. tuberculosis* WhiB1 is an essential [4Fe-4S] protein that acts as a specific NO-sensing DNA-binding protein. The essential nature of the *whiB1* gene and switching of WhiB1 DNA-binding activity in response to NO has implications for the control of *M. tuberculosis* gene expression in the host environment. Although high NO concentrations kill *M. tuberculosis* [52], lower concentrations promote entry into the latent state [53], and thus NO sensing and gene expression reprogramming by WhiB1 could contribute to developmental adaptations in response to host generated NO.

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Abbreviations used

BSA	bovine serum albumin
CRP^{Mt}	cyclic-AMP receptor protein Rv3676

DNIC	dinitrosyl-iron thiol complex
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
EPR	electron paramagnetic resonance
Gent^{R}	gentamycin resistant
Hyg^{R}	hygromycin resistant
IRP1	iron-regulatory protein-1
Kan^{R}	kanamycin resistant
PBS	phosphate buffered saline
P*rpfA*	the *rpfA* promoter region
P*whiB1*	the *whiB1* promoter region
RNAP	RNA polymerase
rpm	revolutions per min
TBE	Tris-borate EDTA
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TraSH	transposon site hybridization
X-Gal	bromo-chloro-indoyl-galactopyranoside

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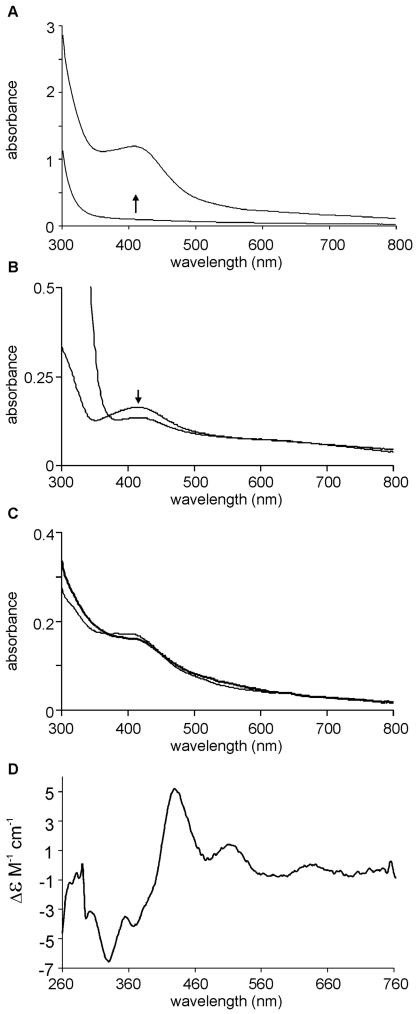

Figure 1. The *M. tuberculosis* WhiB1 protein iron-sulphur cluster can be reconstituted under anaerobic conditions and is O_2 stable

(**A**) Analysis of the WhiB1 (250 μM) reconstitution reaction under anaerobic conditions by UV-visible spectroscopy. The spectra shown are before (lower line) and after (upper line) reconstitution. The arrow indicates the increase in absorbance at 420 nm with time. (**B**) Spectrum of reconstituted WhiB1 (25 μM cluster) in 20 mM sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl and 1 mM DTT before and after treatment with sodium dithionite (0.2 mM for 15 min at 20°C). The arrow indicates the decrease in absorbance at 420 nm. (**C**) Spectra of WhiB1 (10 μM cluster) in 25 mM Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl, 10% glycerol and 1 mM DTT before (thin line) and after (thicker line) incubation for 2 h in the presence of 110 μM O_2. (**D**) CD spectrum of reconstituted WhiB1 (196 μM cluster) in 20 mM sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl.

