Siroheme- and [Fe₄-S₄] -dependent NirA from Mycobacterium tuberculosis Is a Sulfite Reductase with a Covalent Cys-Tyr Bond in the Active Site*

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The nirA gene of Mycobacterium tuberculosis is up-regulated in the persistent state of the bacteria, suggesting that it is a potential target for the development of antituberculosis agents particularly active against the pathogen in its dormant phase. This gene encodes a ferredoxin-dependent sulfite reductase, and the structure of the enzyme has been determined using x-ray crystallography. The enzyme is a monomer comprising 555 amino acids and contains a [Fe₄-S₄] cluster and a siroheme cofactor. The molecule is built up of three domains with an α/β fold. The first domain consists of two ferredoxin-like subdomains, related by a pseudo-2-fold symmetry axis passing through the whole molecule. The other two domains, which provide much of the binding interactions with the cofactors, have a common fold that is unique to the sulfite/nitrite reductase family. These domains form a trilobal structure, with the cofactors and the active site located at the interface of all three domains in the center of the molecule. NirA contains an unusual covalent bond between the side chains of Tyr¹⁶⁹ and Cys¹⁶¹ in the active site, in close proximity to the siroheme cofactor. Removal of this covalent bond by site-directed mutagenesis impairs catalytic activity, suggesting that it is important for the enzymatic reaction. These residues are part of a sequence fingerprint, able to distinguish between ferredoxin-dependent sulfite and nitrite reductases. Comparison of NirA with the structure of the truncated NADPH-dependent sulfite reductase from Escherichia coli suggests a binding site for the external electron donor ferredoxin close to the [Fe₄-S₄] cluster.

Mycobacterium tuberculosis, the causative agent of tuberculosis, poses a major threat to human health. Over the last decade, the number of registered cases has been progressively increasing, resulting presently in approximately 2 million deaths per year (statistics available on the World Wide Web at www.who.int). The emergence of multidrug-resistant strains of M. tuberculosis raises serious concerns about future capabilities to control this pathogen. Chemotherapy is further complicated by the ability of M. tuberculosis to persist in the lungs of infected individuals for decades by switching to a dormant or latent phase (1), which also induces tolerance to current antibiotics (2, 3). Estimates by the WHO suggest that about one-third of the world's population is infected with persistent mycobacteria. Reactivation of these dormant bacteria can occur either spontaneously or as the consequence of an immunocompromised state (e.g. HIV infection/therapy), resulting in active tuberculosis. It is thus clear that a successful long term strategy against M. tuberculosis requires antibiotics targeting the bacteria also in the persistent state.

Latency is associated with nonreplicating or very slow growth of M. tuberculosis, and several experimental in vitro models for the dormant phase of the bacilli have been developed (4–7). Comparison of gene expression profiles and proteome analyses of active versus nonreplicating bacteria have identified a number of genes that are up-regulated in the dormant phase. Genes involved in oxidative stress, anaerobic respiration, and the metabolism of sulfur have consistently been identified as up-regulated in response to limited access to oxygen and nutrient starvation (8–11).

One of the genes active in the dormant phase of M. tuberculosis is nirA (Rv2391) (9, 10). Himar1 transposon mutagenesis has further shown that nirA is an essential gene (12). The amino acid sequence, derived from the nirA gene, shows homology to a family of ferredoxin-dependent sulfite/nitrite reductases. These enzymes are found in archaea, bacteria, fungi, and plants (for a review, see Refs. 13 and 14). The sulfite reductases catalyze the reduction of sulfite to sulfide, one step in the biosynthesis of sulfur-containing amino acids and cofactors. Nitrite reductases participate in the assimilation of nitrogen via nitrate in plants and can also act in anaerobic energy metabolism. This class of sulfite/nitrite reductases generally accepts both nitrite and sulfite as substrate, but the particular metabolic function is reflected in pronounced differences in the kinetic parameters. These enzymes contain a unique combination of cofactors, a [Fe₄-S₄] iron-sulfur cluster and a siroheme (15).

