Simulation-Driven Design of Stabilized SARS-CoV-2 Spike S2 Immunogens

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Abstract

The full-length prefusion-stabilized SARS-CoV-2 spike (S) is the principal antigen of COVID-19 vaccines. Vaccine efficacy has been impacted by emerging variants of concern that accumulate most of the sequence modifications in the immunodominant S1 subunit. S2, in contrast, is the most evolutionarily conserved region of the spike and can elicit broadly neutralizing and protective antibodies. Yet, S2's usage as an alternative vaccine strategy is hampered by its general instability. Here, we use a simulation-driven approach to design highly stable S2-only antigens retaining a closed prefusion conformation. Weighted ensemble simulations provide mechanistic characterization of the S2 trimer's opening, informing the design of tryptophan substitutions that impart kinetic and thermodynamic stabilization. Alchemical free energy perturbation calculations and a corroborating set of experiments confirm that V991W and T998W in the central helices of S2 stabilize the trimer in the closed prefusion conformation, producing an antigen with increased protein expression, superior thermostability, and preserved immunogenicity against sarbecoviruses.

I. Introduction

Coronaviruses (CoVs) are a group of enveloped viruses featuring a positive-strand RNA genome.[1] Several human CoVs (HCoVs) belonging to the α (229E and NL63) and β (OC43 and HKU1) genera endemically circulate every year, causing upper respiratory illness.[2] Over the first two decades of the 21st century, outbreaks of other β-CoVs, such as the Middle East respiratory syndrome (MERS)-CoV and the severe acute respiratory syndrome (SARS)-CoV viruses, led to severe cases of respiratory illnesses characterized by a high mortality rate.[3] More recently, COVID-19—the disease resulting from infection with another β-CoV, SARS-CoV-2—has caused at least 770 million cases and 6.9 million deaths worldwide since the virus was first identified.[4] Vaccines have provided protection against severe COVID-19 disease, hospitalization, and mortality,[5,6] but their efficacy has been challenged by the continual emergence of several SARS-CoV-2 variants of concern (VOCs), including Alpha, Beta, Delta, Gamma, multiple Omicron sublineages BA.1, BA.2, BA.3, BA.4, BA.5, and the currently dominant EG.5, FL.1.5.1, and XBB.1.16.[7] Therefore, while bivalent boosters may help recover some degree of efficacy, a more direct approach of designing pan-coronavirus vaccines is required for combating the relentless evolution of this pathogen.[8]

The primary antigen used in most of the COVID-19 vaccines is an engineered version of the SARS-CoV-2 spike (S), a homotrimeric class I fusion glycoprotein stabilized in the prefusion state.[9,10] Prominently extending from the virus surface and responsible for cell entry,[11] the spike serves as the principal target of the host's humoral immune response. The spike is composed of two subunits, S1 and S2. The former initiates viral entry by engaging host cell receptors, and the latter mediates fusion between viral and host membranes upon cleavage at the S1/S2 furin site, shedding of S1, further proteolytic cleavage at the S2' site, and refolding from a prefusion to postfusion conformation.[9–11] On one hand, S1 is immunodominant as it encompasses the two domains eliciting the majority of anti-CoV neutralizing antibodies,[12–15] the N-terminal domain (NTD) and the receptor-binding domain (RBD). On the other hand, due to selective immune pressure,[16,17] most of the mutations across different CoV strains and SARS-CoV-2 VOCs occur in S1.[2,18–20] As a result, the acquired humoral immunity gained from S1-directed antibodies is often closely related to the specific CoV strain or SARS-CoV-2 VOC responsible for the infection. Conversely, the S2 subunit is highly conserved and less prone to accumulate sequence modifications, offering the opportunity for the design of S2-based immunogens that provide

broader protection against different SARS-CoV-2 VOCs and, possibly, CoV strains.[21–24] Several studies have characterized cross-reactive antibodies targeting the S2 subunit's stem helix,[18,21,23,25] fusion peptide,[24] and the usually occluded "hinge" region at the trimer apex spanning residues 980-1006.[26–28]

Although immunogenically appealing, prefusion S2 is metastable as it irreversibly transitions to a stable, postfusion conformation when S1 is removed.[29] The S2 trimer adopts an elongated baseball-bat-like conformation, where the heptad repeat 1 (HR1) and central helices (CHs) refold at the hinge region, transitioning from a bent hairpin into a continuous, extended coiled-coil helical bundle.[29,30] This structure is framed by the HR2 motif at the helix-helix interfaces, yielding a rigid six-helix bundle in the regions where HR2 maintains its helical fold.[29,30] Introduction of two consecutive prolines within the HR1/CH hinge point (K986P and V987P) in the S2 subunit of the engineered SARS-CoV-2 'S-2P' spike proved a successful strategy to prevent progression to the postfusion state.[9,31] Further stabilization of the prefusion conformation, along with improved cellular expression, has more recently been accomplished upon the incorporation of four additional prolines in the S2 subunit (F817P, A892P, A899P, A942P), resulting in the SARS-CoV-2 'HexaPro' spike.[32] In this construct, the three S1 subunits sit atop and seal together the HexaPro S2 subunits nestled underneath. Interestingly, previous HDX-MS experiments showed that the SARS-CoV-2 HexaPro spike can also sample an open conformation where the S2 trimer interfaces become solvent accessible.[28] This may suggest that, in the absence of S1, the Hexapro S2-only trimer would most likely dissociate into individual monomers. To stabilize the SARS-CoV-2 HexaPro S2-only antigen, McLellan and coworkers introduced an interprotomer disulfide bond via two cysteine substitutions (S704C and K790C) and an interprotomer salt bridge with R765 upon Q957E substitution. We note that at the time of writing, the aforementioned work by McLellan and coworkers is under review for publication. The resulting S2-only construct, renamed HexaPro-SS-Δstalk (Fig. 1), adopted a prefusion conformation where trimer dissociation was prevented. Although stabilized in the prefusion conformation, the HexaPro-SS-Δstalk apex was shown to splay apart, adopting semi-open and open conformations. This suggests the potential to further enhance its stability by shifting the equilibrium towards a closed conformation.

