

Evidence That the *Streptomyces* Developmental Protein WhiD, a Member of the WhiB Family, Binds a [4Fe-4S] Cluster*

Received for publication, November 8, 2004, and in revised form, December 17, 2004
Published, JBC Papers in Press, December 21, 2004, DOI 10.1074/jbc.M412622200

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WhiD is required for the late stages of sporulation in the Gram-positive bacterium *Streptomyces coelicolor*. WhiD is a member of the WhiB-like family of putative transcription factors that are present throughout the actinomycetes but absent from other organisms. This family of proteins has four near-invariant cysteines, suggesting that these residues might act as ligands for a metal cofactor. Overexpressed WhiD, purified from *Escherichia coli*, contained substoichiometric amounts of iron and had an absorption spectrum characteristic of a [2Fe-2S] cluster. After Fe-S cluster reconstitution under anaerobic conditions, WhiD contained ~4 iron atoms/monomer and similar amounts of sulfide ion and gave an absorption spectrum characteristic of a [4Fe-4S] cluster. Reconstituted WhiD gave no electron paramagnetic resonance signal as prepared but, after reduction with dithionite, gave an electron paramagnetic resonance signal ($g \sim 2.06, 1.94$) consistent with a one-electron reduction of a [4Fe-4S]²⁺ cluster to a [4Fe-4S]¹⁺ state with electron spin of $S = 1/2$. The anaerobically reconstituted [4Fe-4S] cluster was oxygen sensitive. Upon exposure to air, absorption at 410 and 505 nm first increased and then showed a steady decrease with time until the protein was colorless in the near UV/visible region. These changes are consistent with an oxygen-induced change from a [4Fe-4S] to a [2Fe-2S] cluster, followed by complete loss of cluster from the protein. Each of the four conserved cysteine residues, Cys-23, -53, -56, and -62, was essential for WhiD function *in vivo*.

Fe-S cluster proteins are an ancient class of proteins (1) that play important roles in electron transfer and in numerous metabolic pathways across all kingdoms of life. The realization that Fe-S cluster proteins can also play vital regulatory roles is comparatively recent, and only four such proteins have been characterized to date. In mammalian cells, the [4Fe-4S] cluster form of the cytosolic isozyme of aconitase catalyzes the isomerization of citrate to isocitrate. However, in its apoform, termed iron regulatory protein, it binds specific mRNAs, in some cases to block their translation and in other cases to enhance it (2, 3). The apoforms of bacterial aconitases have recently also been

found to bind specific mRNAs and function as translational regulators (4, 5). In addition, three transcription factors that have regulatory Fe-S clusters have been characterized, namely, FNR, SoxR, and IscR (reviewed in Ref. 3). All three of these transcription factors were discovered in *E. coli*, but it is now clear that Fe-S-dependent regulatory networks involving these proteins are present in many other bacterial genera. In this report we have presented evidence that WhiD, a member of a family of putative transcription factors previously characterized only at the genetic level, also binds a regulatory Fe-S cluster.

The *whiD* developmental gene was isolated from *Streptomyces coelicolor* by map-based cloning and complementation (6). Sequencing revealed that WhiD is a member of the Wbl (WhiB-like) family of putative transcription factors that are present throughout the actinomycetes but absent from all other organisms so far sequenced (6, 7). The actinomycetes include *Streptomyces*, the genus responsible for the production of two-thirds of the known antibiotics, as well as medically important pathogens like *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae*. Although the biochemical function of Wbl proteins, including WhiD, has not been demonstrated, circumstantial evidence suggests they are transcriptional activators: WhiB3, the WhiD orthologue of *M. tuberculosis*, was identified in a yeast two-hybrid screen in which the C-terminal domain (region 4.2) of the principal, essential RNA polymerase σ factor of *M. tuberculosis* was used as bait. This interaction was confirmed biochemically using surface-enhanced laser desorption and ionization-time of flight mass spectrometry (8). In addition, one gene in *S. coelicolor* (SCP1.161c, carried on the 356-kb plasmid SCP1 (9)) encodes a protein having a Wbl at its N terminus and an RNA polymerase σ factor at its C terminus.

Consistent with the proposed role of Wbl proteins as transcription factors, mutations in different *wbl* genes have been found to cause a wide range of highly pleiotropic phenotypes in both *Streptomyces* and *Mycobacterium*, demonstrating that Wbl proteins play important roles in actinomycete biology. The first member of the *wbl* family to be characterized was the *whiB* developmental gene itself. *S. coelicolor whiB* mutants produce abnormally long, tightly coiled aerial hyphae that are completely blocked in their ability to form sporulation septa (10–12). In contrast, *whiD* mutants can make sporulation septa but form spores that differ from those of the wild-type in being heat sensitive, prone to lysis, highly irregular in size, and extremely variable in spore cell wall deposition (6, 13). In addition, *whiD* spores are frequently partitioned into irregular smaller units through the deposition of additional septa, often laid down in several different planes, very close to the spore poles (6, 13). The orthologue of *S. coelicolor whiB* in *Mycobacterium smegmatis*, *whmD*, is essential, but controlled depletion