NirA also shows weak amino acid sequence similarity to bacterial NADPH-dependent [Fe₄-S₄]- and siroheme-containing sulfite reductases (e.g. 23% sequence identity to CysI from Escherichia coli). Unlike the monomeric ferredoxin-dependent enzymes, the NADPH-dependent sulfite reductases are oligomeric complexes consisting of four or eight subunits of the hemoprotein component (CysI) and eight flavoprotein subunits that deliver electrons derived from NADPH to the redox centers of the hemoprotein subunit (16, 17). In the ferredoxin-dependent enzymes, the electron donor binds transiently and delivers electrons to the [Fe₄-S₄] cluster, one at a time (18). The electrons are then transferred to the siroheme, which coordinates the substrate.
The only available structural information for this enzyme family is the crystal structure of a truncated form of the hemo-protein subunit of NADPH-dependent sulfite reductase from *E. coli*, CysL, where 73 amino acids at the N terminus had been proteolytically removed before crystallization (19, 20). No three-dimensional structure for a representative of the ferro-doxin-dependent sulfite/nitrite reductases is yet available. Here, we show that the *nirA* gene from *M. tuberculosis* encodes [Fe₃S₄] and siroheme-dependent sulfite reductase. The crystal structure of the mycobacterial enzyme, a potential target for novel drugs against human persistent tuberculosis infection, revealed an unexpected covalent bond between the side chains of Tyr²⁹³ and Cys¹⁶¹ in the immediate vicinity of the siroheme cofactor and the substrate binding site. The functional implications of this tyrosyl-cysteine residue in the active site of *NirA* have been probed by site-directed mutagenesis.

**MATERIALS AND METHODS**

**Gene Cloning and Expression Screening**—The gene coding for the predicted *NirA* protein (Rv2391) was amplified from *M. tuberculosis* H₃₇Rv genomic DNA by PCR using *Pfu* Turbo polymerase (Stratagene) with the appropriate upstream primer GATCCATGGCCACTGCACGTTGCTGGTGGGGCCTGTTCACCCAGCGTGAGCAGGGC and GCCCTGCCGCGCATCCTAATGTCGCTGGTCGTTCAAGGG and the downstream primer GATAAGCTTATCGCAGG-TGCGCTGCCGCTGGTCGTTCAAGGG and the bound proteins were eluted by an imidazol step-gradient. The PCR fragments containing upstream Neol and downstream HindIII sites were cloned into the pMOSBlue vector (Amersham Biosciences) and the nucleotide sequence of the PCR fragments was verified by DNA sequencing. Extensive expression screening using a series of expression vectors and several *E. coli* strains at different temperatures, however, only resulted in insoluble protein. Availability of the siroheme cofactor might be limiting for proper folding, and co-expression of *cysG*, coding for an uroporphyrinogen III C-methyltransferase active in the biosynthesis of siroheme, has been shown to support the expression of soluble siroheme-containing proteins (21, 22). The *cysG* gene was therefore amplified from *Salmonella typhimurium* genomic DNA and cloned into the expression vector utilizing the pCAM promoter pACYC origin of replication and compatible with all of our *nirA* expression constructs. Co-expression of *cysG* resulted in partially soluble product (up to ∼20% of the total amount of *NirA* produced) using modified pET28a-*NirA* plasmids containing either a His₅ tag (MDVSHHHHHHG) or an IgG-binding ZZ tag (23) inserted into the Neol site.

**Protein Production and Purification**—The pET28a expression system was chosen to produce the recombinant His₅-NirA enzyme with *E. coli* BL21(DE3) as expression host. Typically, cells were grown in 2 liters of LB medium at 21 °C and induced in mid-log phase by adding 0.1 mM isopropyl-1-thio-β-D-galactopyranoside and 0.05% arabinose and increasing the concentration of FeSO₄ to 1 mM. After 24 h, *E. coli* cells were harvested, lysed by sonication. The insoluble material was removed by centrifugation. The samples were in-gel digested using porcine trypsin (Promega, Madison, WI) (28). The digests were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry on a Bruker Ultraflex TOP TOF spectrometer (Bruker Daltonics, Bremen, Germany) using α-cyanohydroxycinnamic acid as matrix and the instrument setting optimized for analytes up to m/z 3500. Internal calibration was achieved with the autolytic fragments of trypsin. Data were analyzed via GPM AW (Lighthouse Data, Odense, Denmark).