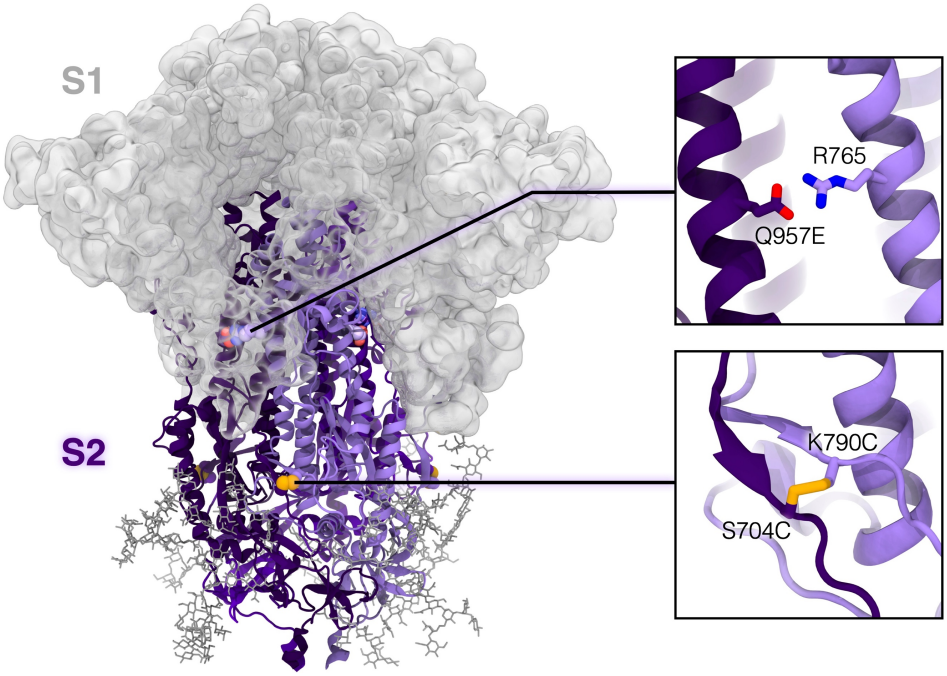

Fig. 1. HexaPro-SS-Δstalk S2-only antigen and context within the SARS-CoV-2 spike protein. HexaPro-SS-Δstalk (cartoon representation with protomers highlighted with different shades of purple on the left panel) is an S2-only construct derived from the prefusion-stabilized SARS-CoV-2 HexaPro spike. The spike's S1 subunit, here depicted with a gray transparent surface, is not present in this construct. N-glycans linked to S2 are shown as dark grey sticks. Glycans linked to S1 are omitted for clarity. The substitutions (S704C, K790C, Q957E) incorporated in HexaPro-SS-Δstalk are highlighted in the right panels. They stabilize S2 in a prefusion trimer assembly, preventing protomer dissociation via formation of interprotomer salt bridges and disulfide bonds.

Opening of the trimer, or "breathing," is a motion observed in several class I fusion glycoproteins, including influenza hemagglutinin (HA),[33,34] HIV-1 Env,[35] respiratory syncytial virus (RSV) F,[36] as well as the full-length SARS-CoV-2 spike.[26–28] Silva et al.[26] showed that accessibility of the full-length SARS-CoV-2 spike's hinge epitope necessitates both RBD transitioning to the 'up' conformation and S2 subunits 'breathing.' This aligns with HDX-MS experiments showing the existence of an open conformation of the full-length spike where the S2 apex is solvent-exposed.[28] However, S2 apex epitopes elicit non-neutralizing antibodies,[26,27] which are dominant in the B cell population[27,37] and can interfere with binding of neutralizing antibodies.[38,39] This is also the case for other trimer interface epitopes in influenza HA[34] and human metapneumovirus (hMPV) F.[37] Hence, preventing the S2 trimer's apex opening may offer the foundations for the design of improved S2-based immunogens that may also afford binding and elicitation of quaternary epitope-specific antibodies.

Here we use a simulation-driven approach to characterize the opening mechanism of the SARS-CoV-2 S2 trimer and design an S2-based immunogen that is stable in the closed prefusion conformation. Weighted ensemble[40] (WE) molecular dynamics (MD) simulations allowed us to characterize how the S2 trimer apex splays open along an asymmetric pathway. We used this dynamical information as a basis to predict two cavity-filling tryptophan substitutions (V991W and T998W) that alter the conformational landscape of S2, stabilizing the closed prefusion conformation. These substitutions also provide enhanced thermostability and cellular expression, allowing complete structural characterization via cryo-EM. Subsequent neutralization assays showed that sera from mice immunized with the newly designed S2 antigen significantly neutralizes recombinant vesicular stomatitis viruses (rVSVs) bearing spikes from SARS-CoV-2 Wuhan-1 and Omicron BA.1. Overall, by integrating cutting-edge computational methodologies and a comprehensive array of experiments, our work offers a thorough understanding of the mechanistic, structural, dynamical, and immunogenic aspects of novel, aptly engineered S2-only antigens. The findings presented here open new avenues for devising improved S2-based immunogens, thereby holding promise for the development of pan-coronavirus vaccines.

II. Results and Discussion

Prefusion-stabilized S2 asymmetrically transitions to a splayed open conformation.

To probe the stability of the prefusion-stabilized HexaPro-SS-Δstalk S2-only base construct, we first sought to characterize the conformational plasticity of the closed conformation within the nanosecond timescale by performing all-atom conventional MD simulations. A model of HexaPro-SS-Δstalk in the closed conformation was built from the S2 domain of the cryo-EM structure of the SARS-CoV-2 HexaPro spike (PDB ID: 6XKL[32]) integrated with the fusion peptide's residue coordinates grafted from the SARS-CoV-2 3Q-2P spike cryo-EM structure (PDB ID: 7JJI[41]). The distinctive substitutions engineered in HexaPro-SS-Δstalk (i.e., S704C, K790C, Q957E) were then incorporated using Visual Molecular Dynamics (VMD),[42] and S2's N-linked glycans were added as described by Casalino et al.[11] The final system (Fig. 1), comprising ~274,000 atoms with explicit water molecules and ions, was subjected to four, ~600 ns-long MD simulation replicates. In these simulations, we observed one protomer's apex transiently swinging away from the other two protomers (Fig. S1); however, the trimer did not adopt an open

conformation where the three protomers are splayed apart. Notably, the slight detachment of one protomer from the others was observed only once throughout a combined sampling of 2.6 μs, suggesting that trimer opening is a rare (or infrequent) event when investigated by means of conventional MD. Thus, to elucidate the complete transition of HexaPro-SS-Δstalk from a closed conformation to an open conformation, an enhanced sampling method was necessary.

Exploration of a system's conformational space, such as the one conducted with conventional MD, is limited by high free energy barriers that separate distinct minima in which a system may remain trapped. As large conformational transitions occurring in the millisecond-to-second timescale are computationally prohibitive via conventional MD, enhanced sampling methods may be employed instead.[40,43,44] Therefore, we first attempted to inquire into the conformational transition of HexaPro-SS-Δstalk from the closed to the open conformation using Gaussian accelerated MD (GaMD). GaMD is a method that applies a harmonic boost potential to smooth energy barriers on the potential energy surface, expanding the accessibility of long-timescale dynamic motions.[44] However, we found that the applied boost potential at the highest acceleration level was insufficient to induce sampling of open conformations (Fig. S1, Tables S1, S2). Hence, supported by our previous success in studying SARS-CoV-2 spike's RBD down-to-up transition,[45] we opted to investigate the opening of the HexaPro-SS-Δstalk S2 trimer using the WE path-sampling method.[40,46] By running many short (0.1 ns) stochastic MD simulations in parallel, and iteratively replicating trajectories that have explored new regions of configurational space defined by appropriately binned progress coordinates, the WE strategy facilitates atomically detailed sampling of large conformational changes without alteration of the system's free energy.[47]

