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

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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)

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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

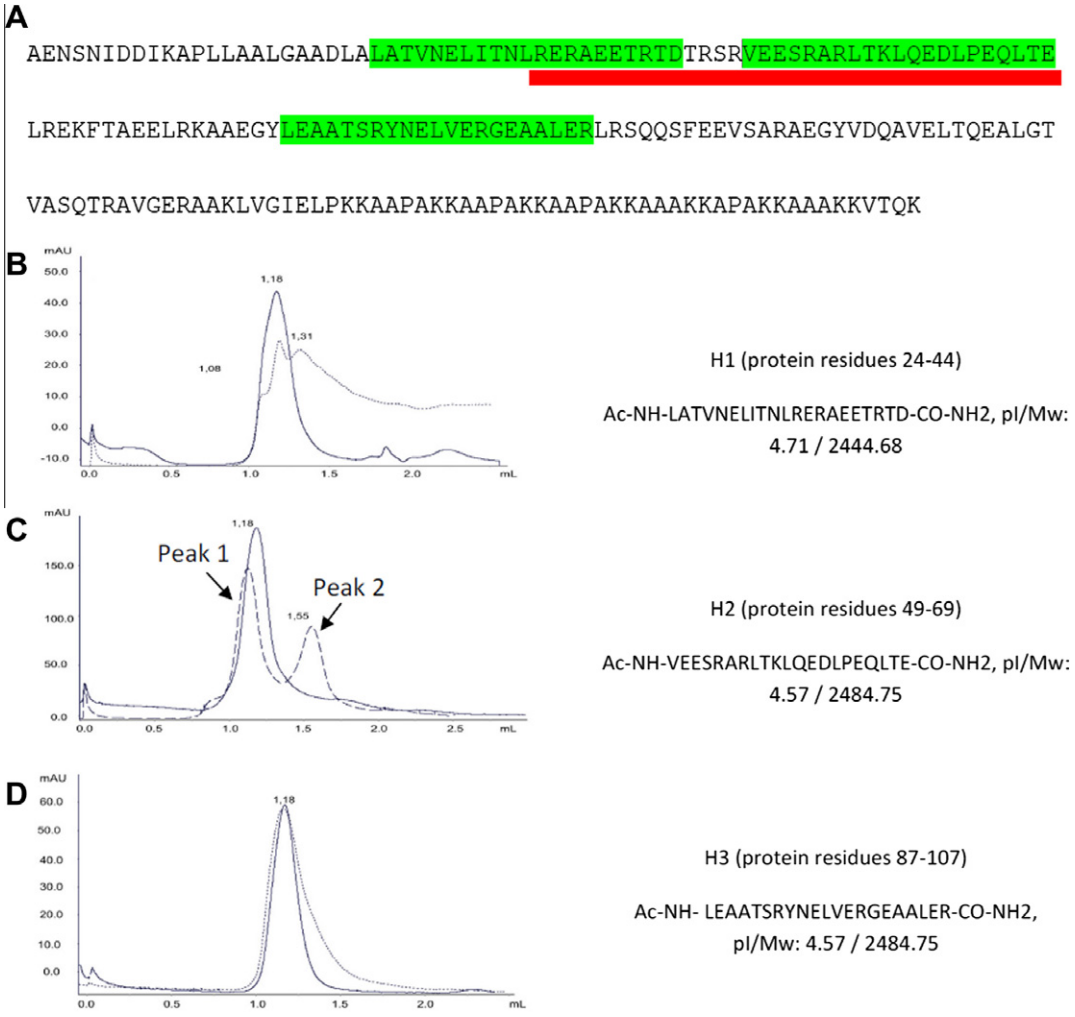

Fig. 1. (A) Sequence of HBHA; the three predicted helices with the highest propensities for coiled coil, as predicted by PCOILS (score > 0.8) [17], are highlighted green, whereas those corresponding to highest propensities for dimeric coiled coil (MULTICOIL2, score > 0.8) [16] are indicated by the red bar. (B–D) Analytic gel filtration chromatography profiles after unfolding–refolding in the presence of peptides H1, H2 and H3, respectively (protein:peptide ratio 1:10). Solid and dashed lines refer to HBHA before and after unfolding, respectively. Sequences of peptides are reported in the right panel.

Table 1
Sequences of synthesized peptides.

