Computational analysis of interior mutations of SARS-CoV-2 Spike protein suggest a balance of protein stability and S2: S1 separation propensity

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ABSTRACT

SARS-CoV-2 variants often include surface mutations in the Spike protein that are important for viruses to recognize host receptors and evade antibody neutralization. The Spike protein also has mutations in the interior of the protein likely to affect the Spike protein S1 – S2 subunit’s separation propensity, the most important of which is the D614G mutation. Remarkably, the Omicron variant contains a large number of internal mutations at the S2: S1 interface, which have not been investigated yet. In this study, we examined the effects of such interfacial mutations on the S2: S1 and subunit domain interactions and on the subunit’s dissociation process. We found that the interaction with S2 is mainly contributed by the three encapsulation domains, named INT, ED1 and ED2 of S1, which are sandwiched between the S1 RBD and N-terminal NTD domain. We found that D614 is the strongest contributor for the S2: S1 interaction which is greatly weakened by the D614G mutation. Surprisingly, we found that, mutations T547K, H655Y, N764K, N856K, N969K, L981F in the Omicron variant largely enhance the S2: ED1 interaction, partially compensating the loss of S2: ED2 interaction due to the D614G mutation. Lastly, these results, together with biological considerations, allow us to suggest that in addition to the binding strength of between the RBD and ACE2, the stability of the Spike protein and the propensity of Spike protein S2: S1 separation are critical factors which likely exist in a balance for a particular infectivity and pathogenicity of the virus.

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1. Introduction

The SARS-CoV-2 coronavirus has four variants so far defined by the WHO as variants of concern, i.e. Beta, Gamma, Delta and Omicron. Of these variants, Delta and Omicron have swept the globe due to their higher infectivity [1–3]. Mutations in the virus envelope Spike protein on the surface of the coronavirus are also closely related to the effectiveness of vaccines and to the antibodies generated. The Beta variant contains five mutations in the Spike protein – K417N, E484K, N501Y, D614G, A701V. The Gamma variant has K417T, E484K, N501Y, D614G, H655Y mutations. The Delta variant has four mutations as L452R, T478K, D614G, and P681R. The Omicron variant (BA. 1) contains as many as 34 Spike protein mutation sites (A67V, del69-70, T95I, del142–144, Y145D, del211, L212I, ins214EPE, G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F). Of all these Spike protein mutations, amino acid substitution D614G is the only mutation common to these variants, suggesting, that by itself, it has a profound role on the structure-dynamics-function relationship of the Spike protein.

At present, for Spike protein variants, there is an extensive and growing literature focusing on the effect of mutations on the binding strength of the mutated Spike proteins to human cell surface receptor ACE2, as well as the effect of mutations on antibody neutralization effects. In general, studies have found that mutations reduce antibody neutralization and evolved for the virus to escape the immune system [3,4,5,6,30]. However, some of the reports are inconsistent with one another as to whether the mutations...
enhance the binding strength of the Spike protein to ACE2 [7,8,11,26,31]. Intriguingly, some mutation sites are located outside the receptor-binding region (RBD, residues 318–541) of the Spike protein, such as D614, suggesting potent allosteric effects [28]. Importantly, ACE2 binding by the Spike protein is only possible when one or several RBD in the timer in an “up” state, “opening” the protein. In accord, studies have shown that the G614 spike variant adopts a more open “RBD up” conformation for ACE2 binding [12]. However, other experiments indicated that the D614G mutation actually little effect on the binding affinity toward recombinant ACE2 [11,14].

The effect of Spike mutations is not limited to the binding between Spike protein and human receptors. The mutations potentially have various effects on different stages of the viral infection process, not just virus-host recognition, but also on virus endocytosis and transport, virus fusion, and virus assembly [13]. At the outset of our study we wanted to understand which Spike protein variants are likely to significantly affect the internal interaction between spike protein S1 and S2 domains. The virus Spike protein is a trimeric complex, and the complex is processed by furin or the between spike protein S1 and S2 domains. The virus Spike protein, such as D614, suggests potent allosteric effects [28]. Importantly, ACE2 binding by the Spike protein is only possible when one or several RBD in the timer in an “up” state, “opening” the protein. In accord, studies have shown that the G614 spike variant adopts a more open “RBD up” conformation for ACE2 binding [12]. However, other experiments indicated that the D614G mutation actually little effect on the binding affinity toward recombinant ACE2 [11,14].

