Only One Protomer Is Active in the Dimer of SARS 3C-like Proteinase

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The severe acute respiratory syndrome coronavirus 3C-like protease has been proposed to be a key target for structurally based drug design against SARS. The enzyme exists as a mixture of dimer and monomer, and only the dimer was considered to be active. In this report, we have investigated, using molecular dynamics simulation and mutational studies, the problems as to why only the dimer is active and whether both of the two protomers in the dimer are active. The molecular dynamics simulations show that the monomers are always inactive, that the two protomers in the dimer are asymmetric, and that only one protomer is active at a time. The enzyme activity of the hybrid severe acute respiratory syndrome coronavirus 3C-like protease of the wild-type protein and the inactive mutant proves that the dimerization is important for enzyme activity and only one active protomer in the dimer is enough for the catalysis. Our simulations also show that the right conformation for catalysis in one protomer can be induced upon dimer formation. These results suggest that the enzyme may follow the association, activation, catalysis, and dissociation mechanism for activity control.

In early 2003, a highly epidemic disease named severe acute respiratory syndrome (SARS) first broke out in China and then quickly spread to other circumjacent countries (1). Research proved that the nosogenis was a novel coronavirus. In the coronavirus life cycle, 3C-like proteinase (3CLpro) is important and indispensable and is a pivotal target in anti-SARS drug design (2).

SARS 3CLpro shares 40 and 44% sequence identity to 3CLpro of human coronaviruses 229E and transmissible gastroenteritis virus (3CLpro), the crystal structures of which have been resolved (2, 3). Several homology models for SARS 3CLpro have been reported (2, 4, 5). More recently, the crystal structures of the enzyme and the inhibitor-enzyme complex have been determined (6–11). All structures are very similar and consist of three domains. The first two domains form a chymotrypsin fold, and the third domain is an extra helix domain that plays an important role in dimerization and enzyme activity (12). All of the proteins are dimeric in the crystal structures, and there exists an equilibrium between the monomer and dimer in solution. In our previous work, we have observed that the activity increases with the increase of enzyme concentration, indicating the dimer is the active form of the proteinase (13). Other groups have studied the function of the N-finger in dimerization and enzyme activity. The N-terminal residues 1–5 delete transmissible gastroenteritis virus 3CLpro, and the N-terminal residues 1–7 delete SARS 3CLpro; both have been reported to have no enzyme activities (3, 14–16). Interestingly, Chen et al. (14) report that the N-finger deletion mutation does not affect the dimerization of SARS 3CLpro. Contrary to this, Hsu et al. (16) has found that the N-4 truncated protease is mainly monomeric and has little enzyme activity, but the N-3 truncated protease is almost the same as the wild-type enzyme. Furthermore, Chou et al. (17) report that the mutation of R44A, E290A, and R44A/E290A weakens the dimerization.

All current experimental evidence agrees that only the dimeric form of proteinase is active. But why the monomer is inactive remains unclear. The other intriguing problem is whether the two protomers in one dimer are both active at the same time, whether only one protomer is active, or whether the two protomers are alternately active at different times. We have used molecular dynamics simulations and hybrid protein experiments to address these problems. Simulations on the dimer and two monomers were done. Our analysis confirms that the monomers are always inactive and that the two protomers in the dimer are asymmetric with probably only one protomer active at one time. We also purposely made a hybrid protein dimer of the wild-type protein and the inactive C145A mutant and measured its enzyme activity. The hybrid protein was shown to be active. This experiment strongly supports that dimerization is essential for enzyme activity and only one active protomer in the dimer is enough for the catalysis.

EXPERIMENTAL PROCEDURES

Molecular Dynamics Simulation—The molecular dynamics simulations were performed using the GROMACS software package version 3.2.1 (18, 19) with the OPLS/AA force field (20). The initial conformations of the dimer (model 1) and monomers (models 2 and 3) all came from the x-ray crystal structure of SARS 3CLpro at pH 8.0 (Protein Data Bank (PDB) code 1uk2; resolution 2.2 Å) (6). Because the two protomers in the crystal structure were asymmetric (r.m.s.d. of heavy atoms was 1.38 Å), the two protomers were calculated separately (model 2 was from the protomer A of the crystal structure, also named as protomer A1 in the following analysis; model 3 was from the protomer B, also named as protomer B1; accordingly, the protomers A and B in model 1 were respectively named protomer A2 and B2). These three models were simulated in a TIP4P (21) water environment. The model systems were rectangular periodic boxes, and the minimum distance between the protein and box boundary was >0.90 nm. To neutralize the charges and simulate a real physiological environment, some water molecules were replaced by Na+ and Cl− ions. In the final models, model 1 had 20,373 water molecules, 63 Na+ ions, and 57 Cl− ions in a 7.9 × 9.58 × 9.52 nm3 box; model 2 had 10,003 water molecules, 31 Na+ ions, and 28 Cl− ions in a 5.55 × 9.58 × 6.57 nm3 box; and model...
had 9,858 water molecules, 31 Na⁺ ions and 28 Cl⁻ ions in a 5.53 × 6.73 × 9.52 nm³ box.