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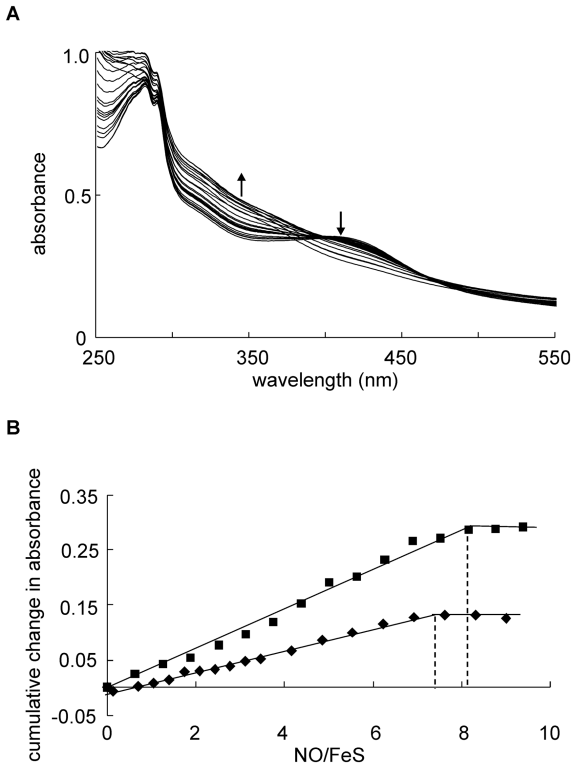

Figure 2. The WhiB1 iron-sulphur cluster reacts with NO

(**A**) Reconstituted WhiB1 (19 μM cluster) was exposed to increasing amounts of proline NONOate under anaerobic conditions and spectra were obtained 10 min after each addition. The arrows indicate the decrease in absorbance at 420 nm and the increase in absorbance at 355 nm as the concentration of proline NONOate was increased. (**B**) The cumulative changes in absorbance at 355 nm (corresponding to the formation of DNICs) from two representative NO titrations under anaerobic conditions in the absence of DTT were calculated taking into account the dilution associated with successive additions of proline NONOate. These values were plotted against the total NO added (μM) divided by the initial concentration of iron-sulphur cluster (μM) in the sample. The dashed vertical lines indicate the points at which further addition of NONOate failed to change the absorbance at 355 nm. All samples were in 25 mM Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl and 10% glycerol.

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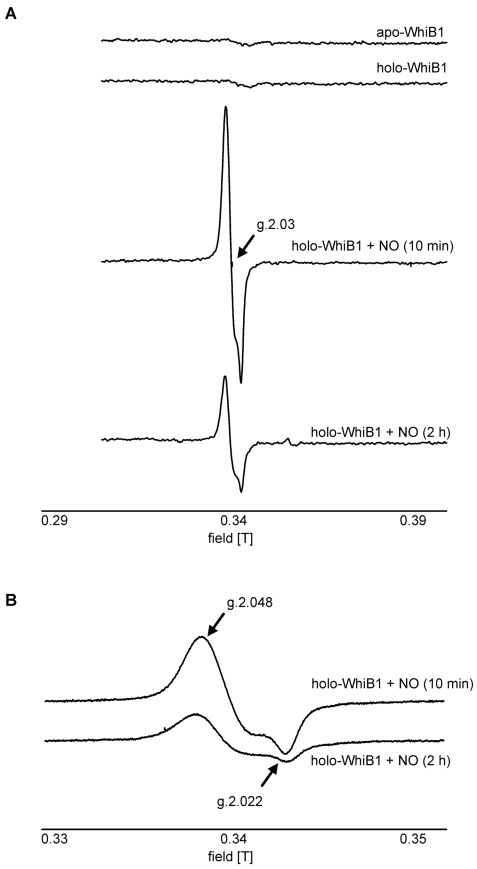

Figure 3. EPR spectra of WhiB1 and the response to NO

(**A**) From top to bottom: apo-WhiB1; anaerobically reconstituted holo-WhiB1; reconstituted WhiB1 treated with a 27-fold molar excess of proline NONOate for 10 min under anaerobic conditions; reconstituted WhiB1 treated with a 27-fold molar excess of proline NONOate for 2 h under anaerobic conditions. Spectra were recorded at 20 K. (**B**) EPR spectra (g = ~2 region) of the EPR active samples shown in panel **A** measured at 77 K for quantification. In all cases the WhiB1 protein concentration was 17 μM. The spectra were obtained using a continuous-wave EPR spectrometer with a microwave frequency of 9.68 GHz operating at a 100 kHz magnetic field modulation. All samples were in 25 mM Tris-HCl buffer (pH 7.4) containing 1 mM DTT.