The RBD of the Spike protein has two orientations relative to the rest of the Spike protein – RBD “up” or “open” and RBD “down” or “closed”. For the wt Spike protein, a state with 2 subunits in the RBD “closed” state and 1 subunit in the RBD “open” state is popular.

The D614G mutation, on the other hand, significantly increases the population of the 2 subunit RBD “open” state [11]. For the Omicron variant, the current electron microscope results indicate the existence of both 2 RBD “open” and 1 RBD “open” states, but the relative ratio is unknown [10].

For the wt Spike protein, the structure was based on the available crystal structure (6vsb and 7a93), ‘1 RBD open’ state of Spike protein was based on PDB entry 6vsb. In CHARMM-GUI, Woo et al constructed and provided the Spike protein structure as the full-length protein by adding missing loops and residues. We used the structures in this study for the simulations [19]. ‘2 RBD open’ state of Spike protein was based on PDB entry 7a93 [12]. Several short missing loops/residues were added by referring to the ‘1 RBD open’ structure. For the G614 variant and Omicron variant, they were all built with ‘2 RBD open’ states. For the G614 variant, amino acid Aspartic acid 614 was mutated to Glycine 614. For the Omicron variant, we used PDB 7t9j as a template to build the structure [9]. However, in this structure only one RBD (in ‘closed’ state) is visible, and there are many missing densities in this electron microscopy structure, including for the other two RBDs. We firstly used Swiss-Model to add missing loops and residues (but not the missing RBDs). In PDB entry 7s1l, a recombinant Omicron RBD is bound to an ACE2 protein. We used this RBD structure and fused it to the Omicron Spike protein by referring (with superimposition) the steric position of the two “open state” RBDs in wt S protein.
structure (PDB 7a93). Glycosylation sites of the Spike protein are distributed on its surface and have no direct influence on interior interaction. Therefore, for simplicity, glycosylation of the Spike protein was not considered (see also Fig. S2). For reference, relevant studies of the role of glycans in Spike protein are found elsewhere [27,32]. Added loops or mutated residues were relaxed with a 1 ns short simulation with the protein backbone fixed.

2.3. Steered molecular dynamics simulation

After the short relaxation simulation, we ran steered molecular dynamics (SMD) simulations in order to investigate the separation of S2 and S1 subdomains. We have carried out similar SMD simulation of S2: S1 separation in the presence and absence of the Spike protein (co-)receptor/host factor neuropilin-1 [23]. Recently, SMD simulations were also used for the investigation of RBD: ACE2 separation [6,33,34]. In the SMD simulation, the long axis of the S2: S1 trimer complex is aligned in the z direction. In the steered MD simulation, a potential bias is added to a simulation system:

\[ U(t) = \frac{1}{2} k \left[ vt - \left( \vec{R}(t) - \vec{R}_0 \right) \cdot \vec{n} \right]^2 \]

where \( \vec{R}(t) \) is the current and \( \vec{R}_0 \) is the initial center of mass of SMD atoms, \( \vec{n} \) for pulling direction, \( k \) for the force constant and \( v \) for moving velocity of virtual reference coordination. In this study, S2 gradually detaches from S1 by pulling S2 in the z-direction. The time step is set to 1 fs. The pulling rate constant \( v \) is set to 0.0000001 nm/fs. The total simulation time is 60 ns, thus S2 has a total movement of 6.0 nm. Each set of simulations was repeated 800.000001 nm/fs. The total simulation time is 60 ns, thus S2 has a total movement of 6.0 nm. Each set of simulations was repeated 8 times with different velocity starting seeds. As before [23] a force constant \( k \) of 0.25 kcal/(mol \cdot nm^2) was used for the harmonic spring that generates the force to pull S2 away from S1. The S1 RBD (res. 321–534) of three Spike protein units were restrained and held during the pulling simulation, the same way as in our previous study [23] mimicking the situation when they are firmly bound by the ACE2 protein.