All simulations were under a constant pressure and temperature ensemble using the Nose-Hoover method (22) for temperature control and the Parrinello-Rahman method (23) for pressure control. Van der Waals interactions were computed using a twin range cutoff method, and electrostatic interactions were computed using the particle-mesh Ewald method (24). All bonds with hydrogen atoms were constrained by the LINCS algorithm (25). Before simulation, all three systems were minimized with the steepest descent algorithm. During simulations, the time step was set to 1.0 fs, and conformations were saved every 10 ps. Thereafter, four 10-ns simulations were run for each system. Furthermore, to investigate the conformation exchange between the two protomers, we built two pseudodimers. Pseudodimer A was built with two protomers A of the crystal structure, whereas pseudodimer B was built with two protomers B. Subsequently, the four simulations were run for 5 ns for pseudodimer A and 2 ns for pseudodimer B, respectively. All simulations were performed at 310 K in 1 atmosphere, and the total of the simulation time for all trajectories was 148 ns.

Calculation of Substrate Binding Energy—The model substrate was built into the trajectories, and the energies were calculated in the CHARMM19 force field (26) using the CHARMM software package (27). The sequence of the model peptide was TSaVLQ, which was the N-terminal P6-P1 sequence of a reported best peptide substrate (13). The initial conformation and the relative geometric position of the enzyme and the substrate were derived from the x-ray crystal structure (PDB code 1uk4; chain G). We then fixed the enzyme conformation and optimized the complex. During the conformation optimization, the distances of the following important atom-atom pairs had been harmonically constrained, namely Cys145 SG-Gln6 C, G166 OE1-Gln6 NE2, His163 NE2-Gln6 OE1, G166 O-Val4 N, and G166 N-Val4 N.

Site-directed Mutagenesis of SARS 3CLpro—The C145A mutant of SARS 3CLpro was prepared with the QuikChange site-directed mutagenesis kit (Stratagene) using pET 3CLP-21h (28) as a template. The mutation was verified by nucleotide sequencing.

The Expression and Purification of SARS 3CLpro—The wild-type and mutational SARS 3CLpro were expressed and purified as described in our previous report (29). The purity was verified by SDS-PAGE to be >95%. The enzyme concentration was calculated from absorbance at 280 nm with 1.04 mg/optical density.

Colorimetric Enzyme Assay for the Wild-type and the Hybrid Protein—The enzyme activity was measured by a colorimetric assay as reported previously (28). In short, 20 μl of pNA substrate stock solution (2 mM Thr-Ser-Ala-Val-Leu-Gln-pNA water solution) was added to 180 μl of 25 °C preheated reaction buffer (40 mM phosphate-buffered saline, 1 mM EDTA, 0.2 mg/ml bovine serum albumin, 3 mM dithiothreitol, pH 7.3), which contained SARS 3CLpro wild-type enzyme and the C145A mutant. The concentration of wild-type enzyme was held at either 0.6 or 0.8 μM, and the concentration of C145A was changed from 2.5 to 500 μM. Colorimetric measurements of enzymatic activity were performed in 96-well microtiter plates using a multiwell ultraviolet spectrometer (Spectra Max 190, Molecular Devices) at 390 nm. Each assay was repeated twice. We also determined the wild-type protease activity at nine concentrations from 0.1 to 3 μM alone or with a high concentration of C145A mutant protein.

RESULTS

Dynamics of the Dimer and Monomers—To find out the differences between the dimer and the monomer, molecular dynamics simulations were performed. For all trajectories, r.m.s.d. values of all heavy atoms were calculated. After 2 ns, all of the three systems were approximately in equilibrium (supplemental Fig. S1). In the following analysis, only the last 8 ns in each trajectory were checked for all of the 12 trajectories.

