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SARS-CoV and SARS-CoV-2 main protease residue interaction networks change when bound to inhibitor N3

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ABSTRACT

COVID-19 is a respiratory disease caused by the coronavirus SARS-CoV-2. SARS-CoV-2 has many similarities with SARS-CoV. Both viruses rely on a protease called the main protease, or Mpro, for replication. Therefore, inhibiting Mpro may be a successful strategy for treating COVID-19. Structures of the main proteases of SARS-CoV and SARS-CoV-2 with and without inhibitor N3 are available in the Protein Data Bank. Comparing these structures revealed residue interaction network changes associated with N3 inhibition. Comparing network clustering with and without inhibitor N3 identified the formation of a cluster of residues 17, 18, 30–33, 70, 95, 98, 103, 117, 122, and 177 as a network change in both viral proteases when bound to inhibitor N3. Betweenness and stress centrality differences as well as differences in bond energies and relative B-factors when comparing free Mpro to inhibitor-bound Mpro identified residues 131, 175, 182, and 185 as possibly conformationally relevant when bound to the inhibitor N3. Taken together, these results provide insight into conformational changes of betacoronavirus Mpros when bound to an inhibitor.

1. Introduction

SARS-CoV-2 is the coronavirus that leads to the respiratory disease COVID-19. Coronaviruses have large, positive-sense, single-stranded RNA genomes (Fehr and Perlman, 2015). The 2020 pandemic and its major world-wide effects underscore the importance of finding effective treatments and preventative measures for COVID-19; research into vaccines and antiviral drugs is desperately needed. One effective antiviral treatment strategy may be to find or design a drug that inhibits the main protease (Mpro) of betacoronaviruses. Mpro is an enzyme coronaviruses use to cleave viral gene products involved in viral replication (Xia and Kang, 2011). Several studies have attempted to inhibit the SARS-CoV Mpro (Bacha et al., 2008; Wong et al., 2014; Yang et al., 2005; Yin et al., 2007), and this drug approach has been proposed as a potential treatment strategy for COVID-19 patients (Morse et al., 2020; Ton et al., 2020). Additional information about structural changes that occur in inhibitor-bound Mpros may eventually contribute to this effort.

The 33.8 kDa betacoronaviral Mpro is necessary for viral replication. The coronavirus genome is transcribed as a polypeptide, and Mpro cleaves the polypeptide in 11 places to give the mature gene products (Xia and Kang, 2011). Therefore, Mpro is active early in the viral replication cycle. The Mpro structure is similar to serine proteases (Yang et al., 2003). However, Mpro has a catalytic dyad (involving His41 and Cys145) instead of a triad (Yang et al., 2003). Mpro has three domains; the first two have mainly beta-sheet structure, while the third is formed mainly from alpha-helices (Yang et al., 2003). Mpro is found in both monomeric and homodimeric forms, but it is thought to only be active as a dimer (Fan et al., 2004). Because Mpro is necessary for viral replication and coronaviruses have similar Mpro structures (Yang et al., 2005), Mpro has been the focus of many studies. Many Mpro structures have been solved and are available for analysis.

Residue interaction networks (RINs) abstract a protein to nodes (residues) and edges (the bonds between the residue side chains); this approach is reviewed by DiPaola and colleagues (DiPaola et al., 2013). Applying techniques of network analysis can provide novel insight into the three-dimensional structure and function of proteins. Studies have used RINs to identify enzyme active sites (Emerson and Louis, 2015), investigate molecular interactions (Liu and Hu, 2011), and study protein folding (Dokholyan et al., 2002). Network metrics can be global (describing the network as a whole) or local (describing individual nodes and their connections). Betweenness is a local metric. The betweenness of a node is the number of shortest paths passing through it. The global network metrics average clustering coefficient, average number of neighbors, characteristic path length,
and centralization were also measured in this study. These metrics are discussed elsewhere (Di Paola et al., 2013; Pavlopoulos et al., 2011; Scardoni and Laudanna, 2012; Steuer and Lopez, 2008; Watts and Strogatz, 1998). They provide an idea of the overall network structure by indicating information such as the extent and nature of connectedness in a network.

