nature portfolio

Peer Review File

Time-resolved X-ray solution scattering unveils the events leading to hemoglobin heme capture by staphylococcal IsdB

Corresponding Author: Professor Luca Ronda

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Bei et al. conducted a study in which they aimed to elucidate the kinetics of the structural changes associated with the formation of IsdB:Hb complex. The authors achieved this using X-ray scattering techniques, including small-angle X-ray scattering (SAXS), wide-angle X-ray scattering (WAXS) and time-resolved X-ray solution scattering (TR–XSS). They performed SAXS/WAXS measurements of IsdB:metHb and IsdB:oxyHb complexes across various protein concentrations. By comparing the obtained SAXS/WAXS patterns and the candidate structural models, they obtained structural insights into the formation of IsdB:metHb and IsdB:oxyHb complexes. Notably, the authors suggested a kinetic model for the interaction between IsdB and Hb using TR–XSS combined with a rapid mixing triggering approach and the spectroscopic techniques, including time-resolved optical absorption (TR-OA) and fluorescence (TR-F) spectroscopy.
The authors have successfully presented a detailed kinetic model for the interaction between IsdB and Hb. This reviewer believes the kinetic information will provide important insights into understanding the physiological properties of the IsdB:Hb complex. However, the current manuscript lacks a detailed structural discussion regarding the formation of the complex. Furthermore, this reviewer encountered challenges in identifying any novel analytical or experimental advancements in this manuscript. For example, in numerous studies employing X-ray solution techniques, the scattering data have been measured in both SAXS and WAXS regions (J. Phys. Chem. Lett., 8, 4413 (2017) and Photochem Photobiol Sci., 17, 874, (2018)). Moreover, several of these studies have applied various approaches such as MD simulations, rigid body modeling and ab-initio modeling to analyze the scattering data, yielding not only information about the global shapes of proteins but also about their local shapes (Nature, 509, 245–248 (2014), Nat. Commun., 12, 3677 (2021), Sci. Adv., 2, e1600920 (2016)). Lastly, the TR–XSS-guided MD simulations have already been successfully applied to protein complexes in a study (Sci. Adv., 8, eabm6278 (2022)). Therefore, this reviewer cannot recommend the publication of this manuscript until the following severe concerns are fully addressed.

1. Although the authors mention that their work can contribute to the design of inhibitors for the interaction between IsdB and Hb, the manuscript lacks detailed structural discussions related to the interaction. Regarding this, this reviewer recommends that the authors provide comprehensive discussions about the following questions using the structures obtained from the structural analysis of SAXS/WAXS and TR–XSS data: (i) what structural changes will occur in IsdB and Hb during the formation of the IsdB:Hb complex. (ii) how the structural changes in IsdB and Hb lead to the formation of the IsdB:Hb complex. Such additional discussions would be beneficial for readers in understanding the process of complex formation.

2. In line 79 - 83, the authors mentioned, ‘Although TR-XSS has been already used in combination with a stopped flow apparatus, all studies have been so far limited to the small-angle X-ray scattering (SAXS) region, which mainly yields information on the overall shape of the protein and its radius of gyration. We improved the structural resolution of this approach by collecting TR-XSS data in the wide-angle X-ray scattering (WAXS) region after rapidly mixing solutions of IsdB and metHb.’ However, it should be noted that in several studies utilizing X-ray solution techniques with a stopped flow setup, the time-resolved scattering data have been measured not only in the SAXS regions but also in the WAXS region (for example, J. Phys. Chem. Lett., 8, 4413 (2017) and Photochem Photobiol Sci., 17, 874, (2018)). Furthermore, some of these studies have applied various approaches, including molecular dynamics (MD) simulations, rigid body modeling and ab-initio modeling, to the scattering data, providing not only information about the global shape of proteins but also atomic models (or low-resolution structure) for their 3D conformations (Nature, 509, 245–248 (2014), Nat. Commun., 12, 3677 (2021), Sci. Adv., 2, e1600920 (2016)). Considering these, it is recommended that the authors revise the related content accordingly by introducing the previous studies.

3. In line 86 - 90, the authors stated, ‘In order to extract structural information from our experimental TR-XSS signals, we have also performed TR-XSS guided molecular dynamics (MD) simulations on the IsdB:Hb complex. We applied for the first time this approach to a protein complex, which couples the intrinsically low-resolution information from X-ray solution scattering with a priori physicochemical knowledge on the protein structure.’. Depending on how protein complexes are defined, this claim can be challenged by those who studied a protein complex made of multiple domains and a protein multimer (for example dimer) using the same set of techniques (Sci. Adv., 8, eabm6278 (2022)). Considering these, it is recommended that the authors revise the content mentioned above accordingly by properly introducing the previous studies.

4. In Fig 2D, as the concentration of IsdB increases, it is observed that the difference between the experimental curves and the theoretical curves calculated form a linear combination of the structural models with various oligomer state, including 2IsdB:βoxyHbtet, 1IsdB:βoxyHbdim and IsdB, becomes more pronounced in the small-angle region. This difference raises the question for this reviewer whether, at higher concentrations of IsdB, complexes with larger oligomer states than those considered in the structural analysis might also form. In this regard, this reviewer recommends that the authors provide comprehensive discussions of the discrepancy between the experimental and theoretical curves.

Reviewer #2

(Remarks to the Author)
The paper “Time-resolved X-ray solution scattering unveils the events leading to hemoglobin heme capture by staphylococcal IsdB” by O. Bei, M. Marchetti, S. Guglielmo, E. Gianquinto, F. Spyrakis, B. Campanini, S. Bettati, M. Levantino, L. Ronda presents a thorough multi-techniques study of protein-protein interaction between Iron-regulated Surface Determinant (IsdB) protein and hemoglobin(Hb). Important process of scavenging iron from hemoglobin is investigated. The scavenging of iron from human blood by infectious entity Staphylococcus aureus is a subject which presents an interest to a general scientific community and as such it is suitable for the Nature Communication audience.
Key Results. In the last years, significant efforts were applied to study the process of the heme cluster transfer from Hb to Isd complex. Several optical studies, sensitive to the local environment of the heme molecule were reported, along with a cryo-EM structure PDB ID 7PCF , and a crystallographic structure PDB ID 5VMM of two stable complexes IsdB:Hb . The current work presents a first study of structural dynamics of scavenger IsdB molecule interaction with human blood molecule Hb. IsdB molecule was mixed with metHb in solution, and then the course of reaction was followed by taking X-Ray images of the reactants as function of the time delay after the mixing event. Time-resolved X-ray solution scattering (TRXSS) is capable of detecting reaction intermediates under physiological conditions albeit with lower resolution than crystallography. The authors combined static and time-resolved X-ray solution scattering with time-resolved optical absorption (TR-OA), fluorescence spectroscopy (TR-F) and molecular dynamic (MD) simulations to determine stoichiometry and course of reaction of IsdB:metHb complex after mixing event. They determined, that IsdB scavenger molecule after binding with tetramer metHb forms a 2IsdB:βmetHbtet intermediate and that the final product of the reaction is 2IsdB:αβmetHbdim dimer (heme cofactor is bound to both αβ chains of Hb), which forms approximately ~ 1 second after the mixing event . By using time-resolved optical methods authors show that heme cofactor transfer occurs after 2IsdB:αβmetHbdim formation from apo-2IsdB: holo αβmetHbdim to holo-2IsdB: apo- αβmetHbdim. Authors proposed a mechanism of dissociation of 2IsdB:βmetHbtet intermediate into 2 dimers IsdB:βmetHbdim and subsequent binding of IsdB scavenger molecule to the α-chain of metHb, thus forming the final molecule. Authors built the kinetic model of the process, determined kinetic constants of the model and proposed that intersubunit communications triggered by IsdB binding to β chains of IsdB:βmetHbdim make α subunits competent for IsdB binding. Further, time-resolved structural scattering signal difference was used as additional driving force for MD simulations. The simulations reveal that in the final process of heme scavenging (1.3 seconds and 24 seconds data) F-helix refolds in α-chains of Hb, but not in β-chains, supporting different dynamics of the two Hb subunits, which the authors proposed.
Originality and Significance. The results reported in the paper are exciting, original, and novel. The dynamic process of how infectious entity Staphylococcus aureus scavenges Fe molecule for it’s nutrients from Hb in human bloodstream is an important health and medical subject. Therefore, the topic is of interest not only for the specialized biomedical community, but also to a general audience from different disciplines.

Data and methodology. Validity of the results.
The manuscript presents an outstanding example of experimental work in time-resolved structural biology. Using static SAXS/WAXS data combined with TRXSS and complimentary TR-OA and TR-F is a reliable approach both technically and methodologically. Time resolution, achieved in the experiment, is adequate to determine reported structural intermediates, formed in the reaction, and to determine kinetic constants.
However, I have problems with some aspects of analysis of static, and time-resolved solution scattering data.
Statistical analysis. Statistical analysis should be improved. Data, presented on the graphs, and raw data attached to the manuscript do not show any error bars. Those should be present. Fits of the time-resolved solution scattering data in Fig. 5A should have fit errors, for example, like the fits of the static solution scattering data with theoretical constructs (Fig. S3, S4).
Static Solution Scattering Data. Rg analysis is not that convincing. In Fig. S1 , A and C one can see deviation from the linear fit as a function of concentration. It is not clear if those deviations are statistically significant. Please show deviation of linear fit from the data. If statistically significant, please indicate, if you have to correct low-q data for aggregation (for example, Cho and co-workers 1) and if not - then why.
Static model curves in Fig.2 A, D deviate from experimental data at higher concentrations. IsdB:oxyHb cannot be described well at low-q region at high concentrations by the model. Is this a sign of an aggregation? What does Molecular Weight estimation show? Interestingly, data for the same molecule in Fig.3 shows excellent fit.
Time-resolved Solution Scattering Data.
Fig.5. On the scale presented in Fig 5, it is not clear how large are differences between individual solution scattering curves.

Please provide direct overlay of several time data points, for example 50 ms, 1.3s and 24 s. Are those differences outside of the error bars?
Authors must be more convincing why they choose the kinetic model described in the text. Was Singular Vector Decomposition (SVD) done on the TRXSS data? How many major components the SVD analysis shows, what time constants can be extracted from the right SVD (time-related) components?
The authors discarded early data time-points from their analysis (10ms, 30 ms). Have they tried to extract an additional solution scattering pattern from these data? According to the kinetic model, the contribution from proposed IsdB:βmetHbtet disappears by 50 ms. Therefore, does this intermediate have to be included into the analysis of the time-resolved scattering data (50 ms and longer time-delays) at all?
According to the model 2IsdB:βmetHbtet → 1IsdB:βmetHbdim transition (dimerization) Kon, 2 is very slow compared to Kon, 1 and Kon, 3 . Do authors see any signature of 1IsdB:βmetHbdim intermediate in the residual scattering intensity of the fits? Can they correlate it with any of the theoretical constructs depicted in Fig.S2?
Time-resolved spectroscopy.
TR-OA spectroscopy (local heme environment) shows 2 intermediates. Assignment of one intermediate to the heme transfer from from apo-2IsdB: holo αβmetHbdim to holo-2IsdB: apo- αβmetHbdim is justifiable and agrees with previously reported in the literature. However, I did not understand what intermediate state is assigned to another major component found in SVD analysis of TR-OA data. Is there any structural state which authors associate with this TR-OA intermediate and why?
Overall, kinetic constants in the paper correlate with the ones published previously by Bowden and co-workers (ref. 6 in the paper) and Gianquinto and co-workers (ref. 7 in the paper). Bowden assigns their kinetic constants k1 to concentration dependent collision events which leads to IsdBN1N2·metHb complex formation, and k2, k3 and k4 with associated with steps in the heme transfer process after IsdBN1N2·metHb complex formation. k2 does not depend on the concentration, while kinetic constant, obtained from the global fit in the current work and compared to k2, r3 (Table S3) is strongly dependent on the concentration. Do they describe the same process and can be compared?

Molecular Dynamic Simulations.
Please provide a convergence parameter as a function of runs/time, if possible (for example, chi for TR-WAXS fits or similar). A visual representation, how calculated data were clustered and which cluster was chosen for the final model, would be helpful to a reader.
Minor Comments.
Figure 1. Please specify abbreviation RBC
Line 251. The difference scattering signal is presented but nor further discussion why structural changes at 0.08 Å-1 observed and what it can be associated with?
Table 1. Please provide a reference for a general audience, how dissociation constants were obtained.
Line 305. Please show TR-OA data (perhaps in Supplemental).
Fig. 6 Please provide holo and apo protein meanings for the general audience.
Line 547: TR-spectra are recorded in 0.05- 2.2 Å-1, compare to line 569 : data analysis is 0.01-1 Å-1
Supplement materials Figure S6. Explain Abbreviation SPR
Tables S1 and S2 are hard to read, perhaps authors should revise their presentation of the global fit coefficients data.

References:
The manuscript references previous literature adequately.
Clarity:
The manuscript is written in clear language, understandable to a general audience. Abstract, Introduction and Conclusions are appropriate.
The manuscript does not contain any inflammatory material or language.
Reviewer expertise: My expertise is in time-resolved X-ray solution scattering and supporting experiments (for example optical spectroscopy) and analysis of time-resolved and static X-ray solution scattering data, in particular for biomolecules in solutions. The topic of this manuscript - IdsB:Hb PPI interactions -does not align directly with the field of my expertise.
My assessment that the manuscript can be published in Nature Communications with revisions, which address above comments.
1. Cho, H. S. et al. Picosecond Photobiology: Watching a Signaling Protein Function in Real Time via Time-Resolved Small- and Wide-Angle X-ray Scattering. J. Am. Chem. Soc. 138, 8815–8823 (2016).

Irina Kosheleva
BioCARS
University of Chicago.

Reviewer #3

(Remarks to the Author)
In the manuscript entitled "Time-resolved X-ray solution scattering unveils the events leading to hemoglobin heme capture by staphylococcal IsdB," the authors explore the interaction between protein B of iron-regulated surface determinant (IsdB) from S. aureus and human hemoglobin (Hb). The research focuses on the molecular events leading to heme extraction from Hb, initiated by the formation of the IsdB:Hb complex. This study is primarily conducted through a combination of static and time-resolved X-ray solution scattering, along with WAXS-driven molecular dynamics (MD) simulations. While the system under investigation is relevant, certain points in the manuscript require improvement before it can be deemed acceptable for publication.

