Correlation of Conservation of Sequence and Structures of Mycobacterial Hemerythrin-like Proteins with Evolutionary Relationship and Host Pathogenicity

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ABSTRACT: The Rv2633c gene of Mycobacterium tuberculosis, which plays a role in infection, encodes a hemerythrin-like protein (HLP). The crystal structure of an orthologue of Rv2633c, the HLP from Mycobacterium kansasii, revealed that it possessed structural features that were distinct from other hemerythrins and HLPs. These and other orthologous proteins comprise a distinct class of non-heme di-iron HLPs that are only found in mycobacteria. This study presents an analysis and comparison of protein sequences, putative structures, and evolutionary relationship of HLPs from 20 mycobacterial species that are known to cause tuberculosis or pulmonary disorders in humans. The results of this analysis allowed correlation of the physicochemical characteristics of amino acid residues that are substituted in these highly conserved sequences with their position in structures, possible effects on function, and evolutionary relationships. The sequences of the proteins from M. tuberculosis, Mycobacterium bovis, and other members of the M. tuberculosis complex, which cause tuberculosis, have substitutions not seen in the other non-tuberculous mycobacteria. Furthermore, groups of species that are closely related, based on phylogenetic analysis, possess substitutions of otherwise conserved residues not seen in other species that are less related. This information is correlated with the occurrence and clinical presentations of these groups of mycobacterial species. The results of this study provide a framework for structure–function studies to determine how subtle differences in the primary sequences of members of this family of proteins correlate with their structures and activities and how this may influence the infectious properties of the host species.

INTRODUCTION

A di-iron hemerythrin-like protein (HLP) encoded by the Rv2633c gene of Mycobacteria tuberculosis (Mtb) was recently characterized.1 Subsequently, the orthologous HLP was isolated from Mycobacteria kansasii (Mka), and its crystal structure was determined.2 Despite the two proteins exhibiting 78% similarity and 86% identity, the two proteins exhibited notable differences in solution properties and enzymatic activity. The Mtb HLP could be isolated only in very low yields and had limited solubility. It was a homodimer that was unstable as it precipitated from solution. This protein did exhibit catalase activity, which was an unprecedented activity for an HLP. In contrast, the Mka HLP was isolated in reasonable yields and was a stable monomer in solution. These properties allowed its crystallization and structure determination. The Mka HLP also exhibited catalase activity, albeit with lower activity than the Mtb HLP, and interacted with nitric oxide. A homology model of the Mtb HLP was generated using the Mka HLP structure as a template.2 The model suggested that the overall structures of the two proteins were very similar with five α-helices connected by relatively short loops. The amino acids that coordinate the two iron ions were completely conserved (Figure 1). An interesting distinction was that the Mtb HLP model contained two proline residues (Pro63 and Pro145 situated at α-helices 3 and 5, respectively) that were not present in the Mka HLP. These substitutions suggested that those helices might be disrupted and alter the true structure of the Mtb HLP in solution.

Phylogenetic analysis of sequence databases revealed that these two HLPs were members of a family of HLPs that is found only in mycobacteria. The mycobacterial HLPs are distinct from true hemerythrins and hemerythrin-like domains of other proteins.1,2 These distinctions were not solely based...
A remarkable feature of this group of mycobacterial HLPs is the high level of conservation of sequence, which suggests conservation of structures. Each protein has approximately 162 amino acid residues with very few small gaps or insertions in sequence. As such, alignment and comparative analysis of the protein sequences are very straightforward, and the likely accuracy of homology models is enhanced. Of particular interest are the positions in sequence of residues that provide the first and second sphere ligands for the iron ions (Figure 1B). The first sphere ligands are residues that interact directly with the two irons in the active site. The second sphere ligands are residues that interact with the first sphere ligands. Determining the extent of conservation of these residues is important because the ligand set observed in the Mka HLP structure had not been observed in any other hemerythrins or HLPs.

Another point of interest is the identification of substitutions of specific amino acid residues in the HLP sequences in certain subsets of this group of mycobacteria. In the present study, conserved residues in this family of mycobacterial proteins are identified, which are not iron ligands but in the proximity of the di-iron site and which suggest specific roles in protein function. The sequences of the proteins from M. tuberculosis and Mycobacterium bovis, which are capable of causing tuberculosis in humans, are shown to have multiple unique features that are distinct from those of the other non-tuberculous mycobacteria (NTM). Among the sequences of the HLPs of the 20 species in this study, substitutions of otherwise conserved residues are correlated with their positions in the protein structure and presence within related species of mycobacteria. Overall, these results provide a much broader view of the subtle but important structural distinctions that are present in certain members of this highly conserved family of proteins and which are correlated with the evolutionary relationship between these mycobacterial species. These results are discussed in the context of reported clinical presentations associated with infection by these mycobacteria.

