Liberation of SARS-CoV main protease from the viral polyprotein: N-terminal autocleavage does not depend on the mature dimerization mode

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ABSTRACT

The main protease (Mpro) plays a vital role in proteolytic processing of the polyproteins in the replicative cycle of SARS coronavirus (SARS-CoV). Dimerization of this enzyme has been shown to be indispensable for trans-cleavage activity. However, the auto-processing mechanism of Mpro, i.e. its own release from the polyproteins through autocleavage, remains unclear. This study elucidates the relationship between the N-terminal autocleavage activity and the dimerization of “immature” Mpro. Three residues (Arg4, Glu290, and Arg298), which contribute to the active dimer conformation of mature Mpro, are selected for mutational analyses. Surprisingly, all three mutants still perform N-terminal autocleavage, while the dimerization of mature protease and trans-cleavage activity following auto-processing are completely inhibited by the E290R and R298E mutations and partially so by the R4E mutation. Furthermore, the mature E290R mutant can resume N-terminal autocleavage activity when mixed with the “immature” C145A/E290R double mutant whereas its trans-cleavage activity remains absent. Therefore, the N-terminal auto-processing of Mpro appears to require only two “immature” monomers approaching one another to form an “intermediate” dimer structure and does not strictly depend on the active dimer conformation existing in mature protease. In conclusion, an auto-release model of Mpro from the polyproteins is proposed, which will help understand the auto-processing mechanism and the difference between the autocleavage and trans-cleavage proteolytic activities of SARS-CoV Mpro.

KEYWORDS SARS-CoV Mpro, N-terminal autocleavage, autocleavage activity, trans-cleavage activity, dimerization

INTRODUCTION

The global outbreak of the highly infectious Severe Acute Respiratory Syndrome (SARS) between November, 2002, and June, 2003, was caused by a new human coronavirus, SARS coronavirus (SARS-CoV) (Drosten et al., 2003; Peiris et al., 2003). SARS-CoV is an enveloped, positive-sense RNA virus and involves the largest viral RNA genome known to date, encoding several structural and auxiliary proteins as well as two large overlapping polyproteins, pp1a (replicase 1a, around 450 kDa) and pp1ab (replicase 1ab, around 750 kDa) necessary for viral RNA synthesis (Marra et al., 2003; Thiel et al., 2003). SARS-CoV is an enveloped, positive-sense RNA virus and involves the largest viral RNA genome known to date, encoding several structural and auxiliary proteins as well as two large overlapping polyproteins, pp1a (replicase 1a, around 450 kDa) and pp1ab (replicase 1ab, around 750 kDa) necessary for viral RNA synthesis (Marra et al., 2003; Thiel et al., 2003). These two polyproteins are cleaved extensively by the main protease (Mpro, also often called 3C-like protease) and the papain-like cysteine protease (PL2pro), both of which are encoded by the viral genome, to yield a multi-subunit protein complex termed “viral replicase-transcriptase” (Thiel et al., 2003; Ziebuhr, 2004; Groneberg et al., 2005). Because of its functional indispensability in viral replication, the main protease (Mpro) has become an attractive
target in developing inhibitors directed at SARS-CoV and other coronaviruses (Anand et al., 2005).

SARS-CoV M<sub>pro</sub> exists as a dimer in all crystal structures of the wild-type enzyme determined so far (Yang et al., 2003; Hsu et al., 2005a; Lee et al., 2005; Tan et al., 2005) and dimerization is also observed in solution, in a concentration-dependent manner (Chou et al., 2004; Fan et al., 2004; Shi et al., 2004; Chen et al., 2005; Graziano et al., 2006b). Structurally, two monomers orient perpendicular to one another to form a dimer. Each monomer contains three domains (Fig. 1A) (Yang et al., 2003): domains I (residues 8–101) and II (residues 102–184) form a chymotrypsin fold and domain III (residues 201–306) is an antiparallel globular cluster of five α-helices connected to domain II by a long loop (residues 185–200). The substrate-binding pocket is located in a cleft between domains I and II and the active site consists of a Cys145–His41 catalytic dyad (Huang et al., 2004). In the active conformation of the dimer, the pocket is accessible for interaction with the respective amino-acid residues of the substrate and the oxyanion loop (residues 138–145) has the correct shape to donate two hydrogen bonds from main-chain amides to stabilize the tetrahedral transition-state of the proteolysis reaction. To date, numerous experimental results have indicated that only the dimer is the biological functional form of SARS-CoV M<sub>pro</sub> and the monomer is considered enzymatically inactive (Chou et al., 2004; Hsu et al., 2005a, b; Barrila et al., 2006; Graziano et al., 2006a; Chen et al., 2008b). It has also been revealed that the activity loss of the dissociated monomer is mainly due to the collapse of the oxyanion hole and the S1 substrate-binding subsite (Tan et al., 2005).

Since the dissociated monomer of M<sub>pro</sub> is inactive, the dimer interface has been suggested as another potential target for inhibitor design (Shi et al., 2004; Hsu et al., 2005a). As revealed by the crystal structure, the dimer interface of the protease mainly involves the following interactions (Fig. 1B–D): (i) between the N-terminal finger (residues 1–7) of one monomer and residues near the S1 subsite of the other monomer, and (ii) between the two A' α-helices of each monomer. The residues selected for site-directed mutations in this study are marked in red.