1) In reference to Figure 2, the interpretation of the SAXS patterns for IsdB:metHb is sufficiently convincing. When considering all the possible guessed arrangements present in solution, only the calculated curve of 2IsdB:Hb_dim demonstrates satisfactory agreement with the experimental curve (Fig. S3). However, for IsdB:oxyHb, the calculated curves of at least two arrangements exhibit a similar level of agreement with the experimental curve (Fig. S4), albeit both being significantly inferior to that observed for IsdB:metHb. It is worth noting, however, that the authors assert the presence of the IsdB:oxyHb complex in solution, primarily favoring a specific arrangement. This aspect requires further clarification in the text, as the rationale employed for interpreting the data of IsdB:metHb loses consistency when comparing IsdB:oxyHb.

2) Returning to Figure 2, as suggested by the authors, the observed dissociation constant of 0.38 ± 1.30 for IsdB:oxyHb might align with the Hb-dimer-tetramer dissociation constant (please report in the text the value published in Ref. 7). However, it is crucial to note that, owing to its high uncertainty, this value could also be consistent with the IsdB-Hb dissociation constant of 1.68 ± 0.62 observed for IsdB:metHb. Further clarification on this aspect is necessary to ensure a comprehensive understanding.

3) The experimental patterns were compared with the patterns calculated using CRYSOL. It raises the question of how the agreement would be if the patterns were calculated from unbiased MD simulations of the complexes in solution, explicitly considering the hydration shell.

4) Concerning Figure 5, there is a need for a clearer elucidation of the rationale behind the adoption of the kinetic model presented in panel c. Moreover, due to the high uncertainty, the fitting procedure had to start from 50 ms, a time point when the majority of kinetic processes had nearly reached equilibrium. This raises concerns about the significance of the values of the kinetic constants reported in Table 1. It is recommended to calculate the uncertainty of each constant for a more comprehensive evaluation.

5) The procedure used for generating TR-WAXS-driven trajectories is, in principle, correct. However, there are considerations to address. The restraint potential may lead the system to adopt spurious conformations if the structural change is too pronounced and the period for turning on the biased potential is too short. To enhance the robustness of the methodology, the authors should: a) compare the WAXS pattern of the biased simulation with the pattern generated from an unbiased simulation, b) calculate the root mean squared deviation of each residue with respect to a reference structure (e.g., unbiased), and c) repeat the TR-WAXS-driven MD simulation by incrementally increasing the WAXS-t-target N times to observe the consistency of the results.

Minor point:
Please provide clarification for the statement “This behavior is in line with the higher dynamics of α-chain with respect to β-chain in metHb” (page 13, lines 396-7).

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In the revised manuscript, the authors have effectively addressed the concerns regarding the lack of detailed structural discussion on the formation of the IsdB:Hb complex. They have provided comprehensive explanations of the structural mechanism for the formation of the complex in the results and discussion. Additionally, they have enhanced the readability of the manuscript by introducing the previous studies based on X-ray scattering methods in the introduction. Moreover, in the discussion, they have suggested that inhibition strategies need not focus solely on blocking the initial binding of IsdB to Hb β-chains but can also target its subsequent binding to Hb α-chains. This approach provides additional insights that could be of interest to future drug design studies. In contrast, the limitations in structural analysis based on SAXS data, caused by the presence of larger oligomeric states of the complex observable from the data, have not been resolved. Nevertheless, they have utilized various experimental techniques, including SAXS, WAXS, TR-XSS, TR-OA, and TR-F, to investigate the formation of the complex. The results obtained from these different experimental methods are consistent with each other, demonstrating that the proposed mechanism provides valuable insights into the formation of the IsdB:Hb complex. Given these considerations, I suggest that this manuscript is acceptable for publication in Nat. Commun. in its current form.

Reviewer #2

(Remarks to the Author)
The paper "Time-resolved X-ray solution scattering unveils the events leading to hemoglobin heme capture by staphylococcal IsdB" by O. Bei, M. Marchetti, S. Guglielmo, E. Gianquinto, F. Spyrakis, B. Campanini, S. Bettati, M. Levantino, L. Ronda presents a thorough multi-techniques study of proteinprotein interaction between Iron-regulated Surface Determinant (IsdB) protein and hemoglobin(Hb).
Important process of scavenging iron from hemoglobin is investigated. The scavenging of iron from human blood by infectious entity Staphylococcus aureus is a subject which presents an interest to a general scientific community and as such it is suitable for the Nature Communication audience.
Key Results. In the last years, significant efforts were applied to study the process of the heme cluster transfer from Hb to Isd complex. Several optical studies, sensitive to the local environment of the heme molecule were reported, along with a cryo-EM structure PDB ID 7PCF , and a crystallographic structure

PDB ID 5VMM of two stable complexes IsdB:Hb . The current work presents a first study of structural dynamics of scavenger IsdB molecule interaction with human blood molecule Hb. IsdB molecule was mixed with metHb in solution, and then the course of reaction was followed by taking X-Ray images of the reactants as function of the time delay after the mixing event. Time-resolved X-ray solution scattering (TRXSS) is capable of detecting reaction intermediates under physiological conditions albeit with lower resolution than crystallography. The authors combined static and time-resolved X-ray solution scattering with time-resolved optical absorption (TR-OA), fluorescence spectroscopy (TR-F) and molecular dynamic (MD) simulations to determine stoichiometry and course of reaction of IsdB:metHb complex after mixing event. They determined, that IsdB scavenger molecule after binding with tetramer metHb forms a 2IsdB:βmetHbtet intermediate and that the final product of the reaction is 2IsdB:αβmetHbdim dimer (heme cofactor is bound to both αβ chains of Hb), which forms approximately ~ 1 second after the mixing event . By using time-resolved optical methods authors show that heme cofactor transfer occurs after 2IsdB:αβmetHbdim formation from apo-2IsdB: holo αβmetHbdim to holo-2IsdB: apo- αβmetHbdim. Authors proposed a mechanism of dissociation of 2IsdB:βmetHbtet intermediate into 2 dimers IsdB:βmetHbdim and subsequent binding of IsdB scavenger molecule to the α-chain of metHb, thus forming the final molecule. Authors built the kinetic model of the process, determined kinetic constants of the model and proposed that intersubunit communications triggered by IsdB binding to β chains of IsdB:βmetHbdim make α subunits competent for IsdB binding. Further, time-resolved structural scattering signal difference was used as additional driving force for MD simulations. The simulations reveal that in the final process of heme scavenging (1.3 seconds and 24 seconds data) F-helix refolds in α-chains of Hb, but not in β-chains, supporting different dynamics of the two Hb subunits, which the authors proposed. Originality and Significance. The results reported in the paper are exciting, original, and novel. The dynamic process of how infectious entity Staphylococcus aureus scavenges Fe molecule for it's nutrients from Hb in human bloodstream is an important health and medical subject. Therefore, the topic is of interest not only for the specialized biomedical community, but also to a general audience from different disciplines.

My comments from the review of an original submission of the manuscript were addressed, I do not have other comments. The manuscript can be published.

Reviewer #3

(Remarks to the Author)
The reviewer wishes to recognize the authors for their efforts in conducting this study and for their responsiveness to reviewer comments. However, while acknowledging the relevance and significance of the study's topic, it is essential to recognize that the quality of research, encompassing data analysis, assumptions, and overall presentation, significantly impacts a paper's suitability for publication in a high-impact journal. Weaknesses or flaws in these aspects can detract from the study's overall appeal and scientific rigor. Therefore, it is imperative to address the critical concerns outlined below.

Major points:

1) The authors justified the choice of the 2IsdB:βHb_{tet} and 2IsdB model over the 2IsdB:Hb_{dim} model for IsdB:oxyHb complexes, arguing that the former model exhibits overall better agreement with the experimental data at higher q-values, even though the agreement at the lowest q-values is inferior. They attribute this discrepancy to the presence of a small contamination of oligomeric species larger than any of the IsdB:Hb models. However, this explanation is rather unconvincing and arbitrary, especially considering that excellent agreement was achieved for IsdB:metHb at any q-value range without invoking the presence of such contamination. Additionally, the reliance on previously published cryoEM results to resolve this question diminishes the novelty of the study, as the new experiments do not unambiguously confirm these findings but rather accept them as given. The reliance on cryoEM results to resolve this issue does indeed raise questions about the novelty and originality of the study, as it gives for granted previous findings rather than presenting novel insights into the system under investigation. It may be beneficial for the authors to provide additional analysis or experiments to support their choice of model and address the discrepancy in agreement between the different complexes.

2) The authors have provided the published K_D value of 0.25 ± 0.05 μM for the Hb tetramer-dimer dissociation of oxyHb as requested for comparison. However, it is questionable to directly substitute this value for the K_D of IsdB:oxyHb complexes, given the differences in systems and binding contexts. In fact, the binding of IsdB to Hb may potentially affect the dissociation constant, albeit slightly. Therefore, even though the K_D obtained for IsdB:oxyHb has a larger error (0.38 ± 1.30 μM), it should still be reported for comparison purposes. Reporting both K_D values allows for a more comprehensive assessment of the binding affinities and provides context for interpreting the results of the study. Additionally, the authors could discuss the potential impact of IsdB binding on the dissociation constant.

3) Despite the high uncertainty in estimating the k_{off} values due to the limited available time points, the authors opted to fix these k_{off} values to proceed with fitting the kinetic model. This procedure seems somewhat arbitrary and lacking smoothness, particularly considering the uncertainties affecting k_{off}. Furthermore, the authors continue to report equilibrium dissociation constants calculated as the ratio k_{off}/k_{on} in the text, despite the uncertainties surrounding k_{off}.
Additionally, the rationale behind adopting the kinetic model presented in Figure 5 panel C is insufficiently elucidated. The experimental points with reasonable uncertainty are available in panel B for only three species in solution (IsdB,

2IsdB:Hb_{dim}, 2IsdB:Hb_{tet}), and it remains unclear why the concentration of the intermediate 1IsdB:Hb_{dim} was not measured. Furthermore, it is ambiguous why the overall kinetic constants for step 1 of the kinetic model (IsdB binding to Hb_{tet}) are equivalent to the step-wise kinetic constants, and why step 2 (dimerization/activation) is considered virtually irreversible without introducing a corresponding k_{off} and equilibrium constant estimation.
Overall, there are several aspects of the kinetic modeling and parameter estimation that require further clarification and justification. The decision to fix k_{off} values despite high uncertainty warrants explanation, and the implications of this choice on the interpretation of dissociation constants should be addressed. Additionally, a clearer rationale for the adoption of the kinetic model and the treatment of intermediate species and irreversible steps is necessary to ensure rigor in the analysis. Providing additional justification and sensitivity analyses could help mitigate these concerns and enhance the credibility of these findings.

4) In WAXS-driven MD simulations, an additional energy term is added to the Hamiltonian to drive and achieve agreement between the WAXS curve calculated on-the-fly and the target experimental WAXS curve. The higher the disagreement between experimental and calculated WAXS curves, the larger the additional energetic term. During the WAXS-driven MD simulations, the evolution of the reduced chi-square was monitored as a function of simulation time, but no actual convergence behavior was observed (Fig S9). This lack of convergence is unusual and unexpected, as even if the structure were transient, it should be relatively stable.
Additionally, it can be noted that after rapidly decreasing, the reduced chi-square reached a minimum followed by a sudden and rapid increase, indicating a catastrophic event where the additional energetic term might have forced the system to sample spurious and unstable conformations that undermine the structure and architecture of the protein (Fig S9B).
Furthermore, spikes observed in some regions for the displacement of alpha carbons between structures extracted from the WAXS-driven simulation trajectory and the starting configurations indicate suspected system instability during the simulation (Fig. S9G-H).
It may be necessary for the authors to investigate the cause of these issues and consider alternative approaches or additional analyses to ensure the robustness and validity of their findings. Addressing these concerns is crucial to maintaining the credibility of the conclusions.

5) The study ultimately fails to provide structural insights that justify the specificity of IsdB for metHb over oxyHb. Despite the extensive analysis and experimental techniques employed, including time-resolved X-ray solution scattering and molecular dynamics simulations, the manuscript falls short in elucidating the underlying structural mechanisms responsible for the observed preference of IsdB for metHb.

Minor points:

1) The upper margin of panel D of Fig. 6 is cut.
2) Compared to the previous version of the manuscript, Fig. 3 includes fits performed with either CRYSOL or OLIGOMER without justification.

Without the resolution of these intrinsic critical concerns regarding data analysis, assumptions, and presentation, I cannot recommend the manuscript for publication in Nature Communications.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Considering the overall evidence and findings presented in this manuscript, the concerns related to local structural instabilities in the WAXS-driven MD simulations are not significantly relevant to the main arguments of the manuscript. Reviewer 3 raises concerns about potential oversights of local structural instabilities in the WAXS-driven MD simulations. Although I agree that these instabilities cannot be completely dismissed in the WAXS-driven MD simulations, the simulations were primarily aimed at evaluating whether oxyHb could act as a structural intermediate during the metHb binding and heme extraction by IsdB, not examining the specificity of IsdB for metHb versus oxyHb. Furthermore, the simulations provided additional insights by identifying structures that well describe the WAXS signal, aligning with their intended purpose. Additionally, the main objective of this paper is to elucidate the kinetics of the structural changes associated with the formation of the IsdB:Hb complex, using various experimental techniques like SAXS, WAXS, TR-XSS, TR-OA, and TR-F. The authors have successfully proposed a valuable mechanism for the kinetics, supporting the main focus of the manuscript. Therefore, I believe the manuscript would be publishable if the authors more clearly defined the purpose of the MD simulations within their text. The third reviewer's comments regarding MD simulations highlight aspects I have not previously considered, and it would be possible that readers might share the same concerns. To aid readers' understanding, it might be helpful to briefly mention, in a single sentence, the purpose of conducting MD simulations.

Reviewer #3

(Remarks to the Author)
1) The authors presented supplementary figures and new models to demonstrate the impact of contamination on their data. They used an ellipsoid model to better fit the experimental data, and this additional evidence appears to support their claim

about the presence of larger oligomeric species. While this approach seems reasonable, the lack of a clear structural rationale for this species remains a concern. Ideally, additional experimental methods could corroborate the presence and size of these oligomers.

Additionally, the authors' explanation that different stoichiometries can lead to varying propensities for oligomerization is plausible. However, this explanation would benefit from more detailed data or literature references supporting such behavior differences in similar systems.

