Mechanism and inhibition of *Streptococcus pneumoniae* IgA1 protease

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Opportunistic pathogens such as *Streptococcus pneumoniae* secrete a giant metalloprotease virulence factor responsible for cleaving host IgA1, yet the molecular mechanism has remained unknown since their discovery nearly 30 years ago despite the potential for developing vaccines that target these enzymes to block infection. Here we show through a series of cryo-electron microscopy single particle reconstructions how the *Streptococcus pneumoniae* IgA1 protease facilitates IgA1 substrate recognition and how this can be inhibited. Specifically, the *Streptococcus pneumoniae* IgA1 protease subscribes to an active-site-gated mechanism where a domain undergoes a 10.0 Å movement to facilitate cleavage. Monoclonal antibody binding inhibits this conformational change, providing a direct means to block infection at the host interface. These structural studies explain decades of biological and biochemical studies and provides a general strategy to block *Streptococcus pneumoniae* IgA1 protease activity to potentially prevent infection.
Streptococcus pneumoniae (S. pneumoniae) is a leading cause of bacterial pneumonia and meningitis in children and adults worldwide\(^1\). According to the World Health Organization (WHO), S. pneumoniae remains a “major global public health problem”. Polysaccharide-based vaccines have proven effective against invasive S. pneumoniae, but less so against mucosal infections, and these vaccines target only a subset of the known serotypes\(^2\). Thus, employing a more widely shared protein virulence factor such as the S. pneumoniae IgA1 protease (IgA1P) as a vaccine target has been advocated, as it is present in all pathogenic strains and active at the respiratory mucosa\(^3\).\(^4\)\(^5\).

S. pneumoniae IgA1P is the prototypical member of the M26 class of bacterial metalloproteases\(^6\), which share virtually no sequence homology to previously characterized proteins\(^8\). Other opportunistic pathogens that secrete similar IgA1 metalloproteases include Streptococcus oralis and Streptococcus sanguinis\(^9\).

The mature forms of these enzymes comprise nearly 2000 amino acids and are covalently linked to their bacterial cell surface by sortase A\(^13\). Beyond the metalloprotease class of IgA1Ps, there are two other structurally distinct IgA1P classes that coordinate the serine proteases\(^14\) and cysteine proteases\(^15\), illustrating the evolutionary need to converge upon mechanisms of IgA1 cleavage in order to thwart the initial host immune response.

All classes of bacterial IgA1Ps cleave host IgA1 within the heavy-chain (HC) hinge region, the linker connecting the IgA1 constant fragment (Fc) to its antigen binding region (Fab) (Fig. 1a), leaving the light chain (LC) intact while separating the Fc and Fab. The shorter linker within IgA2 and IgGs renders them inaccessible to IgA1P cleavage\(^16\). Proteolysis by IgA1Ps serves two purposes: to prevent phagocytic killing by decoupling the IgA1 Fc recognized by neutrophils from the pathogen-recognizing Fab\(^6,17\) and to coat the bacterial surface with non-functional Fab fragments to effectively shield it from immune surveillance\(^18,19\). Despite the discovery of the metalloprotease class of bacterial virulence factors 30 years ago\(^20\), their structure and mechanism of substrate engagement has remained unknown until now.

In this work, we use cryo-electron microscopy (cryo-EM) to elucidate the structure of the S. pneumoniae IgA1P catalytic region alone and in complex with both its IgA1 substrate and a neutralizing monoclonal antibody (mAb), thereby addressing the molecular basis of substrate recognition and enzyme inhibition.

**Results**

The high-resolution structure of the IgA1P catalytic region. In order to identify the S. pneumoniae IgA1P catalytic domain, we engineered several constructs based on our previous results of limited proteolysis on the full, mature IgA1P (residues 154–1963, UniProt accession Q59947; NCBI accession WP_000417171) that corresponds to the common D39 and R6 strains\(^21,22\). Only constructs that began at or prior to residue 665 were accessible to enzymatic cleavage of the MBP tag by thrombin (Supplementary Fig. 1a) and there was an observed reduction in IgA1-cleavage for shorter constructs (Supplementary Fig. 1b). Thus, we focused our cryo-EM studies on a construct of IgA1P spanning residues 665–1963 that could be excised from its MBP tag and had comparable cleavage to the full-length IgA1P (Fig. 1b).