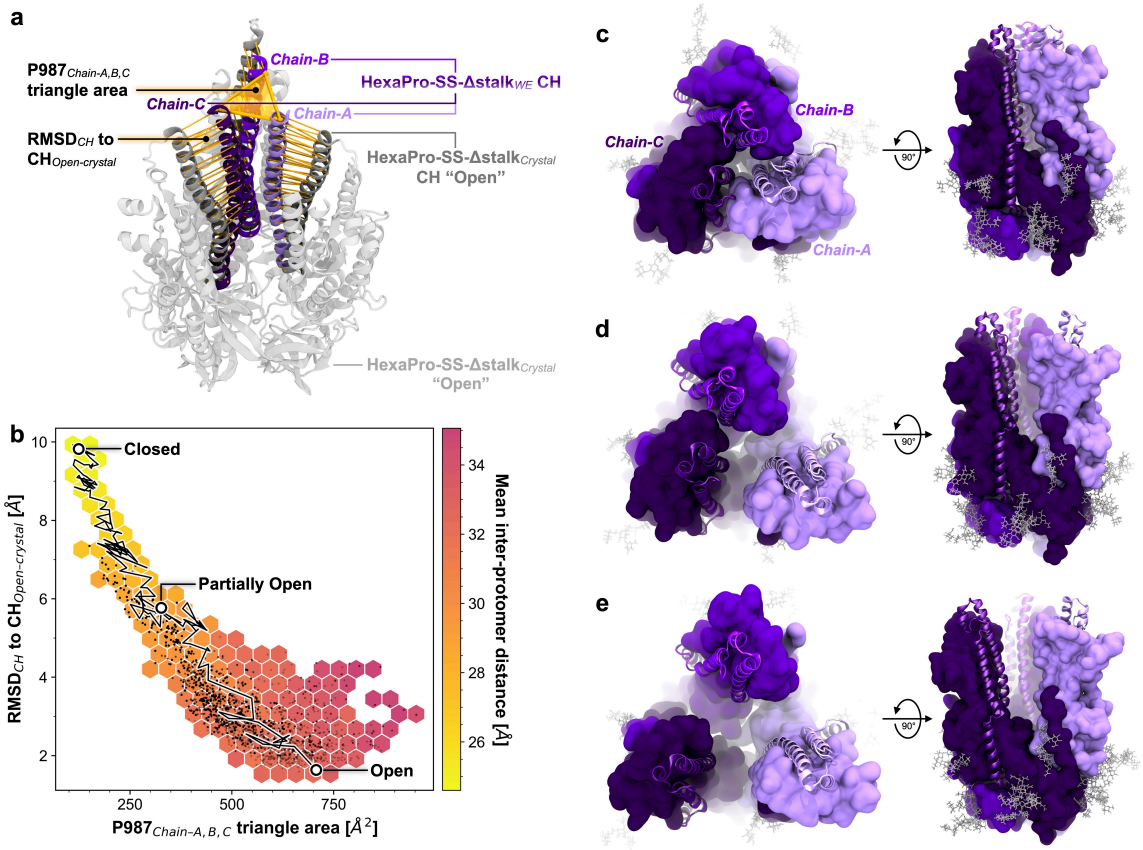

Fig. 2. HexaPro-SS-Δstalk S2 trimer opening. a, Progress coordinates used in the WE simulation of HexaPro-SS-Δstalk, namely P987$_{C\alpha}$ triangle area and RMSD$_{CH}$, are highlighted with yellow shapes (triangle and cylinders, respectively) drawn on top of the molecular structures. The CHs as in the closed conformation of the simulated construct are shown with shades of purple and are overlayed on the open conformation crystal structure (colored in grey). **b**, Distribution of the conformations sampled in the opening pathways obtained from the WE simulation. Each black point represents a conformation sampled along an opening pathway. The RMSD of the CH to CH$_{Open\text{-}crystal}$ (y-axis) is plotted against the area of the triangle formed by the P987$_{C\alpha}$ at the CH apex (x-axis). The hexagonal bin color is scaled to the mean interprotomer distance of the black data points within the respective bin. A trace of one of the successful pathways is shown as a black line with white points corresponding to the closed, partially open, and open conformations. **c-e**, Molecular representation of closed (**c**), partially open (**d**), and open (**e**) conformations as highlighted in (**b**). Chain-A is depicted in light purple, chain-B in purple, and chain-C in dark purple. The CHs and upper helices (UHs, residues 945-977) are illustrated with a cartoon representation, whereas the rest of the chain is shown as an opaque surface. Glycans are shown as grey sticks.

To enhance sampling of open conformations of the initially closed HexaPro-SS-Δstalk construct, we defined two progress coordinates (Fig. 2a,b): i) the area of the triangle formed by the Cα atoms of the three P987 residues (one for each protomer) residing at the apex of the CHs, and ii) the RMSD of the CHs with respect to the CHs as in the open conformation. We remark

that, at the time of writing, the crystal structure of HexaPro-SS-Δstalk in the open conformation, resolved by McLellan and coworkers and made available beforehand for this study, is currently under review. WE simulations of HexaPro-SS-Δstalk were performed with WESTPA 2.0[48,49] and Amber20,[50] generating 180 continuous opening pathways across 45 μs of aggregate sampling time (for a complete description of WE simulations see Materials and Methods in the SI, Fig. S2, Table S3).

The wealth of atomic-level information provided by these simulations allowed us to characterize the mechanism by which the HexaPro-SS-Δstalk S2-only immunogen adopts a splayed open conformation (Video S1). Starting from a closed conformation (Fig. 2c), the initial detachment of chain-A forms an asymmetric, partially open conformation (Fig. 2d) until chain-B and chain-C separate to form the open conformation (Fig. 2e, Video S1). Notably, asymmetric breathing was also recently reported for influenza HA.[33] In the open conformation, the distances between the P987 residues increase from values close to ~20 Å (as in the closed conformation) to values in the range of ~35 to ~40 Å. Interestingly, we also observed further open conformations where at least one of the trimer's protomers kept moving further away (Fig. S3). The opening pathways were used for analyzing the contacts at the interfacial helices in the closed, partially open, and open conformations (Fig. 3a-c and Figs. S4-S6). Using PyContact[51], we examined interprotomer residue-residue interactions involving the CHs (residues 978-1030), upper helices (UHs, residues 738-783), and HR1 helices (residues 945-977). S2 trimer opening occurs upon a progressive breakage of interprotomer residue-residue interactions within the CHs from apex to base, following a trend from hydrophilic to hydrophobic (Fig. 3c). A salt bridge between R995 and D994 breaks before the hydrogen bond network consisting of Q1002, Q1005, T1006, and T1009 is broken until the loss of the hydrophobic interactions between L1012 and I1013 leads to the separation of chain-A. As expected, the helices in chain-A did not form strong interactions in the partially open conformations, and interactions throughout the trimer were almost null in the open conformation (Fig. 3c and Figs. S4-S6). The partially open conformations are characterized by $CH_{Chain\text{-}B}$ forming an extensive interaction interface with the polar residues along $UH_{Chain\text{-}C}$ (Fig. 3c, Figs. S4-S6). Interestingly, residues V991 and T998 in the CHs did not contribute to stabilizing interprotomer contacts in either the closed or partially open conformations (Fig. 3a-c). On the contrary, residues D994, R995, and Q1002, which are one or two helical turns away from V991

and T998, form stabilizing interprotomer interactions in both the closed and partially open conformations (Fig. 3a-c).