**Figure 1.** The overall dimeric structure and detailed dimer interface of mature SARS-CoV M<sub>pro</sub>. (A) A ribbon diagram for the dimer structure of mature SARS-CoV M<sub>pro</sub> (PDB code: 1UK4). Monomers A and B are represented in green and cyan, respectively, and the three domains are labeled. The dimer interface mainly involves the interactions: (B) between the S1 subsite of monomer A (green) and the N-terminal finger (cyan) of monomer B, (C) between the N-terminal finger and the S1 subsite of monomer A and the helical domain III of monomer B, (D) between the two A' α-helices of each monomer. The residues selected for site-directed mutations in this study are marked in red.
monomer, in particular the oxyanion loop, (ii) between the N-terminal finger and the helical domain III from each monomer, (iii) between the two α-helices (residues 10–15), one from each monomer, that immediately follow the N-terminal finger. The contributions of several individual residues on the dimer interface to dimerization and enzymatic activity of Mpro have been identified by several groups (Chou et al., 2004; Hsu et al., 2005b; Barrila et al., 2006; Shi and Song, 2006; Wei et al., 2006; Chen et al., 2008a, b; Lin et al., 2008). Firstly, residues on the N-terminal finger were considered to play an important role in both dimerization and activity (Chen et al., 2005; Hsu et al., 2005b; Chen et al., 2008b). In particular, Arg4 of the N-terminal finger can form a salt-bridge with residue Glu290 of the other monomer, which is vital for stabilizing the dimer structure to maintain the correct conformation of the active site (Chou et al., 2004). Furthermore, residues of domain III have also been found to extensively mediate dimerization and to be responsible for positioning the N-terminal finger to interact with the active site of the neighboring monomer (Shi and Song, 2006; Lin et al., 2008). Residue Arg298 in the C-terminal helix (residues 293–301) has been identified as a key component for maintaining the dimer conformation and its mutation was found to trigger a structural switch from dimer to monomer (Shi et al., 2008). In addition, the α-helix (residues 10–15) is another critical part of the dimer interface since mutation of Gly11 can also result in complete dimer dissociation, as shown by X-ray crystallography (Chen et al., 2008a).

Before proteolytic processing of the viral polyproteins pp1a and pp1ab into a total of 15 or 16 non-structural proteins (Nsp) occurs, SARS-CoV Mpro itself is embedded in these polyproteins as the Nsp5 domain. On both sides, it is flanked by putative transmembrane (TM) domains (Nsp4 and Nsp6) that are anchored to the double-membrane vesicles where viral replication takes place in the infected host cell (Snijder et al., 2003, 2006; Knoops et al., 2008). Therefore, the Mpro has to first liberate itself from the polyproteins through autocleavage, and then the self-released mature Mpr0 would form a dimer and trans-cleave pp1a and pp1ab at other sites. Despite being the first and essential step for viral maturation, the autocleavage mechanism of Mpr0 has only been rarely addressed (Lin et al., 2004; Shan et al., 2004; Hsu et al., 2005a) and remains poorly characterized. On the other hand, the relationship between dimerization and enzymatic activity of the mature Mpr0 is well documented (Chou et al., 2004; Shi et al., 2004; Hsu et al., 2005b; Graziano et al., 2006a, b; Chen et al., 2008a; Lin et al., 2008). Since most of these studies use a protease preparation that resembles the sequence of mature Mpro after auto-processing and formation of the final dimer structure, all these results are only relevant for the trans-cleavage activity of the mature enzyme.

In this paper, we focus on the correlation between the N-terminal autocleavage activity and dimerization of “immature” SARS-CoV Mpr0, which has not been reported yet. A total of three residues involved in maintaining the dimer conformation of mature Mpro are selected for mutational analyses, i.e., Arg4, Glu290, and Arg298 (Fig. 1B and 1C). In the crystal structure of the mature dimer, the side-chain guanidyl of Arg4 forms a salt bridge with the side-chain of Glu290 of the neighboring monomer in the dimer. Mutation of the not absolutely conserved Arg4 shifts the dimer-monomer equilibrium and induces a significant decrease of trans-cleavage activity (Chou et al., 2004). Furthermore, mutation of Arg298 has also been shown to trigger dimer dissociation in both solution and crystal as well as a complete loss of activity (Shi et al., 2008). As the construction strategy of all the plasmids used in the study presented here, a SARS-CoV Mpro autocleavage site (AVLQ↓S) was introduced between the N-terminal tag and the N-terminal residue (Ser1) of the protease. Thus, the N-terminal autocleavage activity of wild type and mutated Mpros can be evaluated by the extent to which the N-terminal tag is removed. Our results surprisingly reveal that all three mutants can still perform autocleavage during protein production and purification. In the following, we also investigate the effects of these mutations on dimerization and trans-cleavage activity of mature SARS-CoV Mpro. Consistent with the published results, dimer formation of mature protease in solution is completely abolished by the E290R and R298E mutations and partially so by the R4E mutation, resulting in an entire or dramatic loss of trans-cleavage activity. Furthermore, the reconstructed cleavage assay indicates that the mature E290R mutant can resume N-terminal autocleavage activity when mixed with the “immature” C145A/E290R double mutant whereas its trans-cleavage activity remains absent. These results indicate that N-terminal autocleavage of SARS-CoV Mpro from the polyproteins only requires two “immature” proteases approaching one another to form an “intermediate” dimer structure and does not depend on the active dimer conformation existing in the mature protease. The present study is expected to help us better understand the maturation mechanism and the difference between autocleavage and trans-cleavage proteolytic processing of SARS-CoV Mpro.