In conclusion, the authors have provided a detailed response that partially addresses the concerns raised. Ideally, the authors might provide further validation to support their claims.

2) The authors presented an explanation and additional data to justify fixing the k_{off} values in their kinetic model. They clarified that these values were obtained from an initial fitting procedure and demonstrated through Supplementary Table 1 that varying k_{off} minimally affects the best-fit values of other parameters. This approach seems methodologically correct.

Considering the uncertainties in the equilibrium dissociation constants, it would be preferable to remove these values to avoid misleading interpretations.

For the reader's convenience, any reference to published data (e.g. from Ref. 7) should be explicitly reported to permit a quick comparison.

Finally, as recommended previously, the authors should provide a more detailed explanation of their model choices, particularly the rationale behind adopting the kinetic model presented in Figure 5, panel C. Specifically, why was the concentration of the intermediate 1IsdB:Hb_{dim}
not measured? Why are the overall kinetic constants for step 1 of the kinetic model (IsdB binding to Hb_{tet}) equivalent to the stepwise kinetic constants? Why is step 2 (dimerization/activation) considered virtually irreversible without introducing a corresponding k_{off} and equilibrium constant estimation?

3) The authors' emphasis on the stabilization of the total potential energy is not entirely convincing. Local structural changes, which might involve only a few kJ/mol, can be masked by the fluctuations in the total potential energy of the entire system (approximately -5.4e6 kJ/mol). Therefore, relying solely on the total potential energy to argue system stability may overlook significant local instabilities.

Moreover, it must be noted that an additional energy term is added to the Hamiltonian to drive the simulations towards the target. While this penalty term is intended to guide the system, the lack of convergence in the reduced chi-square is rather concerning. Typically, even in transient structures, some level of convergence should be observed.

The authors indicate that Fig. S9G-H may have been misinterpreted. However, the observed marked deviations between the WAXS-driven simulation structure and the unbiased structure might still suggest potential local instabilities.

The lack of convergence in the reduced chi-square raises concerns about the robustness and reliability of the WAXS-driven MD simulations in this context. Typically, some level of convergence or stability in the target curve/structure is expected in such simulations.

Given these concerns, I recommended that the authors conduct additional analyses to better understand the lack of convergence in the reduced chi-square. If these issues cannot be satisfactorily addressed, it may be prudent to reconsider the inclusion of the WAXS-driven MD simulation results in the manuscript to maintain the integrity and reliability of the reported findings.

Version 3:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The authors have made an effort to address the concerns raised in my previous review, yet several critical issues remain unresolved. My primary concern continues to be the lack of convergence in the WAXS-driven MD simulations. In these simulations, a penalty term is introduced into the Hamiltonian, designed to drive the system toward a target conformation that reproduces the experimental WAXS curve. The penalty term takes the form of a quadratic potential, proportional to a force constant and the difference between the calculated WAXS curve and the experimental one (typically measured through the chi-squared metric). Ideally, if the target conformation corresponds to a free energy minimum in the unbiased system, the penalty term should vanish as the system approaches the target, regardless of the value of the force constant. However, the fact that increasing the force constant leads to convergence only when unphysical distortions occur strongly suggests that no native conformation adequately reproduces the target WAXS curve. Instead, as the penalty term becomes dominant, the system is artificially driven into non-native states.

This interpretation explains why, with a moderate force constant, the system initially moves toward the target but then

diverges, failing to reach full convergence. This might occur because the bias introduced by the penalty term is eventually outweighed by the natural dynamics of the system, which drives it into an incorrect basin. Convergence is achieved only when the penalty term becomes overwhelmingly large, but this results in unphysical changes, such as the unfolding of stable secondary structure elements like the IsdB linker helices. This points to a deeper issue in the applicability of WAXS-driven MD for this system. In short, the fact that unphysical changes occur at higher force constants indicates that the experimental WAXS curve cannot be matched by a native conformation of the system.

Furthermore, the authors argue that the results of the simulations are reproducible, as repeated simulations yield consistent results. However, reproducibility alone is not a validation of the approach. Rather, it suggests that there might be a systematic error in the procedure. If the simulations are consistently driven toward non-native states, then repeating the simulations will likely yield similarly incorrect results. Therefore, this reproducibility should not be taken as evidence of robustness but rather as a potential sign of a systematic issue in the simulations.

The authors also emphasize that WAXS data are sensitive only to large-scale, concerted motions involving thousands of atoms, and that localized structural changes are not significant. While this is partially true, when the WAXS-driven simulations fail to find a native conformation that matches the experimental WAXS curve, the system often undergoes artificial reshaping to force a match. This reshaping frequently leads to local distortions, such as the unfolding of stable helices, as observed here. Thus, the fact that the penalty term is relatively small compared to the total energy of the system does not necessarily validate the results. Even a small bias can potentially lead to unphysical changes when the system is pushed to match a target that the native conformation cannot achieve.

Moreover, if the system is being repeatedly driven towards incorrect states, it becomes increasingly difficult to differentiate between legitimate structural changes and those that are artifacts of the simulation. The complexity of the system, coupled with the concerted motions of many atoms, makes this challenge even more pronounced. While the authors have clearly stated that the goal of the TR-WAXS-driven MD simulations was not to achieve atomic resolution, this does not mitigate the fact that the lack of convergence and the introduction of unphysical distortions cast doubt on the validity of the results. If the available WAXS data do not allow for convergence, then any conclusions drawn from these simulations must be treated with caution. As mentioned in Reviewer #1’s comments, the main goal of this study is to elucidate the kinetics of the structural changes involved in the formation of the IsdB complex. While the experimental data are sufficient to support the conclusions related to the kinetics, they are not accurate enough to validate structural changes at an atomic level using TR-WAXS-driven MD simulations.

For these reasons, I recommend removing the WAXS-driven MD simulation results from the manuscript to maintain the integrity and reliability of the findings. Excluding these results would not affect the main arguments or the significance of the paper, but would ensure that only the most robust and reliable data are presented.

Reviewer #1

Bei et al. conducted a study in which they aimed to elucidate the kinetics of the structural changes associated with the formation of IsdB:Hb complex. The authors achieved this using X-ray scattering techniques, including small-angle X-ray scattering (SAXS), wide-angle X-ray scattering (WAXS) and time-resolved X-ray solution scattering (TR–XSS). They performed SAXS/WAXS measurements of IsdB:metHb and IsdB:oxyHb complexes across various protein concentrations. By comparing the obtained SAXS/WAXS patterns and the candidate structural models, they obtained structural insights into the formation of IsdB:metHb and IsdB:oxyHb complexes. Notably, the authors suggested a kinetic model for the interaction between IsdB and Hb using TR–XSS combined with a rapid mixing triggering approach and the spectroscopic techniques, including time-resolved optical absorption (TR-OA) and fluorescence (TR-F) spectroscopy.

The authors have successfully presented a detailed kinetic model for the interaction between IsdB and Hb. This reviewer believes the kinetic information will provide important insights into understanding the physiological properties of the IsdB:Hb complex. However, the current manuscript lacks a detailed structural discussion regarding the formation of the complex.

We thank the reviewer for positively evaluating our work and stressing the relevance of our results for the understanding of the physiological properties of the IsdB:Hb complex.

In the revised version of the manuscript, we have expanded the description of the structural information that we have obtained. In particular, in the comments relative to Fig. 7, we have better described the structural changes observed at 1.3 and 24 s from mixing. As described in the text, these two time-delays are particularly relevant as they correspond to states in which essentially a single molecular species is populated (as our kinetic analyses show) and in that they are representative of the complex structure before (1.3 s) and after (24 s) heme extraction. As it is now better highlighted in the text, the observed structural changes are not only limited to a partial unfolding of F helices in Hb molecules (necessary to allow the breakage of the coordination bond between the proximal histidine and the heme iron), but also include several structural rearrangements of IsdB in the NEAT2 domain and the linker region. Concerning specifically the formation of the complex, although we provide evidence of the sequence of events (kinetic steps and stoichiometry) leading from isolated IsdB and Hb molecules to a stable complex, the main focus of the present work was not to investigate in detail the structure of the initial IsdB:metHb complex (formed in the tens of milliseconds). In view of the limited time resolution of our time-resolved WAXS measurements and the complexity of the biological reaction investigated (which involves the interaction between different proteins forming different types of complex states), we believe that further more detailed analyses would require more data at shorter time-delays with higher time-resolution in order to cope with a system in which several different molecular species are present simultaneously. Our data clearly yield crucial information on the IsdB:Hb complex, clarifying the stoichiometry of the interaction, elucidating important kinetic steps, and revealing structural rearrangements that are required for the heme extraction process to take place. In the revised version of the manuscript, we have tried to better clarify the main outcomes of our work and, in particular, we have better described the structural information that our data and our analysis provide.

Furthermore, this reviewer encountered challenges in identifying any novel analytical or experimental advancements in this manuscript. For example, in numerous studies employing X-ray solution techniques, the scattering data have been measured in both SAXS and WAXS regions (J. Phys. Chem. Lett., 8, 4413 (2017) and Photochem Photobiol Sci., 17, 874, (2018)). Moreover, several of these studies have applied various approaches such as MD simulations, rigid body modeling and ab-initio modeling to analyze the scattering data, yielding not only information about the global shapes of proteins but also about their local

shapes (Nature, 509, 245–248 (2014), Nat. Commun., 12, 3677 (2021), Sci. Adv., 2, e1600920 (2016)). Lastly, the TR–XSS-guided MD simulations have already been successfully applied to protein complexes in a study (Sci. Adv., 8, eabm6278 (2022)).

While we agree with the reviewer in that there are examples in the literature of the combined use of SAXS and WAXS or of the use of MD simulations to interpret time-resolved XSS data, this is the first time that all these approaches have been applied together to a rather sophisticated biological process involving several different intermediates and the formation of a protein complex between two different protein species. On top of that, this is the first time that TR-WAXS has been successfully used in combination with rapid mixing, which opens the path to the study of a broad range of other biological systems.

We have modified the text of the manuscript to better clarify the above point and have also added citations to the references mentioned by the reviewer. Nonetheless, we would like to stress that works #1 and #2 (J. Phys. Chem. Lett., 8, 4413, 2017 and Photochem Photobiol Sci., 17, 874, 2018) report TR-XSS data collected after a temperature jump. Works #3, #5 and #6 (Nature, 509, 245–248, 2014, Sci. Adv., 2, e1600920, 2016, and Sci. Adv., 8, eabm6278, 2022) present both TR-XSS data and static SAXS data collected at a dedicated beamline, but they deal with intramolecular motions of intrinsically photosensitive proteins (phytochromes). Work #4 (Nat. Commun., 12, 3677, 2021) focuses on photoinduced ultrafast structural changes of homodimeric hemoglobin. So, none of the above works presents TR-XSS combined with rapid mixing experiments to investigate the interaction of two distinct proteins.

Therefore, this reviewer cannot recommend the publication of this manuscript until the following severe concerns are fully addressed.

1. Although the authors mention that their work can contribute to the design of inhibitors for the interaction between IsdB and Hb, the manuscript lacks detailed structural discussions related to the interaction. Regarding this, this reviewer recommends that the authors provide comprehensive discussions about the following questions using the structures obtained from the structural analysis of SAXS/WAXS and TR–XSS data: (i) what structural changes will occur in IsdB and Hb during the formation of the IsdB:Hb complex. (ii) how the structural changes in IsdB and Hb lead to the formation of the IsdB:Hb complex. Such additional discussions would be beneficial for readers in understanding the process of complex formation.

As already mentioned above, our data indicate that the IsdB:Hb complex undergoes significant structural rearrangements both at the Hb and IsdB level associated with heme extraction and in agreement with other reports (Bowden et al., J. Biol. Chem. 293, 177, 2018; De Bei et al., Proc. Natl. Acad. Sci USA, 119, e2116708119, 2022). In particular, our WAXS-driven MD simulations allowed us to detect relevant motions and changes in the intrinsic dynamics of specific domains of IsdB. We have expanded the manuscript text to better describe the most relevant structural changes observed (see also previous related answer above).

Regarding the reviewer first comment about the importance of these analyses for the identification of new inhibitors, we would like to stress that such a complex interaction, made by subsequent binding, change of oligomerization state, and cofactors movements requires not only a knowledge of the molecular details of the final complex but also of the sequence of biological events and conformational changes occurring along the pathway leading to the final structure, where hemes are on the hemophores. Through the experiments reported here, we have been able to define the sequence of events carrying to the final stoichiometry of the complex and to recognize both Hb chains as possible targets for the design of interface inhibitors since the heme extraction process requires IsdB binding to both globin chains in a Hb dimer. We also characterized the subtle changes occurring in the relevant intermediate at 1.3 s by performing molecular dynamics (MD) simulations driven by the TR-WAXS signal, where the final complex is formed, but the hemes are still on Hb. All these pieces of information are of great interest and relevance in the perspective of identifying molecules able to interact with specific regions of Hb or IsdB, by means

of structure-based drug design, at different levels before heme extraction occurs. We have expanded the text in the revised version of the manuscript in order to better explain the above points.

2. In line 79 - 83, the authors mentioned, 'Although TR-XSS has been already used in combination with a stopped flow apparatus, all studies have been so far limited to the small-angle X-ray scattering (SAXS) region, which mainly yields information on the overall shape of the protein and its radius of gyration. We improved the structural resolution of this approach by collecting TR-XSS data in the wide-angle X-ray scattering (WAXS) region after rapidly mixing solutions of IsdB and metHb.' However, it should be noted that in several studies utilizing X-ray solution techniques with a stopped flow setup, the time-resolved scattering data have been measured not only in the SAXS regions but also in the WAXS region (for example, J. Phys. Chem. Lett., 8, 4413 (2017) and Photochem Photobiol Sci., 17, 874, (2018)).

We don't agree with the reviewer on this point. Indeed, as already noted above, the two mentioned works (J. Phys. Chem. Lett., 8, 4413, 2017 and Photochem Photobiol Sci., 17, 874, 2018) have not employed rapid mixing methods, but rather a laser-induced temperature jump.

Furthermore, some of these studies have applied various approaches, including molecular dynamics (MD) simulations, rigid body modeling and ab-initio modeling, to the scattering data, providing not only information about the global shape of proteins but also atomic models (or low-resolution structure) for their 3D conformations (Nature, 509, 245–248 (2014), Nat. Commun., 12, 3677 (2021), Sci. Adv., 2, e1600920 (2016)). Considering these, it is recommended that the authors revise the related content accordingly by introducing the previous studies.

