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Unmasking the Conformational Stability and Inhibitor Binding to SARS-CoV-2 Main Protease Active Site Mutants and Miniprecursor

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Abstract

We recently demonstrated that inhibitor binding reorganizes the oxyanion loop of a monomeric catalytic domain of SARS CoV-2 main protease (MPro) from an unwound (E) to a wound (active, E*) conformation, independent of dimerization. Here we assess the effect of the flanking N-terminal residues, to imitate the MPro precursor prior to its autoprocessing, on conformational equilibria rendering stability and inhibitor binding. Thermal denaturation (T_m) of C145A mutant, unlike H41A, increases by 6.8 °C, relative to wild-type mature dimer. An inactivating H41A mutation to maintain a miniprecursor containing TSAVL[Q or E] of the flanking nsp4 sequence in an intact form (−6)MProH41A and (−6*)MProH41A, respectively, and its corresponding mature MProH41A were systematically examined. While the H41A mutation exerts negligible effect on T_m and dimer dissociation constant (K_dimer) of MProH41A, relative to the wild type MPro, both miniprecursors show a 4–5 °C decrease in T_m and > 85-fold increase in K_dimer as compared to MProH41A. The K_d for the binding of the covalent inhibitor GC373 to (−6*)MProH41A increases ~12-fold, relative to MProH41A, concomitant with its dimerization. While the inhibitor-free dimer exhibits a state in transit from E to E* with a conformational asymmetry of the protomers’ oxyanion loops and helical domains, inhibitor binding restores the asymmetry to mature-like oxyanion loop conformations (E*) but not of the helical domains. Disorder of the terminal residues 1–2 and 302–306 observed in both structures suggest that N-terminal autoprocessing is tightly coupled to the E-E* equilibrium and stable dimer formation.

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Introduction

SARS CoV-2, a positive-sense single stranded RNA virus, encodes its proteins for its maturation and propagation in at least 12 open reading frames.1–2 Two-thirds of the ~30 kb genome is translated as 2 large polyproteins, pp1a and pp1ab.3–5 Polyproteins pp1a and pp1ab span the non-structural proteins (nsp) nsp1-nsp10 and nsp1-nsp16, respectively.3 A single copy of the main protease (MPro) is synthesized as part (nsp5) of these polyproteins proximal to membrane spanning segments within nsp4 and nsp6.6 It catalyzes its own release (termed autoprocessing)
promoting cleavages at its termini and the various sites in the polyproteins between nsp6 and nsp16. Together with a papain-like protease, encoded within nsp3, which cleaves the polyproteins to release nsp1-nsp3, the mature nsps assemble to form the replication/transcription complex essential to produce the mature progeny virus.

Mature MPro, also known as 3C-like protease, is a homodimeric cysteine protease. Each protomer (306 amino acids) comprises 3 domains. Domains 1 (residues 8–101) and II (residues 102–184) together form the catalytic region exhibiting a chymotrypsin-like fold. This region is connected to domain III (residues 201–306), which encompasses 5 helices, through a long loop region (residues 185–200). Each protomer includes the non-canonical active site dyad, C145 and H41 residues, with a characteristic oxyanion loop conformation. The active sites of the dimer are catalytically equivalent.

Since the emergence of the SARS CoV-2 pandemic, MPro has been a renewed target for developing potent oral inhibitors for the treatment of COVID-19. Developing such inhibitors is even more urgent considering emerging variants that escape the current vaccine treatments. The chemical structure and specificity of inhibitors mimic the substrate making critical contacts with the binding pockets of the enzyme. They include various chemically reactive groups such as a carbonyl or nitrile, which form a covalent bond with the active site C145 residue. Known inhibitors include nirmatrelvir, which is currently in use for the treatment of COVID-19 under an emergency use authorization by the FDA, and the recently described covalent and non-covalent inhibitors. Consistent with this observation, it is noteworthy that the majority of these inhibitors are designed to act on the mature MPro and only limited knowledge exists regarding the efficacy of binding of such inhibitors to the active site of MPro precursor prior to its maturation at its termini or to the monomeric form of mature MPro in vivo.

In vitro experiments to determine the order of cleavage at the termini of MPro require intact model precursors containing the nsp4 and nsp6 sequences flanking the MPro. However, lack of accumulation of such precursors due to their rapid autoprocessing of MPro upon expression in E. coli precludes their accumulation and isolation. Insolubility of the constructs also limits such endeavors (our unpublished results). A precise mechanism by which an MPro protomer or a dimer catalyzes cleavages at its termini, either through an intra- or intermolecular mechanism, is not fully understood. Moreover, few such studies permit only to the previous SARS CoV isolate and not to the recent SARS CoV-2 MPro. A recent study aiming to address the conformation of the flanking C-terminal residues of nsp4 (SAVLQ) appended to MPro resulted in observing only the cleaved product SAVLQ bound to the dimeric MPro in the crystal structure. Structures of the dimeric MPro bearing a C145A mutation displaying the C-terminal residues of one dimer bound to the active site of a second dimer have led to the proposal that C-terminal autoprocessing occurs via an intermolecular process. From a handful of previous studies of SARS CoV MPro, a consensus emerges suggesting that cleavage at the N-terminus precedes the C-terminal cleavage and that the N-terminal cleavage is accompanied by the conformational reorganization of 1) the active site oxyanion loop and 2) the interface promoted by the free N-terminal residues through intra- and inter-protomer contacts with domains II and III in conjunction with a distinctive reorientation of the latter.