### RESULTS

**Sequence and Phylogenetic Analysis.** A BLAST search using the sequence of the Mtb HLP as the template was performed with the current database. The parameters were set for a maximum e-value of $1 \times 10^{-46}$ and the search yielded 262 sequences. All of the sequences were of proteins from bacteria in the Mycobacteriaceae family. The sequences of proteins from 20 of these mycobacterial species were chosen for further analysis. A criterion for choosing these mycobacterial species is that each have been reported to cause tuberculosis or pulmonary disorders in humans. Furthermore, these sequences were highly conserved as they exhibited e values less than $5 \times 10^{-63}$. An alignment of the sequences of these 20 mycobacterial HLPs is present in Figure 2. Key residues are highlighted to facilitate comparison.

A phylogenetic tree of the 20 host species of the mycobacterial HLPs, which is based on the sequence alignment in Figure 2, is shown in Figure 3A. Another phylogenetic tree of these 20 species, which is based 16s rRNA gene sequences, is shown in Figure 3B.

**Homology Modeling.** In order to estimate the extent of the conservation of the structure of the mycobacterial orthologues of the Mka HLP, homology models of each of the proteins were constructed using Swiss-model homology modeling online software (Table 1). The QMEAN Z-value is
a measure of the quality of the structural model. A value near zero reflects strong agreement between the structural model and known structures of similar size. A value less than \(-4.0\) is considered to be of poor quality.\(^{12}\) The GMQE score is a value between 0 and 1 with a value of 1 reflecting the highest level of reliability of the model.\(^{13}\)

Figure 2. Alignment of the sequences of HLPs. The residues that provide ligands for the iron in the di-iron site are colored red and those that are second sphere ligands are colored green. Residues that are common to \(M.\) tuberculosis and \(M.\) bovis, while different in other species, are colored blue.
the parameters shown in Table 1 provide the best estimates of the goodness of the model, which will reflect the similarity in structure to the known Mka HLP structure. While the modeling cannot predict exactly what the structure will look like, the importance of the parameters in Table 1 is that they provide a measure of the extent to which the structures of the other HLPs deviate from that of the Mka HLP. These parameters indicate that the overall structures are in fact very similar. This suggests that differences in solution properties and function are related to minor changes in the structure or substitution of amino acid residues in key positions that alter the physicochemical properties of that residue.

**Correlation of Conservation of Sequence and Structure with Evolutionary Relationship. Residues near the di-iron Site.** Inspection of the structure of the di-iron site of the Mka HLP (Figure 1B) describes the first and second sphere ligands of the iron ions. The first sphere ligands, residues that provide ligands for the two irons in the active site, are completely conserved in each of the sequences (colored red in Figure 2). A key feature of the structure of the Mka
HLP$^2$ is the presence of a Tyr ligand for an iron in this position in the di-iron site where His provides the ligand in true hemerythins. This Tyr54 is conserved in the sequences of each of these mycobacterial orthologues. The structure of the Mka HLP also shows residues that provide second sphere ligands for the di-iron site. These residues interact with the primary ligands. Each of the residues that provided these second sphere ligands are also conserved in every sequence (colored green). Thus, any observed differences in the physical properties or functions of the HLPs from the different species are not due to differences in residues that define the structure of the oxo-bridged di-iron site.

Phe46 is another amino acid residue in the vicinity of the di-iron site that is highly conserved in sequence and in structure in the homology models. Phe46 is present in all but four of the protein sequences. It resides approximately 4.0 Å from Fe2 (Figure 4). The position of the Phe side-chain in the structure could block access of hydrophilic species that could potentially react with iron. This idea is supported by the observation that the side of Phe46 opposite to that of the iron (Figure 4). The four sequences that do not have this Phe instead have a Met in this position. These sequences are from the HLPs from Mycobacterium arupensis, Mycobacterium heraklionense, Mycobacterium fortuitum, and Mycobacterium abscessus. This Met residue could be performing a similar function or signify a difference in reactivity as these may influence the position of the Tyr ligand or interact with water. Another interesting substitution seen only in Mtb and M. bovis is the Val at position 108. In all of the other sequences, the 108 position is occupied by a Glu, and it is the first of three consecutive Glu residues, which are then followed by an Asp. These residues are located on the α-helix 4 and strongly influence the di-iron site. The side chain of Glu109 provides ligands to both Fe1 and Fe2, and Glu110 is a second sphere ligand that interacts with the His11 ligand of Fe1. If the substitution of Glu108 with Val has even a subtle influence on Glu109 and Glu110, this could influence the position of the Tyr ligand and interact with water.

