Structural basis for replicase polyprotein cleavage and substrate specificity of main protease from SARS-CoV-2

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The main protease (Mpro) of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a key enzyme, which extensively digests CoV replicase polyproteins essential for viral replication and transcription, making it an attractive target for antiviral drug development. However, the molecular mechanism of how Mpro of SARS-CoV-2 digests replicase polyproteins, releasing the nonstructural proteins (nsp), and its substrate specificity remain largely unknown. Here, we determine the high-resolution structures of SARS-CoV-2 Mpro in its resting state, precleavage state, and postcleavage state, constituting a full cycle of substrate cleavage. The structures show the delicate conformational changes that occur during polyprotein processing. Further, we solve the structures of the SARS-CoV-2 Mpro mutant (H41A) in complex with six native cleavage substrates from replicase polyproteins, and demonstrate that SARS-CoV-2 Mpro can recognize sequences as long as 10 residues but only have special selectivity for four subsites. These structural data provide a basis to develop potent new inhibitors against SARS-CoV-2.

SARS-CoV-2 | main protease | cleavage cycle | substrate selectivity

Coronaviruses (CoVs) are prevalent pathogens that infect both humans and animals, which can lead to severe health problems and economic losses. CoVs cause a wide range of diseases, leading to damage to the respiratory, gastrointestinal, hepatic, and nervous systems (1–3). From the beginning of this century, there have been three CoVs that have crossed the species barrier and caused outbreaks of severe respiratory diseases in humans. These include severe acute respiratory syndrome (SARS) in 2002 (4), Middle East respiratory syndrome (MERS) in 2012 (5), and COVID-19 in 2019. The etiological agent for COVID-19 has been identified to be a new CoV referred to as SARS-CoV-2 (6, 7). The clinical features of COVID-19 include fever, cough, shortness of breath, and fatigue (8). SARS-CoV-2 infection can injure organs including lung, heart, liver, kidney, brain, and intestines. Older patients with baseline comorbidities are more likely to exhibit poor clinical prognosis, including death, from the infection (8). To date, there have been 480 million confirmed cases globally and more than 6.12 million deaths from this disease (9).

CoVs are positive-sense RNA viruses with the largest viral RNA genomes ranging from 25,500 nt to 32,000 nt. The genomic length of SARS-CoV-2 is around 30,000 nt and shows a high similarity to that of SARS-CoV (6, 7). After viral entry, two overlapping polyproteins, pp1a (490 kDa) and pp1ab (794 kDa), are translated by the translational read-through (8). To date, there have been 480 million confirmed cases globally and more than 6.12 million deaths from this disease (9).

The main protease (Mpro) of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a key enzyme, which extensively digests CoV replicase polyproteins essential for viral replication and transcription, making it an attractive target for antiviral drug development. However, the molecular mechanism of how Mpro of SARS-CoV-2 digests replicase polyproteins, releasing the nonstructural proteins (nsp), and its substrate specificity remain largely unknown. Here, we determine the high-resolution structures of SARS-CoV-2 Mpro in its resting state, precleavage state, and postcleavage state, constituting a full cycle of substrate cleavage. The structures show the delicate conformational changes that occur during polyprotein processing. Further, we solve the structures of the SARS-CoV-2 Mpro mutant (H41A) in complex with six native cleavage substrates from replicase polyproteins, and demonstrate that SARS-CoV-2 Mpro can recognize sequences as long as 10 residues but only have special selectivity for four subsites. These structural data provide a basis to develop potent new inhibitors against SARS-CoV-2.

Significance

COVID-19 is a deadly rampaging infectious disease with over 480 million cases worldwide. Unfortunately, effective therapies remain very limited. Novel antiviral agents are urgently needed to combat this global healthcare crisis. Here, we elucidate the structural basis for replicase polyprotein cleavage and substrate specificity of SARS-CoV-2 main protease (Mpro). Through analyzing a series of high-resolution structures of SARS-CoV-2 Mpro throughout the proteolytic process, we demonstrate the molecular mechanism of Mpro in proteolytic processing that confers substrate specificity. Substrate selectivity is revealed using structures of the H41A mutant in complex with six individual native cleavage substrates. Our study underscores the mechanistic function of Mpro in the viral life cycle, which provides structural insights to develop effective inhibitors against this essential target of SARS-CoV-2.
Here, we determined the high-resolution structures of SARS-CoV-2 Mpro in its resting state, precleavage state, and postcleavage state, providing a comprehensive view of the full catalytic cycle. Further, we solved the structures of SARS-CoV-2 Mpro mutant (H41A) in complex with six cleavage substrates from replicase polyproteins. Our studies provide insights into the discovery of potent inhibitors against SARS-CoV-2 Mpro.