**Crystallization and Data Collection**—Crystals of *NirA* were obtained by the hanging drop vapor diffusion method. 2 µl of the protein solution (10–14 mg/ml in 25 mM Tris buffer, pH 8.0, 150 mM sodium chloride) were mixed with 2 µl of well solution, containing 0.1 M Tris-HCl, pH range 8.2–8.7, 0.2 M MgCl₂, and 30% polyethylene glycol 4000, and equilibrated against 1.0 ml of the well solution. Clusters of needle-shaped, brown crystals appeared after 5 days at room temperature. Red-shaped single crystals suitable for x-ray structure analysis were obtained by streak seeding of freshly made drops (protein concentration 7 mg/ml) equilibrated for 1 day against the above mother liquor.

X-ray data were collected from crystals after direct transfer into a nitrogen gas stream at 110 K, at station ID14–1, ESRF (Grenoble, France) and beam line ID711 at MAX-lab (Lund, Sweden). The x-ray data were processed and scaled with the programs MOSFLM and SCALA from the CCP4 suite (29). Crystals belong to the monoclinic space group P2₁, with cell dimensions a = 59.9 Å, b = 83.3 Å, c = 108.8 Å, and β = 102.2°. A second crystal form in the orthorhombic space group P2₁2₁2₁, with cell dimensions a = 84.25 Å, b = 115.5 Å, c = 114.7 Å was obtained under identical conditions. In both cases, the asymmetric unit contains two molecules, with a solvent content of ∼45%. The statistics of the data sets are given in Table II.

**Molecular Replacement and Crystallographic Refinement**—The structure was solved by molecular replacement using the program MOLREP (30), initially in space group P2₁. A polyserine model of CysI

1 There is an annotation ambiguity regarding the exact definition of the open reading frame coding for *NirA* (Rv2391) in the genome sequence of *M. tuberculosis* H₃₇Rv. The Swiss-Prot data base entry P71753 assigns the first methionine of the sequence MSATK of TARPARKNERG as the N terminus, whereas in the GenBank entry AAK46756, the second methionine is defined as start of the *NirA* protein. The latter appears more likely, since it results in a better Shine-Dalgarno sequence, and therefore a construct starting with the second methionine was chosen for further work.

2 The abbreviations used are: MV, methyl viologen; r.m.s., root mean square.
from *E. coli* (19) (Protein Data Bank accession code 2gep) with residues 158–172, 200–208, 268–274, and 554–569 omitted was used as a search model. The best solution for the monoclinic NirA data set had a correlation coefficient of 0.266 and an R-factor of 55% with two molecules in the asymmetric unit. Initial rounds of rigid body refinement using Refmac5 (31) resulted in a drop of the R-factor by 5%. The siroheme molecule and the iron-sulfur cluster were excluded from these refinement cycles, and the correctness of the molecular replacement solution was confirmed by electron density for the cofactors appearing at the expected positions.

Manual rebuilding of the model was carried out with the program O (32), based on weighted 2Fo – Fc and Fo – Fc electron density maps (33), and refinement was continued with the program Refmac5 (31). Tight noncrystallographic symmetry restraints were applied in order to limit the number of parameters, except for a few loop regions with slightly different conformations. The structure determination for the orthorhombic NirA crystal form was carried out in a similar way, except that an initial NirA model from the monoclinic data set was used for the molecular replacement with residues 10–94 excluded from the search model. This stretch of the polypeptide chain was gradually rebuilt during refinement. CNS (34) was employed to calculate composite omit maps at the end of refinement. The protein models were analyzed with PROCHECK (35) in order to monitor the stereochemistry. Details of the refinement are given in Table I.

Sequence alignments were carried out using ClustalW (36). Structural comparisons were done with the programs DALI (37), TOP (38), and the LSQ option in O (32), applying default parameters. Figures were made using BOBSCRIPT (39) and rendered with RASTER3D (40).

The atomic coordinates and structure factors for NirA have been deposited with the Protein Data Bank with accession numbers 1zj8 (space group P212121) and 1zj9 (space group P21).