We have added citations to these studies (as requested by the reviewer) as examples showing different approaches for the interpretation of TR-XSS data. However, as already stated above, we would like to stress that these studies are focused on systems containing in-solution species that do not change their oligomerization state during the kinetics. Obtaining detailed structural information on a sophisticated biological process like the one reported here is an important novelty of our work.

3. In line 86 - 90, the authors stated, 'In order to extract structural information from our experimental TR-XSS signals, we have also performed TR-XSS guided molecular dynamics (MD) simulations on the IsdB:Hb complex. We applied for the first time this approach to a protein complex, which couples the intrinsically low-resolution information from X-ray solution scattering with a priori physicochemical knowledge on the protein structure.'. Depending on how protein complexes are defined, this claim can be challenged by those who studied a protein complex made of multiple domains and a protein multimer (for example dimer) using the same set of techniques (Sci. Adv., 8, eabm6278 (2022)). Considering these, it is recommended that the authors revise the content mentioned above accordingly by properly introducing the previous studies.

We have better clarified in the text what we mean by "protein complex" and cited the work on homodimeric hemoglobin as suggested by the reviewer.

4. In Fig 2D, as the concentration of IsdB increases, it is observed that the difference between the experimental curves and the theoretical curves calculated form a linear combination of the structural models with various oligomer state, including 2IsdB:βoxyHbtet, 1IsdB:βoxyHbdim and IsdB, becomes more pronounced in the small-angle region. This difference raises the question for this reviewer whether, at higher concentrations of IsdB, complexes with larger oligomer states than those considered in the structural analysis might also form. In this regard, this reviewer recommends that the authors provide comprehensive discussions of the discrepancy between the experimental and theoretical curves.

We thank the reviewer for stimulating us to better clarify this important point. We have now better explained in the text that, indeed, we believe that a small fraction (few percent) of larger objects than the ones described in Fig. S2 are present in solution. We have modified Fig. S5 that now has 4 panels. In the new

panels A and B we demonstrate this point and estimate the approximate size of these objects. While the presence of larger oligomeric states could limit the information that can be extracted from the SAXS patterns, the strength of our approach is that we don't rely on SAXS data only, but rather exploit the high-resolution scattering data extending up to 1 $Å^{-1}$ to identify the most relevant oligomeric species in solution.

Reviewer #2

The paper "Time-resolved X-ray solution scattering unveils the events leading to hemoglobin heme capture by staphylococcal IsdB" by O. Bei, M. Marchetti, S. Guglielmo, E. Gianquinto, F. Spyrakis, B. Campanini, S. Bettati, M. Levantino, L. Ronda presents a thorough multi-techniques study of protein-protein interaction between Iron-regulated Surface Determinant (IsdB) protein and hemoglobin(Hb). Important process of scavenging iron from hemoglobin is investigated. The scavenging of iron from human blood by infectious entity Staphylococcus aureus is a subject which presents an interest to a general scientific community and as such it is suitable for the Nature Communication audience.

Key Results. In the last years, significant efforts were applied to study the process of the heme cluster transfer from Hb to Isd complex. Several optical studies, sensitive to the local environment of the heme molecule were reported, along with a cryo-EM structure PDB ID 7PCF , and a crystallographic structure PDB ID 5VMM of two stable complexes IsdB:Hb . The current work presents a first study of structural dynamics of scavenger IsdB molecule interaction with human blood molecule Hb. IsdB molecule was mixed with metHb in solution, and then the course of reaction was followed by taking X-Ray images of the reactants as function of the time delay after the mixing event. Time-resolved X-ray solution scattering (TRXSS) is capable of detecting reaction intermediates under physiological conditions albeit with lower resolution than crystallography. The authors combined static and time-resolved X-ray solution scattering with time-resolved optical absorption (TR-OA), fluorescence spectroscopy (TR-F) and molecular dynamic (MD) simulations to determine stoichiometry and course of reaction of IsdB:metHb complex after mixing event. They determined, that IsdB scavenger molecule after binding with tetramer metHb forms a 2IsdB:βmetHbtet intermediate and that the final product of the reaction is 2IsdB:αβmetHbdim dimer (heme cofactor is bound to both αβ chains of Hb), which forms approximately ~ 1 second after the mixing event . By using time-resolved optical methods authors show that heme cofactor transfer occurs after 2IsdB:αβmetHbdim formation from apo-2IsdB: holo αβmetHbdim to holo-2IsdB: apo- αβmetHbdim. Authors proposed a mechanism of dissociation of 2IsdB:βmetHbtet intermediate into 2 dimers IsdB:βmetHbdim and subsequent binding of IsdB scavenger molecule to the α-chain of metHb, thus forming the final molecule. Authors built the kinetic model of the process, determined kinetic constants of the model and proposed that intersubunit communications triggered by IsdB binding to β chains of IsdB:βmetHbdim make α subunits competent for IsdB binding. Further, time-resolved structural scattering signal difference was used as additional driving force for MD simulations. The simulations reveal that in the final process of heme scavenging (1.3 seconds and 24 seconds data) F-helix refolds in α-chains of Hb, but not in β-chains, supporting different dynamics of the two Hb subunits, which the authors proposed.

Originality and Significance. The results reported in the paper are exciting, original, and novel. The dynamic process of how infectious entity Staphylococcus aureus scavenges Fe molecule for it's nutrients from Hb in human bloodstream is an important health and medical subject. Therefore, the topic is of interest not only for the specialized biomedical community, but also to a general audience from different disciplines.

We thank the reviewer for his/her positive evaluation of the key results, originality, and significance of the manuscript and for pointing out that the work is of potential interest for a general audience from different disciplines.

Data and methodology. Validity of the results. The manuscript presents an outstanding example of experimental work in time-resolved structural biology. Using static SAXS/WAXS data combined with TRXSS and complimentary TR-OA and TR-F is a reliable approach both technically and methodologically. Time resolution, achieved in the experiment, is adequate to determine reported structural intermediates, formed in the reaction, and to determine kinetic constants.

However, I have problems with some aspects of analysis of static, and time-resolved solution scattering data.

We appreciate the very positive feedback from the reviewer on the adequacy of our experimental approach to investigate the structural dynamics of the IsdB:Hb interaction. In the revised version of the manuscript, we have addressed the reviewer's concerns regarding the data analysis (statistical analysis, treatment of data errors, MD simulations) point-by-point by performing additional analyses, providing more details on the analyses of the first submission, and/or by improving the figures/tables (see details below).

Statistical analysis. Statistical analysis should be improved. Data, presented on the graphs, and raw data attached to the manuscript do not show any error bars. Those should be present. Fits of the time-resolved solution scattering data in Fig. 5A should have fit errors, for example, like the fits of the static solution scattering data with theoretical constructs (Fig. S3, S4).

Following the reviewer's recommendation, we have dedicated particular attention to improving the presentation of data and associated errors in the revised version of the manuscript. We have made sure that all data files made available for readers contain the associated errors. We have better clarified throughout the text what parameters have been estimated through a fit and reported best-fit values with the associated fitting error (see e.g. revised Table 1) or reduced chi-square values when relevant. We have added error bars to data reported in Fig. 5B and clearly stated that errors are smaller than the symbol size in the case of Fig. 2, 3 and 5A. In the case of spectroscopic data (Fig. 6), we provided further information on the error in the Figure legend and the Materials and Methods section.

Static Solution Scattering Data. Rg analysis is not that convincing. In Fig. S1 , A and C one can see deviation from the linear fit as a function of concentration. It is not clear if those deviations are statistically significant. Please show deviation of linear fit from the data. If statistically significant, please indicate, if you have to correct low-q data for aggregation (for example, Cho and co-workers 1) and if not - then why. Static model curves in Fig.2 A, D deviate from experimental data at higher concentrations. IsdB:oxyHb cannot be described well at low-q region at high concentrations by the model. Is this a sign of an aggregation? What does Molecular Weight estimation show? Interestingly, data for the same molecule in Fig.3 shows an excellent fit.

The goal of the Guinier analysis reported in Fig. S1 was indeed not that of convincing the reader of the reliability of our Rg estimation. Rather, the main goal was to show that Rg values, both in the case of IsdB:metHb and IsdB:oxyHb, depend on concentration and this is due to an equilibrium that shifts towards larger objects in solution as the total protein concentration increases (as described in the text). The values reported in Fig. S1B and S1D should thus be taken as apparent Rg values. We acknowledge that this was not clear in the text of the manuscript and supplementary information that we have now modified accordingly.

Concerning the deviations at the lowest q-values, they are statistically significant (see new panel E in the revised Fig. S1). However, the correction of low-q data as the one done by Cho and coworkers (J. Am. Chem. Soc. 138, 8815–8823, 2016) cannot be applied in our case. Indeed, such analysis is based on the assumption that there is a single species of molecules in solution and that any deviation in the low-q region is the result of the interactions between identical molecules. If the above assumptions are correct, scattering patterns at any concentration can be reproduced as the product of a "form factor" and a concentration-dependent "structure factor" (see e.g. Bonette et al., J. Cryst. Growth 196 403-414, 1999), which can be directly estimated from the SAXS concentration series. As demonstrated by the new supplementary figure Fig. S1, panel F, the differences in the IsdB:Hb patterns reported in our work cannot be simply described as the result of the structure factor contribution and this further demonstrates that the assumption of a single molecular species in solution would not be correct.

The origin of the misfit at the lowest q-value and highest concentration rather indicates that a small fraction of objects significantly larger than the ones discussed in the text are present in our samples (as already pointed out in the answer to Reviewer #1). These objects are not large aggregates (which would result in a much larger increase of the signal at the lowest q-values), are not X-ray radiation-induced (as we have obtained identical patterns by reducing the exposure time by a factor 2 or 4), and do not correspond to any of the structural models reported in Fig. S2. Stimulated by the reviewer's comment (and similar comment by Reviewer #1, see above), we have performed further analyses with the goal of estimating the size of these objects. The results of these analyses are reported in the new Fig. S5 to which two new panels have been added. The new panel A of Fig. S5 shows that the description of the IsdB:oxyHb SAXS data at low-q values and high protein concentration (186.9 μM) in terms of a linear combination of the 1IsdB:Hb_{dim} and the 2IsdB:Hb_{tet} contributions (as in the main text) is not improved even if we assume that the largest possible IsdB:Hb complex (4IsdB:Hb_{tet}, which is unlikely to form in solution because of steric hindrance between adjacent IsdB molecules) is present in solution. As a second check, we have estimated the average size of objects that could give rise to the missing contribution by performing a Guinier analysis of the residual data (the difference between the experimental curve and the corresponding OLIGOMER fit reported in Fig. 2D of the main text). The result, which is reported in new panel B of Fig. S5, shows that the average size of objects that are not taken into account in the analysis reported in the main text is of the order of 90 Å (the fitted radius of gyration is 86 +/- 3 Å). This kind of radius of gyration is large even with respect to the 4IsdB:Hb_{tet} (Rg~40 Å), but not big enough to correspond to a large aggregate. Although this analysis does not demonstrate which objects are indeed present in solution, it strongly suggests that we are dealing with a small fraction of oligomers composed of either 2-3 complexes or of a small number of IsdB/Hb molecules.

Based on the above arguments, we believe that in our samples at the highest protein concentration, there is a small contamination of oligomeric species. As these species are relatively big with respect to any other species present in solution, their fraction must be rather small (few percent), otherwise the SAXS signal would be dominated by their contribution, which is clearly not the case. In line with the above, the combined SAXS/WAXS data reported in Fig. 3 were obtained by merging the SAXS data measured at an intermediate protein concentration (3.049 mg/mL in the case of IsdB:metHb and 2.117 mg/mL for IsdB:oxyHb) with WAXS data measured at higher protein concentration (16.355 mg/ml for IsdB:metHb and 14.454 mg/mL for IsdB:oxyHb), after normalization for the different concentrations. Although, in principle, it would have been best to measure the WAXS patterns at the same concentration of SAXS data (2-3 mg/mL), this is practically not possible as the signal in the WAXS region is orders of magnitude smaller than that in the SAXS region. The rather good agreement of the scattering patterns calculated from our hypothesized IsdB:Hb complex structures with SAXS/WAXS data extending over a rather large q-range (up to 1 $Å^{-1}$) demonstrates that these structures are indeed representative of the vast majority of molecules in solution.

Time-resolved Solution Scattering Data. Fig.5. On the scale presented in Fig 5, it is not clear how large are differences between individual solution scattering curves. Please provide direct overlay of several time data points, for example 50 ms, 1.3s and 24 s. Are those differences outside of the error bars?
Authors must be more convincing why they choose the kinetic model described in the text. Was Singular Vector Decomposition (SVD) done on the TRXSS data? How many major components the SVD analysis shows, what time constants can be extracted from the right SVD (time-related) components?
According to the kinetic model, the contribution from proposed IsdB:βmetHbtet disappears by 50 ms. Therefore, does this intermediate have to be included into the analysis of the time-resolved scattering data (50 ms and longer time-delays) at all?

The evolution of TR-XSS patterns can be better evaluated from the difference patterns reported in Fig. 4C. The signal changes are above the errors (now reported in the data repository deposited files) which, as stated above, are of the order of the symbol size in Fig. 5A. As far as the kinetic model is concerned, the referee is right that error bars are necessary to judge how convincing the choice of our kinetic model is. We have added them in the revised Fig. 5B.

The proposed kinetic model has been chosen as the simplest scenario based on the evidence obtained from static XSS data: 1) in the case of IsdB:oxyHb, IsdB binds to the β-subunits of the Hb tetramer; it is thus reasonable to assume that such state is also an intermediate in the case of IsdB-metHb interaction; 2) our static XSS data shows that the final IsdB:metHb complex is made by an Hb dimer bound to two IsdB molecules; this implies that Hb dimerization must occur in order to account for the observed stoichiometry.