2.4. Simulation condition and parameters

We applied Generalized Born Implicit Solvent method to investigate the S2: S1 separation [22,23]. While explicit water models are generally more suitable for molecular dynamics simulations, here, we applied an implicit water model for the steered molecular dynamics for the following reasons: In order to pull S2 out of S1, we need to reserve a region of 6 nm in the z-direction (totally about 33 nm) in the simulation box, so that the S2 can be displaced in the Z direction. The number of atoms contained in this system would be very large, estimated around 860,000, which would have been beyond our computational resources. More importantly, the size of S2 is relatively large. Different from pulling a small molecule, the movement of a sizeable S2 subunit on one hand needs to push away a lot of water molecules, which would generate excessive pressure on the simulation systems in a size-limited simulation box. On the other hand, the movement of S2 leaves a large void region between S1 and S2 subunits. The water molecules will find it difficult to fill the void space left by S2 in the short simulation time in a manner which would appear to be at equilibrium. Thus, we expect the movement of such waters would generate a significant level of noise. Therefore, we think that the use of an implicit water model is a reasonable choice for studying the process of studying S2: S1 separation. Last, but not least, we wanted to build on our earlier work [23] which used the same simulation protocol. In the implicit solvent simulation, a cut-off of 1.4 nm was used for calculating the interactions. Ion concentration was set as 150 mM NaCl. A cut-off of 1.2 nm was used for calculating the Born radius. The simulations were performed at 310 K. The CHARM36m force field [21] was used throughout and all simulations were performed with the NAMD/2.12 package [20].

### Table 1

The four sets of simulation systems used in this study.

| Systems | Sequence | Variant | RBD state | PDB template(s) |
|---------|----------|---------|-----------|-----------------|
| System 1 | (S1: 1–685 and S2: 700–1146 for all) | WT       | 2 ‘open’  | 7a93            |
| System 2 |          | WT       | 1 ‘open’  | 6vsc            |
| System 3 |          | G614     | 2 ‘open’  | 7a93            |
| System 4 |          | Omicron  | 2 ‘open’  | 769j719l        |

3. Results

3.1. Definition of critical domains for maintaining S2: S1 interface

SARS-CoV-2 Spike or S protein includes the S1 subunit and the S2 subunit. S1 subunit contains the NTD (N-terminal domain, residues 1–292) and the RBD (receptor binding domain, res. 331–530), which are well folded and spatially isolated (Fig. 1). Two linker sequences connect the NTD to RBD, and RBD to the N-terminus of S2 subunit respectively (Fig. 1a). As described below in this study, we found that the two linker regions sandwiched between NTD and the RBD domains are the critical regions that are responsible for maintaining the S1 and S2 binding interactions (Fig. 1b). However, these linker regions are largely understudied in previous reports. At present, the amino acid terminals of the linker sequence at both ends as well as the naming of the two linkers are inconsistent in different publications [e.g. [11,24,25,26]]. For the convenience of analysis of the S1: S2 interaction, and given the importance of these regions on encapsulating S2 subunits, here we divided the two linker regions (NTD-RBD linker and RBD-S2 linker) into 3 different subdomains (Fig. 1a and Table 2): the intermediate domain (INT, residues 293–330), encapsulation domain 1 (ED1, residues 531–590) and encapsulation domain 2 (ED2, residues 591–685).

Despite being distant in sequence, INT 310–318 and ED2 593–599 form two adjacent β-strands. Similarly, INT 324–329 and ED1 540–545 form two adjacent β-strands (Fig. 1c). It is worth noting that the interactions between the three subunits of S proteins are staggered, i.e., S2 of unit A interacts with S1 of unit B, S2 of unit B interacts with S1 of unit C, and S2 of unit C interacts with S1 of unit A (Fig. 1d).

