The Redox State Regulates the Conformation of Rv2466c to Activate the Antitubercular Prodrug TP053

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Rv2466c is a key oxidoreductase that mediates the reductive activation of TP053, a thienopyrimidine derivative that kills replicating and non-replicating Mycobacterium tuberculosis, but whose mode of action remains enigmatic. Rv2466c is a homodimer in which each subunit displays a modular architecture comprising a canonical thioredoxin-fold with a Cys19- Pro20-Trp21-Cys22 motif, and an insertion consisting of a four α-helical bundle and a short α-helical hairpin. Strong evidence is provided for dramatic conformational changes during the Rv2466c redox cycle, which are essential for TP053 activity. Strikingly, a new crystal structure of the reduced form of Rv2466c revealed the binding of a C-terminal extension in α-helical conformation to a pocket next to the active site cysteine pair at the interface between the thioredoxin domain and the helical insertion domain. The ab initio low-resolution envelopes obtained from small angle x-ray scattering showed that the fully reduced form of Rv2466c adopts a “closed” compact conformation in solution, similar to that observed in the crystal structure. In contrast, the oxidized form of Rv2466c displays an “open” conformation, where tertiary structural changes in the α-helical subdomain suffice to account for the observed conformational transitions. Altogether our structural, biochemical, and biophysical data strongly support a model in which the formation of the catalytic disulfide bond upon TP053 reduction triggers local structural changes that open the substrate binding site of Rv2466c allowing the release of the activated, reduced form of TP053. Our studies suggest that similar structural changes might have a functional role in other members of the thioredoxin-fold superfamily.

Among infectious human diseases, tuberculosis (TB) is the second greatest killer worldwide due to a single infectious agent, and remains a major challenge to human health care. In 2013, there were about 9 million new cases and 1.5 million deaths from TB, with an estimated one-third of the human population carrying a latent infection (1). First-line treatment for drug-susceptible TB requires the administration of a combination of four drugs during a period of 6 months: isoniazid, rifampicin, ethambutol, and pyrazinamide. Lengthy treatment regimens, unpleasant side effects, and patient noncompliance have provided conditions for the generation of multidrug-resistant and extensively drug-resistant cases of TB (2). Thus, the discovery and development of novel anti-TB drugs with bactericidal mechanisms different from those of currently available agents has become an urgent need. In that context, bedaquiline, a diarylquinoline that inhibits the c subunit of ATP synthase from Mycobacterium tuberculosis, has been recently approved by the Food and Drug Administration (FDA) for the treatment of multidrug-resistant TB in adults (3–5). Moreover, several candidate molecules are currently in preclinical studies, phase II and III clinical trials (6, 7). However, up to date, only a few drugs are capable of effectively killing non-replicating M. tuberculosis, thus allowing TB reactivation (8). Interestingly, we have recently discovered a new series of thienopyrimidine (TP) compounds that kill both replicating and non-replicating bacilli (Fig. 1) (9). Structure-activity relationship analysis demonstrated that a NO2 group at position C6 is essential for anti-TB activity as any alternative substitution, including NH2, carboxitriile, carboxylate, or carboxamide, resulted in an inactive TP compound. In addition, the most active compound TP053, displayed a hydrophobic phenyl and N-methyl groups at positions 2 and 4, respectively, accounting for a minimum inhibitory con-
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**Figure 1.** Rv2466c mediates the reduction of TP053 to kill M. tuberculosis. Model of the catalytic cycle of Rv2466c. Reduced Rv2466c reacts with TP053 to oxidize Rv2466c and reduced reaction products. Oxidized Rv2466c is then recycled to its reduced form by reduced DTT via disulfide exchange.

### Experimental Procedures

**Methods—**Recombinant Rv2466c wild-type and mutants were produced in *Escherichia coli* and purified to apparent homogeneity as previously described (9). Rv2466c-C195, Rv2466c-C225, and Rv2466c-H99A, mutants were synthesized by GenScript using the pET29a-Rv2466c construct as template. Activity measurements were performed as previously described (9).

Rv2466c-CT-His Expression and Purification—The Rv2466c gene was amplified by standard PCR using oligonucleotide primers Rv2466c_CT_His_Nde1_TEV_Fwd (5′-GGAATTCCTATATGTCTGAGAGCCGCCGAGGCTCGGTGCG-3′) and Rv2466c_CT_His_HindIII_TeV_Rev (5′-CCCAAGCTTGGTCGAACGTAGGCGGCGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGC-3′), Phusion DNA Polymerase (New England Biolabs), and genomic DNA from *M. tuberculosis* H37Rv as DNA template. The PCR fragment was digested with Ndel and HindIII and ligated to the expression vector pET23b (Novagen) generating pET23b-Rv2466c-CT-His. The recombinant Rv2466c-CT-His (220 residues) has an additional peptide of 13 amino acids (KLAAALEHHHHHH) at the C terminus that includes a histidine tag (Fig. 3). *E. coli* BL21(DE3)pLyS cells transformed with pET23b-Rv2466c-CT-His were grown in 3000 ml of 2× YT medium supplemented with 100 µg/ml of carbenicillin and 34 µg/ml of chloramphenicol at 37 °C. When the culture reached an *A*₅₆₂₅₀ value of 0.8, the Rv2466c-CT-His expression was induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (MIP). After ~16 h at 18 °C, cells were harvested and resuspended in 40 ml of 20 mM imidazole, 50 mM Tris-HCl, pH 7.5, 500 mM NaCl (solution A), containing protease inhibitors (Complete EDTA-free, Roche). Cells were disrupted by sonication (five cycles of 1 min) and the suspension was centrifuged for 20 min at 10,000 × g. Supernatant was applied to a HisTrap chelating column (1 ml, GE HealthCare) equilibrated with solution A. The column was washed with solution A until no absorbance at 280 nm was detected. Elution was performed with a linear gradient of 20–500 mM imidazole in 40 ml of solution A at 1 ml/min. Fractions containing Rv2466c-CT-His were pooled and loaded into a HiLoad 16/60 Superdex 200 column (GE HealthCare) equilibrated in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl (solution B). Aliquots of 12 µl were mixed with 3 µl of 250 mM Tris-HCl, pH 8.0.