The Conformation of Catalytic Dyad—We have shown that SARS 3CLpro underwent a general serine protease catalysis mechanism, and His₄¹ and Cys₁₄₅ comprised the catalytic dyad (28). The number of occurrences of the right conformation for the dyad was counted in all trajectories. Here the right conformation meant that the SG atoms in Cys₁₄₅ and the ND1/NE2 atom in His₄¹ forms a hydrogen bond as recognized by HBPLUS (30). The results were summarized in Table 1. The dyad have chances to be in the right catalytic conformation in protomer A, in protomer B, and in protomer BM, whereas no suitable conformation for catalysis was found in all four trajectories of protomer AM.

The Conformation of the Substrate Binding Pocket—Combining the analysis of transmissible gastroenteritis virus 3CLpro by Anand et al. (3) and of SARS 3CLpro (6) by Yang et al. (6), we summarize the following key structure elements for catalysis as follows: 1) the oxyanion hole, 2) the Y-X-H motif, 3) the hydrophobic packing of His₁₆₃ and Phe₁₄₀, and 4) conformations around Glu₁₆₆. All of these features were checked in the simulations. For the crystal structure, protomer A passed all four tests, and protomer B failed in tests 1 and 4 (6).

Oxyanion Hole—The distances between the main chain oxygen atom of the Gln reside at the P1 position of the substrate and the main chain amides of Gly₁₄₅, Ser₁₄₆, and Cys₁₄₅ were calculated. The coordinates of the substrate came from the modeling result in the later section. When all three distances were <4.5 Å, we defined it as a good oxyanion hole. The results were listed in supplemental Table S1. Protomers A and B behaved quite differently. Both in the dimer and in stand-alone monomer, protomer B was always bad and protomer A was always good.

Tyr-Xaa-His Motif—The hydrogen bond of Tyr₁₆₁ OH and His₁₆₃ ND1 is listed in supplemental Table SII. The results indicated that the Y-X-H motif was much more stable in protomer A than in protomer B, both in the dimer and in the monomer.

Hydrophobic Packing between His₁₆₃ and Phe₁₄₀—The centroid distance and the cosine of the dihedral angle between the imidazole ring of His₁₆₃ and the phenyl ring of Phe₁₄₀ were monitored. Here the angle was the dihedral angle between the plane that was defined by atoms CG, ND1, and NE2 in the imidazole ring of His₁₆₃ and the plane that was defined by atoms CG, CE1, and CE2 in the phenyl ring of Phe₁₄₀. The results were shown in supplemental Fig. S2, A and B. The hydrophobic packing between His₁₆₃ and Phe₁₄₀ in protomer A was much better than that in protomer B.

Conformation around Glu₁₆₆—The hydrogen bond of Glu₁₆₆ OE1/OE2 was also monitored and listed in supplemental Table SII. In the monomers, it can form a hydrogen bond with His₁₆₃ (in protomer A) or Asn₁₄₂ (in protomer B) from the same protomer. Both interactions block the binding of substrate.

Furthermore, we also monitored the hydrogen bond of the atoms around the active sites and the binding pocket along the trajectories. These atoms include Cys₁₄₅ SG, His₁₄¹ NE2/ND1, Gly₁₆₆ O, Tyr₁₆₁ OH, Asp₁₈₁ OD1/OD2, and Arg₉ O/N. The results were also listed in supplemental Table SII. We noticed that the hydrogen bonds of Gly₁₆₃

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Number of hydrogen bonds between Cys-145 SG and His-41 ND1/NE2</th>
</tr>
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<tbody>
<tr>
<td>Protomer A</td>
<td>Protomer B</td>
</tr>
<tr>
<td>1⁺</td>
<td>2⁺</td>
</tr>
<tr>
<td>428</td>
<td>15</td>
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<tr>
<td>44</td>
<td>176</td>
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</tr>
<tr>
<td>334</td>
<td>33</td>
</tr>
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</table>

* The numbers 1, 2, 3, and 4 represent the four trajectories for each system.
Dimerization and Enzyme Activity of SARS 3C-like Proteinase

