Host and viral proteins involved in SARS-CoV-2 infection differentially bind heme

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Abstract
In most severe cases, SARS-CoV-2-induced autoimmune reactions have been associated with hemolytic complications. Hemolysis-derived heme from ruptured red blood cells has been shown to trigger a variety of fatal proinflammatory and procoagulant effects, which might deteriorate the progression of COVID-19. In addition, the virus itself can induce proinflammatory signals via the accessory protein 7a. Direct heme binding to the SARS-CoV-2 protein 7a ectodomain and other COVID-19-related proteins has been suggested earlier. Here, we report the experimental analysis of heme binding to the viral proteins spike glycoprotein, protein 7a as well as the host protein ACE2. Thus, protein 7a chemical synthesis was established, including an in-depth analysis of the three different disulfide-bonded isomers. Surface plasmon resonance spectroscopy and in silico studies confirm a transient, biphasic binding behavior, and heme-binding affinities in the nano- to low micromolar range. These results confirm the presence of the earlier identified heme-binding motifs and emphasize the relevance for consideration of labile heme in preexisting or SARS-CoV-2-induced hemolytic conditions in COVID-19 patients.

KEYWORDS
ACE2, COVID-19, heme, hemolysis, protein 7a, SARS-CoV-2, spike glycoprotein

Abbreviations: 2,5-DHAP, 2,5-dihydroxyacetophenone; ACE2, angiotensin-converting enzyme 2; AKI, acute kidney injury; ARDS, acute respiratory distress syndrome; CD, cluster of differentiation; COVID-19, Coronavirus Disease 2019; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); ECD, ectodomain; EDTA, ethylenediaminetetraacetic acid; Fmoc, 9-fluorenlymethoxy carbonyl; GSH, reduced glutathione; GSSG, oxidized glutathione; Hb, hemoglobin; HBM, heme-binding motif; HBTU, O-(Benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate; HCl, hydrochloric acid; HPLC, high-performance liquid chromatography; IAA, 2-iodoacetamide; ICAM-3, intercellular adhesion molecule 3; IL, interleukin; kassoc, rate of association; KD, equilibrium dissociation constant; kdis, rate of dissociation; LFA-1, lymphocyte function-associated antigen 1; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MBHA, 4-methylbenzhydrylamine; MD, molecular dynamics; NaOH, sodium hydroxide; NSP, nonstructural proteins; PNH, paroxysmal nocturnal hemoglobinuria; PTH, phenylthiohydantoin; PVDF, polyvinylidene difluoride; RAS, renin–angiotensin system; RMSD, root mean square deviation; RMSF, root mean square fluctuation; RP-HPLC, reversed-phase high-performance liquid chromatography; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SCD, sickle cell disease; SPPS, solid-phase peptide synthesis; tBu, tert-butyl; TFA, trifluoroacetic acid; TLR4, Toll-like receptor 4; TMPRSS2, transmembrane serine protease 2; TNF, tumor necrosis factor.
1 | INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which emerged by the end of 2019 in Wuhan, China, has quickly spread around the whole world and is still a matter of clinical concern. To date, more than 240 million cases of infections and more than 5 million deaths due to severe disease progression of the SARS-CoV-2-associated disease COVID-19 (“Coronavirus Disease 2019”) were confirmed globally. Deeply within the pharynx, SARS-CoV-2 settles down at nasal and throat mucosa (later also lung epithelium), thereby entering the host cells through docking of the S1 domain of its surface-exposed homotrimeric spike glycoprotein to the transmembrane metallocarboxypeptidase angiotensin-converting enzyme 2 (ACE2), which is usually responsible for the regulation of blood pressure as part of the renin-angiotensin system (RAS). In parallel, the S2 domain of the spike glycoprotein directs membrane fusion, leading to the release of viral RNA into the intracellular compartment of the infected host cells. The in-house replication machinery of the host cell is then used for viral replication, which causes exhaustion and cell death of the infected host cells. Subsequent infiltration of proinflammatory cells of the innate immune system to the injured sites provokes a cytokine storm (e.g., IL-1β, IL-6, IL-8, and TNF-α), immunothrombosis, and the development of autoimmune reactions, which characterize and drive the common symptoms (e.g., fever, cough, and breathing shortness) and complications in most severe progression of COVID-19 infections, such as acute respiratory distress syndrome (ARDS), acute kidney injury (AKI), and death. Furthermore, SARS-CoV-2 is capable of immune modulation through its transmembrane accessory protein 7a that has been shown to interact with CD14+ monocytes through an immunoglobulin-like ectodomain (ECD), consequently activating NF-κB signaling and upregulating cytokine expression. The induction of autoimmune reactions has been reported to trigger hemolytic and/or hemorrhagic events as well. In turn, preexisting hemolytic disorders, such as sickle cell disease (SCD) and paroxysmal nocturnal hemoglobinuria (PNH) have been reported to increase the risk of hospitalization and mortality. Furthermore, a variety of common blood biomarkers in hemolytic and SARS-CoV-2-driven (COVID-19) pathophysiology were identified that mainly comprise elevated components of the immune and blood coagulation system, which might account for a potentiation of pathophysiological effects in COVID-19 in case of hemolytic disorders. However, independent of autoimmune hemolytic reactions, decreased levels of hemoglobin (Hb) and albumin as well as increased levels of the Hb-binding protein haptoglobin and the heme-scavenging protein hemopexin were monitored in COVID-19 patients. Beside accumulating heme precursor levels (i.e., uroporphyrin and coproporphyrin) in patients with severe COVID-19 progression, elevated heme levels (~20 μM) were reported. Thus, as described for other viruses, such as human immunodeficiency virus 1 and Zika virus, a potential heme-driven effect in COVID-19 infections has been discussed and analyzed in vitro with contradicting results. While heme has been shown to inhibit SARS-CoV-2 attachment to endothelial cells and/or intracellular replication in kidney epithelial cells in a dose-dependent manner, which was mainly attributed to heme-driven upregulation of heme oxygenase 1 expression, an antiviral potency of heme toward SARS-CoV-2 infection has been disproven by others as demonstrated in kidney and airway epithelial cell lines. Beyond an impact of heme on the attachment and replication of SARS-CoV-2, direct interaction of heme with SARS-CoV-2 proteins of the intravirion and extravirion (i.e., spike glycoprotein, nucleoprotein, membrane protein, the nonstructural proteins [NSP] 3 and 7, and protein 7a) as well as heme binding to the in SARS-CoV-2 infection participating human host cell proteins TMPRSS2 and ACE2 has been proposed. Suitable heme-binding motifs were identified and experimentally studied only for spike glycoprotein, protein 7a, TMPRSS2, and ACE2. However, approval of heme binding at the protein level is still lacking. In the intravirion nucleoprotein, a heme-binding site has been suggested as well but only by applying in silico techniques. From a biochemical point of view, however, a fast association of hemoysis-derived heme and consequently a physiologically relevant interaction is only conceivable for extravirion parts of the proteins on the surface of the virus.