Results

Resting State of SARS-CoV Mpro. In order to delineate the full catalytic cycle of substrate hydrolysis for SARS-CoV-2 Mpro, we determined the crystal structures of this peptidase in its resting state, precleavage state, and postcleavage state. We first solved the 2.0-Å apo structure of SARS-CoV-2 Mpro, which represents its resting state. In the apo structure, there is only one protomer in an asymmetric unit, and a functional dimer is formed via C2 crystal symmetry (Fig. 1 and SI Appendix, Table S1). Each protomer consists of three domains (Fig. 1C). The substrate binding pocket and the catalytic dyad are located at the cleft between two β-barrel fold domains (domain I and domain II) as previously described (15, 18). Domain III is relatively independent and is connected to domain II through a long loop region (residues 185 to 200). Four subsites, S10, S1, S2, and S4, are well defined (Fig. 1D), and can accommodate P1, P1, P2, and P4 positions, respectively, of the cleavage substrate. Sequence alignment shows that these subsites have preference for specific residues among the 11 cleavage sites (SI Appendix, Fig. S1C).

In the apo structure (resting state), the S1 subsite in each protomer has an absolute preference for glutamine at P1 site. This subsite is composed of the side chains of F140, N142, S144, H163, E166, and H172, and the main chain atoms of F140, L141, N142, M165, and the first residue from a neighboring protomer. The S1’ is formed by the side chains of T25, L27, H41, and C145, as well as the backbone atoms of T26 and C145. This is a shallow subsite, which can only accommodate residues with short side chains. S2 subsite is a highly hydrophobic subpocket, which consists of the side chains of H41, M49, Y54, and M165 and the alkyl portion of the side chain of Asp187. S4 is a semienclosed subsite, which is constituted by the side chains of M165, L167, F185, and Q192 and the backbone of Q189 (SI Appendix, Fig. S1D and E).

Precleavage State of SARS-CoV-2 Mpro. To investigate the precleavage state of SARS-CoV-2 Mpro, we designed an inactive mutant H41A to capture the nsp5/6 cleavage substrate (residues from S3564 to R3574 at pp1ab; SI Appendix, Fig. S1B). Then we determined the crystal structure of apo H41A at a resolution of 1.5 Å (SI Appendix, Fig. S2A). This structure is almost identical to the resting-state structure except for its flexible C terminus (SI Appendix, Fig. S2B). Next, we solved the crystal structure of the H41A mutant in complex with the nsp5/6 substrate (H41A–nsp5/6) at 1.5 Å (Fig. 2A). The complex structure is similar to that of the mutant alone with an rmsd of 0.259 Å for all Cα atoms (SI Appendix, Fig. S2A). The most significant movement can be observed for α46–50 and loop188–191 in each protomer, which is likely induced by nsp5/6 substrate binding (SI Appendix, Fig. S2B). The substrate binding pocket in the complex structure is slightly expanded compared with that in the apo mutant structure (SI Appendix, Fig. S2A). P6 to P3’ (from S3564 to V3572) can also be seen bound in the substrate binding pocket (Fig. 2B–D). A similar structure in the precleavage state was observed in SARS-CoV Mpro. The structure of SARS-CoV Mpro in the
Preincision state is similar to that of SARS-CoV-2 Mpro, with an rmsd of 0.646 Å for all Cα atoms. The major difference between these two binding modes lies in the P2', P3', and P4' positions, as we can see for SARS-CoV-2 Mpro in recognizing its various peptidyl substrates. (SI Appendix, Fig. S3 A and B) (26).

In the S1 subsite, which is the most selective subsite, Q3569 at P1 position occupies this subpocket. This corresponds to the lactam ring in the Michael acceptor inhibitors reported in other complex structures (15, 18, 19). In our structure, the side chain of the glutamine is stabilized by three hydrogen bonds formed by the Oε1 atom of Q3569 and Nε2 atom of H163, the Nε2 atom of Q3569 and carbonyl oxygen of F140, and the Nε2 atom of Q3569 and an ordered water molecule (SI Appendix, Fig. S2 C). These three hydrogen bonds impose an absolute requirement for glutamine in the S1 subsite. The main chain carbonyl oxygen of Q3569 occupies the oxyanion hole, which is stabilized by the amide groups of G143 and C145 (SI Appendix, Fig. S2 C).