The 3D cryo-EM reconstruction of the S. pneumoniae IgA1P (residues 665–1963) resulted in a 3.8 Å resolution map (resolution shells are shown in Fig. 1c and structural data parameters and refinement statistics are presented in Supplementary Fig. 2 and Supplementary Table 1). The overall structure reveals that S. pneumoniae IgA1P is a multi-domain enzyme (Fig. 1d), broadly comprised of N-terminal (NTD; residues 665–1070), middle (MD; 1071–1611), and C-terminal domains (CTD; 1612–1963). The NTD can be further divided into a small subdomain (residues 665–769) attached by a long linker to a β-helix (residues 781–1070), a common structural motif found in several bacterial proteins\(^23\). A defining feature of this S. pneumoniae β-helix is both its relatively small size and the lack of protruding secondary structure elements from within the domain itself. For example, the β-helix of the functionally similar but structurally unrelated serine IgA1P from Haemophilus influenzae is about twice as long and has multiple domains that emanate from and return to the β-helical core\(^24\). The IgA1P MD and CTD have no structural similarity to any known protein to date, as indicated by the lack of structural similarity using DALI searches. The active site is formed between the MD and CTD domains that bifurcate the zinc-coordinating residues of the HEMTH motif (residues 1604–1608 in the MD) and a downstream E (E1628 in the CTD) (Fig. 1d, e).

Despite the unique structural folds of the IgA1P MD and CTD, the S. pneumoniae IgA1P active site comprises catalytically important residues within similar positions to that of the prototypical metalloprotease, thermolysin (Fig. 1f), which supports a conserved catalytic mechanism that is consistent with our previous biochemical studies\(^21\). Specifically, there are two side chains from IgA1P H1604, H1608, and the downstream E1628 coordinate the Zn atom and are homologous with thermolysin residues H142, H146, and E166, respectively. The Zn both polarizes the carbonyl group of the scissile peptide bond and activates the nucleophilic water molecule that is deprotonated by a fourth residue, E1605 (E143 in thermolysin)\(^25\).

Trapping an active IgA1P/IgA1 complex. In order to trap the S. pneumoniae IgA1P in complex with its IgA1 substrate, several challenges were overcome. We engineered an active-site mutant in the context of IgA1P 665–1963 that removes one of the zinc-coordinating residues, referred to as IgA1P-E1605A. While this mutation abrogates catalysis under catalytic concentrations of the enzyme (Supplementary Fig. 1b), catalysis was still observed during incubation at stoichiometric concentrations of the IgA1P-E1605A/IgA1 complex (Supplementary Fig. 1c). As EDTA was found to slow catalysis (Supplementary Fig. 1c), the IgA1P-E1605A/IgA1 complex was purified for cryo-EM studies in the presence of EDTA (Supplementary Fig. 1d). A cryo-EM 3D reconstruction of the complex resulted in a 3.5 Å resolution map (Resolution shells are shown in Fig. 2a and structural data parameters and refinement statistics in Supplementary Fig. 2 and Supplementary Table 1), which facilitated fits of the individual domains with subsequent refinement.

**Experimental Procedures**

**Trapping an active IgA1P/IgA1 complex.** In order to trap the S. pneumoniae IgA1P in complex with its IgA1 substrate, several challenges were overcome. We engineered an active-site mutant in the context of IgA1P 665–1963 that removes one of the zinc-coordinating residues, referred to as IgA1P-E1605A. While this mutation abrogates catalysis under catalytic concentrations of the enzyme (Supplementary Fig. 1b), catalysis was still observed during incubation at stoichiometric concentrations of the IgA1P-E1605A/IgA1 complex (Supplementary Fig. 1c). As EDTA was found to slow catalysis (Supplementary Fig. 1c), the IgA1P-E1605A/IgA1 complex was purified for cryo-EM studies in the presence of EDTA (Supplementary Fig. 1d). A cryo-EM 3D reconstruction of the complex resulted in a 3.5 Å resolution map (Resolution shells are shown in Fig. 2a and structural data parameters and refinement statistics in Supplementary Fig. 2 and Supplementary Table 1), which facilitated fits of the individual domains with subsequent refinement.