RINGs represent connections between residues; they are most useful when combined with sequence and additional three-dimensional structural information for a more complete picture of protein structure (Amiati et al., 2004). This study combines additional structural data and RIN analysis to identify conformational changes of the main proteases of SARS-CoV and SARS-CoV-2 when bound to the inhibitor N3. The structures of SARS-CoV and the SARS-CoV-2 main proteases are available from the Protein Data Bank (PDB IDs: 2H2Z of SARS-CoV (Xue et al., 2007); 2HOB of SARS-CoV with inhibitor N3 (Xue et al., 2007); 6Y84 of SARS-CoV-2 (unpublished); and 6LU7 of SARS-CoV-2 with inhibitor N3 (Jin et al., 2020). N3 is an irreversible Michael acceptor inhibitor (Yang et al., 2005). Michael acceptor inhibitors form covalent bonds with the target protein through the Michael reaction. As a peptidomimetic inhibitor, N3 has several peptide bonds as well as a Michael acceptor (Xue et al., 2007); N3 fits in the active site much like the natural target of M\text{pro}. The covalent bond that N3 forms with M\text{pro} blocks the active site, preventing further catalysis. N3 has been shown to irreversibly bind to the active site of the M\text{pro} (Jin et al., 2020). Since M\text{pro} structures for SARS-CoV and SARS-CoV-2 are available in the free and N3 inhibitor-bound states, it is possible to compare the structures to find differences in the active versus inhibitor-bound forms. It is not known if structural changes to M\text{pro} in locations other than the active site tend to produce functional changes (demonstrate allostery), but this study reveals changes that could be further explored for inhibitory relevance.

2. Methods

2.1. Creating residue interaction networks and visualizing 3D protein structure

The Residue Interaction Network Generator (RING) v2.0.1 (Piovesan et al., 2016) was used to create residue interaction networks from Protein Data Bank (PDB) (Berman et al., 2000) files. All networks were limited to chain A with a strict distance threshold. Water and hetero atoms and ligands were skipped, and one edge per type of interaction was drawn between the closest atoms on residues separated in sequence by at least two other residues. The resulting XML files were imported into Cytoscape v3.6.1 (Shannon et al., 2003), exported as SIF files, and re-imported to Cytoscape. To visualize the three-dimensional structure of proteins, PDB files were imported to UCSF Chimera v1.13 (Pettersen et al., 2004). All atoms except those in chain A were deleted, and the structures were aligned using the Matchmaker tool (Meng et al., 2006) in Chimera. To compare the amino acid sequences, the MatchAlign tool in Chimera was used.

2.2. Calculating RING metrics

The Network Analyzer program (Assenov et al., 2008) in Cytoscape was used to calculate basic global network metrics such as average clustering coefficient, average number of neighbors, characteristic path length, and centralization. The same program calculated the local metrics betweenness and stress. The MCODE plugin (Bader and Hogue, 2003) in Cytoscape was used to find clusters within the network. Clusters containing five or more nodes were selected for inclusion in this study.

2.3. Identifying key residues using stress and betweenness metrics

The calculated betweenness and stress centralities were exported from Cytoscape to Microsoft Excel. To find key residues, the Z-score of each residue was calculated after data normalization; if the absolute value of the Z-score was \( \geq 2 \), the residue was considered of interest. This Z-score cutoff was used because it roughly corresponds to a 95% confidence interval in a normally distributed data set. Because the Z-score method assumes normality, the betweenness values were transformed by taking the arcsine of the cube root of the betweenness value for each residue. For stress, data were transformed by taking the cube root of the stress value for each residue. The Kolmogorov-Smirnoff test for normality was used after the transformations. The data was considered close enough to normal for further analysis if it passed the KS test with a P-value of \( \geq 0.005 \). The residues of interest identified using betweenness and stress were compared for each structure, and the overlap of the two sets contained the residues considered key.

