An extended conformation of SARS-CoV-2 main protease reveals allosteric targets

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The coronavirus main protease (Mpro) is required for viral replication and has enzymatic activity as a homodimer. Thus, targeting its dimerization is an effective strategy for developing allosteric inhibitors to suppress mutation escape. In this study, we obtained the extended conformation of the native monomer of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Mpro by trapping it with nanobodies, and found that the catalytic domain and the helix domain dissociate, revealing allosteric targets. We also found another state, a compact conformation, similar to the dimeric form. Our data support that the Mpro may be in equilibrium among the monomeric extended conformation as the precursor of all other states, the compact conformation as the intermediate state, and the dimeric conformation as the active state. We designed an innovative Nanoluc Binary Technology (NanoBiT)-based high-throughput allosteric inhibitor assay based on the rearranged conformation. In addition, we identified a set of allosteric inhibitory nanobodies against Mpro, one of which is also a competitive inhibitor of Mpro. Our results provide insight into the maturation of the coronavirus Mpro and a way to develop antiviral drugs through targeting the folding process to inhibit the autocleavage of the main protease.

Significance

The coronavirus main protease (Mpro) is required for viral replication. Here, we obtained the extended conformation of the native monomer of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Mpro by trapping it with nanobodies and found that the catalytic domain and the helix domain dissociate, revealing allosteric targets. Another monomeric state is termed compact conformation and is similar to one protomer of the dimeric form. We designed a Nanoluc Binary Technology (NanoBiT)-based high-throughput allosteric inhibitor assay based on structural conformational change. Our results provide insight into the maturation, dimerization, and catalysis of the coronavirus Mpro and pave a way to develop an antiviral drug through targeting the maturation process to inhibit the autocleavage of Mpro.
luciferase reporter system, Nanoluc Binary Technology (NanoBiT) (16). The luminescent signal from NanoBiT provides an accurate indication of the interaction dynamics. The NanoBiT system has been successfully used to detect protein–protein interactions and to design biosensors based on the conformational changes, establishing high-throughput screening approaches to identify allosteric modulators (17, 18).

Here, we first identified a set of allosteric inhibitory nanobodies against Mpro, and then determined two authentic monomeric transient structures of SARS-CoV-2 Mpro trapped by two nanobodies (NB1A2 and NB2B4). One is the extended state stabilized by NB2B4, in which the catalytic domain and extra helix domain is dissociated, and reveals the allosteric target. It is the precursor of all other states. The other conformation is similar to that of the dimer, and displays a substantial compaction compared with the extended structure, and is thus termed the compact conformation. Both nanobodies can target dimerization of Mpro and dissociate the dimer into monomers, thereby exerting an inhibitory effect on Mpro; thus, they are allosteric inhibitors. The epitope of NB1A2 on Mpro overlaps with the catalytic and substrate binding sites, so it is also a competitive inhibitor. In addition, we established an innovative NanoBiT-based high-throughput allosteric inhibitor assay based on the conformational changes of monomeric Mpro, and this kind of allosteric modulator can inhibit autocleavage.

Results

Generation and Characterization of Cameld Inhibitory Nanobodies against SARS-CoV-2 Mpro. We developed nanobodies with potent inhibitory activity against the SARS-CoV-2 main protease. We first obtained 11 high-affinity nanobodies from the Mpro-immunized camel using the phage display library (Table 1), and identified seven nanobodies with a potent inhibitory effect on the catalytic activity of Mpro; the IC50 values ranged from 102.04 ± 16.935 nM to 186.633 ± 23.081 nM (Fig. 1 A–D and SI Appendix, Fig. S1 A–D). The most potent nanobodies, NB1A2 and NB2B4, were 186.633 ± 23.081 nM and 122.00 ± 7.711 nM, respectively. We measured their binding kinetics using biolayer interferometry, and NB1A2 and NB2B4 had binding affinities of 2.426 ± 0.020 nM and 0.461 ± 0.007 nM, respectively (Fig. 1 E–H and SI Appendix, Fig. S1 E–K). Interestingly, the profiles from size-exclusion chromatography (SEC) showed that the result of these inhibitory nanobodies binding to the dimeric Mpro was dissociation to catalytically inactive monomers (Fig. 1 I–L). Moreover, NB1A2 and NB2B4 have different epitopes on Mpro because they can simultaneously bind to Mpro, which indicates that both nanobodies are allosteric inhibitors, which can convert the active dimeric Mpro into the inactive monomeric Mpro and each has a different allosteric inhibitory mechanism for SARS-CoV-2 Mpro (SI Appendix, Fig. S1I).