An SVD analysis of the data was indeed done and it yields 3 significant components (basis patterns) that we report now in the Supplementary Information (Fig. S13). We have now also performed a kinetic analysis of the right-singular vectors (V columns) and verified that at least 3 exponentials are needed. A fit obtained using the same exponential rate constants (0.18, 2.8 and 180 s^{-1}) for all the first three SVD right-singular vectors is reported in Fig. S13 panels B, C and D. The obtained values overlap well with the rate constants resulting from the global fit in terms of the kinetic model reported in Fig. 5C and with the rate constants estimated from the combined global fit of TR-WAXS, TR-OA and TR-F data (Table S1). One point that warrants further clarification is that the TR-WAXS data decomposition reported in Fig. 5B (closed circles) does not include the 1IsdB:βmetHb$_{tet}$ which disappears by 50 ms. Indeed, the closed circles in Fig. 5B are the concentrations obtained from the analysis of the TR-WAXS patterns as a linear combination of only 4 species: IsdB, Hbtet, 2IsdB:Hbtet, and 2IsdB:Hbdim, corresponding to 3 different states (isolated IsdB and tetrameric Hb, 2IsdB:Hbtet and 2IsdB:Hbdim). From the kinetic model point of view, once the above 4 species are assumed to get populated, then also the 1IsdB:Hbtet and 1IsdB:Hbdim species must necessarily be included and that is why their concentration (simulated through SymBiology) is reported in Fig. 5B (continuous lines).

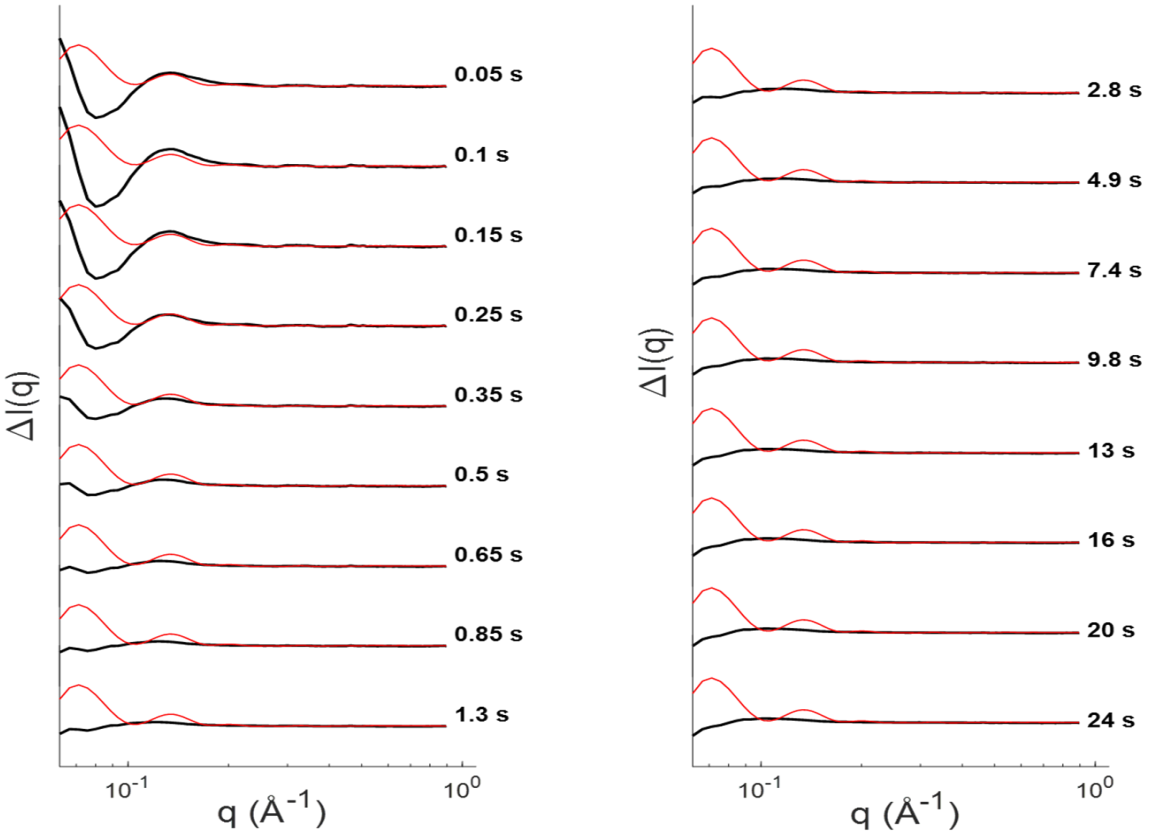
The authors discarded early data time-points from their analysis (10ms, 30 ms). Have they tried to extract an additional solution scattering pattern from these data?

The data at the earliest time-points have not been used in our analysis, since the analysis is based on the use of experimental static WAXS patterns and no equilibrium WAXS pattern representative of the intermediate species formed in the tens of ms timescale was available. In principle, the early time-points could be exploited by performing an analysis similar to the one reported by Cammarata et al. (J. Mol. Biol. 400, 951, 2010) or Levantino et al. (PNAS, 109, 14894, 2012) to investigate the structural changes induced by photolysis of carbonmonoxide hemoglobin. In this kind of approach, it is possible to analyze a TR-WAXS dataset as a linear combination of basis patterns extracted directly from the data assuming a well-defined kinetic model. In view of the limited time-resolution of our TR-XSS experiments, which allowed us to retrieve only few time points before the formation of the IsdB:Hb complex, and taking into account the complexity of the systems investigated (involving different proteins and different molecular complexes in solution), we believe that our data do not allow us to employ reliably such kind of approach, which anyway requires to make *a priori* assumptions on the correspondence between the concentration of molecular species in solution and the basis patterns. Further investigations with higher time resolution could potentially yield enough TR-WAXS patterns at shorter time-delays to justify a more sophisticated analysis approach. This however goes beyond the scope of the present work.

According to the model 2IsdB:βmetHbtet → 1IsdB:βmetHbdim transition (dimerization) $k_{on,2}$ is very slow compared to $k_{on,1}$ and $k_{on,3}$. Do authors see any signature of 1IsdB:βmetHbdim intermediate in the residual scattering intensity of the fits? Can they correlate it with any of the theoretical constructs depicted in Fig.S2?

Based on the $k_{on,1}$, $k_{on,2}$ and $k_{on,3}$ values, we do not expect 1IsdB:βmetHbdim accumulation, as also shown in Fig. 6C (gray line). Testing directly the reviewer's hypothesis is not trivial as obtaining an experimental reference WAXS pattern of the 1IsdB:βmetHbdim species would be extremely challenging. Nevertheless, in order to allow the reviewer to better judge our claim, we have compared the residual intensity (difference

between experimental and reconstructed patterns in Fig. 5A) at all time-delays with the difference between the calculated pattern of 2IsdB:Hbdim (calculated pattern in Fig. S3B) and that of 1IsdB:βmetHbdim (calculated pattern in Fig. S3H). As can be judged by the figure reported below, the subtle differences between the experimental traces and their corresponding reconstructions (black lines, highly magnified here for clarity) cannot be explained as the formation of the 1IsdB:βmetHbdim intermediate (red lines) at any time-delay.



Time-resolved spectroscopy. TR-OA spectroscopy (local heme environment) shows 2 intermediates. Assignment of one intermediate to the heme transfer from apo-2IsdB: holo αβmetHbdim to holo-2IsdB: apo- αβmetHbdim is justifiable and agrees with previously reported in the literature. However, I did not understand what intermediate state is assigned to another major component found in SVD analysis of TR-OA data. Is there any structural state which authors associate with this TR-OA intermediate and why?

In TR-OA our probe is the heme, which reports changes in its coordination state and environment. While the attribution of the first TR-OA component was more straightforward and was assigned as mainly arising from the heme transfer, the second TR-OA component attribution is less trivial. We observed that its amplitude starts to vary with the first and second IsdB binding and, upon final complex formation, its signal shows a partial reversion which is kinetically coupled to the heme transfer. Since we know that 1) during IsdB binding NEAT2 domain approaches the heme pocket for promoting the transfer, and 2) the comparison between structures upon WAXS-driven MD at 1.3 and 24 s highlights NEAT2 detachment from Hb heme pocket upon heme extraction, we tentatively assign this component to the distance between Hb and NEAT2 sensed by the heme. We have mentioned this tentative assignment in the revised version of the text.

Overall, kinetic constants in the paper correlate with the ones published previously by Bowden and co-workers (ref. 6 in the paper) and Gianquinto and co-workers (ref. 7 in the paper). Bowden assigns their kinetic constants k1 to concentration dependent collision events which leads to IsdBN1N2·metHb complex formation, and k2, k3 and k4 with associated with steps in the heme transfer process after IsdBN1N2·metHb complex formation. k2 does not depend on the concentration, while kinetic constant, obtained from the global fit in the current work and compared to k2, r3 (Table S3) is strongly dependent on the concentration. Do they describe the same process and can be compared?

The kinetic constants reported by Bowden et al. (J. Biol. Chem. 293, 177, 2018) were obtained from the analysis of absorption changes at a single wavelength (406 nm) at which several intermediate species may contribute (the SVD analysis of our data reported in Fig. S7 shows indeed that at least two meaningful components are present). Moreover, the data reported in Figure S2 of the same paper shows that a concentration dependence of k_2 cannot be excluded based on those data. Within the limits of the comparison between experiments performed in quite different conditions, there is an overall reasonably good correlation with previously published data (as the reviewer points out) and we do not see any strong evidence suggesting that these processes are different than those reported in that paper or by Gianquinto et al. (Sci Rep 9, 18629, 2019).

Molecular Dynamic Simulations. Please provide a convergence parameter as a function of runs/time, if possible (for example, chi for TR-WAXS fits or similar). A visual representation, how calculated data were clustered and which cluster was chosen for the final model, would be helpful to a reader.

In view of the reviewer's comment and of analogous comments and requests from Reviewer #3, we have repeated our MD simulations and performed them at different values of the *WAXS-t-target* parameter (see Reviewer #3's report and corresponding answers), for a total amount of 200 ns. As correctly suggested by the reviewer, we calculated the reduced chi-square and used it for comparing computed WAXS profiles with experimental ones. The evolution of the reduced chi-square was monitored as a function of the simulation time, as now reported in Fig. S9, panels A and B. Although we could not observe an actual convergence behavior, which is not unexpected since we are dealing with transient structures, the identification of minima regions of the reduced chi-square parameter allowed us to select representative structures with the best accordance to the experimental data. Based on the trend of the reduced chi-square, we selected a trajectory interval for each system (highlighted in red in Fig. S9, panels A and B); from this ensemble of structures, the representative ones were extracted after clustering using the Gromos clustering algorithm, as the medoid of the most populated cluster. Each cluster was obtained based on an alpha carbons RMSD cuf-off of 1.5 Å.
A visual representation of clustering and how a specific cluster was chosen is not trivial. As this was anyway obtained using standard methods (Gromos clustering algorithm available in GROMACS), we believe that adding a better explanation and reference to the GROMACS package is enough to properly clarify the employed methodology to the reader.
We have updated Figure 7 accordingly, highlighting a slightly different behavior of IsdB NEAT2 and the last part of the linker with respect to the previous representation. Even if we still clearly observe the detachment of NEAT2 from hemoglobin, the roto-translation occurs following a different vector, as highlighted in Fig. 7 panels C and E. With the aim of presenting the most representative structure of the trajectories, in the first version we only based our analysis on the clustering according to gromos standard procedure; here, having significantly extended the simulations, we have been able to observe regions of trajectories more in agreement with the experimental data (see Fig. S9 panels A and B). We indeed believe this might be a more rigorous and reliable approach, in particular in quite complicated and large systems like the one here investigated.

Minor Comments:

- Figure 1. Please specify abbreviation RBC
  We have specified the abbreviation in the figure legend.

- Line 251. The difference scattering signal is presented but nor further discussion why structural changes at 0.08 $Å^{-1}$ observed and what it can be associated with?
  As we have now explained in the text, although it is not possible to exactly pinpoint signal changes at a given q-value to one specific structural change (several processes contribute to the overall shape of the WAXS pattern at the same time), changes at 0.08 $Å^{-1}$ are typically due to changes in

the overall shape of a protein or to interactions with other proteins, while changes at 0.5 and 0.65 $Å^{-1}$ are typically arising from changes in the relative position of secondary structure elements (e.g. helices).

- Table 1. Please provide a reference for a general audience, how dissociation constants were obtained.
  We have clarified in the main text how dissociation constants were calculated. The values are now reported in the main text and not anymore in Table 1 as they are not fitting parameters.

- Line 305. Please show TR-OA data (perhaps in Supplemental).
  TR-OA data are now reported in the first panel of Fig. S7, panel A.

- Fig. 6 Please provide holo and apo protein meanings for the general audience.
  We have defined the meaning of holo and apo protein at its first occurrence (Small Angle X-ray Scattering section).

- Line 547: TR-spectra are recorded in 0.05- 2.2 Å-1, compare to line 569 : data analysis is 0.01-1 Å-1
  We thank the reviewer for spotting this typo, which we have corrected.

- Supplement materials Figure S6. Explain Abbreviation SPR
  We have added the definition of the abbreviation in the figure legend.

- Tables S1 and S2 are hard to read, perhaps authors should revise their presentation of the global fit coefficients data.
  We agree with the reviewer that Tables S1 and S2 were not easy to interpret. We have prepared completely new tables removing color codes and reporting amplitude values rather than percentages. Also, offset values were added for completeness.

References. The manuscript references previous literature adequately.

Clarity. The manuscript is written in clear language, understandable to a general audience. Abstract, Introduction and Conclusions are appropriate. The manuscript does not contain any inflammatory material or language. Reviewer expertise: My expertise is in time-resolved X-ray solution scattering and supporting experiments (for example optical spectroscopy) and analysis of time-resolved and static X-ray solution scattering data, in particular for biomolecules in solutions. The topic of this manuscript - IdsB:Hb PPI interactions -does not align directly with the field of my expertise.
My assessment that the manuscript can be published in Nature Communications with revisions, which address above comments.
1. Cho, H. S. et al. Picosecond Photobiology: Watching a Signaling Protein Function in Real Time via Time-Resolved Small- and Wide-Angle X-ray Scattering. J. Am. Chem. Soc. 138, 8815–8823 (216).

We thank the reviewer for his/her in-depth assessment of our work and for stimulating us to revise several relevant points.