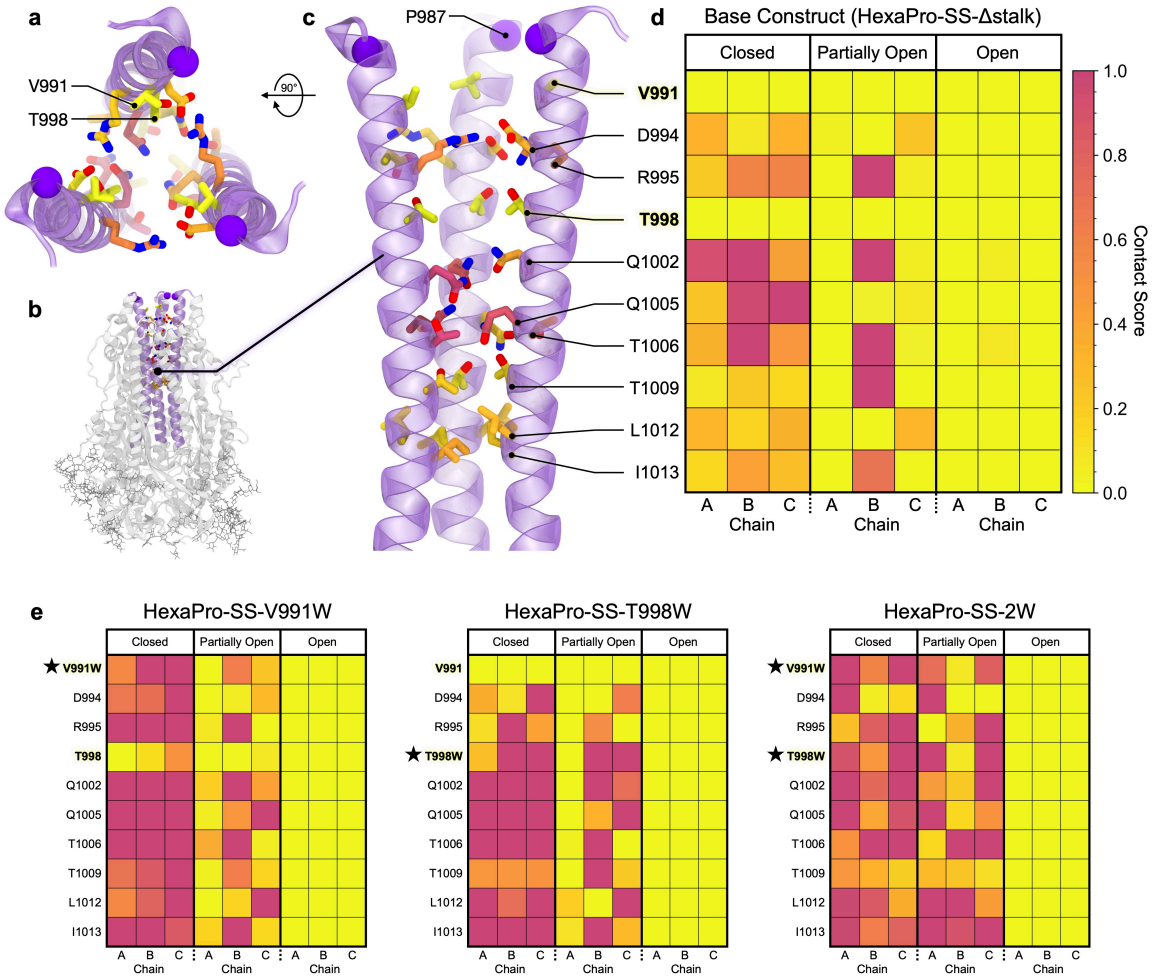

Fig. 3. Contacts between residues in the central helices during S2 opening. a-c, HexaPro-SS-Δstalk CHs in the closed conformation from a top-down view (**a**), side view in the context of the S2 trimer (gray cartoons) (**b**), and side view (**c**). CHs are highlighted with purple cartoons. The sidechains of the residue pointing toward the interior of the CHs are shown as sticks with the C atoms colored by the corresponding residue contact score as in the closed conformation of the HexaPro-SS-Δstalk. **d,e,** Contact score heat maps for the HexaPro-SS-Δstalk base construct (**d**) and HexaPro-SS-Δstalk tryptophan substitutions (**e**) in the closed, partially open, and open states. The scale ranges from 0 (weak or transient contacts) to 1 (persistent or extensive contacts, corresponding to the 95th percentile value of the HexaPro-SS-Δstalk contact scores). The star indicates the substituted residue.

The opening mechanism of HexaPro-SS-Δstalk underscored two residues located in the proximity of the CH apex, V991 and T998, as best candidates for cavity-filling substitutions aimed at improving the stability of the closed conformation. Cavity-filling is a common strategy utilized to stabilize proteins by introducing hydrophobic side chains to occupy hollow regions within the

protein.[52,53] This approach has been previously applied to other viral glycoproteins such as RSV F, HIV-1 Env,[53,54] and SARS-CoV-2 S1.[55] Intrigued by this observation, we hypothesized that introducing a bulky hydrophobic sidechain at positions 991 and 998, such as in tryptophan, may contribute to forming novel, interprotomer, stabilizing interactions, which, in turn, could slow down opening or fully stabilize S2 trimer in the closed prefusion conformation.

Tryptophan cavity-filling substitutions slow down prefusion-stabilized S2 trimer opening.

Aiming to stabilize the S2 trimer in the closed prefusion conformation, we designed three HexaPro-SS-Δstalk tryptophan variants. Three engineered models in the closed conformation were generated from the base construct: i) *Hexapro-SS-V991W*, incorporating V991W substitution; ii) *Hexapro-SS-T998W*, including T998W substitution; iii) a double substitution variant, named *Hexapro-SS-2W*, containing both V991W and T998W substitutions. In a similar way to the base construct, we performed WE simulations of the three HexaPro-SS-Δstalk tryptophan variants to interrogate their stability and, possibly, opening mechanism. Interestingly, each engineered S2 trimer swung away from the initial closed conformation to sample partially open and open conformations. We collected 149, 187 and 195 opening pathways across 35, 47, and 56 μs of aggregate sampling for Hexapro-SS-V991W, Hexapro-SS-T998W, and Hexapro-SS-2W, respectively (see Table S3, and Figs. S7-S10). The closed-to-open transition is consistent across all four simulated S2 constructs, with chain-chain separation proceeding stepwise. Although opening occurred, it is important to note that WE is a path-sampling approach used to enhance the sampling efficiency of conformational transitions that are rarely observed by means of conventional MD.[40] That is, sampling an open conformation does not itself imply that the closed conformation is unstable; rather, the WE method allowed us to observe a slow dynamic behavior that is physically accessible to the protein.

Our WE simulations indicate that trimer opening is impacted by the introduction of the V991W and T998W substitutions. There are differences in the aggregate sampling time to observe the first opening event during WE simulations (i.e., including the time spent in the closed state) (Fig. S11a), with Hexapro-SS-T998W and Hexapro-SS-2W requiring an additional ~6.3 μs and ~4.9 μs of sampling, respectively, and a corresponding shift toward overall longer molecular times to opening (Fig. S11b). This may also suggest more time spent in the closed state. In contrast, Hexapro-SS-V991W necessitates less sampling to open for the first time, approximately ~1.5 μs

less than the base construct. Analysis of opening event durations as a measure of the molecular time required by the system to transition from the closed state to the open state (i.e., excluding the time spent within the closed state), shows a distribution slightly shifted toward longer durations for the three tryptophan variants (Fig. S12), with Hexapro-SS-T998W exhibiting the longer mean duration followed by Hexapro-SS-2W (Table S3). In this regard, no significant differences are observed for Hexapro-SS-V991W with respect to the base construct, suggesting that V991W might not affect the opening kinetics. One advantage of WE simulations is the possibility of estimating kinetics quantities such as rate constants underlying the investigated transition.[48,56–58] In our case, by iteratively reweighting the WE trajectories using the WE Equilibrium Dynamics (WEED)[56,58] protocol within WESTPA 2.0[48] to facilitate the convergence of WE simulations toward equilibrium (Figs. S2, S7-S9),[56,57] we were able to estimate the rate constant of the opening transition ($k_{opening}$) for each simulated system (see Material and Methods in the SI for a detailed description, Fig. S13, Table S3). We estimated that the opening of the HexaPro-SS-Δstalk S2 trimer occurs on the order of fractions of milliseconds [$k_{opening} = (4.5 \pm 1.5) \times 10^4$ s^{-1}]. Although the rate constant does not significantly change for Hexapro-SS-V991W [$k_{opening} = (1.1 \pm 0.5) \times 10^5$ s^{-1}], it becomes smaller for Hexapro-SS-2W [$k_{opening} = (7.0 \pm 2.3) \times 10^3$ s^{-1}] and Hexapro-SS-T998W [$k_{opening} = (3.4 \pm 0.1) \times 10^3$ s^{-1}], indicating that T998W induces a roughly 10-fold slower trimer opening. V991W alone does not appear to affect opening kinetics. Although we acknowledge that these estimations might be affected by sampling limitations, the calculated values align well with each other and single-molecule fluorescence resonance energy transfer (smFRET) studies reporting similar domain movements of class I fusion glycoproteins occurring in the millisecond-to-second time scales.[59–61]