These rearrangements lead to a significant decrease in the dimer dissociation constant and appearance of mature-like catalytic activity. Thus, mutational, kinetics and structural studies have focused mostly on the previous SARS CoV isolate examining the relationship of catalytic activity to conformational rearrangements/dimerization of the mature MPro and not of the MPro precursor. It is evident from various investigations that substrate or inhibitor binding reorganizes the active site oxyanion loop from an inactive (E) to an active (E*) state that is typical of the catalytically active conformation of the mature dimeric MPro. This transition is concomitant with the monomer–dimer equilibrium shifting to the dimer form. Furthermore, by using a construct that is exclusively monomeric consisting of only the catalytic domain and loop region (residues 1–199, MPro), we recently showed that inhibitor binding transitions the E state to E*I state independent of dimerization. Consistent with this observation, a miniprecursor of MPro containing the N-terminal flanking region undergoes autoprocessing suggesting that precursor processing may also be governed by the binding of the N-terminal cleavage site sequence modulating the E-E* equilibrium. The above result is in accordance with earlier observations showing that monomeric model precursors, in which the N-terminal flanking residues of nsp4 are appended to the full-length MPro E290A/R298A mutant, also undergo N-terminal autoprocessing, albeit at a slower rate than the wild-type.

It is evident from the above observations that biochemical and structural studies of model precursors of MPro are feasible only through either mutating the active site or cleavage site residues to permit their accumulation and isolation for in vitro studies. Here we report the results of a systematic study towards understanding the effect of active site mutations and the presence of N-terminal flanking region sequence, to mimic an MPro precursor prior to the N-terminal cleavage, on the dimer dissociation constant, conformational stability, and thermodynamics of inhibitor binding.

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As monomers did not yield crystals and crystal growth was selective only for dimers, two dimeric structures of (-6*)MProH41A with and without GC373 (the reactive aldehyde form of GC376),

are described and compared with the corresponding mature MPro structures.

**Results**

**Active site mutations and the N-terminal flanking sequence TSAVLQ alter the conformational stability and dimer dissociation constant of MPro**

Differential scanning fluorimetry (DSF) was utilized to assess the effect of active site mutations on the overall conformational stability of MPro. Proteins were subjected to DSF at 10 µM in buffer B (pH 7) containing 0.1 % DMSO. Tm values along with the estimated dimer dissociation constants (Kdimer) of the various constructs are listed in Table 1 (Figures 1, 2 and S2). MProC145A and MProH41A are mainly dimeric at 10 µM (25 °C) based on the apparent Kdimer by SV-AUC and SEC-MALS analyses resembling that of MProWT (Figure 1(A), (B) and (D)). The mid-point (Tm) for the two-state unfolding transition of MProWT is 53.9 °C. This Tm is consistent with the value reported by differential scanning calorimetry (DSC) for SARS CoV MProWT, which is 96 % identical in sequence to SARS CoV-2 MProWT.31

Circular dichroism (CD) profiles of dimeric MProWT, MProH41A and MProC145A at 10 µM concentration in buffer B at 25 °C are similar (Figure 3(A)). Surprisingly, the Tm for MProC145A is 6.8 °C higher than that observed for MProH41A, which displays a nearly identical Tm as that of MProWT (Table 1 and Figure 2). Consistent with the DSF data, thermal denaturation CD profiles also return a Tm difference (ΔTm) of 6.8 °C between MProWT (or MProH41A) and MProC145A (Figure 3(B)). To ascertain, if this two-state behavior is exclusive to a dimer form as well as evaluate the contribution of the dimer interface to the overall conformational stability, a previously described construct, MProM was analyzed. MProM is predominantly monomeric, with a Kdimer of 6600 µM,10 because of two critical interface mutations E290A and R298A, leading to MPro dimer dissociation.10 The observed Tm of MProM is almost the same (Table 1 and Figure 2(A)) as MProWT. This suggests that the Tm signifies the unfolding transition of the monomer fold of MProWT and MProH41A. In this context, it is worth noting that the secondary structure CD profiles as well as the tertiary fold of monomer and dimer forms are similar.8,10,32–33

As H41A mutation does not alter the Tm or the Kdimer, relative to the wild type, but is expected to inactivate the enzyme, MProH41A was chosen to engineer a construct containing 6 amino acids of the flanking nsp4 sequence TSAVLQ to mimic a miniprecursor of MPro prior to its cleavage at its N-terminus, termed (-6*)MProH41A (Figure S1). .

Appending these residues decreases the Tm by 4.6 °C and increases the Kdimer drastically by > 85-fold (Table 1 and Figure 1(E) and S2). However, when subjecting this construct to prolonged incubation at ~300 µM for > 2 weeks at ambient temperature, it exhibits very slow cleavage at the nsp4/nsp5 junction. A product corresponding to mature MProH41A was identified by mass spectrometry indicative of this cleavage at the nsp4/nsp5 junction. The crystal structure of MProWT determined by neutron diffraction shows that both the side chains of C145 and H41 are ionized and form an ion pair.34 The substitution of the basic H41 residue could result in a change in the ionization state of the sulfur atom of C145 from thiolate to thiol which is a less effective nucleophile and thus, accounts for the observed low catalytic activity. To further limit autoprocessing, the conserved Q residue in the P1 position of the nsp4/nsp5 junction was substituted to E26,35 (denoted by an asterisk), the resulting construct was termed (-6*)MProH41A. The Tm of (-6*)MProH41A is about the same as (-6*)MProH41A, but the Kdimer is predicted to be much higher than 340 µM as no dimer was detectable by SV-AUC at 40 µM (Figure 1(E) and (F)). There is evidence for very weak self-association at 80 µM, unlike (-6*)MProH41A, which clearly exhibits a species indicative of a monomer/dimer equilibrium boundary at a similar concentration of 90 µM and a Kdimer of 340 ± 50 µM (Figure 1(E) and S2). Thus, the influence of the presence of the N-terminal flanking sequence is reflected in a decreased Tm of 4–5 °C for (-6*)MProH41A and (-6*)MProH41A. Of significance is the observation that the P1 Q to E mutation results in an even larger increase in Kdimer. As expected, no N-terminal autoprocessing of (-6*)MProH41A was observed after prolonged periods of incubation at high concentrations.