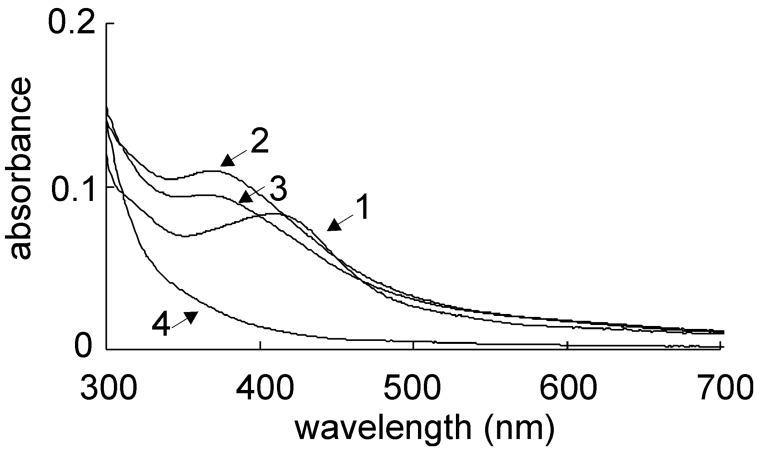

Figure 4. Stability of the DNIC form of WhiB1

Trace 1 shows the spectrum of holo-WhiB1 (4.2 μM cluster). This protein was treated with 400 μM proline NONOate under anaerobic conditions resulting in spectrum 2 (~10 mM DNIC), which was unchanged after 2 h. The cuvette was then uncapped and the contents were exposed to air yielding spectrum 3 after 2 h and spectrum 4 after >24 h. The temperature was 25°C, and all samples were in 25 mM Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl, 1 mM DTT and 10% glycerol.

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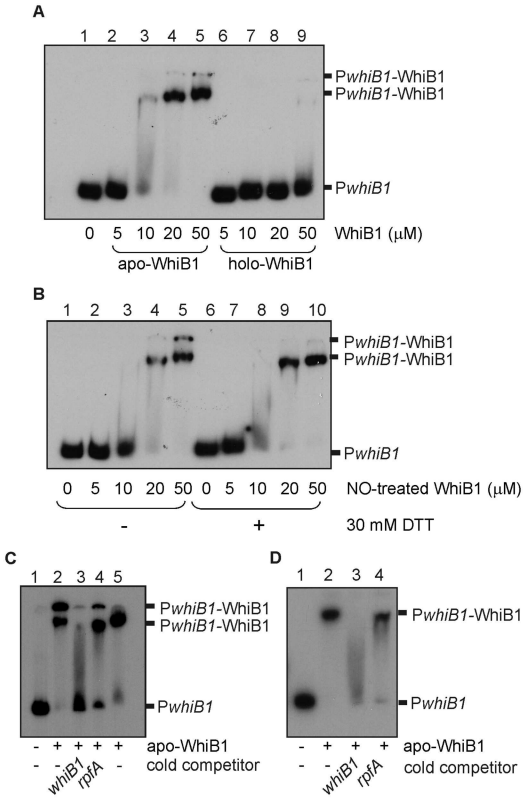

Figure 5. WhiB1 is a DNA-binding protein

(**A**) Binding of WhiB1 at the *whiB1* promoter (P*whiB1*) region. Radiolabelled P*whiB1* was incubated with the indicated amounts of apo- or holo-WhiB1 before separation of protein-DNA complexes (P*whiB1*-WhiB1) by electrophoresis. (**B**) Binding of NO-treated holo-WhiB1 to P*whiB1*. A 20-fold molar excess of NO (as proline NONOate) was added to holo-WhiB1 and after incubation for 1 min at 20°C an aliquot was removed and DTT was added to a final concentration of 30 mM. After further incubation at 20°C for 3 min EMSAs were done using the DTT-treated and untreated samples as described in *Materials and Methods*. The final concentrations of WhiB1 protein are indicated. (**C**) Oxidation of WhiB1 results in the formation of a second retarded species. Binding of oxidized apo-WhiB1 (20 μM, pre-treated with 5 mM diamide) to radiolabelled P*whiB1* (lane 2) was challenged by the presence of 100-fold excess unlabelled P*whiB1* DNA (lane 3) or 100-fold excess unlabeled P*rpfA* DNA (lane 4). Lane 5 shows the EMSA with reduced (1 mM DTT) apo-WhiB1 and P*whiB1*. (**D**) Apo-WhiB1 binding at P*whiB1* is specific. Binding of apo-WhiB1 (20 μM) in the presence of DTT (1 mM) to radiolabelled P*whiB1* (lane 2) was challenged by the presence of 100-fold excess unlabelled P*whiB1* DNA (lane 3) or 100-fold excess unlabeled P*rpfA* DNA (lane 4).