* This work was supported by a grant-in-aid to the John Innes Centre from the Biotechnology and Biological Sciences Research Council, by a Joint Infrastructure Fund award from the Wellcome Trust (to A. J. T.), and by National Institutes of Health Grant R01 AI 51668 (to W. R. B. and K. F. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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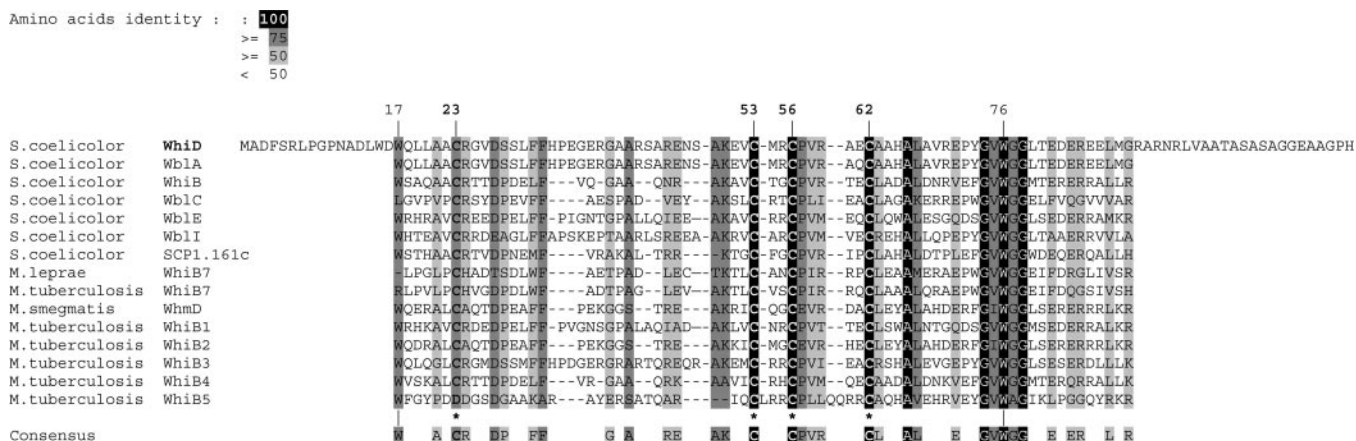


FIG. 1. Alignment of WhiD with selected other members of the WhiB-like (Wbl) family from the genera *Streptomyces* and *Mycobacterium*. Note that only the conserved region of the proteins is shown. The four near-invariant cysteines and the two highly conserved tryptophans discussed in the text are highlighted.

experiments showed that WhmD is required for proper septation and cell division (14). Emphasizing the important and diverse roles of Wbl proteins, the *wblC* locus of *S. coelicolor* was identified genetically in a screen for mutants pleiotropically sensitive to a wide range of chemically and functionally unrelated, clinically important antibiotics. WblC has now been shown to control innate multidrug resistance, not only in *S. coelicolor* but also in the major human pathogen, *M. tuberculosis* (15). Overexpression of *wblC* leads to enhanced multidrug resistance (15). The remarkable antibiotic tolerance of *M. tuberculosis* is the main cause of treatment failure, relapse, and acquired drug resistance in patients with tuberculosis, making WblC an attractive target for therapeutic intervention (15). Thus, the Wbl family of proteins is novel and of academic and medical significance.

To date, genome sequencing and directed approaches have identified >80 *wbl* genes in the actinomycete genera *Streptomyces*, *Mycobacterium*, *Streptovorticillium*, and *Rhodococcus* (e.g. Fig. 1). In addition to *whiB* and *whiD*, this includes 11 further *wbl* genes in *S. coelicolor* itself, 8 on the chromosome and 3 on the giant linear plasmid, SCP1 (9, 16). With the exception of the Wbl σ factor fusion protein (SCP1.161c), all the Wbl proteins are small, varying in length from 81 to 122 residues. A striking feature of the Wbl family is four cysteine residues (Cys-23, -53, -56, and -62 in WhiD) that are perfectly conserved in all members, with the exception of only four proteins in which Cys-23 is replaced by aspartate (Fig. 1). This suggests that these residues might act as ligands for a metal cofactor. Here we have presented evidence that WhiD can bind a [4Fe-4S] cluster that reacts with oxygen to generate a [2Fe-2S] cluster.

EXPERIMENTAL PROCEDURES

Overexpression of WhiD—A 0.43-kb fragment carrying an NdeI site overlapping the ATG start codon of *whiD*, an EcoRI site at the 5'-end, and a HindIII site at the 3'-end was generated by PCR using pIJ6626 (6) as template. The PCR program was 5 min at 95 °C, followed by 25 cycles of 1 min at 95 °C, 30 s at 68 °C, and 30 s at 72 °C before a final 10-min incubation at 72 °C. The primers were 5'-GCTACAAGGGAATTCATATGGCAGATTTCTCCCG-3' introducing the NdeI and EcoRI sites and 5'-GACGCGCTGACCGCGTGGGAAGCTTGGCGGGCC-3' introducing the HindIII site. The PCR product was digested with EcoRI and HindIII and cloned into EcoRI-HindIII-cut pIJ2925 (17) to create pIJ6630. The resulting allele of *whiD* was sequenced over its entire length to ensure that only the desired change had been introduced. Finally, the *whiD* overexpression allele was excised as a 0.43-kb NdeI-BglII fragment and cloned into the expression vector pET11c (Novagen) cut with NdeI and BamHI, generating pIJ6990.