3.2. Key S2: S1 interactions within the encapsulation pocket of the WT Spike protein

We first performed pulling simulations for the ‘2 RBD open’ state. Under the action of the pulling force, S2 gradually dissociates from S1’s encapsulation pocket (Fig. 2). In the beginning, the average interaction between S2 and S1 is the largest (Fig. 3a), the interaction energy was binned every 0.1 nm interval). After the native contacts between the S2: S1 amino acids are initially stretched while maintaining their interaction energy, the intensity of the interaction decreases rapidly (Fig. 3a). We further analyzed the
S2: S1 interaction at the subdomain- as well as residue level during the initial dissociation (0–0.5 nm displacement) process. By decomposing the interaction electrostatic energy into individual subdomains (Fig. 3b), we found that ED2 makes the highest contribution to the total interactions (53 %), INT contributes about 30 %, while the ED1 contributes approx. 10 % of the total energy and the contribution of the RBDs is only about 5 % and involvement in the NTDs in the S1:S2 interaction is negligible. Looking at the atomistic level, we found that ED2 has the most amino acids which contribute to the S2: S1 interaction (Fig. 3c–e). In particular, ED2 D614 forms a strong interaction with K854. In addition, INT E309/R319 and ED2 R646 form strong interactions with S2 R765/ D745/E748 and K835/D867/E868 respectively (Fig. 3c, d).

We calculated the number of residue-residue contacts between the S2 and S1 domains (Fig. 3e). We found that in S2’s exposure to S1, the interactions with subdomains INT, RBD, and ED1 is rapidly reduced, while S2’s exposure to S1 ED2 lasts longer. In this calculation, ED2 moves with S2, so the contact between the two lasts longer. This is because on one hand there is a stronger interaction between ED2 and S2, and on the other hand ED2 is localized initially further down on the S2 structure and can be thought of as

Table 2

| Structural subdomain (definition, this work) | Residues | Other names (in literatures) |
|---------------------------------------------|----------|-----------------------------|
| NTD                                         | 1–292    | –                           |
| INT                                         | 293–330  | INT1, SD1                   |
| RBD                                         | 331–530  | –                           |
| ED1                                         | 531–590  | INT2, SD2, SD1              |
| ED2                                         | 591–685  | CTD, SD2                    |
| S2                                          | 686–1146 | –                           |

Fig. 1. Definition of structural subdomains for maintaining S2: S1 binding interactions. a) Structural subdomains. b) Complex highlighting the NTD, RBD, INT, ED2 and ED1 structural domain. c) Complex highlighting the INT, ED2 and ED1 structural domain. d) A top view (structure of b), 90° rotated around x-axis), of S2: S1 showing staggered interactions between S1 ED2/ED1 and S2.

Fig. 2. A representative dissociation pathway of S2: S1. Note that the INT region is small and hidden from view.
being on the exit pathway of S2. Thus, it forms non-specific contact interactions during the dissociation process. Note that in none of the simulations, S1 and S2 are completely separated at 6 nm distance as the ED2 can be stretched considerably.

3.3. The opening and closure of RBD only slightly affects S2: S1 interactions

Any RBD, in the “closed” state, contacts and caps the top of S2, although these contacts are fewer and less strong than those of ED2. We compared the ‘1 RBD open’ with the ‘2 RBD open’ states (Fig. 4a). As the former has one more RBD capping the S2 region, RBDs should be able to establish more interactions with S2. However, in terms of the total interactions, we do not see a noticeable difference (Fig. 4b). At the RBD: S2 interface, amino acids K376 and K386 are major contributors for the RBD: S2 interaction, which interact with D985/E988 and D979 respectively (Fig. 4c). The decomposition of interactions shows that there are stronger interactions between the RBD and the S2 in ‘1 RBD open’ state, but the increase of interaction is compensated by a decrease in interaction between ED2 and S2 (Fig. 4d). Overall, the RBD: S2 interaction contributes to the total energy, but the proportion is small.

3.4. The G614 variant reduces the separation barrier by reducing the S2: ED2 interaction

Examining the S2: S1 separation for the G614 variant, the energy barrier for S2: S1 separation is significantly reduced (Fig. 5a, b). Comparing the interaction of D614/G614 with S2, we find that G614 has only a weak interaction with S2. It largely reduces the S2: ED2 interaction (Fig. 5c). Unlike aspartic acid, glycine is not able to form a strong interaction with adjacent positively charged amino acids. Thus, the original electrostatic interactions between D614 and positively charged residues are absent (Fig. 5d). The residue-residue contact between S2 and S1 are similar between 614G variant and WT protein (Fig. 5e). S2 has much weaker interaction with S1 comparing G614 variant with WT protein (Fig. 5b). As discussed above, ED2 is at the exit pathway of S2. With the D614G mutation, the obvious decrease in the S2: ED2 interaction largely smoothens the separation process. Thus, the separation process of S2 and S1 becomes easier with the D614G mutation. Many studies emphasized a potent distal influence of D614G mutation on RBD: ACE2 binding. However, our study indicates that the D614G mutation, first of all, has significant local influence on the S2: S1 interaction.