The S2 subsite is a deep hydrophobic pocket, which is slightly expanded to accommodate the bulky side chain of F3568 at the P2 site compared with the apo mutant structure. M49, M165, and the alkyl portion of D187, R188, and Q189 are involved in hydrophobic interactions (Fig. 2 C). S3 is solvent exposed so that it can tolerate a wide range of functional groups at P3 site. Two hydrogen bonds formed by the amide group of T3567 at P3 with the carbonyl oxygen of E166 and the carbonyl oxygen of T3567 with the amide group of E166 stabilized the substrate (SI Appendix, Fig. S2 C). S4 is occupied by a small residue, V3566 (Fig. 2 B–D). Hydrophobic interaction between the side chain of V3566 and the side chains of M165, L167, and Q192 contributes to substrate recognition (Fig. 2 C and SI Appendix, Fig. S2 B). It is interesting to note that Q189 is not only involved in S2 subsite
formation but also participates in stabilization of the S4 subsite. Due to the steric hindrance of the benzyl group of F3568 at the P2 site, Q189 moves outward to interact with the main chain at the P4 site (SI Appendix, Fig. S2 B and C). Two pairs of hydrogen bonds are formed by the amide group of V3566 and the Oε1 atom of Q189, and by the carbonyl oxygen of V3566 and the Ne2 atom of Q189 (SI Appendix, Fig. S2 C). It is observed that α46−50 and loop188−191 move outward from the binding site (SI Appendix, Fig. S2 A and B). The P5 and P6 sites are partially exposed to the solvent, and interact with P168 and A191 of the protease through van der Waals interactions (Fig. 2 C and SI Appendix, Fig. S2 B).

S1′ is a shallow subsite, which can only hold small residues such as S3570 at P1′ (Fig. 2 C). Here, the carbonyl oxygen of S3570 and the amide group of G143 form a hydrogen bond (SI Appendix, Fig. S2 C). The side chains of T25, L27, and C145 interact with S3570 via van der Waals interactions. At the S2′ subsite, the main chain of A3571 at P2′ is stabilized by two hydrogen bonds, which are formed between the amide group of A3571 and the carbonyl oxygen of T26, and between the carbonyl oxygen of A3571 and the amide group of T26 (SI Appendix, Fig. S2 C). The side chain of A3571 is exposed to solvent, so it does not contribute significantly to substrate binding (Fig. 2 B and C). At the S3′ subsite, V3572 is at P3′ and is also solvent exposed. The S3′ subsite shows low sequence conservation among 11 cleavage sites (SI Appendix, Fig. S1 B). It only forms weak van der Waals interactions with P168 (Fig. 2 C and SI Appendix, Fig. S2 B). The last two residues of the substrate cannot be traced, due to the poor electron density in this region.

Postcleavage State of SARS-CoV-2 Mpro. In an attempt to crystallize wild-type (WT) apo Mpro, we obtained a crystal form of the enzyme that belongs to the P1 space group (SI Appendix, Table S1). We determined this crystal structure at 2.2 Å, and it showed there are two pairs of Mpro dimers (AB and A′B′) in an asymmetric unit (Fig. 3 A). The AB dimer is almost identical to the A′B′ dimer, with an rmsd of 0.065 Å for all Cα atoms. Interestingly, the C-terminal residues S301 to Q306 (corresponding to S3564 to Q3569 in the polyprotein) from the neighboring protomer A′ in the A′B′ dimer are observed to fill the substrate binding pocket of protomer A (Fig. 3 B). This arrangement of amino acids in the catalytic dyad. (E) A cartoon visualization of the cleavage cycle of SARS-CoV-2 Mpro.