	Peptide sequences
First generation peptides	
H1	Ac-NH-LATVNELITNLRERAEETRTD-CO-NH_2
H2	Ac-NH-VEESRARLTKLQEDLPEQLTE-CO-NH_2
H3	Ac-NH-LEAATSRYNELVERGEAALER-CO-NH_2
Second generation peptides	
	Helix stabilization
H4	Ac-NH-VEESR<u>K</u>RLTKLQEDL<u>E</u>EQL<u>KK</u>-CO-NH_2
H5	Ac-NH-VEE<u>L</u>R<u>K</u>RLTKLQEDL<u>E</u>EQL<u>KK</u>-CO-NH_2
	Length modifications
H1ext	Ac-NH-APLLAALGAADLALATVNELITNLRERAEE-CO-NH_2
H2ext	Ac-NH-TRTDTRSRVEESRARLTKLQEDLPEQLTEL-CO-NH_2
H2shift	Ac-NH-RERAEETRTDTRSRVEESRARLTKLQED-CO-NH_2

peptide was completely ineffective during HBHAΔC refolding (Fig. 1D). Differently, two well separated peaks were obtained for mixtures of HBHAΔC with the peptide H2: after unfolding and refolding in the presence of H2, size exclusion chromatography clearly evidences the formation of a species with molecular weight corresponding to half of that of HBHA dimers (Peak 2, Fig. 1C). These data suggested the formation of a hybrid species constituted by HBHA monomers complexed with the H2 peptide.

3.2. Second-generation peptides development by mutation and C-terminal capping

CD spectroscopy was used to investigate the structural content of synthesized peptides. As a result, we observed that the peptide H2, like H1 and H3, did not contain a significant amount of α-helix (Fig. 2). Therefore, we designed second generation peptides to enhance peptide helicity, based on the idea that an enhancement of helicity of the H2 peptide may provide greater propensity for its association with HBHA monomers. Both C-terminal capping and specific amino acids substitutions which typically stabilize α-helices were adopted (Fig. 2A, Table 1).

Coiled-coil sequences are characterized by heptad repeats 'abcdefg', with a marked preference for hydrophobic amino acids at the 'a' and 'd' positions and hydrophilic residues on the external 'b', 'c', 'e', 'f' and 'g' positions. Thus, based on the H2 helical-wheel diagram (Fig. 2A), we designed two peptide analogs with the following criteria: (i) maintenance of amino acid residues critical for interaction (positions 'a','d'); (ii) replacement of non-conserved residues located at the solvent-accessible sites (positions 'b', 'c', 'f' and 'g') by charged and hydrophilic glutamic acid (E) or lysine (K) and introduction of C-terminal capping; (iii) modification of hydrophilic core residues with hydrophobic ones. We thus