### Table 1 Thermal denaturation (Tm) and relationship to Kdimer of various MPro constructs in the absence and presence of GC373.

| Constructs | Tm (°C) | ΔTm (°C) | M/D status (at 10 µM, 25 °C) | ~Kdimer (µM, 25 °C) |
|------------|---------|----------|-----------------------------|---------------------|
| MProWT     | 53.9    | 0.0      | dimer                       | ~330 µM             |
| MProH41A   | 54.3    | 0.6      | monomer                     | 6600 µM             |
| MProC145A  | 60.0    | -0.8     | dimer                       | ~1 µM               |
| (-6*)MProH41A | 53.9   | 0        | monomer                     | <4 µM               |
| (-6*)MProH41A | 49.1   | -4.8     | monomer                     | 340 ± 50 µM         |
| MPro      | 49.8    | -4.2     | dimer                       | <40 µM              |
| (-6*)MPro + 20x GC373 | 53.1  | 19.2     | dimer                       | ~1 µM               |
| MProH41A + 20x GC373 | 50.3  | 19.1     | dimer                       | ~1 µM               |

Tm was determined by DSF at a concentration of 10 µM in buffer B. M/D denotes monomer/dimer. a estimated by SV-AUC. b monitored by SEC-MALS. c cited from references.6,10 DSF scans are shown in Figure 2. Green and red denoting the increase and decrease, respectively, of Tm and ΔTm, relative to the corresponding mature MPro construct.
enabling the determination of crystal structures described below.

GC373 exhibits weaker binding to the miniprecursor mimetic relative to its mature counterpart

The reversible covalent inhibitor GC373 binds to MProWT with a dissociation constant ($K_d$) of 0.15 μM (Table 2). This $K_d$ equates to a $T_m$ of 70 °C with twofold (2x) molar excess of GC373, matching the same protein to GC373 ratio at the completion of the ITC experiment, and a $T_m$ increase ($\Delta T_m$) of 16.1 °C relative to the inhibitor-free MProWT (Table 1). This suggests that inhibitor binding with high affinity enhances the conformational stability of the dimer. It is well-known that increased thermal stability correlates with inhibitor binding affinity. A gradual increase in $T_m$ is observed with increasing GC373 concentration (Figure 2(C)), and at 20x GC373, $\Delta T_m$ increases to 19.8 °C (Table 1). At 50 μM MProC145A, no thermal response was observed when titrating with GC373 indicative of its weak binding. Even though, H41 lacks close contacts with GC373, the $K_d$ for the binding of GC373 to MProH41A increases by 9-fold ($K_d = 1.4$ μM, Table 2 and Figure 4) due to the lower reactivity of the protonated sulfur of C145 compared to the unprotonated of the wild type. This relates to a $T_m$ of 70.8 °C, which is a difference in $T_m$ of 2.9 °C and 16.9 °C compared to MProWT + 20x GC373 and inhibitor-free MProH41A, respectively.

In contrast to the binding of GC373 to the MProH41A dimer, even when the $K_d$ increases by 9-fold with only a small decrease in $T_m$ ($\Delta T_m$) of 3 °C relative to MProWT + 20x GC373, the binding of GC373 to (-6*)MProH41A results only in a very small $T_m$ increase of 0.4 °C as compared to
inhibitor-free \((-\text{6})\text{MProH}^{\text{H41A}}\). This result parallels an even weaker binding of GC373 to \((-\text{6})\text{MProH}^{\text{H41A}}\). The $K_d$ for GC373 binding to \((-\text{6})\text{MProH}^{\text{H41A}}\) is $\times 12$ times larger than MPro H41A (Table 2 and Figure 4). Notably, addition of GC373 promotes dimerization of \((-\text{6})\text{MProH}^{\text{H41A}}\) with a $50:50$ distribution of dimer to monomer population when 60 $\mu$M \((-\text{6})\text{MProH}^{\text{H41A}}\) was mixed and incubated for 60 min with GC373 at 5-times the concentration of protein prior to SEC-MALS as shown in Figure 1C. The estimated $K_{\text{dimer}}$ for \((-\text{6})\text{MProH}^{\text{H41A}}\) with GC373 is 38 ± 5 $\mu$M (Figure S3). In the absence of GC373, \((-\text{6})\text{MProH}^{\text{H41A}}\) is mainly monomeric with a $K_{\text{dimer}}$ much higher than 340 $\mu$M. Thus, the single binding isotherm observed for the titration of GC373 to \((-\text{6})\text{MProH}^{\text{H41A}}\) reflects the two processes of binding concomitant with dimerization, evident from the slow thermal response (Figure 4) and the large increase in entropy ($\Delta S$) which offsets the decrease in $\Delta H$ (Table 2). Here the $\Delta H$ likely signifies the weaker interaction of GC373 with the active site and $\Delta S$, the conformational and hydration changes associated with dimer formation. Of these two processes, namely inhibitor binding and shift in the M-D equilibrium to the dimer, P1-Q to E mutation largely accounts for the increase in $K_{\text{dimer}}$, which indirectly suggests that P1 Q plays a role in enhancing dimer stability. We have recently shown that the active site oxyanion loop exists mainly in the unwound (inactive, E) state in the monomeric form and that it switches to the wound/mature-like (active, E*) state upon inhibitor binding, the latter resembling inhibitor-free \((-\text{5})\text{MProH}^{\text{H41A}}\). This result parallels an even weaker binding of GC373 to \((-\text{5})\text{MProH}^{\text{H41A}}\). The $K_d$ for GC373 binding to \((-\text{6})\text{MProH}^{\text{H41A}}\) is $\times 12$ times larger than MPro H41A (Table 2 and Figure 4). Notably, addition of GC373 promotes dimerization of \((-\text{6})\text{MProH}^{\text{H41A}}\) with a $50:50$ distribution of dimer to monomer population when 60 $\mu$M \((-\text{6})\text{MProH}^{\text{H41A}}\) was mixed and incubated for 60 min with GC373 at 5-times the concentration of protein prior to SEC-MALS as shown in Figure 1C. The estimated $K_{\text{dimer}}$ for \((-\text{6})\text{MProH}^{\text{H41A}}\) with GC373 is 38 ± 5 $\mu$M (Figure S3). 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the loop conformation of the mature dimer. The E-E* loop equilibrium being mainly in the unwound E state, as expected to occur in the monomeric (-6*)MProH41A miniprecursor, is likely to account for the increased $K_d$ ($/C24/12$-fold) of GC373 as compared to its mature counterpart, MProH41A.

