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FRETing over SARS-CoV-2: Conformational Dynamics of the Spike Glycoprotein

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In this issue of *Cell Host & Microbe*, Lu et al. utilize single-molecule FRET to reveal the conformation dynamics of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike protein, showing transitions from a closed ground state to the open receptor-accessible conformation via an on-path intermediate. These insights into spike conformations will facilitate rational immunogen design.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) entry is mediated by its surface viral spike (S) glycoprotein. The ~540-kDa trimeric S protein is a multifunctional molecular machine, structurally organized as two subunits that are proteolytically separated during biogenesis. In record time, mere weeks after the initial sequencing of the virus, the first structures of the pre-fusion S ectodomain were determined by single-particle (SPA) cryo-electron microscopy (cryo-EM) (Zhou et al., 2020). Since then, additional structures of S have been determined by crystallography and by both SPA and cryo-electron tomography subtomogram averaging (STA) (Cai et al., 2020; Ke et al., 2020; Turoňová et al., 2020; Walls et al., 2020; Wrapp et al., 2020). These structural snapshots of the SARS-CoV-2 S have revealed an unprecedented amount of information regarding how the S protein facilitates viral entry, and distinct S protein conformations involved in the process have been characterized.

The 3D reconstruction of the pre-fusion state revealed major structural differences between protomers in the S1 receptor-binding domain (RBD). A hinge-like movement results in at least two distinct conformational forms (closed and open states). In the closed ground-state trimer, all three RBDs are pointed "downward," largely occluding the receptor-binding site, whereas, in the open pre-fusion conformation, one or more of the RBDs are oriented "upward" in a receptor-accessible conformation (Cai et al., 2020; Walls et al., 2020). Cryo-electron tomography revealed that full-length S is mainly in the closed conformation on the virion surface, which, in combination with its extensive glycosylation, could hamper antibody recognition (Ke et al., 2020; Turoňová et al., 2020).

A complete understanding of the function of SARS-CoV-2 S in viral entry and its sensitivities to immune surveillance demands the characterization of the dynamic intermediate states between the open and closed conformations. All structural characterizations presented to date have technical limitations that prevent a complete understanding of these intermediates. Crystal structures are static snapshots trapped in a crystalline lattice and often provide limited information on flexible regions. Likewise, SPA might neglect rare conformations during the 2D and/or 3D classification process, only revealing the "views" that are most readily observed in a particular sample preparation. Lastly, STA has, on average, lower resolution than other structural approaches, which limits its usage for detailed analysis of ensembles of conformational states.

Single-molecule Förster resonance energy transfer (smFRET) has emerged as an effective technique for the study of protein conformational dynamics, especially intermediate states that are transient in nature. A major advantage is the ability of smFRET to detect discrete protein intermediate states that are concealed in averaged population-based data. Moreover, smFRET allows real-time *in situ* visualization of conformers in the timescale of biomolecular motions under physiological conditions. This technique has now been used to understand the conformational dynamics of several viral fusion glycoprotein systems—such as HIV-1 gp160 and influenza A HA—revealing the presence of intermediates different than the pre- and post-fusion end states (Das et al., 2018; Munro et al., 2014).

In this issue of *Cell Host & Microbe*, Lu et al. (2020) used smFRET to study the *in situ* conformational dynamics of the SARS-CoV-2 S glycoprotein on the surface of lentivirus pseudotyped and coronavirus-like particles. Remarkably, smFRET revealed that S protein on the surface of viral particles is highly dynamic, sampling of at least four different conformational states detected by a spectrum of FRET efficiencies (low, 0.1; intermediates, 0.3 and 0.5; and high, 0.8) (Figure 1A). The identities of the four conformational states were not initially clear. A multimodal-Gaussian distribution showed that the intermediate conformation (0.5 FRET) was most abundant. Moreover, this conformation was stabilized by the introduction of a disulfide bond between Cys383 and Cys985. SPA previously showed that the Cys383-Cys985 disulfide stabilized the S protein in a closed trimer with all three RBDs pointed down (Henderson et al., 2020). Taken together, these findings identify this conformation as the RBD-inaccessible closed trimer.