The high-resolution structure of the IgA1P/IgA1 complex. The structure of the IgA1P-E1605A/IgA1 complex reveals key details regarding their interaction that includes a gating mechanism that is described further below. Regarding the complex structure, the IgA1P NTD binds to the substrate IgA1 Fab (of the HC) on one end while the IgA1 PTD engages both IgA1 Fc monomers at the other end (Fig. 2b, c). Thus, the stoichiometry of a 1:1 complex can now be understood by the fact that a single IgA1P requires interactions with both IgA1 Fc monomers and therefore prevents two IgA1Ps from symmetrically binding two substrate IgA1s. In fact, weak density can be ascribed to the unbound Fab (Fig. 2a), supporting the fact that despite two IgA1 hinge sites may be available, only one is accessed at a time by IgA1P. The ability of IgA1P to broadly cleave polyclonal IgA1 is also explained by this structure, as IgA1P does not interact with the variable region of the IgA1 substrate (Supplementary Fig. 3).

As the highest resolution information on the complex was found within the active site, most of the IgA1 hinge backbone of the IgA1 hinge could be confidently modeled (Fig. 2d, e and Supplementary Fig. 2d). Following the numbering scheme of the...
initial fit IgA1 Fab and Fc models\textsuperscript{26,27}, the HC comprising residues 1–221 within the initial Fab model could be extended through the active site of IgA1P to residue S232 (Fig. 2d). Although there is no visible density from residues 233–240, which connects the remaining hinge within the IgA1 HC, it is clear that IgA1 residues P227 and T228 are properly aligned for cleavage within the IgA1P cleavage site consistent with previous biochemical studies\textsuperscript{28}. Thus, we were able to capture the intact active site within the active complex.

The IgA1P gating mechanism. Potentially the most striking finding of the \textit{S. pneumoniae} IgA1P/IgA1 complex is the conformational change associated with substrate binding (Fig. 3), which underlies an active-site gating mechanism. This large-scale movement is best illustrated by a superposition of the \textit{S. pneumoniae} IgA1P free and bound states (Fig. 3a), which results in a 7.2 Å RMSD for the whole enzyme but only a 1.5 Å RMSD when the NTD \( \beta \)-helix is excluded from the RMSD calculation. Thus, it is the NTD that repositions from a “closed” state in the absence of substrate to an “open” state allowing for binding of the IgA1 hinge region. In order to facilitate this domain rearrangement, two flexible loops are involved: IgA1P residues 770–783 and a 66-residue linker that connects the IgA1P NTD to the CTD (residues 1051–1116). These loops act as flexible tethers to the entire \( \beta \)-helix (779–1050), providing enough slack to allow for a shift of the \( \beta \)-helix by approximately 10 Å relative to the rest of the protease (Fig. 3b).

The number of interactions between the IgA1P NTD and MD are diminished upon this rearrangement, providing an energetic rationale for why the enzyme “snaps” back after product release. Such findings also led us to hypothesize that the IgA1P NTD may be soluble alone, similar to its G5 domain and CTD that have been previously shown to be independently folded\textsuperscript{21,22}. Indeed, we were able to engineer an NTD construct that gives rise to a well-dispersed \textsuperscript{15}N-HSQC analogous to the independently folded IgA1P CTD and the G5 domain (Supplementary Fig. 4), supporting our findings that the NTD is capable of decoupling from the MD during substrate binding. Thus, \textit{S. pneumoniae} IgA1P exhibits an active-site-gating mechanism in which the NTD \( \beta \)-helix plays a crucial role. Interestingly, a gating
mechanism has been proposed for the evolutionarily distinct serine-type IgA1P from *Haemophilus influenzae* IgA1P, but our structure of the *S. pneumoniae* IgA1P/IgA1 complex here provides direct experimental proof that the metalloprotease subgroup of IgA1Ps must undergo such a conformational change to facilitate substrate binding.

Blocking IgA1P active-site-gating through mAb binding. Considering the clinical potential of utilizing immunogenic regions of IgA1P to develop vaccines preventing *S. pneumoniae* infection, we present here evidence validating that such a strategy has the potential to block IgA1P activity. A comparison of the neutralizing activity of a previously developed mAb used to detect secreted IgA1P, referred to as mAb #1, and a currently produced mAb, referred to as mAb #2, reveals that the latter potently blocks IgA1P cleavage of IgA1 and this occurs at stoichiometric concentrations (Fig. 4a). Moreover, this neutralizing mAb also blocks IgA1 substrate binding (Fig. 4b). A 3D reconstruction of IgA1P in the presence of the neutralizing mAb Fab fragment (mAb #2) resulted in a 4.8 Å resolution map (resolution shells are shown in Fig. 4c and structural parameters and refinement statistics in Supplementary Fig. 2 and Supplementary Table 1), which facilitated an initial rigid body fit of the mAb #2 Fab with likely delineation of the individual VL-CL (variable light-constant light) and VH-CH1 (variable heavy-constant heavy 1) chains (Supplementary Fig. 5). The final refined model reveals that the neutralizing mAb simultaneously engages both the IgA1P NTD and MD domains (Fig. 4d, e), which constricts IgA1P to its closed conformation through intimate contacts with both domains (Fig. 4f, g) and thereby occludes substrate binding. Thus, these studies provide a proof-of-concept that IgA1P activity may be blocked by mAbs.
Discussion