## RESULTS AND DISCUSSION

Characterization of Recombinant NirA—Recombinant NirA from *M. tuberculosis* has a brown color and shows the characteristic absorption spectrum of [Fe₄S₄]⁻ and siroheme-containing sulfite/nitrite reductases in the oxidized state (15, 22, 41) (Fig. 1).

**FIG. 1.** Absorption spectrum of oxidized recombinant NirA from *M. tuberculosis*. The spectrum was recorded in 25 mM Tris-HCl buffer, 150 mM NaCl, pH 8.0, at an enzyme concentration of 39.4 μM. The asymmetry in the band at 381 nm, observed in all preparations of NirA, could be due to a mixture of two states of the enzyme.

### Table I

| Parameter | Value | Value |
|-----------|-------|-------|
|           | Space group P2₁ | Space group P₂₁₂₁₂₁ |
| Data collection | | |
| Beam line | ID14–1 (ESRF) | 711 (MAX-lab) |
| Resolution (Å) | 2.9 | 2.8 |
| No. of observed reflections | 73,907 | 176,803 |
| No. of unique reflections | 23,209 | 28,218 |
| Completeness | 12.4 (34%) | 17.7 (3.5) |
| R/s | 99.1% (98.8%) | 100% (99.9%) |
| Rsym | 9.6% (33.8%) | 10.9% (46.8%) |
| B-factor from Wilson plot (Å²) | 53.3 | 55.3 |
| Refinement | | |
| Rfree | 28.7% (40.8%) | 29.2% (38.6%) |
| Rfactor | 21.3% (30.1%) | 21.4% (31.2%) |
| No. of protein atoms | 8832 | 8832 |
| No. of cofactor atoms | 144 | 144 |
| Overall B-factor (Å²) | 38.9 | 43.8 |
| r.m.s. deviation bonds (Å) | 0.009 | 0.013 |
| r.m.s. deviation angles (degrees) | 1.33 | 1.50 |
| Ramachandran plot | | |
| Percentage of non-glycine residues in most favorable regions | 85.0 | 87.0 |
| Percentage of non-glycine residues in additional allowed regions | 15.0 | 13.0 |

a Numbers in parentheses are for the highest resolution shell.

b 5% of the reflections were used to monitor Rfree and were not included in the refinement.
FIG. 2. **Overall structure of NirA.** A, ribbon representation of the ferredoxin-dependent sulfite reductase from *M. tuberculosis*. Blue, parachute domain (the N-terminal segment comprising residues 10–90 is shown in darker blue); green, middle domain; red, C-terminal domain. The [Fe₄S₄] cluster and the siroheme molecule are shown as ball-and-stick models. B, folding diagram of the domains of NirA. L, position of an unusual left-handed connection in the β-sheet. The color coding is as in A. C, contribution of the N-terminal segment of NirA (residues 10–90, shown in blue) to active site topology. The location of the active site is shown by the bound chloride ion (green sphere) and the active site residues Tyr⁶⁹ and Cys¹⁶¹, shown in orange. D, stereo view of the superposition of the N-terminal half (residues 67–312) (green) with the C-terminal half (residues 332–555) (red) of NirA illustrating the internal 2-fold pseudosymmetry indicative of gene duplication.
The enzyme has a specific activity of 2.0 units/mg protein, comparable with sulfite reductases from other organisms (13, 15). We could not detect any decrease in the concentration of nitrite in this assay. Reduction of the artificial electron donor methyl viologen by dithionite results in the formation of equimolar amounts of sulfite as a by-product, which will compete with nitrite as a substrate. The failure of the enzyme to reduce nitrite under these conditions, even when added in 10-fold excess over sodium dithionite, shows that NirA has a pronounced preference for sulfite over nitrite as substrate.3

Gel filtration chromatography and dynamic light scattering suggest that NirA is a monomer in solution (data not shown), a finding that is also consistent with the crystallographic analysis (see below).