Fig. 3. Structure of SARS-CoV-2 Mpro in the postcleavage state. (A) Two Mpro dimers are associated together, representing the postcleavage state. One dimer pair is colored in salmon (protomer A) and gray (protomer B). A second dimer is formed by protomer A′ and B′ (bright orange and light gray). The C terminus of protomer A′ is inserted into the substrate binding pocket of protomer A. (B) The zoom-in view of the C terminus of protomer A′. The residues S301 to Q306 are shown as ball-and-stick models. Residues involved in substrate binding are shown as sticks. (C) A comparison of the H41A−nsp5:6 complex structure and Mpro in postcleavage state structure. The H41A−nsp5:6 complex is colored in cyan and green (protease in cyan and peptidyl substrate nsp5:6 in green), and Mpro in postcleavage state is colored in salmon and bright orange (protease in salmon and C-terminal protomer A′ in bright orange). Substrates located in substrate binding pocket are shown as ball-and-stick models. Residues involved in substrate binding are shown as sticks. (D) The arrangement of amino acids in the catalytic dyad. (E) A cartoon visualization of the cleavage cycle of SARS-CoV-2 Mpro.
binding pocket of protomer A in the AB dimer (Fig. 3 A and B). Similar conformation was observed in SARS-CoV Mpro, suggesting this is an important intermediate state for these two viral proteases in the cleavage cycle (SI Appendix, Figs. S3 C and D) (27). This structure therefore represents the postcleavage state for SARS-CoV-2 Mpro in the processing of the nsp5|6 substrate, where the P10 to P4’ portion has already been released. The P1 to P6 sites take on conformations similar to those in the precleavage state (Fig. 3C). At the active center, the complete carboxyl terminus of Q1569 (P1 site) is close to the thiol group of the C145 nucleophile whose thiol sulfur is 3.8 Å from the Ne2 of the base H41 (Fig. 3D). Thus, it is possible to envisage the full cycle of substrate hydrolysis (Fig. 3A). Additional interactions between protomer A’ and protomer B (SI Appendix, Fig. S4) are also observed which may play a role in the cleavage event.

It has been observed that domain III of each protomer is flexible (SI Appendix, Fig. S5A), and the most flexible region is located at its C terminus (SI Appendix, Fig. S5B). The alignment of protomer A and protomer B shows that the loop connecting domains I and II serves as a hinge, which allows domain III to rotate by ~5° (SI Appendix, Fig. S5C). These conformational changes may be important in facilitating efficient substrate recognition and catalysis.

### Substrate Specificity of SARS-CoV-2 Mpro
In order to understand the substrate preference of Mpro in processing the polyproteins, we determined the structure of Mpro mutant H41A in complex with a series of substrates derived from multiple cleavage sites on polyproteins. In addition to the aforementioned crystal structure of H41A–nsp5|6 peptide, we successfully solved the structures of H41A complexed with nsp4|5, nsp6|7, nsp9|10, nsp14|15, and nsp15|16 substrates, respectively (Figs. 4A and 5 and SI Appendix, Fig. S6 and Table S1). The overall structure is almost identical in all six complex structures, with r.m.s.d. values from 0.118 Å to 0.324 Å for all Ca atoms (Fig. 4A). Interestingly, superposition of these structures shows that the Ca positions of P6 to P1′ for different peptidyl substrates are well aligned, indicating that SARS-CoV-2 Mpro has strong selectivity at these cleavage sites (Fig. 4 B and C). In contrast, Ca positions from P2′ to P4′ sites are sharply divergent (Fig. 4D), which suggests that S2′ to S4′ substrates in SARS-CoV-2 Mpro would not have a stringent preference on substrate side chains, reflecting their higher degree of plasticity compared with S6 to S1′ subsites.

A closer look at the subsites in these six H41A–substrate complex structures reveals that S1 is only occupied by glutamine because the side chain of P1 glutamine can form three hydrogen bonds with the side chain of H163, the main chain of F140, and an ordered water molecule, respectively (Fig. 5 and SI Appendix, Fig. S7). The S2 subsite is a deep hydrophobic subsite which prefers hydrophobic residues, and hydrophobic interactions dominate the interplay between the protease and the substrate at this subsite. This subsite is highly malleable and can tolerate the side chain of each residue is shown as a colored sphere. This can be seen in the H41A–nsp4|5, H41A–nsp9|10, H41A–nsp14|15, and H41A–nsp15|16 complex structures. In contrast, valine (in the nsp6|7 complex structure), with a smaller side chain, cannot reach to the bottom of S2. Distinct from the nsp5|6 complex structure, where Q189 moves outward to interact with the main chain of P4 site due to the steric hindrance as

![Image](Image)

**Fig. 4.** Structure of H41A mutant in complex with peptidyl substrates. (A) A comparison of the structures of six peptidyl substrates in complex with the H41A mutant. The structures of H41A–nsp4|5, H41A–nsp5|6, H41A–nsp9|10, H41A–nsp14|15, and H41A–nsp15|16 are shown in stick models. (D) Ca positions are divergent from P2′ to P4′ sites. (E) Positively charged residues at P3 position make extra interactions with H41A. The interacting residues are shown as stick models. Water molecules are shown as green spheres and named W1 and W2, respectively. (G) MST assay curve of the binding affinity between H41A and six peptidyl substrates. Data from three independent experiments are presented as the mean values with their SD.
mentioned above (SI Appendix, Fig. S2B), in the other five complex structures, Q189 participates in positioning these five substrates through a hydrogen bond formed by its own Oe1 atom and the amine moiety of the amide group at all these P2 sites (SI Appendix, Fig. S7).

