Crystal structure of the conserved hypothetical protein Rv1155 from *Mycobacterium tuberculosis*

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1. Introduction

Tuberculosis is one of the most devastating bacterial diseases and kills two to three million people around the world each year. It is further estimated that around one-third of the world’s population will be infected as a result of ability of the organism to persist for many years in a semidormant or latent form inside activated macrophages [1]. In addition, current therapies are long and difficult and multidrug resistance has appeared, resulting in a resurgence of interest from the scientific community to unveil factors responsible for the pathogenicity of this organism. Most importantly, the unusual ability of *M. tuberculosis* to persist in the host for long periods of time suggests that mycobacteria may use unique pathogenic mechanisms, possibly involving specific proteic factors, compared to other common bacterial pathogens.

The sequence information of the *M. tuberculosis* H37Rv genome has considerably increased our knowledge about the protein families that are present in mycobacteria [2]. The ~4000 gene products have been classified into 11 functional groups but a known or putative function can be assigned at the sequence level for only 52% of those, while only a few 150 have been experimentally characterized in mycobacteria [3,4]. Therefore, the remaining 48% of the gene products, which consist of conserved hypothetical proteins belonging to the functional class 11, remain to be fully investigated. More recently, 194 gene products were found to be essential for mycobacteria survival in vivo [5]: ~30% of these have yet unknown function, an observation that emphasizes structure-function relationship studies of these conserved hypothetical proteins.

We are attempting to solve new crystal structures of proteins from *M. tuberculosis* that are exclusively distributed in mycobacteria as well as amongst the *Actinomyces* sub-group of bacteria. To this aim, we have focused our attention on 95 conserved hypothetical proteins that were selected based on solubility criteria as detected by bio-informatic analyses out of 376 proteins that are unique to the *Actinomyces* sub-group of bacteria based on currently available genomes [4]. Currently, no three-dimensional structure is available for this class of proteins in *M. tuberculosis*.

In order to shed light on its function by revealing possible homologies that cannot be detected at the sequence level, we have determined the crystal structure of Rv1155 at 1.8 Å resolution. Rv1155 is a conserved hypothetical protein of 147 residues with a theoretical molecular weight of ~16.3 kDa and a pl value of 6.5. The amino acid sequence of Rv1155 has no detectable homology with any other proteins of known structure, but its three-dimensional structure unambiguously reveals that Rv1155 is structurally related to the large family of flavin mononucleotide-binding protein (FMN-bp), including pyridoxamine 5’-phosphate oxidase (PINOx). In fact, Rv1155 not only shares a two-domain β-barrel fold organization as seen for members of this family but also the dimeric assembly that characterizes PINOx. At the dimer interface, a large cleft is lined with conserved residues that may have biological implications in Rv1155. Consequently, the presence of these functional consensus motifs within close homologs of...
Rv1155 permitted the identification of a new family of Rv1155-like proteins that is structurally related to FMN-bp and PNPOx but does not bind FMN as demonstrated by fluorescence titration.

2. Materials and methods

2.1. Construction of the His-pKMS96-Rv1155 expression vector

The open reading frame encoding for Rv1155 was amplified by PCR from the MTCI65 cosmids [3,6] using the forward primer 5'-gggaaac- acctgtacctcgggtgCGCGCGGAAATCCGACGAC-3' (TEV cleavage site is underlined) and the reverse primer 5'-TTAGGCGCATAC- CGGGGCGCAG-3' containing the attB1 and attB2 recombination sites at the 5' and 3' end, respectively. The PCR product was first cloned into the pDONR 201 plasmid and subsequently into the expression vector His-pKMS96 [7,8], following the manufacturer’s protocol (Gateway, Invitrogen). The presence of a TEV site between His6- MBP and Rv1155 allowed release of recombinant Rv1155 after proteolysis.

2.2. Expression and purification of recombinant Rv1155

Escherichia coli BL21 pLys S (Novagen) cells were transformed with the His-pKMS96-Rv1155 plasmid and cultured overnight in LB broth with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. The culture was diluted 20-fold with minimal M9 medium and grown at 37 °C. Protein expression was induced with 0.5 mM isopropyl-1-thiogalactopyranoside (IPTG) when the OD600 had reached a value in the 0.6–0.8 range. After induction, 1 ml of protein solution with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 3 mg/ml DNAse and 20 mM MgSO4 (final concentration). Cells were disrupted by ultrasonication and cell debris were removed by centrifugation. Rv1155 was purified by Ni2+-affinity and size exclusion chromatographies in buffer made of 5 mM HEPES, pH 7.5, and 150 mM NaCl. Rv1155 was cleaved from the MBP fusion by TEV digestion and purified by exclusion on a Ni2+-affinity column, taking advantage of the presence of the 6His tag on the MBP N-terminus. Purified Rv1155 was further characterized by circular dichroism and dynamic light scattering and SeMet incorporation was verified by MALDI-TOF mass spectrometry. SeMet-labeled Rv1155 was concentrated up to 5.7 mg/ml and stored at −80 °C.