Two Transient States of Monomeric Mpro Are Trapped by Two Different Allosteric Inhibitory Nanobodies. To obtain the structure of authentic monomeric Mpro, we selected two nonoverlapping nanobodies (NB2B4 and NB1A2) and then crystallized Mpro in complex with NB2B4 and NB1A2, respectively. We determined two complex structures at a resolution of 2.0 Å and 1.75 Å by molecular replacement (Fig. 2 A and B and Table 2).

The structure of Mpro in complex with NB2B4 shows that it binds to the α-helical domain of Mpro, and is far from the catalytic site and substrate binding sites (Fig. 2A). NB2B4 binding causes the separation of the α-helical domain and antiparallel-barrel fold, the hairpin loop linking domains II and III fully unfolds, the α-helical domain swings 100° from one side of the β-barrel structure to the other side, and there is a change from bound state to free state (Fig. 2C). The NB2B4 binding did not result in overall conformational change to either the α-helical and β-barrel catalytic domains as two independent fold units, compared with that of dimeric Mpro. The conformation of monomeric Mpro is designated as the extended state.

The crystal structure of Mpro in complex with NB1A2 shows that the epitope of NB1A2 overlaps with the substrate binding site and catalytic site (Fig. 2D). The catalytic domain and helix domain contact each other, representing the compact conformation of monomeric Mpro compared with the above extended conformation, and the whole architecture is similar to that of one promoter of dimeric Mpro. However, the α-helical domain displaces from the catalytic domain about 2 Å (Fig. 2D). The structure also differs from the previously reported structures of monomeric mutants in which the catalytic domain and helix domain rotated about 40° compared with the compact monomer and the dimeric Mpro (19–22 (SI Appendix, Fig. S2A). The monomeric structure shows that the interface between the C-terminal and N-terminal domains is mainly composed of loop–loop interactions, including the salt bridge formed by R131 with D197, D289, and E290, and a hydrogen bond between N133 and D197 (SI Appendix, Fig. S2B), which indicate the C-terminal and N-terminal domains are easily dissociated. In addition to the two distinct conformations of SARS-CoV-2 monomeric Mpro stabilized by two nonoverlapping nanobodies, the mutant monomeric structures of SARS-CoV Mpro were previously reported (19–22). They adopted three completely different conformations, which indicates that Mpro in the inactive monomer has conformational heterogeneity (SI Appendix, Fig. S2A). Our data support that the compact state may be an intermediate and the monomeric extended state may be the parent state of two other monomeric forms. The two folding units of extended monomeric state come together to form the compact monomeric states, and then dimerize into the active dimer (Fig. 2E).

Mechanism of Allosteric Inhibition of SARS-CoV-2 Mpro by NB2B4. The epitope of NB2B4 on the C-terminal domain of the monomeric Mpro consists of the F helix and the loop connecting the E and F helices (Figs. 2A and 3A). The aromatic ring of residues P291 from the loop between the E and F helices inserts into the hydrophobic pocket composed of L107, L109, P110, and Y59 of NB2B4 (Fig. 3B). Mutation of P291 to Ala destroys the hydrophobic interaction, thereby eliminating the binding of NB2B4 to Mpro (SI Appendix, Fig. S3A). At the periphery of the hydrophobic core, there are multiple

Table 1. The CDR sequences of the nanobodies

| CDR1 | CDR2 | CDR3 |
|------|------|------|
| NB1A2 | GVTASSVYY | INTVGTYT | AATYLLRFAASLSATNPYY |
| NB2B4 | GYTYSSKCC | IYTGGSST | AASGAAGIAIGRLCLPHGTYTY |
| NB1H2 | GATASSVVL | INTVGYYT | AATYLRPFALGATDFPP |
| NB1F1 | RYTFSSVOC | IYPGGSST | AAPSAACTCRSRLGMOVFYS |
| NB1A1 | GFTSSSDYN | IITSTMR | ATDDSQDG |
| NB1D05 | GVTASSVYY | INTNGRT | AAHDLGYGGLRCVGVAR |
| NB1D10 | GYSSCVYD | IDSNRT | KVGGIASVPPVEVSCPPAPFGY |
| NB1B12 | GYSSCVYD | IDSNRT | KVGGIASVPPVEVSCPPAPFGY |
| NB2D10 | GHYSSCVYD | IDSNRT | AATHYTRGGLFRLLLAPFGY |
| NB2E3 | GFIAATSCA | ITTDGGT | KLCCGQQYCA |
| NB2H4 | GVAISSLV | INAVGYT | AATYLLRTAASLSAGNPYY |
hydrophilic interactions between NB2B4 and M\textsuperscript{pro} (Fig. 3C). First, R106 of CDR3 forms a salt bridge with the carbonyl group of sidechain of E290 from the loop between the E and F helices, and donates the hydrogen bond with the sidechain carbonyl group of helix F D295 (Fig. 3C). Second, the mainchains of L107 and L109 from CDR3 form double hydrogen bonds with the sidechain of Q299 from helix F. Third, an electrostatic interaction between the sidechain of R298 and the mainchain of A103 of NB2B4 is observed (Fig. 3C). The sidechain–sidechain interaction is between T113 from NB2B4 and N214 of the C-terminal domain (Fig. 3C). In addition, the hydroxyl group of Y59 on NB2B4 also forms one hydrogen bond with E288 from the C-terminal domain. The binding of NB2B4 to M\textsuperscript{pro} disrupts the important elements of enzyme dimerization, and also destroys the interactions between the N-terminal and C-terminal domains. Therefore, NB2B4 can stabilize the monomeric extended conformation and prevent the formation of the monomer compact state and dimer state.

