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Mapping key interactions in the dimerization process of HBHA from *Mycobacterium tuberculosis*, insights into bacterial agglutination

Carla Esposito [a,1], Marco Cantisani [b,1], Gabriella D'Auria [c], Lucia Falcigno [c], Emilia Pedone [a], Stefania Galdiero [a,b], Rita Berisio [a,*]

[a] *Institute of Biostructures and Bioimaging, CNR, Via Mezzocannone 16, I-80134 Napoli, Italy*
[b] *Department of Biological Sciences, Via Mezzocannone 16, I-80134 Napoli, Italy*
[c] *Department of Chemistry, University of Naples "Federico II", I-80126, Via Cinthia 45, Napoli, Italy*

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ABSTRACT

HBHA is a cell-surface protein implicated in the dissemination of *Mycobacterium tuberculosis* (Mtb) from the site of primary infection. Its N-terminal coiled-coil region is also involved in bacterial agglutination. However, despite the importance of HBHA dimerization in agglutination, protein regions involved in dimerization are hitherto not known. Here, we mapped these regions by coupling peptide synthesis, biochemical and computational analyses, and identified structural determinants for HBHA monomer–monomer recognition. Importantly, we obtained the first molecule able to induce HBHA dimer disaggregation at 37 °C, the typical growth temperature of Mtb. This result provides new opportunities towards the development of Mtb anti-aggregation molecules with therapeutic interest.

Structured summary of protein interactions:
HBHA and **HBHA** bind by molecular sieving (View interaction)
HBHA and **H1 peptide** bind by competition binding (View Interaction)
HBHA and **H1ext peptide** bind by competition binding (View Interaction)
HBHA and **H2ext peptide** bind by competition binding (View Interaction)
HBHA and **H2 peptide** bind by competition binding (View Interaction)
HBHA and **H2ext peptide** bind by competition binding (View Interaction)
HBHA and **HBHA** bind by blue native page (View interaction)

1. Introduction

Mycobacterium tuberculosis (Mtb), the etiologic agent of Tuberculosis (TB), is one of the deadliest human pathogens, infecting more than one third of the human population. Peculiar of Mtb is its ability to survive in harsh conditions, replicate in host cells and disseminate from the site of primary infection to potentially any organ, where it can persist for decades and eventually reactivate to cause active TB [1–3]. Dissemination from the site of primary infection involves interactions of Mtb with epithelial cells through a surface protein called heparin-binding hemagglutinin, HBHA [4–6]. Indeed, HBHA mediates binding of mycobacteria to epithelial cells and to extracellular matrix components.

HBHA contains three functional domains: a transmembrane domain of 15–20 amino acids located near the N-terminus of the protein; an α-helical coiled coil region which may be involved in protein oligomerization and a C-terminal region containing methylated lys-pro-ala-rich motifs. Interactions of HBHA with host components such as cell surface sulfated glycoconjugates and proteoglycans, which are also abundant in interstitial lung tissue, is mediated via the HBHA C-terminal domain [5,7,8]. Similar to other adhesins, HBHA is also capable to promote bacterial agglutination, a function due to HBHA N-terminal part (residues 1–160) [8]. Using single-molecule atomic-force microscopy, it was shown that HBHA coiled coil domain is responsible for protein multimerization [9]. Small-angle X-ray scattering studies and other biophysical techniques have further confirmed the role of the coiled coil domain in protein oligomerization. More precisely, these studies showed that HBHA has a dimeric coiled coil structure with an elongated

Abbreviations: HBHA, heparin binding hemagglutinin A; Mtb, *Mycobacterium tuberculosis*; CD, circular dichroism; PDB, protein data bank; HBHAΔC, HBHA deprived of its C-terminal arm (residues 161–198)

* Corresponding author. Fax: +39 0812536642.
E-mail address: rita.berisio@unina.it (R. Berisio).

[1] These authors equally contributed to this work.

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shape and that HBHA dimerization is key to its structural integrity [10,11]. Coiled coil proteins are notably capable of dynamic switching of monomer subunits [12]. In this scenario, HBHA is likely to form reversible bridge-like structures connecting bacteria through the N-terminal coiled coil domain [11]. This hypothesis is supported by atomic force microscopy studies, which measured homophilic HBHA–HBHA interacting forces occurring at the bacterial surface of live mycobacteria [9].

HBHA-mediated aggregation of bacteria leads to clumping of bacilli, an extremely simple, yet effective, defense mechanism. Indeed, clustering by aggregation enables bacteria to generate a robust spatial structure with a high local cell density. These multicellular aggregates can easily resist a toxic onslaught from chemicals such as antibiotics [13]. The existing correlation between Mtb clumping and HBHA oligomerization has prompted us to investigate the interaction mode between HBHA subunits to form dimers. This feature of HBHA, so for not understood, is of great relevance to the understanding of Mtb aggregation phenomena. Here, we successfully identified these regions by combining peptide synthesis with biochemical experiments and molecular modeling analyses. Together with mapping critical regions for protein dimerization, we discovered a peptide molecule able to disaggregate HBHA dimers, with the production of a well-structured monomeric protein–peptide hybrid. Our findings provide the first molecular entities able to interfere with HBHA dimerization and, likely, with Mtb agglutination. The ability of our best working peptide, here named as H2ext, to disaggregate HBHA dimers at 37 °C will likely provide a strong contribution to the formulation of anti-agglutination molecular entities of therapeutic interest.