2.3. FMN-binding assay

Fluorometry data were acquired using a Cary Eclipse Fluorescence Spectrophotometer (Varian) and analyzed using the Cary Eclipse software. Emission spectra were generated using an excitation wavelength of 450 nm. Quenching of FMN fluorescence was determined from the fluorescence emission integrated over the 470–650 nm wavelength range. Standard assay conditions were measured at room temperature with 2 μM of FMN (Sigma) incubated with various amounts of Rv1155 (0.3–15 μM) in 1 ml of buffer solution (5 mM HEPES, pH 7.5, and 150 mM NaCl). Measurements were also performed in function of time (0 and 24 h). Flavodoxin from Desulfovibrio vulgaris was used as a positive control. Briefly, apo-flavodoxin was purified as described previously [10] and bound FMN was completely removed by mixing cold trichloroacetic acid (10% w/v) to the protein solution in 5 mM HEPES, pH 7.5, and 150 mM NaCl (buffer A) at 4 °C. After centrifugation, the precipitate was dissolved in buffer A and this procedure was repeated once; traces of trichloroacetic acid were removed by dialyzing overnight against the same buffer. Protein concentration was estimated using the Bradford assay.

2.4. Crystallization and X-ray data collection

Initial crystals of Rv1155 were obtained at room temperature from solution 21 of the SeMet-labeled Rv1155 were grown using the hanging drop method by mixing 1 μl of protein solution with 1 μl of precipitant solution consisting of 0.1 M HEPES, pH 7.5, and 14–16% PEG 6 K. Thin plate crystals grew within 5–6 days. Crystals were transferred to a cryo-protectant solution consisting of reservoir solution supplemented with 30% (v/v) ethylene glycol and flash frozen in a nitrogen gas stream at 100 °K. Crystals belong to the monoclinic space group P21 with cell dimensions a = 46.9 Å, b = 55.1 Å, c = 80 Å and β = 90.8°. A monohydrate was used to name a 2.5 Å model corresponding to 41% solvent. A 3-wavelength multiple-wavelength anomalous dispersion (MAD) data set was collected up to 1.8 Å resolution on the SLS beam line X06SA (Switzerland). Data were integrated and scaled and merged with SCALA [13].

2.5. Structure determination and refinement

Initial phases were obtained using SOLVE [14] that located the four Se atoms present in the asymmetric unit. The initial MAD phases were improved by solvent flattening and non-crystallographic symmetry averaging using RESOLVE at 1.8 Å resolution [15] and an initial model consisting of 242 out of the 289 residues present in the final model was automatically traced. Further manual rebuilding was done with the graphics program TURBO-FRODO [16] and refinement was carried out with REFMAC [17]. Solvent molecules were automatically added using ARP/wARP [18]. The final model consists of residues Phe6 to Arg147 and Val5 to Arg147 for each of the two subunits present in the asymmetric unit. The stereochemistry of the final model was verified with PROCHECK [19]. The r.m.s.d. value between the two subunits is 0.8 Å for 115 Ca atoms. Data collection, refinement and structure quality statistics are shown in Table 1. Coordinates of SeMet-labeled Rv1155 have been deposited in the Protein Data Bank, with Accession No. 1W9A. Figures were produced with PyMOL [20].

3. Results and discussion

3.1. Cloning, expression and purification

Attempts to express Rv1155 as a soluble protein using the pDEST 17 expression vector (Gateway, Invitrogen) were unsuccessful. To overcome this problem, the gene was inserted downstream of various fusion proteins (MBP, Trx, GST and NusA) known to favor the expression of soluble proteins in E. coli [21]. As a result, large amounts of Rv1155 could be expressed in soluble form using the MBP and Trx fusion constructs, but release of Rv1155 using the TEV protease was more efficiently achieved from the MBP fusion protein. Further characterizations were assessed by circular dichroism and dynamic light scattering and have shown that Rv1155 is a monodisperse α/β protein in solution (data not shown). The overall yield of purified SeMet-labeled Rv1155 after TEV cleavage is 1.9 mg/L of culture medium, corresponding to 60% of the cleaved MBP fusion protein.