Reviewer #3

In the manuscript entitled "Time-resolved X-ray solution scattering unveils the events leading to hemoglobin heme capture by staphylococcal IsdB," the authors explore the interaction between protein B of iron-regulated surface determinant (IsdB) from S. aureus and human hemoglobin (Hb). The research focuses on the molecular events leading to heme extraction from Hb, initiated by the formation of the IsdB:Hb complex. This study is primarily conducted through a combination of static and time-resolved X-ray solution scattering, along with WAXS-driven molecular dynamics (MD) simulations. While the system under investigation is relevant, certain points in the manuscript require improvement before it can be deemed acceptable for publication.

1) In reference to Figure 2, the interpretation of the SAXS patterns for IsdB:metHb is sufficiently convincing. When considering all the possible guessed arrangements present in solution, only the calculated curve of 2IsdB:Hb_dim demonstrates satisfactory agreement with the experimental curve (Fig. S3). However, for IsdB:oxyHb, the calculated curves of at least two arrangements exhibit a similar level of agreement with the experimental curve (Fig. S4), albeit both being significantly inferior to that observed for IsdB:metHb. It is worth noting, however, that the authors assert the presence of the IsdB:oxyHb complex in solution, primarily favoring a specific arrangement. This aspect requires further clarification in the text, as the rationale employed for interpreting the data of IsdB:metHb loses consistency when comparing IsdB:oxyHb.

The reviewer highlights here a relevant point. It is indeed true that, in the case of IsdB:oxyHb complexes, the calculated pattern from the 2IsdB:Hb_{dim} model (Fig. S4B) gives a similar agreement to the data as the calculated pattern obtained assuming that the species in solution are 2IsdB:βHb_{tet} and 2IsdB (Fig. S4F). The reason we favor the second model is two-fold: 1) cryoEM data on IsdB:HbCO complexes show that 2IsdB:βHb_{tet} complexes are formed; although cryoEM data on IsdB:oxyHb are not available, it is reasonable to assume that complexes involving ligated Hb species should form the same oligomeric complex state; 2) for the reasons already discussed in the answers to the first 2 reviewers (presence of a contamination of oligomeric species in solution, see above and revised manuscript), it would be misleading to base our structural attribution on the SAXS data at the lowest q-values, which has a big impact on the χ^2 parameter reported in those figures; the agreement at intermediate and large q-values is clearly better in the case of Fig. S4F than in that of Fig. S4B and this indicates that the 2IsdB: βHb_{tet} better describes the experimental data at higher structural resolution. Following the reviewer's suggestion, we have modified the text to better explain/justify our structural attribution of the IsdB:oxyHb complexes.

2) Returning to Figure 2, as suggested by the authors, the observed dissociation constant of 0.38 ± 1.30 for IsdB:oxyHb might align with the Hb-dimer-tetramer dissociation constant (please report in the text the value published in Ref. 7). However, it is crucial to note that, owing to its high uncertainty, this value could also be consistent with the IsdB-Hb dissociation constant of 1.68 ± 0.62 observed for IsdB:metHb. Further clarification on this aspect is necessary to ensure a comprehensive understanding.

We thank the reviewer for motivating us to improve the clarity of these aspects. We have now reported in the text the published K_D = 0.25 ± 0.05 μM for the Hb tetramer-dimer dissociation of oxyHb (Gianquinto et al., Sci Rep 9, 18629, 2019) as requested. Moreover, to avoid any ambiguity/misinterpretation, we have modified Fig. 2F and used also in this case (as in the case of the IsdB:metHb data in Fig. 2C) molar concentration expressed on a heme basis in the horizontal axes of the plot. Incidentally, we have also corrected a mistake in Fig. 2C: in the previous version of Fig. 2C, the fraction corresponding to an IsdB:metHb molar concentration of 30.2 μM (in heme) was erroneously reported as that at 12.7 μM (two different fraction values were thus reported for the same protein concentration by mistake).

We concur with the reviewer on the substantial uncertainty associated with the fitting in Fig. 2F: we have removed the fitting of the data in Fig. 2F and replaced it with the theoretical prediction (Eq. 1 in the

Supplementary Information) obtained using the published value of the Hb tetramer-dimer dissociation constant in the absence of IsdB (0.25 μM). However, we don't agree with the reviewer's suggestion that the data in Fig. 2D could be potentially compatible with an IsdB dissociation from Hb of 1.68 μM as in the case of IsdB:metHb data. Indeed, while heme extraction occurs in IsdB:metHb complexes and lowers the affinity of IsdB for Hb (corresponding to an increase of K_D), the same process does not occur for IsdB:oxyHb complexes. Although we cannot exclude IsdB dissociation from oxyHb with a K_D << 1.68 μM, this process is unlikely to occur with a K_D value comparable or higher than that reported for oxyHb tetramer-to-dimer dissociation.

3) The experimental patterns were compared with the patterns calculated using CRYSOL. It raises the question of how the agreement would be if the patterns were calculated from unbiased MD simulations of the complexes in solution, explicitly considering the hydration shell.

In order to check the specific point raised by the reviewer, we have compared results obtained with CRYSOL and with WAXSiS. The latter software computes unbiased restrained MD simulations and treats the hydration shell contribution explicitly. The comparison reported in the Supplementary Information (Fig. S11), demonstrates that the two approaches yield similar results in our case. We have decided to use CRYSOL mainly to be able to compare it more directly with results obtained with OLIGOMER (a software from the same package). In any case, we thank the reviewer for giving us the opportunity to show that our data interpretation is confirmed even when the hydration shell contribution is explicitly taken into account.

4) Concerning Figure 5, there is a need for a clearer elucidation of the rationale behind the adoption of the kinetic model presented in panel c. Moreover, due to the high uncertainty, the fitting procedure had to start from 50 ms, a time point when the majority of kinetic processes had nearly reached equilibrium. This raises concerns about the significance of the values of the kinetic constants reported in Table 1. It is recommended to calculate the uncertainty of each constant for a more comprehensive evaluation.

The reviewer is right that further clarification is needed to explain our approach and clearly assess its limits and points of strength. As the reviewer notes, we have high uncertainty in the estimation of the k_{off} values in view of the available time points in our analysis. We have clearly stated in the revised version of the Materials and Methods section, that k_{off} values are not well determined in our fits. For this reason, we have decided to fix their values to $1x10^{-2}$ and $0.5x10^{-2}$ s^{-1} (values obtained during the first fitting run) in the last fitting run. We note however that the other parameters are much better determined as demonstrated by the errors on the best fit values which are reported in the revised version of Table 1. Moreover, we have verified that even a change of $k_{off,1}$ and $k_{off,3}$ by a factor of 3 does not affect the best fit values obtained for the other parameters.

Coming back to the values of $k_{off,1}$ and $k_{off,3}$, we note that the obtained values are reasonable in that they are of the same order of magnitude of the IsdB dissociation from Hb β-subunits ($3.7x10^{-2}$ s^{-1}) obtained by Gianquinto et al. (Sci Rep 9, 18629, 2019) through SPR measurements. A further point which comforts us in the validity of our estimated rate is the comparison with results obtained from spectroscopic data by Bowden et al. (Biol. Chem. 293, 177, 2018). Although these authors have performed their experiments in quite different conditions (in particular, much lower protein concentration), by using our estimated kinetic constants we can reproduce kinetic evolutions close to those reported by Bowden et al. The corresponding simulated time traces are reported in Fig. S10 (Fig. S9 in the original version of the Supplementary Information) and a comparison of the rates reported by Bowden et al. with apparent rates extrapolated from our data is in Table S3.

5) The procedure used for generating TR-WAXS-driven trajectories is, in principle, correct. However, there are considerations to address. The restraint potential may lead the system to adopt spurious conformations if the structural change is too pronounced and the period for turning on the biased potential is too short. To enhance the robustness of the methodology, the authors should: a) compare the WAXS pattern of the biased simulation with the pattern generated from an unbiased simulation, b) calculate the

root mean squared deviation of each residue with respect to a reference structure (e.g., unbiased), and c) repeat the TR-WAXS-driven MD simulation by incrementally increasing the WAXS-t-target N times to observe the consistency of the results.

We thank the reviewer for stimulating us to add further tests and to more exhaustively present WAXS-driven MD simulations. As already mentioned in the answer to Reviewer #2, we have performed new simulations (40 ns long) using different values of the WAXS-t-target (2000, 4000, 6000, 8000 and 10000 ps) as requested by the reviewer (point c). All the simulations proved to be stable, and in no cases we observed inconsistent structural changes. With the aim of presenting the most representative structure of the trajectories, in the first version we only based our analysis on the clustering according to gromos standard procedure; here, having significantly extended the simulations, we have been able to observe regions of trajectories more in agreement with the experimental data (see Fig. S9 panels A and B). Indeed, we believe this might be a more rigorous and reliable approach, especially in rather complicated and large systems such as the one investigated here. We have updated Fig. 7 which shows an overall similar behavior of the system with respect to previous analysis, with slight changes in the detaching motion of IsdB NEAT2 from hemoglobin.

The patterns calculated from the WAXS-driven simulations are now compared with unbiased simulations in Fig. S9, panels E and F (point a of the reviewer's comment). Finally, the displacement of alpha carbons for each residue between structures extracted from the WAXS-driven simulation trajectory and the starting configurations (structure selected through clustering analysis of the preparatory unbiased simulation trajectory) were calculated and are now reported in Fig. S9, panels G and H (point b of the reviewer's comment); as a global metric the RMSD values between WAXS-driven and unbiased structures were also calculated over alpha carbons and are 5.4 and 6.5 Angstrom for 1.3 s and 24 s delay respectively.

As already stated, the new simulations are in overall agreement with the previous ones. We believe that the new analysis and new plots allow the reader to better evaluate the quality of the employed methodology and the most relevant structural changes that can be extracted from WAXS-driven simulations.

Minor point:
Please provide clarification for the statement "This behavior is in line with the higher dynamics of α-chain with respect to β-chain in metHb" (page 13, lines 396-7).

We have more clearly restated this sentence - which was indeed too synthetic - as follows:

"This behavior is not surprising as it is known that β-chains of metHb have larger equilibrium fluctuations of atoms around their equilibrium position than α-chains, as shown by hydrogen/deuterium exchange mass spectroscopy measurements[49]."

Response to reviewers' comments

Reviewers #1 and #2 did not raise any additional comments in the second review round. In the following, point-to-point answers to reviewer #3 comments are reported in blue.

Reviewer #3

The reviewer wishes to recognize the authors for their efforts in conducting this study and for their responsiveness to reviewer comments. However, while acknowledging the relevance and significance of the study's topic, it is essential to recognize that the quality of research, encompassing data analysis, assumptions, and overall presentation, significantly impacts a paper's suitability for publication in a high-impact journal. Weaknesses or flaws in these aspects can detract from the study's overall appeal and scientific rigor. Therefore, it is imperative to address the critical concerns outlined below.

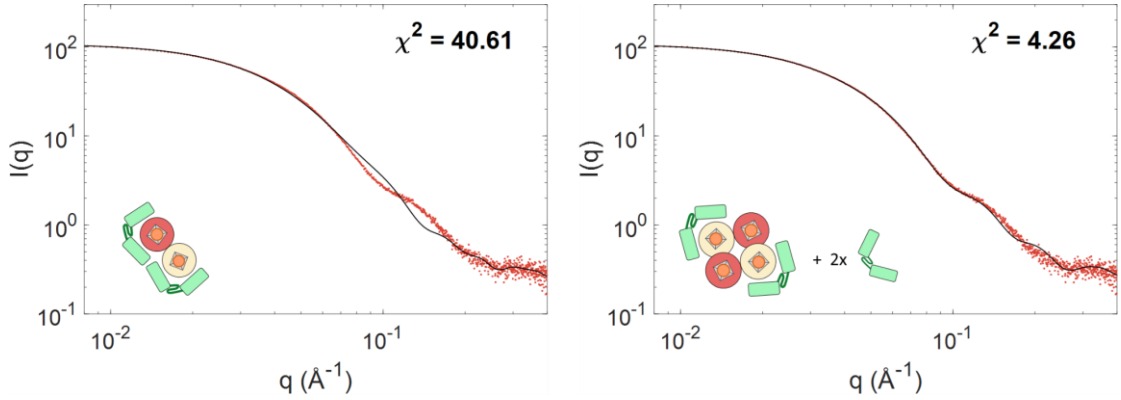
We thank the reviewer for acknowledging the efforts made in conducting our research and addressing all the reviewer's previous comments. In the following, we have answered all further reviewer's comments.

Major points:

1) The authors justified the choice of the 2IsdB:βHbtet and 2IsdB model over the 2IsdB:Hbdim model for IsdB:oxyHb complexes, arguing that the former model exhibits overall better agreement with the experimental data at higher q-values, even though the agreement at the lowest q-values is inferior. They attribute this discrepancy to the presence of a small contamination of oligomeric species larger than any of the IsdB:Hb models. However, this explanation is rather unconvincing and arbitrary, especially considering that excellent agreement was achieved for IsdB:metHb at any q-value range without invoking the presence of such contamination. Additionally, the reliance on previously published cryoEM results to resolve this question diminishes the novelty of the study, as the new experiments do not unambiguously confirm these findings but rather accept them as given. The reliance on cryoEM results to resolve this issue does indeed raise questions about the novelty and originality of the study, as it gives for granted previous findings rather than presenting novel insights into the system under investigation. It may be beneficial for the authors to provide additional analysis or experiments to support their choice of model and address the discrepancy in agreement between the different complexes.

The reviewer's comment focuses on the analysis and interpretation of IsdB:oxyHb SAXS patterns measured at different protein concentrations (Fig. 2, panels D, E and F). In his/her original report, the reviewer acknowledged that the analysis of IsdB:metHb data is convincing and raised concerns about the analysis of IsdB:oxyHb data. According to us, this last dataset is unambiguously affected by a contamination of oligomeric species much larger than any of the IsdB:Hb models as clearly shown by panels A and B that we have added to Supplementary Fig. 5 in response to the previous reviewer's criticism.

Here we provide further evidence that supports our claim. In particular, we have analyzed our data by adding an additional component that simulates the contribution of a molecular species in solution having a radius of gyration (R_g) of 86 Å. While we cannot affordably propose a rationale for structurally detailing this species, since this size is larger than the larger complex we are considering for a structural evaluation (i.e. a Hb tetramer with 4 bound IsdB molecules), we have modeled this contribution as an ellipsoid having R_g = 86 Å. By adding this contribution to the 2 most probable structural candidates (see for comparison Supplementary Fig. 4B and 4F), it is possible to eliminate in both cases the misfit at the lowest q-values as shown in the following plots.