It has been assumed in the literature that the solvent-accessible S3 is not selective (15, 18), so it has not been considered to play a significant role in drug development. Among all cleavage sites on the replicase polyproteins, this position can be occupied by five different residues, including hydrophobic residues (valine and methionine), polar residue (threonine), and positively charged residues (lysine and arginine) (SI Appendix, Fig. S1B). In the complex structures we have determined, there are four residues that appear at this position (valine in nsp4/5, threonine in nsp5/6 and nsp6/7, arginine in nsp9/10 and nsp14/15, and lysine in nsp15/16). All the main chains are positioned by two hydrogen bonds as has been described in the H41A–nsp5/6 complex structure (SI Appendix, Fig. S7). It is notable that the side chains of positively charged residues (arginine or lysine) at the P3 position are able to form hydrogen bonds with H41A, thus contributing to substrate binding. In the structure of H41A in complex with nsp9/10, the side chain of R452I at P3 forms two hydrogen bonds with Q189 and N142 (Fig. 4E and SI Appendix, Fig. S7C); in the structure of H41A in complex with nsp15/16, the side chain of K6796 at the corresponding site forms a hydrogen bond with a surrounding water molecule which bridges both K6796 from the substrate and Q189 from the protease (Fig. 4E and SI Appendix, Fig. S7E). These findings suggest that a suitable functional group at the P3 site can be created to assist in increasing inhibitor potency and selectivity. Compared with the S3 subsite that can tolerate a variety of residues, S4 is a small subpocket which can only accommodate residues with small side chains. In the complex structures, four different residues with small side chains (alanine, valine, proline, and threonine) can be seen to bind to this subsite (SI Appendix, Fig. S1B). Compared with the H41A–nsp5/6 complex structure, where Q189 interacts with the main chain of the P4 site, only the carbonyl oxygen of T190 is involved in the substrate positioning by forming a hydrogen bond with the amide nitrogen group from the residue at the P4 site in H41A–nsp4/5, H41A–nsp6/7, H41A–nsp9/10, and H41A–nsp14/15 complex structures (SI Appendix, Figs. S2C and S7 A–D). Interestingly, the carbonyl oxygen of T190 and the amide nitrogen oxygen of T190. Similar to the structure of H41A–nsp5/6 peptide, residues at the P5 and P6 sites are solvent exposed in the other five complex structures, thus having limited selectivity. In these structures, only the carbonyl oxygen from the P5 position can form a hydrogen bond with the protease through a bridging water molecule (H41A–nsp4/5, H41A–nsp6/7, and H41A–nsp9/10). The side chain of S5269 also forms a hydrogen bond with the carbonyl oxygen of Q189 through a bridging water molecule in the H41A–nsp4/5 complex structure (SI Appendix, Fig. S7A). Except for P6793 at the P6 position that cannot be traced in the structure of H41A–nsp15/16 complex, the residues at this position in other complexes are visible (Fig. 4B and SI Appendix, Fig. S6).

As S1 is a shallow pocket, it prefers small residues. All six structures show that serine (in nsp4/5, nsp5/6, nsp6/7, nsp14/15, and nsp15/16) and alanine (in nsp9/10) can be accommodated at this subsite, while serine is the most common residue appearing at the P1 position in the substrate (SI Appendix, Fig. S1B). A water molecule bridges the side chain of serine from the substrate to the main chain of A41 through hydrogen bonds, which may account for serine being frequently observed at this site (SI Appendix, Fig. S7 A, B, D, and E). The side chain at P1' also interacts with the side chains of T25, L27, and C145 through van der Waals interactions. The backbone atoms of P2' are confined to a relatively fixed position through two hydrogen bonds formed by the amide group of P2' with the carbonyl oxygen of T26 and the carbonyl oxygen of P2' residue with the amide group of T26 (SI Appendix, Fig. S7). The side chains of P2' are also exposed to the solvent, and thus S2' is not expected to have a strong substrate selectivity. Only in the H41A–nsp15/16 complex structure, the side chain of S6800 at P2' position forms hydrogen bonds with N119 and G143 through water molecules (SI Appendix, Fig. S7E).