We have determined the structure of a member of the M26 metalloprotease IgA1P subfamily of bacterial IgA1Ps both in isolation as well as in complex with its human IgA1 substrate and solved its structure with a neutralizing mAb that provides insight into blocking S. pneumoniae IgA1P activity. Other than the IgA1P NTD that comprises a β-helix identified in several bacterial proteins, both the IgA1P MD and CTD exhibit no structural similarity to any other previously solved proteins, much less any other metalloprotease. Despite this architecture, catalytic residues contributed by both the IgA1P MD and CTD that include H1604, E1605, H1608, and E1628 are directly comparable in their structural positioning to the same active site residues within the prototypical metalloprotease, thermolysin. The most striking discovery here is how the modular nature of this unique IgA1P facilitates a conformational change that acts as an active-site gate. Specifically, this conformational change can be described as “closed” for the free enzyme and “open” to allow for IgA1 substrate binding, whereby the mAb occludes substrate binding by binding the IgA1P in the “closed” conformation (Fig. 5). Our studies therefore provide the underlying mechanism by which this unique class of IgA1Ps engages IgA1 and reveals how IgA1Ps can broadly engage IgA1 through contacts with the constant region of the substrate IgA1 HC.

The complex structure with IgA1 also provides an understanding for how IgA1P can cleave the secreted forms of IgA1 (sIgA1) that are joined by the J-chain. Specifically, the Fc portions of each IgA1 that are coupled by the J-chain leave the IgA1 Fc binding site for IgA1P accessible for cleavage, as shown here by superimposing the bound IgA1 Fc within the IgA1P/IgA1 complex onto the recently solved structure of one of the sIgA1 Fc regions (Fig. 5).

Considering the sequence similarity of the S. pneumoniae IgA1P to many other proteins found within invasive bacteria and across multiple strains (Supplementary Fig. 6a), these studies provide an important foundation for understanding how multiple...
opportunistic pathogens that employ similar IgA1 metalloproteases cleave host IgA1 (Supplementary Fig. 6b). While our cryo-EM models demonstrate the specific site of the current neutralizing mAb is not completely conserved across such strains, there are several highly conserved epitopes that could serve for the creation of broad-spectrum vaccines or multiple exposed epitopes can now be developed to block multiple strains. Lastly, the similarity in the predicted domain structure of this IgA1P in comparison to proteases in the ZmpB and ZmpC classes of secreted metalloproteases suggests that the structural data provided here can generally inform upon the mechanisms of cleavage mediated by these related enzymes with possess divergent substrate selectivity.

Methods

Protein expression and purification. All S. pneumoniae IgA1P constructs of the full catalytic region were engineered and purified similar to that previously described for the mature IgA1P (residues 154–1963), with the exception that the MBP and 6xHis tags were swapped from their original positions21. The kanamycin-resistant plasmid, pJ401 (DNA2.0/Atum), was used as the backbone with the open reading frame coding for an N-terminal MBP, a thrombin cleavage site, IgA1P (residues 665–1963) was excised from the full antibody by use of immobilized Ficin (Thermo Scientific) and diluted using PBS. The substrate IgA1 Fc (PDB accession number 1OW0), and the substrate IgA1 Fab (PDB accession 3M8O), which was further refined (Fig. 2). As the IgA1 used was polyclonal, all variable residues within the Fab region of both its HC and LC were modeled as alanine residues (Supplementary Fig. 3). This did not alter the model of the IgA1P-E1605A/IgA1 complex were processed in both RELION31 and cryoSPARC23,31. Models for both complexes were built using Chimera. For IgA1P alone, the movies were motion corrected using MotionCor232 and their contrast transfer functions were estimated using Gctf34. Micrographs with resolution worse than 6 Å were discarded. ~1500 particles were manually picked and classified into 10 classes. Meaningful 5 representative class averages were selected and used for template picking. The particles were extracted with a box size of 336 × 336 pixels and subjected to 2D classification. Class averages were selected and subjected to ab initio reconstruction, 3D classification, and refinement. After particle polishing and CTF refinement, the final model for the substrate IgA1 Fab (PDB accession 3M8O), which was further refined (Fig. 2). As the IgA1 used was polyclonal, all variable residues within the Fab region of both its HC and LC were modeled as alanine residues (Supplementary Fig. 3). This did not alter the model of the IgA1P-E1605A/IgA1 complex.