The crystal structure of reduced Rv2466c has been solved recently, revealing a unique homodimer in which a β-strand is swapped between the thioredoxin domains of each subunit, and an α-helical subdomain inserted into each thioredoxin domain (9). A large, mostly hydrophobic groove harbors a redox active-site motif CPWC, typical of the thioredoxin superfamily of oxidoreductases (13–16). Using a combination of x-ray crystallography, small angle x-ray scattering, limited proteolysis, circular dichroism, site-directed mutagenesis and activity measurements, we show that Rv2466c undergoes significant conformational changes upon formation of its oxidized state, providing unprecedented insight into the molecular mechanism of TP053 activation.

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The experimental data support a model in which the oxidized derivatives (9), as these products are extremely toxic, readily damage unknown cellular targets including proteins, membrane lipids, and DNA and likely leading to *M. tuberculosis* death (9).

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6.8, 10% SDS, 50% glycerol, 500 mM DTT, and 0.01% bromphenol blue at the indicated times. Samples were boiled for 3 min and run onto a NuPAGE® 4–12% gel (Invitrogen). Protein bands were visualized by staining the gel with SimplyBlue™ SafeStain (Invitrogen). The resulting preparation displayed a single protein band. Purified Rv2466c-CT-His was concentrated to 10–50 mg ml⁻¹ in 10 mM Tris-HCl, pH 7.5, and stored at −80 °C.

Rv2466c-CT-His Crystallization and Data Collection—Rv2466c-CT-His was crystallized in the presence of the 13-residue long C-terminal tag containing 6 histidine residues (Fig. 3). Two crystal structures were obtained, referred thereafter as Rv2466c-HT1 and Rv2466c-HT2. Rv2466c-HT1 was obtained by mixing 0.25 μl of Rv2466c-CT-His at 10 mg/ml in 20 mM Tris-HCl, pH 7.5, with 0.25 μl of a solution containing 100 mM sodium acetate, pH 5.5, 0.3 M sodium acetate, 25% (w/v) PEG 20,000. Crystals grew in 7–8 days as rhombic prisms to maximum dimensions of 140 × 80 × 80 μm and cryo-cooled in liquid nitrogen using 100 mM sodium acetate, pH 5.5, 0.3 M sodium acetate, 10% (w/v) PEG 20,000, and 25% (v/v) ethylene glycol. X-ray diffraction data were collected at beamline ID29 (European Synchrotron Radiation Facility, Grenoble, France) and processed with XDS program (17) to a maximum resolution of 1.70 Å. Rv2466c-HT1 crystallized in space group P2₁ with two independent molecules in the asymmetric unit (Table 1). The second structure, Rv2466c-HT2, was obtained by mixing 0.25 μl of Rv2466c at 10.0 mg/ml with 0.25 μl of 0.1 M Tris-HCl, pH 8.5, 0.1 M magnesium chloride, and 17% (w/v) PEG 20,000. Crystals grew in 1–2 days as rhombic prisms to maximum dimensions of 100 × 50 × 50 μm. Prior to data collection the crystals were cryo-cooled in liquid nitrogen by using 0.1 M Tris-HCl, pH 8.5, 0.1 M magnesium chloride, and 10% (w/v) PEG 20,000 and 20% ethylene glycol as cryo-protectant solution. X-ray diffraction data were collected at beamline I04 (Diamond Light Source, United Kingdom) and processed with XDS program (17). Crystals diffracted to a maximum resolution of 1.51 Å. Rv2466c-HT2 crystallized in P2₁ with two independent molecule in the asymmetric unit (Table 1).

Rv2466c-CT-His Structure Determination and Refinement—Structure determination of Rv2466c-CT-His was carried out by molecular replacement using Phaser (18) and the PHENIX suite (21). The final manual refinement was accomplished with 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma) and post-transition baselines, respectively, T is the temperature (°C) and Hm is the enthalpy change of unfolding at T°. Transitions were tentatively fitted according to Equation 1 to obtain the apparent melting temperature (Tm). Equation 1

\[ y = \left( y_i + m_i \times T \right) + \left( y_u + m_u \times T \right) \times e^{(\Delta Hm/RT)} \times e^{((T - Tm)/Tm)} \times (1 + e^{((T - Tm)/Tm)}) \]

where y represents the observed CD signal at 222 nm, y₁ and y₂ are the y axis intercepts, and m₁ and m₂ are the slopes of the pre- and post-transition baselines, respectively. T is the temperature in K. Tm is the melting temperature, and ΔHm is the enthalpy change of unfolding at T°. Curve fitting was performed with Prism GraphPad.