**RESULTS AND DISCUSSION**

**Autocleavage of the N-terminally tagged wild-type and mutated SARS-CoV Mpr0 during lysate preparation**

The goal of this study was to elucidate the relationship between the N-terminal autocleavage activity and dimerization of “immature” SARS-CoV Mpro. Three residues (Arg4, Glu290, and Arg298), which are involved in maintaining the integrity of the dimer interface of mature SARS-CoV Mpro (Fig. 1B and 1C), were selected for mutational analysis. The Arg4→Glu290 salt bridge has been demonstrated to be a key
element in dimerization of mature M\textsuperscript{pro} and residues on domain III (e.g., Arg298, Gln299 et al.) have also been shown to be vital regulators of dimerization and trans-cleavage activity (Chou et al., 2004; Shi and Song, 2006; Lin et al., 2008). Structurally, domain III of monomer B in the dimer not only directly interacts with the opposite S1 subsite (Gln299B...Ser139A, Arg298B...Ser123A; Fig. 1C), but also helps its own N-terminal finger to properly insert into the counterpart monomer A by making both inter-monomer (Arg4B...Glu290A) and intra-monomer (Arg298B...Met6B) interactions (Fig. 1B). Since these residues contribute to the active dimer conformation of mature M\textsuperscript{pro} mainly by electrostatic interactions, we mutated them into oppositely charged residues and evaluated the influence of these mutations on the N-terminal autocleavage activity of M\textsuperscript{pro}. An M\textsuperscript{pro} autocleavage site (AVLQ↓S), corresponding to the C-terminus of Nsp4 which precedes the M\textsuperscript{pro} (Nsp5) in the viral polyproteins pp1a and pp1ab, was inserted between the N-terminal GST tag and the first residue (Ser1) of the protease (Fig. 2A) to resemble the “immature” M\textsuperscript{pro} before autocleavage. In addition, 24 nucleotides coding for eight extra residues (GPH6) were added at the C-terminus of M\textsuperscript{pro} for purification convenience. Using this construct, the N-terminal autocleavage activity of M\textsuperscript{pro} was evaluated in terms of the extent of removal of the GST tag during gene expression and protein purification. As shown by SDS-PAGE analysis.

![Figure 2](image.png)

**Figure 2.** SDS-PAGE analyses of the autocleavage activity of N-terminally tagged SARS-CoV M\textsuperscript{pro}. (A) Schematic plots of N-terminal GST- or Trx-tagged SARS-CoV M\textsuperscript{pro} constructs designed in this study. The arrow with "↓" represents the autocleavage site of M\textsuperscript{pro} and the arrow alone indicates the cleavage site of PreScission protease. (B-F) Purification of WT, C145A, R4E, E290R and R298E mutants of GST-tagged M\textsuperscript{pro}. Lane 1, protein molecular-mass marker; lanes 2–5 represent the proteases eluted by 500 mM imidazole from the Ni-NTA column. (G-H) Purification of WT and R298E mutant of Trx-tagged M\textsuperscript{pro}. Lanes 2–3 and lanes 2–5 represent the 500 mM imidazole eluants from Ni-NTA column respectively.
results clearly indicated that Glu290 and Arg298 are key residues for the mature Mpro (WT) from the Ni-NTA affinity column, well in agreement with the molar mass calculated from the amino-acid sequence (33.8 kDa) of mature Mpro. This indicated that the N-terminal GST fusion tag (~26 kDa) had been removed by WT protease through autocleavage. As a negative control (Fig. 2C), we mutated the catalytic residue Cys145 to Ala. The C145A mutant exhibited a band with an N-terminal GST fusion tag (~26 kDa) had been removed completely. It has been reported (Chou et al., 2004) that mutation of the highly, but not absolutely conserved Arg4 to Ala can result in unstable dimers while the mutant still maintains a trans-cleavage activity comparable to that of WT protease. Thus, it is possible that the “immature” R4E mutant can still perform efficiently in N-terminal autocleavage during expression, as a small amount of unstable dimers might form. On the other hand, mutation of the totally conserved Glu290 to Ala was reported to induce complete dimer dissociation of Mpro in solution (Shi and Song, 2006) and the Arg298Ala mutation also produced a monomeric structure in the crystal (Shi et al., 2008), thereby inducing complete loss of trans-cleavage activity. These results clearly indicated that Glu290 and Arg298 are key factors in maintaining the dimeric form of mature Mpro. Surprisingly, as shown in Fig. 2E and 2F, both the E290R and R298E mutants displayed no obvious difference of autocleavage behavior compared to WT protease. Since mutation of either of these two residues were reported to completely abolish the dimer of mature Mpro, our finding raises the intriguing question of how the E290R and R298E mutants can auto-process their N-terminal GST tags when they are unable to form the active dimer structure. In order to better explain these results and elucidate the autocleavage mechanism of Mpro, the contributions of these residues to dimerization and trans-cleavage activity of mature Mpro needed to be further investigated.

The GST protein alone also exists as a homodimer in the crystal (Nishida et al., 1998) and the N-terminal autocleavage of the E290R or R298E mutant might possibly be due to dimerization of the GST-fused Mpro through the N-terminal GST tag. For excluding this possibility, WT Mpro and all the mutants were also subcloned into the vector pET-32a (Fig. 2A). This construct encodes an N-terminal thioredoxin (Trx) tag, which does not form dimers by itself (Jeng et al., 1994). As shown by SDS-PAGE (Fig. 2G and 2H), two purified protein bands corresponding to cleaved Mpro (~33 kDa) and Trx (~19 kDa including an additional in-frame sequence from the vector) were observed for both WT and R298E mutant, suggesting that the Trx-tagged R298E mutant maintains autocleavage activity comparable to WT protease. These findings therefore clearly indicate that the N-terminal fusion tag has no impact on autocleavage of Mpro and further validate the reliability of our autocleavage assay.

