Insight into the Activity of SARS Main Protease: Molecular Dynamics Study of Dimeric and Monomeric Form of Enzyme

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ABSTRACT The phenomenon that SARS coronavirus main protease (SARS M^{pro}) dimer is the main functional form has been confirmed by experiment. However, because of the absence of structural information of the monomer, the reasons for this remain unknown. To investigate it, two molecular dynamics (MD) simulations in water for dimer and monomer models have been carried out, using the crystal structure of protomer A of the dimer as the starting structure for the monomer. During the MD simulation of dimer, three interest phenomena of protomer A have been observed: (i) the distance between NE2 of His41 and SG of Cys145 averages 3.72 Å, which agrees well with the experimental observations made by X-ray crystallography; (ii) His163 and Glu166 form the “tooth” conformational properties, resulting in the specificity for glutamine at substrate P1 site; and (iii) the substrate-binding pocket formed by loop 140–146 and loop 184–197 is large enough to accommodate the substrate analog. However, during the MD simulation of the monomer complex, the three structural characteristics are all absent, which results directly in the inactivation of the monomer. Throughout the MD simulation of the dimer, the N-terminus of protomer B forms stable hydrogen bonds with Phe140 and Glu166, through which His163, Glu166, and loop 140–146 are kept active form. Furthermore, a water-bridge has been found between the N-terminus of protomer B and Gly170, which stabilizes His172 and avoids it moving toward Tyr161 to disrupt the H-bond between Tyr161 and His163, stabilizing the conformation of His163. The interactions between the N-terminus and another monomer maintain the activity of dimer. *Proteins* 2007;66:467–479. © 2006 Wiley-Liss, Inc.

Key words: SARS coronavirus; main protease; molecular dynamics; dimer; monomer; intermolecular interaction

INTRODUCTION

Severe Acute Respiratory Syndrome (SARS) has resulted in huge damage, since it burst out in China. SARS coronavirus (SARS-CoV), a previously unrecognized coronavirus, was discovered by the etiological agent of the disease,^{1–4} which encodes all the sequences of functional proteins required for viral replication and transcription. It is also known that the functional polypeptides are released from polyprotein by extensive proteolytic processing, primarily by the SARS coronavirus main protease (SARS M^{pro}). This protease, with a chymotrypsin fold, has similarity with the coronavirus main protease in sharing the cleavage specificity for glutamine at substrate P1 site.^{5,6} For the functional importance of SARS M^{pro} in the viral life cycle, it becomes an attractive target in drug development directly against SARS disease.^{7–12} However, presently, neither a vaccine nor an efficacious therapy is available. So, it is still urgent to design the potential therapeutic agents against SARS.

Through the specific intermolecular interactions between monomers,¹³ SARS M^{pro} assembles together to form an asymmetrical dimer (shown in Fig. 1). Both HCoV (Human Coronavirus) and TGEV (Transmissible Gastroenteritis Virus) M^{pro} exist as a mixture of monomer and dimer in diluted solutions.¹⁴ Dissection of the enzyme results in the enzyme inactive and the dimer is the main function form of the enzyme.^{15,16} However, the reason for this remains unknown. The structural infor-

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Fig. 1. Schematic ribbon diagram of SARS M^{pro} (a) dimer and (b) monomer complexed with a substrate-analog hexapeptidyl CMK inhibitor and the structures of the active site and the substrate-analog binding site of the enzyme. α -Helices are shown as spirals and β -strands as sheets and inhibitor molecules as stick models. The catalytic site (His41 and Cys145) is shown as a ball-and-stick model and colored as pink. The substrate-analog-binding site is defined by loop 140–146, loop 184–197 and β -strand 163–172 and colored as blue. Some residues key to the activity of SARS M^{pro} in the binding site are colored as the “Oxyanion hole” (143–145) and S3 pocket (187–189) in brown, the residues of His163 and Glu166 (shown as a ball-and-stick model), specific for glutamine at substrate P1 site, in yellow. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mation about the monomer and dimer, especially for the residues of the active pocket, could provide some insights into the functional differences. The crystal structure of SARS M^{pro} dimer has been solved,¹⁷ while, for the monomer form, only a theoretical model of its 3D structure, based on the structures of the HCoV and TGEV M^{pro} , has been described.¹⁸