Small Angle X-ray Scattering Measurements—Synchrotron x-ray diffraction data for recombinant purified WT Rv2466c and mutants were collected on the P12 beamline of the European Molecular Biology Laboratory (storage ring Petra-3, DESY, Hamburg) on a pixel Pilatus 2M detector. Scattering
patterns were measured with a 1-s exposure time for protein samples at a minimum of three different protein concentrations ranging from 1 to 10 mg/ml. Samples were incubated with 2 mM DTT or 2 mM ascorbic acid in the case of WT Rv2466cOX before data collection. To check for radiation damage, 20 50-ms exposures were compared; no radiation damage was observed. Using the sample-to-detector distance of 3.1 m, the range of momentum transfer values is 0.008 < q < 0.47 Å⁻¹ (s = 4 π sin(θ)/λ where 29 is the scattering angle and λ = 1.5 Å is the x-ray wavelength). Data were processed using standard procedures and extrapolated to infinite dilution by the program package PRIMUS (26). The forward scattering (I(0)) was evaluated using the Guinier approximation (27) assuming the intensity is represented as I(s) = I(0)exp(-(qR₀)²/3) for a very small range of momentum transfer values (s < 1.3/R₀). The maximum dimensions (Dmax), the interatomic distance distribution functions (P(r)), and the radii of gyration (Rg) were computed using GNOM (28). The molecular mass of the protein was evaluated by comparison of the forward scattering with that from a reference solution of bovine serum albumin.

**Ab Initio Shape Determination**—The low-resolution structures of WT Rv2466c and mutants were calculated *ab initio* by using DAMMIF (29). Structure clustering and averaging were carried out with DAMAVER and DAMCLAUST (30). The results and statistics are summarized in Table 2. For each data set, pairwise alignment of structures was carried out, and the normalized spatial discrepancy (NSD) was calculated (31). The clustering process groups structures with lower NSD. In general, NSD values close to 1 indicate that the two structures are similar; structures with NSD larger than 2 are taken as outliers and removed. Average NSD values within clusters were between 0.6 and 1, indicating a very good structural agreement for each cluster. Furthermore, average NSD values for structures of different clusters are between 0.8 and 1.1 also indicating similarity between the clusters.

**Fitting the Crystal Structure to Small Angle X-ray Scattering (SAXS) Envelopes**—The crystal structure of Rv2466c (PDB code 4NXI) was fitted to the SAXS envelopes using Chimera. The video (supplemental Video S1) shows a swinging back-and-forth movement of the helical bundle. To calculate the morph trajectory, we first used Modeler (34) to construct *ab initio* all missing side chains in the crystallographic structures of the Rv2466c reduced state (PDB code 4NXI). The hybrid SASREF model of the Rv2466c oxidized state was calculated following the methodology described under “Fitting the Crystal Structure to SAXS Envelopes.” After superposition of their thioredoxin domains, we used Chimera (UCSF) to generate coarse conformational intermediates by interpolating the atomic positions. The interpolation was based on the Cockscrew method, as implemented in Chimera (32). We show 171 linear interpolation steps calculated between the reduced and oxidized Rv2466c forms. For each interpolation step, the hydrogen-bonding network was calculated and displayed. The conformational intermediates of only one monomer in the dimer are shown for clarity (chain A).

**Results and Discussion**

Rv2466c Displays a Modular Architecture with Structural Variability at an α-Helical Subdomain—The crystal structure of Rv2466c in its reduced state revealed a modular architecture comprising a canonical thioredoxin-fold (residues Met¹ to Val⁵⁰ and Asp¹³ to Ser¹⁸⁹), an insertion consisting of an antiparallel four α-helical bundle (residues Trp⁶⁹ to Tyr¹³²; α₃⁺α₄-α₅-a6) and a short α-helical hairpin (residues Met⁴⁵ to Ala⁶⁸; α₂-α₃; Fig. 2A)(9, 14). Importantly, the thioredoxin domain and the α-helical bundle communicate with each other through an extensive interface of 1380 Å². This interface comprises residues Phe¹⁵, Asp¹⁶, Pro¹⁷, and Leu¹⁸ (β₁-α₁ loop), Trp²³, Arg²⁷, and Leu²⁹ (α₁), Phe⁴₂, and Val⁴⁴ (β₂; from the thioredoxin-fold domain); and Val⁷² and Ile⁷⁶ (α₃), Leu⁷⁷, Asp⁸⁸, Tyr⁹¹, Thr⁹³, Asn⁹⁶, Ile⁹⁸, His¹⁰⁰ (α₄; located in the α-helical subdomain; 9). The last 17 residues of the protein (Tyr¹⁹⁰ to Asp²⁰⁷) drives protein dimerization via a β-strand (β₅) swapping mechanism (Fig. 2A) (9). The Cys¹⁹⁻Pro²⁰⁻Trp²¹⁻Cys²² active-site motif is located on top of α₁ in the thioredoxin-fold, and in close proximity to the α₂-α₃ hairpin and the four-helix bundle of the insertion domain. It is worth noting that the lateral chain of Trp²⁸ makes hydrophobic interactions with three secondary structural elements of the core of the thioredoxin domain of Rv2466c. The mutation of W28S predicted structural defects in the thioredoxin-fold, likely including the destabilization of the dimerization core and the Cys¹⁹⁻Pro²⁰⁻Trp²¹⁻Cys²² motif, impairing the activity of Rv2466c against TP053. Structural superposition of the two monomers of the protein dimer suggested that the helical subdomain might display structural flexibility (9).