Herein, we report the chemical synthesis of SARS-CoV-2 accessory protein 7a ECD for the first time, providing novel insights into the disulfide bond connectivity of the protein as well as an alternative to the earlier described elaborative expression of protein 7a ECD in E. coli. Moreover, we show heme binding to the protein 7a ECD with an affinity that is comparable to that of heme binding to the ECDs of SARS-CoV-2 spike glycoprotein trimer and the human host cell receptor for SARS-CoV-2, ACE2. In silico studies support the potential for heme binding to the earlier predicted and experimentally proven (HBM) in protein 7a, two HBMs in spike glycoprotein, and three HBMs in ACE2.

2 | RESULTS

2.1 | Synthesis and elucidation of the disulfide bond connectivity of SARS-CoV-2 protein 7a ECD

The synthesis of protein 7a ECD was performed by solid-phase peptide synthesis (SPPS) and subsequent oxidative
self-folding (Table S1). The crude product of protein 7a ECD linear precursor (H-ELYHYQEC6VRGTVLLK-EPC19SSGYGNSPFHPLDNKALT45FSTQFA-FAC52PDGVKHYQLRARSVSPKLFIRQEEVQENH2) was obtained in a yield of \(~37\)%, which is acceptable for an 80mer protein and straightforward SPPS.\(^{32}\) The linear precursor was characterized by RP-HPLC and elutes at \(~42\)% of 0.1% TFA/acetonitrile in a gradient system. Beyond that successful synthesis was confirmed by MALDI-TOF-MS ([M + H]\(^+\) \(~9,115\) m/z) and automated Edman sequencing of the N-terminus up to position 10. Amino acid analysis revealed a peptide content of 74.7%. Due to negligible contamination, the crude product (10 \(\mu\)M) was directly subjected to oxidative self-folding in a redox-active Tris-EDTA buffer system containing GSH/GSSG (2 mM/1 mM).\(^{32}\) The oxidized product had a similar elution profile as the linear precursor (\(t_r\) 42.88 min; Figure S1). Therefore, the oxidation process could not be monitored by HPLC reaction control. Automated Edman sequencing of the oxidized product revealed that at least the first two cysteines (C\(^8\) and C\(^{20}\)) were involved in disulfide bonds due to the observation of a large degree of signal loss after the respective cysteines. Complete conversion of the linear precursor to the oxidized protein was confirmed by MALDI-TOF-MS ([M + H]\(^+\) \(~9,111\) m/z; Figure S1), iodoacetamide derivatization and subsequent MALDI-TOF-MS, as well as the Ellman’s test.

2.2 | In silico investigation of the stability of the three protein 7a ECD isomers

The native disulfide bond pattern inevitably correlates with the stability, structural integrity, and function of a peptide/protein and often represents the thermodynamically stable form of the peptide/protein,\(^{33,34}\) emphasizing the relevance for studying the different possible disulfide-bonded isomers of protein 7a ECD (A–C). As a result of the MD simulation of the disulfide-bonded isomers B and C (B: C\(^8\)–C\(^{20}\)/C\(^{43}\)–C\(^{52}\) and C: C\(^8\)–C\(^{52}\)/C\(^{20}\)–C\(^{43}\)) in comparison to the earlier, as the native form suggested,\(^{11}\) disulfide-bonded isomer A (C\(^8\)–C\(^{43}\)/C\(^{20}\)–C\(^{52}\)), nearly complete unfolding of the secondary structure was observed during the energy minimization phase in case of the isomers B and C (Figure 1a–d; Figure S2; Table S2). In addition, the isomers B and C were characterized by a higher flexibility which is evident from a higher root mean square fluctuation (RMSF) over the MD simulation trajectory (Figure 1e).