The Electron Density Map and Quality of the Model—The structure of NirA was solved by molecular replacement in two crystal forms, each containing two molecules in the asymmetric unit (Table I). Most parts of the polypeptide chain, including the iron-sulfur cluster and the siroheme cofactor, are well defined in electron density. The real space correlation to the electron density maps is in the range of 0.87–0.92 for both protein chains and space groups. The N-terminal His tag and the first nine residues of NirA are not seen in the electron density map. Less well-defined regions of the polypeptide chain include several surface loops (residues 32–35, 51–55, 192–200, and 487–490). The final model consists of 8643 nonhydrogen protein atoms (two chains comprising residues 10–555) of NirA, two [Fe₄-S₄] clusters, two siroheme molecules, two chloride ions, and 20 water molecules. The stereochemistry of the model is as expected for this resolution (Table I). Superposition of the refined models of NirA from the two space groups gives an r.m.s. deviation of 0.45 Å, with the deviations mainly located in the weakly defined loop regions.

Overall Structure of M. tuberculosis NirA—In solution and in the crystal, NirA is present as a monomer. The buried accessible surface areas for the enzyme molecules in both crystal forms are on the order of 550 Å², corresponding to 2% of the total accessible surface area. This value is typical for crystal contacts rather than for a dimer interface.

The NirA molecule consists of three α/β domains: the parachute (19) domain (residues 1–159 and 331–405), the middle domain (residues 160–330), and the [Fe₄-S₄]-binding C-terminal domain (residues 406–555) (Fig. 2, A and B). Before entering the core of the parachute domain, the polypeptide chain packs against the surface of the middle domain and interacts through a number of hydrogen bonds and salt bridges with residues from that domain. The core of the parachute domain is built up of two modules with a ferredoxin-like fold (42) related by a pseudo-2-fold symmetry (Fig. 2, B and D) (see below). Each module consists of an antiparallel four-stranded β-sheet, flanked by two helices on one side. The two subdomains interact tightly to form the parachute domain. A major interface region involves residues following the second β-strands in each module, which form an additional antiparallel edge β-strand to the adjacent module (βp3 and βp3′, respectively), thus extending each sheet to a total of five β-strands (Fig. 2B). Residues 72–92 of this domain form a long loop between βp1 and βp2, packing against the C-terminal domain. Helix αp0 and β-strand βp1 limit access to the active site, and βp1 provides residues that are in van der Waals contact to the siroheme cofactor and thus form part of the active site (Fig. 2C).

The middle and C-terminal domains are very similar in structure. Each domain consists of a central five-stranded mixed β-sheet with the fourth strand antiparallel to the others (Fig. 2B). One side of the sheet forms part of the binding site for the cofactor, siroheme, or the [Fe₄-S₄] cluster, respectively, whereas the other side of the sheet is covered by four α-helices facing the surface of the molecule. The fold of these domains is unique to the sultite/nitrite reductase family.

A striking feature of the structure of NirA is the internal pseudo-2-fold symmetry. This feature was also noted in the crystal structure of CysI and led to the proposal that the enzyme has arisen as a result of gene duplication (19). Residues 67–312 of NirA can be superimposed on residues 332–555 with an r.m.s. deviation of 1.95 Å for 162 equivalent Ca atoms (Fig. 2D). The internal pseudo-2-fold symmetry is more pronounced in NirA than in E. coli CysI. A similar superposition of the CysI structure gives only 100 equivalent residues with an r.m.s. deviation value of 1.99 Å. The lower number of equivalent residues is in part due to the proteolytic digestion of the N terminus in CysI, which may have removed β-strand βp1 from the core of the parachute domain. The NirA gene thus also appears to have arisen as a result of gene duplication, although with 17% of identical residues it can barely be recognized at the amino acid sequence level. Of the 27 and 16 residues in NirA and CysI, respectively, that are identical between the N- and C-terminal halves, only four are conserved in both enzymes, Gly102/Ser, Gln113/Asp, Gly244/Gly, and Gly245/Gly. These residues are located at the N-terminal or C-terminal end of β-strands and appear to be conserved for structural reasons.

Comparison with Sulfite Reductase from E. coli—Despite the low degree of sequence conservation between NirA and CysI (23% identity) (Fig. 3), the overall structures are very similar. Superposition of the two protein models gave an r.m.s. deviation of 1.7 Å, based on 361 equivalent Ca atoms. Significant structural differences are found at the very C terminus of the polypeptide chain and are otherwise confined to several surface loop regions, where either insertions or deletions have occurred (Fig. 3).