Here we determined the crystal structure of a second construct of Rv2466c (Rv2466c-CT-His; 220 residues) in its...
reduced form (Rv2466c\textsubscript{RED}), containing an additional peptide of 13 amino acids at the C terminus that includes a hexahistidine tag (208KLAAALEHHHHHH220), at 1.51-Å resolution (Fig. 2 and Table 1). The close inspection of the electron density maps revealed that the C-terminal peptide extension binds to the active site pocket, being in direct contact with the Cys\textsuperscript{19}-Pro\textsuperscript{20}-Trp\textsuperscript{21}-Cys\textsuperscript{22} motif (Fig. 2, B and C). Strikingly, the peptide adopts an α-helix conformation (α9; Fig. 2C). The protein-peptide interaction promotes several conformational changes in Rv2466c, including the reorientation by about 20 degrees of the α2-α3' hairpin, which opens the groove next to the active thiol pair that accommodates the peptide (r.m.s. deviation for residue range 45–68 is 2.8–3.0 Å; Fig. 2D). A short kink (residues 64 to 67) located between α3' and α3'' helices adopts an α-helical conformation allowing these two helices to merge into an elongated α3 helix of about 38 Å in length (r.m.s. deviation for residue range 59–82 is 1.9 Å; Fig. 2D) (35). In addition, the connecting loop α7-β3, located at the central hydrophobic groove becomes partially disordered in both monomers.
More recently, the crystal structure of the reduced, unliganded form of a Rv2466c homologue from Mycobacterium leprae has been solved at 2.3-Å resolution (PDB code 4WKW; 80.6% amino acid sequence identity; Z-score of 13.0; r.m.s. deviation value of 1.26 for 177 aligned residues for monomer B, and Z-score of 12.3; r.m.s. deviation value of 1.35 for 175 aligned residues for monomer A). Structural comparison of Rv2466c-CT-His with the M. leprae homologue revealed that (i) the Cys<sup>19</sup>-Pro<sup>20</sup>-Trp<sup>21</sup>-Cys<sup>22</sup> active-site motif and (ii) the residues located at the interface between the thioredoxin-fold and the α-helical subdomain are strictly conserved in both proteins. However, very important differences were observed in the conformation of the α-helical subdomain in both monomers of the protein dimer, indicating structural variability and a tendency of C2 symmetry breakage (Fig. 2, E and F). Interestingly, the α-helical hairpin was partially disordered in monomer A, as observed in the crystal structure of the untagged Rv2466c (9). Taken together, the experimental data suggest that the artificial C-terminal tag in Rv2466c binds next to the active-site disulfide in a manner that mimics the structure of a complex between Rv2466c and a putative peptide substrate and that the substrate is accommodated via a conformational change in the helical bundle as a result of the intrinsic flexibility within the helical bundle. In addition, the structural data suggest that the interface between the thioredoxin domain and the α-helical subdomain might play an important role during substrate recognition and catalysis.

### TABLE 1

| X-ray crystallography data | Rv2466c-His |
|---------------------------|-------------|
| Beamline                   | 104 (DLS)   |
| Wavelength (Å)            | 0.952700    |
| Resolution range (Å)      | 40.26—1.507 (1.561—1.507) |
| Space group               | P<sub>2</sub> |
| Unit cell                 | 40.649 46.814 104.308 90 97.97 90 |
| Total reflections          | 249,842 (19424) |
| Unique reflections         | 60,540 (5200) |
| Multiplicity              | 4.1 (3.7)   |
| Completeness (%)          | 98.29 (85.07) |
| Mean I/σ(I)               | 10.47 (2.11) |
| Wilson B-factor           | 11.86       |
| R<sub>sym</sub>           | 0.09497 (0.6503) |
| R-factor                  | 0.1314 (0.1958) |
| R<sub>free</sub>          | 0.1762 (0.2530) |
| Number of non-H atoms     | 3861        |
| Macromolecules            | 3415        |
| Ligands                   | 1           |
| Water                     | 426         |
| Protein residues          | 419         |
| R.m.s. (bonds)            | 0.009       |
| R.m.s. (angles)           | 1.15        |
| Ramach. favored (%)       | 100         |
| Ramach. outliers (%)      | 0           |
| Clashscore                | 3.00        |
| Average B-factor          | 19.40       |
| Macromolecules            | 17.70       |
| Ligands                   | 38.30       |
| Solvent                   | 32.00       |