The positions for the Cαs become diverse beyond the P2' site (i.e., out to P3' and P4'). The directions of the backbones for these substrates diverge into two different directions at the P3' position, noting that, in the H41A–nsp14/15 complex, the backbone can only be traced to P2' (Figs. 4B and 5 G and H). In the structures for nsp4/5, nsp5/6, nsp9/10, and nsp15/16, their backbone extension directions share a common feature. In contrast, in the structure of H41A–nsp6/7 complex, M3862 at P3' has a sharp turn of nearly 90°, causing the deviation of its backbone direction from those in the other complexes (Fig. 4 B and D). Only in the H41A–nsp15/16 complex, the density for the side chain of Q6801 at P3' can be observed (Fig. 5 I and J). In this structure, the main chain carbonyl oxygen 15 forms a hydrogen bond with a water molecule, which takes on the bridge to T24 and T26. In addition, the Ne2 from its side chain makes a hydrogen bond with T25 Oγ (SI Appendix, Fig. S7E).

The density can be traced to P4' merely in three substrate complex structures (H41A–nsp4/5, H41A–nsp6/7, and H41A–nsp9/10) (SI Appendix, Fig. S6). In the H41A–nsp4/5 complex, the main chain NH group of R3267 interacts with T24 through a hydrogen bond. Two water molecules form a bridge with the side chains of Q19, T21, L67, and Q69 through hydrogen bonds (Figs. 4F and 5 A and B and SI Appendix, Fig. S7A). In the H41A–nsp9/10 structure, only one hydrogen bond is formed between the main chain NH group of A257 and T24 (SI Appendix, Fig. S7C). The Cε position of S3863 at the P4' position in the nsp6/7 substrate is 6 Å from those at the P4' site in the other two complex structures (Fig. 4B). An ordered water molecule forms hydrogen bonds with the main chain NH group of S3863, T24, and T25 (Figs. 4G and 5 C and D and SI Appendix, Fig. S7B). Another water molecule forms hydrogen bonds with the main chain carbonyl group of S3863 and S46 (Figs. 4G and 5 C and D and SI Appendix, Fig. S7B).

We next measured the binding affinity between the H41A mutant and the six substrates. The Kd values ranges from 28.1 μM to 2.7 mM (Fig. 4H). Nsp4/5, nsp6/7, and nsp9/10 have the strongest binding affinity for H41A, with Kd values of 28.1 μM, 41.2 μM, and 115.8 μM, respectively. This is consistent with the fact that these substrate backbones can be traced to as long as 10 residues (P6 to P4') in the density maps. Two other cleavage substrates (nsp14/15 and nsp15/16) have lower binding affinity, with Kd values of 1.5 mM and 262.4 μM. In these two complexes, eight residues can be seen, including the P5 to P3' sites for nsp14/15 and the P6 to P2' sites for nsp15/16. However, serine at P2' and glutamine at P3' in the nsp15/16 substrate form three extra hydrogen bonds compared with the nsp14/15 substrate complex. Hence, the nsp15/16 cleavage substrate is able to form stronger binding to H41A.
involved in viral replication and transcription (14, 15, 28). Evolutionarily conserved nonstructural proteins, which are cleaved from the polyprotein in the event of replicase assembly, which implies that viral protease might have a preference to release certain nsps in the process. Significantly, the cleavage substrates and H41A suggest that viral protease might have a preference to release certain nsps from the polyprotein in the event of replicase assembly, which can be taken advantage of to design more potent inhibitors.

Discussion

New drugs are urgently needed to treat SARS-CoV-2 infections. Significant efforts have been focused on targeting the evolutionarily conserved nonstructural proteins, which are involved in viral replication and transcription (14, 15, 28–30). Among them, Mpro is one of the most attractive targets, due to its pivotal role in replicase polyprotein cleavage and the fact that it is evolutionarily conserved across all CoVs (12, 13). Highly selective inhibitors against Mpro could be potential broad-spectrum drug candidates to fight against CoV infections not only for today but also in the future (12). One step toward the rational design of potent and selective inhibitors that target Mpro can be achieved by discovering the detailed mechanisms for the process of proteolysis and substrate recognition. In this work, we determined the structures of viral protease at the resting state, precleavage state, and postcleavage state (Fig. 3E). Our findings showed that domain III of Mpro is the most flexible domain. It can adjust its conformation to allow its C terminus to access the substrate binding pockets from its surrounding active Mpros (SI Appendix, Fig. S5A). The most flexible region lies at C terminus (S301 to Q306) of Mpro, which has three preferred orientations (SI Appendix, Fig. S5B). The flexibility of this region is important for regulation of its C-terminal autocleavage process. Particularly, the Mpro C terminus in orientation 2 may allow itself to favorably insert into the substrate binding pocket of its neighbor Mpro during C-terminal proteolysis. This suggests that small molecules which can block the conformational change of domain III may serve as allosteric inhibitors for Mpro (31).