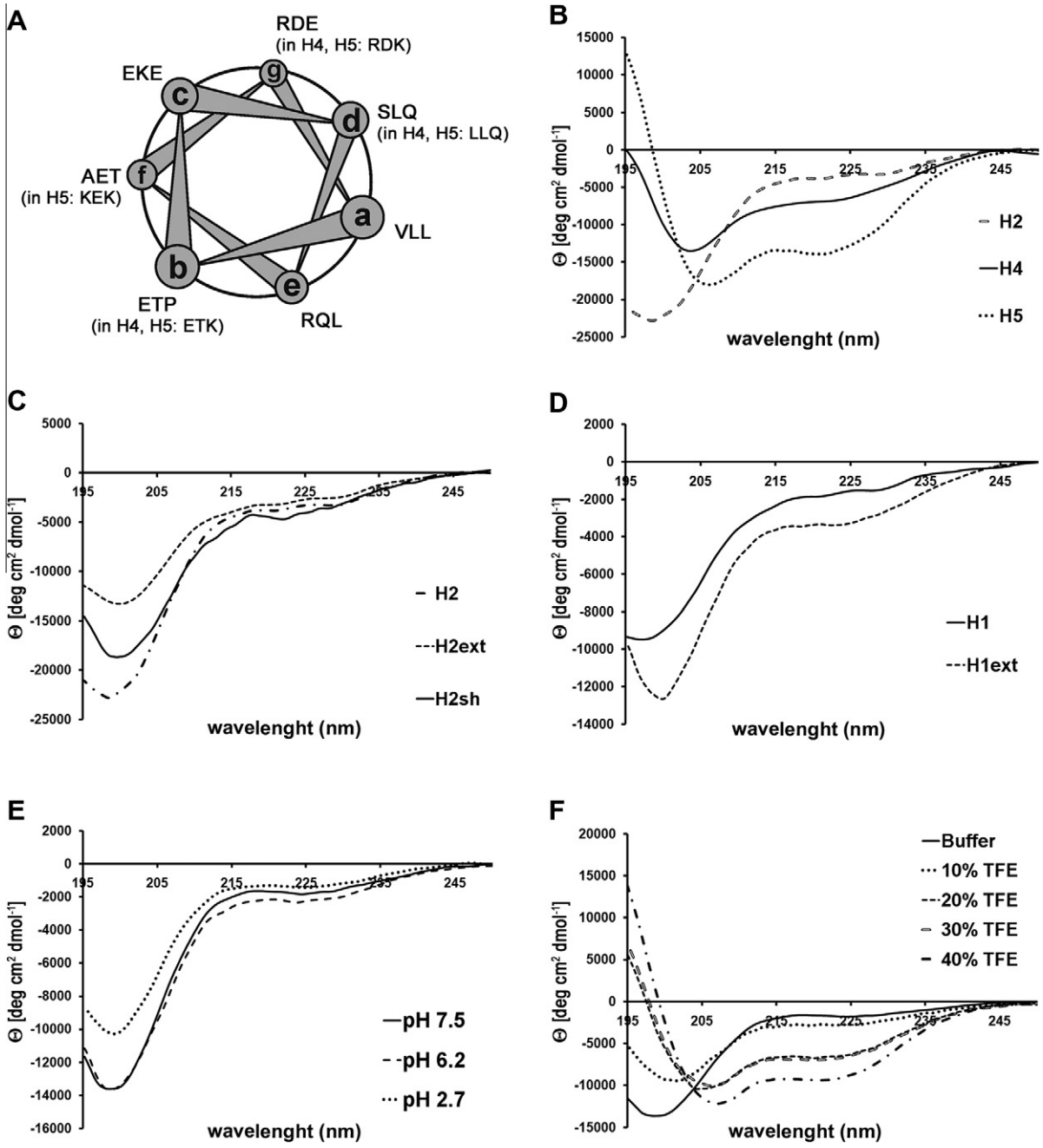

Fig. 2. (A) Helical wheel diagram showing aminoacid content of H2 helix and its mutants. (B) Circular dichroism spectra of native and second generation peptides H2 and H4. CD spectra in buffer of peptides designed for helix stabilization H2, H4, H5. (C) CD spectra of elongated peptides H2ext, H2shift. (D) CD spectra of H1 and its elongated version H1ext. (E) CD spectra of H2ext at different pH values. (F) CD spectra of H2ext in the presence of increasing TFE concentrations. Similar spectra were obtained for H2shift and H1ext.

obtained the two analogues shown in Table 1 (H4, H5). CD spectra of peptides H4, and H5, shown in Fig. 2B, clearly indicate a higher tendency of second generation peptides, compared to H2, to adopt an α-helical structure in phosphate buffer 5 mM, pH 7.4. A higher level of structural content of H4 and H5 peptides was also confirmed by NMR experiments (data not shown). Therefore, the ability of peptides H4 and H5 to disaggregate HBHAΔC dimers was tested using to the same unfolding–refolding protocol used for H1-H3. Surprisingly, we observed that peptides H4 and H5 present a lower ability to disaggregate HBHA dimers, compared to the starting H2 sequence (Fig. 3A). These results clearly showed that the obtained improvement of peptide α helix structure content (Fig. 2) does not imply a better interaction with the protein. This prompted us to design more natural peptides by retaining the protein sequence and extending their lengths.

3.3. Second-generation peptides development by sequence extension

Our results show (Fig. 1A–C) that peptides H2 and, to a minor extent, H1 are able to interfere with HBHAΔC dimerization. This is in accordance to MULTICOIL prediction (Fig. 1A) that helix H2 and part of H1 are involved in the formation of a dimeric coiled coil. Therefore, we extended the sequence of the peptide H2 to cover also part of the H1 helix (Table 1 and Fig. 1A). A further peptide

was synthesized by shortening the C-terminus and further elongating the N-terminus, to eliminate the proline residue (P64 in HBHA sequence) in the peptide sequence, since prolines are known helix breaker residues (H2shift, Table 1). We also analyzed the effects produced after peptide elongation of H1. The peptide H1ext was significantly elongated at the N-terminus (Table 1). CD spectra of peptides H2ext, H2shift, H1ext, shown in Fig. 2, clearly show that sequence extension did not improve peptide structuration. Indeed, CD spectra recorded at different pH values evidenced a random conformation for all peptides (Fig. 2E). When we used increasing percentages of TFE, a helical enhancer, CD spectra show the typical features of α-helix conformations, this indicating a tendency of peptides to form α-helix conformations (Fig. 2F).

All synthesized peptides were checked for their ability to disaggregate HBHAΔC dimers. Interestingly, elongation of H1 (peptide H1ext) provided only a small improvement in HBHA disaggregation (Fig. 3B), whereas the elongation of H2 to obtain H2shift produced no effect at all (Fig. 3D). Differently, elongation of H2 to obtain H2ext resulted in a striking enhancement of ratios between disaggregated and dimeric HBHA forms (Fig. 3C). Indeed, H2ext was able to disrupt more than 80% of HBHA dimers, to form HBHA–peptide hybrids.