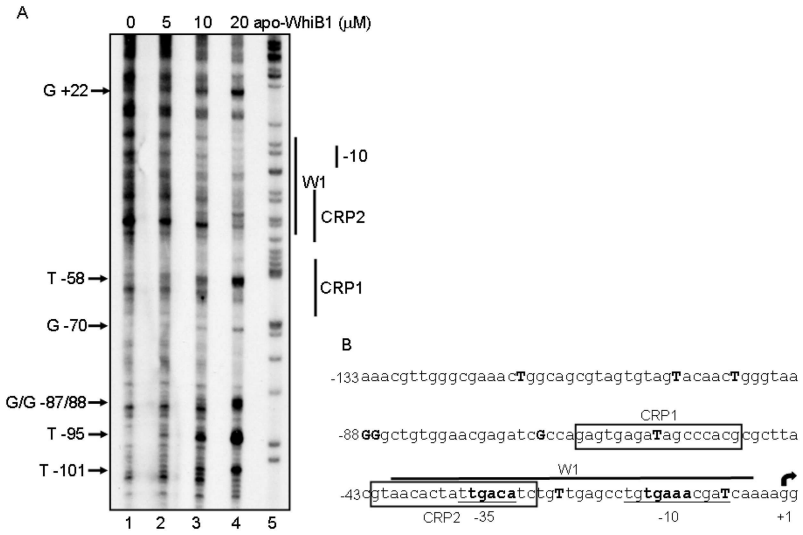

Figure 6. DNase I footprint of apo-WhiB1 at P*whiB1*

(**A**) Increasing concentrations of apo-WhiB1, as indicated above each lane, were incubated with P*whiB1* before DNase I digestion as described in *Materials and Methods*. Hypersensitive sites associated with interaction with apo-WhiB1 are arrowed; numbering is relative to the transcriptional start site. The locations of apo-WhiB1 interaction (W1), the previously identified Rv3676 (CRP^{Mt}) binding sites (CRP1 and CRP2), and the −10 element are indicated. Lane 5 shows a Maxam and Gilbert G track. (**B**) Nucleotide sequence of P*whiB1* showing: the transcript start (+1, block arrow), the −10 and −35 elements (underlined), the tandem Rv3676 (CRP^{Mt}) binding sites (boxed, CRP1 and CRP2), the hypersensitive sites that characterize interaction with apo-WhiB1 (bold upper case), the location of the protected region (W1; overlined), and two related DNA sequences within the protected region (bold lower case) are indicated.

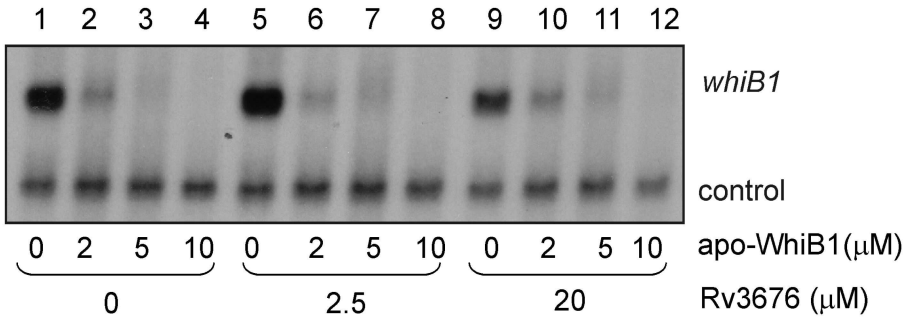

Figure 7. Apo-WhiB1 inhibits transcription of *whiB1 in vitro*

Reactions contained: 0.1 pmoles P*whiB1*, 1 pmole *M. smegmatis* RNAP, 40 mM Tris-Cl pH 8.0, 10 mM $MgCl_2$, 70 mM NaCl, 1 mM EDTA, 1 mM DTT, 250 μg ml^{-1} BSA, 5% glycerol. Proteins (Rv3676 and apo-WhiB1) were pre-incubated with P*whiB1* for 10 min at 37°C before addition of nucleotide triphosphates and further incubation for 10 min at 37°C. The location of the *whiB1* transcript, the loading control, and the concentrations of the Rv3676 and apo-WhiB1 proteins, are indicated.