PCR Mutagenesis and Complementation—To incorporate single point mutations into *whiD*, two complementary primers, both containing the

mutation, were used in an amplification reaction with plasmid DNA as the template. Reaction conditions were 94 °C for 5 min and then 25 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 10 min, followed by 72 °C for 15 min. The reaction mixtures were transferred to 1.5-ml tubes and treated with 10 units of DpnI for 30 min at 37 °C to destroy the methylated template DNA and then 72 °C for 30 min to inactivate DpnI. After cooling on ice, each reaction mixture was treated with 2 units of T4 DNA ligase for 1 h at room temperature and used to transform competent *E. coli* DH5 α . After sequencing on both strands to confirm that only the intended cysteine to alanine codon changes had been introduced, mutant alleles were isolated as 1.3-kb BamHI-SphI fragments and cloned into pSET152 (18) to create a series of constructs otherwise identical to pIJ6627 (carrying the wild-type *whiD* allele) (6). Plasmids were transferred from *E. coli* to the *whiD* null mutant strain J2152 (6) by conjugation, as described (19).

Cell Growth—Aerobic *E. coli* cultures were routinely grown in Luria Bertani medium, supplemented with 50 μ M iron, zinc, and copper ions, at 37 °C in 2-liter flasks with shaking at 230 rpm. Carbenicillin (100 μ g ml⁻¹) was required for maintenance of pIJ6990. Exponentially growing cells of *E. coli* BL21-CodonPlus-RIL (Stratagene) bearing pIJ6990 were induced with 0.5 mM isopropyl-1-thio- β -D-galactopyranoside for 5 h at 37 °C, harvested by centrifugation, washed with 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and frozen. Protein expression was checked by SDS-PAGE of whole-cell lysates.

Purification of WhiD—After thawing, the cell paste derived from growth of BL21-CodonPlus-RIL pIJ6990 was resuspended in 40 ml of buffer C (20 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing an EDTA-free protease inhibitor mixture (Roche Applied Science). Cells were disrupted in a French press, and the lysate was clarified by centrifugation. WhiD is found in the insoluble fraction as inclusion bodies. Therefore, the pellet fraction was washed with 30 ml of buffer D (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20), harvested by centrifugation, and solubilized by slow agitation in 8 M urea at room temperature using buffer E (10 mM Tris-HCl, pH 8.0, 150 mM sodium phosphate, 10 mM dithiothreitol (DTT),¹ 8 M urea). The solution was clarified by centrifugation, and the supernatant was dialyzed at 4 °C overnight against buffer F (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM DTT, 5% glycerol). The dialyzed material was clarified by centrifugation and concentrated using a Centri-prep concentrator (10-kDa cutoff; Millipore). The purity of the protein was analyzed by SDS-PAGE and the concentration determined (Bio-Rad protein assay).

Reconstitution of an Iron-Sulfur Cluster—All steps of the reconstitution procedure were performed anaerobically inside a glovebox (Belle Technology) in an N₂ atmosphere containing less than 2 ppm O₂. Buffer F, Buffer G (50 mM Tris-HCl, pH 7.5, 150 mM NaCl), and H₂O were purged with N₂ and kept anaerobic for more than 24 h before use. Fe(NH₄)₂(SO₄)₂, FeCl₃, L-Cys, and DTT were transferred into the glovebox as weighed aliquots and dissolved in H₂O inside the glovebox to create 10 \times stock solutions. In Reconstitution Method I, WhiD was anaerobically equilibrated in Buffer G and treated with DTT (10 mM final concentration), followed by Fe(NH₄)₂(SO₄)₂ or FeCl₃ in 5-fold mo-

¹ The abbreviations used are: DTT, dithiothreitol; EPR, electron paramagnetic resonance.

FIG. 2. Absorption spectrum of WhiD after solubilization with urea and dialysis but without Fe-S cluster reconstitution (0.3 mM WhiD in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM DTT, 5% glycerol).

