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Cryo-EM undiscloses structural and mechanistic details on iron hijacking by *Staphylococcus aureus*: An insight into the interaction of IsdB hemophore with human hemoglobin(*)

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Summary. — Iron is an essential nutrient for almost all organisms. In the human body, it is primarily bound to the heme cofactor of hemoglobin (Hb), myoglobin and other heme-binding proteins. During infection, *Staphylococcus aureus* exploits Hb heme pool as its favored iron source, capturing and internalizing it by cell wall hemophores. The first step is performed by IsdB, which intercepts free Hb and extracts heme. IsdB, a proven virulence factor, is an attractive putative target for antimicrobials development but its mechanism of action needs to be further detailed. To this aim, we used cryo-EM single-particle analysis to study IsdB:Hb complex formation and heme extraction. The key complexes before and after heme extraction were solved at 2.9 Å and 5.8 Å resolution using carboxyHb, resistant to heme removal, and oxidized Hb, the physiologic IsdB substrate. IsdB first binds to Hb β-chains, enhancing Hb dimerization to favor a second IsdB molecule binding to α-chains before extraction. These results greatly improve our current knowledge of IsdB structural and functional dynamics, thus promoting future studies on new potential antimicrobials aimed at impairing *S. aureus* iron acquisition.

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1. – Introduction

Antimicrobial resistance is a concerning phenomenon rapidly spreading among microbial species and threatening the efficacy of antibiotics, with a toll of 700000 deaths per year that could increase more than tenfold by 2050 if no effective actions are undertaken [1]. In the World Health Organization global list of resistant pathogens, *Staphylococcus aureus* is classified as a high-priority target, with particular regard to its vancomycin- (VRSA) and methicillin-resistant (MRSA) strains [2]. *S. aureus* needs the acquisition of iron to support host colonization and infection settlement. In the human host, iron is almost totally bound to storage and carrier proteins (*e.g.*, ferritin and transferrin) or is a component of protein cofactors such as heme. The most abundant and preferential iron source for bacteria is represented by the hemoglobin (Hb) heme. To face its iron demand and gain access to Hb, *S. aureus* secretes hemolysins, toxins able to disrupt the red blood cells membrane; Hb is then captured by the Iron surface determinant (Isd) system, a nine-hemophore gear able to extract heme and transfer it into the bacterium. The first step in hemic iron acquisition consists in heme extraction from Hb, mediated by the two cell wall-exposed receptors IsdB and IsdH. These hemophores share a high structural and functional similarity, but only IsdB was found to sustain bacterial growth *in vitro* and is a proven virulence factor [3]. Therefore, the interference with IsdB functions can be a promising approach for the development of new antimicrobials. IsdB is a modular, dumbbell-shaped protein formed of two NEAr iron Transporter (NEAT) domains, with an immunoglobulin-like folding, separated by a flexible linker domain. IsdB binds Hb through NEAT1 (N1) thanks to the surface-exposed loop 2; NEAT2 (N2), carrying the heme-binding motif ^{440}YDGQY^{444} can then be placed near the Hb binding pocket and extract heme. The cofactor will be afterward passed to the next hemophore IsdA, in charge of managing the following steps for heme internalization. IsdB can extract the ferric heme from cell-free, oxidized Hb (methemoglobin, metHb), naturally present as a dimer, but not from the ferrous oxygenated tetrameric form (oxyHb) present inside red blood cells. IsdB binding can accelerate heme release from Hb up to 2000-fold and heme transfer can be spectroscopically monitored [4, 5].

2. – Results and discussion

Despite the numerous catalytic and spectroscopic data available, X-ray crystallography has never allowed obtaining a satisfactory and detailed description of the conformational dynamics likely responsible for complex formation and heme extraction. We then decided to apply single-particle cryo-electron microscopy (cryo-EM) to trap different steps along the complex formation/heme extraction pathway. CarboxyHb (HbCO) was exploited to try to isolate the initial complex formation before heme extraction (IsdB cannot extract heme when iron is in the ferrous state), while metHb was used to obtain the structure of the final state after extraction completion. HbCO was preferred over the oxyHb form because of its higher stability to oxidation in the experimental conditions. The single-particle analysis (SPA) of IsdB:HbCO complex led to the identification of two major compositional states, corresponding to one (IsdB:HbCO*) or two (IsdB:HbCO) IsdB molecules bound to a Hb tetramer (fig. 1(A)). Their three-dimensional (3D) cryo-EM maps were reconstructed at 3.62 Å (PDB ID 7pcq) and 2.89 Å (PDB ID 7pch), respectively. The IsdB:HbCO complex reconstruction at 2.89 Å is the highest-resolution structure obtained so far for a complex between a staphylococcal hemophore and human Hb. Interestingly, IsdB preferentially first binds to the β-Hb subunits, which are