It was previously reported that N-finger was crucial for the dimerization and enzyme activity (19, 23, 24). The density of N-terminal residues (resides 1–11) in the extended monomeric M\textsuperscript{pro} is completely missing compare with the active dimeric M\textsuperscript{pro}, which is due to the loss of contact with its own C-terminal domain and the interaction from other protomers. 

NB2B4 to the helical domain III disrupts the association between two protomers. NB2B4 also interferes with the interaction between domain III and domain II (SI Appendix, Fig. S2B). Second, the mainchains of L107 and L109 from CDR3 form double hydrogen bonds with the sidechain of Q299 from helix F. Third, an electrostatic interaction between the sidechain of R298 and the mainchain of A103 of NB2B4 is observed (Fig. 3C). The sidechain–sidechain interaction is between T113 from NB2B4 and N214 of the C-terminal domain (Fig. 3C). In addition, the hydroxyl group of Y59 on NB2B4 also forms one hydrogen bond with E288 from the C-terminal domain. The binding of NB2B4 to M\textsuperscript{pro} disrupts the important elements of enzyme dimerization, and also destroys the interactions between the N-terminal and C-terminal domains. Therefore, NB2B4 can stabilize the monomeric extended conformation and prevent the formation of the monomer compact state and dimer state.

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**NB1A2 Is Not Only an Allosteric Inhibitor, but Also a Competitive Inhibitor.** The structure reveals that NB1A2 employs its CDR3, inserting into the cleft of the substrate binding site and the catalytic site as a competitive inhibitor (Fig. 2B). This creates extensive intimate interactions through hydrogen bond networks (Fig. 3D). The sidechain of R124 on CDR3 forms hydrophilic interactions with the catalytic site residue H41 and the two residues C44 and S46 from the substrate binding pocket. A hydrogen bond is formed between the carboxy group of T131 on CDR3 and the sidechain hydroxy group of S46 from helix A. Q189 from the loop connecting domains II and III interacts with residues T120, Y121, and L122 of CDR3 through four different hydrogen bonds. These interactions can block the substrate binding. The sidechain of N142 donates a hydrogen bond to the sidechain of S127 of CDR3. In addition, a hydrogen bond is formed between the mainchain of E166 and the mainchain of L123 from NB1A2. However, the two residues, N142 and E166, are crucial to form the oxyanion hole and the subsite S1 pocket. Moreover, E166 also plays an important role in the enzyme dimerization (25, 26).

The N-terminal finger in the compact monomer shows that it swings about 21.5° relative to that of active dimer and the orientation of the sidechain of M6 and R4 changes greatly (SI Appendix, Fig. S4A). It is possible that the N-terminal finger of
the monomer is not restrained by the interaction with the other protomer of dimeric form. The architecture of monomeric MPRO supports the view that the N-finger plays a key role for the maturation, dimerization, and enzyme activity (19, 23, 24).

In dimeric MPRO structure, three hydrogen bonds are formed among the C-terminal residues Q299, V303, and F305 from the A protomer and the residues P122, S123, and S139 from the B protomer (5–7, 9, 10). Thus, the orientation of the A protomer C terminus is stabilized by the B protomer. Then, three hydrogen bonds are formed between the residues Q306 from the C terminal and the residues K12, Y154, and D155 from the catalytic domain, which fixes the distance between the C-terminal and N-terminal domains (5–7, 9, 10) (SI Appendix, Fig. S4 B and C). However, these interactions are lost in the monomer, and the constraints to the C terminal are released; indeed, the density of the residues of the C terminal is not observed in the monomer. Therefore, the orientation and distance between the α-helical domain and the catalytic domain is related with MPRO dimerization. The two domains present a different rearrangement in the monomer because the C-terminal residues are not restricted, and there are at least two different structures: our compact structure and the monomeric mutant structure, which indicates that the C-terminal residues play a vital role for the dimerization and catalytic activity.