TABLE 2

<table>
<thead>
<tr>
<th>Substrate binding to the dimer and the monomer</th>
<th>Vdw</th>
<th>Elec</th>
<th>Inner</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>The full substrate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DIMER</strong></td>
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<td></td>
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<tr>
<td>Protomer A&lt;sup&gt;α&lt;/sup&gt;</td>
<td>15.57</td>
<td>890.00</td>
<td>326.44</td>
</tr>
<tr>
<td>Protomer B&lt;sup&gt;α&lt;/sup&gt;</td>
<td>4.34</td>
<td>860.44</td>
<td>162.96</td>
</tr>
<tr>
<td><strong>MONO</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Protomer A&lt;sup&gt;α&lt;/sup&gt;</td>
<td>6.60</td>
<td>850.55</td>
<td>134.96</td>
</tr>
<tr>
<td>Protomer B&lt;sup&gt;α&lt;/sup&gt;</td>
<td>4.45</td>
<td>828.80</td>
<td>128.93</td>
</tr>
<tr>
<td><strong>P1 position of substrate</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>DIMER</strong></td>
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<td></td>
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<td>Protomer A&lt;sup&gt;α&lt;/sup&gt;</td>
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<tr>
<td><strong>MONO</strong></td>
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<tr>
<td>Protomer A&lt;sup&gt;α&lt;/sup&gt;</td>
<td>1.82</td>
<td>219.83</td>
<td>40.20</td>
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<tr>
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<td>4.17</td>
<td>208.58</td>
<td>41.82</td>
</tr>
</tbody>
</table>

<sup>a</sup> The van der Waals energy between the substrate and the enzyme.
<sup>b</sup> The electrostatic energy between the substrate and the enzyme.
<sup>c</sup> The internal energy of the substrate.

O–Asn<sup>28</sup> ND2 and Ser<sup>139</sup> N–Tyr<sup>126</sup> OH were very stable in protomer A but disappeared or were less stable in protomer B. We also noticed that, at the dimeric interface, Ser<sup>139</sup> OG of protomer A can form a stable hydrogen bond with the main chain of Arg<sup>7</sup> in protomer B, whereas Ser<sup>139</sup> OG of protomer B cannot.

**Binding of Substrate to the Dimer and Monomers**—We built the model substrate into the trajectories and calculated the binding energies. The results were summarized in Table 2. The median of the van der Waals interaction energy in the P1 pocket for the two protomers in the dimer and the two monomers were −5.04, −0.42, −1.82, and 4.17 kcal/mol, respectively. It is clear that protomer A<sup>α</sup> has much stronger binding ability than all of the others.

**Conformation Transition between Protomers A and B**—To see whether protomers A and B in the dimer can exchange conformation, two additional simulations were done. Thereafter, the r.m.s.d. values of the binding pocket between the trajectories and the protomer A of the crystal structure were calculated and summarized in Table 3. Here the r.m.s.d. values of two protomers were 1.41 and 2.06 Å (Fig. 1A). In pseudodimer B, we noticed that only one protomer could stay in the original conformation. For example, in trajectory 1, the average r.m.s.d. values of two protomers were 1.41 and 2.06 Å (Fig. 1A). In pseudodimer B, we observed a clear transition; in trajectory 3, the average r.m.s.d. value of one protomer was lowered to 1.47 from 1.95 Å (Fig. 1B). This means that proper conformation for catalysis can be induced by dimer formation.

**Enzymatic Activity of the Wild-type and the Hybrid SARS 3CL<sup>pro</sup>**—In the previous study, we have reported that the active site mutation C145A gave an inactive enzyme (28). The mutant did not show any visible enzyme activity at concentrations as high as 100 μM, respectively. It is clear that protomer AD has much stronger binding ability than protomer BD and the two stand-alone monomers.

**DISCUSSION**

**Difference between the Dimer and Monomers in Simulations**—To reveal the differences between the dimer and the monomers, the total of the molecular dynamics simulations for the dimer and monomers run under physiological conditions was 148 ns. Thereafter, key structure features necessary for catalysis were checked. Similar to the previous analysis based on the crystal structure (6), protomer BD was also found to be unsuited for catalysis in our simulations. Moreover, we found that some structure features required by catalysis were destroyed in protomer AM, such as 1) no suitable conformation for catalytic dyad and 2) Glu<sup>166</sup> forming a hydrogen bond with His<sup>162</sup> and blocking the substrate binding. By the conformation analysis of the trajectories, two binding-related hydrogen bonds, which behaved very differently between protomers A and B, were found. Furthermore, the binding energy of the model substrate with the enzyme also shows that protomer A<sup>α</sup> has much stronger binding ability than protomer B<sup>α</sup> and the two stand-alone monomers.