**FIGURE 3.** Limited proteolysis experiments show significant conformational differences between the redox forms of Rv2466c. A, SDS-PAGE showing the trypsin cleavage profile for Rv2466c<sub>OX</sub>, Rv2466c<sub>RED</sub>, and Rv2466c<sub>RED</sub> in the presence of TP053. B, the peptide bonds that are cleaved specifically by trypsin are shown with arrows. The N-terminal sequences of selected proteolytic fragments are underlined in the amino acid sequence and labeled in dark green in the schematic representation of Rv2466c.
TABLE 2
SAXS data and refinement parameters

| Data collection parameters | Rv2466c reduced | Rv2466c C19S | Rv2466c C22S | Rv2466c oxidized | Rv2466c H99A |
|----------------------------|-----------------|--------------|--------------|-----------------|--------------|
| Instrument                 | Beamline P12 (Petra-3) | Beamline P12 (Petra-3) | Beamline P12 (Petra-3) | Beamline P12 (Petra-3) | Beamline P12 (Petra-3) |
| Wavelength (Å)             | 1.5             | 1.5          | 1.5          | 1.5             | 1.5          |
| S range (Å⁻¹)              | 0.007–0.370     | 0.008–0.322  | 0.014–0.307  | 0.009–0.370     | 0.015–0.316  |
| Exposure time (s)          | 1               | 1            | 1            | 1               | 1            |
| Concentration range (mg ml⁻¹) | 1–10          | 1–10         | 1–10         | 1–10            | 1–10         |
| Temperature (K)            | 283             | 283          | 283          | 283             | 283          |

Structural parameters
- \( I(0) \) (cm⁻¹) (from \( P(r) \))
  - Rv2466c reduced: 37.43(1)
  - Rv2466c C19S: 35.99(1)
  - Rv2466c C22S: 37.72(1)
  - Rv2466c oxidized: 38.52(1)
  - Rv2466c H99A: 37.60(1)
- \( R_g \) (Å) (from \( P(r) \))
  - Rv2466c reduced: 25.7(1)
  - Rv2466c C19S: 25.4(1)
  - Rv2466c C22S: 25.7(1)
  - Rv2466c oxidized: 27.2(1)
  - Rv2466c H99A: 26.4(1)
- \( I(0) \) (cm⁻¹) (from Guinier)
  - Rv2466c reduced: 37.65(7)
  - Rv2466c C19S: 35.91(2)
  - Rv2466c C22S: 37.62(5)
  - Rv2466c oxidized: 38.5(1)
  - Rv2466c H99A: 37.57(3)
- \( D_{max} \) (Å)
  - Rv2466c reduced: 81.0
  - Rv2466c C19S: 81.3
  - Rv2466c C22S: 84.9
  - Rv2466c oxidized: 90.0
  - Rv2466c H99A: 89.5
- Porod volume estimate (Å³)
  - Rv2466c reduced: 27,900
  - Rv2466c C19S: 27,900
  - Rv2466c C22S: 27,900
  - Rv2466c oxidized: 27,900
  - Rv2466c H99A: 27,900
- \( Ab\ ini\ initio\ modeling (x^2\ value)\)
  - Rv2466c reduced: 0.973(1)
  - Rv2466c C19S: 1.179(7)
  - Rv2466c C22S: 0.890(3)
  - Rv2466c oxidized: 0.747(1)
  - Rv2466c H99A: 0.965(1)
- Average NSD between clusters
  - Rv2466c reduced: 0.91(2)
  - Rv2466c C19S: 0.8(1)
  - Rv2466c C22S: 0.86(1)
  - Rv2466c oxidized: 0.9(2)
  - Rv2466c H99A: 0.89(1)
- Average NSD within clusters
  - Rv2466c reduced: 0.6(1)
  - Rv2466c C19S: 0.6(1)
  - Rv2466c C22S: 0.58(1)
  - Rv2466c oxidized: 0.59(8)
  - Rv2466c H99A: 0.58(9)

Molecular mass determination
- Molecular mass (from \( I(0) \)) (kDa)
  - Rv2466c reduced: 45(3)
  - Rv2466c C19S: 43(3)
  - Rv2466c C22S: 45(3)
  - Rv2466c oxidized: 46(3)
  - Rv2466c H99A: 45(3)
- Calculated monomeric from sequence (kDa)
  - Rv2466c reduced: 23.1
  - Rv2466c C19S: 23.1
  - Rv2466c C22S: 23.1
  - Rv2466c oxidized: 23.1
  - Rv2466c H99A: 23.1

Software employed
- Primary data reduction
  - SASFLOW
- Data processing
  - PRIMUS
- Ab initio analysis
  - DAMMIF
- Validation and averaging
  - DAMAVER
- Rigid body modeling
  - SASREF
- Computation of model intensities
  - CRYSOL

* S-range used for calculation of \( P(r) \) function and \( ab\ ini\ initio\ modeling.
* Values in parentheses are estimated errors approximated to the last decimal place.
* Two clusters have been identified, thus no standard deviation reported.
* Molecular mass estimated by calculation of \( I(0) \) and comparison against bovine serum albumin.