HBHA dimer disaggregation after unfolding–refolding of HBHAΔC:H2ext mixture was also analyzed using native gel electrophoresis, with protein:peptide ratios ranging from 1:6 to 1:12 (Fig. 3E). As a result, native gels show two extra bands, compared to the native gel of the sole protein (Fig. 3E). The upper band corresponds to a monomeric state of HBHA whereas the intermediate band likely corresponds to a different conformational state of HBHA dimers, induced by H2ext (Fig. 3E). Preparative scale purification of the protein–peptide hybrid was carried out using the unfolding–refolding protocol described above (See Section 2). The size exclusion chromatography experiments, performed using a Superdex 75 10/30 column, provided a profile with two well separated peaks (1 and 2 in Fig. 3F), which were collected and used for both HPLC chromatography and far-ultraviolet (UV) CD analysis. Using HPLC

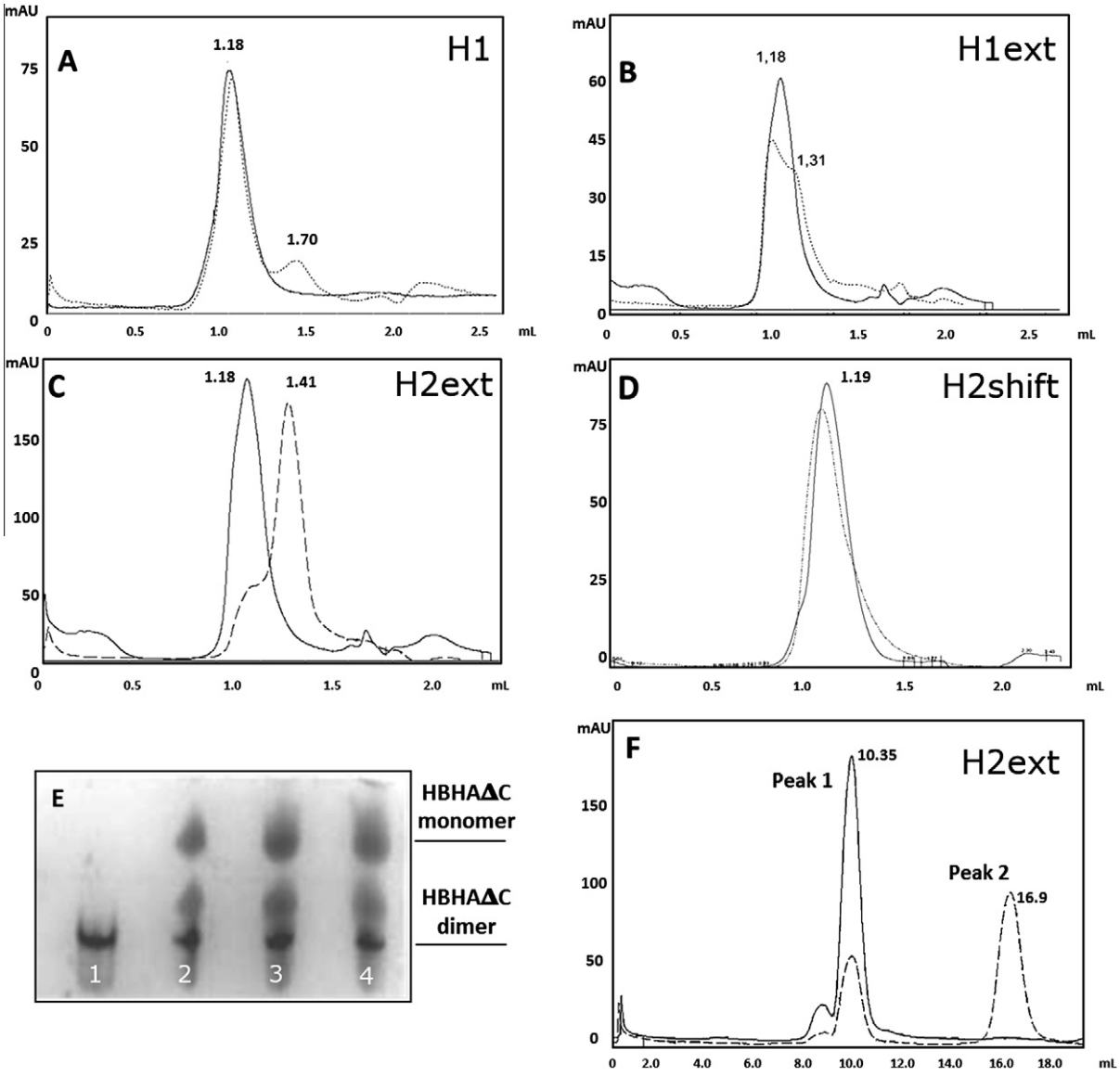

Fig. 3. Analytic gel filtration chromatography profiles after unfolding–refolding in the presence of peptides H4 (A), H1ext (B) H2ext (C) and H2shift (D) using a protein: peptide ratio of 1:10. Solid and dashed lines refer to HBHA before and after unfolding, respectively. (E) Native gel electrophoresis of HBHAΔC mixture after the unfolding–refolding protocol, using protein:H2ext ratios 1:6 (lane 2), 1:8 (lane 3), 1:12 (lane 4). The reference lane (lane 1) refers to HBHAΔC alone. (F) Gel filtration chromatography profiles after unfolding–refolding in the presence of peptides H2ext on a preparative Superdex 75 10/30 column.

chromatography, we observed that the peak corresponding to the HBHA dimer (Peak 1 in Fig. 3F) was constituted solely by protein, whereas Peak 2 contained both HBHA and the peptide H2ext (Fig. 4). These results unambiguously showed that the peptide H2ext is able to interact with HBHAΔC and lock it in a monomeric state. In parallel, we adopted CD spectroscopy to investigate the secondary structure content of the protein–H2ext hybrid. As a result, CD spectra evidenced that protein–peptide hybrid (Peak 2 in Fig. 3F) is well folded and presents the typical features of α-helical coiled-coil structures, with minima at 208 and 222 nm (Fig. 4D). Also, the CD spectrum of Peak 1 presents the typical features of that of HBHAΔC dimers [10]. The spectra of HBHA dimer (Peak 1) and of the protein:peptide hybrid (Peak 2) are in significant agreement, beside a slightly different ratio between ϴ222 and ϴ208 nm (Fig. 4D).

3.4. Modeling of helix 2 interactions