Lu et al. then sought to determine which conformation corresponds to the open, receptor-accessible trimer. SARS-CoV-2 S recognizes and binds the human angiotensin-converting enzyme-2 (ACE2) receptor with high affinity (Walls et al., 2020). Thus, Lu and colleagues added monomeric and a more potent dimeric ACE2 receptor to S-pseudotyped particles. In both cases, they observed a stabilization of the low-efficiency FRET signal (0.1 FRET). This clearly identified the low-FRET signal as the activated, open conformation with all RBDs oriented up. An increase in this signal was also

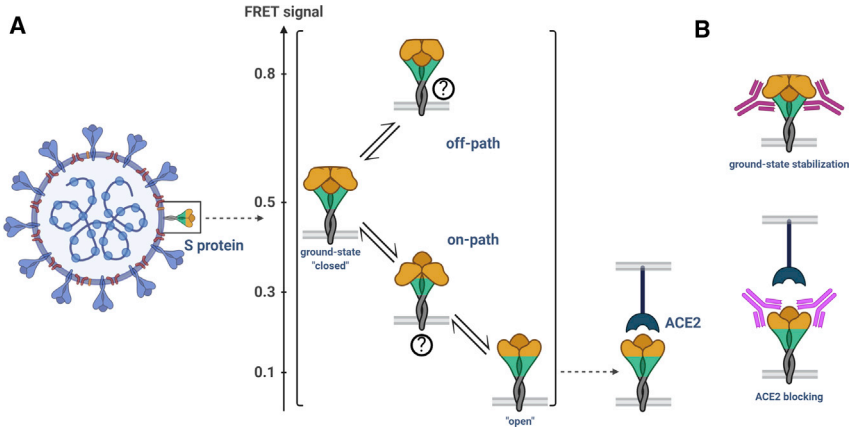

Figure 1. Conformational Dynamics of the SARS-CoV-2 S Glycoprotein

(A) Schematic showing the SARS-CoV-2 S protein intermediate conformations observed by smFRET. (B) smFRET studies suggest that antibodies block SARS-CoV-2 entry via two mechanisms. (1) Some antibodies bind to and stabilize the ground state to prevent the conversion of the closed trimer to the open conformation capable of binding the host ACE2 receptor. (2) Other antibodies bind the open conformation to compete with ACE2 binding. Created with BioRender.com.

observed when the particles were treated with trypsin, which mimics the native serine protease TMPRSS2 involved in priming the S protein for virus entry. ACE2 binding to the trypsin-cleaved S protein also promoted the formation of the open state.

Quantitative analysis of the discreet FRET transitions allowed Lu et al. to provide insights into the order and timing of the S protein conformational change. First, the unbound S protein on the surface of the virus is in equilibrium between conformations. Moreover, there was a defined sequence of structural transitions. The authors suggest that the unbound closed conformation readily transitions to the receptor-bound open conformation via a previously uncharacterized intermediate at 0.3 FRET signal. ACE2 binding to S increased the rate of the transition from the closed ground state to the intermediate (0.3 FRET) and the open (0.1 FRET) states. Lu et al. also detected an off-pathway transition where the closed ground-state conformation reversibly accesses an infrequent conformation at 0.8 FRET.

The understanding of the SARS-CoV-2 S protein intermediates is critical to understanding human antibody responses. By using their smFRET platform, Lu et al. analyzed the effects of two plasma samples with neutralizing activity from convalescent patients (S002 and S006) to understand the neutralization potentials of the antibodies. Interestingly, the convalescent plasma samples recognized the S protein through seemingly different mechanisms. Antibodies from the S006 plasma stabilized S in an RBD-upward open conformation (0.1 FRET), similar to the effects of ACE2 binding. In contrast, S002 plasma stabilized the RBD-down closed ground-state conformation (0.5 FRET). smFRET analysis of the S protein with other neutralizing antibodies (H4, 2-43, and 2-4) and the nanobody VHH72 revealed that some stabilized the open conformation and others the closed S protein conformation. Therefore, the authors hypothesize that SARS-CoV-2 can be neutralized by one of two strategies: either direct competition for ACE2 binding or via an allosteric mechanism where the ground-state closed trimeric conformation is stabilized to prevent the open conformation from binding the receptor (Figure 1B).

smFRET is a powerful tool for the characterization of transient structural intermediates. The paper by Lu et al., as well as studies by others, clearly show that the SARS-CoV-2 S protein is displayed on the surface of the virus in an equilibrium of conformations. Furthermore, this study demonstrated that the transition from the most abundant, closed ground-state conformation to the open receptor-accessible conformation goes through at least one previously undescribed on-path intermediate state. Understanding the full ensemble of viral fusion glycoprotein intermediates and dynamics will guide the rational design of immunogens for vaccination and the design of therapeutic antibodies and small-molecule inhibitors that block fusion. Based on the results in this paper, designing spike proteins to elicit antibodies that trap the S protein into the closed ground state represents an appealing strategy to jam the SARS-CoV-2 viral entry machinery.

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