3.5. The Omicron variant has a strongly enhanced S2: ED1 association

The Omicron variant has 34 mutations. Of these mutations, D614G, T547K, H655Y in S1 domain and S2 N764K, N856K, N969K, L981F are at the S2: S1 interface. These mutations create four lysine residues. Because the Spike protein contains three subunits, so these mutations will increase the number of positive charges by 12. By plotting the electrostatic potential energy of the S2 region, we found that the mutation drastically changes the surface electrostatic potential energy of S2 (Fig. 6a).
For the Omicron variant, the total S2: S1 interaction is almost the same in comparison to the S2: S1 interaction of WT (Fig. 6b), but the constituent of the total energy is dramatically different in comparison to the D614G variant. Amazingly, we found that the Omicron variant has significantly increased interactions between S2 and ED1 (Fig. 6c). This is because the T547K mutation establishes new interactions with S2 D745 and D979 (Fig. 6d, f). In the meantime, D571 and D568 establishes additional interactions with residue S2 856 which is also mutated to lysine (Fig. 6e, f). Altogether, these mutations enhance the association strength of S2 to S1, largely counteracting the weakening of S2 and S1 interactions caused by the D614G mutation (Fig. 5). The S2: ED2 interaction is reduced for the Omicron variant. This is similar to the G614 variant as the Omicron variant also has the D614G mutation. Due to the weakening of the S2: ED2 interaction, the S2: S1 dissociation is relatively smooth in comparison to wild type Spike protein (Fig. 6g).

To summarize, the G614 variant largely diminishes the S2: S1 interaction, but the Omicron variant has a small influence on the total S2: S1 interaction energy. However, it dramatically alters the balance of the S2: ED1 and S2: ED2 interaction. The Omicron variant has a large decrease in S2: ED2 interaction energy, but on the other hand, the interaction of S2: ED1 contacts are significantly enhanced. S2, has less effect on the total S2: S1 interaction. We found that ED2 makes the biggest contribution to maintaining the S2: S1 interface for the WT virus. Of all the amino acids, D614 in ED2 is one of the key contributors to the S2: S1 interaction. Importantly, the D614G mutation dramatically reduces the S2: S1 interaction. Furthermore, in addition to D614G, the Omicron variant has six other mutations (T547K, H655Y, N764K, N856K, N969K, L981F) at the S2: S1 binding interface. These kinds of mutations are new compared to the previous widely distributed variants. Of these mutations, 4 amino acids are mutated to lysine, which greatly alters the electrostatic surface potential of the S2 protein and enhances the binding strength of S2 to S1 ED1. The enhancement of inter-domain and inter-subunit packing likely helps stabilize an open conformation of RBD, as proposed in the study by Ye et al [29]. However, a 614G variant itself already has a role in increasing the percentage of ‘RBD open’ conformation [11]. So, from this perspective, it has been unclear what made Omicron different from the other variants.

Glycosylation of the Spike protein is implicated for host recognition or immune evasion by shielding the protein surface from detection by antibodies. Importantly, the glycosylation sites are distributed all over the surface of the Spike protein (Fig. S2). Our research concerns the interior interactions between S2 and S1, but there are no glycosylation sites on the interior S2: S1 dissociation path. Therefore, we believe that glycosylation will not have a direct effect on the S2: S1 interaction and separation.