Figure 5. Positions of Val108 and Thr67 substitutions in the Mtb model. Portions of the Mka structure and Mtb model are superimposed. Carbon colors: gray for Mka residues and green for Mtb residues. Nitrogens are colored blue, and oxygens including waters and the one bridging the irons are colored red. Interactions described in the text are shown as dashed lines. The distances between the waters and Glu108 are given. The structure shown uses the coordinates in PDB entry 6Q09 for Mka HLP and the homology model of the Mtb HLP.

As can be seen in Figure 3, these species are grouped together in a major branch at the bottom of the phylogenetic trees, separate from the other 16 species.

Residues Present Only in HLPs from Species that Cause Tuberculosis. Mtb and M. bovis are two closely related (see Figure 3) and highly pathogenic species of mycobacteria. The analysis in Figure 2 reveals several residues that are only present in the sequences from these two species (colored blue in Figure 2) and highly conserved as different amino acids in the other species. It was previously noted that two Pro residues in the sequence of the Mtb protein in positions that are in α-helices in the structure of the Mka HLP$^2$. The same substitutions of two Pro residues at positions 63 and 145 are only found in the sequence from M. bovis. All other sequences have a Leu at position 63. The sequence from M. abscessus does have a Pro at position 145, while the other sequences show a variety of residues at this position, Ala, Thr, Ser, or Lys. The sequences of Mtb and M. bovis also substitute a Thr for Ala67, which is conserved in all of the other 18 sequences. This residue is positioned 3.5 Å from the Tyr54 side chain (Figures 1B and 5), which provides an iron ligand, on the side opposite that which faces Fe2. The larger size of the Thr side chain, and its potential ability to form a hydrogen bond, could be related to reactivity as these may influence the position of the Tyr ligand or interact with water. Another interesting substitution seen only in Mtb and M. bovis is the Val at position 108. In all of the other sequences, the 108 position is occupied by a Glu, and it is the first of three consecutive Glu residues, which are then followed by an Asp. These residues are located on the α-helix 4 and strongly influence the di-iron site. The side chain of Glu109 provides ligands to both Fe1 and Fe2, and Glu110 is a second sphere ligand that interacts with the His11 ligand of Fe1. If the substitution of Glu108 with Val has even a subtle influence on Glu109 and Glu110, this could influence properties of the di-iron center. Furthermore, Glu108 forms a hydrogen bond with one of two waters that approach the di-iron site (Figure 5). Thus, the replacement with Val may also affect access from the surface to the di-iron site.

Mtb and M. bovis are members of the Mycobacterium tuberculosis complex (MTBC), a group of mycobacteria that are known to cause tuberculosis. The BLAST search that identified the proteins in Figure 2 also identified proteins from other members of the MTBC, which were listed as subspecies of Mtb. These mycobacteria with the accession numbers for the sequences, in parentheses, are Mycobacterium africanum (AMC64977.1), Mycobacterium canettii (CCK64818.1), Mycobacterium caprae (APU26589.1), Mycobacterium microti (AMC60342.1), and Mycobacterium pinnipedii (PRH90547.1). These HLP sequences are not included in Figure 2 because each of these protein sequences was identical to that of the Mtb HLP. In fact, the only non-identical sequence of the MTBC species is that from the M. bovis
protein, which has only one difference, a Val instead of a Leu at residue 102. Thus, the multiple substitutions described above in Mtb and M. bovis are completely conserved in the sequences of proteins of other species that cause tuberculosis and do not appear in any of the NTM species.