The superposition of the NB1A2 binding monomer and one protomer of dimeric MPRO shows that the NB1A2 has a steric clash with the B helix, D helix, and C terminal of another protomer of dimeric MPRO (SI Appendix, Fig. S4D). This is one of the reasons why NB1A2 can target dimerization of MPRO. Therefore, NB1A2 also acts as an allosteric inhibitor of the enzyme.

The Conformation of Monomeric MPRO Is Incompatible with Substrate Binding. Compared with the active dimer, the extended monomer displays an upward rotation of the β-turn composed of residues 166 to 172 (Fig. 4A), and the β-turn approach the active loop, consisting of residues 145 to 137, because E166, H172, G170, and T169 from the β-turn form hydrogen bond networks with N142, G138, and K137 from the active loop, resulting in the closure of the S1 subsite (Fig. 4B). The β-turn also shifts to the left and the volume of the S2-S4 binding pocket shrinks down, which is not suitable for the substrate binding (Fig. 4C). The amplitude of upward movement of the β-turn in the compact monomer is a little smaller than that of the extended monomer because the pattern of the hydrogen bonds pattern has slightly changed: some hydrogen bonds are untied (such as the hydrogen bond between the sidechain of N142 and H172, the hydrogen bond...
between the mainchain of G138 and G170, and the hydrogen bond formed by K137 and T169), which releases some constraints between the \( \beta \)-turn and active loop, and provides more space for the P1 binding. However, the S1 subsite is still closed due to the existence of hydrogen bond formed by N142 and E166 (Fig. 4D and SI Appendix, Fig. S5A). Alignment of the \( \beta \)-turn in different states shows that it gradually displaces, rendering more and more space for substrate binding, from the extended monomer to the compact monomer, then to the inactive protomer of the dimer, and finally to the active protomer of the dimer (SI Appendix, Fig. S5 B–G). The volume of substrate bound pocket in all different states except for the active protomer is insufficient to accommodate the substrate; therefore, the substrate binding pocket is opened step by step with the maturation of the enzyme.

The orientation of the active loop composed of residues 137 to 145 is crucial for enzyme activity (5–7, 9, 10). All residues in the active loop of the monomer have large structural displacement, compared with the active loop conformation of dimeric M\(^{\text{pro}}\). In the active dimer of M\(^{\text{pro}}\), the aromatic ring stacking for Phe140 and His163 and the two hydrogen bonds between the carboxylate groups of Glu166 and Phe140 and the NH-group of Ser1 from another protomer stabilize the right conformation of the oxyanion loop and S1 pocket of the substrate-binding site (25). But, in the monomeric M\(^{\text{pro}}\), the interactions between two rings of F140 and Y126 replace

| Table 2. Data collection and refinement statistics |
|-----------------------------------------------|
| **M\(^{\text{pro}}\)_NB2B4**                      |
| Wavelength                                      |
| 0.979                                          |
| Resolution range                               |
| 33.21–2.0 (2.03–2.0)                           |
| Space group                                    |
| P 21 21 2                                      |
| Unit cell                                      |
| 254.6 33.5 48.7 90 90 90                      |
| Unique reflections                             |
| 29,088 (1,394)                                 |
| Multiplicity                                   |
| 12.1 (10.8)                                    |
| Completeness (%)                               |
| 98.6 (95.9)                                    |
| Mean I/\( \sigma \)(I)                        |
| 26.73 (3.28)                                   |
| Wilson B-factor(\( \AA^2 \))                   |
| 23.44                                         |
| \( R \)merge                                    |
| 0.089(0.719)                                   |
| \( R \)meas                                    |
| 0.093 (0.755)                                  |
| \( R \)pim                                     |
| 0.026(0.222)                                   |
| CC1/2                                         |
| 0.997 (0.846)                                  |
| Reflections used in refinement                 |
| 28,360                                        |
| Reflections used for \( R \)\_free             |
| 1,421                                         |
| \( R \)work                                    |
| 0.23                                          |
| \( R \)free                                    |
| 0.26                                          |
| No. of nonhydrogen atoms                       |
| 3,449                                         |
| RMS (bonds) (\( \AA \))                        |
| 0.004                                         |
| RMS (angles) (\(^\circ\))                     |
| 0.70                                          |
| Ramachandran favored (%)                       |
| 96.59                                         |
| Ramachandran allowed (%)                       |
| 3.41                                          |
| Ramachandran outliers (%)                      |
| 0.00                                          |
| **M\(^{\text{pro}}\)_NB1A2**                   |
| Wavelength                                      |
| 0.979                                          |
| Resolution range                               |
| 29.88–1.75 (1.78–1.75)                        |
| Space group                                    |
| P 43 21 2                                      |
| Unit cell                                      |
| 61.7 61.7 239.0 90 90 90                      |
| Unique reflections                             |
| 48,189 (2,351)                                 |
| Multiplicity                                   |
| 23.3 (19.0)                                    |
| Completeness (%)                               |
| 100 (100)                                      |
| Mean I/\( \sigma \)(I)                        |
| 20.3 (2.25)                                    |
| Wilson B-factor(\( \AA^2 \))                   |
| 18.50                                         |
| \( R \)merge                                    |
| 0.292 (2.378)                                  |
| \( R \)meas                                    |
| 0.298 (2.442)                                  |
| \( R \)pim                                     |
| 0.060 (0.550)                                  |
| CC1/2                                         |
| 0.999 (0.930)                                  |
| Reflections used in refinement                 |
| 47,780                                        |
| Reflections used for \( R \)\_free             |
| 2,305                                         |
| \( R \)work                                    |
| 0.19                                          |
| \( R \)free                                    |
| 0.22                                          |
| No. of nonhydrogen atoms                       |
| 3,700                                         |
| RMS (bonds) (\( \AA \))                        |
| 0.004                                         |
| RMS (angles) (\(^\circ\))                     |
| 0.71                                          |
| Ramachandran favored (%)                       |
| 98.55                                         |
| Ramachandran allowed (%)                       |
| 1.45                                          |
| Ramachandran outliers (%)                      |
| 0.00                                          |