Although many screened or designed inhibitors that target the substrate binding pocket of Mpro have been discovered or developed (15, 17, 18), potent inhibitors with clinical potential are still needed. In this study, we have solved the crystal structures of Mpro mutant H41A in complex with six cleavage substrates, respectively. These structures reveal that SARS-CoV-2 Mpro can recognize substrates as long as 10 residues but generally have special selectivity for four subsites (S1, S1′, S2, and S4). In addition to the most conserved S1 subsite, the highly plastic S2 subsite is also important for drug design. The S2 subsite is a deep hydrophobic pocket which prefers hydrophobic residues. Interestingly, this subsite is highly malleable, a factor that can be taken into account for drug development. Although it has been assumed that P3 does not have selectivity, in the structures of H41A–nsp9/10, H41A–nsp14/15, and H41A–nsp15/16 complexes, arginine or lysine at P3 can form hydrogen bonds with the protease through their long side chains (Fig. 5 F, H, and J). Thus, the P3 position can serve as a subsite to enhance protease inhibitor binding. These structures also indicate that the P2′ to P4′ sites demonstrate a higher conformational diversity than those for the P6 to P1′ sites, due to the lack of protease substrate specificity at these positions. We compared several published Mpro–inhibitor complex structures (15, 16, 18, 19, 21, 32–34) with the structure of Mpro in complex with nsp4/5, which has the strongest binding affinity to Mpro among the substrates we tested (SI Appendix, Fig. S8). N3, 11a, 11b, 13b, boceprevir, and nirmatrelvir (PF-07321332) are peptidomimetic inhibitors which mainly target S1, S1′, S2, and S4 sites. All of them form a covalent bond to C145, although the structure of each warhead can be different. N3 occupies S1, S2, and S4 and partially occupies S1′ (SI Appendix, Fig. S8A). Inhibitors 11a and 11b occupy S1, S2, and S4 (SI Appendix, Fig. S8 B and C). The cyclohexyl moiety of 11a and the 3-fluorophenyl group of 11b at P2 sites can both be snugly accommodated by the bulky S2 subsite. Inhibitor 13b is bound to the viral protease in a similar mode to 11a and 11b, but its Boc group does not occupy the S4 subsite (SI Appendix, Fig. S8D). This is the likely explanation for the lower potency of 13b. In the Mpro–boceprevir complex structure, although cyclobutylalanine at the P1 site can fit in the S1 subsite, it does not form as strong an interaction as other inhibitors (SI Appendix, Fig. S8F). In contrast, nirmatrelvir (PF-07321332) is a
new oral clinical drug that shares a similar rigid dimethylcyclpropopropylproline group of its P2 site but has a classical (S)-γ-lactam ring at the P1 site, and demonstrates a strong potency as an oral therapeutic for COVID-19 (SI Appendix, Fig. S8F) (33, 34). Compound 23 is a perampanel-derived noncovalent inhibitor that binds to S1, S1′, S2, and S4 sites and demonstrates promising antiviral activity (SI Appendix, Fig. S8G) (32). Peficitinib, which exhibits a strong antiviral activity, binds to a new allosteric site close to the most flexible region (S301 to Q306) in domain III and hampers the movement of the C-terminal tail (SI Appendix, Fig. S8H) (31).

In summary, our comprehensive structural studies provide valuable insights for drug design. We demonstrate that the subpockets (S1, S1′, S2, and S4) which are endowed with an exceptional selectivity are important for selective and potent binding. Additionally, other subsites such as S3 and S2′ to S4′, which have less specificity for substrate recognition, may also be used to enhance inhibitor binding and to specifically target Mpro.