**Dimerization and Enzyme Activity**—After the above analysis, we now have an overview about the dimerization and enzyme activity. In the dimer, only protomer A is active and protomer B can help protomer A maintain the correct catalytic conformation. However, exactly how protomer B makes protomer A stable in the correct conformation for catalysis is still unclear. Anand et al. (3) ascribed it to the hydrogen bond of Glu<sup>166</sup> OE1/ OE2 and Ser<sup>1</sup> N in the other protomer, and recently Tan et al. (10) ascribed it to the hydrogen bond between the N terminus of the other protomer and the main chain of Phe<sup>80</sup>. However, the mutation experiment showed that the N-3 truncated protease was also active and remained dimeric (16). In our simulations, we noticed that, in the dimeric interface, hydrogen bond S139A OG–A4B O/N was stable, whereas S139B OG–A4A O/N did not exist. The mutation experiment also showed that the N-4 truncated protease was inactive (16). Therefore, this interaction was probably also very important. Furthermore, the stabilization may only depend on the dynamic behavior of the whole structure.

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Moreover, we measured the enzyme activity of the hybrid SARS 3CL\textsuperscript{pro}. The experiments showed that the enzyme activity increased dramatically by adding the inactive mutant C145A. This proved two things. 1) C145A could form the hybrid dimer with the wild-type enzyme; otherwise the enzyme activity could not have been changed. 2) The hybrid dimer also had enzyme activity; otherwise along with the formation of the hybrid dimer, the enzyme activity would be decreased. Combining with our analysis from molecular dynamics simulations, we proposed that, in the hybrid dimer, the C145A monomer can perform the function of protomer BD and help the wild-type enzyme protomer stay in the correct conformation. In conclusion, only one active protomer is required for the activity of the SARS 3CL\textsuperscript{pro} dimer. Furthermore, by the quantitative analysis under the enzyme assay condition, the apparent $K_d$ was $\approx 0.81$ $\mu M$, which is much less than the $K_d$ reported before for the pure enzyme ($\approx 14$ $\mu M$) (29). It means that the substrate can stabilize the dimer, or in other words, the dimer has much stronger ability to bind the substrate than the monomer. The $k_{cat}$ of the wild-type dimer and the hybrid dimer could also been deduced. It is interesting that the hybrid dimer has about one-half of the wild-type dimer activity. However, this phenomenon does not conflict with our previous conclusion. We concluded that only one protomer in the dimer was active at one time. For the pure wild-type enzyme dimer, there is always one protomer that stays in the active conformation. While in the hybrid dimer, the wild-type protomer may stay either in the active form or in the inactive form. Thus the hybrid dimer has only about one-half of the wild-type protein activity.

Conformation Transition between Protomers A and B—In the previous analysis, we found the two protomers in one dimer were asymmetric and only one protomer was active. Because the stand-alone monomer cannot maintain the active conformation, this asymmetry was because of dimerization. There are three possible mechanisms. 1) The two protomers in the dimer can exchange conformation. 2) During the association process of the two protomers, one becomes active and the other remains inactive. 3) A mixed mechanism of both items 1) and 2). In the simulations at normal body temperature, the conformational transition cannot be observed in our time scale. When we placed the two pro-
Dimerization and Enzyme Activity of SARS 3C-like Proteinase

This supports that dimerization is important for the enzyme activity, and in the dimer, only one active protomer is enough for the catalysis. Furthermore, we studied the mechanism for activity control in the dimer. Our simulations show that the most probable mechanism is the right conformation, as catalysis can be induced upon dimer formation. As the dimer is relatively weak, the enzyme may follow the association, activation, catalysis, dissociation mechanism for activity control, and no conformational exchange is necessary within one dimer. Much more needs to be studied about why dimerization can induce and maintain the right conformation for catalysis. Our simulations and experiments confirm that dimerization was the essential requirement for enzyme activity and only one monomer is active in the dimer.

REFERENCES


CONCLUSION

We have used molecular dynamics simulations and enzyme activity measurement of the hybrid protein to study why only the dimer of SARS 3CLpro is active. Analysis of the simulation trajectories showed that stand-alone monomers cannot play the enzymatic role correctly, and only one protomer in the dimer is in the right state for catalysis. The experiments on the hybrid SARS 3CLpro between the wild-type enzyme and the inactive mutant C145A revealed an increased enzyme activity.
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