**Folding behavior and dimerization features of mature WT and mutated SARS-CoV Mpro**

As described above, all three mutants maintain efficient N-terminal autocleavage activity despite the residues we selected for mutation, especially E290 and R298, have been reported to be indispensable for dimerization of mature Mpro (Shi and Song, 2006; Shi et al., 2008). In view of this apparent inconsistency, we further investigated the folding behavior and dimerization features of all mature mutants after autocleavage. Fig. 3A shows the fluorescence emission spectra of mature WT and the mutants of Mpro. The emission wavelength max (λ<sub>max</sub>) of WT Mpro is around 330 nm. Similar to WT protease, all three mutants show only minor differences in the emission spectrum max (varying from 329 to 331 nm), demonstrating that mutation of a single residue on the dimer interface into one carrying the opposite charge has not changed the fold of the protease. In addition, the far-UV CD spectra of mature WT and mutated Mpro are also similar to one another (Fig. 3B). All spectra feature a positive peak around 200 nm and dual negative peaks at 209 and 222 nm, typical of proteins containing significant amounts of α-helix and β-sheet. These data suggest that all three mutants have well-defined secondary structure and exclude the possibility of misfolding caused by the mutation of individual residues.

The dimerization of mature Mpro has been successfully characterized by various biochemical and biophysical methods (Shi et al., 2004; Chen et al., 2005; Hsu et al., 2005b; Graziano et al., 2006b). According to a published method (Prakash et al., 2002), we performed a chemical cross-linking analysis of mature WT Mpro. When incubated with 0.01% glutaraldehyde, the protease at a concentration of 0.1 mg/mL predominantly displayed the monomeric form near 33.0 kDa, with a minor band around 66.0 kDa corresponding to the dimer (Fig. 4A, lane 5). With increasing protein concentration, the dimeric form increased and was almost equivalent to the amount of the monomeric form at 1 mg/mL (Fig. 4A, lanes 6–8). When using a higher concentration of glutaraldehyde (0.1%), a similar cross-linking pattern of the protease was observed with slightly higher efficiency, as the dimer had become the predominant species at the same protein concentrations (Fig. 4A, lanes 1–4). These results further demonstrate that mature WT Mpro possesses a dimer-monomer equilibrium in solution and its dimerization is concentration-dependent, in good agreement with the literatures (Chou et al., 2004; Shi et al., 2004; Chen et al., 2005; Graziano et al., 2006b). Nevertheless, the possibility of minor
artificial cross-linking effects might still exist due to the appearance of high-order multimers in SDS-PAGE (Fig. 4A). However, since the cross-linking analyses of all three mutants can be performed under exactly the same experimental conditions, this method is still very useful for examining the effects of these mutations on dimerization of mature Mpro. As indicated in Fig. 4B, the R4E mutant showed an obvious tendency to monomer and the amount of the dimer decreased significantly at lower protein concentrations compared to WT protease, which is consistent with the published result that mutation of Arg4 would result in weakened dimerization of Mpro (Chou et al., 2004). For the E290R and R298E mutants (Fig. 4C and 4D), dimerization seemed to be disrupted even more severely than for the R4E mutant since almost no dimer can be observed at low protein concentrations, further demonstrating the indispensability of Glu290 and Arg298 for maintaining the dimeric structure. However, a very small amount of dimeric form of the E290R and R298E mutants were visible in SDS-PAGE at relatively high protein concentrations (Fig. 4C and 4D, lanes 7, 8). This might be the real dimer of Mpro or just a dimeric product caused by minor artificial cross-linking effects as mentioned above.

Subsequently, size-exclusion chromatography (SEC) analyses were carried out to evaluate the dimer-monomer
equilibria of the three mutants more precisely. In view of the appearance of a dimeric form of the mutants at high concentration in the cross-linking analyses, we used a protein concentration of 5 mg/mL for each SEC run, which is much higher than the highest concentration used for cross-linking. The physical state corresponding to the native monomer or dimer of M\textsuperscript{pro} in the gel-filtration column was then monitored. As shown in Fig. 5, WT M\textsuperscript{pro} eluted as a single peak with a retention volume of 57.01 mL. The elution profiles of four molecular-mass marker proteins confirmed that the peak represented the dimeric species of M\textsuperscript{pro} (Table 1, estimated Mr: ~66.7 kDa), implying that in solution, WT protease exists almost exclusively as a dimer at relatively high protein concentration. In comparison, the R4E mutant eluted at a higher volume (61.86 mL), consistent with it being a mixture of dimer and monomer, with the monomer being the predominant species (Table 1, estimated Mr: ~47.6 kDa). This further supports the cross-linking result and the literature report that Arg4 affects dimerization only to a moderate extent (Chou et al., 2004). For E290R and R298E mutants, the elution volumes dramatically shifted to 66.47 and 64.79 mL, corresponding to estimated Mr values of 34.6 kDa and 38.9 kDa, respectively, clearly indicating a monomeric state in solution. The data therefore demonstrate that individual mutation of Glu290 or Arg298 is sufficient to prevent dimerization of mature M\textsuperscript{pro} even at a high protein concentration, which convincingly supports the published results (Shi and Song, 2006; Shi et al., 2008) and also suggests that the very minor amounts of E290R and R298E dimers that appeared in SDS-PAGE after cross-linking (Fig. 4C and 4D) is likely due to a small extent of nonspecific cross-linking.