We used molecular modeling to analyze interactions between H2 helices of HBHA, those most involved in HBHA dimerization (Fig. 1A). These helices are predicted by MULTICOIL to form a parallel dimeric coiled coil [16]. Therefore, we modeled a parallel coiled coil embedding the H2 sequence, starting from the structure of a typical coiled coil protein (PDB code 3HNW). It is worth noting that the sequence of the H2 helix, VEESRARLTKLQEDLPEQLTE, presents two peculiar features. First, it embeds a proline residue (Pro64 in HBHA sequence), which typically induces distortions of α-helices. Also, the sequence of the H2 helix presents a clear irregularity in its heptads composition. Indeed, the C-terminal heptad of H2, of sequence LPEQLTE, presents an atypically hydrophilic residue, Gln66, at the 'd' position, followed by a leucine, Leu67, at the 'e' position, a position which is typically occupied by hydrophilic residues. We modeled the typical distortion induced by proline in α-helices, i.e. the formation of a wobble, and a consequent kink of the helices. This model was energetically minimized using the Steepest Descent and Conjugate Gradient Algorithms implemented in GROMACS [14]. The resulting model was validated, using the software SOCKET [15], which analyzes the typical structural features of coiled coils, named as knobs-into-holes. As shown in Fig. 5, the effect of a wobble induced by proline brings Leu67 residues of the two contacting helices at the typical position occupied by 'a' residues in coiled coils. Also, Gln66 of the two helices, which would point to each other in regular coiled coils (as 'd' residues in typical coiled coil sequences, Fig. 5A) occupy the location typically adopted by 'c' residues (Fig. 5B). The SOCKET analysis of this mode shows that the helix packing of the coiled coil model is tight-knit. Indeed, like in typical parallel helices, 'a' knobs dock into holes formed by $d^{-1}\,g^{-1}$ad residues, whereas 'd' knobs interact with $adea^{+1}$ holes (superscript numbers refer to either the following (+) or the preceding (−) heptad of the adjacent helix) (Table 2). Due to the distortion of the coiled coil induced by Pro64, Leu67 residues do not form proper knobs (Table 2A); however, they establish hydrophobic interactions with Leu63 of the same chain and with both Leu63 and Leu67 of the adjacent chain (2B). Notably, in a non-distorted model (Fig. 5A), helices are not well packed, as they establish only a single knobs-into-holes interaction (knob Leu59). This is likely due to the steric hindrance induced by the presence of glutamine, Gln66, at 'd' position. Therefore, the wobble induced by proline in helix H2 (Fig. 5) is an important structural requirement for the stabilization of a distorted coiled coil through the typical knobs-into-holes interactions.