3.2. Structure quality and overall monomer fold

The crystal structure of Rv1155 was solved using the MAD method from the Se anomalous signal. The excellent quality of the 1.8 Å resolution experimental electron density maps permitted to build automatically 242 residues, out of the 289 present in the final model, that were subsequently refined against data collected at the remote energy wavelength (Table 1). The final model, which has good stereochemistry, consists of residues Val5/Phe6 to Arg147 for each of the two subunits present in the asymmetric unit and 371 solvent molecules. A Rv1155 subunit is folded into two separate domains. The larger domain (domain 1) encompasses two discontinuous regions formed by residues Phe6 to Pro90 and Arg128 to Arg147 and consists of a central curved six-stranded anti-parallel β-barrel (β1 to β6) flanked by two helices (α1,α2). The smaller domain (domain 2), which is composed of residues
Table 1
Data collection and refinement statistics

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Refinement

| Resolution range (Å)     | 20–1.8 (1.85–1.80) |
| Protein/solvent atoms    | 2301/371           |
| Rmerge/Rmerge (%)         | 14.1 (17.2)/17.5 (22.0) |
| Average B main/side/solvent (Å²) | 14.7/17.3/31.2 |

Rms deviations

| Bond distances (Å)   | 0.011 |
| Bond angles (°)      | 1.28  |
| Chiral volume (Å³)   | 0.08  |
| Main/side chain ΔB (Å²) | 0.56/1.67 |
| Ramachandran outliers| None  |

Values in parentheses are for the highest resolution shell.

*Rmerge = \sum_{i=1}^{N} \sum_{hkl} \left| F_{\text{obs}} - \langle F_{\text{hkl}} \rangle \right| / \sum_{i=1}^{N} \sum_{hkl} \left| \langle F_{\text{hkl}} \rangle \right|.

Pro91 to Asp127, forms a helical hairpin made of α3 and α4 inserted between β5 and β6 and is located opposite to the concave face of the central β-sheet (Fig. 1). Helix 4 within domain 2 lies almost perpendicular to the direction of strands β2, β3 and β5 in domain 1 and largely participates to the domain interface. Indeed, helix 4 residues Thr99, Leu103, Leu106 and Ile110 tightly interact with Thr89, Ser50, Leu131, Gln48 and His39 from the central β-sheet.

3.3. Dimeric assembly

Two Rv1155 subunits associate in the asymmetric unit of the crystal to form a tight dimer that adopts an elongated shape with dimensions of 60 Å × 35 Å × 35 Å (Fig. 1). This dimeric assembly is consistent with the apparent molecular size of 31.6 kDa obtained by gel filtration (data not shown) and thus may reflect the biological assembly of Rv1155 in solution. The dimer interface, which buries 1350 Å² to a 1.6 Å probe radius on each subunit, involves ~20 conserved residues located within strands β1, β2, β4 and β5 located on the concave face of the β-barrel. In contrast, residues within each of the two domains 2 do not contribute to dimer stabilization, since these domains are projected away from each extremity of the dimer interface and are distant by 50 Å.

3.4. Putative ligand binding site

The molecular surface of Rv1155 evidences a curve-shaped cleft, 6 Å deep and 25 Å long, as a possible functional ligand binding site built up by charged and polar residues from both subunits. The bottom of the binding cleft is entirely defined by residues from strand β2, while distinct arrays of residues form the lateral walls of the cleft (Fig. 1). Residues within the β3–β4 loop in domain 1 and the two α3 and α4 helices in domain 2 are recruited to form one wall, while the opposite wall is made with residues in strand β6 from the second subunit. While the section of the cleft is rather narrow near the dimer interface, its volume significantly increases at the domain junction to form a larger binding pocket. It is likely that Ser50 that lies at the bottom of the pocket may have an important role for ligand recognition. Other residues protruding into the cleft and thus likely to be crucial for ligand binding are Gln37, Arg55, Lys57 and Thr58 within domain 1 along with residues Tyr107, His114, Asp119, Tyr120, Met124, Asp127 and Arg129 within domain 2 on one side of the cleft and residues Trp77, Tyr79, Tyr140, and Arg147 from the second subunit at the opposite wall of the binding cleft. Interestingly, these latter residues located in the β4–β5 loop and at the very C-terminus, respectively, undergo a concerted motion in one subunit with respect to the other, leading to remarkable structural differences at one side of the cleft. This, together with an inter-domain motion of 10° of the small domain 2 relative to domain 1, rendering one of the binding clefts more narrow than the other, leads to speculate that a considerable conformational flexibility exists in solution.