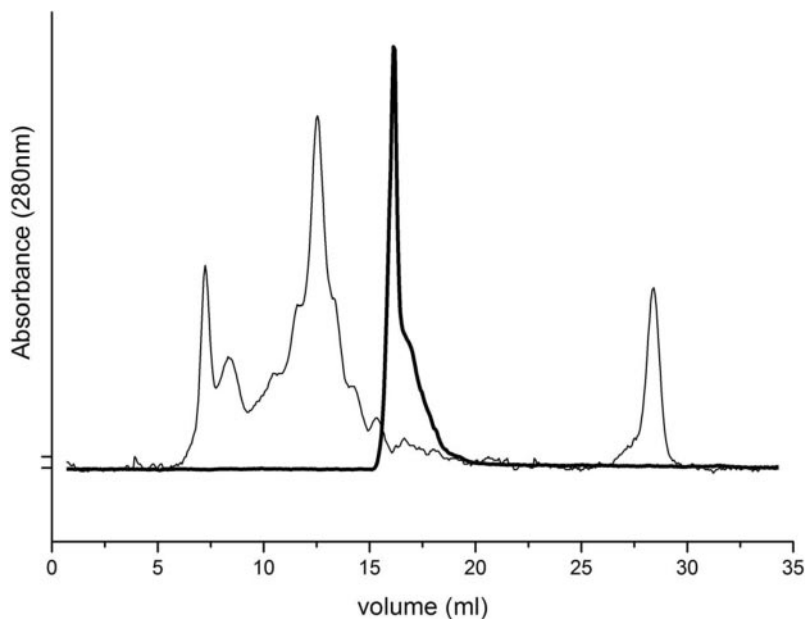
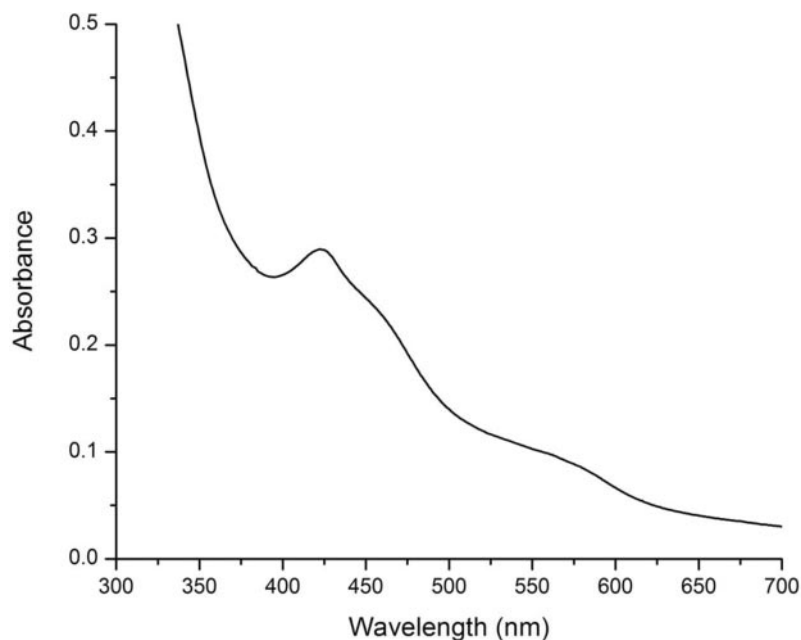


FIG. 3. Effect of Fe-S cluster reconstitution on the oligomeric state of WhiD. Samples of WhiD taken before (*thin line*) and after (*thick line*) Fe-S cluster reconstitution were subjected to analytical gel filtration on a 30-cm S75 Sephadex gel filtration column. There is a one order of magnitude difference in scale between the two traces, reflecting the increased solubility of the reconstituted monomeric species. The column was equilibrated with 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 2 mM sodium dithionite. Freshly prepared buffer was degassed and purged with N_2 before the addition of dithionite, and N_2 flow was maintained over the buffer solution during chromatography.

lar excess over WhiD, and finally with L-cysteine (2 mM final concentration) and 2.5 μ g of NifS (a kind gift from Dr. Sara Austin). The reaction mixture was incubated at room temperature overnight. In Reconstitution Method II, anaerobically equilibrated WhiD was incubated with a 1.5-fold molar excess of Na_2S and the same excess of either $Fe(NH_4)_2(SO_4)_2$ or $FeCl_3$ in 2 ml of buffer F at 22 °C for 4 h. The reconstituted WhiD protein was separated from low molecular mass material using a PD-10 desalting column (equilibrated and eluted with buffer G for Method I or buffer F for Method II), inside the glovebox and concentrated to ~ 11 mg ml $^{-1}$ using a Centri-prep concentrator (10-kDa cutoff; Millipore). Protein concentration was determined using the Bio-Rad protein assay.

EPR Spectroscopy—Perpendicular mode X-band electron paramagnetic resonance (EPR) spectra were recorded on an EPR spectrometer comprising an ER-200D electromagnet and microwave bridge interfaced to an EMX control system (Bruker Spectrospin) and fitted with a liquid helium flow-cryostat (ESR-9; Oxford Instruments) and a dual-mode X-band cavity (Bruker type ER4116DM).