Table 1 Estimation of protein multimeric states of mature WT and mutated SARS-CoV M\textsuperscript{pro} based upon gel filtration data

|        | WT | R4E | E290R | R298E |
|--------|----|-----|-------|-------|
| Ve (mL)| 57.01 | 61.86 | 66.47 | 64.79 |
| K\text{av} | 0.21 | 0.27 | 0.33 | 0.31 |
| estimated Mr | 66.96 | 47.61 | 34.58 | 38.85 |
| monomer Mr | 34.82 | 34.79 | 34.85 | 34.79 |
| estimated monomer Mr | 1.92 | 1.37 | 0.99 | 1.12 |
| solution state | dimer | mixture of dimer and monomer | monomer | monomer |

Note: Ve is the elution volume and K\text{av} is the gel-phase distribution coefficient. Calibration of the column determined the relationship between K\text{av} and Mr as: K\text{av} = -0.4109 \log Mr + 2.1949. Full values were used in all calculations, but were rounded to the nearest two decimals for tabulation.
Trans-cleavage activity of mature WT and mutated SARS-CoV M\textsuperscript{pro}

Numerous published data have proposed that only the dimer is the biologically functional form of mature M\textsuperscript{pro} and the dissociated monomer has no trans-cleavage activity. Since mutation of a single residue on the dimer interface could result in partial or complete dimer dissociation of mature M\textsuperscript{pro} as demonstrated above, the three mutants were expected to show decreased or no trans-cleavage activity. To verify this prediction, we performed trans-cleavage assays of mature WT protease and the mutants using a fluorogenic peptide substrate (Verschueren et al., 2008). As shown in Fig. 6, the fluorescence increase following hydrolysis of the substrate by WT M\textsuperscript{pro} was significant and time-dependent, implying that the protease trans-cleaved the substrate efficiently, whilst the C145A mutant showed negligible activity against the substrate, thus proving the validity of the trans-cleavage assay. Similar to the C145A mutant, the trans-cleavage activities of the three “dimerization mutants” at a concentration of 0.1 μM were all below the detection limit of the assay during the entire measuring process (Fig. 6), even when the reaction time was prolonged to overnight (data not shown). Next, we increased the concentration of the mutants up to 1 μM, and the data showed that the R4E mutant displayed a slight increase of activity but still no measurable trans-cleavage reactivity was detected for the E290R and R298E mutants (see inset of Fig. 6). These results clearly reveal that mutation of Glu290 or Arg298 induces a complete loss of trans-cleavage activity of mature M\textsuperscript{pro}, correlating well with the complete dimer dissociation of the two mutants determined by SEC analyses. On the other hand, the R4E mutant exhibits a weak activity when increasing protein concentration, well in agreement with its unstable dimerization in solution.

Reconstruction of N-terminal autocleavage of M\textsuperscript{pro} with mature E290R and “immature” C145A/E290R mutants

According to all the results described above, mutation of residues E290 or R298 seemed to have no obvious influence on the N-terminal autocleavage of “immature” M\textsuperscript{pro}, although it did cause complete dimer dissociation and disruption of trans-cleavage activity of the mature form of the mutated M\textsuperscript{pro}. This surprising observation led to the interesting conclusion that the N-terminal autocleavage of SARS-CoV M\textsuperscript{pro} is not dependent on dimerization, at least it does not require the final dimer conformation existing in the mature protease. On the other hand, this final dimer conformation is indispensable for trans-cleavage activity of mature M\textsuperscript{pro}. In order to prove this hypothesis, an N-terminal autocleavage assay was constructed. The inactive C145A/E290R double mutant, which cannot perform autocleavage and still contains the N-terminal GST tag, was prepared as an “immature” substrate for mature WT M\textsuperscript{pro} and the E290R mutant. The cleavage
The process can be easily followed from the appearance of free GST in SDS-PAGE. As shown in Fig. 7A, GST-C145A/E290R was cleaved efficiently by mature WT M\textsuperscript{pro}. The band (~26 kDa) corresponding to the cleaved GST tag appeared after 5 min of incubation time (lane 2) but not in the substrate-only lane (lane 1), and the cleavage was totally completed within 2 hours (lanes 3–6). Since mature WT M\textsuperscript{pro} can form the active dimer even at a low concentration in solution, the free GST tag is the product of trans-cleavage of the “immature” substrate by the WT M\textsuperscript{pro} dimer (Fig. 7C). As a control, an unrelated protein (the effector domain of influenza A virus nonstructural protein 1, H1N1 NS1 ED, Fig. 7A, lane 8) containing an N-terminal His tag and an M\textsuperscript{pro} cleavage site (AVLQ↓S), was also prepared as a trans-cleavage substrate for WT M\textsuperscript{pro}. Compared to GST-C145A/E290R, a similar cleavage pattern was observed for His-H1N1 NS1 ED and the cleaved substrate (~17 kDa) was clearly visible in SDS-PAGE (lanes 9–13), further demonstrating the high trans-cleavage activity of the WT M\textsuperscript{pro} dimer. With the mature E290R mutant, quite a different cleavage behavior was observed between His-H1N1 NS1 ED and GST-C145A/E290R (Fig. 7B). The mature E290R mutant showed no detectable cleavage activity with His-H1N1 NS1 ED during the entire measuring procedure (lanes 9–13). As His-H1N1 NS1 ED is a trans-cleavage substrate for M\textsuperscript{pro} (see above; Fig. 7D), this result is well in agreement with the conclusion that the mature E290R mutant exists as a monomer in solution as revealed by SEC analysis (Fig. 5), without any trans-cleavage activity (Fig. 6). On the other hand, when mixing the E290R mutant with GST-C145A/E290R, the cleaved GST tag appeared after 2 h (lane 3) and cleavage was almost complete after overnight incubation (lanes 4–6), indicating that the mature E290R mutant can still cleave “immature” GST-C145A/E290R, although the efficiency is much lower than for WT protease. Since the mature E290R monomer cannot trans-cleave His-H1N1 NS1 ED (Fig. 7B) nor the M\textsuperscript{pro} peptide substrate (Fig. 6) at all, the cleavage of GST-C145A/E290R by the mature E290R monomer might be due to the reconstruction of only the N-terminal autocleavage by these two M\textsuperscript{pro} monomers (Fig. 7D). This strongly supports the idea that autocleavage of coronavirus M\textsuperscript{pro} occurs in trans (Anand et al., 2003). Furthermore, as the E290R mutation completely abolishes formation of the mature dimer in solution (Fig. 5), these data strongly support the hypothesis that the active dimer conformation existing in mature WT protease is unnecessary for N-terminal autocleavage of M\textsuperscript{pro}.