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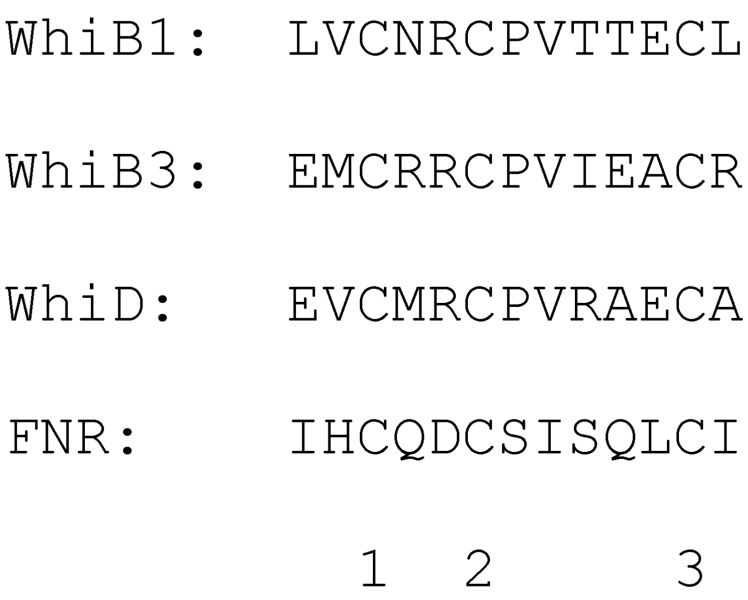

Figure 8. Alignment of the conserved cysteine-rich motif in Wbl proteins and the O_2-sensor FNR
The protein sequences aligned are the Wbl-family proteins WhiB1 and WhiB3 from *M. tuberculosis*, WhiD from *S. coelicolor*, and the O_2-sensing transcription factor FNR from *E. coli*. The sequences are shown in single letter amino acid code and the Cys residues are labelled 1-3 for ease of identification in the text.

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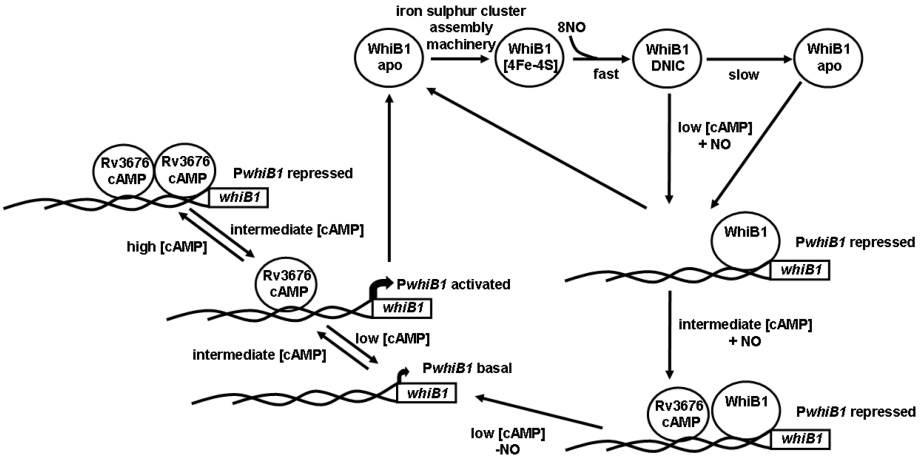