2. Methods

2.1. Cloning, expression and purification

HBHAΔC (lacking residues 161–199) was cloned, expressed and purified as previously reported [10,11].

2.2. Peptide design

Peptides of the first generation (H1, H2, H3) were designed based on bioinformatics predictions of helix boundaries and coiled coil dimerization interface. These peptides were acetylated and amidated at their N- and C-terminal ends, respectively. Peptides of the second generation were designed by either extending them at their N- and C-terminal ends (H2ext, H1ext, H2shift) or by modifying the peptide sequence (H4, H5) in order to stabilize their helix propensities.

2.3. Peptide synthesis

Peptides were synthesized by solid-phase 9-fluorenylmethoxycarbonyl (Fmoc) method. Peptides were fully deprotected and cleaved from the resin with trifluoroacetic acid (TFA) with 5% thioanisole, 3% ethandithiol, and 2% anisole. The peptides were purified to homogeneity by preparative reverse-phase high-pressure liquid chromatography (RP-HPLC). Identity of the purified peptides was confirmed by Thermo Electron Surveyor MSQ RP-HPLC-electro spray ionization-mass spectrometer.

2.4. Analysis of HBHA dimer disaggregation—unfolding/refolding protocol

HBHA was incubated with peptide and slowly denatured by increasing the temperature up to 90 °C. After denaturation, the protein:peptide mixture was slowly cooled down to 20 °C. For analytical preparations, the refolded mixture was loaded on a Superdex 75 pc 3.2/30 size exclusion chromatography column. The extent of HBHA dimer disaggregation was evaluated as the percentage of decrease of the peak area corresponding to the molecular weight of HBHA dimers. For preparative scale purification, the refolded mixture was analyzed using a Superdex 75 10/30 column.

2.5. Analysis of HBHA dimer disaggregation at 37 °C

HBHA dimer disaggregation was also evaluated at 37 °C by incubating the HBHA protein with peptides at increasing time intervals (from 2 to 16 h) and with protein:peptide ratios ranging from 1:6 to 1:12. Incubates were analyzed using native gel electrophoresis using 8% non-denaturating polyacrylamide gel in Tris–glycine buffer (pH 8.8). Electrophoresis was performed at 10 mA for 2 h.

2.6. HPLC analysis

Analysis of hybrid protein–peptide complex collected fraction was performed with analytical RP-HPLC ESI-MS, using a linear gradient from 15% to 80% of B solvent (as reported previously) over 15 min at a flow rate of 1 ml/min.

2.7. Molecular modeling

Modeling of the coiled coil embedding helix H2 was performed using the structure of the basic coiled coil protein from Eubacterium eligens (PDB 3HNW) as a template. The program "O" was used to model the proline induced wobble of helices. Energy minimization of the generated 3D-model was done through GROMACS [14] by using Steepest Descent and Conjugate Gradient Algorithms. The coiled coil model was validated using the software SOCKET [15].

3. Results

3.1. Peptides as mimics of coiled coil regions of HBHA

Coiled-coil motifs represent a natural mechanism for guiding and cementing protein–protein interactions. Several evidences have suggested that dimerization between HBHA monomers, an event that dictates bacterial agglutination, proceeds through coiled coil recognition [9–11]. Using coiled coil predicting softwares, like PCOILS [16] and MULTICOIL [17], we identified potential interacting coiled coil regions (Fig. 1A). HBHA coiled coil domain (HBHAΔC) contains three main coiled coil helices (Fig. 1A), here denoted as H1 (residues 24–44), H2 (residues 49–69) and H3 (residues 87–107). Starting from this information, we synthesized three peptides corresponding to sequences H1, H2 and H3 (Fig. 1 and Table 1). To check the potential ability of the peptides to disrupt HBHAΔC dimers by mimicking interaction sites, we took advantage of the fully reversible unfolding of HBHAΔC, as we previously evidenced both using CD spectroscopy and differential scanning calorimetry [10,11]. Therefore, we adopted an unfolding–refolding protocol in the presence of each peptide and followed species formation using size exclusion chromatography. Unfolding–refolding experiments of mixtures of HBHA with either H1, H2 or H3 provided different results for each peptide species (Fig. 1). Indeed, chromatographic profiles after protein unfolding–refolding in the presence of the peptide H1 indicate some level of interactions between HBHAΔC and the peptide H1, although peaks are not resolved (Fig. 1B). Consistent with bioinformatics analyses, predicting that the helix H3 does not form dimeric coiled coils (Fig. 1A), the presence of H3