As previously performed for the base construct (Fig. 3d), we looked at the interprotomer residue-residue interactions for the tryptophan variants to gain a mechanistic understanding of the impact of V991W and T998W on the opening pathways. As highlighted from the contact heatmaps in Fig. 3e and Figs. S4-S6, both V991W and T998W substitutions boost interprotomer interactions in the closed and partially open conformations with respect to the base construct. These interactions promote engagement at the respective substituted positions and the nearby residues located a few helical turns away (Fig. 3e). The increased interhelix interplay observed in the partially open conformations may underlie the longer opening durations observed in Hexapro-SS-T998W and Hexapro-SS-2W. Interestingly, when only position 991 is substituted to tryptophan

(i.e., in Hexapro-SS-V991W), it does not induce slower opening kinetics despite furthering interprotomer engagement. This could be ascribed to the apical position of W991, which is more solvent-exposed than W998 and, in the absence of a partner hydrophobic residue underneath (i.e., W998 in Hexapro-SS-2W), is less kinetically stabilizing than W998.

V991W and T998W substitutions stabilize S2 in a closed prefusion conformation.

Beyond characterizing the trimer opening kinetics for the tryptophan variants, we sought to investigate whether V991W and T998W substitutions energetically stabilize HexaPro-SS-Δstalk in the prefusion, closed conformation. Computationally, this can be probed through "alchemical" FEP calculations where an amino acid (i.e., V or T) is gradually morphed into another one (i.e., W) in a set of non-equilibrium MD simulations, both in the context of the folded and unfolded protein (see Material and Methods in the SI for a complete description).[62] Relying on the notion that the free energy of a system is a state variable, it is possible to calculate the difference in the free energy of folding ($\Delta\Delta G_{mutation\text{-}folding}$) between the investigated variant and the base construct (HexaPro-SS-Δstalk), as illustrated in the thermodynamic cycle in Fig. S14. Here, starting from an equilibrated closed conformation of HexaPro-SS-Δstalk, we performed alchemical FEP calculations to predict the energetic contribution of V991W and/or T998W in the three engineered tryptophan variants (Fig. S15). As a result, in all three cases, the introduced substitutions are stabilizing (Fig. 4a). In ascending order (i.e., from the least to the most stabilizing), calculated $\Delta\Delta G_{mutation\text{-}folding}$ for Hexapro-SS-V991W, Hexapro-SS-T998W, and Hexapro-SS-2W, are -2.76 ± 0.11, -8.91 ± 0.26, and -12.18 ± 0.34 kcal/mol (Fig. 4a, Table S3). Hence, these independent simulations predicted the double substitution variant, Hexapro-SS-2W, to be the most stable in the closed prefusion conformation.

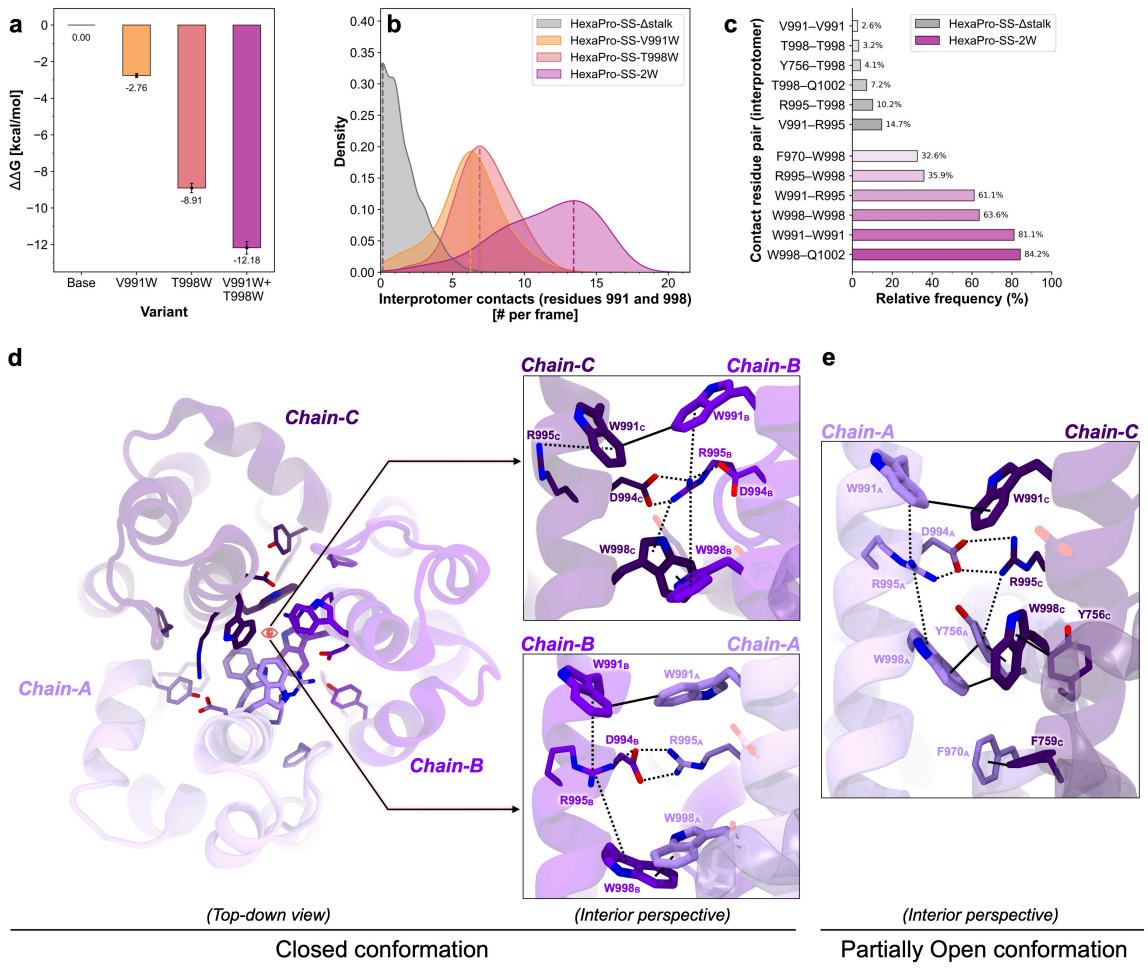

Fig. 4. V991W and T998W stabilize HexaPro-SS-Δstalk in the closed conformation. a, Thermodynamic stabilization of the closed conformation as imparted by each tryptophan variant relative to the base system (HexaPro-SS-Δstalk). Values of $\Delta\Delta G_{mutation\text{-}folding}$ are calculated via alchemical FEP calculations and reported in kcal/mol. Error bars denote uncertainties calculated via bootstrapping. **b,** Distribution of the number of interprotomer contacts established per frame by residues at positions 991+998 from the ensemble of closed conformations extracted from respective WE simulations. Distributions are shown as kernel densities. **c,** Relative frequency of occurrence (%) of the top six residue–residue interprotomer contacts occurring in the closed conformations observed in HexaPro-SS-Δstalk and HexaPro-SS-2W WE simulations. Relative frequencies are calculated with respect to the total number of closed conformations extracted from the respective WE simulation. **d,e,** Molecular representation of the most important interprotomer contacts in HexaPro-SS-2W in (**d**) the closed conformation and (**e**) the partially open conformation. Chain-A is depicted in light purple, chain-B in purple, chain-C in dark purple, and interacting residues are highlighted with sticks. Solid lines connect residues that form hydrophobic (π–π) interactions, whereas dashed lines indicate electrostatic (cation–π and salt bridge) interactions. A representative closed conformation of HexaPro-SS-2W was selected for this purpose upon clustering of closed conformations retrieved from the respective WE simulation (Fig. S16).