3.5. H2ext disrupts HBHA dimers at 37 °C

M. tuberculosis typically grows at 37 °C. Therefore, we analyzed the effects of H2ext in the disaggregation of HBHA dimers at 37 °C. To achieve this result, we incubated HBHA with the peptide for increasing incubation times and with a range of protein:peptide ratios (See Section 2). After incubation, we adopted native gel electrophoresis to detect the amounts of formed protein–peptide forms. Results clearly show that the peptide H2ext is able to

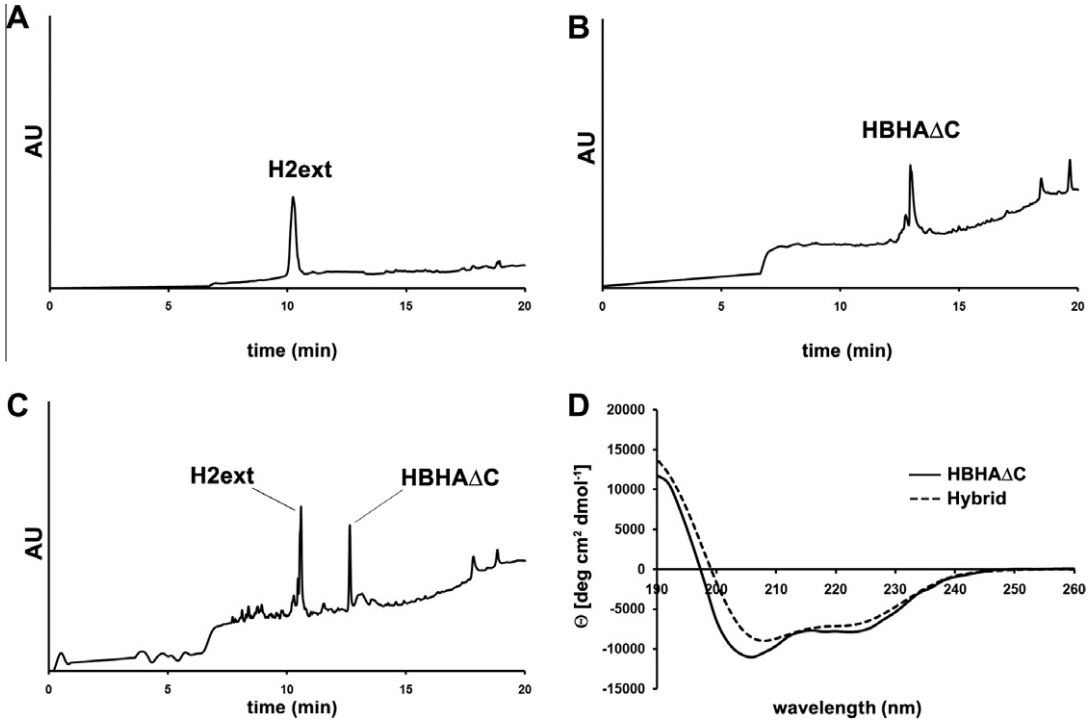

Fig. 4. (A) Hplc profile of H2ext peptide; (B) Hplc profile of HBHAΔC; (C) Hplc profile of gel filtration Peak 2 (Fig. 3F). (D) Overlay of CD spectra of (continuous line) HBHAΔC and of (dashed line) the protein:H2ext hybrid, Peak2 in Fig. 3F.