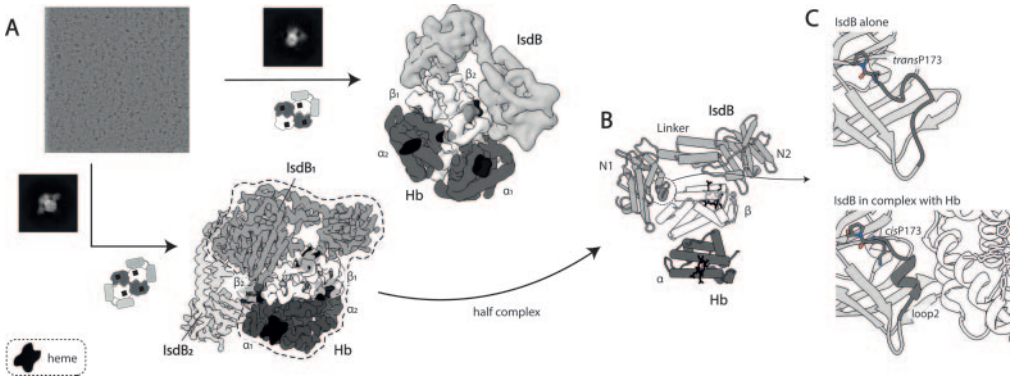

Fig. 1. – IsdB:HbCO and IsdB:HbCO* complexes structures from cryo-EM. (A) (left to right), a representative micrograph, two reference-free 2D classes, schematic representations, and the cryo-EM maps. The density map of heme on Hb is represented in black. (B) Asymmetric unit of the IsdB:HbCO complex. (C) Comparison of isolated IsdB N1 (PDB ID 2moq, top) with IsdB in complex with Hb (PDB ID 7pcq, bottom). Structures are semi-transparent, while the K172-P173 peptide bond and the regions involved in folding upon binding (loop 2) are in dark grey.

naturally more prone than α-chains to release heme. Four regions of interaction were identified between the hemophore and Hb (fig. 1(B)). In the first one, N1 is located near the Hb A and H helices and the EF loop; here, the near-atomic resolution of the complex allowed appreciating the *cis* conformation of the K172-P173 peptide bond in close proximity to the folded loop 2 on IsdB (fig. 1(C)). The comparison with the X-ray structure of the isolated N1 (PDB ID 2moq), where loop 2 is not folded and the Pro-containing peptide is in *trans* conformation, could indicate isomerization as the driving force for IsdB association/dissociation with Hb. The second contact area is established by the IsdB linker and the N2 domain with the F helix, here still folded, but whose distortion represents the central step in the heme extraction process. The last two regions of interaction show N2 at the entrance of the heme-binding pocket, where the cofactor is still bound, preparing the hemophore for heme extraction. The IsdB:HbCO* complex could represent an intermediate step along the formation of a saturated assembly, as can be

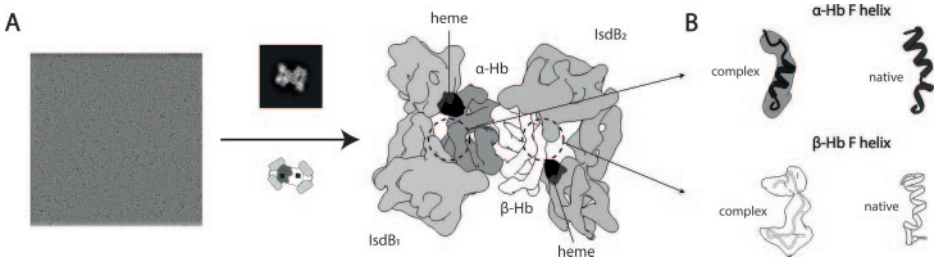

Fig. 2. – IsdB:metHb complex structures from cryo-EM. (A) (left to right) a representative micrograph, a reference-free 2D class, schematic representation and the cryo-EM map. (B) Comparison between F helices in either α- (top) or β-Hb (bottom) subunits within IsdB:metHb complex and native helices in isolated Hb structure (PDB ID 3p5q).

assumed from the absence of some interactions between the residues of the two proteins, but present in the IsdB:HbCO structure. The cryo-EM map of IsdB:metHb complex was solved at 5.8 Å (PDB ID 7pcf). The low resolution only consented to accommodate the secondary structure into the map by aligning an *ad hoc* model, without positioning the amino acid side chains. It was however possible to trap an oligomeric state never observed before, where two IsdB molecules bind to a Hb dimer (fig. 2(A)). Here, heme molecules are bound to the two N2 domains and the F helices have lost their native fold, representing a snapshot of the concluded extraction process (fig. 2(B)).

3. – Conclusions and future perspectives

The determination of key structural states allowed proposing a model describing the sequential events along the IsdB:Hb complex formation and heme extraction process. After a first IsdB binding to a Hb tetramer β-chain (PDB ID 7pcq), a second hemophore molecule is recruited on the other homologous subunit (PDB ID 7pch). This step promotes the Hb tetramer dimerization, enabling binding another IsdB unit on the available α-chain (PDB ID 7pcf); this second binding on the dimer is likely the key step driving heme extraction, before the dissociation of the complex.

The structural information derived in this study strengthens the knowledge of the key steps of IsdB:Hb protein-protein interaction (PPI) dynamics. These structural and mechanistic insights could be of great importance for the design of molecules aiming at interfering with IsdB:Hb PPI as a novel antimicrobial strategy.

4. – Materials and methods

IsdB:Hb complexes were imaged in a Titan Krios cryo-electron microscope (Thermo Fisher Scientific) at 300 kV, with a Gatan K3 direct electron detector working at the calibrated magnification of $\times$130000, yielding a pixel size of 0.326 Å. A total of about 3000 movie stacks were recorded by EPU software (Thermo Fisher Scientific), with a defocus range of $-0.8/-3.1$, an exposure time of 1.1 s, and a dose rate of 15.3 e^-/s. The IsdB:HbCO complex was analyzed using WARP (`http://www.warpem.com/warp/`) to identify 460000 particles, then processed using cryoSPARC (`https://cryosparc.com/`). The 2D classification allowed separating the complexes where an HbCO tetramer is bound by one (IsdB:HbCO*) or two IsdB molecules (IsdB:HbCO). The IsdB:metHb complex SPA was carried out using RELION (`https://relion.readthedocs.io/en/release-3.1/`) with a final selection of roughly 100000 particles. Cryo-EM data processing yielded 2.9, 3.6, and 5.8 Å maps for the IsdB:HbCO, IsdB:HbCO*, and IsdB:metHb complexes, respectively.

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