Revelations of the minor distorted and asymmetric conformations of the MPro miniprecursor dimer in the absence and presence of GC373

Because (-6*)MProH41A is monomeric in solution up to a concentration of 340 μM (~11 mg/mL), we were interested in obtaining its crystal structure in the inhibitor-free form. The room-temperature structure was obtained at 1.90 μm resolution. (-6*)MProH41A crystallizes in the P21 space group. Unexpectedly, we found a homodimer instead of a monomer in the asymmetric unit, with the dimer’s quaternary structure resembling that of the inhibitor-free mature MProWT dimer (Figure 5(A)). We modeled residues 3–301 in (-6*)MProH41A structure, whereas the rest of the N-terminal and C-terminal residues are disordered and are not visible in the electron density map.

To evaluate how similar the inhibitor-free (-6*)MProH41A and MProWT dimers are, we superimposed each protomer molecule of (-6*)MProH41A with the monomer of MProWT (PDB ID 7JUN). Of note, MProWT crystallizes with one protomer in the asymmetric unit, and the homodimer is created through a crystallographic twofold symmetry axis. In the MProWT dimer, the protomers A and B have identical conformations. The root mean square deviations (RMSDs) on the main-chain atoms are 0.53 μm and 1.34 μm, respectively, when protomers A and B of (-6*)MProH41A are each aligned with an MPro WT protomer. This result demonstrates the structural similarity of protomer A but points to a significant difference between the tertiary structures of protomer B in (-6*)MProH41A compared to MProWT. Notably, it also signifies asymmetry in the quaternary structure of the (-6*)MProH41A dimer. This is demonstrated in Figure 5 (B), which shows the superposition of inhibitor-free (-6*)MProH41A and MProWT, in which protomer A of the miniprecursor was aligned with one of the protomers of the wild-type mature enzyme. There is a major conformational shift of the helical domain of protomer B in (-6*)MProH41A, where some helices move by 5–7 μm from their positions in the native MProWT structure. As a whole, the helical domain of protomer B moves away from the corresponding domain of protomer A by ~2 Å adopting a more open conformation relative to MProWT (Figure 5 (C)). At the helical domains interface the closest residues Ala285 are separated by 7.3 μm in (-6*)MProH41A versus 5.2 Å in MProWT. A similar opening of the helical domains interface was previously observed in a MPro construct with non-native Gly-Ala-Met residues fused to the N-terminal Ser1, termed IMT MPro (MProIMT hereafter, PDB ID 7KFI). In MProIMT, however, the helical domains move away from each other symmetrically, making the distance between the interfacing Ala285 resi-

Table 2 Estimated thermodynamic parameters of GC373 binding to MProWT, its active site H41A mutant and miniprecursor as determined by ITC.

| Construct  | N    | $K_d = K_i$ (μM) | ΔH (kcal/mol) | ΔS (cal/mol/K) | ΔG (kcal/mol) |
|------------|------|-----------------|---------------|---------------|---------------|
| MProWT     | 0.99 ± 0.01 | 0.15 ± 0.03 | −6.7 ± 0.1 | 9.1 | −9.4 |
| MProH41A   | no response | | | | |
| MProH41A   | 0.97 ± 0.03 | 1.4 ± 0.5 | −5.6 ± 0.2 | 8.3 | −8.1 |
| (-6*)MProH41A | 0.94 ± 0.04 | 16.7 ± 4.2 | −0.8 ± 0.06 | 19.2 | −6.6 |