Fig. 3. Structural mechanism of inhibition of SARS-CoV-2 M\(^{\text{pro}}\) by NB2B4 and NB1A2. (A) The epitope of NB2B4 on the C-terminal domain of SARS-CoV-2 M\(^{\text{pro}}\), with the C-terminal domain colored in blue and NB2B4 colored in orange, respectively. (B) The residues F291 of the F helix from the C-terminal domain are inserted into the hydrophobic pocket composed of residues Y59, L107, and I09 of NB2B4. Residue F291 is shown as a stick model; residues Y59, L107, and I09 of NB2B4 are shown as the electrostatic surface to illustrate the hydrophobic pocket. (C) Close-up views of the interface between SARS-CoV-2 extended M\(^{\text{pro}}\) (blue) and NB2B4 (orange). (D) Close-up views of the interface between SARS-CoV-2 compact M\(^{\text{pro}}\) (cyan) and NB1A2 (purple). The key residues involved in interaction are shown as stick models. Polar interactions are indicated with black dashed lines.
the aromatic ring stacking for Phe140 and His163, and the N-terminal finger displaces (SI Appendix, Fig. S6) (25). As a result, the active loop consisting of residues 137 to 145 shifts to the right and has significant conformational changes, resulting in the collapses of the oxyanion hole and the closure of the S1 pocket (SI Appendix, Fig. S6). The whole conformation of the loop in the monomeric Mpro is very similar to that of the inactive protomer of the dimer and the monomeric mutants (13, 14, 21, 22). Therefore, the active site in the monomeric Mpro does not adopt the right conformation for the substrate binding.

Development of a NanoBiT-Based Conformational Sensor for the Monomeric SARS-CoV-2 Mpro. As revealed in the structural analysis, there is significant conformational rearrangement between the extended and compact monomeric Mpro. The interaction between the catalytic domain and helix domain is accompanied by the proximity of the N terminal and C terminal of monomeric Mpro. The LgBiT (large subunit) and SmBiT (small subunit) fused to the N terminal and C terminal of monomeric Mpro, respectively (Fig. 5A). Complement luminescence is used to specifically monitor the association of the catalytic domain and helix domain. Because the residues extension in native sequence to the N and C termini significantly increase the dimerization $K_d$ compared with the mature Mpro (27), the profile from SEC showed that the purified NanoBiT-Mpro (LgBiT-Mpro-SmBiT) is a monomer (SI Appendix, Fig. S7). As expected, the results showed that the luminescent signal was strong in the absence of NB2B4 and the reducing luminescence was observed in the presence of NB2B4, which clarifies the conformational changes associated with the action of NB2B4 (Fig. 5A and SI Appendix, Fig. S8A). The inhibitory effect of NB2B4 on the Mpro was determined by the NanoBiT sensor assay, with a pIC$_{50}$ of 4.94 nM (Fig. 5B). We also tested other strongly inhibitory nanobodies we identified. The results showed that in the presence of NB2B4, NB1H2, or NB1A1, the luminescence decreases, while in the presence of NB2H4, NB1D5, NB1A2, or NB1F1, the luminescence signal increases and the effects are different (SI Appendix, Fig. S8A). This confirmed that there was equilibrium between the Mpro extended conformation and compact conformation in solution. Therefore, in solution, it is possible that there is an equilibrium among the extended state, the compact state, and the active dimer.