The second approach focused on the investigation of serine mutants of protein 7a ECD, including the mutants C\(^8\)S–C\(^{20}\)S (1), C\(^{43}\)S–C\(^{52}\)S (2), C\(^8\)S–C\(^{43}\)S (3), C\(^{20}\)S–C\(^{52}\)S (4), C\(^8\)S–C\(^{52}\)S (5), and C\(^{20}\)S–C\(^{52}\)S (6), which further supported the importance of the disulfide bonding. The simulation of these six mutants (1–6) revealed that there is only low structural similarity between them with an overall root mean square deviation (RMSD) ranging from 12.14 Å (4, C\(^{20}\)S–C\(^{52}\)S mutant) to 20.13 Å (1, C\(^8\)S–C\(^{20}\)S mutant) (Figure S3; Table S3). Thereby, the mutants that hamper the formation of the isomer with the connectivity C\(^8\)–C\(^{20}\)/C\(^{43}\)–C\(^{52}\), that is, 1 (C\(^8\)S–C\(^{20}\)S) and 2 (C\(^{43}\)S–C\(^{52}\)S), showed the highest overall RMSD compared with the parent structure (Figure S3; Table S3). Due to the mutation of the respective cysteines in 1 and 2, the remaining cysteines are not close enough to each other anymore, which prevents disulfide bond formation. Only the C\(^{20}\)S–C\(^{52}\)S mutated form, with the lowest overall RMSD when superimposed with isomer A, still retained C\(^8\) and C\(^{43}\) close enough for disulfide bond formation (Figure S3; Table S3).

2.3 | SARS-CoV-2 protein 7a binds heme

SPR measurements were performed to investigate heme binding to protein 7a (Figure 2a,d). Heme binding to accessory protein 7a occurs transiently, which is characterized by a rapid association (\(k_a = (4.17 \pm 0.13) \times 10^4 \text{M}^{-1} \text{s}^{-1}\)) and dissociation (\(k_d = (1.95 \pm 0.06) \times 10^{-2} \text{s}^{-1}\)). According to the best fit, a nanomolar binding affinity was determined (\(K_D = 469 \pm 27 \text{nM}\)). Furthermore, heme binding to protein 7a occurred in a biphasic and heterogeneous manner, suggesting the presence of a second heme-binding site, which only allows for a slower association with less affinity (\(K_D = 740 \pm 73 \text{nM}\)) toward heme.

2.4 | SARS-CoV-2 spike glycoprotein trimer and human ACE2 bind heme

Direct heme binding to spike glycoprotein trimer and ACE2 was analyzed by SPR under the same conditions as described for accessory protein 7a (Figure 2b–d). The interaction of heme with ACE2 and spike glycoprotein is of a biphasic nature. First, a transient rapid binding event occurs \([k_{a1} = (2.68 \pm 0.10) \times 10^4 \text{M}^{-1} \text{s}^{-1}\) (spike glycoprotein); \(k_{a1} = (4.33 \pm 0.17) \times 10^4 \text{M}^{-1} \text{s}^{-1}\) (ACE2)], followed by a second \([k_{a2} = (4.91 \pm 0.29) \times 10^2 \text{M}^{-1} \text{s}^{-1}\) (spike glycoprotein); \(k_{a2} = (7.40 \pm 1.20) \times 10^2 \text{M}^{-1} \text{s}^{-1}\) (ACE2)], that is characterized by slow binding kinetics. In case of both proteins, the first heme association occurs with nanomolar affinity [\(K_{D1} = 650 \pm 21 \text{nM}\) (spike glycoprotein); \(K_{D1} = 659 \pm 35 \text{nM}\) (ACE2)], while the
second is characterized by a higher $K_D$ [$K_{D2} = 1.86 \pm 0.10$ μM (spike glycoprotein); $K_{D2} = 1.54 \pm 0.25$ μM (ACE2)]. The hyperstoichiometric binding signals indicate a multivalent binding behavior, which supports the presence of more than one heme-binding site in SARS-CoV-2 spike glycoprotein and human ACE2.
2.5 | In silico binding studies confirm heme binding to HBMs in protein 7a, spike glycoprotein, and ACE2

Initially, molecular dynamics (MD) simulation of protein 7a (ECD), the S1 domain of spike glycoprotein as well as ACE2 (extracellular domain) were performed for 350 and 150 ns, respectively, which revealed a high degree of structural stability (RMSD difference of ~0.50 Å; Figure S4a). In case of the large proteins spike glycoprotein (S1 domain) and ACE2, equilibrium in RMSD was observed after 90 and 45 ns with an RMSD difference of ~4.50 Å and ~1.50 Å, respectively (Figure S4b,c).