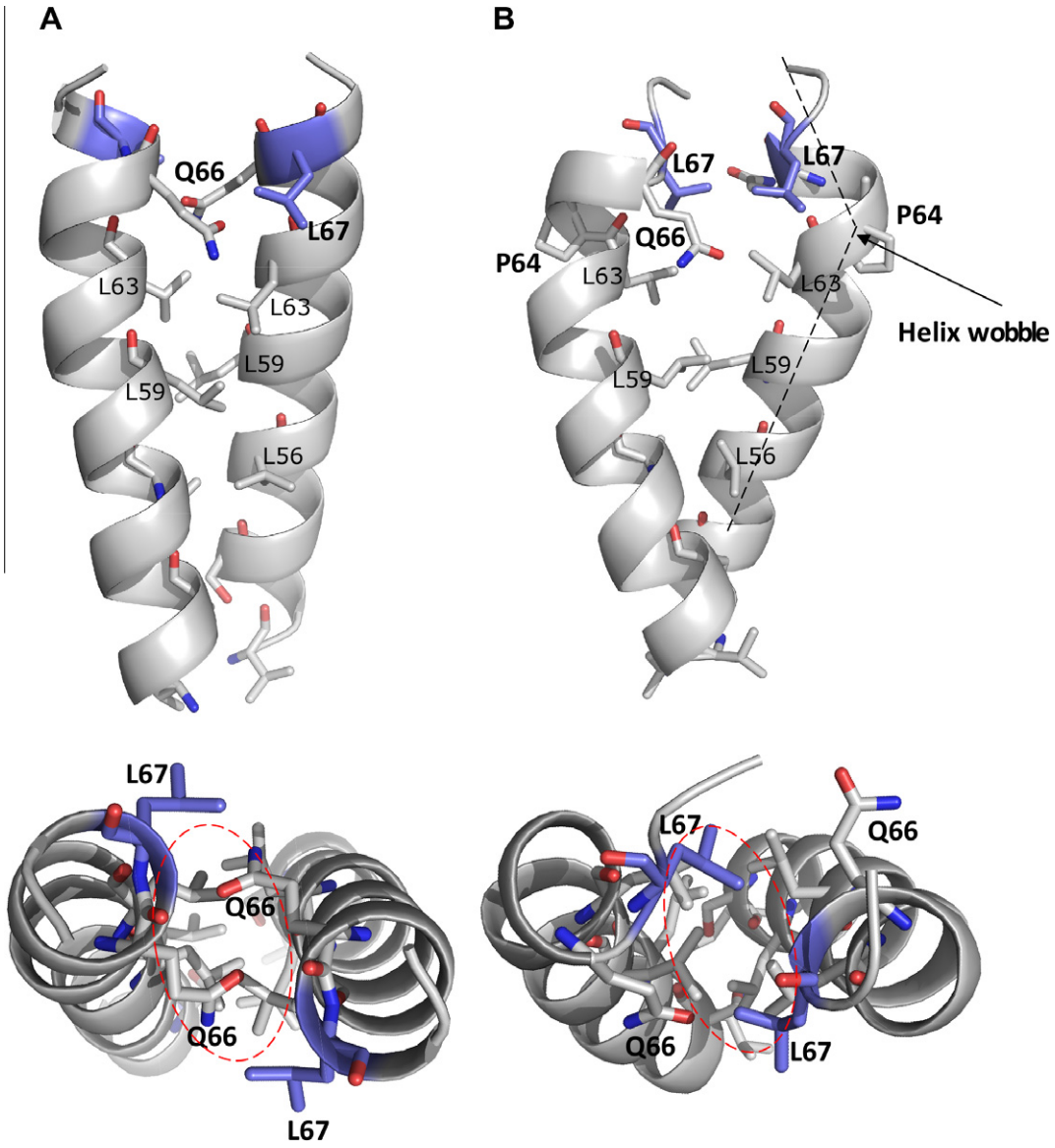

Fig. 5. (A) Side view (top) and top view (bottom) of a hypothetical non-distorted coiled coil. (B) Side view (top) and top view (bottom) of the wobbled coiled coil. Red circles in bottom panels indicate typical positions occupied by 'a' and 'd' residues in coiled coils.

Table 2
(A) Knobs-into-hole interactions in the coiled coil model; (B) Hydrophobic core interactions.

(A)	
Knobs (chain A)	Holes (chain B)
Leu56 (a)	Ser52 (a), Arg55 (g), Leu56 (a), Leu59 (d)
Leu59 (d)	Leu56 (a), Leu59 (d), Gln60 (e), Leu63 (a)
Knobs (chain B)	Holes (chain A)
Leu56 (a)	Ser52 (a), Arg55 (g), Leu56 (a), Leu59 (d)
Leu59 (d)	Leu56 (a), Leu59 (d), Gln60 (e), Leu63 (a)
(B)	
Leu56 (chain A)	Leu56 (chain B), Leu59 (chain B)
Leu59 (chain A)	Leu59 (chain B), Leu63 (chain B)
Leu63 (chain A)	Leu59 (chain B), Leu63 (chain B), Leu67 (chain A)
Leu67 (chain A)	Leu63 (chain B), Leu63 (chain A), Leu67 (chain B)

disrupt HBHA dimers also at 37 °C (Fig. 6A) and significant amounts of hybrid species are evident also after short incubation times (about 2 h). Parallel experiments carried out with peptides H4 and H5 did not produce detectable amounts of protein peptide hybrids (Fig. 6B). Notably, the native gel obtained after incubation of the protein with H2ext at 37 °C (Fig. 6A) presents the same features observed after unfolding–refolding (Fig. 3E). This suggests that the unfolding–refolding procedure well reproduces the conformational states generated at 37 °C by the presence of H2ext.

4. Discussion

Bacterial aggregation and cell adhesion mediated by HBHA are two important steps of TB pathogenesis. Mtb is able to escape from the phagosome and translocate into the cytosol, where it can be found in tightly packed clumps [18] . Whether aggregation occurs during the intracellular life or once inside the cytosol is hitherto not clear. However, HBHA-mediated aggregation is a strong instrument for the formation of clumps of bacilli, which more effectively adhere and invade epithelial cells than single bacilli. It has been suggested that HBHA may interact with cytosolic components of the host cells such as actin [19]. Also in this case, bacterial aggregation likely ensures a more efficient binding of bacterial cells to their host targets.