Cryo-EM data processing and structural modeling. Data for the free S. pneumoniae IgA1P (residues 665–1963) were processed in RELION31 and that of the S. pneumoniae IgA1P/E1605A/IgA1 complex were processed in both RELION and cryoSPARC23,31. The final model was complete in the PHENIX final model was complete in the PHENIX37,38.

NMR sample preparation and data collection. All NMR data were collected on a Varian 900 equipped with a cryoprobe. Samples were produced as previously described using 2H2O M9 minimal media for the CTD (residues 1611–1963) and the NTD (residues 665–1010). Final size exclusion was performed in NMR buffer (50 mM NaHPO4, pH 6.5, and 150 mM NaCl).
Protease neutralization assay. 96 well microtiter plates were coated with anti-IgA1 CH3 (catalog number ab17747, Abcam; Cambridge, MA) (2 mg/ml overnight at 4 °C, washed, and blocked with PBS-TBSA for 2 h at room temperature. Wild type IgA1P residues 154–1963 (286 ng/ml; 1.3 nM) was incubated overnight with the indicated doses of the neutralizing mAb at 4 °C. Human IgA1 substrate (catalog number MBS18189, mybioscience; San Diego, CA) alone or with equal volumes of the mAb/IgA1 mixture were incubated for 1 h at 37 °C and added to the plates for 2 h at room temperature. After washing, alkaline phosphatase-labeled goat anti-human Kappa (catalog number A38913, Sigma; St. Louis, MO) or goat HRP-labeled anti-human IgA CH3 (Abcam; Cambridge, MA) was added for an hour at room temperature. Intact or cleaved IgA1 was detected by the presence or absence of bound kappa light chain using alkaline phosphatase-labeled goat anti-human Kappa (Sigma; St. Louis, MO), which is lost by IgA1 cleavage. IgA1 binding was normalized with HRP-labeled goat anti-human Fca. Both are read at 405 nm in separate wells. Plates were either developed with p-nitrophenyl phosphate substrate-AP (Sigma) or ABTS-HRP, respectively.

IgA1 binding assay. 96 well Nunc Maxisorp plates were coated with 1 μg/ml (4.6 nM) IgA1P-E1605A residues 154–1963 in PBS overnight at 4 °C, blocked for 2 h with PBS-Tween-20-Thimerol-0.5% BSA (PBS-TT-BSA) and incubated with 100 ng/ml human IgA1 (mybioscience; San Diego, CA), which was followed by the addition of 2 μg/ml mAb (14.6 nM). Human IgA1 that bound to the protease was detected with HRP-labeled goat anti-human IgA, developed with peroxide and ABTS, and optical densities measured at 405 nm with Veramax ELISA plate reader.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The cryo-EM maps are deposited in the Electron Microscopy Data Bank under accession codes “EMD-22205” (IgA1P residues 665–1963), “EMD-22204” (IgA1P residues 665–1963 with the single E1605A mutation in complex with IgA1), “EMD-23228” (IgA1P residues 665–1963 in complex with the mAb). Structure coordinates are deposited at the Protein Data Bank with accession codes 6XIA (IgA1P residues 665–1963), 6XIA (IgA1P residues 665–1963 with the single E1605A mutation in complex with IgA1), 7G7P (IgA1P residues 665–1963 in complex with the mAb). Source data is provided with this paper.

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Author contributions
Z.W. and H.Z. determined the 3D reconstructions and Z.W. refined IgA1P alone. N.T., T.H. and E.Z.E. refined both complexes. Y.C.C. began the initial cloning of the IgA1P with E.Z.E., J.R. and E.J. produced the mAb with J.R. and E.Z.E. purifying the product. J.S.R. and E.Z.E. purified all proteins and complexes.

Competing interests
The authors declare no competing interests.

Additional information
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