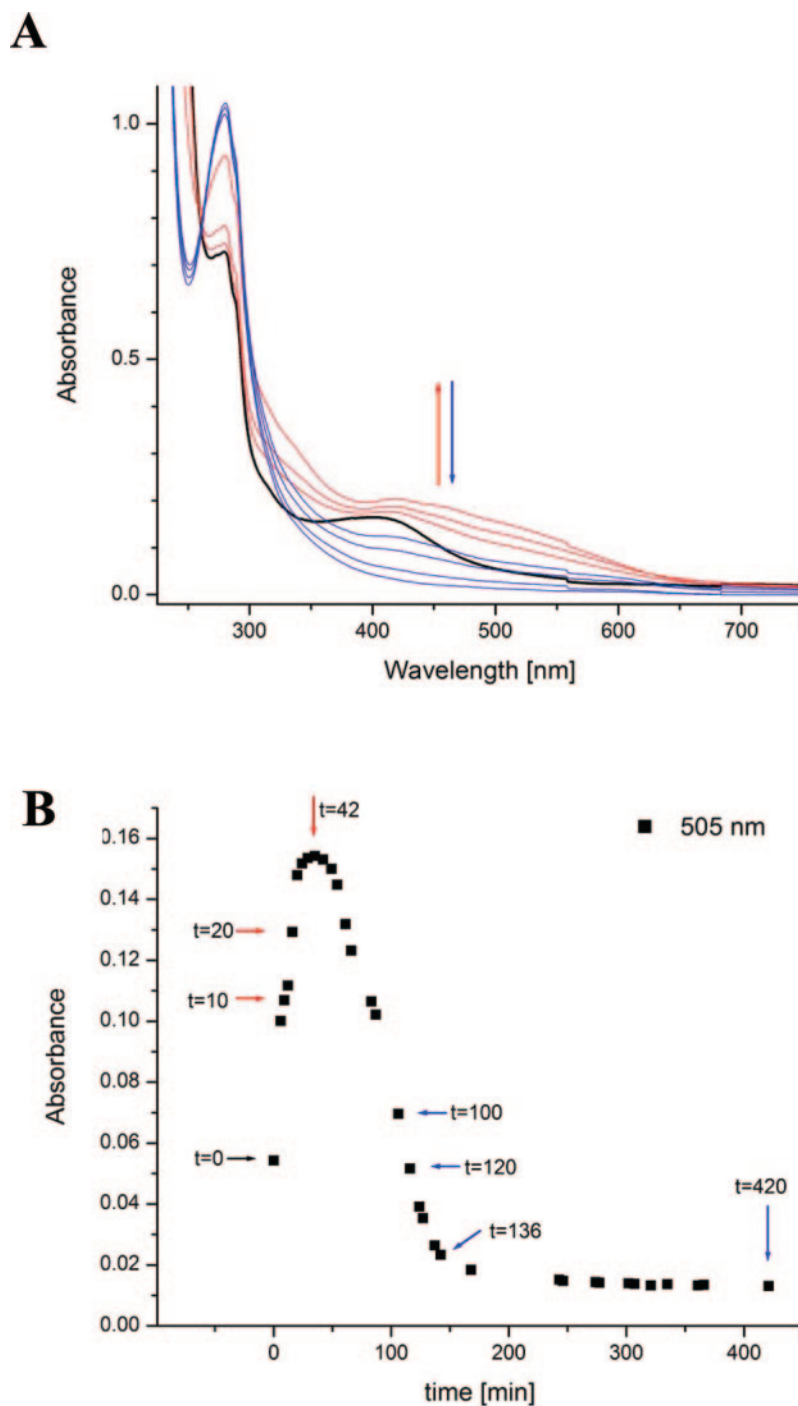
Other Analytical Techniques—UV-visible absorption spectra were collected using a Lambda 35 UV/VIS spectrometer (PerkinElmer) in the 230–700 nm wavelength range. Measurements were carried out at room temperature in a quartz cuvette with a path length of 10 mm.

Fluorescence of WhiD samples was recorded at room temperature using an LS55 luminescence spectrometer (PerkinElmer) with an excitation wavelength of 280 nm and an emission range of 300–450 nm with a scan rate of 500 nm min $^{-1}$. The circular dichroism spectra were measured with a JASCO model J-810. The iron content of WhiD was determined using inductively coupled plasma atomic emission spectroscopy (Southern Analytical, Brighton, UK). The iron content of WhiD was also estimated using the molar extinction coefficient of 4000 M $^{-1}$ cm $^{-1}$ /mole of iron at 400 nm for an Fe-S cluster (20). Sulfide content was determined as described (21).

RESULTS

WhiD, without any affinity tags, was expressed in *E. coli* grown in medium supplemented with 50 μ M iron, zinc, and copper ions. WhiD was present in highly colored inclusion bodies from which it could be purified by solubilization in 8 M urea followed by dialysis to induce protein refolding. WhiD prepared in this way retained its red-brown color. The absorption spectrum showed a band at 420 nm and two shoulders at 450 and 550 nm, strongly suggestive of a [2Fe-2S] cluster (Fig.

FIG. 4. Oxygen-induced transformation and degradation of the Fe-S clusters in WhiD. *A*, absorption spectra of the anaerobically reconstituted protein before (black line) and after exposure to air (2–42 min, red lines; 47–420 min, blue lines; the exact time points are indicated by the arrows in panel *B*). *B*, absorption of WhiD at 505 nm versus time after exposure to air at time 0. WhiD (20 μM) in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM DTT.



2) (22). It is noteworthy that this cluster appears to have, at least partially, survived exposure to 8 M urea. This form of WhiD was >98% pure as judged by SDS-PAGE and contained iron (but not nickel, zinc, copper, or cobalt) according to metal analysis (inductively coupled plasma atomic emission spectroscopy). The iron:WhiD ratio varied between 0.3 and 0.75, depending on the preparation, showing that the cluster was substoichiometric (15–37% occupancy for a [2Fe-2S] cluster). This may have arisen because all purification steps were carried out aerobically.

Therefore, WhiD, purified in this way, was subjected to attempts at Fe-S cluster reconstitution under anaerobic conditions. Two methods were used. Reactions were carried out either using Na_2S alone or by generating sulfide ion enzymatically using L-cysteine and NifS (L-cysteine desulfurase). Reac-

tions were carried out at room temperature, and progress was monitored by UV-visible spectroscopy in an anaerobically sealed cuvette. In both cases, Fe-S cluster reconstitution was complete in 3–6 h, and both methods led to identical absorption spectra (data not shown). The resulting samples were purified by anaerobic gel filtration and found to contain 3.67–4.2 iron atoms/WhiD monomer and similar amounts of sulfide ion. The visible spectrum of reconstituted WhiD gave a molar absorption coefficient (ϵ) at 400 nm of $\sim 4000 \text{ M}^{-1} \text{ cm}^{-1}$ /mol of iron, a value characteristic of an Fe-S cluster. The absorption spectrum of the reconstituted protein has a single broad peak at 420 nm and no other resolved features at longer wavelength. It is different from that of the cluster in the protein purified aerobically from inclusion bodies. The absorption spectrum, together with the absence of an EPR signal in the $g \sim 2$ region

(see below), is characteristic of a [4Fe-4S] cluster (22).