The main difference between the monomer and the dimer is that there exist some specific intermolecular interactions between the two monomers within the dimer, while not for the isolated monomer. There exist a number of intermolecular interactions between the two monomers within the dimer, that is, the hydrogen bonds between active pocket and the N-terminus of another monomer, the salt bridge between domain III and N-terminus of another monomer and the specific electrostatic and hydrophobic interactions between the domains III of monomers in dimer. Since any mutation or deletion of these residues involving the interactions have resulted in an inactive enzyme,^{14,19} these interactions are vital to maintain the active conformation of SARS M^{pro} dimer and might be the ultimate reasons for the activity differences between dimer and monomer, which can be treated as new targets for the drug design against SARS-CoV. However, because of the absence of conformational information about monomer, the key functional roles of these intermolecular interactions in how to fix the dimer at active structure are still vague.

Molecular dynamics (MD) simulations are widely used to simulate the motion of molecules, which provides useful information of the chemical reactions, phase transitions, and other physical phenomena due to molecular interactions. So, MD simulation can be used as a powerful tool

for studying the structural variations and motions of SARS M^{pro} . Many MD studies on the enzyme have been reported^{20–22} that none of them target the differences of the structure and dynamics of the active site residues of monomer relative to dimer. Using the X-ray structure of the enzyme dimer form and the protomer A deleting protomer B within the dimer as the starting point of dimer and monomer model, we have performed two MD simulations of dimer and monomer. Because of the absence of the crystal structure of enzyme complexed with substrate for substrate hydrolysis by SARS M^{pro} , the substrate-analog hexapeptidyl CMK inhibitor, which cannot be hydrolyzed by enzyme, was present in the structures of both MD simulations to investigate the substrate binding.

In this research, by comparing the process of the MD simulation of monomer with dimer, our aim is to (i) find the structural variations and dynamics of the active site residues in SARS M^{pro} monomer in contrast to the dimer, which result in an inactive monomer and provide useful information for receptor based drug design; (ii) investigate the detailed specific interactions involving the two monomers within the dimer and its functional roles in maintaining the activity of the dimer, which provides insights for the design of specific protease inhibitors using the interface of the dimer as a new target.

METHODS

Preparation of Enzyme-Bound Complexes

The crystal structure of SARS M^{pro} (PDB code 1UK4), containing protomer A and protomer B complexed with the CMK inhibitor, and the crystal structure of protomer

Fig. 2. Root-mean-square deviations (rmsd) of the heavy atoms of the backbone of (a) protomer A within dimer and (b) monomer relative to the SARS M^{Pro} crystal structure during simulation.

A deleting protomer B were used as the starting structures of dimer and monomer model, respectively. Considering the residues around His163, the imidazole of His163 is protonated at the NE2 atom. The experiment of Huang et al.²³ have supported the view that the nucleophile in this proteinase is the uncharged thiol, with His41 acting as a general base. Therefore, Cys145 and His41 of the catalytic dyad were left uncharged in the two MD simulations in this work.

Molecular Dynamics Simulations

Two independent 10 ns MD simulations were performed by the program AMBER7.0²⁴ with the force field reported by Cornell et al.²⁵ The same MD simulation protocol was applied for both simulations. For these two MD simulations, we used the NPT ensemble (pressure = 1.025×10^5 Pa) with explicit solvent and periodic boundary conditions. All covalent bonds involving hydrogens were fixed with the SHAKE algorithm,²⁶ and the integration of the equations of the motion was done with the leap-frog algorithm.²⁷ The long-range electrostatic interactions were treated with particle-mesh Ewald (PME²⁸) with a short-range spherical cutoff of 9 Å.