A striking difference in structure is the accessibility of the active site. The N-terminal stretch of the polypeptide chain, which had been truncated in CysI and could therefore not be observed in the CysI crystals, folds over the substrate channel in NirA and limits the access to the active site from the solution. In particular, strand β1 (residues 67–72) completes the fold of the ferredoxin-like module 1 of the parachute domain, contributes to the architecture of the active site, and is positioned close to the siroheme cofactor (Fig. 2C). In full-length CysI, the N-terminal 73 residues could fold in a similar way.

Siroheme Binding—Siroheme binds tightly in the cleft formed at the interface between the three domains of NirA (Fig. 2A). The cofactor is buried in the protein interior, and only two oxygen atoms from one of the propionate moieties are accessible to solvent. There are numerous interactions of the siroheme cofactor with protein residues. All siroheme atoms, which can act as donor or acceptor, are engaged in hydrogen bonds or salt bridges (Fig. 4A). Eight basic residues (Arg30, Arg31, Lys112, Lys119, Arg297, Lys244, Arg245, and Arg246) form salt bridges to the carboxylate groups of the siroheme and contribute to charge compensation (residues that are invariant in the Nir-SIR enzyme family are underlined). In addition, several other residues form hydrogen bonds with the siroheme carboxylates (Ser128, Asp129, Asn132, Gln134, His136, Leu247 (main chain atoms), Ser248, Gln379, and Ser418). A crucial interaction is the coordination bond between the iron atom of the [Fe₄-S₄] cluster and the oxygen atom of the s指纹e cofactor.
tween the iron of the siroheme and the thiolate of Cys$^{467}$ (Fig. 4B). This invariant cysteine residue is also coordinated to one of the iron ions of the [Fe$_4$S$_4$] cluster. It thus provides the structural basis for the electronic coupling of the two redox cofactors, shown in previous studies of sulfite/nitrite reductases using a variety of techniques such as Mössbauer (43), EPR (44), $^{57}$Fe ENDOR (45), and NMR (46) spectroscopy. Most likely, the thiolate bridge directly participates...
electron transfer from the iron-sulfur center to the siroheme.

**Iron-Sulfur Cluster Binding**—The \( \text{[Fe}_{4}\text{-S}_{4}] \) cluster is bound exclusively by residues from the C-terminal domain and is located at the proximal side of the siroheme (Fig. 2A). Although the cluster is buried in the protein interior, it is rather close to the protein surface. The four iron ions are coordinated via covalent bonds to the thiolates of four conserved cysteine residues, Cys\text{417}, Cys\text{423}, Cys\text{463}, and Cys\text{467} (Fig. 4B). These residues are located on two regions of the polypeptide chain, the end of the first \( \beta \)-strand of the C-terminal domain and the following loop (C\text{173-SGIEFC}\text{423}) and a conserved loop (GC\text{463PNSC}\text{467A}), part of the left-hand connection between the second and third strand. This loop carries one of the conserved sequence fingerprints of the sulfite/nitrite reductase family (13) and wraps tightly around the cluster, shielding it from the solvent. The side chain of the invariant residue Asn\text{465} is accessible from the outside and could be involved in electron transfer from ferredoxin to the \( \text{[Fe}_{4}\text{-S}_{4}] \) cluster.

**The Substrate-binding Site Contains a Covalent Tyr-Cys Bond**—The distal face of the siroheme is accessible from the outside through a narrow channel that is dominated by basic residues. The positive electrostatic potential at the entrance of this channel, which extends into the active site, facilitates the encounter of the enzyme with the negatively charged substrate molecules and guides the diffusion of these anions into the interior of the substrate binding cleft (Fig. 5A).

At the distal face of the siroheme cofactor, residual density was observed during refinement, indicating an axial ligand bound to the iron ion (Fig. 5B). We have modeled this electron density as a chloride ion, due to the shape of the density and the fact that chloride anions were present during purification and crystallization in high concentration (400 mM). This anion-binding site is located at the position where substrate and other anions are bound in Cys\text{i} (19, 20). Residues close to the axial ligand in NirA are Arg\text{97}, Arg\text{120}, Arg\text{166}, Lys\text{207}, and Lys\text{209} (Fig. 5C). In Cys\text{i}, the corresponding residues are involved in binding of the substrate, and both lysine residues might also be involved in proton transfer during catalysis. All of these positively charged residues, with one exception, are conserved in the family of sulfite/nitrite reductases. The substitution of Lys\text{209} by an asparagine has been associated with a switch in substrate preference from sulfite to nitrite (20).