Oligomeric State of WhiD—To determine the possible effect of Fe-S cluster reconstitution on the oligomeric state of WhiD, samples of the protein were examined by analytical gel filtration under anaerobic conditions. Reconstituted WhiD gave a homogenous monomeric peak, whereas the partially iron-loaded material solubilized from inclusion bodies was markedly heterogeneous with no monomeric peak (Fig. 3). Further, the far UV CD spectrum gave evidence that reconstituted WhiD was structured. Consistent with these observations, the maximum concentration achievable in buffered solution for the reconstituted protein ($\sim 50 \text{ mg ml}^{-1}$) was much higher than for the partially iron-loaded material solubilized from inclusion bodies.

Oxygen-induced Changes in the Fe-S Cluster—Upon exposure to air, the absorption spectrum of anaerobically reconstituted WhiD changes. A sample of protein carried in a sealed anaerobic cuvette was exposed to oxygen by opening the cuvette. The sample was mixed with air by pipetting, the cuvette was resealed, and subsequent changes in the absorption spectrum were measured *versus* time (Fig. 4A). A plot of absorbance changes at 505 nm *versus* time (Fig. 4B) shows two phases, an increase over the first 50 min followed by a steady decrease over the next 100 min until the protein solution is colorless in the near UV/visible region. The absorption spectrum after ~ 50 min was very similar to that of the [2Fe-2S] cluster present in the material solubilized from inclusion bodies. These changes are consistent with an oxygen-induced change from a [4Fe-4S] to a [2Fe-2S] cluster followed by the complete loss of cluster from the protein. The rate of each phase was dependent on a number of factors, including the degree of exposure to oxygen and the presence of DTT. If no DTT were present, the phase corresponding to the [4Fe-4S] to [2Fe-2S] conversion was much faster, becoming difficult to observe by absorption spectroscopy.

These changes could also be followed by observation of the fluorescence spectrum of the tryptophan residues. WhiD contains three tryptophans (Trp-15, -17, and -76), at least two of which must be close to cysteine residues Cys-23 and -62 that likely bind the cluster. As the oxygen reaction proceeded, the protein tryptophan fluorescence intensity first dropped on the time scale corresponding to the conversion of the [4Fe-4S] cluster to the [2Fe-2S] form and then increased as all cluster was lost from the protein (data not shown). Fe(III)-S charge transfer absorption bands overlap the emission maximum for tryptophan fluorescence. As a consequence, Fe-S clusters absorb strongly in the 360-nm region. The [4Fe-4S] and [2Fe-2S] clusters clearly quench the tryptophan fluorescence to different degrees, resulting in an initial decrease and subsequent increase in fluorescence intensity. On complete loss of the cluster, the fluorescence intensity was at a maximum.

EPR Spectroscopy of WhiD—Reconstituted WhiD, which had been purified anaerobically by gel filtration, was analyzed by EPR spectroscopy, both as prepared and after reduction with sodium dithionite (Fig. 5). Reduction caused the red-brown color of the sample to bleach partially, showing a loss of the broad shoulder between 400–420 nm in the absorption spectrum (Fig. 5A). This is characteristic of the reduction of [4Fe-4S]²⁺ to [4Fe-4S]¹⁺. Almost no EPR signal was apparent in the spectrum at liquid helium temperatures of the sample as prepared, but intense EPR signals were seen at $g = 2.06$, 1.94 after reduction with dithionite (Fig. 5B). These observations are consistent with a one-electron reduction of a [4Fe-4S]²⁺ cluster, that is EPR silent (electron spin $S = 0$), to a [4Fe-4S]¹⁺ state with electron spin $S = \frac{1}{2}$. The temperature dependence and the microwave power saturation properties of these signals were