Subsequent focused docking to the earlier experimentally identified HBM Y3KHVYQLRA64 in protein 7a confirmed coordinative binding to H58 with a distance of 3.86 Å between the Fe3+ ion of the heme molecule and the deprotonated nitrogen atom in H58 (Figure S5a,b). The stability of the protein 7a-heme complex was further confirmed by three independent 50 ns simulation runs, in which heme moved even closer to H58 with an average distance of ~2.90 Å and an estimated Poisson-Boltzmann binding energy of −331.12 ± 59.87 kJ/mol (Figure 3a; Figure S6a). Heme binding to this motif led to a decreased flexibility of the N-terminus of the protein (Figure S5b). Since the experimental SPR binding studies suggested the presence of a second heme-binding site with less affinity, docking of a second heme molecule to protein 7a was attempted. Docking studies revealed the N-terminal Y3 as a further potential heme-binding site, which was supported by a 50 ns MD simulation run, in which heme moved even closer to the coordinating residue (distance of 2.36 Å; binding energy of −553.84 ± 90.58 kJ/mol; Figure 3a; Figure S5c,d; Figure S7a). Interestingly, binding of a second heme molecule to this side led to a further stabilization of the other heme-binding region, the C-terminus of the protein, which was indicated by a lower RMSF (Figure S5d).

For the spike glycoprotein S1 domain, focused docking to the earlier identified HBMs (F140LGVYVYHKN148 and 1̅20̅3̅YSKHTPIN211)20 with moderate heme-binding affinity was performed. For both motifs, histidine serves as the coordinating residue with a distance of 2.86 and 2.88 Å between the Fe3+ ion of heme and the deprotonated nitrogen atom of H146 and H207, respectively. During the 50 ns simulation runs, heme stayed stably bound to both motifs, as indicated by the low RMSD change (Figure S7b; Figure S8). In addition, heme moved more closely toward the coordinating histidine residues with average distances of ~2.20 and ~2.13 Å as well as a binding energy of −248.34 ± 60.45 kJ/mol and −579.90 ± 96.47 kJ/mol, respectively (Figures 3b, S6b, and S8). Although both HBMs are located at the N-terminus of the S1 domain of the spike glycoprotein, heme binding to each of the motifs led to the stabilization of the C-terminus of the domain (Figure S7b), which was observed by the decreased RMSF of the C-terminal residues (Figure S8).

Focused docking of heme to the earlier found HBMs in ACE2 that exhibited moderate to high heme-binding affinity (P235LYEHLHAY243 and S511FIRYTTYRT519)20 led to ACE2-heme complexes with distances of 3.46 and 3.56 Å between the Fe3+ ion of heme and side chain nitrogen atom of the coordinating histidines (H239 and Y515, respectively). In previous studies, also A650MRQYFLKV658 turned out as a moderate HBM. However, molecular docking to the motif on the protein level failed, since the motif is not well exposed, and thus, heme binding is sterically hindered. MD simulation studies of heme bound to ACE2 at P235LYEHLHAY243 and S511FIRYTTYRT519 demonstrated the transient nature of heme binding to the protein (Figure 3c). While heme left the first motif (P235LYEHLHAY243) already after 1.6 ns of the simulation run, in the case of the second motif (S511FIRYTTYRT519), it was observed that heme slides to neighboring residues and then starts leaving the binding pocket after 2 ns (Figure 3c; Figure S7c; Figure S9). The protein itself remained stable in its initial conformation (Figure S7c; Figure S9).

3 | DISCUSSION

Earlier studies suggested several intra- and extraviral SARS-CoV-2 proteins as heme-binding proteins (e.g., nucleoprotein,28 membrane protein,28 and spike glycoprotein20,28,31), lacking evidence on the molecular level. Recently, we experimentally identified (HBM) in two SARS-CoV-2 proteins, the transmembrane spike glycoprotein and the accessory protein 7a, as well as in the human host cell receptor for SARS-CoV-2, ACE2, by exploring the heme-binding capacity of protein-derived peptides.20 However, heme binding to the ECDs of spike glycoprotein, protein 7a, and ACE2 on the protein level has not been proven yet.

For that purpose, the chemical synthesis of untagged SARS-CoV-2 protein 7a ECD by SPPS and subsequent oxidative self-folding, as has been described for other cysteine-rich peptides/miniproteins (e.g., the coagulation factor XIII inhibitor tridegin) before, was successfully performed herein.32,35,36 Up to date, the production of the SARS-CoV-2 protein 7a ECD was only conducted in bacterial expression systems (e.g., E. coli BL2111). However, these studies were impaired by the insolubility of bacterially expressed recombinant protein 7a, which led to the formation of inclusion bodies,11,37 suggesting the
chemical synthesis of protein 7a ECD as a suitable alternative. The recombinant protein 7a ECD has been structurally characterized by X-ray crystallography (PDBs: 6W37 and 7CI3). In this structure, two disulfide bonds were manually assigned between C8 and C43 as well as C20 and C52 (A). Our in silico studies with the three

FIGURE 3  In silico studies confirm binding of heme to two SARS-CoV-2 proteins and the human host cell receptor ACE2. (a) During two independent 50 ns simulation runs, heme (red) stayed bound to accessory protein 7a ECD to the earlier confirmed HBM (VKHVYQLRA) and to two HBMs (green) at once, respectively, coordinating heme via H38 and Y9. (b) In the S1 domain of the SARS-CoV-2 spike glycoprotein, heme stably bound to two HBMs (green) around H146 and H207. (c) Heme coordination by ACE2 via H239 and Y515 was confirmed as well. However, in comparison to the viral proteins accessory protein 7a and spike glycoprotein, MD simulation of heme binding to ACE2 demonstrated the transient nature of heme binding to the host cell receptor, which was observed by step-wise migrating out of the binding pocket over the surface of the protein.
different disulfide-bonded isomers of the protein further supported this assignment of the native disulfide bond pattern (A), since the other isomers, that is, C8–C20, C43–C52 (B) and C8–C52, C20–C43 (C), showed strongly decreased stability during the 50 ns simulation runs, which was also evident by the nearly complete loss of the secondary structure elements (including the characteristic β-sheet pattern of protein 7a).