The preliminary test showed that in addition to NB2B4, NB1H2 also has a strong inhibitory effect on the luminescence signal. So, we further tested that the inhibitory effective curve of NB1H2 and its pIC$_{50}$ value was 5.48 nM (Fig. 5C). We also tested the dose-dependent enhancement curves of NB1F1, NB2H4, NB1A2, and NB1D5 that have effect of increasing the luminescent signal with the pEC 50 about 12.61 nM, 15.68 μM, 15.76 nM, and 41.26 nM (Fig. 5D and SI Appendix, Fig. S8B–D).

The interaction between the catalytic domain and helix domain is necessary for the compact state of monomeric Mpro, which is a prerequisite state for the active dimeric Mpro. Therefore, the allosteric modulators blocking the interaction of the two domains could be therapeutically valuable. We developed

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Fig. 4. The conformation of monomeric Mpro is not suitable for the substrate binding. (A) Superposition of the extended monomeric structure and one protomer of dimeric structure based on the N-terminal domain, with the extended structure colored in blue, and dimer structure colored in magenta (Left) and close-up view of the conformational change of the β-turn (Right). (B) The interaction between the β-turn (E166-H172) and the active loop (K137-N142) of the extended monomeric structure (blue). (C) The model of the extended monomer Mpro and substrate (TSAVLQ, derived from the N-terminal autocleavage sequence of the viral protease), the volume of substrate bound pocket is insufficient to accommodate the substrate. The extended monomer Mpro and substrate are shown as surface, with the monomeric Mpro colored in blue and substrate colored in brown. (D) The interaction between the β-turn (E166-H172) and the active loop (K136-N142) of the compact monomeric structure (cyan). The key residues involved in interaction are shown as stick models. Polar interactions are indicated with black dashed lines.
the innovative NanoBiT-based high-throughput allosteric inhibitor assay based on the conformational transition between the monomeric $\text{M}^\text{pro}$ extended state and the monomeric $\text{M}^\text{pro}$ compact state, not based on the conformational rearrangement between the monomeric $\text{M}^\text{pro}$ compact state and the active dimer. Therefore, the variation of luminescence signal indicates the conformation transition between the monomeric $\text{M}^\text{pro}$ extended state and the monomeric $\text{M}^\text{pro}$ compact state. The decrease of luminescence signal means that the inhibitor stabilizes the monomeric $\text{M}^\text{pro}$ extended conformation, while the increase of luminescence signal indicates that the inhibitor stabilizes the monomeric $\text{M}^\text{pro}$ compact state. The high-throughput screening method is feasible for screening allosteric inhibitory nanobodies or compounds through targeting the folding process to inhibit the activity of dimeric $\text{M}^\text{pro}$ and prevent the $\text{M}^\text{pro}$ in the polyprotein assembling into any active form and inhibit the self-cleavage.

**Conclusion**

In this study, we identify a set of allosteric inhibitory nanobodies against $\text{M}^\text{pro}$, and determined the two crystal structures of $\text{M}^\text{pro}$ with two noncompeting nanobodies to clarify their inhibitory mechanism. The two nanobodies captured two distinct transient conformations of $\text{M}^\text{pro}$ in the inactive monomeric state. One monomer has an extended conformation with its N-terminal and C-terminal domain dissociated, and the other monomer has the same structure as that of the protomer of the well-characterized dimer. Complemented with previously reported numbers of dimeric structure (5–7, 9, 10, 28, 29), these results allow us to understand the structural framework and essential events during the maturation of $\text{M}^\text{pro}$ (Fig. 2E). The extended state and compact state should exist in an equilibrium in the polyprotein, and based on the structural rearrangement, the screening inhibitor can prevent $\text{M}^\text{pro}$ autocleavage.

The maintained dissociation of the N- and C-terminal domains is an early stage interfering with enzyme maturation, which can prevent the $\text{M}^\text{pro}$ in the polyprotein assembling into any active form and inhibit the self-cleavage. Therefore, targeting early maturation of the main protease is an effective strategy for the development of antiviral drugs. Based on the structural rearrangement, an innovative NanoBiT-based high-throughput
allosteric inhibitor assay was developed to suppress the auto-
cleavage of Mpro in the viral polyprotein. In addition, the allo-
steric nanobodies unveil novel promising allosteric targets for
the structure-based design of allosterically small molecular
inhibitors.