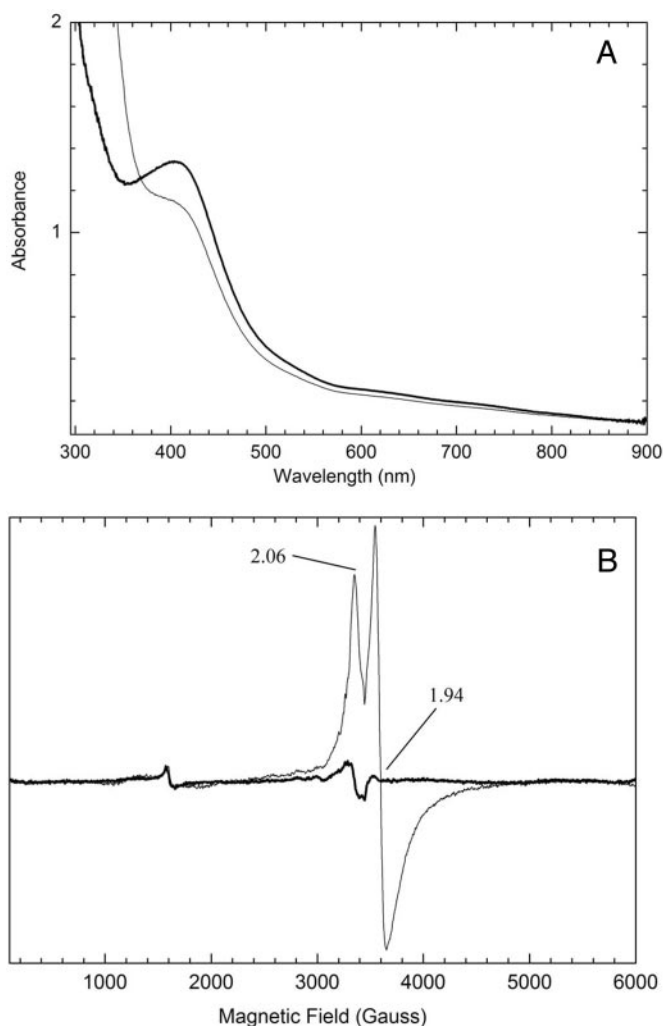


FIG. 5. A, UV-visible spectra of anaerobically reconstituted WhiD (1.25 mM) in 25 mM Tris-HCl, pH 7.5, 50% glycerol before (*thick line*) and after (*thin line*) reduction with sodium dithionite (10 mM). B, EPR spectra of WhiD before (*thick line*) and after (*thin line*) reduction with dithionite. EPR spectra were recorded at 10 K, with a microwave power of 2 milliwatts, a modulation amplitude of 10 G, and a microwave frequency 9.67 GHz.

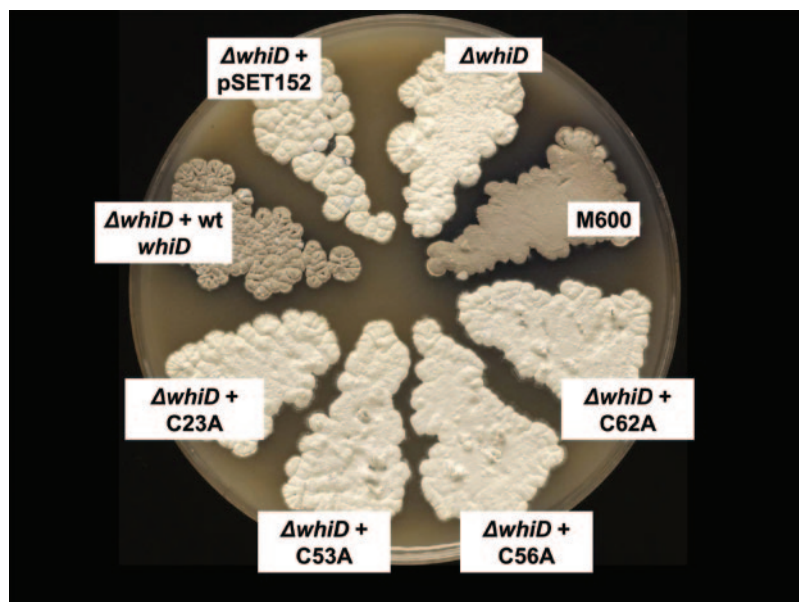
also consistent with a [4Fe-4S]¹⁺ center rather than a [2Fe-2S]¹⁺ cluster (23).

Four Invariant Cysteines Are Essential for WhiD Function *in Vivo*—To assess the importance of Cys-23, -53, -56, and -62 for WhiD function *in vivo*, each cysteine was individually mutated to alanine and the four resulting *whiD* mutant alleles and the wild-type allele were used to generate five otherwise identical constructs, based on the integrative vector pSET152 (21). Each was introduced into the constructed *whiD* null mutant J2152 (9), and its ability to complement the mutant phenotype was assessed (Fig. 6, and data not shown). As expected, J2152 was fully complemented by the wild-type *whiD* allele. In contrast, introduction of any of the C23A, C53A, C56A, and C62A mutant alleles had no influence on the *whiD* null mutant phenotype, showing that each of the four cysteines was essential for WhiD function *in vivo* (Fig. 6, and data not shown).

DISCUSSION

This work has shown that the WhiD protein of *S. coelicolor* can bind either [4Fe-4S] or [2Fe-2S] iron-sulfur cluster cores. Expression in *E. coli* produces a protein that is not properly folded, contains substoichiometric amounts of iron, and has an absorption spectrum characteristic of a [2Fe-2S] cluster. How-

FIG. 6. The four invariant cysteines are essential for WhiD function *in vivo*. The wild-type *whiD* allele (carried on pIJ6627), the C23A, C53A, C56A, and C62A mutant alleles, and the vector (pSET152) alone were introduced into the *whiD* null mutant J2152. Only the wild-type *whiD* allele complemented the mutant phenotype (indicated here by restoration of the synthesis of the gray spore pigment).



ever, cluster reconstitution causes a [4Fe-4S] core cluster to become bound and yields a monomeric form of the protein that runs as a single, narrow peak on anaerobic analytical gel filtration. Hence, cluster insertion causes the protein to fold into a single conformer. Evidence for the [4Fe-4S] core is provided by a characteristic absorption spectrum together with the ability to undergo reduction to a paramagnetic state, $S = 1/2$, giving rise to an EPR signal typical of such a cluster core. Reaction of the [4Fe-4S]²⁺ form of the protein with air leads to transformation first to a [2Fe-2S]²⁺ core followed by complete cluster loss. Cluster transformation with air-saturated buffer ($[O_2] \sim 230 \mu M$ at 25 °C) (24) has a half-life of about 25 min in the presence of 1 mM DTT, which may be slow for a regulating process. However, in the absence of DTT the transformation becomes much more rapid, being difficult to detect with conventional spectrophotometry. Stopped-flow kinetic methods will be required to follow the process under these conditions.