**Deletions and Insertions of Residues.** While 15 of the 20 sequences have 161 residues, 5 have residues deleted or inserted. The most extreme case is seen in the sequences from *Mycobacterium heckeshornense* and *Mycobacterium xenopi*. Each contain a gap of three missing residues in the same position in the sequences and thus they have only 158 residues. These two closely related species comprise a branch of the phylogenetic trees that is distinct from the other species (see Figure 3). The position of these gaps in residues in the Mka HLP structure (residues 27–29) correspond to the last two residues in the loop that connects α-helices 1 and 2 and the first residue of α-helix 2 (see Figure 1A). This suggests a shorter loop in these proteins. One sequence from *Mycobacterium kyorinense* is missing the final residue and is only 160 residues long. This species is isolated in the phylogenetic trees from any other species (Figure 3). In contrast to the sequences with missing residues, the sequences from *M. arupense* and *M. heraklionense* each have an additional residue inserted after the initial Met. These species, which are closely related, comprise the bottom branch of the trees.

## DISCUSSION

Mycobacterial species continue to be identified, and over 190 species have been reported.\(^{16,17}\) The majority of tuberculosis cases in humans are caused by *Mtb*. Tuberculosis in humans can also be caused by *M. bovis*\(^{18}\) and members of the MTBC.\(^{14,15}\) Many other mycobacterial species are categorized as NTM. While these may be considered potentially pathogenic, the NTM do not typically cause severe disease in healthy individuals. However, certain NTM, which are included in this study, are known to cause pulmonary disease in humans.\(^{19}\) Phylogenetic analyses of bacteria are typically based on comparison of polymeric molecules such as RNA, DNA, or particular proteins. In the present study, the amino acid sequences of the HLPs were used to determine the evolutionary relationship of the species that encode this protein. For comparison, a phylogenetic tree was also constructed using the 16s rRNA genes sequences. The results of this analysis allowed correlation of the physicochemical characteristics of amino acid residues that are substituted in these highly conserved sequences with their position in structure, possible effects on function, and evolutionary relationships.

The sequences of the HLPs from *Mtb*, *M. bovis*, and other MTBC species exhibit distinct differences from the other 18 NTM sequences. These are the only two sequences with Pro residues present at both positions 63 and 145. These positions correspond to residues within α-helices in the Mka HLP structure. This finding suggests that the actual structures may deviate from those observed in the homology models and could affect their solution properties. Alterations in structures such as this could explain why the *Mtb* HLP is poorly soluble and precipitates, whereas the Mka HLP is readily soluble and stable in solution.\(^{12}\) The proteins from *Mtb* and *M. bovis* are also the only two that substitute a Thr for the Ala67 near the di-iron site. This substitution could affect the properties of Tyr54, the iron ligand that is unique to mycobacterial HLPs. A substitution of Val for the conserved Glu108 may also influence the properties of the di-iron site and access for water to that site. Thus, the HLPs from mycobacteria that cause tuberculosis contain multiple substitutions in the protein sequence that are conserved within this subset of mycobacteria and replace different amino acids which are highly conserved in the NTM which are infectious but do not cause tuberculosis. This information provides a basis for further experimental studies to determine precisely what effects these sequence substitutions, which are unique to the MTBC, have on the properties of their HLPs *in vitro* and to gain insights into how these HLPs may contribute to the unique ability of the MTBC species to cause tuberculosis.

The sequences of the HLPs of the four species in the major branch at the bottom of the phylogentic trees, *M. arupense*, *M. heraklionense*, *M. fortuitum*, and *M. abscessus*, are distinct from the other 16 in having a Met substituted for Phe46, which is close to the di-iron site and may restrict the access of potential hydrophilic reactants. *M. abscessus* accounts for a significant number of NTM infections.\(^{20}\) It typically causes chronic lung infection, skin and soft tissue infection, and tenosynovitis. This group is subdivided into two branches of the phylogenetic tree. The members of one branch *M. arupense* and *M. heraklionense* have a further distinction of each having an additional residue inserted after the initial Met.

Only two species exhibited a three-residue gap in the sequence. These species, *M. xenopi* and *M. heckeshornense*, comprise a distinct branch of each of the phylogenetic trees. *M. xenopi* exhibits high prevalence and increasing clinical importance and is a leading cause of NTM lung disease in Europe and Canada.\(^{21}\) It is seen in patients with and without underlying chronic lung disease. The closely related *M. heckeshornense* exhibits even greater pathogenicity.\(^{22}\)

The *M. kyorinense* sequence is distinct among these mycobacterial HLPs as it is the only one missing the final residue. It occupies a similar position in each phylogenetic tree that is separated from any other species. *M. kyorinense* has been isolated from immunocompetent patients with a history of pulmonary disease, primarily in Japan,\(^{23}\) and causes significant respiratory disease. More recently, it was also described in Brazilian\(^{24}\) and Australian\(^{25}\) patients.