Materials and Methods

Cloning, Expression, and Purification of SARS-CoV-2 Mpro. The cDNA of full-
length SARS-CoV-2 Mpro was code-optimized and inserted into pGEX4T-1 vector
by GenScript, which encoded the SARS-CoV-2 Mpro protease (accession no.: 
MN908947.3, ORF1ab polyprotein residues 3262 to 3569), and the VVPRGS was
inserted into the N terminus of the Mpro as the linking sequence between the
GST and Mpro, which is the specific substrate recognition sequence of thrombin.
After transformation into BL21(DE3), cells were cultured in the 2YT medium sup-
plement with ampicillin (50 μg/mL) and induced with isopropyl β-D-thiogalacto-
pyranoside (IPTG, final concentration 0.1 mM) at 16 °C for 10 h, when the value of
OD600 reached 0.6. Then, the cells were sedimented by centrifuge and resus-
pended in the lysis buffer (1×PBS) with protease inhibitor, and lysed by sonica-
tion. Next, they were centrifuged at 10,000 rpm for 45 min, the supernatant was
harvested and loaded into the equilibrated GST-column and washed with PBS for 30 mL,
and the GST-tagged Mpro was eluted by adding reduced glutathione to the elution
buffer. The elution was concentrated and the GST-tag was cleaved off using a
thrombin protease (Yeasen, Cat#:20402ES03). Finally, it was purified by gel-
filtration chromatography using a HiLoad 16/600 Superdex 75 pg column (Cytiva,
Cat#:2899333) and stored into liquid nitrogen.

To obtain homodimeric Mpro, the construction method of expression vector was
as described previously (10, 30). The gene encoding Mpro was cloned into the
pGEX6p-1 vector, and AVLQSGFR was inserted into the N terminus of the
Mpro gene as the linking sequence between the GST gene and Mpro gene, which
is the specific substrate recognition sequence of SARS-CoV-2 Mpro, the N proximitative nanobody would become available by autocleavage during protein expression.
Additionally, the GPHHHHHH were inserted into C terminus, which can be
claved by the rhinovirus 3C protease to generate the native C terminus of Mpro.
The expression purification strategy was as described above.

Construction of Camelid Nanobody Phage-Display Library. Camel immu-
nizations and nanobody library generation were performed as described
previously (31). Animal work was conducted under the supervision of Shanghai
Institute of Materia Medica, Chinese Academy of Sciences. Two camels were
immunized with the purified Mpro at doses of 1 mg once a week seven times.
After immunization, the peripheral blood lymphocytes were isolated from the
whole blood using Ficoll-Paque Plus according to the manufacturer’s instruc-
tions. Total RNA from the peripheral blood lymphocytes was extracted and
converted into cDNA using a Super-Script III First-Strand SUPERSCRIPT Mix
(Invitrogen). The VHH encoding sequences were amplified with two
enriched-nested PCR using VHH-speci
fic primers and cloned into pMECS vector
and transformed into electro-competent E. coli BL21(DE3), and the LVPRGS was
inserted into the N terminus of the Mpro as the linking sequence between the
GST and Mpro, which is the specific substrate recognition sequence of SARS-CoV-2 Mpro, the N proximitative nanobody would become available by autocleavage during protein expression.
Additionally, the GPHHHHHH were inserted into C terminus, which can be
claved by the rhinovirus 3C protease to generate the native C terminus of Mpro.
The expression purification strategy was as described above.

Expression and Purification of Nanobodies. The specific nanobody of Mpro
was cloned into pMECS vector and transformed into Top10F
°
cells (Huayueyang Biotech) to inoculated in 2YT media containing 100 μg/mL ampicillin, 0.1%
(wt/vol) glucose, and 1 mM MgCl2. When the OD600 reached 0.6 to 0.8, the
IPTG was added, and when the work concentration was 1 mM, after 16 h, we harvested the cells and resuspended them in the lysis buffer and they were lysed by sonication. The supernatant was load onto Ni-NTA beads and eluted with elu-
tion buffer, which contained 20 mM Hepes pH 7.5, 150 mM NaCl, and 300 mM
imidazole. The elution was concentrated by the 3-kDa concentrated tube of
Amicon Ultra Filter and polished by gel-filtration chromatography using a HiLoad 16/600 Superdex 75-pg column (Cytiva, Cat#:2899333) and
stocked into liquid nitrogen.

Analysis of Nanobody Binding Epitope on Mpro. In order to analyze the
nanobody binding epitope on Mpro, the purified dimeric Mpro and purified na-
obodies were mixed and incubated on ice for 30 min. The SEC experiment was
performed and the peak position of the mixture was detected by HPLC (Agilent
1260) with the Superdex 200 increase 5/150 GL (Cytiva).