3.5. Structural comparison

A DALI search for close structural homologs of Rv1155 within a non-redundant set of protein structures from the PDB revealed top-ranked hits for the FMN-binding proteins (D. vulgaris FMN-bp) (Accession No. 1AXJ [22], Z score value 9.5, r.m.s.d. of 3.0 Å for 107 Cα atoms), the PNPOx from Sac-
charomyces cerevisiae (1CI0, 8.7, 3.4 Å for 115 Cα atoms) involved in the de novo synthesis of pyridoxine (vitamin B6) and pyridoxal phosphate. Although these proteins share a similar structural topology, Rv1155 displays only 19% and 11% sequence identities with FMN-bp and PNPOx, respectively. FMN-bp represents the smallest FMN-binding protein known to date with only 122 amino acid residues compared to 205 residues found in PNPOx. Whereas the core of the β-barrel made of six β-strands is well conserved between these proteins, the largest structural differences reside in the overall architecture of domain 2, which consists of 15 and 55 residues in FMN-bp and PNPOx, respectively, compared to 37 in Rv1155 (Fig. 2). In domain 1, the conformations of the α1–β1 loop and the two β1–β2 and β3–β4 loops forming the FMN-binding site in FMN-bp and the corresponding loop regions in PNPOx are also markedly different in Rv1155.

Whereas FMN-bp is monomeric in solution [22], Rv1155 shares the dimeric assembly that characterizes members of the PNPOx family, for which three-dimensional structures have been determined so far for E. coli [23], human [24] and yeast enzymes (unpublished data, PDB 1CI0) (Fig. 2). In addition to the structural differences identified between Rv1155 and FMN-bp, members of the PNPOx family are characterized by the extension of 10 and 25 residues in the N- and C-terminal region, respectively. Consequently, the size of the dimer interface through a transparent molecular surface in the left subunit.

Fig. 1. Overall fold of Rv1155. (A) Ribbon diagram of the dimer viewed along the approximate twofold axis with domain 1 from the two subunits shown in cyan and yellow, while the two domains 2 are shown in green. The β4–β5 loop and the C-terminal region are shown in orange. The secondary structure elements are indicated. (B) View oriented 90° from (a) and colored as in (a) showing the large cleft at the dimer interface through a transparent molecular surface in the left subunit.

Fig. 2. Structural comparison. (A) Ribbon diagram of the D. vulgaris FMN-bp (Accession No. 1AXJ) with bound FMN (magenta) showing a single conformer (model 1) out of 20 and oriented and colored as the left subunit in Fig. 1A. (B) Ribbon diagram of the S. cerevisiae PNPOx dimer (Accession No. 1CI0) with bound FMN (magenta) with the central β-sheet of the left subunit oriented and colored as in Fig. 1A. The additional regions in PNPOx compared to Rv1155 and FMN-bp are shown in red, while those that significantly differ between Rv1155, FMN-bp and PNPOx are indicated in orange. In the left subunit, the FMN-binding site at the dimer interface is shown through a transparent molecular surface.

In both FMN-bp and PNPOx, the FMN ligand is tightly bound within a shallow cleft located between β2 and the loop connecting β3–β4 as found in Rv1155. Moreover, there are numerous FMN- and phosphate-mediated hydrogen bonds linking the two PNPOx monomers, suggesting a similar scenario for Rv1155, where conserved residues from both subunits might have an important role in ligand recognition. The fact that only a single residue (PNPOx Arg95/FMN-bp Lys53) in contact with FMN is conserved between D. vulgaris FMN-bp and S. cerevisiae PNPOx indicates that FMN does not require specific signature motifs and is thus able to be accommodated by diverse proteins. However, a structural overlap evidences several clashes between the FMN isalloxazine ring of both FMN-bp and PNPOx and the Rv1155 conserved residues Trp77, Tyr79 in the β4–β5 loop and the C-terminal Arg147, suggesting that Rv1155 may lack the ability to bind to FMN or the related FAD and riboflavin compounds.