**How does “immature” M\textsuperscript{pro} release itself from the precursor polyproteins without forming the mature dimer structure?**

What we find puzzling is why the N-terminal autocleavage activity of M\textsuperscript{pro} is independent from the dimer structure that is vital to trans-cleavage activity of the protease? What is the
difference between the proteolytic autocleavage and trans-cleavage mechanisms of SARS-CoV M^{pro}? It has been reported (Shi et al., 2004) that domain III of M^{pro} itself possesses an intrinsic tendency to form a dimer even at a very low concentration. Recently, the crystal structure of domain III alone has been determined, revealing a novel 3D domain-swapped dimer structure (Zhong et al., 2009). Furthermore, it was reported (Zhong et al., 2008) that the N-terminal finger deletion mutant of M^{pro} also forms a novel stable dimer through domain III-domain III interactions, which is different from the dimer interface observed in the WT M^{pro} dimer. These results indicate that in addition to the dimer conformation in the crystal structure of WT protease, M^{pro} might have other possible dimerization modes. M^{pro} is still a part of pp1a and pp1ab when the polyproteins are produced in coronavirus-infected cells. Through the hydrophobic domains of Nsp4 and Nsp6 that flank the M^{pro} (Nsp5) domain, the unprocessed polyproteins are anchored to the double-membrane vesicles found in infected host cells. Subsequently, when these precursor proteins accumulate to high concentration, the “immature” M^{pro} can release itself from the polyproteins by inter-molecular cleavage, and then the self-released mature M^{pro} triggers the trans-cleavage processing of the polyproteins (Anand et al., 2003). In the dimer structure of mature M^{pro}, the N-terminal finger of one monomer can form intensive interactions with the S1 subsite and Domain III (the C-terminal helix) of the other monomer (Fig. 1B and 1C), which are key residues for maintaining the active dimer conformation. It is very unlikely that the “immature” M^{pro} would form such dimer because of the two TM domains flanking it at both N- and C-termini before self-releasing (Oostra et al., 2008). In our proposed model (Fig. 8), the N-terminal autocleavage might only need two “immature” M^{pro} domains in monomeric polyproteins approach one
another to form an "intermediate" dimer, possibly through Domain III-III dimerization, which also agrees well with the plausible dimer formation pathway proposed for the Mpro previously (Chen et al., 2008a; Hu et al., 2009). The formation of the "intermediate" dimer could trigger the rotation of their domains I/II relative to domains III and thereby insert their "uncleaved" N-termini into the substrate-binding pockets of the opposite monomer, which might induce the active conformation of the S1 subsites through an induced-fit catalytic mechanism. Once the autocleavage is finished, the "cleaved" N-terminal fingers should slip away from the active sites and switch to their final spatial positions, which is observed in the mature dimer of WT Mpro (Fig. 1B), thereby locking the dimer in a catalytic competitive state. The conformational shift of the N-terminal finger from the auto-processed state to the final "cleaved" state is also supported by the crystal structure of the Mpro H41A mutant in complex with an N-terminal auto-cleavage substrate (Xue et al., 2008), indicating an estimated distance of ~20 Å between residues P1′-P5′ (SGFRK) of the bound substrate and the free N-terminal finger of the mature dimer (Fig. 9A). Meanwhile, Domains III should also glide to their final positions to produce the mature active dimer since the last helices of domains III are tethered with the N-terminal fingers in the mature dimer structure (Fig. 1C). The dimer conformation existing in mature Mpro is not the cause but more likely the product of N-terminal auto-processing of "immature" protease from polyproteins. Thus mutation of residues that are indispensable for dimerization of mature Mpro has little effect on N-terminal autocleavage since the conformation of the

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**Figure 7.** Reconstruction of N-terminal autocleavage of Mpro by mature E290R and "immature" C145A/E290R mutants. SDS-PAGE analyses of cleavage by mature WT Mpro (A) and E290R mutant (B). The inactive C145A/E290R double mutant (to prevent autocleavage) containing an N-terminal GST tag and autocleavage site was prepared as an "immature" substrate for mature WT Mpro and E290R mutant. The substrate protein at a concentration of 20 μM (lane 1) was mixed with 5 μM of WT Mpro or E290R mutant at 25°C and the products were monitored after specified periods of incubation time shown on the top of the figure (lanes 2-6). Meanwhile, the effector domain of influenza A virus nonstructural protein 1 (H1N1 NS1 ED, lane 8) containing an N-terminal His tag and Mpro cleavage site (AVLQ\_5) was also prepared as a trans-cleavage substrate for WT Mpro and E290R mutant, and the cleavage assay was performed under the same experimental conditions (lanes 9-13). Lane 7, protein molecular-mass marker. (C) The proposed trans-cleavage scheme of mature WT Mpro. (D) The proposed scheme of autocleavage reconstruction of mature E290R with "immature" C145A/E290R mutant.
“intermediate” dimer is different from that of mature dimer. On the other hand, the trans-cleavage activity of the mature dimer is much higher than the autocleavage activity of the “intermediate” dimer, as indicated in Fig. 7A and 7B. However, the “intermediate” dimer might be the only feasible structure for “immature” M<sup>pro</sup> to form within pp1a and pp1ab. In order to perform the first step of autocleavage from the polyproteins, “immature” M<sup>pro</sup> possibly tolerates a less-than-ideal dimer. Once the mature dimer is formed after N-terminal self-releasing, the unprocessed C-terminus of one mature dimer can insert into an active site of another mature dimer for trans-cleavage (Fig. 8), as revealed by a product-bound crystal structure of the M<sup>pro</sup> C145A mutant (Hsu et al., 2005a) (Fig. 9B and 9C). After the formation of the final mature M<sup>pro</sup> dimer with authentic N- and C-termini, trans-cleavage of the polyproteins at other sites could subsequently be triggered at a high rate. In conclusion, our current work is expected to provide more insights into the auto-processing mechanism of SARS-CoV M<sup>pro</sup>. In addition, the proposed auto-releasing model should be further verified by X-ray crystallographic determination of the “intermediate” dimer structure.