An unexpected feature in the structure of NirA is the covalent bond formed between the thiolate of Cys\text{161} and the C\( \varepsilon \) carbon atom of Tyr\text{69} (Fig. 5B). Attempts to verify this covalent link by mass spectrometry were inconclusive. The mass spectrometric analysis of tryptic peptides of NirA showed a fairly good coverage of the sequence (53%). Neither the peptide fragments expected in the absence of the Tyr-Cys bond nor a fragment consistent with the calculated mass of the peptide containing the covalent Tyr-Cys linkage could be identified.

This type of thioether-bridged tyrosyl-cysteine has been found in the active site of another redox enzyme, galactose oxidase (47). The covalently linked amino acids are located adjacent to a redox center, in this case copper ions, and act as a secondary cofactor in the stabilization of tyrosyl radicals (47, 48). The observation of such a covalently modified tyrosine side chain within van der Waals distance to the siroheme redox cofactor in NirA prompted us to further investigate the functional implications of this amino acid modification. The hydroxy group of Tyr\text{69} is in close proximity to the substrate-binding site, further indicating a possible role of this residue in catalysis. Tyr\text{69} was replaced by phenylalanine and alanine, whereas Cys\text{161} was mutated to serine and alanine. Mass spectrometric analysis of tryptic peptides verified the mutations and gave a fragmentation pattern consistent with the absence of the covalent linkage. The absence of the covalent bond also resulted in the same systematic shift in mobility of the mutant versus wild-type enzyme in SDS-PAGE as observed in galactose oxidase (49).

The absorption spectra of the four mutants showed minor but significant differences (data not shown), indicating that the electronic states of the redox cofactors are influenced by these amino acid substitutions. The mutants were catalytically impaired to various degrees, with the Y69F and C161S mutations being the most deleterious (Fig. 6). The significant decrease in activity indicates that the covalent linkage between these two residues in NirA is of functional importance, although not absolutely crucial for catalysis. It should be kept in mind, however, that the artificial electron donor methyl viologen has been used in the assay, and the situation may well be different in the case of the physiological electron donor. The residual activity of the Y69F mutant further indicates that this residue, albeit within proximity of the bound anion at the substrate-binding site, most likely does not participate in acid/base chemistry.

**The Putative Ferredoxin-binding Site**—In sulfite/nitrite reductases, the electrons flow from the external electron donor via the \( \text{[Fe}_{4}\text{-S}_{4}] \) cluster to the siroheme (18), and the most likely position for the ferredoxin-binding site is therefore in the vicinity of the iron-sulfur cluster. This redox center is located just beneath the enzyme surface, with the closest atom about 4 Å from the surface. A comparison of the structures of NirA and its NADPH-dependent homologue Cys\text{i} shows that several of the major structural differences between these two enzymes are located in this region, possibly reflecting the difference in the structure of the external electron donors. In particular, two loop regions at the enzyme surface are involved. The \( \beta \)-hairpin loop, which connects \( \beta \text{3} \) with \( \beta \text{4} \) in the C-terminal domain, is considerably longer, whereas the spatially adjacent loop between \( \beta \text{4} \) and \( \beta \text{5} \) is shorter in NirA by seven residues (Figs. 3 and 5A). It is noteworthy that the pattern of insertion/deletions for these two loops is also preserved in other bacterial ferredoxin-dependent sulfite/nitrite reductases when compared with Cys\text{i}. We propose that the putative binding site for the external electron donor is located at the enzyme surface in the

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**FIG. 4. Cofactor binding sites in NirA from *M. tuberculosis*. A. stereo view of the surroundings of the siroheme moiety. B. stereo view of the binding site of the \( \text{[Fe}_{4}\text{-S}_{4}] \) cluster. SRM, the siroheme molecule.
vicinity of these two surface loops, which are also close to the invariant surface residue Asn\textsuperscript{465} in contact with the iron-sulfur cluster. This region is, aside from the substrate channel, the only extended patch on the enzyme surface with a positive electrostatic potential (Fig. 5A). Since all putative ferredoxins from \textit{M. tuberculosis} are acidic proteins, the preponderance of positively charged residues might facilitate binding of reduced ferredoxin for electron transfer.