In this study, we confirmed heme binding to two SARS-CoV-2 proteins (spike glycoprotein ECD trimer and the in-house produced protein 7a ECD) and the extracellular part of the human host protein ACE2 with rapid association and dissociation rates as well as binding affinities that are characteristic for transient heme-binding proteins.38–40 For protein 7a, two heme-binding events were observed that were characterized by nanomolar heme-binding affinity. Thereby, heme binding to the earlier identified HBM (VKH58VYQLRA)20 with H58 as the coordinating residue was confirmed by molecular docking and simulation studies. Furthermore, the possibility of simultaneous heme binding to a second site (Y3) at the N-terminus of protein 7a was demonstrated in silico. With respect to the binding distances and energies, both HBMs are equally suited, explaining the similar binding affinities in the nanomolar range. However, it might be conceivable that heme binding to the second motif is only possible when the first heme molecule is already bound to H58, since this first binding event showed a stabilization of the N-terminus, which might support heme binding to the N-terminus of the protein.

The spike glycoprotein ECD trimer exhibited similar heme-binding properties with a biphasic binding behavior and at least two binding events with nanomolar affinity. The earlier investigated HBMs (F140LGVYYHKN148 and I203YSKHTPIN211)20 were confirmed on the protein level by in silico studies in this study, suggesting SFIR44,45 as the high-affinity binding site for heme in our previous study and confirmed herein. However, the other HBM (around H136) seems to be more important for the protein, and thus, of higher potential physiological relevance concerning heme binding. Mutations in this motif (e.g., of Y144 and K147) were described to lead to vaccine resistance, bearing a higher risk for immune evasion.42 The same might be provoked by shielding the motif through heme binding, which should be investigated in the future and considered in patients suffering from the comorbidity of COVID-19 and a hemolytic disorder.

Interestingly, the human host cell receptor for SARS-CoV-2 and thus the counterpart of spike glycoprotein, ACE2, shares a very similar heme-binding behavior with the spike glycoprotein, characterized by two binding events with nanomolar and micromolar affinities. The respective heme-binding sites were earlier predicted and characterized on the peptide level, showing moderate (PLYEH239LHAY) to high (SFIRY315YTRT) heme-binding affinities.20 Herein, heme binding to PLYEH239LHAY and SFIRY315YTRT was also confirmed by in silico studies. However, during a 20 ns MD simulation of the ACE2-heme complexes, heme moved away from the binding sites, explaining the transient fashion of heme binding to ACE2. Both, the earlier experimental studies on the peptide level as well as the MD simulation studies in this study, suggest SFIRY315YTRT as the high-affinity binding site in ACE2 and PLYEH239LHAY as the site for the second binding event possessing a lower affinity.

Taken together the recent and present results, the physiological relevance of heme binding to these proteins needs to be considered, yet requires further analysis ex vitro and in vivo by employing suitable functional and pathophysiological assay systems. In conditions of coexisting SARS-CoV-2 and an increased amount of heme in the vascular compartment, as might be the case in patients suffering from a hemolytic disorder and a concomitant SARS-CoV-2 infection, it is conceivable that SARS-CoV-2 may scavenge heme on the surface of the virus by binding heme to spike glycoprotein and protein 7a. Thereby heme could be transferred, for example, to endothelial cells, platelets or immune cells, comparable to what has been earlier described for erythrocyte-derived microvesicles (Figure 4a).43 This subsequently may lead to an increased activation of these cells, accelerating inflammation and thrombosis in patients.44,45 Both, SARS-CoV-2 and heme were reported to be capable of
activating the proinflammatory signaling. In the case of SARS-CoV-2, this activation is performed by direct interaction of its spike glycoprotein with toll-like receptor 4 (TLR4). Since direct heme binding to TLR4 was only suggested so far, it remains to be clarified which of both ligands bind to TLR4 with higher affinity to predict a
spatial and temporal hierarchy of TLR4 activation under these conditions. Interestingly, protein 7a binds to CD14+ cells, which might further support the activation of the CD14-TLR4 axis, leading to a dual burden of the immune system. Although the heme-binding sites of the viral spike glycoprotein and the human receptor ACE2 are not found on the direct interface of both proteins (Figure 4b), heme binding to both proteins might affect the viral uptake into the host cell, which may explain the earlier observed replication suppression of SARS-CoV-2 in the presence of heme. Apart from COVID-19, heme binding to ACE2 should be analyzed in the context of the earlier described heme-triggered hypotension, since ACE2 displays not only the key receptor for SARS-CoV-2 but also regulates the blood pressure as part of the RAS. 