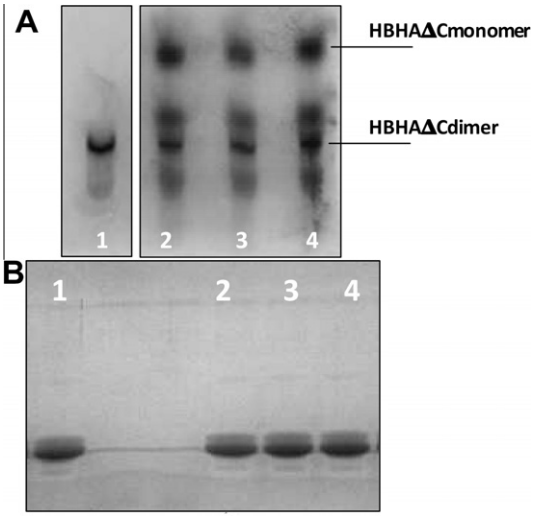

Fig. 6. Native gel electrophoresis at 37 °C (A) 60 μg of HBHADC (lane 1) were incubated for two hours with protein: H2ext ratios 1:6 (lane 2), 1:8 (lane 3), 1:12 (lane 4). (B) 60 μg of HBHADC (lane 1) were incubated overnight with peptides H4 (lane 2), H5 (lane 3) and H2shift (lane 4) with 1:8 protein:peptide ratio.

It was previously shown that homophilic HBHA–HBHA interacting forces occur at the bacterial surface of live mycobacteria [9]. Several studies have suggested that these interactions are mediated by the coiled coil regions of HBHA [10,11], which likely stabilize HBHA dimer formation. Therefore, a strong correlation exists between HBHA dimerization and its ability to induce bacterial aggregation. In this framework, we here mapped molecular determinants of HBHA dimerization by combining peptide synthesis with biochemical and computational studies. We designed and synthesized several peptides which mimicked the most critical predicted regions in HBHA dimerization. As a result, we identified a peptide, covering a specific region of HBHA sequence (residues 41–70, peptide H2ext), which strongly competes with protein dimerization by mimicking the protein dimerization interface. This peptide is able to disaggregate HBHA dimers during the protein refolding process, with the formation of species with a lower molecular weight (Figs. 3C,E,F). We proved that this species is a hybrid protein–peptide form (HPLC analysis, Fig. 4A–C) with a well-preserved secondary structure (CD spectroscopy, Fig. 4D). Notably, the ability of H2ext to disaggregate HBHA dimers and form a hybrid form is preserved at 37 °C, the growth temperature of Mtb.

To rationalize the different behavior of our synthesized peptides and to identify critical regions of HBHA monomer–monomer recognition, we performed molecular modeling of the helix which is most involved in HBHA dimerization (helix H2). These studies clearly identified a peculiar structural feature of H2, which embeds a Pro residue at its C-terminus. The proline residue, traditionally considered as a 'helix breaker', is well represented in helices of transmembrane proteins [20], where it plays the crucial role of conferring flexibility in the local conformational dynamics of transmembrane segments. We modeled a coiled coil formed by kinked helices, in which a wobble angle exists between the postproline helix segment, with respect to the preproline segment. Energy minimization of this model provided a well-packed coiled coil, which explains the anomalous presence of Gln66 at the 'd' position and of Leu67 at the 'e' of the 'abcdef' typical coiled coil heptad (Fig. 5). A helical kink induced by proline was previously observed in the coiled coil portion of Dynein, a motor protein that transports cellular cargo along the microtubules within the cytoplasm [21]. Notably, as observed for Dynein, the proline residue responsible for the helical kink, Pro64, is strictly conserved in bacteria (data not shown). These considerations strongly suggest that the kink of H2 helices is an important structural determinant for HBHA monomer–monomer recognition. To enforce this observation, peptides H4 and H5, which contain a proline mutation, are barely able to disaggregate HBHA dimers (Fig. 6), despite their higher helical content, compared to H2 and H2ext (Fig. 2). Similar considerations apply to the peptide H2shift, which embeds the protein sequence immediately preceding Pro64 (Table 1 and Fig. 3A, C, and D). In conclusion, we identified the first molecule (H2ext) able to disaggregate HBHA dimers, and likely to severely interfere with Mtb agglutination. The ability of the peptide H2ext (embedding Pro64) to disaggregate HBHA dimers at the growing temperature of Mtb has a strong implication for the development of anti-agglutinating molecules with therapeutic interest. These molecules will likely weaken Mtb agglutination-driven defense mechanisms and have a strong impact on the Mtb adhesion to epithelial cells.

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Appendix A. Supplementary data

Supplementary data (Supplementary Materials and Methods) associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2012.01.047.

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