2.4. Identifying predicted bond energies of key residue interactions

RING predicts the presence and energy of bonds between residues in a protein. The data for the edges was downloaded from RING and imported to Excel. The files were searched for the key residues, and the total energies of the bonds the key residues were predicted to be involved in were calculated. If there was a consistent and large difference in energies when comparing the free and inhibitor-bound forms of the M\text{pro} structure, the residues were selected for further study.

2.5. Comparing B-factors of residues involved in the largest cluster or bonding with key residues

B-factor data for the alpha carbons of the residues were obtained from the PDB files for the proteins. The B-factors for select key residues and the residues with which they interact were compared using a paired t-test; P-values less than 0.05 were considered statistically significant. If a key residue interacted with another residue in both the inhibitor-bound state and the free state, it was included in the B-factor comparison; only the consensus interactions were included. The B-factors were compared for the largest cluster. Only the residues included in the largest cluster in both structures with the inhibitor were included in the B-factor comparisons. In all cases, B-factors were compared between structures with the inhibitor vs without the inhibitor. Because of the differences in average B-factor when comparing structures as a whole, all B-factor values were divided by the average B-factor for that structure to express the B-factor relative to the whole.

3. Results

3.1. Three-dimensional structure, amino acid sequences, and global network metrics were similar

The three-dimensional structures were all similar to that of the SARS-CoV-2 M\text{pro} with N3 shown in Fig. 1A. Residue interaction networks were generated for M\text{pro} structures for SARS-CoV and SARS-CoV-2 with and without an inhibitor. Fig. 1B is a representative network. The comparison between the free form and the inhibitor-bound form of M\text{pro} is of particular interest to this study SARS-CoV-2 (Fig. 1C) and SARS-CoV-2 (Fig. 1D) are shown overlapped with the inhibitor-bound forms in Fig. 1. Comparing the M\text{pro} structures of SARS-CoV to SARS-CoV-2 gave an average RMSD of 0.65 Å for the backbone of each residue close enough to be compared in Chimera; for SARS-CoV with and without N3, 0.15 Å; for SARS-CoV-2 with and without N3, 0.54 Å. Of the 306 residues of the main protease amino acid sequence, only 12 differed between SARS-CoV and SARS-CoV-2 (Fig. 2), a difference of about 4%. Of the differences, RING only predicts residue 46 to have a nonspecific interaction with N3 in both SARS-CoV and SARS-CoV-2. The structures chosen for SARS-CoV and SARS-CoV-2 each have the same amino acid sequence as their respective inhibitor-bound form. Overall, several global network metrics were also similar (Table 1).
3.2. More clusters with greater overlap in RINs of inhibitor-bound Mpro’s

The MCODE scores for clusters with five or more residues and the members of the clusters are shown in Table 2 for structures without the inhibitor and in Table 3 for structures with the inhibitor. For both SARS-CoV and SARS-CoV-2, binding to the inhibitor led to the formation of more clusters with higher MCODE scores. The clusters were not only more numerous, but they also had more members. The greatest overlap in cluster members was in the clusters in structures with the inhibitor and containing residues 17, 18, 30–33, 70, 95, 98, 103, 117, 122, and 177. This group is considered the consensus cluster.

3.3. Residues 131, 185, and 203 were key in both Mpro’s without inhibitor N3

SARS-CoV-2 with no inhibitor RIN betweenness analysis identified
residues 130, 131, 134, 150, 182, 185, and 203 as residues of interest (Fig. 3A). The stress metric identified 131, 134, 181, 185, 187, 203, and 204 as of interest (Fig. 3B). Key residues are the overlap of these two sets. For SARS-CoV with no inhibitor, betweenness residues of interest included 131, 135, 161, 175, 182, 185, and 203 (Fig. 3C). Stress residues of interest included 131, 135, 181, 182, 185, 203, and 204 (Fig. 3D). The key residue agreement for these structures identified 131, 185, and 203 as of particular interest in the inhibitor-free form of Mpros.