FIGURE 4. Conformation of oxidized and reduced Rv2466c, as characterized by SAXS. A, upper panel: scattering curves of Rv2466c_red and Rv2466c_ox. Lower panel: \( P(r) \) function distributions of Rv2466c_red and Rv2466c_ox. B and C, low resolution models of Rv2466c_red and Rv2466c_ox in solution. Average low resolution structure of Rv2466c_red (B) and Rv2466c_ox (C) with the high resolution crystal structure of Rv2466c_red (Protein Data Bank code 4NXI) fitted by rigid body docking, respectively.
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fragments of 13 and 9 kDa. The 13-kDa band proved to be a mixture of 4 fragments with the N-terminal sequences EGMAR, AATS, SHHAG, and KSHHA, whereas the 8-kDa fragment showed the sequence AATS located in the last α-helix of the bundle. Moreover, whereas TP053 becomes reduced, oxidized Rv2466c was formed (70% of Rv2466cOX after 150 min incubation with TP053), adopting a similar proteolytic pattern to that observed for Rv2466cOX.

Second, the overall shapes of Rv2466cOX and Rv2466cRED in solution were obtained by SAXS, a powerful technique capable of providing structural information on flexible and dynamic proteins in solution (38, 39) (Table 2; see Fig. 4A for experimental SAXS data and DAMMIF-calculated fits from corresponding overall solution structures). The interatomic distance distribution functions \((P(r))\) computed for Rv2466cOX and Rv2466cRED are shown in Fig. 4A. The molecular mass determined from the scattering data confirmed that the protein is a homodimer in solution with size exclusion chromatography and dynamic light scattering (9). The radius of gyration \((R_g)\) values obtained for Rv2466cOX (27.2(1) Å) and Rv2466cRED (25.7(1) Å) revealed a reduction in \(R_g\) \((\Delta R_g)\) of about -1.5 Å, indicating that Rv2466cOX is in an extended conformation compared with the reduced state.

The ab initio low-resolution envelopes of Rv2466cOX and Rv2466cRED were reconstructed using SAXS data (Fig. 4; Table 2). The crystal structure (PDB 4NXI) readily fits into the ab initio low-resolution envelope of Rv2466cRED (Fig. 4B). It is worth noting that even though symmetry constrains were not applied in the ab initio reconstruction, the low-resolution envelopes of Rv2466cRED clearly show internal symmetry, in agreement with its homodimeric nature. In contrast, the ab initio shape of Rv2466cOX appears asymmetric and significantly differs from the crystal structure of Rv2466cRED (Fig. 4C) pointing to major differences between the redox states. The structural differences are most remarkable in the region of the α-helical domains; in the case of Rv2466cRED both apical lobes display a similar compact conformation, in which the crystallographic conformation readily fits. To reconcile the structure of Rv2466cOX observed in the crystal and the oxidized state suggested by SAXS we performed a refinement as implemented in the program SASREF (see “Experimental Procedures” for details). The method employed consists in allowing a certain degree of flexibility within α-helical domains, whereas fixing the thioredoxin domain. Using this refinement strategy, it was possible to construct a hybrid model agreeing with the solution SAXS envelope and fitting the experimental SAXS data from Rv2466cRED with discrepancy \(\chi^2\) around 1.0. These results indicate that tertiary structural changes in the α-helical subdomain are sufficient to account for the observed variability (Fig. 4C).

To further explore this hypothesis, we investigated the secondary and tertiary structure changes of Rv2466c under reducing and oxidizing conditions by near-UV circular dichroism (CD). The near-UV CD spectra revealed clear differences in the local environment of the aromatic side chains in Rv2466cOX and Rv2466cRED (Fig. 5A). In addition, thermal unfolding followed by the far-UV CD signal at 222 nm indicated dramatic differences in protein stability between the two states (Fig. 5B). Specifically, the apparent melting temperatures \(T_m\) of Rv2466cOX and Rv2466cRED were 41.5 and 63.4 °C, indicating that the reduced state is about 22 °C more stable than the oxidized form. In summary, CD analysis indicates that the formation of the catalytic disulfide bond in Rv2466cOX leads to large tertiary structure changes and a less stable conformation. These results are in good agreement with the predicted opening of the α-helix subdomain of the protein in oxidative conditions, as observed by SAXS.

The Redox State of the CPWC Motif Regulates Rv2466c Conformation and Activity—Rv2466c displays a catalytic disulfide located in the Cys19-Pro20-Trp21-Cys22 motif. In Rv2466cRED, the N-terminal cysteine (Cys19) is solvent-exposed, whereas the C-terminal cysteine (Cys22) is buried, as observed in other members of the thiol-disulfide oxidoreductase superfamily (Fig. 6A) (40). Interestingly, Rv2466cRED adopts a “closed” conformation and is able to metabolize TP053, whereas Rv2466cOX adopts an “open” conformation, and is unable to activate TP053. To gain further insight into the relevance of the redox state on the conformational changes triggered in Rv2466c and
its ability to metabolize TP053, we studied the single-cysteine variants C19S and C22S. As depicted in Fig. 6B, the C19S mutant proved to be completely inactive, whereas the C22S mutant displayed only 6% residual activity. This is consistent with the fact that Cys19 is the active-site cysteine responsible for the initial nucleophilic attack during the reduction of TP053 (13, 40). Interestingly, the near-UV CD spectra of the C19S and C22S variants. As depicted in Fig. 6B, the C19S mutant proved to be completely inactive, whereas the C22S mutant displayed only 6% residual activity. This is consistent with the fact that Cys19 is the active-site cysteine responsible for the initial nucleophilic attack during the reduction of TP053 (13, 40). Interestingly, the near-UV CD spectra of the C19S and C22S variants.