For each system, we extracted all the closed conformations that were sampled during the respective WE simulation, including those that did not result in an opening event. Using this multi-microsecond-long conformational ensemble of closed conformations, we then analyzed interprotomer contacts involving CHs, UHs, and HR1 helices to decipher, at the molecular level, the stabilizing effect imparted by the tryptophan substitutions (Figs. S17-S19). Introducing V991W and T998W into HexaPro-SS-Δstalk directly contributes to novel stabilizing contacts, enhancing electrostatic and hydrophobic interaction networks (Figs. 4b-e, S17-S19, Video S2). The tryptophan side chains at positions 991 and 998 self-associate to form π–π hydrophobic clusters at the S2 apex surrounding R995 (Fig. 4c-e, Video S2). Notably, alongside forming a salt bridge with D994 from the adjacent protomer, the side chain of R995 can also adopt an inward-oriented conformation, where it engages in a π–cation–π stack with W991 and W998 from both the same and neighboring protomers (Figs. 4d-e, S17, S18, Video S2). W998 is sometimes observed to swivel slightly outward, interacting with Y756 and F759 of the same chain and F970 from the adjacent chain. Interestingly, an extensive network of interactions is also preserved in the partially open conformations obtained from the WE simulation, where the presence of intra-protomer π–π and cation–π interactions modulate the orientation of the tryptophans' sidechain (Fig. 4e). Most importantly, the extensive network of π–π and cation–π interactions at the S2 apex is absent in the HexaPro-SS-Δstalk base construct (Figs. 4c, S17, S18). As these interactions in the closed state are not chain-specific, this suggests that the synergistic residue coordination in the S2 interior can dynamically exchange interactions to stabilize a closed conformation.

To experimentally probe the computational predictions, all three tryptophan variants were assessed relative to HexaPro-SS-Δstalk for expression yield in FreeStyle 293F cells (Fig. 5a,b) and thermostability through differential scanning fluorimetry (DSF) (Fig. 5c). In line with the FEP-predicted free energy trend, expression of HexaPro-SS-2W and, to a lesser extent, HexaPro-SS-T998W, led to higher yields than the parent. In addition, the slightly right-shifted peaks of their size-exclusion chromatography (SEC) profiles hint to more compact structures (Fig. 5b). V991W alone does not significantly improve the expression yield of HexaPro-SS-V991W relative to the base construct. A similar trend is observed from the thermostability profile (Fig. 5c), where HexaPro-SS-2W exhibits the highest melting temperature relative to the control systems (~ +6 °C shift), followed by HexaPro-SS-T998W, and HexaPro-SS-V991W.

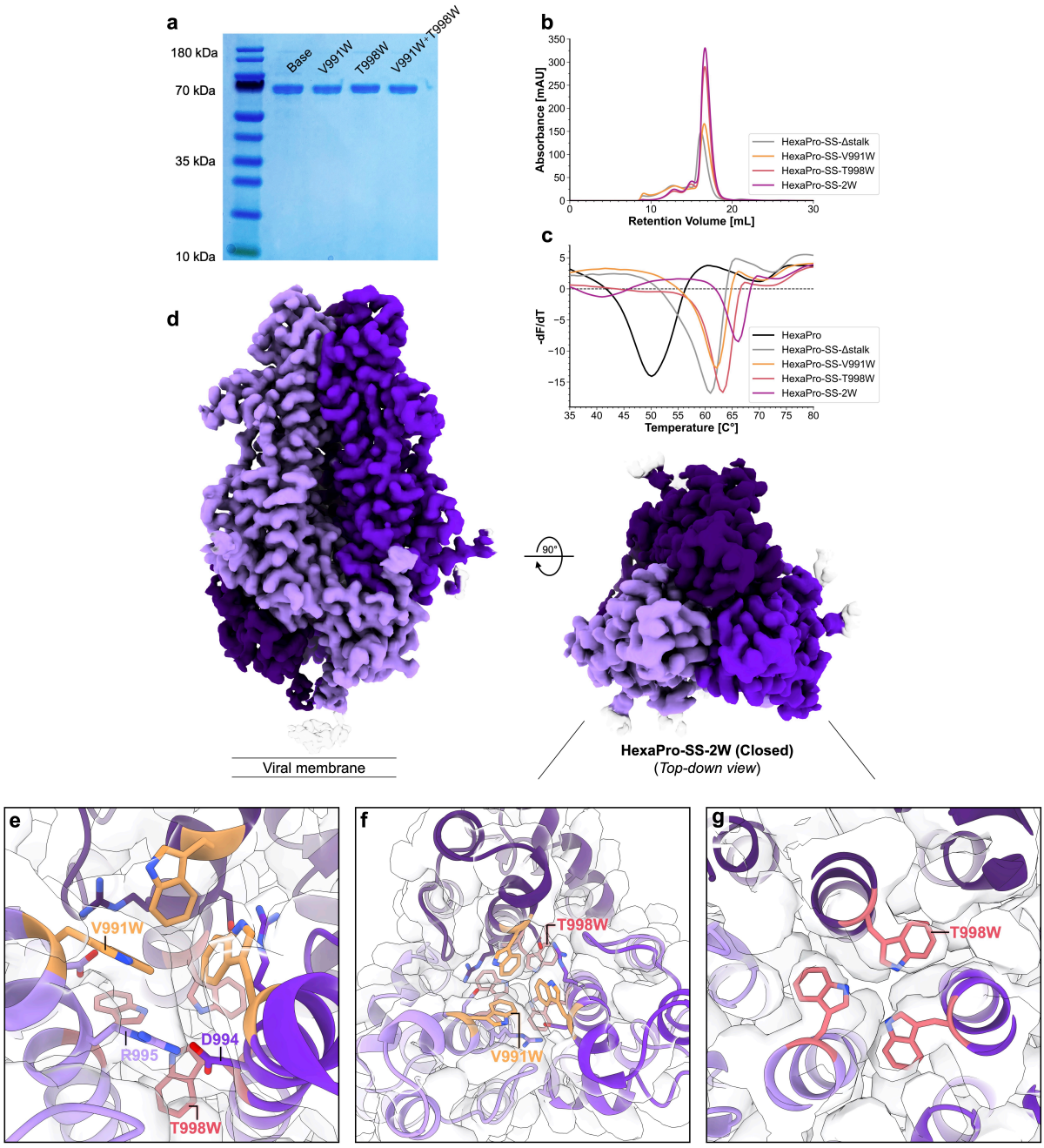

Fig. 5. Cellular expression, thermostability, and structural characterization of HexaPro-SS-2W. a, SDS-PAGE of purified S2 constructs (HexaPro-SS-Δstalk, HexaPro-SS-V991W, HexaPro-SS-T998W, and HexaPro-SS-2W). **b**, Size-exclusion chromatography (SEC) of purified S2 constructs. Both tryptophan substitutions increase protein expression yield, with V991W+T998W and T998W resulting in a greater increase. **c,** Differential scanning fluorimetry of S2 constructs, including the original HexaPro (S1+S2). HexaPro-SS-2W exhibits superior thermal stability to all HexaPro constructs, with a ~16 °C increase in T_m relative to HexaPro. **d,** Cryo-EM map of closed prefusion state HexaPro-SS-2W (Δstalk) from side (left) and top-down (right) perspectives. **e-g**, Top-down perspective of HexaPro-SS-2W structure highlighting V991W (orange) and T998W (red) packing within the S2 interior; the

tryptophan sidechains self-associate between chains in offset edge-to-edge and edge-to-face π–π stacking orientations. The EM map is shown as a transparent gray volume.