Table 1
Global network metrics for Mpro with and without inhibitor N3.

| Virus       | PDB ID | N3 | Clustering Coefficient | Characteristic Path Length | Average Number of Neighbors | Centralization |
|-------------|--------|----|-------------------------|---------------------------|----------------------------|----------------|
| SARS-CoV    | 2H2Z   | No | 0.138                   | 7.187                     | 3.692                      | 0.028          |
|             | 2H0B   | Yes| 0.142                   | 7.314                     | 3.723                      | 0.028          |
| SARS-CoV-2  | 6Y84   | No | 0.162                   | 7.125                     | 3.899                      | 0.022          |
|             | 6LU7   | Yes| 0.134                   | 7.175                     | 3.638                      | 0.024          |

Table 2
Residues involved in MCODE clusters in Mpro without inhibitor N3.

| Virus       | PDB ID | MCODE Cluster Score | Residues Involved* |
|-------------|--------|---------------------|--------------------|
| SARS-CoV    | 2H2Z   | 2.857               | 111, 129, 131, 135, 167, 171, 185, 290 |
|             |        | 2.500               | 63, 68, 77, 80, 89  |
| SARS-CoV-2  | 6Y84   | 2.875               | 111, 129, 131, 132, 198, 199, 221, 223, 233, 239, 240, 266, 269, 270, 272, 273, 290 |
|             |        | 2.800               | 3, 202, 206, 210, 282, 293 |

* Involved residues in common between SARS-CoV and SARS-CoV-2 are in bold.
3.4. Residues 131 and 182 were key in both Mpros within inhibitor N3 with inhibitor RIN betweenness analysis identified residues 109, 130, 131, 162, 175, 182, 203, and 207 as of interest (Fig. 4A). The stress metric identified residues 130, 131, 135, 175, 182, 185, 203, and 204 as of interest (Fig. 4B). As above, the intersection of these sets includes the residues considered key. For SARS-CoV with the inhibitor, the betweenness metric identified residues 111, 131, 135, 175, 182, 185, 203, and 206 (Fig. 4C). The stress metric identified residues 131, 135, 181, 182, 185, and 204 (Fig. 4D). The key residue agreement for these structures identified residues 131 and 182 as of particular interest for the inhibitor-bound form of Mpros.

3.5. Residues 175, 182, and 185 had bond energy increases within inhibitor N3

Of the key residues identified using betweenness and stress centralities, some were selected for further analysis. All residues part of a key residue consensus (131, 182, 185, and 203) were selected. Furthermore, residues with complete differences (neither identified in betweenness or stress in one structure but in both betweenness and stress in the other) when comparing the same Mpros with or without the inhibitor were also selected. This added residues 134 and 175 to the list of residues to further investigate. The total bond energy for the bonds in which each of these residues was involved for all structures was calculated and is shown in Table 4. Residues 175, 182, and 185 each had a large bond energy increase in structures with the inhibitor compared to structures without.

### Table 3

Residues involved in MCODE clusters in Mpros with inhibitor N3.

| Virus     | PDB ID | MCODE Cluster Score | Residues Involved* |
|-----------|--------|---------------------|--------------------|
| SARS-CoV  | 2HOB   | 4.500               | 208, 211, 219, 271, 281 |
|           |        | 3.143               | 200, 202–204, 206, 289, 293, 296 |
|           |        | 2.857               | 111, 129, 131, 135, 167, 185, 192, 290 |
|           |        | 2.714               | 13, 17, 18, 30–33, 70, 86, 95, 98, 103, 112, 115, 117, 122 |
|           |        | 2.500               | 63, 68, 77, 80, 89 |
| SARS-CoV-2| 6LU7   | 4.000               | 3, 206, 210, 282, 296, 300 |
|           |        | 2.833               | 20, 27, 36, 38, 40–42, 85, 87, 89, 164, 175, 187 |
|           |        | 2.429               | 14, 17, 18, 30–33, 70, 95, 98, 101, 103, 117, 122, 177 |

* Involved residues in common between SARS-CoV and SARS-CoV-2 are in bold.