Titrations were performed in buffer C with 30–50 μM protein in the cell and inhibitor in the syringe at 10 times the concentration of the protein. As no thermal response was observed for (-6*)MProH41A at 50 μM, as was also the case with MProC145A, the concentration of (-6*)MProH41A was increased to 150 μM protein and 1.5 mM GC373 to attain meaningful thermal response and thermodynamic parameters. MProWT data is listed here from reference for ease of comparison.
dues of about 10 Å. Therefore, (-6*)MProH41A conformation can be considered ‘semi-open’, whereas that found in MProIMT can be viewed as ‘open’. In addition, the presence of non-native residues at the N-terminus of MProIMT results in major structural alterations in the oxyanion hole and subsites S1, S2 and S4 caused by the cascade effect to avoid steric clashes by the N-terminal residues with Phe140, Glu166 and Pro168. In contrast, there is no substantial reshaping of the oxyanion loop or the substrate-binding subsites in (-6*)MProH41A protomer A (Figure 6A and B). A significant change, however, occurs for the conformation of Glu166 in protomer B of (-6*)MProH41A where it flips down to form a new hydrogen bond of 3.1 Å with His163, which is absent in MProWT. The Glu166 movement attracts the Leu141 side chain that rotates closer to Glu166, making hydrophobic interactions, leading to a somewhat distorted oxyanion loop geometry and apparent closure of the S1 subsite. A similar conformational change of Glu166 side chain was observed in the recently described structure of MPro1-199 which lacks the helical domain.30 But, in MPro1-199 the oxyanion loop unwinding and reshaping into a 3_10-helix resulted in Glu166 hydrogen bonding with the main chain amide of Gly143. Due to the formation of the Asp166...His163 hydrogen bond in (-6*)MProH41A, we can speculate that His163 becomes positively charged (i.e., doubly protonated) to neutralize the negative charge on the Glu166 carboxylate, whereas this histidine was found neutral (i.e., singly protonated) in MProWT.34 The considerable conformational plasticity of the MPro helical domain was also previously noted in the crystal structures of the fully monomeric enzyme containing single mutations G11A, S139A, and R298A.32–33,38 However, in these structures the substantial geometry change of the helical domain may be partially or solely due to the monomeric nature of the mutant enzymes crystallographic studies, which were carried out under cryogenic conditions.

To determine whether inhibitor binding to the miniprecursor enzyme restores its quaternary structure to the native state we obtained a roomtemperature X-ray crystal structure of (-6*)MProH41A-GC373 at 1.80 Å resolution. (-6*)MProH41A-GC373 crystallizes with the isomorphous unit cell parameters relative to the inhibitor-free (-6*)MProH41A, indicating that their quaternary structures are similar. Indeed, superimpositions of the corresponding protomers in the inhibitor-free and GC373-bound structures result in RMSDs of 0.23 Å for protomers A and 0.27 Å for protomers B. GC373 is found in the active sites of both protomers in (-6*)MProH41A-GC373 (Figure 7A).

Figure 4. Binding isotherm of GC373 to MProH41A and its miniprecursor. Titrations were carried out in buffer C at 28 °C. The thermodynamic parameters are listed in Table 2.
Figure 5. Room-temperature structure of (-6*)MProH41A and comparison with MProWT. (A) Cartoon representation of the homodimeric (-6*)MProH41A. Catalytic C145 and the site of H41A mutation are shown as spheres. Helical and catalytic domains are colored in light violet and light orange, respectively. (B and C) Superposition of (-6*)MProH41A (colored like in A) and MProWT (light grey). Movement of the helical domain of protomer B in (-6*)MProH41A is shown in panel C by the increased distances between corresponding Ala285 residues.
GC373 binding, however, has no effect on the protomer B tertiary structure that maintains the semi-open conformation of its helical domain observed in the inhibitor-free (-6*)MProH41A structure. Conversely, the presence of GC373 in the protomer B active site fully restores the geometry of the oxyanion loop and the Glu166 orientation. Thus, covalent inhibitor binding reverts the oxyanion loop and S1 subsite geometries to their active configurations. GC373 binding to (-6*)MProH41A is nearly identical to its binding to MProWT; the inhibitor makes similar hydrogen bonding interactions with the active sites even when H41A is present (Figure 7(B), (C), and S4). Hence, the structural analysis demonstrates that covalent binding of GC373 leads to conformational changes in the oxyanion loop and the S1 subsite geometries to their active configurations.

Discussion

In this study, we partially succeeded in our attempt to characterize a miniprecursor MPro mimetic prior to the cleavage at its N-terminus. The miniprecursor consisted of 6 amino acids (-6) of the flanking nsp4 sequence appended to the N-terminus of MPro. An effective approach to retain the N-terminal flanking sequence for biochemical and structural analyses requires inactivating the enzyme by mutating either one of the catalytic dyad residues, H41 or C145. As the thermal denaturation profile is different for MProC145A, contrary to MProH41A which closely resembles the wild type, H41A mutation was the logical choice for creating the miniprecursor mimic (-6*)MProH41A. However, as H41A mutation did not completely abolish catalytic activity, which resulted in N-terminal autoprocessing during prolonged periods at high concentrations of (-6*)MProH41A, a second mutation was introduced. The second miniprecursor construct, (-6*)MProH41A bearing the substitution mutation P1-Q to E of the residue flanking the N-terminus of MProH41A and is devoid of cleavage, was systematically analyzed. Clearly, the lower stability of the miniprecursor also points to the importance of the N-terminal cleavage and the requirement of the free N-terminal residues, which form the interface for stable dimer formation. The Tm changes from 49.3-49.7°C for the miniprecursors to 53.9°C for MProH41A. This Tm difference (∆Tm) of 4.5°C correlates with the total interface forming upon dimerization. Of note, the enhanced thermal stability observed for MProC145A raises the question if this mutant is indeed an ideal surrogate for biochemical studies. Further experiments are needed to explain if the C145A mutation exerts an effect on global stability by perturbing the oxyanion loop equilibrium which is coupled to dimer formation.