Cysteine residues Cys-23, -53, -56, and C62, each of which is essential for protein function *in vivo*, are candidate cluster ligands. These four residues are perfectly conserved in all ~80 members of the Wbl family, with the exception of just four Wbl proteins in which Cys-23 is replaced by an aspartate (Fig. 1). However, there are examples of [4Fe-4S] clusters coordinated by three thiolate side chains of cysteines and one carboxylate of aspartate (25). It is therefore tempting to speculate that all members of the Wbl family can bind a [4Fe-4S] cluster.

In the transcription factors SoxR, FNR, and IscR, the Fe-S cluster plays a key regulatory role. SoxR senses superoxide stress, stimulating transcription of the gene encoding SoxS, which in turn activates >50 genes encoding enzymes that detoxify superoxide (*e.g.* superoxide dismutase) and repair oxidative damage in the cell. In the absence of oxidative stress, SoxR is a homodimer with one [2Fe-2S]¹⁺ cluster/monomer; this form of the protein binds the *soxS* promoter but does not activate transcription. In the presence of superoxide, the cluster is oxidized to its [2Fe-2S]²⁺ state in which it is competent to stimulate transcription of *soxS* (3). FNR, a homodimer containing one [4Fe-4S]²⁺ cluster/monomer, regulates >100 genes in response to oxygen limitation, repressing genes involved in aerobic respiration and activating genes concerned with the use of alternative terminal electron acceptors. These functions of dimerization, site-specific DNA binding, and transcriptional activation require an intact [4Fe-4S]²⁺ cluster, and exposure of whole cells or the purified protein to oxygen causes its rapid

conversion to a [2Fe-2S]²⁺ center and resultant inactivation of the protein (3, 26). IscR represses expression of the *isc* operon that specifies the Fe-S cluster assembly pathway in *E. coli*, and EPR spectroscopy shows that IscR contains a [2Fe-2S] cluster. Current evidence suggests a homeostatic feedback model in which the repressor activity of IscR is lost under Fe-S cluster-limiting conditions (3).

How might a [4Fe-4S] cluster contribute to the biological function of WhiD and, by implication, the function of other members of the Wbl family? One possibility would be that the cluster plays a purely structural role. For example, the [4Fe-4S] cluster of *E. coli* endonuclease III appears to play a key role in stabilizing a cluster-bound loop that interacts with the DNA backbone (27, 28). However, whereas the [4Fe-4S] cluster of endonuclease III is redox inert, the [4Fe-4S] cluster of WhiD can undergo single electron oxidation/reduction and is oxygen-labile, suggesting that the activity of WhiD and, by implication, those of other members of the Wbl family, might be redox regulated *in vivo*. *Streptomyces* species have traditionally been regarded as obligate aerobes, but one of the most interesting and unexpected outcomes of the *S. coelicolor* genome sequence was the discovery of a number of genes and operons encoding enzymes that, in facultative anaerobes such as *E. coli* and *B. subtilis*, are typically involved in microaerobic and anaerobic metabolism. These enzymes include respiratory nitrate and nitrite reductases, formate dehydrogenase, NarK, and cytochrome *d* oxidase (16, 29). Further, several transcriptional regulators controlled by redox switches have been identified in *Streptomyces* and characterized to varying degrees, including σ^R -RsrA (reviewed in Ref. 30), Rex (31), OxyR (32), CatR (33), FurA and OhrR (34). The oxygen-induced 4Fe to 2Fe switch in WhiD is reminiscent of the regulation of FNR by oxygen in facultative anaerobes such as *E. coli*. However, WhiD and WhiB are positive regulators of sporulation. They are believed to function in the aerial mycelium, which is presumably an oxygen-sufficient cell type, making the suggestion that they function principally as oxygen sensors unlikely (unless the aerial mycelium is in fact oxygen deficient because of the hydrophobic coating that protects it from desiccation (35)). Instead, we suggest that WhiD and WhiB proteins might respond to redox changes that occur as an inescapable part of alterations in cellular metabolism accompanying the developmental process, for example during the downshift from a metabolism associated with rapid aerial growth to that of non-growing

hyphae about to undergo consecutive stages of sporulation. Although the underlying mechanism is not understood, there is at least one published observation connecting redox biology and differentiation in *S. coelicolor*: constitutive overexpression of the disulfide stress regulon, caused by loss of the σ^R -specific anti- σ factor RsrA, blocks the initiation of sporulation (36).

Our current understanding of the biochemical roles of WhiB-like proteins is limited: challenges for the future will be to identify genes that are down-regulated in *whiD* mutants, to determine whether WhiD affects expression of these genes by direct DNA binding or through some other mechanism, and to investigate how the state of the WhiD Fe-S cluster affects these functions.

Acknowledgments—We thank Virginie Molle for making the WhiD overexpression construct, Shirley Fairhurst for help with preliminary EPR experiments, Sara Austin for the gift of NifS, Patricia Kiley, Monica Chander, Matt Hutchings, and Marie Elliot for helpful advice, and Gary Sawers and Mark Paget for comments on the manuscript.

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