**Figure 8. A proposed mode of SARS-CoV M<sup>pro</sup> auto-release from the precursor polyproteins.** The chymotrypsin fold (domains I and II) and domain III of M<sup>pro</sup> monomers in two polyproteins are shown as boxes and cylinders, respectively. The N- and C-termini are also labeled. The auto-release mode involves four steps. **Step 1,** two “immature” M<sup>pro</sup> monomers approach one another and their domains III form an “intermediate” dimer structure, which triggers the rotations of their chymotrypsin folds and insert their “uncleaved” N-termini into the substrate-binding pockets of the opposite monomers. **Step 2,** with an induced-fit catalytic mechanism, the active site of M<sup>pro</sup> could be activated and catalyze the N-terminal autocleavage. Afterwards, the “cleaved” N-terminal fingers slip away from the active sites and switch to their final spatial positions, as well as domains III, locking the mature dimer with “uncleaved” C-termini at the active state. **Step 3,** the “uncleaved” C-terminus of one mature dimer can insert into an active site of another mature dimer, as supported by a product-bound crystal structure of the M<sup>pro</sup> C145A mutant (Hsu et al., 2005a). **Step 4,** once the C-terminus is processed by trans-cleavage, the final mature dimer with authentic N- and C-termini is formed, which is observed in the crystal structure of WT M<sup>pro</sup> (Xue et al., 2007).
MATERIALS AND METHODS

Expression and purification

The plasmid of WT SARS-CoV M\textsuperscript{pro} was a gift from Prof. Z. Rao (Xue et al., 2007). Briefly, 12 nucleotides coding for the four amino acids AVLQ (corresponding to the P4−P1 residues of the N-terminal autocleavage site of SARS-CoV M\textsuperscript{pro}) were added to precede the N-terminal Ser1 residue of the protease. 24 nucleotides coding for eight extra residues (GPH\textsubscript{6}) were added at the C-terminus of M\textsuperscript{pro}. The resulting coding sequence was inserted into BamHI and XhoI sites of the pGEX-6P-1 plasmid. The plasmid was then transformed into E.coli BL21 (DE3) cells, and the N-terminal GST fusion protein with a C-terminal His tag was produced by introducing IPTG to 0.5 mM at 25°C for 6 h.
Because of the plasmid construction strategy, the N-terminal GST tag can be cleaved off by autocleavage of active M\textsuperscript{pro}. The purification of the C-terminally GPH\textsubscript{6} tagged product was performed according to the published method (Xue et al., 2007). The protein was first subjected to Ni-NTA affinity chromatography and concentrated in PreScission protease cleavage buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1 mM DTT). The PreScission protease was then added to cleave off the C-terminal GPH\textsubscript{6} tag, producing an authentic mature SARS-CoV M\textsuperscript{pro}. For the inactive M\textsuperscript{pro} C145A mutant, which cannot perform autocleavage, the product was first purified by Ni-NTA column, followed by adding the PreScission protease to remove both the N-terminal GST and the C-terminal GPH\textsubscript{6} tags. All the cleaved proteins were further purified using anion-exchange chromatography. Finally, the purified and concentrated protease was dialyzed against 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM DTT, and stored at −20°C.

**Site-directed mutagenesis of SARS-CoV M\textsuperscript{pro}**

Site-directed mutagenesis of SARS-CoV M\textsuperscript{pro} was processed by a modified recombinant PCR method. The mutated proteases (Cys145Ala, Arg4Glu, Glu290Arg single mutants, and Cys145Ala/Glu290Arg double mutant) were prepared using the QuikChange II site-directed mutagenesis kit (Stratagene) using pGEX-6p-1-SARS-CoV M\textsuperscript{pro} as a template. The nucleotide sequences of the primers used for mutation are given in Table 2. All the gene fragments coding for WT and mutated proteases were amplified using a procedure similar to that described above for WT protease. The purity and structural integrity of the mutated proteases were analyzed by SDS-PAGE.

All the gene fragments coding for WT and mutated proteases were also cleaved off from the plasmids of pGEX-6p-1-SARS-CoV M\textsuperscript{pro} using BamH\textsubscript{I} and XhoI, and subcloned into the vector pET-32a with N-terminal thioredoxin (Trx) tag. The resulting plasmids were verified by sequencing and transformed into E. coli BL21 (DE3) cells. The proteins were produced and purified using a procedure similar to that described above for WT protease. The N-terminal GST or Trx tag and the first Ser1 residue of the protease (Fig. 2A). The N-terminal GST or Trx tag can be removed by autocleavage of active M\textsuperscript{pro}. Thus, the N-terminal autocleavage activity of WT and mutated M\textsuperscript{pro}s can be evaluated by the removal extent of GST or Trx tag during the process of protein production and purification. The tag-cleaved protease was then checked using SDS-PAGE after Ni-NTA affinity chromatography purification.