An effect of heme on protein 7a is more speculative since the functions of protein 7a are not yet fully understood. Due to structural similarity with immunoglobulins and cell adhesion proteins, the regulation of virus–cell adhesion was suggested to be one further role of protein 7a beyond the immunomodulating functions. Through heme binding to the small protein the interaction with adhesion molecules might be disturbed (Figure 4c). However, heme itself is capable of upregulating the expression of adhesion molecules, which might further support the adhesion of the virus to host cells.

4 CONCLUSION

In conclusion, the present study provides insights into the molecular basis of the interaction of heme with three proteins that are relevant for the viral entry of SARS-CoV-2 and its immunomodulating functions, namely, protein 7a, spike glycoprotein, and human ACE2, all binding heme with nano- to micromolar binding affinities. The identified binding sites explain recent observations of the interaction of heme with SARS-CoV-2 and can be the starting point for further studies on accelerated immune response in SARS-CoV-2 patients with pre-existing hemolytic disorders.

5 MATERIALS AND METHODS

5.1 Reagents

Fmoc-Rink-Amid MBHA resin, the coupling reagent HBTU, Fmoc-amino acids as well as other reagents for solid-phase peptide synthesis (SPPS), for example, piperidine, N,N-dimethylformamide, and N-methylmorpholine were obtained from IRIS Biotech, ORPEGEN Peptide Chemicals, Novabiochem, VWR, FLUKA Chemicals, and Sigma-Aldrich. Trifluoroacetic acid (TFA) for peptide cleavage was kindly provided by Solvay. HPLC and LC–MS-grade solvents (e.g., acetonitrile, water, methanol, diethyl ether) were purchased from Fisher Scientific and VWR, while buffer components (e.g., HCl, Tris–HCl, EDTA, and NaOH) were obtained from Carl Roth GmbH and VWR. Hemin was acquired from Frontier Scientific. Untagged human ACE2 and SARS-CoV-2 spike glycoprotein trimer (both only extracellular part) were purchased from trentzyme GmbH. Other chemicals, including 2-iodoacetamide (IAA), 2,5-dihydroxyacetophenone (2,5-DHAP), glutathione, and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman’s Reagent) as well as buffers and ninhydrin solution for amino acid analysis were obtained from Sigma-Aldrich, Carl Roth GmbH, Thermofisher, and Laborservice Onken GmbH. Chemicals (e.g., PTH-amino acid standards, acetonitrile, mobile phase, N-methylpiperidine) and polyvinylidene difluoride (PVDF) membranes for protein sequencing were obtained from FUJIFILM Wako Chemicals and Shimadzu. The Peptide Calibration Standard II for mass spectrometry was obtained from Bruker Daltonics GmbH.

5.2 Synthesis of the linear precursor of the extracellular domain of SARS-CoV-2 protein 7a

The linear precursor of ECD of protein 7a with the sequence H-ELYHYQECVRGTTVLLKEPCSSGTYEGNS PFHPLADNKFALTCFSTQAFACPDGVKHYYQLRARS VSPKLFIRQEEVQEH2-iodoacetamide (IAA), 2,5-dihydroxyacetophenone (2,5-DHAP), glutathione, and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman’s Reagent) as well as buffers and ninhydrin solution for amino acid analysis were obtained from Sigma-Aldrich, Carl Roth GmbH, Thermofisher, and Laborservice Onken GmbH. Chemicals (e.g., PTH-amino acid standards, acetonitrile, mobile phase, N-methylpiperidine) and polyvinylidene difluoride (PVDF) membranes for protein sequencing were obtained from FUJIFILM Wako Chemicals and Shimadzu. The Peptide Calibration Standard II for mass spectrometry was obtained from Bruker Daltonics GmbH.

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The linear precursor of ECD of protein 7a with the sequence H-ELYHYQECVRGTTVLLKEPCSSGTYEGNS PFHPLADNKFALTCFSTQAFACPDGVKHYYQLRARS VSPKLFIRQEEVQEH2-iodoacetamide (IAA), 2,5-dihydroxyacetophenone (2,5-DHAP), glutathione, and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman’s Reagent) as well as buffers and ninhydrin solution for amino acid analysis were obtained from Sigma-Aldrich, Carl Roth GmbH, Thermofisher, and Laborservice Onken GmbH. Chemicals (e.g., PTH-amino acid standards, acetonitrile, mobile phase, N-methylpiperidine) and polyvinylidene difluoride (PVDF) membranes for protein sequencing were obtained from FUJIFILM Wako Chemicals and Shimadzu. The Peptide Calibration Standard II for mass spectrometry was obtained from Bruker Daltonics GmbH.

In conclusion, the present study provides insights into the molecular basis of the interaction of heme with three proteins that are relevant for the viral entry of SARS-CoV-2 and its immunomodulating functions, namely, protein 7a, spike glycoprotein, and human ACE2, all binding heme with nano- to micromolar binding affinities. The identified binding sites explain recent observations of the interaction of heme with SARS-CoV-2 and can be the starting point for further studies on accelerated immune response in SARS-CoV-2 patients with pre-existing hemolytic disorders.