Crystallization and Data Collection. The purified Mpro and NB2B4 or NB1A2
were incubated at 4 °C for 30 min, then polished by the HiLoad 16/600 Super-
dex 75-pg column, and the peak collected for crystallization. The complex of the
Mpro and NB2B4 was crystallized using the sitting-drop vapor-diffusion method at
20 °C for 2 wk. The crystals were obtained in conditions containing 9% PEG
20,000, 0.1 M MES (pH5.5). The crystals of the Mpro in complex with NB1A2 were
grown in 0.2 M (NH4)2HPO4, 20% PEG3530, pH 8.0. X-ray diffraction data were
collected at 100K on the beamline 19U1 at the National Synchrotron Radia-
tion Research Center, China (λ = 0.978 Å) (32). Data were processed using
HKL3000 suite.

Structure Determination. The structure determination of the complex of the
Mpro and NB2B4 or NB1A2 was solved with the molecular replacement using
Phaser in the PHENIX package (33), and the model (PDB ID code 7LMC) of
SARS-CoV-2 Mpro was used. Model building was carried out by Coot (34).
The refinement of the structure was done with PHENIX suite. Finally, stereochemical
quality and final validation of the model were performed using Mol Probity
(35). The final statistics of data collection and structural refinement are shown in
Table 2. Figures were prepared in PyMOL (https://pymol.org/2/).

Enzymatic Activity and Inhibition Assay. The activity of SARS-CoV-2 Mpro
was measured by a continuous kinetic assay with the substrate MCA-peptide
(MCA AVGSGFR-Lys [Dnp]-Lys-NH2), a peptide similar to the N-terminal or
C-terminal amino acid sequence of the nsp2 to nsp16 of SARS-CoV-2, which
was synthesized by GL Biochem Ltd. Inhibition kinetics were performed by incubating
200 nM enzyme with 20 nM to 5 μM inhibitor in assay buffer at 4 °C with gentle
shaking for 30 min, and then adding into the half-96-well plate, 50 μL of 40 μM
substrate into the well to initiate reactions. The fluorescence signal
changes of MCA were detected at the excitation wavelength 320 nm and
emission wavelength 405 nm. The results were compared with the control sam-
ple without inhibitor, and the nanobodies with certain inhibitory effect were
screened out. The initial rate of the reaction was obtained, and then the IC50
was calculated by GraphPad Prism7 software.

Under experimental conditions, the inhibition percentage of the reaction was
calculated compared with the activity of Mpro in the absence of nanobodies.
The formula for calculating IC50 is v = a/(1 + ([I]/IC50)). In the formula, v is the initial
reaction rate of the reaction in the presence of nanobodies, a is the enzymatic
reaction rate in the absence of nanobodies, [I] is the working concentration of
nanobodies, and IC50 is the concentration of nanobodies when the activity of
the enzyme is inhibited by 50%. Three independent experiments were performed
in triplicate.

Development of a NanoBiT-Based Conformational Sensor for SARS-
CoV-2 Monomeric Mpro. The LgBiT and SmBiT were fused to the N and C
termini of monomeric Mpro, respectively. The expression construct of LgBiT-Mpro,
SmBiT was subcloned in to pGEX4T-1 vector, which coded the LgBiT-Mpro,SmBiT
with the GST-tag in the N-terminal, and then expressed with the E. coli BL21(DE3),
purified by GST-column, and removed the GST-tag by thrombin enzyme.

The complement luminescence of NanoBiT system has previously been
described (5, 25, 26). In brief, in a 100-μL reaction system, 10 nM LgBiT-Mpro,
SmBiT and different nanobodies are mixed and added into a black half-area
96-well plate; the concentration of the nanobodies ranges from 0.02 nM to
2.5 μM. After gently shaking and mixing, incubating at 4 °C for 30 min, adding
10 μM furimazine (GlpBio, Cat#: GC32913) to initiate reaction, the data are proc-
cessed by GraphPad Prism7 software. All experiments were performed in
triplicate.
Data Availability. The atomic coordinates have been deposited in the Protein Data Bank (PDB ID codes 7VFA and 7VFB).

ACKNOWLEDGMENTS. We thank the staff of the BL19U1 beamline of the National Facility for Protein Science in Shanghai at the Shanghai Synchrotron Radiation Facility for assistance during data collection, and Prof. Xu Yechun for her help and suggestions on the enzymatic activity and inhibition assays. This work is supported by the National Natural Science Foundation of China (31670743); the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA12040326); the Science and Technology Commission of Shanghai Municipality (18JC1415400); the Joint Research Fund for Overseas, Hong Kong, and Macao Scholars (81628013); the Natural Science Foundation of Shanghai (16ZR1442900); the National Science Foundation for Young Scholar Projects (81803599); a grant from the Zhejiang University Covid-19 Special Project (2020XGZX092); and grants from the Shanghai Institute of Materia Medica, the Chinese Academy of Sciences (CASIMM0120164013, SIMM1606YZ-06, SIMM1601KF-06, 55201631121116101, 55201631121108000, 5112345601, and 2015123456005).

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