The monomer/dimer complexes were immersed in an orthorhombic box of dimensions ($77 \times 98 \times 74$ Å) and ($84 \times 100 \times 99$ Å), respectively, and filled with TIP3 model water molecules. To neutralize the total charge of the solvated systems, two and five Na⁺ ions were randomly placed far away from the surface of the monomer/dimer complexes. For the simulation with the monomer complex, the entire system consisted of 4722 solute atoms (enzyme, inhibitor, and two Na⁺ ions) and 41,496 solvent atoms, whereas for the dimer complex system, the number of solute and solvent atoms were 9411 and 67,260, respectively. The two systems relaxed by performing 100 steps of steepest descent and 1000 steps of conjugate gradient energy minimization to reduce steric

conflicts between water molecules and the solute before the MD trajectory.

During the equilibration, the solute (enzyme, inhibitor, and Na⁺) was constrained and the solvent (water molecules) was equilibrated for 50 ps simulation at 298 K using an integral time step of 1 fs. Then, the constraints on the solute were removed and the entire system was heated gradually from 0 to 298 K, at increments of 50 K, each for 15 ps with a 1 fs time step. Finally, the whole solvated model was subjected to a free equilibrium simulation for 10 ns, using an integral time step of 2 fs with a temperature of 298 K kept constant by a Berendsen²⁹ temperature coupling constant of 0.1 ps.

The parameters of hexapeptidyl inhibitor CMK required for dynamic simulation were generated with the program AMBER7.0.

Structural Analysis

The coordinates of the system were saved at every 1 ps from the trajectories of the MD simulations of the dimer and monomer complexes of SARS M^{Pro} for subsequent analyses. For the calculation of the root-mean-square deviation (rmsd) values, the structures were least-squares fit using the C α atoms. In case of the dimer, only the rmsd value of protomer A is calculated. The averaged structures were obtained by least-square fitting of all the atoms of the 10,000 complexes saved at a 1 ps interval. The averaged structures were minimized by 500 steps of conjugate gradient energy minimization.

RESULTS AND DISCUSSION

The rmsd of the backbone heavy atoms during MD simulations of protomer A within the SARS M^{Pro} dimer model and monomer model relative to the crystal structure are shown in Figure 2. In the first 0.5 ns, the rmsd value increases quickly, which means that the structure of the enzyme dissolved in solution relaxes and is differ-

ent from the crystal structure. From the plot, the fluctuation of rmsd values is low after 1 ns, fluctuating around 1.8–2.2 Å in protomer A within dimer and higher values around 1.7–2.7 Å in monomer. It can be deduced that the magnitude of changes in the conformation of monomer is larger than that in protomer A within dimer, which means that the monomer shows greater motions than protomer A.

These two models exhibit different dynamics behavior: the dimer model displays conformational rigidity higher than that of the monomer model, which has been further confirmed by the root-mean-square fluctuations of the C α atoms for each residue during MD simulations. Figure 3 compares the calculated B-factors with the experimental ones from the X-ray structure. It is obvious from the plot in Figure 3 that the B-factors from the dimer MD simulation are little higher than the crystallographic B-factors except for segment 120:170, where the calculated fluctuations are close to the experimental ones. The 120:170 segment corresponds to the domain II of SARS M^{pro} (the interfacial region of SARS M^{pro} dimer), which is buried in the dimer interior thus not exposed to the solvent. This may explain a better agreement of the calculated mobility with the crystal. While for the monomer model, the B-factors of the whole protein, including the segment 120:170, show considerable larger fluctuations than the dimer model. This is expected because, for the loss of the counter monomer, the whole protein exposes to and interacts strongly with solvent.