**Materials and Methods**

**Cloning, Protein Expression, and Purification of COVID-19 Virus Mpro.** The cell cultures were grown and the protein was expressed according to a previous report (15). The cell pellets were resuspended in lysis buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 5% glycerol), lysed by high-pressure homogenization, and then centrifuged at 25,000 × g for 30 min. The supernatant was loaded onto a nickel-nitritolauric acid (Ni-NTA) affinity column (Qiagen), and washed in lysis buffer containing 20 mM imidazole. The His-tagged Mpro was eluted using lysis buffer containing 300 mM imidazole. The imidazole was then removed through desalting. Human rhinovirus 3C protease was added to remove the C-terminal His tag. SARS-CoV-2 Mpro was further purified by ion exchange chromatography. The purified Mpro was transferred to 10 mM Tris-HCl pH 8.0 by desalting and stored at −80 °C until needed. The H41A construct design was based on the WT Mpro reported earlier (15). It was then cloned into a modified pET32a vector with an N-terminal thioredoxin tag and a SARS-CoV Mpro cleavage site. The vector was transferred into BL21 (DE3) and induced with isopropyl β-D-thiogalactoside for protein expression. The affinity purification was the same as for WT. The elution solution was concentrated, and imidazole was removed without desalting. Next, the thioredoxin tag was cleaved by human rhinovirus 3C protease overnight at 4 °C. Next, a Ni2+ affinity purification step was used to remove thioredoxin tag and SARS-CoV Mpro. The eluant was then loaded onto a desalting column to remove imidazole. The eluant was collected and cleaved by human rhinovirus 3C protease overnight at 19 °C. Finally, another Ni2+ affinity purification step was used to separate His tag cleaved and uncleaved protein. The flow-through sample was concentrated, and further purified by ion exchange chromatography. Then the purified protein was transferred to 10 mM Tris-HCl pH 8.0.

**Crystallization, Data Collection, and Structure Determination.** SARS-CoV-2 Mpro was concentrated to 5 mg/mL and mixed in a 1:1 (vol/vol) ratio with the well buffer, then crystallized by the hanging drop vapor diffusion method at 20 °C. The best crystals were grown using a well buffer containing 0.1 M imidazole pH 8.1, 10% polyethylene glycol (PEG) 3000, 0.2 M LiSO4. The cryoprotectant solution was the same as the reservoir but with 20% glycerol added.

The procedure for the SARS-CoV-2 Mpro H41A mutant was the same as for the WT Mpro, except the best crystals were grown using a well buffer containing 0.1 M MES (pH 5.4 to 5.8), 7% PEG 6000, and 6% dimethyl sulfoxide (DMSO). The cryoprotectant solution was the same as the reservoir but with 20% glycerol added.

The synthesized peptides (GL Biochem) were dissolved in a well buffer (0.01 M MES pH 5.6, 3.5% PEG 6000, and 3% DMSO) at 10 mM. Next, the dissolved peptide was added to the drop that contained crystals in a 1:1 (vol/vol) ratio and soaked for 12 h. The cryoprotectant solution was the same as for the Mpro H41A mutant.

X-ray data were collected on beamlines BL17U1 and BL19U1, Shanghai Synchrotron Radiation Facility (SSRF) at 100 K using an Eiger X 16M image plate detector or Pilatus3 6M image plate detector. Data integration and scaling were performed using the programs XDS (35) and xia2 (36). The apo structure was determined by molecular replacement (MR) with PHASER (37), a program inside the Phenix 1.17.1 package (38). The COVID-19 virus Mpro (Protein Data Bank [PDB] ID: 6LU7) was used as a search template. The model from MR was subsequently subjected to iterative cycles of manual model adjustment with Coot 0.8 (39), and refinement was completed with Phenix REFINEM (40). The substrates were built according to the omit map. The phasing and refinement statistics are summarized in SI Appendix, Table S1.

**Microscale Thermophoresis Assay.** The microscale thermophoresis (MST) assays were carried out according to the method previously reported (41). Binding affinities of peptide substrates with SARS-CoV-2 Mpro (H41A) were measured using Monolith NT-Automated (NanoTemper Technologies). H41A was fluorescently labeled according to the manufacturer’s procedure. Protein was kept in MST Buffer (20 mM Hepes pH 7.0, 300 mM NaCl, 5% glycerol), and the concentration was adjusted to 4 μM. Next, RED-Tris NTA-second-generation dye was added, mixed, and incubated for 30 min at 25 °C in the dark. The final molar ratio of protein to dye was 80:1. Each of the unlabelled peptide substrates at 12 different serially diluted concentrations was then mixed with the same volume of labeled protein at room temperature. The samples were then loaded into standard treated capillaries (NanoTemper Technologies) and measured at 25 °C, 40% LED power, and medium MST power. Each assay was repeated three times. Data analyses were performed using MO.Afinity Analysis v.2.2.4 software (NanoTemper Technologies). All of the final plots were made using GraphPad Prism 8.0.

**Data Availability.** All study data are included in the article and/or SI Appendix. The coordinates and structure factors have been deposited in the PDB, under the following PDB codes: 6M03 for Mpro, 7UH for H41A, TESK for Mpro post cleavage state, 7DVJ for H41A-nsp14, 7DWW for H41A-nsp5, 7DXW for H41A-nsp7, 7DVY for H41A-nsp9, 7D6W for H41A-nsp14[15], 7DWO for H41A-nsp15[16].

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