**Fluorescence spectroscopy**

The fluorescence measurements were performed on a Cary Eclipse fluorescence spectrophotometer. The protease samples were prepared in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1 mM DTT, with a protein concentration of 5 μM. The fluorescence emission spectra from 300 to 420 nm were recorded after excitation at 280 nm, with a spectral slit width of 5 nm for excitation and emission. Fluorescence spectra of mature WT and mutated SARS-CoV M\textsuperscript{pro}s were measured in a 1-ml quartz cuvette at 25°C. All final spectra were corrected for the buffer contribution, and were the average of three parallel measurements.

**Circular dichroism (CD) spectroscopy**

Far-UV CD spectra from 195 to 250 nm were recorded on a JASCO-715 spectropolarimeter. The protein samples were prepared in 50 mM Tris-HCl pH 7.5, 1 mM EDTA at 25°C, with a concentration of 10 μM. The spectra were collected with a 1 nm band width using a 0.1-cm path length cuvette, and normalized by subtracting the baseline recorded for the buffer. Each measurement was repeated five times and the final result was the average of five independent scans. The CD spectra of mature mutated proteases were compared to that of WT SARS-CoV M\textsuperscript{pro} to exclude the possibility of misfolding caused by mutation.

**Glutaraldehyde cross-linking**

Chemical cross-linking was performed for mature WT and mutated SARS-CoV M\textsuperscript{pro}s (final concentrations from 0.1 to 1.0 mg/mL in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA) with glutaraldehyde. An aliquot of 25% (v/v) glutaraldehyde was added to the protein samples to give a final concentration of 0.1% or 0.01%, and the samples were incubated at 25°C for 20 min followed by quenching the reaction with the addition of 1.0 M Tris-HCl pH 8.0 (0.5%, v/v). Orthophosphoric acid was thereafter added to the

**Table 2  Nucleotide sequences of the primers used for site-directed mutagenesis of SARS-CoV M\textsuperscript{pro}**

| oligonucleotide sequence (5'→3') | polarity | mutation introduced |
|---------------------------------|----------|---------------------|
| CTTCCCTTAATGGATGACCTGTTGAGTTGTTTAAAC | forward | SARS-CoV M\textsuperscript{pro} Cys145Ala |
| GTTAAACCAACACTACAGCTGATCCATAAGGAAG | reverse | SARS-CoV M\textsuperscript{pro} Cys145Ala |
| GTGTTGACAGATGTTGTTCCGAAAAATGCGGATCCC | forward | SARS-CoV M\textsuperscript{pro} Arg4Glu |
| CGGGAAATGCGAATTTCTCGAAAACACTCTGCAACAC | reverse | SARS-CoV M\textsuperscript{pro} Arg4Glu |
| GTGAGCAGTATTTTGAAGATACGGTTTACCACTGATGTTG | forward | SARS-CoV M\textsuperscript{pro} Glu290Arg |
| CAACATCAATGGTGAAACCCTATCTTCTAAATAGGCTACC | reverse | SARS-CoV M\textsuperscript{pro} Glu290Arg |
| CCAATTAGTGTGGGAACAAATGCTGTTGTTG | forward | SARS-CoV M\textsuperscript{pro} Arg298Glu |
| CACACCAGACGATTCAACAACATCAAATGG | reverse | SARS-CoV M\textsuperscript{pro} Arg298Glu |

**Note:** The mutant codons in the oligonucleotide sequences are highlighted in boldface. SARS-CoV M\textsuperscript{pro} amino acids are numbered continuously from the N-terminal residue, Ser1, to the C-terminal residue, Gln306.
reaction mixture to result in precipitation of the cross-linked proteins. After centrifugation (12,000 rpm, 4°C), the precipitate was redissolved in loading buffer and heated at 70°C for 10 min. SDS-PAGE was run with 12.5% acrylamide gels.

**Size-exclusion Chromatography (SEC) analysis**

The dimer-monomer equilibria of mature WT and mutated SARS-CoV Mpros were measured using size-exclusion chromatography on a HiLoad 16/60 Superdex 75 prep grade column through an ÄKTA FPLC system. Buffer used was 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM DTT, and 1 mM EDTA. Protein samples with a concentration of 5 mg/mL were loaded onto the column and then eluted with the buffer by detection of absorbance at 280 nm. The column was calibrated with four molecular-mass marker proteins (labeled in the inset of Fig. 5): (A) cytochrome c (11.8 kDa), (B) chymotrypsinogen A (25.7 kDa), (C) ovalbumin (42.9 kDa), and (D) albumin (69.3 kDa). A linear relationship between the gel-phase distribution coefficient (Kav) and the average molecular weight (Mr) was fit by the equation Kav = 0.4109 log Mr + 2.1949.

**Trans-cleavage assay**

A FRET-based assay was used to assess the trans-cleavage activity of mature WT and mutated SARS-CoV Mpros (Verschueren et al., 2008). The assay made use of a 14-amino acid fluorogenic substrate, Dabcyl-KTSAVLQ↓SGFRKME-EDANS (95% purity, Biosyntan GmbH, Berlin, Germany), which contains an optimal Mpro cleavage site (indicated by the arrow). The enhanced emission fluorescence due to cleavage of this substrate as catalyzed by the protease was monitored as a function of time at 490 nm with excitation at 340 nm, due to cleavage of this substrate as catalyzed by the protease was performed as a control and the SEC, size-exclusion chromatography; TM, transmembrane; Trx, thioredoxin; WT, wild type

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