The low Kd of MProWT with GC373 of 0.15 μM is mainly a contribution of the hemithioacetal formation between the inhibitor’s aldehyde carbon and the sulfur of C145. Even though there are no contacts < 3.5 Å between the H41 residue and GC373 in MProWT-GC373 complex (PDB ID A. Kovalevsky, L. Coates, D.W. Kneller, et al. Journal of Molecular Biology 434 (2022) 167876
H41A mutation leads to a ~9-fold increase in $K_d$ for GC373 binding to MProH41A probably due to the difference in the chemical reactivity between thiolate anion and thiol. This trend in $K_d$ is reflected by increases in $T_m$ for MProWT-GC373 ($\sim 20 \, ^\circ C$) and MProH41A-GC373 ($\sim 17 \, ^\circ C$) complexes. This difference is even more when comparing mature MProH41A-GC373 and miniprecursor (-6*)MProH41A-GC373 complexes such that a 12-fold weaker binding of GC373 to the miniprecursor accounts for a $\Delta T_m$ of $21 \, ^\circ C$. In accordance with the lack of thermal response by ITC, indicative of very weak binding, no increase in $T_m$ was observed for MProC145A in the presence of 20-fold molar excess GC373, relative to inhibitor-free MProC145A (Figure 2 (A)), again indicating the importance of the reactive group in the inhibitor, which forms a covalent bond with the active site C145 leading to enhanced affinity.

Catalytic activity is governed by an equilibrium in which the oxyanion loop transitions from an unwound state (E), as observed in the monomer, to a wound mature-like state (E*), which is accompanied with interface conformational rearrangements leading to dimer stabilization. The conformational stability assessed by DSF and SEC-MALS indicates that both the N-terminal flanking sequence and the P1-Q to E mutation exert an additive effect by decreasing the $T_m$ by 4–5 $^\circ C$ and increasing the $K_{dimer}$ by $\geq 85$-fold. Notably, just the P1-Q to E mutation contributes to a further increase in $K_{dimer}$ to $> 340 \, \mu M$ and $K_d$ by 12-fold as compared to (-6*)MProH41A suggesting that prior to N-terminal processing, P1-Q influences the M-D equilibrium by reshaping (induced fit) the oxyanion loop to an E* state. However, addition of the inhibitor GC373 restores dimerization clearly indicating that E to E*
transition is coupled to dimerization, as shown recently for a full-length MPro\(^{10}\) and a dissected catalytic domain of MPro, MPro\(^{1-199}\).\(^{30}\) Notably, the large increase in \(T_m\) is observed only for the dimeric protein in which the oxyanion loop conformation is mainly in the E\(^*\) state and readily available for binding. Thus, an inhibitor that is designed to bind to the unwound E conformation selectively and prevent its switching to the E\(^*\) state may restrict autoprocessing itself. In this context, knowing that inhibitors promote dimer formation and enhance catalytic activity through an E-\(E^*\) transition \textit{in vitro}, warrants an understanding of how such inhibitors may alter MPro autoprocessing and the virus maturation itself in space and time \textit{in vivo}.

Even though the \(K_d\) for \((-6^*)\text{MProH41A}\) is > 340 \(\mu M\) in the absence of GC373, under the conditions of crystal growth at \(\sim 9\) mg/ml (\(\sim 260\) \(\mu M\)), selective crystallization of only the dimeric population was observed even when the majority of the protein is monomeric. Therefore, failing to attain a monomeric structure of \((-6^*)\text{MProH41A}\), only the dimers of inhibitor-free \((-6^*)\text{MProH41A}\) and GC373 bound \((-6^*)\text{MProH41A}\) complex could be compared. As seen in both structures, disorder of the terminal residues may account for the lower conformational stability, which again emphasizes the importance of the N-terminal cleavage for stable dimer formation. Unlike MPro\(^{WT}\), \((-6^*)\text{MProH41A}\) dimer is asymmetrical, one major difference arising from a significant movement of the helical domain of protomer B away from the corresponding helical domain of protomer A, thus, defining a \textit{semi-open} quaternary state. Also, of note is a unique downward flip of the E166 residue in protomer B which plays a critical role in substrate binding.\(^{39}\) This movement and associated changes may relate to the transitioning of the oxyanion loop from the E to E\(^*\) state and could partly be attributed to the much weaker binding of GC373 to \((-6^*)\text{MProH41A}\), relative to its mature counterpart. Accordingly, \((-6^*)\text{MPro}^{\text{H41A}}\) complex shows a completely restored oxyanion loop conformation, symmetric relative to each other, except for the helical domain, which stays in a semi-open state pointing to the requirement of the N-terminal cleavage to restore the symmetry for this region through interactions of S1 and G2 residues with subsite S1 of the opposite protomer as seen for mature MPro\(^{WT}\). In \((-6^*)\text{MPro}^{\text{H41A}}\) and its complex with GC373, the N-terminal flanking residues S1 and G2 are disordered and not visible in the electron density maps. A closer examination reveals lack of one of the two hydrogen bonds between R4 of one protomer and E290 of the other, as seen in the mature wild type MPro\(^{34}\) which may partly account for the increased \(K_d\) and semi-open conformation in the precursor form of MPro prior to its N-terminal maturation.

In conclusion, being a large multidomain dimeric protein, the appearance of catalytic activity of MPro is governed collectively by several equilibria and conformational changes during its conversion from a polyprotein to a mature fully active dimer as illustrated in Figure 8. Our results suggest that inhibitors designed to the mature active site oxyanion loop (E\(^*\)) will bind weakly to monomeric MPro and its miniprecursor, thus, providing an insight that the E state of monomeric MPro could be a strategic target for inhibitor design to effectively restrict MPro maturation from its polyprotein precursor.