4. Discussion

4.1. Amino acid mutations and the S2: S1 interaction

In this study, we investigated – surprisingly it appears for the first time – the detailed interactions between the S2 and S1 subdomains, INT, RBD, ED1 and ED2. The RBD has relatively weak interactions with S2, and its opening and closure relative to the rest of S2, has less effect on the total S2: S1 interaction. We found that ED2 makes the biggest contribution to maintaining the S2: S1 interface for the WT virus. Of all the amino acids, D614 in ED2 is one of the key contributors to the S2: S1 interaction. Importantly, the D614G mutation dramatically reduces the S2: S1 interaction. Furthermore, in addition to D614G, the Omicron variant has six other mutations (T547K, H655Y, N764K, N856K, N969K, L981F) at the S2: S1 binding interface. These kinds of mutations are new compared to the previous widely distributed variants. Of these mutations, 4 amino acids are mutated to lysine, which greatly alters the electrostatic surface potential of the S2 protein and enhances the binding strength of S2 to S1 ED1. The enhancement of inter-domain and inter-subunit packing likely helps stabilize an open conformation of RBD, as proposed in the study by Ye et al [29]. However, a 614G variant itself already has a role in increasing the percentage of ‘RBD open’ conformation [11]. So, from this perspective, it has been unclear what made Omicron different from the other variants.

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4.2. What make Omicron variant differs from 614G variant like Delta variant?

All the other circulating variants (such as Beta and Delta variants) only have the D614G mutation localized to the S2: S1
interface. However, Omicron has multiple other mutations present at the interface between S2 and S1. The 614G variant dramatically weakens the S2: S1 interaction in comparison to the WT virus. However, for the Omicron variant the extent of decrease in S2: S1 interaction is not as significant as in the other 614G variants. By decomposing the interaction of S2 and S1, we found that similar to the other 614G containing variants, the S2: ED2 interaction is reduced, but the reduction in S2: ED2 interaction is largely compensated by an increase in interaction between S2: ED1. ED1 is next to the RBD and likely has a direct role in regulating RBD orientation. ED2 is distal from the RBD, but it forms more contacts with S2 and is at the exiting pathway of the S2 subunit.

4.3. S2:S1 interaction in biological context

The strength of the S2: S1 interaction is related to the stability of the Spike protein itself, but it is also closely related to the fission–fusion process (fission, i.e. separation of S2 and S1 and fusion of S2 –and the virus- with the host cell membrane). A strong S2: S1 interaction enhances protein stability, but is likely detrimental to protein dissociation. In contrast, a weak S2: S1 interaction would lead to a higher shedding effect of spike protein, which is harmful to virus assembly and maturation [14,15]. Ideally, the Spike protein should be stable enough before host receptor recognition, and easy to separate after binding with the host receptor. Therefore, a balance between Spike protein stability, S2: S1 separation propensity, and potentially S1: ACE2 binding affinity should be reached.

From a perspective of protein stability and protein separation propensity, we infer that the change in the relative strength of S2: ED1 and S2: ED2 interactions are likely biologically meaningful. The S2: ED1 interaction for the Spike protein of the Omicron variant is enhanced in comparison to the wild type Spike protein. However, the enhancement of the S2: ED1 interaction is largely compensated by the reduction of the S2: ED2 interaction (Fig. 7). Thus, the total interactions are of a similar strength. However, ED1 and ED2 are spatially distributed in different locations, and have different roles in maintaining protein stability and modulating protein dissociation. Specifically, ED1 locates in proximity to the RBD, and it has a pivotal role on gathering three loose RBDs on top of the S2 subunits. This prevents the RBD and the total S1 shedding from S2, especially when the RBDs are in the open conformation. On the other hand, ED2 is on the pathway of S2 dissociation, so the contact between S2 and ED2 exists throughout the dissociation process, thus the S2: ED2 interaction has a larger weight for modulating the dissociation of S2. Therefore, although the S2: ED1 interaction has been enhanced, the Omicron variant dissociates more easily compared to the WT protein as a result of the weakened S2: ED2 interaction.

So far, we still know very little about the effects of viral spike protein mutations on the entire process of virus infection. In this study, we investigated the potential role of S2: S1 interface mutations in affecting the S2: S1 interaction, and we suggest that a balance of S2: S1 stability and separation propensity should be an
important factor related to virus infectivity. Such a balance may be achieved in the context of protein structural variations, by balancing the relative binding strength of S2 with the different S1 subdomain ED1 and ED2.

**CRediT authorship contribution statement**

**Zhen-lu Li:** Conceptualization, Methodology, Software, Data curation, Writing – original draft, Writing – review & editing.
Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contribution

Z.L. and M.B designed the studies, interpreted the data and wrote the manuscript. Z.L. performed the sequence analysis and computational modeling.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2022.10.044.

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