Within the limits of this analysis, the above figures shows that:

- the claim that a contamination of objects having a size much larger than any IsdB:Hb model is not arbitrary (otherwise we would not have obtained such good fits)
- the claim that the 2IsdB:βHb_{tet} + 2 IsdB model is the most likely candidate to explain our data is also not arbitrary: it is based on a better agreement of the model to the data at all scattering angles that are less affected by the contamination of larger oligomeric species

The reviewer's current remark that the same contamination is not observed for IsdB:metHb samples does not prove that our claim is incorrect. Considering the difference in stoichiometry between IsdB:oxyHb and IsdB:metHb, it is not surprising that the system may have a different propensity to form oligomers.

Concerning the novelty of our results in relation to the previously published cryoEM results, we would like to stress that determining the structure of the IsdB:oxyHb complex is not the main goal (or one of the main goals) of the present manuscript, which is focused on elucidating the kinetic steps and stoichiometry of the interaction between IsdB and metHb. The available crystallography or cryoEM data do not provide any information on the kinetics of the IsdB:metHb interaction and/or on intermediate species.

The main motivation of collecting XSS data on IsdB:oxyHb was to have a reference scattering pattern that could be used to model one of the intermediate species in the path towards the formation of the IsdB:metHb complex. Indeed, as explained in the manuscript, it is well-known that heme extraction cannot occur when IsdB interacts with oxyHb.

2) The authors have provided the published K_D value of 0.25 ± 0.05 µM for the Hb tetramer-dimer dissociation of oxyHb as requested for comparison. However, it is questionable to directly substitute this value for the K_D of IsdB:oxyHb complexes, given the differences in systems and binding contexts. In fact, the binding of IsdB to Hb may potentially affect the dissociation constant, albeit slightly. Therefore, even though the K_D obtained for IsdB:oxyHb has a larger error (0.38 ± 1.30 µM), it should still be reported for comparison purposes. Reporting both K_D values allows for a more comprehensive assessment of the binding affinities and provides context for interpreting the results of the study. Additionally, the authors could discuss the potential impact of IsdB binding on the dissociation constant.

We agree with the reviewer that reporting both K_D values allows for a more comprehensive assessment of the binding affinities. We have modified the manuscript accordingly.

3) Despite the high uncertainty in estimating the k_{off} values due to the limited available time points, the authors opted to fix these k_{off} values to proceed with fitting the kinetic model. This procedure seems somewhat arbitrary and lacking smoothness, particularly considering the uncertainties affecting k_{off}. Furthermore, the authors continue to report equilibrium dissociation constants calculated as the ratio k_{off}/k_{on} in the text, despite the uncertainties surrounding k_{off}.

Additionally, the rationale behind adopting the kinetic model presented in Figure 5 panel C is insufficiently elucidated. The experimental points with reasonable uncertainty are available in panel B for only three species in solution (IsdB, 2IsdB:Hb_{dim}, 2IsdB:Hb_{tet}), and it remains unclear why the concentration of the intermediate 1IsdB:Hb_{dim} was not measured. Furthermore, it is ambiguous why the overall kinetic constants for step 1 of the kinetic model (IsdB binding to Hb_{tet}) are equivalent to the step-wise kinetic constants, and why step 2 (dimerization/activation) is considered virtually irreversible without introducing a corresponding koff and equilibrium constant estimation.
Overall, there are several aspects of the kinetic modeling and parameter estimation that require further clarification and justification. The decision to fix k_{off} values despite high uncertainty warrants explanation, and the implications of this choice on the interpretation of dissociation constants should be addressed. Additionally, a clearer rationale for the adoption of the kinetic model and the treatment of intermediate species and irreversible steps is necessary to ensure rigor in the analysis. Providing additional justification and sensitivity analyses could help mitigate these concerns and enhance the credibility of these findings.

The reviewer raises here concerns about the choice of fixing the k_{off} parameter and the influence that this might have had on the entire analysis and on the best-fit values obtained for the other fitting parameters in particular. Although this is indeed a valid point, we believe that we have already addressed this point. Indeed, in the manuscript we have clearly stated (Materials and Methods / TR-WAXS data analysis):

"After performing an initial SimBiology fit data run, in which published data[6] were used as initialization value, a second fitting run was performed with $k_{off,1}$ and $k_{off,3}$ fixed at the best-fit values obtained in the first round. A different choice of $k_{off,1}$ and $k_{off,3}$ values minimally affects the best fit values obtained for the other parameters (Supplementary Table 1)."

In other works, k_{off} values have not been fixed arbitrarily; we have rather obtained them from a fitting procedure. This initial fit run demonstrates however that the k_{off} values are not well-determined. We clearly warn the readers about this point and state that this does not affect the determination of the other parameters.

Given the reviewer's remark, we have decided to add a further Supplementary Table (reported below) that shows how, even a change of a factor 3 in either of the $k_{off,1}$ or $k_{off,3}$ values does not affect any of the other fitting parameters.

	STEP 1 (IsdB binding to Hb_{tet})		**STEP 2 (Hb dimerization / activation)**	**STEP 3 (IsdB binding to Hb_{dim})**	
parameter	$k_{on,1}$ (M^{-1} s^{-1})	$k_{off,1}$(s^{-1})	$k_{on,2}$ (s^{-1})	$k_{on,3}$ (M^{-1} s^{-1})	$k_{off,3}$ (s^{-1})
best fit value	$(1.4 \pm 0.4) \cdot 10^{6}$	$\mathbf{3 \cdot 10^{-2}}$	20 ± 2	$(1.8 \pm 0.3) \cdot 10^{6}$	$0.5 \cdot 10^{-2}$
best fit value	$(1.4 \pm 0.5) \cdot 10^{6}$	$1 \cdot 10^{-2}$	20 ± 2	$(1.8 \pm 0.9) \cdot 10^{6}$	$\mathbf{1.5 \cdot 10^{-2}}$

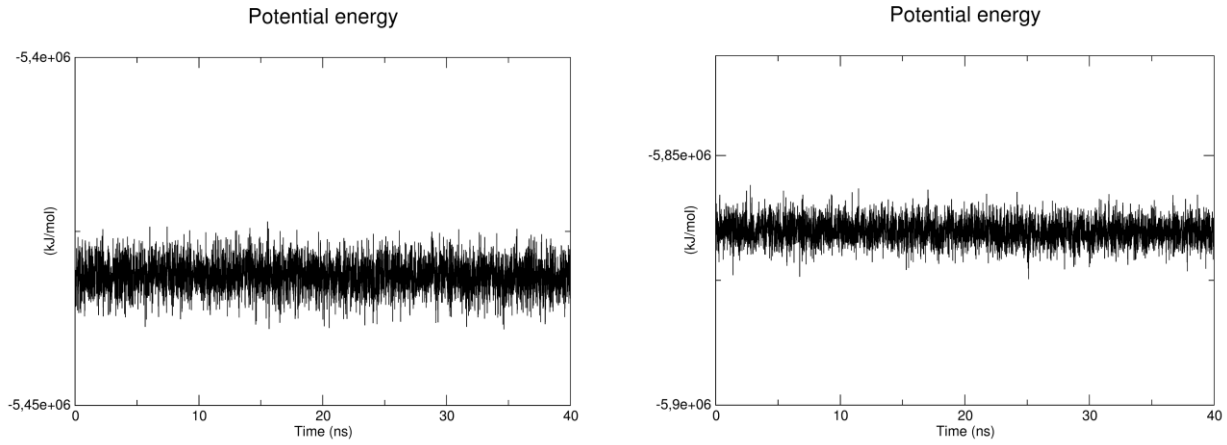
Since k_{off} values are not well-determined, the resulting fitting errors obtained in the first fit run are very large (as large as the corresponding parameter value). In spite of the high uncertainty on the determination of k_{off} values reported in the text, using these values and the fitted k_{on} values, we obtain reasonable equilibrium constants close to those reported in the literature. This is why we state in the manuscript:

"... from the estimated kinetic parameters the resulting equilibrium dissociation constants (calculated as k_{off}/k_{on}) resulted to be 7 and 3 nM for the first (to the β-chains) and second (to the α-chains) binding, respectively, in agreement with previous affinity data reported for IsdB and Hb[7]"

We believe that this information is useful for the reader, but might consider omitting it if the reviewer would judge that this information is incorrect or misleading.

4) In WAXS-driven MD simulations, an additional energy term is added to the Hamiltonian to drive and achieve agreement between the WAXS curve calculated on-the-fly and the target experimental WAXS curve. The higher the disagreement between experimental and calculated WAXS curves, the larger the additional energetic term. During the WAXS-driven MD simulations, the evolution of the reduced chi-square was monitored as a function of simulation time, but no actual convergence behavior was observed (Fig S9). This lack of convergence is unusual and unexpected, as even if the structure were transient, it should be relatively stable. Additionally, it can be noted that after rapidly decreasing, the reduced chi-square reached a minimum followed by a sudden and rapid increase, indicating a catastrophic event where the additional energetic term might have forced the system to sample spurious and unstable conformations that undermine the structure and architecture of the protein (Fig S9B).

We do not agree with the referee on this point: the lack of convergence of the reduced chi-square is not unexpected. During any MD simulation, what is minimized is not the reduced chi-square but rather the total potential energy of the system. What is thus expected is that the total potential energy stabilizes around a minimum if the system is close to an equilibrium state and if the simulation is long enough. As a matter of fact, this is what we observe in our simulation (in spite of the fact that we are trying to simulate a transient state) as can be seen in the following plots of the potential energy as a function of the simulation time for the md1.3 and md24 simulations, respectively:



The bias energy term (associated with the reduced chi-square between calculated and experimental patterns) is a small contribution to the overall energy and it is thus not surprising that it can decrease and increase during the simulation. In order to obtain a convergence of the bias term (reduced chi-square) we would have had to increase by orders of magnitude the weight of the bias term, but this would have perturbed the force field to an unphysical level. In our case, we have rather added a very mild advantage for the system to explore configurations that result in a lower reduced chi-square and then selected those configurations as the most likely to represent the structure of the system in solution.

Furthermore, spikes observed in some regions for the displacement of alpha carbons between structures extracted from the WAXS-driven simulation trajectory and the starting configurations indicate suspected system instability during the simulation (Fig. S9G-H).

We are afraid that the reviewer might have misinterpreted Fig. S9G-H (Supplementary Fig. 9G-H). This figure does not report “displacement of alpha carbons between structures extracted from the WAXS-driven simulation trajectory and the starting configurations”. Indeed, displacements are not reported as a function of time or simulation step. We believe the “spikes” the reviewer refers to are the deviations of

the order of 1.5 Å observed when comparing the structure selected from the WAXS-driven simulation with the structure selected through clustering analysis of the unbiased simulation (deviations of approx 1.5 Å are for example obtained around positions 480-660 and 820-1031, corresponding to IsdB residues interacting with Hb α-chain and with Hb β-chain, respectively). As this kind of plot compares the difference between two structures it can hardly indicate any kind of instability during the simulation.

It may be necessary for the authors to investigate the cause of these issues and consider alternative approaches or additional analyses to ensure the robustness and validity of their findings. Addressing these concerns is crucial to maintaining the credibility of the conclusions.

We acknowledge that ensuring the robustness of our findings is critical for the credibility of our conclusions. However, we failed to detect any indication of anomalous behavior in our simulations. As far as we can tell, the TR-WAXS guided MD simulations that we have performed are state-of-the-art for extracting information on the intermediate states of a complex biological system like the concerned one. Demanding to use alternative approaches or additional analysis is unrealistic and anyway unfeasible.

5) The study ultimately fails to provide structural insights that justify the specificity of IsdB for metHb over oxyHb. Despite the extensive analysis and experimental techniques employed, including time-resolved X-ray solution scattering and molecular dynamics simulations, the manuscript falls short in elucidating the underlying structural mechanisms responsible for the observed preference of IsdB for metHb.

Indeed, the goal of the present manuscript is not to investigate the specificity of IsdB for metHb over oxyHb (which likely resides in the effect of oxidation and coordination state of the heme as highlighted in hydrogen/deuterium exchange mass spectroscopy experiments cited in ref. 42), but rather to evaluate if oxyHb can be exploited as a suitable structural intermediate on the pathway of metHb binding and heme extraction by IsdB. The fact that oxyHb binds to IsdB, but the heme extraction process does not occur, suggests that the XSS pattern of this equilibrium state may be used as a model for one of the intermediate states leading to the final IsdB:metHb complex. Our data support this hypothesis. Indeed, we were able to obtain good fits of our TR-XSS data using only 4 equilibrium XSS patterns (those corresponding to Hb, IsdB, IsdB:oxyHb and IsdB:metHb).

We note *en passant* that we do not observe a preference of IsdB for metHb (binding affinity from Surface Plasmon Resonance data in ref. 7 for metHb and oxyHb are comparable), but a different response of Hb variants upon first binding.

Minor points:

1) The upper margin of panel D of Fig. 6 is cut.

OK. We have modified the figure.

2) Compared to the previous version of the manuscript, Fig. 3 includes fits performed with either CRYSOL or OLIGOMER without justification.

Fig. 3 indeed includes fits performed with either CRYSOL or OLIGOMER. This was the case also in the previous version of the manuscript only the labels have changed. Although this was written in the text, we have decided to modify the labels to make it even more evident.

The reason why CRYSOL could not be used in the case of the IsdB:oxyHb complex is that, by hypothesis, there are at least 2 species in solution (as already recalled in the answer to point 1). Indeed, if we assume that the 2IsdB:oxyHb$_{tet}$ model is the most likely to represent the status of the complex in

solution, then by simple stoichiometry, there must be an excess of IsdB molecules in solution. CRYSOL cannot model SAXS/WAXS patterns corresponding to a mixture of states (2IsdB:βHb_{tet} + 2 IsdB). We then used OLIGOMER (which is based on CRYSOL) to analyze the SAXS/WAXS pattern of IsdB:oxyHb as a mixture of the two above mentioned states.

We believe that the small cartoon at the bottom of Fig. 3B, which lacks the excess IsdB, may lead the reader to confusion. We have thus modified the figure in the revised version of the manuscript to correct this omission.

Without the resolution of these intrinsic critical concerns regarding data analysis, assumptions, and presentation, I cannot recommend the manuscript for publication in Nature Communications.