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hydrogen citrate solution) was applied onto the ground steel target by the dried droplet method (as guided by Bruker Daltonics). As described earlier,\textsuperscript{36,51} the protein content was determined by amino acid analysis. For that purpose, the crude protein was hydrolyzed in 6 N HCl at 110°C for 24 hr, followed by the amino acid analysis using an Eppendorf Biotronik LC3000 equipped with a cation exchange column (CK10 M resin, 4 μm particle size, 10% cross-linkage, type H; Mitsubishi) and detection at 570 nm and 440 nm. To further prove the accurate synthesis, 10 cycles of N-terminal protein sequencing through automated Edman degradation (PPSQ-53A, Shimadzu) of the linear precursor on bead and after cleavage from the resin were performed. For that purpose, one bead (for on-bead approach) and ~50 pmol (in 50% acetonitrile/water +0.1% TFA) of the linear precursor were spotted on a glass fiber disc (polybrene pretreated) and PVDF membrane, respectively, according to the manufacturer's guidelines (Shimadzu). PTH-amino acids were separated isocratically by a coupled RP-HPLC system and detected at 270 nm.

### 5.3 Oxidation of the linear precursor of protein 7a ECD

As described earlier,\textsuperscript{36} the crude product of the protein (yield: ~37%) was subjected to oxidative folding for disulfide bond formation. Thus, the protein (final concentration: ~10 μM) dissolved in pure TFA (final: 0.01%) was added to the reaction mixture containing 1 mM oxidized glutathione (GSGG) and 2 mM reduced glutathione (GSH) in 0.1 M Tris, 1 mM EDTA, pH 8.7. The reaction was performed at 4°C under an inert atmosphere of argon for 24 hr. After freeze-drying, the oxidized protein was purified by semipreparative RP-HPLC (PU-987, JASCO) equipped with a Knauer Eurospher 100 column (C18, 250 × 32 mm, 5 μm particle size, 100 Å pore size) and a gradient elution system with 0.1% TFA in water (eluents A) and 0.1% TFA in 90% acetonitrile/water (eluents B). Elution was reached with a gradient of 20–70% eluent B over eluent A in 120 min at a flow rate of 10 ml/min and monitored at 220 nm.

### 5.4 Analytical characterization of oxidized protein 7a ECD

The purified protein was characterized by means of analytical RP-HPLC, MALDI-TOF-MS, and amino acid analysis. Analytical RP-HPLC was performed with a LC-20A system with a Vydac column (C18, 250 × 4.6 mm, 5 μm particle size, 300 Å pore size). Elution was achieved following a gradient of 20–80% eluent B (0.1% TFA in acetonitrile) over eluent A (0.1% TFA in water) in 60 min at a flow rate of 1 ml/min and monitored at 220 nm. The molecular mass of the protein was detected with a MALDI-TOF-MS (Autoflex II, Bruker Daltonics). 2,5-DHAP served as the matrix. As described for the crude linear precursor, the protein content of the oxidized product was determined by amino acid analysis.

### 5.5 Qualitative determination of disulfide bonds in the ECD of protein 7a

For confirmation of the involvement of all four cysteines in disulfide bonds, the Ellman's test and iodoacetamide derivatization were performed. As described earlier,\textsuperscript{52,53} the Ellman's test was carried out with the oxidized ECD of protein 7a and its linear precursor, as well as cysteine (as positive control) and cystine (as negative control). 100 μl Ellman's reagent (1.76 mg/ml 5,5′-dithiobis-(2-nitrobenzoic acid) in 0.2 M sodium phosphate buffer, pH 8.0) was added to 150 μl of the sample solutions (~70–150 μM in 0.2 M sodium phosphate buffer, pH 8.0). After 10 min of incubation at room temperature, the absorbance was recorded at 410 nm. In addition, protein sequencing of the linear precursor and the oxidized product was performed by automated Edman degradation (PPSQ-53A, Shimadzu; as described above). Finally, the linear precursor and the oxidized product (both final: ~50 μM) were mixed and incubated for 2 hr with the alkylating reagent 2-iodoacetamide (final: 2 mM) in 10 mM phosphate buffer, pH 7.4.\textsuperscript{51} Subsequently, samples were analyzed with MALDI-TOF-MS (as described above) in the linear mode.

In silico construction and molecular dynamics (MD) simulation of the different possible disulfide-bonded isomers of the protein 7a ECD. To study the stability of the different disulfide bond patterns that are theoretically possible for the protein 7a ECD, two approaches were followed. First, the structures of the original and six mutated forms (1–6) of the protein 7a ECD were predicted using AlphaFold (2.1.0) available in the form of google colab in ChimeraX (version 1.3).\textsuperscript{54,55} Cysteines were mutated by serine to avoid disulfide bond formation. The best predicted model from each sequence was selected to perform structure alignment using MUSTANG algorithm available on YASARA (version 21.8.27).\textsuperscript{36} Second, the alternative disulfide bond patterns (B: C8–C20/C43–C52 and C: C8–C52/C20–C43) were assigned by manual removal of the parent disulfide bonds from the crystal structure (PDB:
6W37) and addition of the intended bonds in YASARA, as described earlier.\textsuperscript{56,57} Subsequently, each isomer (including the described form A: C⁸–C⁴⁷/C²⁰–C⁵²) was subjected to three independent 50 ns MD simulations at pH 7.4 and 298 K using the AMBER14 force field and the md_run.mcr macro with default values in YASARA to analyze the folding process.\textsuperscript{57,58}