As shown in Figure 1(b), the active site of SARS M^{pro} comprises loop 140–146, loop 184–197, and β -strand 163–172. There exist some residues important to the substrate binding and hydrolysis in these loops and β -strand, with the “oxanyan hole” and the catalytic site Cys145 in loop 140–146; His163 and Glu166 residues specific for glutamine at substrate P1 site in β -strand 163–173; and potential catalytic site Asp187 and other important S3 subsite of substrate binding in loop 184–197. Other residues in and around the substrate-binding pocket, such as Tyr161 and the N-terminus of the other monomer, in the position to form hydrogen bonds with His163 and Glu166, are also important for enzyme activity. These three segments are all located at the dimer interfacial region 120:170 and are affected by the intermolecular interactions with the counter monomer (see Fig. 3). Noticeably, only the conformational variations of protomer A during the MD simulation of SARS M^{pro} dimer complex are used to compare with that of monomer.

Dynamic Behavior of the Catalytic Site

The catalytic site of coronavirus M^{pro} is similar to those of the picornavirus 3C proteinase and assumed to have two properties, (i) unlike proteinases of the papain family in which an asparagine is the third member of the catalytic triad, a buried water molecule is found in the place that normally would be occupied by the side chain of the third member of the catalytic triad in a

Fig. 3. Fluctuation of SARS M^{pro} C α atoms computed from dynamics simulations (blue line corresponding to protomer A of the dimer complexes and red line corresponding to the monomer) and from crystallographic data (black line). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

number of crystal structures of SARS M^{pro} [Fig. 4(a,b)]. Thus, SARS M^{pro} is assumed to be a viral cysteine proteinase employing only a catalytic dyad¹⁴; and (ii) in the various crystal structures of SARS M^{pro}, the cysteine-histidine distance is between 3.6 and 3.9 Å, which might be important to the substrate hydrolysis by SARS M^{pro} by a general-base mechanism.²³

In the MD simulation of SARS M^{pro} dimer complex, the distance between NE2 of His41 and SG of Cys145 fluctuates in the range of 3.5–4.5 Å [shown in Fig. 5(a)], which agrees well with the experimental observations made by X-ray crystallography. The dihedral angle CA-CB-CG-CD2 of His41 averages 73.4° and the averaged distance between NE2 of His41 and SG of Cys145 is 3.72 Å, indicating the presence of a weak hydrogen bond [occupancy of 36.4%, shown in Fig. 4(c)]. In the crystal structure, a water molecule entrapped by Asp187 and His41 shields Asp187 from the catalytic dyad of SARS M^{pro} [Fig. 4(a,b)]. However, an interesting phenomenon found in the MD simulation is that the distance between ND1 of His41 and OE2 of Asp187 averages 2.79 Å, supporting the presence of a stable hydrogen bond with occupancy of 83.2% (Table I). Monitoring the orientation of the His41 flip by the dihedral angle CG-CB-CA-N, it is shown that this angle remains nearly constantly around 58.2° during the MD simulation of the dimer complex. In contrast to the angle of ~90° in crystal structure, the imidazole ring of His41 turns toward Asp187. This turn helps to form a stable hydrogen bond between His41 and Asp187 [Fig. 4(c)]. So, it can be seen from the MD simulation that, in solution, when the active pocket of SARS M^{pro} combines with substrate, to bind substrate tightly and hydrolyze it efficiently, His41 might regulate itself to turn toward Asp187 and squeeze the water molecule out, thus converting the catalytic site from the dyad (His41 and Cys145) to a more efficient catalytic triad (His41, Cys145, and Asp187). Although it still has not been confirmed by experiment, it can be hypothesized that the catalytic efficiency of the SARS M^{pro} is regulated by substrate binding, which is in accordance with the results found in the MD simulations of SARS M^{pro} modeled by homology complexed with its designed substrate²² and is helpful in inhibitor design for SARS M^{pro}.

There is a similar turn of His41 toward Asp187 in the MD simulation of monomer complexed with substrate analog. A stable hydrogen bond is formed between the

Fig. 4. The structures of the active site of SARS M^{Pro}. Comparison of the active site in the (a) crystal structure (PDB code 1Q2W) with neither inhibitor nor substrate complexed with, (b) crystal structure (PDB code 1UK4), (c) MD averaged structure of protomer A within dimer and (d) monomer complexed with substrate analog.