We hope to have managed to reassure the reviewer on the further concerns raised in his/her second report. All modifications made to answer the reviewer/s comments or to avoid any confusion/misinterpretation of our claims are highlighted in blue in the “marked” version of revised manuscript.

Response to reviewers' comments

In the following, point-to-point answers to Reviewer # 1 and #3 comments are reported in blue. The revised version of the manuscript and supplementary information have been modified accordingly. We note that, in order to complete the MD simulation checklist requested by the editor, some further modifications to the manuscript and supplementary information have been introduced.

Reviewer #1:

Considering the overall evidence and findings presented in this manuscript, the concerns related to local structural instabilities in the WAXS-driven MD simulations are not significantly relevant to the main arguments of the manuscript. Reviewer 3 raises concerns about potential oversights of local structural instabilities in the WAXS-driven MD simulations. Although I agree that these instabilities cannot be completely dismissed in the WAXS-driven MD simulations, the simulations were primarily aimed at evaluating whether oxyHb could act as a structural intermediate during the metHb binding and heme extraction by IsdB, not examining the specificity of IsdB for metHb versus oxyHb. Furthermore, the simulations provided additional insights by identifying structures that well describe the WAXS signal, aligning with their intended purpose. Additionally, the main objective of this paper is to elucidate the kinetics of the structural changes associated with the formation of the IsdB:Hb complex, using various experimental techniques like SAXS, WAXS, TR-XSS, TR-OA, and TR-F. The authors have successfully proposed a valuable mechanism for the kinetics, supporting the main focus of the manuscript. Therefore, I believe the manuscript would be publishable if the authors more clearly defined the purpose of the MD simulations within their text. The third reviewer's comments regarding MD simulations highlight aspects I have not previously considered, and it would be possible that readers might share the same concerns. To aid readers' understanding, it might be helpful to briefly mention, in a single sentence, the purpose of conducting MD simulations.

We thank Reviewer #1 for pointing out that potential local structural instabilities in the WAXS-driven MD simulations are not significantly relevant to the main arguments of the manuscript and that the main objective of this paper is to elucidate the kinetics of structural changes associated with the formation of the IsdB:metHb complex. In the revised version of the manuscript, we have tried to address the concerns of Reviewer #3 as detailed below. We have also added the following sentence in the Results section of the main text to clearly state the purpose of conducting MD simulations following Reviewer #1's suggestions:

"The main purpose of performing TR-WAXS driven MD simulations is to elucidate the structural changes associated with the formation of the IsdB:metHb complex, rather than

obtaining structural details at atomic resolution, which would go beyond the sensitivity of the experimental TR-WAXS patterns."

Reviewer #3:

1) The authors presented supplementary figures and new models to demonstrate the impact of contamination on their data. They used an ellipsoid model to better fit the experimental data, and this additional evidence appears to support their claim about the presence of larger oligomeric species. While this approach seems reasonable, the lack of a clear structural rationale for this species remains a concern. Ideally, additional experimental methods could corroborate the presence and size of these oligomers.

Additionally, the authors' explanation that different stoichiometries can lead to varying propensities for oligomerization is plausible. However, this explanation would benefit from more detailed data or literature references supporting such behavior differences in similar systems.

In conclusion, the authors have provided a detailed response that partially addresses the concerns raised. Ideally, the authors might provide further validation to support their claims.

We have done everything that we could within the precision of the available experimental data. Although further experiments could potentially provide better validation, we think that the analysis and tests that we have presented give already good support to our claim, which, as already discussed, is not a central claim of the manuscript.

2) The authors presented an explanation and additional data to justify fixing the k_{off} values in their kinetic model. They clarified that these values were obtained from an initial fitting procedure and demonstrated through Supplementary Table 1 that varying k_{off} minimally affects the best-fit values of other parameters. This approach seems methodologically correct.

Considering the uncertainties in the equilibrium dissociation constants, it would be preferable to remove these values to avoid misleading interpretations.

For the reader's convenience, any reference to published data (e.g. from Ref. 7) should be explicitly reported to permit a quick comparison.

As requested by the reviewer, we have removed the equilibrium dissociation constants that were based on k_{off} values with large uncertainties.

Finally, as recommended previously, the authors should provide a more detailed explanation of their model choices, particularly the rationale behind adopting the kinetic model presented in Figure 5, panel C. Specifically, why was the concentration of the intermediate 1IsdB:Hb_{dim}

not measured? Why are the overall kinetic constants for step 1 of the kinetic model (IsdB binding to Hb_{tet}) equivalent to the stepwise kinetic constants? Why is step 2 (dimerization/activation) considered virtually irreversible without introducing a corresponding k_{off} and equilibrium constant estimation?

When modeling complicated kinetic phenomena, there are two possible approaches. One is to start from first principles and try to develop a full model based on clearly defined hypotheses. A second approach is to devise an empirical model with the minimum number of kinetic species and kinetic rate constants that are justified by the time evolution of the data (thus avoiding data overfitting). In this second approach, which is the one we applied, one admittedly disregards many different possibilities that, in principle, could occur.

Although we can argue and explain why, for example, we did not consider a k_{off} for dimerization/activation, the most relevant point is that the data can be fitted well without it and they are not fitted better by introducing it. Concerning the concentration of the intermediate 1IsdB:Hb_{dim}, our fitting suggests that no significant accumulation of this species occurs. In any case, this concentration could not be measured since this species is not directly observable through static WAXS (as it is a transient structure). Finally, as the overall kinetic constant for step 1 is concerned, we have made the simplifying assumption that no cooperative binding occurs, which makes the use of two different constants unnecessary. Again, the data are fitted well with this simplifying assumption. We have now explicitly stated in the main text that our kinetic model is an empirical one. Simplifying assumptions are justified to avoid overfitting of the data as pointed out in the text and previously discussed in response to a point raised previously by Reviewer #2.

3) The authors' emphasis on the stabilization of the total potential energy is not entirely convincing. Local structural changes, which might involve only a few kJ/mol, can be masked by the fluctuations in the total potential energy of the entire system (approximately -5.4e6 kJ/mol). Therefore, relying solely on the total potential energy to argue system stability may overlook significant local instabilities.

Moreover, it must be noted that an additional energy term is added to the Hamiltonian to drive the simulations towards the target. While this penalty term is intended to guide the system, the lack of convergence in the reduced chi-square is rather concerning. Typically, even in transient structures, some level of convergence should be observed.

The authors indicate that Fig. S9G-H may have been misinterpreted. However, the observed marked deviations between the WAXS-driven simulation structure and the unbiased structure might still suggest potential local instabilities.

The lack of convergence in the reduced chi-square raises concerns about the robustness and reliability of the WAXS-driven MD simulations in this context. Typically, some level of convergence or stability in the target curve/structure is expected in such simulations.

Given these concerns, I recommended that the authors conduct additional analyses to better understand the lack of convergence in the reduced chi-square. If these issues cannot be satisfactorily addressed, it may be prudent to reconsider the inclusion of the WAXS-driven MD simulation results in the manuscript to maintain the integrity and reliability of the reported findings.

The reviewer is right in pointing out that the effect of local structural changes might be masked in the potential energy of the entire system (solute+solvent). Nevertheless, even if we compare the bias energy term with the potential energy of the solute alone, the bias term remains a small contribution. With a choice of the parameter waxs-fc=1, this corresponds to approx -20 kJ/mol as opposed to approx -42000 kJ/mol. This condition is desirable as we don't want to introduce a bias that dominates over all the other energy terms that are based on real physical interactions. A bias term of -20 kJ/mol is not necessarily much larger than the energy associated to the structural changes we are discussing here, which - we recall - involves the motion of the heme from hemoglobin to IsdB. Indeed, other localized structural changes like the rotation or changes in the position of amino acid side chains are definitely possible, but are not relevant in our case as solution X-ray scattering, even in the WAXS region, is not sensitive to that kind of motions. The only structural changes WAXS data are sensitive to are concerted motions of thousands of atoms (like the motion of an helix with respect to the other), which might involve, for example, the breaking (or formation) of many hydrogen bonds. For the above reasons, we insist that getting a full convergence of the reduced chi-square in our conditions is not trivial.

The reviewer, who is clearly an expert in the field, indicates that some level of convergence of the reduced chi-square can be observed in TR-WAXS driven MD simulation on transient structures. We acknowledge that this is not the case for our simulations (even if the simulation time is prolonged beyond 40 ns) and we have now clearly stated this point in the main text.

In order to address the reviewer's further concerns we have:

1. performed MD simulations at higher values of the bias term scaling factor (waxs-fc = 10, 15 and 20) to verify if convergence of the reduced chi-square could be obtained without generating unphysical structural changes;

2. performed MD simulations replicas of both the md1.3 simulation and the md24 one (x3) to evaluate the robustness and reliability of our conclusions on the main features of the structural transition deduced from the simulations.

The simulations at higher waxs-fc values indicate that a fully convergent behavior could be observed only in case of waxs-fc=20. However, this resulted in unrealistic structural transitions such as the unfolding of the IsdB linker helices, which is known to be highly stable. Also in the case of waxs-fc=10, while full convergence is not obtained, partial unfolding of secondary structure elements was observed.

To evaluate the robustness and reliability of our results, we have carried out two further TR-WAXS driven simulation replicas for each system with the same settings of the original ones. With the same approach of analysis (clustering around local minima of the reduced chi-square), we extracted representative structures that proved comparable to the ones already reported and are reasonably close to each other as demonstrated by a comparison of the RMSD values (see new Supplementary Figure 14). There is no evidence of local structural instabilities in any of the trajectories simulated at waxs-fc=1 as also indicated by the RMSD values.

The main features of the structural transition associated with the formation of the IsdB:metHb complex (namely the relative motion of the IsdB linker and NEAT2 domains with respect to Hb in the contact area located near NEAT2) are essentially the same in all three replicas. In particular, we have compared the key distances highlighted in the main text across different replicas and have obtained the following average values and associated standard deviations:

- md1_3
  - His58 - Tyr444 (Hb α-chain) = 12.3 ± 0.5 Å
  - His63 - Val449 (Hb β-chain) = 20.2 ± 1.0 Å
- md24
  - His58 - Tyr444 (Hb α-chain) = 14.6 ± 0.8 Å
  - His63 - Val449 (Hb β-chain) = 24.5 ± 1.6 Å

Thus, in spite of the lack of convergence of the reduced chi-square, the observation of consistent and reproducible results on the main structural transition associated to the formation of the IsdB:metHb complex, suggests that our method of isolating the structures corresponding to local minima of the reduced chi-square is reliable. We anyway clearly disclose all the methodological details about our simulations (see also attached simulation checklist) and explicitly inform the reader of the lack of convergence of the reduced chi-square so that the reader has all the needed information to critically evaluate our results.

We appreciate the reviewer’s input and hope to have managed to satisfactorily address his/her concerns.

Response to reviewers' comments

Reviewer #3 (Remarks to the Author):

The authors have made an effort to address the concerns raised in my previous review, yet several critical issues remain unresolved. My primary concern continues to be the lack of convergence in the WAXS-driven MD simulations. In these simulations, a penalty term is introduced into the Hamiltonian, designed to drive the system toward a target conformation that reproduces the experimental WAXS curve. The penalty term takes the form of a quadratic potential, proportional to a force constant and the difference between the calculated WAXS curve and the experimental one (typically measured through the chi-squared metric). Ideally, if the target conformation corresponds to a free energy minimum in the unbiased system, the penalty term should vanish as the system approaches the target, regardless of the value of the force constant. However, the fact that increasing the force constant leads to convergence only when unphysical distortions occur strongly suggests that no native conformation adequately reproduces the target WAXS curve. Instead, as the penalty term becomes dominant, the system is artificially driven into non-native states.

This interpretation explains why, with a moderate force constant, the system initially moves toward the target but then diverges, failing to reach full convergence. This might occur because the bias introduced by the penalty term is eventually outweighed by the natural dynamics of the system, which drives it into an incorrect basin. Convergence is achieved only when the penalty term becomes overwhelmingly large, but this results in unphysical changes, such as the unfolding of stable secondary structure elements like the IsdB linker helices. This points to a deeper issue in the applicability of WAXS-driven MD for this system. In short, the fact that unphysical changes occur at higher force constants indicates that the experimental WAXS curve cannot be matched by a native conformation of the system.

Furthermore, the authors argue that the results of the simulations are reproducible, as repeated simulations yield consistent results. However, reproducibility alone is not a validation of the approach. Rather, it suggests that there might be a systematic error in the procedure. If the simulations are consistently driven toward non-native states, then repeating the simulations will likely yield similarly incorrect results. Therefore, this reproducibility should not be taken as evidence of robustness but rather as a potential sign of a systematic issue in the simulations.

The authors also emphasize that WAXS data are sensitive only to large-scale, concerted motions involving thousands of atoms, and that localized structural changes are not significant. While this is partially true, when the WAXS-driven simulations fail to find a native conformation that matches the experimental WAXS curve, the system often undergoes artificial reshaping to force a match. This reshaping frequently leads to local distortions, such

as the unfolding of stable helices, as observed here. Thus, the fact that the penalty term is relatively small compared to the total energy of the system does not necessarily validate the results. Even a small bias can potentially lead to unphysical changes when the system is pushed to match a target that the native conformation cannot achieve.

Moreover, if the system is being repeatedly driven towards incorrect states, it becomes increasingly difficult to differentiate between legitimate structural changes and those that are artifacts of the simulation. The complexity of the system, coupled with the concerted motions of many atoms, makes this challenge even more pronounced. While the authors have clearly stated that the goal of the TR-WAXS-driven MD simulations was not to achieve atomic resolution, this does not mitigate the fact that the lack of convergence and the introduction of unphysical distortions cast doubt on the validity of the results. If the available WAXS data do not allow for convergence, then any conclusions drawn from these simulations must be treated with caution. As mentioned in Reviewer #1's comments, the main goal of this study is to elucidate the kinetics of the structural changes involved in the formation of the IsdB complex. While the experimental data are sufficient to support the conclusions related to the kinetics, they are not accurate enough to validate structural changes at an atomic level using TR-WAXS-driven MD simulations.

For these reasons, I recommend removing the WAXS-driven MD simulation results from the manuscript to maintain the integrity and reliability of the findings. Excluding these results would not affect the main arguments or the significance of the paper, but would ensure that only the most robust and reliable data are presented.

As requested by the reviewer, we have removed the WAXS-driven MD simulation results from the main text and from the supplementary information as well as related discussions.