Figure 9. Nitric oxide- and cAMP-mediated regulation of *whiB1* expression

The forms of WhiB1 are represented by labelled circles, where no specific form is indicated this represents a mixture of oxidized and reduced apo-WhiB1 and DNIC-WhiB1. The *whiB1* promoter DNA (P*whiB1*), Rv3676 (labelled circles), NO and iron-sulphur cluster biosynthesis machinery are also indicated. From bottom left, when cAMP levels are low and NO is absent, transcription of *whiB1* occurs at basal levels. At intermediate concentrations of cAMP, expression of *whiB1* is activated by the *M. tuberculosis* CRP protein (Rv3676) binding at class I site to allow increased synthesis of apo-WhiB1 [5]. At high concentrations of cAMP, Rv3676 occupies tandem sites at P*whiB1* and represses *whiB1* expression [5]. Apo-WhiB1 protein acquires a $[4Fe\text{-}4S]^{2+}$ cluster by the action of the iron-sulphur cluster biosynthesis system (Figure 1), resulting in the non-DNA-binding form. Each WhiB1 $[4Fe\text{-}4S]^{2+}$ cluster reacts extremely rapidly with 8NO (Figure 2) to produce a mixture of mononuclear and dinuclear dinitrosyl-iron complexes (DNIC; Figures 2 and 3). In the presence of O_2, the DNIC form of WhiB1 slowly degrades to yield apo-WhiB1 (Figure 4). DNA-binding activity has been demonstrated for reduced and oxidized apo-WhiB1 and NO-treated WhiB1 (Figures 5 and 6), but it is not yet known whether the minority mononuclear and majority dinuclear DNIC forms possess DNA-binding activity, or whether they are intermediate species that yield the proven DNA-binding form, apo-WhiB1; however, the relative stability of the DNIC form (Figure 4) and the EPR spectra (Figure 3), suggest that the EMSAs with NO-treated WhiB1 contain mostly dinuclear DNIC WhiB1. The DNA-binding apo-WhiB1 severely represses *whiB1* expression *in vitro* in the presence and absence of the *M. tuberculosis* CRP, Rv3676, by interactions at site(s) overlapping the −10 and −35 elements of the promoter (Figures 6 and 7). Apo-WhiB1 can be switched off by the action of the iron-sulphur cluster assembly machinery to regenerate the non-DNA-binding holo-WhiB1 resulting in derepression of *whiB1* expression and synthesis of apoWhiB1 protein.

Table 1

Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
E. coli strains		
BL21 (λDE3)	Lysogen of λDE3 carrying a copy of the T7 RNAP under the control of the IPTG-inducible *lacUV5* promoter	Novagen
JRG6050	BL21 (λDE3) pGS2164	This work
E. coli plasmids		
pET28a	His_6-tag overexpression vector; Kan^R	Novagen
pGS2060	pCR4Blunt-TOPO containing 285 bp upstream of the *whiB1* coding region	[5]
pGS2164	pET28a derivative encoding His_6-WhiB1 fusion protein; Kan^R	This work
M. tuberculosis strains		
H37Rv	Wild-type virulent strain	[28]
Δ*whiB1*/pDMH1	Complemented *whiB1* null mutant	This work
Δ*whiB1*/pDM2	Complemented *whiB1* null mutant (more 5′)	This work
Δ*whiB1*/pDM3	Complemented *whiB1* null mutant (more 5′ and 3′)	This work
M. tuberculosis shuttle plasmids		
p2NIL	Suicide gene delivery vector, *oriE*, *kan*	[21]
p*whiB1*-KO	Final *whiB1* knock out p2NIL derivative (see *Materials and Methods*)	This work
pKP203	Integrase-negative derivative of the integrating vector pMV306, $Gent^R$	K.G. Papavinasasundaram
pKP186	Integrase-negative derivative of the integrating vector pMV306, Kan^R	K.G. Papavinasasundaram
pBS-int	Integrase-positive plasmid lacking a *M. tuberculosis* origin of replication	B. Springer
pDMH1	*whiB1* complementing derivative of pKP203 (293 bp 5′-14 bp 3′)	This work
pDMH2	*whiB1* complementing derivative of pKP186 (1192 bp 5′-14 bp 3′)	This work
pDMH3	*whiB1* complementing derivative of pKP186 (1192 bp 5′-535 bp 3′)	This work

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Table 2

General protein disulphide reductase activity of WhiB1

The data shown are the mean values from three or four experiments with mean standard errors of less than ±0.04.

Protein	Concentration (μM)	Rate (ΔA_{600} min^{-2}) (× 10^{-3})
none	-	0.07
thioredoxin	2.5	1.30
apo-WhiB1	5.0	0.10
apo-WhiB1	10	0.43
apo-WhiB1	20	0.45
holo-WhiB1	5.0	0.07
apo-WhiB1 after treatment with NONOate (100 μM)	5.0	0.07
holo-WhiB1 after treatment with NONOate (100 μM)	5.0	0.02