Fig. 3. Betweenness and stress centralities for Mpro structures without inhibitor N3 for SARS-CoV-2 (A and B) SARS-CoV (C and D). Red data points have Z-score absolute values ≥ 2 for normalized data. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Residues 131 and 182 were key in both Mpro’s with inhibitor N3

SARS-CoV-2 with inhibitor RIN betweenness analysis identified residues 109, 130, 131, 162, 175, 182, 203, and 207 as of interest (Fig. 4A). The stress metric identified residues 130, 131, 135, 175, 182, 185, 203, and 204 as of interest (Fig. 4B). As above, the intersection of these sets includes the residues considered key. For SARS-CoV with the inhibitor, the betweenness metric identified residues 111, 131, 135, 175, 182, 185, 203, and 206 (Fig. 4C). The stress metric identified residues 131, 135, 181, 182, 185, and 204 (Fig. 4D). The key residue agreement for these structures identified residues 131 and 182 as of particular interest for the inhibitor-bound form of Mpro’s.

3.5. Residues 175, 182, and 185 had bond energy increases with inhibitor N3

The relative B-factors of the consensus cluster or residues involved in bonds with key residues were compared in Mpro structures with or without the inhibitor (Figs. 5 and 6). For SARS-CoV, statistically significant differences were found only when comparing residue 182 and its bonding residues (Fig. 5D); there was a slight increase in the relative B-factor of the alpha carbons. For SARS-CoV-2, there were two statistically significant changes: an increase in the relative B-factor of the residues in the consensus cluster (Fig. 6B) and a slight decrease in 182 and its bonding residues (Fig. 6D).
4. Discussion

This study uses residue interaction networks to identify residues outside of the M\textsuperscript{pro} active site that changed residue interactions when bound to inhibitor N3. A network representation of the SARS-CoV-2 M\textsuperscript{pro} is shown in Fig. 1B. Comparing M\textsuperscript{pro} structures in the active form to those bound to inhibitor N3 may reveal structural and complex network changes associated with decreased enzyme activity. Decreasing M\textsuperscript{pro} activity could be an effective treatment strategy to limit coronavirus replication in COVID-19 and SARS patients. There were few obvious differences in the three-dimensional structures of M\textsuperscript{pro} when free or bound to inhibitor N3. A network representation of the SARS-CoV-2 M\textsuperscript{pro} is shown in Fig. 1B. Comparing M\textsuperscript{pro} structures in the active form to those bound to inhibitor N3 may reveal structural and complex network changes associated with decreased enzyme activity. Decreasing M\textsuperscript{pro} activity could be an effective treatment strategy to limit coronavirus replication in COVID-19 and SARS patients. There were few obvious differences in the three-dimensional structures of M\textsuperscript{pro} when free or bound to the inhibitor (Fig. 1C and 1B). The M\textsuperscript{pro}’s for SARS-CoV and SARS-CoV-2 also show remarkable similarity. The less than 4\% difference in the amino acid sequences may account for the structural similarity (Fig. 2).

Combining two different (although related) metrics to identify key residues may reduce the number of false positive hits. The betweenness and stress metrics for each node are shown in Fig. 3 (no inhibitor) and Fig. 4 (inhibitor). Although the global network metrics are similar for all the structures, there are differences on the local level. Similarities in global metrics are expected when comparing proteins so structurally similar. Differences in the key residues identified by betweenness and stress metrics may indicate structurally important differences when an inhibitor is present, so these residues were more closely examined. Interestingly, residue 131 was key in all four structures, indicating it may play a particularly important role in network structure in M\textsuperscript{pro}s generally.

An edge in a RIN represents a chemical bond between residues. Quantifying the bond energy is a way to measure the strength of the connections in which a residue is involved. The bond energies of the residues of particular interest identified above were estimated from the data output from RING. Three residues (175, 182, and 185) had consistent changes of 20 kJ/mol or more when comparing the free structures to the inhibitor-bound structures.