**Materials and Methods**

**Construction and designation of MPro constructs**

The expression and purification of MPro\(^{WT}\) (GenBank ID: MN908947.3) were carried out as described.\(^{10,13}\) Except for MPro\(^{WT}\), the rest of the constructs were designed to contain the 6 \textit{nsp4} residues flanking the N-terminus of MPro. The inactive oxyanion loop (E, unwound yellow) existing in equilibrium with active E\(^*\) state (wound black) is shown as ribbons. Open and closed red boxes also denote the E and E\(^*\) states, respectively. Blue and red arrows signify the change in \(K_d\) and \(T_m\), respectively (Tables 1 and 2).

![Figure 8. Conformational equilibria for MPro maturation and inhibition.](image-url)
constructs were expressed without a fusion partner flanking the N-terminus of MPro. All constructs were synthesized and cloned into pJ414 vector (ATUM, Newark, CA). \( ^{(-6)}\text{MPro}^{H41A} \) consists of 6 amino acids of the flanking nsp4 sequence appended to the active site mutant, \( \text{MPro}^{H41A} \). To restrict residual of the flanking nsp4 sequence appended to the active site MPro, \( \text{MPro}^{H41A} \) was engineered, which contains a substitution mutation of residue Q in the P1 position (denoted with an asterisk) of the nsp4/nsp5 cleavage site to E and termed \( ^{(-6*)}\text{MPro}^{H41A} \). All constructs contain a GP-6His tag at the C-terminus of MPro to facilitate initial purification followed by removal of GP-6His via human rhinovirus 3C (HRV-3C) protease cleavage. Amino acid sequence and designations of all MPro constructs used in this study are listed in Figure S1.

**Expression and purification**

Plasmids were transformed into BL21-DE3 cells (Agilent) and induced for expression at 0.7–0.8 optical density with 1 mM isopropyl \( \beta\)-d-thiogalactopyranoside typically for 3 hrs. Proteins were purified from the cell lysate by nickel-affinity chromatography (NAC, step 1). The bound fraction was subjected to isocratic fractionation on Superose-12 column (step 2, Cytiva Life Sciences) and HRV-3C protease cleavage (step 3, purchased from Sigma-Aldrich) overnight at 4 °C followed by repeating NAC and step 2 in a final buffer of 25 mM Tris-HCl, pH 7 or 7.6, 150 mM NaCl and 1 mM TCEP (buffer A). The full-length wild type (\( \text{MPro}^{WT} \)) was expressed and purified similar in strategy to that described previously\(^3\) except for substituting the fusion partner GST with maltose binding protein (MBP) followed by a 36 amino acid spacer sequence corresponding to the immunoglobulin binding domain B1 of protein G (\( \Delta\text{GB1} \)).\(^{10,13,21}\) Peak fractions were pooled and concentrated to the desired concentration using Amicon Ultra – 15 or 0.5 ml centrifugal filters (Merck Millipore ltd.) and stored in aliquots at \(-30 \) °C and for long term storage at \(-80 \) °C. Purity was verified both by SDS-PAGE on 4–20 % gradient mini-protein TGX precast gel (Bio-Rad) and reverse-phase liquid chromatography with in-line electrospray ionization mass spectrometry.\(^{30}\) Protein concentrations were measured before storage and prior to the experiment at least in duplicate based on the extinction coefficient (Accelrys Gene v2.0) at 280 nm on a Perkin Elmer Lambda 35 or 40 UV/Vis spectrometer.

**Size exclusion chromatography with multi-angle light scattering (SEC-MALS)**

Molecular mass was estimated by analytical SEC with in-line MALS (DAWN Heleos-II, Wyatt Technology Inc., Santa Barbara, CA), refractive index (Optilab T-rEX, Wyatt Technology Inc.) and UV (Waters 2487, Waters Corporation, Milford, MA) detectors. Sample (125 \( \mu\)l) was applied onto a pre-equilibrated Superose-12 column (1.0 × 30 cm, Cytiva) and eluted at a flow rate of 0.5 ml/min in buffer A at 25 °C. Molecular mass was calculated using the Astra software provided with the instrument.

**Sedimentation velocity analytical ultracentrifugation (SV-AUC)**

Various constructs were subjected to SV-AUC either in buffer B (25 mM Tris-HCl, pH 7, 50 mM NaCl and 1 mM TCEP) or buffer C (25 mM Tris-HCl, pH 7.2, 20 mM NaCl and 1 mM TCEP), respectively. Samples containing the inhibitor were prepared using a 10 mM stock solution of GC373 in buffer C to achieve the desired protein and inhibitor ratios and incubated for a period of 1–2 hours prior to filling the cells.

Sedimentation velocity experiments were conducted at 50,000 rpm and 25 °C on a Beckman Coulter ProteomeLab XL-I or Beckman Optima XL-A analytical ultracentrifuge following standard protocols.\(^{40}\) Samples were loaded in 2-channel centerpiece cells and scans were collected using both the absorbance (280 nm) and Rayleigh interference (655 nm, when available) optical detection systems. Sedimentation data were time-corrected and analyzed in SEDFIT 16.1C\(^{41}\) in terms of a continuous c(s) distribution of Lamm equation solutions. Solution densities \( \rho \), solution viscosities \( \eta \), and protein partial specific volumes were calculated in SEDNTERP.\(^{42}\) To estimate the dimer dissociation constant for \( ^{(-6)}\text{MPro}^{H41A} \) and \( ^{(-6*)}\text{MPro}^{H41A} \) with GC373, absorbance sedimentation velocity data collected at various concentrations were analyzed globally using Lamm equation modeling in SEDPHAT 15.2b.\(^{43}\) A monomer–dimer self-association model was used and the presence of both monomer and dimer species was confirmed in the analysis. Absorbance extinction coefficients were calculated in SEDNTERP. Data were plotted in GUSSI.\(^{44}\)