The remaining NTM that were analyzed and were most similar to *M. kansasii* include some members of the *Mycobacterium avium* complex. These are frequent causative agents of chronic pulmonary infections in adults, lymphadenitis in children, and extrapulmonary and disseminated infections in immunocompromised patients.\(^{26}\) The members of this complex that are included in this analysis are *M. avium*, *Mycobacterium colombiense* and *Mycobacterium intracellulare*. While not a formal member of this group, *M. kansasii* is one of the most prevalent causes of NTM disease and is the second most frequent NTM found in HIV-infected patients, after the *M. avium* complex.\(^{27}\)

It is reasonable to infer from these results that these orthologous mycobacterial HLPs are relevant to infectivity and pathogenicity of the host species. The Rv2633c HLP from *Mtb* was initially studied because the Rv2633c gene was upregulated during infection following phagocytosis by macrophages,\(^{28,29}\) and a transposon mutant screen indicated that *Mtb* was significantly attenuated by Tn insertions that inactivated Rv2633c.\(^{30}\) It is also very interesting to see multiple substitutions that appear in the sequences of *Mtb*, *M. bovis*, and the MTBC species, which are different and completely conserved in the other 18 species. Thus, the two most

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Pathogenic species have multiple unique features that are distinct from those of the other infectious NTM. Furthermore, groups of species that are determined to be related based on the sequence of the orthologous HLPs seem to also be related based on clinical presentations. Together, these data strongly suggest that these HLPs play relevant roles in the infectious properties and clinical outcomes of Mtb, M. bovis, and NTMs that cause pulmonary disease in humans.

While the phylogenetic trees in Figure 3 are not identical, they are quite similar. The groups of species that were identified to possess substitutions in sequence that were not seen in other species each occupy the same positions in both trees. Thus, the evolution of the sequences of the HLPs tracks with the overall evolution of the species. This finding suggests that these HLPs were not introduced into mycobacteria via gene transfer but evolved independently. The sum of these data supports the designation of these proteins as a family distinct from true hemerythrins and other HLPs. The results of this study provide a framework for structure function studies to correlate how the subtle differences in the protein sequences affect their structures and activities and how these influence the infectious properties of the host species.

■ METHODS

Basic Local Alignment Search Tool (BLAST) searches were performed using the blastp suite provided by the NCBI. The amino acid sequence of the hemerythrin-like Rv2633c HLP of Mtb was used as the query sequence. NCBI COBALT was used to create the multiple sequence alignment of proteins. A phylogenetic tree of the 20 host organisms of the proteins under analysis was constructed, which is based on the JTT matrix-based model and general time reversible model and the evolutionary history was inferred using the maximum composite likelihood method that was based on the JTT matrix-based model and general time reversible model. The evolutionary analysis of the protein sequences was conducted using MEGAX.

The tree with the highest log likelihood is drawn to scale, with branch lengths measured in the number of substitutions per site. Another phylogenetic tree of the 20 host organisms was constructed, which is based on their 16s rRNA sequences. This analysis involved 20 nucleotide sequences. There were a total of 1449 positions in the final data set. All of the sequences were obtained from GenBank. The methods for analysis were the same as described above for the protein sequence-based tree. Homology models of the 19 proteins of the unknown structure were generated using the Swiss-model homology modeling online software (https://swissmodel.expasy.org), with the structure of Mka protein (PDB entry 6Q09) as the template. All structural figures presented here were prepared using PyMol (http://www.pymol.org).

■ ASSOCIATED CONTENT

Accession Codes

PDB: 6Q09. UniProtKB: WP_023372785.1, WP_007774632.1, WP_046184253.1, WP_007172067.1, WP_104182873.1, WP_065482948.1, WP_036468396.1, WP_064939987.1, WP_085670467.1, WP_055756538.1, WP_036355112.1, WP_003919947.1, WP_048892962.1, WP_004136251.1, WP_023372785.1, WP_065482948.1, WP_036468396.1, WP_064939987.1, WP_085670467.1, WP_055756538.1, WP_036355112.1, WP_003919947.1, WP_048892962.1, WP_004136251.1, KAN9011.1, WP_065015270.1, WP_074334317.1, WP_064897428.1, WP_065040284.1, WP_046187572.1, AMCE6977.1, CCK64818.1, APU26589.1, AMCE60342.1, PRH90547.1.
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