Based on the SEC data that indicated a ~2-fold increase in purified protein, we attempted to characterize the 3D structures of both HexaPro-SS-T998W (Δstalk) and HexaPro-SS-2W (Δstalk) using cryo-EM. For the former, we did not obtain a high-resolution cryo-EM structure; indeed, the low-resolution 3D ab-initio map shows twisted S2 single protomers (data not shown). On the other hand, for HexaPro-SS-2W we successfully generated a high-resolution cryo-EM map from 1,622 exposures collected on a 200 kV Talos Glacios. The cryo-EM map of the HexaPro-SS-2W was processed with C3 symmetry and DeepEMhancer[63] sharpening. 2D classes, especially the top-down views, indicate that the closed prefusion conformation is predominant in HexaPro-SS-2W (Fig. S20). This result is consistent with FEP calculations and DSF experiments. The structure highlights key residue interactions also observed in the WE simulation, showing a densely packed cluster of hydrophobic residues at the S2 trimer's apex (Figs. 5e-g). In this region, an extended network of π–π interactions encompassing W991, W998, Y756, F759, and F970 surrounds an ordered salt bridge (D994–R995) (Figs. 5e, S21). Examining the cryo-EM density of each mutation, V991W at the very apex of the S2 trimer shows clear π–π interactions with R995 (Fig. 5f). T998W shows clear cryo-EM density of the side chain, filling the cavity in between the CHs of the S2 trimer (Fig. 5g). By stabilizing the S2 trimer's apex with V991W and T998W, we were also able to resolve the fusion peptide proximal region (FPPR, residues 835-857). This region had been challenging to characterize in the full-length spike or HexaPro-SS due to its inherent flexibility.

Sera of mice immunized with closed prefusion-stabilized S2-only antigen neutralize rVSV-SARS-CoV-2 variants.

The impact of tryptophan substitutions on S2-only antigen immunogenicity was investigated. We immunized mice with the HexaPro-SS and HexaPro-SS-2W antigens (including the stalk region) and assayed the sera for neutralization against recombinant vesicular stomatitis viruses (rVSVs) bearing the coronavirus spikes of either SARS-CoV-2 Wuhan-1 or Omicron BA.1 variant (Figs. 6, S22). Against rVSV-SARS-CoV-2 Wuhan-1, both sera immunized with HexaPro-

SS and HexaPro-SS-2W show a significant increase in neutralization (Fig. 6b) as compared to PBS-immunized sera, but do not differ with respect to each other. In contrast, only mice immunized with HexaPro-SS-2W demonstrated a significant increase in the neutralization of rVSV-SARS-CoV-2 Omicron BA.1 compared to PBS-immunized sera. These experiments demonstrate the ability of the Hexapro-SS-2W antigen to elicit a neutralizing response against sarbecoviruses, although further testing is needed to assess the neutralizing response across a broader panel of beta-coronaviruses including MERS-CoV, HKU-1, OC43, SARS-CoV, and additional SARS-CoV-2 variants.

To evaluate the impact of the tryptophan substitutions on the preservation of the epitopes on the spike S2 subunit, we performed biolayer interferometry (BLI) experiments. We tested the binding of previously reported neutralizing and non-neutralizing S2-directed antibodies that target the highly conserved stem helix (IgG22,[2] S2P6[23] and CC40.8[25]), fusion peptide (CoV44-79 and CoV91-27[24]), and apex (B3-1, manuscript under review) against HexaPro-SS (Fig. 6c), HexaPro-SS-2W (Fig. 6c), and HexaPro-SS-T998W (Fig. S23) constructs (including the stalk region). An antibody targeting the RBD of S1 was used as a control (N3-1, manuscript under review). BLI shows that the binding affinity of antibodies that target the stem helix or fusion peptide is not affected in HexaPro-SS-2W when compared to HexaPro-SS. However, binding affinity of the non-neutralizing antibody B3-1 targeting the apex of the S2 trimer as in HexaPro-SS-2W was reduced by half with respect to HexaPro-SS (Fig. 6c). This indicates that the opening of the S2 apex is important for B3-1 binding. Interestingly, B3-1 binding is not fully abrogated, suggesting that Hexapro-SS-2W may infrequently undergo opening motions as shown from WE simulations. Reducing the binding affinity of the non-neutralizing antibodies targeting S2 may further indicate that closing the S2 trimer with 2W can bias immunization responses towards neutralizing epitopes such as stem helix and fusion peptide as opposed to non-neutralizing epitopes such as the apex.

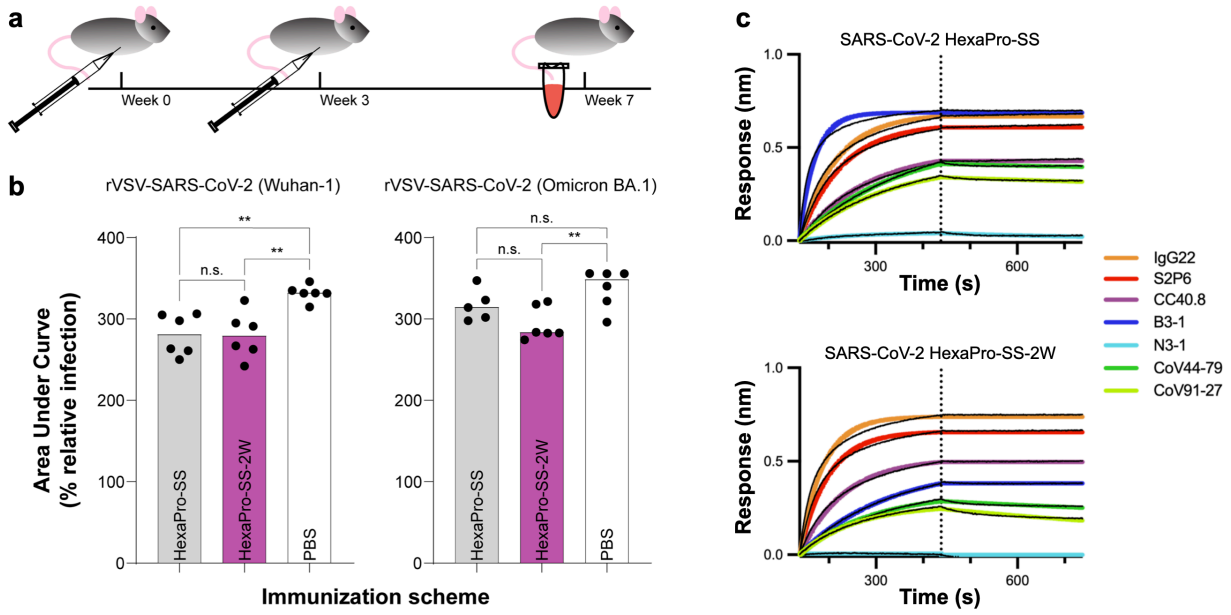

Fig. 6. Immunogenicity of Hexapro-SS and Hexapro-SS-2W constructs. a,b, Sera from mice immunized with HexaPro-SS-2W can neutralize rVSV-CoV-2 variants. **a,** 6–8-week-old female C57BL/6J mice (n = 6) were primed (week 0) and boosted (week 3) with 10 µg of immunogens HexaPro-SS, HexaPro-SS-2W, or PBS. **b**, Sera was isolated at week 7 and evaluated for neutralization capacity against rVSVs bearing the spike proteins of SARS-CoV-2 Wuhan-1 or SARS-CoV-2 Omicron BA.1. Lower area under the curve (AUC) values correspond to better sera neutralizing capability. AUCs were compared across groups by ordinary one-way ANOVA with Tukey's multiple comparisons test (** $p < 0.01$; n.s. not significant). **c**, Biolayer interferometry sensorgrams showing binding responses (colored lines indicate 1:1 binding fit, black lines indicate reference subtracted response) of previously reported neutralizing or non-neutralizing antibodies to epitopes on the S2 subunit in HexaPro-SS (top) and HexaPro-SS-2W (bottom) constructs.