The redox state of the CPWC motif regulates Rv2466c conformation and activity. A, localization of Cys19 and Cys22 in the active site of Rv2466c (top of α1 helix). B, activity measurements of Rv2466c WT and Rv2466c variants against TP053 (9). C, near-UV CD spectra of the C19S and C22S variants. D, thermal unfolding of the C19S and C22S variants as monitored by CD spectroscopy. E, low resolution models of C19S and C22S mutants in solution. Average low resolution structure of C19S and C22S mutants with the high resolution crystal structure of Rv2466cRED (Protein Data Bank code 4NXI) fitted by rigid body docking, respectively.

A

B

C

D

E

FIGURE 6. The redox state of the CPWC motif regulates Rv2466c conformation and activity. A, localization of Cys19 and Cys22 in the active site of Rv2466c (top of α1 helix). B, activity measurements of Rv2466c WT and Rv2466c variants against TP053 (9). C, near-UV CD spectra of the C19S and C22S variants. D, thermal unfolding of the C19S and C22S variants as monitored by CD spectroscopy. E, low resolution models of C19S and C22S mutants in solution. Average low resolution structure of C19S and C22S mutants with the high resolution crystal structure of Rv2466cRED (Protein Data Bank code 4NXI) fitted by rigid body docking, respectively.

The Open to Closed Motion Is Mediated by Conformational Changes at the Interface between the Thioredoxin Domain and the α-Helical Subdomain—The α-helical subdomain of Rv2466c comprises an antiparallel four-helix bundle (α3′–α4–α5–α6) and a short α-helical hairpin (α2–α3′). The α3′, α4, α5, and α6 helices display an amphipathic character with short connecting loops, and a topology that diverges from a canonical four α-helical bundle. This deviation is accounted by steric restrictions and intramolecular interactions imposed by the neighboring thioredoxin-fold. Specifically, the structural elements β1, β2, and α1 located in the thioredoxin-fold seem to be important to maintain the stability of the α-helical bundle (Fig. 7A). The lateral chain of Tyr91 (α4) establishes stacking interactions with the lateral chains of Phe15 (β1), Trp23 (α1), and Phe42 (β2). In addition, the phenolic hydroxyl group of Tyr91 is hydrogen bonded with the lateral chain of Ser26 (α1) and with
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hydrophobic interactions, and including residues Pro\textsuperscript{17} and Leu\textsuperscript{18} (loop β1-α1), Pro\textsuperscript{27} and Val\textsuperscript{22} (α3), Met\textsuperscript{94} and Ile\textsuperscript{98} (α4), and Leu\textsuperscript{106} (α5; Fig. 7A). The α4 is further stabilized by contacts with the N-terminal region of α1. Specifically, the lateral chain of His\textsuperscript{99} (α4) makes a hydrogen bond with the main chain amino group of Pro\textsuperscript{17}, and stacks with the lateral chain of Trp\textsuperscript{23} (α1), which in turn is also in close contact with the main chain atoms of Gly\textsuperscript{96} and Asn\textsuperscript{96}. Finally, the guanidinium group of Arg\textsuperscript{27} hydrogen bonds with the lateral chain hydroxyl group of Thr\textsuperscript{92}.

To further advance on the understanding of the molecular mechanism of TP053 reduction by Rv2466c, we investigated the role of His\textsuperscript{99}, located at the interface between the thioredoxin domain and the α-helical subdomain. As depicted in Fig. 6B, when His\textsuperscript{99} was replaced by alanine, the mutant undergoes a dramatic decrease on its ability to metabolize TP053, retaining only 18% of activity. The near-UV CD spectra of the H99A mutant recorded under reduced and oxidized conditions revealed differences in the tertiary structure with the Rv2466c\textsubscript{RED} state (Fig. 7B). Thermal unfolding followed by CD indicate that the reduced H99A variant has a stability similar to that of the oxidized mimics WT (Fig. 7C). In accordance with the above CD spectra, experimental SAXS data comparison of H99A and WT oxidized state are similar (DATCMP-calculated χ\textsuperscript{2} value of 1.35), and the R\textsubscript{g} value obtained for H99A (26.4(1) Å) is comparable with that observed for the oxidized form of Rv2466c\textsubscript{OX} (27.2(1) Å), indicating that the mutant is in a more open conformation than Rv2466c\textsubscript{RED} (DATCMP-calculated χ\textsuperscript{2} value of 1.52; Table 2). In that sense, the comparison of the crystal structure of Rv2466c\textsubscript{RED} with the solution structures obtained by SAXS for H99A revealed differences in the conformation of the protein as visualized for Rv2466c\textsubscript{OX} (Fig. 7D). Thus, structural changes in the interdomain interface deregulate the transition from an open to a closed state, impairing the capacity of Rv2466c to metabolize TP053.

A Model of TP053 Activation by Rv2466c—Taken together, the experimental data support a model in which local structural changes in the thioredoxin-fold that result from Cys\textsuperscript{19}-Cys\textsuperscript{22} disulfide bond formation are sufficient to alter the fine equilibrium of the α-helical subdomain, triggering its conformational arrangement from a closed to an open state (supplemental Video S1; see “Experimental Procedures” for details). Specifically, once TP053 binds to Rv2466c\textsubscript{RED}, it becomes reduced and Rv2466c\textsubscript{OX} is formed. The formation of the catalytic disulfide bond triggers a conformational transition from the closed to the open conformation that in turn allows the release of the reaction product.