**Physiological Function of NirA—** We have shown here that the \textit{nirA} gene of \textit{M. tuberculosis} encodes a [Fe\textsubscript{4}-S\textsubscript{4}] and siroheme-dependent sulfite reductase. It should also be noted that NirA is the only protein sequence of \textit{M. tuberculosis} that is...
homologous to CysI of E. coli. This suggests that NirA participates in the assimilation of sulfur for the biosynthesis of sulfur-containing amino acids. A comparison of the localization of some of the cys genes in E. coli and M. tuberculosis also supports this proposal. In E. coli, the genes for the flavoprotein subunit CysJ and the hemoprotein component CysI of sulfite reductase are followed by CysH, encoding adenosyl phosphosulfate reductase. In M. tuberculosis, NirA is similarly followed by CysH, and no gene corresponding to the flavoprotein subunit cysJ of the NAPDH-dependent sulfite reductase of E. coli is found at this locus. A BLASTX search (50) of the whole genome of M. tuberculosis (51) for homologues of the cysJ gene from E. coli did not result in any hits, suggesting that in M. tuberculosis, the NAPDH-dependent sulfite reductase is replaced by a ferredoxin-dependent homologue. Furthermore, in a gene expression study of dormant M. tuberculosis, not only nirA but also several other genes involved in cysteine biosynthesis were shown to be up-regulated (10). One of these genes is cysG, encoding an enzyme of siroheme biosynthesis, further emphasizing the link between siroheme-dependent NirA and the cysteine biosynthetic pathway.

A Sulfite Versus Nitrite Reductase Fingerprint—A sequence alignment of ferredoxin-dependent nitrite/sulfite reductases suggests that these enzymes can be divided into two subfamilies. In one set of amino acid sequences, Tyr69 and Cys161, involved in the covalent linkage, and, in common with the NAPDH-dependent sulfite reductases, residues His136 and Lys209, interacting with the siroheme, are invariant. In the other class, these residues are consistently replaced by phenylalanine, glycine, arginine, and asparagine, respectively (Fig. 3). We propose that these two sequence classes correspond to enzymes with a preference for sulfite and nitrite as substrate, respectively. This proposal agrees with available biochemical data on substrate specificity of members of this family (i.e. all established sulfite reductase belong to the first subfamily, whereas nitrite reductases fall within the second subfamily). This fingerprint could guide annotation of genes of the ferredoxin-dependent nitrite/sulfite reductase family as nir or sir genes.

Conclusions—The crystallographic study of full-length M. tuberculosis NirA provides the first three-dimensional structure of a ferredoxin-dependent sulfite/nitrite reductase. Despite the low degree of sequence conservation, the core of the molecule is similar in structure to the NAPDH-dependent sulfite reductase from E. coli. The N-terminal part of the enzyme, comprising the first 90 amino acids, previously unknown in structure, does not form a distinct separate domain but is part of the first ferredoxin-like module of the parachute domain. This segment of the polypeptide chain also contributes to the formation of the active site. It limits access to the distal face of the siroheme and provides residues lining the active site. The cofavalent bond between the side chains of residues Tyr69 and Cys161 in the active site of NirA facilitates catalysis but is not essential for the enzymatic reaction with artificial electron donors. This situation appears different from that found in copper-dependent galactose oxidase where such a cofavalent bond is essential for enzyme function (48). We suggest that this structural feature is typical for ferredoxin-dependent sulfite reductases as opposed to nitrite reductases. The putative ferredoxin-binding site is located at a positively charged surface patch of the enzyme, close to the iron-sulfur cluster.

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Siroheme- and [Fe₄-S₄]-dependent NirA from Mycobacterium tuberculosis Is a Sulfite Reductase with a Covalent Cys-Tyr Bond in the Active Site
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