III. Conclusions

In this work, we present the simulation-driven design of SARS-CoV-2 S2-only antigens stabilized in the closed prefusion conformation. We first used WE path-sampling MD simulations to investigate the conformational plasticity of HexaPro-SS-Δstalk, a prefusion-stabilized S2 construct that exhibits flexibility at the apex, leading to splayed open conformations. We discovered that the prefusion-stabilized S2 trimer transitions from a closed to open conformation through an asymmetric protomer-protomer separation. Contact map analysis of the interfacial helices (CHs, UHs, and HR1) reveals key residue-residue interactions taking place in the opening pathway. To improve interprotomer stabilization, we identified two residues located in the CH apex, V991 and T998, as candidates for cavity-filling substitutions with tryptophan. We next

designed three variants of HexaPro-SS-Δstalk incorporating V991W, T998W, and V991W+T998W substitutions, namely HexaPro-SS-V991, HexaPro-SS-T998, and HexaPro-SS-2W. Additional WE simulations of the three newly engineered S2 variants showed that T998W and V991W+T998W provide kinetic stabilization by slowing down the S2 trimer opening. Subsequent FEP calculations revealed a negative $\Delta\Delta G_{mutation\text{-}folding}$ imparted by the tryptophan substitutions, evincing increased stability of the closed prefusion conformation for all three S2-engineered variants, with HexaPro-SS-2W exhibiting superior stability. Strikingly, cellular expression and thermostability characterization of the newly stabilized S2 constructs confirmed the computational predictions, showing higher expression yields and melting temperatures for the three tryptophan variants, with HexaPro-SS-2W surpassing all the other constructs. The improved stability of HexaPro-SS-2W allowed the high-resolution characterization of its closed prefusion structure via cryo-EM. The resolved cryo-EM structure highlights key residue interactions also observed in our WE simulations. Importantly, immunogenicity assays on sera from mice immunized with HexaPro-SS-2W demonstrated that this stabilized S2-only antigen can neutralize different rVSV-SARS-CoV-2 variants. Protection data will further illuminate the potential of the HexaPro-SS-2W antigen as a vaccine candidate.

These studies underscore the advantage of using dynamic information from molecular simulations to inform immunogen design. Static-structure methods depend on energetic calculations of protein structures in cryogenic conditions or rely on the structural homology of proteins with minor conformational variance. In contrast, our simulations capture dynamic information from an ensemble of conformations at reproduced physiological conditions. In addition, mechanistic insights from protein dynamics could drive vaccine design efforts to neutralize challenging and elusive clinical targets. The computational approach used here enabled the design of novel, highly stable SARS-CoV-2 S2-only immunogens that could serve as the structural basis for future pan-coronavirus vaccine development efforts and may be generally applicable to structure-based vaccine design.

Data availability

Trajectories of S2 trimer opening pathways, WE configuration files, and representative closed prefusion conformations of HexaPro-SS-2W are included in the article as supplementary information data. The atomic coordinates of HexaPro-SS-2W are available in the Protein Data Bank under PDB ID: XXXX. The cryo-EM map is available from the Electron Microscopy Data Bank under accession code EMDB-XXXXX.

Code availability

This study utilized the standard builds of the simulation software WESTPA 2.0 (https://github.com/westpa/westpa), AMBER 20 (https://ambermd.org), NAMD 2.14 / NAMD 3 (https://www.ks.uiuc.edu/Research/namd/), PMX (https://github.com/deGrootLab/pmx), and Gromacs 2022.1 (https://manual.gromacs.org/2022.1/release-notes/2022/2022.1.html) according to best practices with no special modifications.

Acknowledgments

We thank Dr. Lillian T. Chong and Dr. Daniel Zuckerman for helpful discussions about WE simulations. L.C. thanks Dr. Anthony Bogetti for the support with WESTPA software. M.S thanks Dr. Vytautas Gapsys for the support with FEP calculations. All the simulations were run on the Triton Shared Computing Cluster (TSCC) at the San Diego Supercomputer Center (SDCC). We thank them for their support and assistance. This work was funded in part by the Bill & Melinda Gates Foundation INV-031624 (K.C., J.S.M., R.E.A). S.A. acknowledges support from the Department of Chemical Engineering at the University of California, Davis. This work was funded in part by Welch Foundation grant number F-0003-19620604 (J.S.M.).

Author Contributions

X.N. and L.C. contributed equally to this work. X.N. and L.C. designed the S2 cavity-filling substitutions (V991W and T998W). X.N. and L.C. prepared all the simulation models. X.N.

designed and performed conventional MD and GaMD simulations. L.C. designed and performed WE simulations. X.N. and L.C. designed and performed all simulation analyses. M.S., A.A.O., F.L.K., M.A.R., M.S., and R.E.A. critically helped design simulation analyses. M.S. designed and performed FEP calculations and related analyses and figures. X.N. and L.C. made all the figures and videos. S.A. provided guidance with the WESTPA setup and simulation scripts. A.A.O. helped configure and design WE progress coordinates. R.E.A. designed and oversaw all the simulations. L.Z. designed and performed DNA cloning, expression tests, expression for mouse studies, cryo-EM, and BLI experiments, and made related figures. C.M.A. performed expressions and DSF experiments. J.S.M. designed and oversaw all the experiments. A.W., A.L.T, and E.H.M. designed and performed mouse immunizations, rVSV-SARS-CoV-2 viral rescues, and neutralization assays, and made related figures. K.C. designed and oversaw immunogenic and viral assays. R.E.A. oversaw the project. X.N. and L.C. wrote the original draft of the manuscript, and all the authors contributed to reviewing and editing.

Competing Interests

J.S.M., L.Z., R.E.A., X.N., L.C., M.S. are inventors on a U.S. patent application describing the use of stabilized SARS-CoV-2 S proteins as vaccine antigens (Stabilized SARS-CoV-2 S Antigens, 63/583,090). K.C. is a member of the scientific advisory board of Integrum Scientific LLC and has consulted for Axon Advisors, LLC. K.C. owns shares in Integrum Scientific and Eitr Biologics, Inc.

Supplementary Information

Supplementary Information: Supplementary Material and Methods, Figs. S1–S23, Tables S1-S4 and legends for Videos S1, S2.

Supplementary Video 1: Opening transition of HexaPro-SS-Δstalk S2 trimer.

Supplementary Video 2: Stabilization of the S2 trimer's closed prefusion conformation in HexaPro-SS-2W

Supplementary Data 1: S2 trimer's WE opening trajectories (.dcd, one per system) and traces (.txt, all successful pathways), WE configuration files (.cfg), summary of WE simulations (.txt), and representative closed prefusion conformations of HexaPro-SS-2W obtained from the WE simulation (.dcd).

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