The thioredoxin-fold is an ubiquitous protein folding motif in all organisms and found in enzymes catalyzing thiol-disulfide exchange reactions, including protein disulfide reduction (thioredoxin; 41), disulfide formation (disulfide oxidoreductase DsBa; 42, 43), disulfide isomerization (protein-disulfide isomerase; 44), and glutathionylation (glutathione S-transferase, GST; 45). Interestingly, the functional diversity in some thioredoxin family members seems to be due to some extent by the presence of an α-helical insertion between the canonical second β-strand and the second α-helix (corresponding to residues Met\textsuperscript{45} to Tyr\textsuperscript{132} in Rv2466c) of the thioredoxin-fold. For
example, GSTs are a family of enzymes that primarily catalyze the thiol of glutathione (GSH) to a variety of hydrophobic electrophiles in the cellular detoxification of cytotoxic and genotoxic compounds (46). Human \( \kappa \) class GST, the closest homologue with known structure to Rv2466c (Dali Z score 16.4, sequence ID 14%), contains a large \( \alpha \)-helical region (Ser\textsubscript{42} to Pro\textsubscript{184}) that also undergoes conformational changes upon substrate binding (Fig. 8A). Thus, flexibility of the \( \alpha \)-helical subdomain allows substrate-dependent conformational changes that lead to assembly of the GSH and acceptor binding sites. Strikingly, the region of the \( \alpha \)-helical subdomain in GST that is most involved in substrate binding (Ser\textsubscript{42}–Ser\textsubscript{184}) corresponds to the region in the \( \alpha \)-helical subdomain of Rv2466c that participates in recognition of the C-terminal tag (Met\textsubscript{45}–Trp\textsubscript{69}), suggesting a conserved mechanism of substrate binding between both members of the thioredoxin family. DsbA is another family member that contains an inserted \( \alpha \)-helical bundle (Val\textsubscript{61} to Pro\textsubscript{151} in E. coli DsbA). The crystal structures of the DsbB-DsbA complex revealed how DsbA recognizes DsbB, an E. coli membrane protein that maintains DsbA in its oxidized active form (47, 48). Interestingly, the hydrophobic groove of DsbA interacting with DsbB contains two loops within the \( \alpha \)-helical bundle that show conformational variability upon substrate binding (Fig. 8B; loop 1, residues Val\textsubscript{61}–Gly\textsubscript{65}; loop 2, residues Leu\textsubscript{147}–Pro\textsubscript{151}). Importantly, the equivalent regions to loops 1 and 2 in Rv2466c and GST are involved in peptide/substrate binding. Thus, these similarities suggest a conserved mechanism of substrate recognition and product release.

Supporting this notion, the NMR structures of oxidized and reduced human and E. coli thioredoxin also revealed subtle structural differences in the active site and the surrounding regions triggered by a change in redox state (49, 50). The structural variability and available biochemical data (51), prompted to postulate that thioredoxin uses a chaperone-like mechanism of conformational changes to bind a diverse group of proteins and fast thiol-disulfide exchange chemistry in a hydrophobic environment to promote high rates of disulfide reduction (13). It is worth noting that helical bundles have been observed to undergo important functional conformational changes, including hemagglutinin (HA) protein and apolipoporphin III (52–59).

The interdomain analysis indicates that the stability of the \( \alpha \)-helix bundle is very much dependent on the thioredoxin domain, in particular (i) changes in the conformation the \( \chi_1 \) rotational angle of Cys\textsubscript{27} and (ii) changes in \( \alpha_1 \), which share multiple contacts with \( \alpha_4 \), could suffice to destabilize the \( \alpha \)-helix bundle close conformation, allowing the opening into a less stable state. We postulate Rv2466c exploits the subtle structural changes in the thioredoxin-fold during disulfide bond formation to trigger a conformational change from a closed to an open state.

**Concluding Remarks**—The discovery of TP053 and the mechanism that Rv2466c employs to activate it represents a step forward toward the development of novel antibiotics to fight TB. Rv2466c is a member of the thiol-disulfide oxidoreductase superfamily that is responsible for the reduction of TP053, killing replicating and non-replicating M. tuberculosis (9). Rv2466c utilizes a chaperone-like mechanism of conforma-
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Redox-dependent conformational changes to recognize TP053 and redox exchange chemistry in a hydrophobic environment to promote compound reduction. Rv2466c is able to undergo drastic conformational changes involving partial unfolding of an α-helical subdomain, comprising an α-helix bundle and a short α-helical hairpin. Importantly, the conformational state of this α-helical subdomain is synchronized with the redox state of the thioredoxin domain. When the Cys19-Cys22 disulfide bond is present, Rv2466c adopts an open inactive conformation, whereas upon reduction it returns to its active closed conformation. Local conformational changes mainly in the CXXC active site motif of the thioredoxin domain, alter the surface contact between the two domains, and trigger the open conformation, facilitating product release. Our studies contribute to the understanding of the molecular mechanisms of redox reactions in bacteria, suggesting that similar changes may also have a functional role in other members of the thioredoxin-fold superfamily.

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The Redox State Regulates the Conformation of Rv2466c to Activate the Antitubercular Prodrug TP053

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