Analysis of direct heme binding to the ECDs of protein 7a, spike glycoprotein, and ACE2 by surface plasmon resonance (SPR) spectroscopy. As described earlier,\textsuperscript{39,40} binding studies were carried out on a Biacore T200 (Cytiva). The ECD of ACE2, SARS-CoV-2 spike glycoprotein trimer, and SARS-CoV-2 protein 7a were dissolved in 10 mM sodium acetate (pH 4.5 for ACE2, pH 5.5 for spike glycoprotein, and pH 6.0 for protein 7a). The proteins were immobilized by amine coupling on a CM3 chip (Cytiva) to response units of 474 RU (protein 7a ECD), 826 RU (ACE2 ECD), and 2,333 RU (spike glycoprotein trimer ECD). Hemin (designated as “heme” in the following) was prepared as described previously,\textsuperscript{39,40} and diluted in running buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.05% Tween 20). For the determination of binding kinetics, heme was titrated in five consecutive injections of different concentrations (187.5, 375, 750, 1,500, and 3,000 nM). Measurements were performed with a flow rate of 30 µl/min and at 25°C using the standard single-cycle kinetics method (Biacore T200 control software, GE Healthcare). As described below,\textsuperscript{40} regeneration of the sensor chip surfaces was achieved by an injection of 10 mM NaOH. All measurements were performed as a triplicate. Evaluation of binding kinetics was conducted by global fitting with the Biaevaluation software (GE Healthcare). For the SPR data presentation GraphPad Prism 9.3.1 was used.

5.6 Molecular docking simulations of heme binding to the ECDs of protein 7a, spike glycoprotein, and hACE2

As described earlier for other proteins,\textsuperscript{39,40} the available structures of the protein 7a ECD (PDB: 6W37), the S1 domain of spike glycoprotein (extracted from the C-I-Tasser model QHD4341\textsuperscript{59,60} due to a lack of the motif FLG\textsubscript{Y144}Y\textsubscript{145}H\textsubscript{146}KN in the available structures), and the ECD of ACE2 (extracted from: PDB 6M17) were subjected to a 350, 150, and 150 ns MD simulation at pH 7.4 and 298 K using the AMBER14 force field and the md_run.mcr macro with default values, respectively, in YASARA (version 21.8.27).\textsuperscript{56,61} to generate equilibration of the conformation of the protein structures. Snapshots were saved every 100 ps. For all three proteins, conformational equilibrium was reached after 280 ns with a RMSD change of <0.5 Å in case of protein 7a ECD and after 100 ns with an RMSD change of <5 Å in the case of the spike glycoprotein S1 domain and the extra-cellular domain of ACE2. Snapshots were selected from the production phase from the final 100 ns of the MD simulation and analyzed for the surface accessibility of the earlier described HBMs (i.e., in protein 7a: V\textsubscript{KH58}–Y\textsubscript{60}QLA; in spike glycoprotein: FLG\textsubscript{Y144}Y\textsubscript{145}H\textsubscript{146}KN and Y\textsubscript{204}SKH\textsubscript{207}TPIN; in ACE2: PLY\textsubscript{237}E\textsubscript{239}LH\textsubscript{244}Y\textsubscript{243}; SF\textsubscript{Y515}Y\textsubscript{516}–TR, and AMR-Q\textsubscript{Y65}FLK).\textsuperscript{20} These poses were then subjected to molecular focused docking studies of heme to the respective HBMs by using YASARA, as described in detail for other protein/peptide–heme complexes before.\textsuperscript{39,40,53,56,60,62} The ligand heme was downloaded from the ChemSpider database (ID: 16739951).\textsuperscript{63} For each pose, a cubic simulation cell (10 × 10 × 10 Å) was built around the expected coordinating residue of the respective HBMs. As described before,\textsuperscript{40} the ensemble docking approach followed the AutoDock Vina algorithm in YASARA.\textsuperscript{64} The resulting poses were filtered by the lowest distance between the coordinating residue and the iron ion of heme. Following a previously established protocol,\textsuperscript{40} select heme–protein complex poses were subsequently MD simulated thrice for 50 ns (with the same parameters as described above) to analyze the stability of heme binding to the respective HBMs. Analysis of the MD trajectories (e.g., RMSD, RMSF, and binding energies) was automatically conducted in YASARA by using the md_analyze.mcr macro.\textsuperscript{56,58,65} Binding energies were obtained applying the Poisson-Boltzmann method via the md_analyzebindenergy.mcr macro in YASARA,\textsuperscript{64,66} relying on the equations described in detail before.\textsuperscript{34} Figures were generated using UCSF Chimera (1.13.1),\textsuperscript{67} and plots were generated with GraphPad prism (9.3.1).

AUTHOR CONTRIBUTIONS

Marie-T. Hopp: Conceptualization; data curation; funding acquisition; methodology; project administration; supervision; visualization; writing – original draft; writing – review and editing. Dhruv C. Rathod: Data curation; methodology; software; visualization; writing – original draft. Diana Imhof: Conceptualization; project administration; supervision; writing – review and editing.

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DATA AVAILABILITY STATEMENT
Data are available in article supplementary material.

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SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.

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