**Differential Scanning Fluorimetry (DSF)**

Samples in duplicate were prepared with SYPRO orange dye (5000x, Millipore Sigma product number S5692) to yield a final concentration of 10 \( \mu\)M protein and 5x dye in 25 \( \mu\)l of buffer B (25 mM Tris-HCl, pH 7, 50 mM NaCl and 1 mM TCEP) and 0.1 % DMSO. The FRET signal was monitored as a function of temperature in a Bio-Rad C1000 Touch Thermal Cycler, and data was processed with the provided software and plotted using Sigmaplot (Systat Software Inc.). Experiments were repeated at least twice.
Isothermal titration calorimetry (ITC)

Purified proteins were diluted from a stock solution to slightly above the desired concentration and dialyzed extensively against buffer C (25 mM Tris-HCl, pH 7.2, 20 mM NaCl and 1 mM TCEP). Concentrations were estimated after dialysis based on their 280 nm absorbance at least twice. Stock solutions of inhibitors in buffer C were diluted in the same buffer to the desired concentration. Titrations were performed with proteins (30 to 150 μM) kept in the cell and inhibitors at 10-times the concentration of the protein in the syringe at 28 °C on iTC200 microcalorimeter (Malvern Instruments Inc., Westborough, MA). Data were processed using the Origin software provided with the instrument. For competitive inhibitors that bind at only one site, the dissociation constant (K_d = 1/K_a) is equivalent to the inhibition constant measured by enzyme kinetics (K_i).

Circular dichroism

CD spectra were recorded using 10 μM protein in buffer B (25 mM Tris-HCl, pH 7, 50 mM NaCl and 1 mM TCEP) at 25 °C on a JASCO J-810 spectropolarimeter using Spectra Manager software version 2 (Jasco Analytical Instruments, Easton, MD) and a 0.1 cm pathlength cell. Spectra were processed using the same software. The bandwidth was set to 1 nm with an integration time of 0.5 s and 100 nm/min scanning speed. Ten scans were averaged and subtracted from a buffer scan acquired the same way. For thermal denaturation measurements, the change in the CD signal at 222 nm was measured as a function of temperature from 25-60 °C for MPro WT and MProH41A and 25–75 °C for MPro C145A at 1 °C intervals. The spectra were normalized to the mean residue ellipticity.

Protein crystallization and room-temperature X-ray crystallography

(-6*)MProH41A protein sample was concentrated to 9 mg/ml. GC376 stock was prepared at 10 mM concentration in buffer C (25 mM Tris-HCl, pH 7.2, 20 mM NaCl and 1 mM TCEP) for crystallization purposes and stored at −30 °C. For cocrystallization, (-6*)MProH41A was mixed with GC373 at 1:5 molar ratio and incubated at room temperature for at least 30 minutes before setting up crystal trays. Crystals of inhibitor-free and GC373-bound (-6*)MProH41A were grown by sitting drop vapor diffusion methodology with 18–21 % PEG3350, 0.1 M Bis-Tris pH 6.5 or pH 7.0 (1 mL) as the precipitant solution. Crystallization drops of 20 μL at 1:1 ratio were seed struck using the crystals of the native MPro in complex with a covalent ligand NBH2 as described.13,21 Crystals appeared after several days and grew to the final size in about 1 month at 14 °C. The crystals suitable for X-ray diffraction measurements were mounted in MiTeGen (Ithaca, NY) room-temperature capillary setups for data collection.

All room temperature X-ray crystallographic data were collected on a Rigaku HighFlux HomeLab instrument equipped with a MicroMax-007 HF X-ray generator, Osmic VariMax optics, and a DECTRIS Eiger R 4 M hybrid photon counting detector. X-ray diffraction data were integrated using the CrysAlis Pro software suite (Rigaku Inc., The Woodlands, TX) then reduced and scaled using Aimless45 from the CCP4 suite.46 Structures were solved by molecular replacement using Phaser.47 MPro WT structure (PDB code 6WQF,9 was used as a search model to solve the structures of inhibitor-free (-6*)MProH41A and (-6*)MProH41A-GC373 complex. Each model was iteratively refined with phenix.refine from the PHENIX suite48 and COOT.49 Geometry validation was aided by Molprobit.50 GC373 restraints were generated with eLBOW51 using geometry optimized by quantum mechanical calculations in Gaussian16 at B3LYP/6–31 g(d,p) level of theory.52 Final data collection and refinement statistics can be found in Table S1.

Accession numbers

PDB ID 8E4J, inhibitor-free (-6*)MProH41A; PDB ID 8E4R, (-6*)MProH41A-GC373 complex.

Author contributions

A.Y.K and J.M.L. Conceptualization, Methodology, Investigation, Writing. L.C., D.K., R. G., A.A., N.T.N. Methodology, Investigation, Editing.

DECLARATION OF COMPETING INTEREST

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

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2022.167876.

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SARS-CoV-2 main protease; monomer–dimer equilibrium; conformational stability; inhibitor binding; room-temperature X-ray crystallography

Abbreviations:
SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; COVID-19, COrona Virus Disease; nsp, non-structural protein; MPro, main protease; DSF, differential scanning fluorimetry; SEC-MALS, size-exclusion chromatography-multilangle light scattering; SV-AUC, sedimentation velocity analytical ultracentrifugation; CD, circular dichroism; T_m, mid-point of thermal denaturation; K_d, inhibitor dissociation constant; K_{dimer}, dimer dissociation constant; ΔT_m, T_m difference

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