While the shape of the FMN-binding site in PNPOx is funnel-like, the architecture of the putative ligand binding site in Rv1155 resembles a deep cleft and is thus more accessible to
solvent as also observed in FMN-bp [22]. This difference in shape of the binding site is mainly due to the presence of a shorter domain 2 combined with the lack of the two last β-strands in FMN-bp and Rv1155 compared to PNPOx (Fig. 2). To validate our structural interpretation, we have explored the ability of CASTp (http://cast.engr.uic.edu/cast) to automatically identify surface pockets as putative ligand binding sites [25]. CASTp successfully identified the shallow pocket located at the dimer interface of Rv1155. This pocket has a surface area of 780 Å² and a volume of 1573 Å³, consistent with the size of the FMN-binding pocket (1300 and 1812 Å³) previously identified from crystallographic analysis of PNPOx and in agreement with the differences observed for the architecture of the cavity between these two protein families. Altogether, these observations support the idea of a sequence divergence of residues forming the binding cleft in Rv1155, leading to a new specificity for a yet unknown ligand.

3.6. A new family of Rv1155-like proteins with no FMN-binding activity

As expected, BLAST searches using the Rv1155 sequence as a template identified top-ranked homologs with e-values in the \(10^{-80}\) range exclusively within mycobacteria species while others, with e values in the \(10^{-40}\) range, were only found in two Streptomyces species (S. coelicolor and S. avermitilis) and in the Kineococcus radiotolerans bacteria. Overall, these close Rv1155 homologs have sequence identities in the 52–91% range. The sequence alignment clearly indicates that invariant residues are confined at the dimer interface and within the cleft (Fig. 3), consistent with our hypothesis on the importance of the dimeric assembly and the large cleft for the biological function of Rv1155. In addition, Rv1155 shares 27% of sequence identity with another conserved hypothetical protein from M. tuberculosis, Rv1875, that also shares all the putative functional residues identified in the binding cleft of Rv1155 (Fig. 3). Surprisingly, Rv1155 shows a significant homology with a putative ATP/GTP-binding protein from S. coelicolor although the P-loop motif [AG]-X[-4]-G-K-[ST] (PROSITE pattern PS00017) that directly interacts with one of the ATP/ GTP phosphate groups is not conserved in Rv1155, suggesting that Rv1155 is unable to bind to ATP or GTP. Two other M. tuberculosis gene products, Rv0121c and Rv2074, have also been annotated as putative homologs of Rv1155 in the Tuberculist database (http://genolist.pasteur.fr/Tuberculist), but, again, these two homologs do not possess the key functional residues of Rv1155 and may have thus a distinct biological function.

Although Rv1155 has been annotated as a conserved hypothetical protein, searches within the Pfam database unambiguously identified residues Phe6 to Ala93 from domain 1 which

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![Fig. 3. Sequence alignment of members of the Rv1155-like protein family. Alignment of M. tuberculosis Rv1155 with close orthologs from Mycobacterium bovis (Mb1186), Mycobacterium avium (AAS04947), Mycobacterium leprae (ML1508), Mycobacterium smegmatis (contig-3563), K. radiotolerans (contig-3273), S. avermitilis (SAV1780) and S. coelicolor (SCO5312). The sequences of two related proteins from M. tuberculosis (Rv1875) and S. coelicolor (SCO4026, putative ATP binding protein), respectively, are also indicated. Invariant and conserved residues between these proteins are highlighted with a black background and a boxed area, respectively. Secondary structure elements of Rv1155 are shown above the sequences. Residues that are lining either the dimer interface, the putative ligand binding site or both are indicated underneath by (▲), (●) and (★), respectively.](image-url)
belong to the PNPOx family (Accession Nos. PF01243, COG0259), consistent with our structural analysis. However, a structural alignment between Rv1155 and members of this Pfam family clearly indicates that a large number of key residues for binding FMN are not conserved in Rv1155 (see above), suggesting that Rv1155 may have thus evolved towards a different biological function. Such diversity within the large FMN-bp family has been recently extended with the identification of WrbA, a protein of yet unknown function in *E. coli*, that shows a weak but specific FMN-binding activity associated to a marked sequence divergence within the α/β fold [26]. We thus sought to detect a possible interaction between Rv1155 and FMN in solution by fluorescence titration. In contrast to *D. vulgaris* FMN-bp, Rv1155 is not able to bind FMN since no significant quenching between FMN and various amounts of Rv1155 could be observed (Fig. 4) (see Section 2).

4. Conclusion

Recent proteomic studies combining two-dimensional electrophoresis and mass spectrometry have shown that Rv1155 is expressed in the cellular fraction of the *M. tuberculosis* H37Rv strain [27,28], indicating that this conserved hypothetical protein may have a functional role in vivo. Therefore, with our structural information, it would now be interesting to identify the nature of the ligand of Rv1155 in order to assess the biological function of these Rv1155-like proteins in mycobacteria.

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References