To provide further structural insight for the residues identified by RIN analysis, the B-factors of the regions of interest were compared. The B-factor represents the flexibility of a region: The larger the B-factor, the more flexible the structure (Yuan et al., 2005). The average B-factor for the structures without the inhibitor were significantly smaller than the average B-factor for structures with the inhibitor (Fig. 5A and Fig. 6A). This may mean that the inhibitor destabilizes the overall protein. However, some studies indicate that there is a correlation between the B-factor and the resolution of a structure (Carugo, 2018).

Table 4

| Residue | Bond Energies (kJ/mol) |
|---------|------------------------|
|         | SARS-CoV | SARS-CoV-2 | SARS-CoV | SARS-CoV-2 |
|         | No Inhibitor | Inhibitor N3 | No Inhibitor | Inhibitor N3 |
| 131     | 117 | 123 | 143 | 123 |
| 134     | 35 | 35 | 41 | 47 |
| 175     | 64 | 117 | 64 | 129 |
| 182     | 79.4 | 112 | 79.4 | 109.4 |
| 185     | 30 | 60 | 36 | 60 |
| 205     | 92 | 110 | 92 | 116 |

* Residues with large and consistent bond energy changes selected for further analysis are in bold.
Fig. 5. Comparing B-factors for SARS-CoV Mpro structures without and with inhibitor N3 (A); comparing relative B-factors for the consensus cluster without and with inhibitor N3 (B); and comparing relative B-factors for key residues 175 (C), 182 (D), and 185 (E) and their bonding partners. * indicates P-value < 0.05 with a paired t-test.

Fig. 6. Comparing B-factors for SARS-CoV-2 Mpro structures without and with inhibitor N3 (A); comparing relative B-factors for the consensus cluster without and with inhibitor N3 (B); and comparing relative B-factors for key residues 175 (C), 182 (D), and 185 (E) and their bonding partners. * indicates P-value < 0.05 with a paired t-test.
in this study indicates that disrupting it may fundamentally alter the Mpro structure in both SARS-CoV and SARS-CoV-2. Some residues identified in this study are shown on Mpro for SARS-CoV-2 in Fig. 7. The residues involved in the catalytic dyad (Cys145 and His41) are shown in green. The residues identified in this study are mostly separate from the active site. Notably, residue 175 is buried in the structure. Mpro is a dimeric protein (Chou et al., 2004). RINs in this study were created from single polypeptide chains. Future studies should investigate the role of the quaternary structure of Mpro on the structural and network changes reported in this study. It is possible that creating networks from dimerized proteins could affect the network connections of residues at the interface.

5. Study limitations and conclusions

Next steps should include experimental verification of the role of these residues in network metrics, three-dimensional structures, and overall enzyme function. Targeted mutagenesis could provide verification of the structural role of residues identified in this study. According to the PDB files, all four structures were solved for crystals formed under similar conditions: solvents containing DMSO (3%–5%), PEG (15% PEG 4000 or 2% PEG 6000) and 0.1 M MES buffer at pH 6.0 or pH 6.5. Temperatures of crystallization were close, ranging from 291 K to 293 K. Researchers who submitted the structures performed all experiments at 100 K. It is known that Mpro is sensitive to pH changes (Tan et al., 2005), but it is unclear if the small pH difference in the crystallization conditions of the proteins included in this study could alter the RIN metrics or three-dimensional structures in biologically significant ways. Some researchers have observed that pH 6.0 leads to structures that are not as active as at neutral pH (Hilgenfeld, 2014), so the relevance of analyses of these structures to physiological enzymatic function is not entirely clear. Finally, while there is a body of literature on the usefulness of structural applications of RINs, more research needs to be done to determine the extent of the usefulness of these methods. One cluster and four residues (131, 175, 182, and 185) were found to be involved in conformational and network changes when bound to the inhibitor N3. Information on structural and RIN changes when bound to an inhibitor may be useful for understanding how to inhibit SARS-CoV and SARS-CoV-2 Mpros.

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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