Fig. 5. Time-dependent variation of distance between NE2 of His41 and SG of Cys145 in (a) SARS M^{Pro} protomer A complex within dimer and (b) monomer complex.

TABLE I. Hydrogen Bonds Average Distances (Å) and Occupancy (%) of the Active Pocket of MD Structures of the Protomer A Complex within SARS M^{Pro} Dimer

Hydrogen bond	Distances (Å)	Occupancy (%)
HSGCys145...NE2His41	3.72 ± 0.19	36.4
HND1His41...OD2Asp187	2.79 ± 0.14	83.2
HNE2His163...OE1Gln-P1	2.74 ± 0.10	99.9
HOHTyr161...ND1His163	3.07 ± 0.27	95.9
HNGlu166...OSer-P4	2.94 ± 0.18	99.9
HE2NE2Gln-P1...OE2Glu166	3.01 ± 0.34	65.0
HNGly143...OGln-P1	3.40 ± 0.22	97.8
HNGly143...OXTGln-P1	2.85 ± 0.13	99.8
HNSer144...OGln-P1	2.80 ± 0.12	92.3
HNCys145...OGln-P1	2.92 ± 0.17	99.9
HGOGSer1B...OE2Glu166	2.99 ± 0.56	61.3
HNSer1B...OPhe140	2.76 ± 0.09	65.9
HNPhe140...NSer1B	2.90 ± 0.13	100.0
HNPhe3B...OWat829	3.23 ± 0.32	89.5
HOWat829...OGly170	2.62 ± 0.15	70.5
HNArg4B...OE1Glu290	2.71 ± 0.09	100.0

two entities (average distance of 2.95 Å, occupancy of 89.5%, Table II). However, the average dihedral angle CA-CB-CG-CD2 of His41 is 91.3°, flipping ~20° relative to the dimer [Fig. 4(d)]. In this turned orientation, the imidazole ring of His41 rotates away from the side chain of Cys145, and as a consequence, the imidazole moiety NE2 of His41 is on average 4.75 Å from the SG atom of Cys145 [Fig. 5(b)], resulting in a disruption of the weak hydrogen bond between the two entities (occupancy of 6.3%, Table II). This longer distance deviates the experimental observations made by X-ray crystallography so much that the capability of substrate hydrolysis by SARS M^{Pro} monomer might be decreased.

Conformational Characteristics of His163 and Glu166

It has been shown that, in the coronavirus M^{Pro}, the imidazole side chain of histidine interacts with the P1 carboxamide side chain of the substrate, which is generally accepted to determine the coronavirus M^{Pro} specificity for glutamine at P1.¹⁴ During the MD simulation of the SARS M^{Pro} dimer complex, the distance between the NE2 of His163 and the OE1 of glutamine at the substrate analog P1 site shows little fluctuation around time-averaged distance 2.76 Å (see Fig. 6). NE2 of His163 donates a proton to OE1 of glutamine at the substrate analog P1 site to form a strong hydrogen bond [Fig. 7(a)], with the occupancy of 99.9%. From plots of dihedral angle CA-CB-CG-CD2 of His163 versus times [Fig. 8(a)], it is obvious that the imidazole ring of His163 flip remains in the orientation required for the NE2(His163)...OE1(Gln-P1) hydrogen bond throughout the MD simulation of SARS M^{Pro} dimer complex.

In the average structure of protomer A within the dimer, the carboxylate group of Glu166 is also found to approach the carboxamide side chain of glutamine at

TABLE II. Hydrogen Bonds Average Distances (Å) and Occupancy (%) of the Active Pocket of MD Structures of SARS M^{Pro} Monomer Complex

Hydrogen bond	Distances (Å)	Occupancy (%)
HSGCys145...NE2His41	4.75 ± 0.20	6.3
HND1His41...OD2Asp187	2.95 ± 0.25	89.5
HNE2His163...OE1Gln-P1	7.90 ± 0.35	0.0
HOHTyr161...ND1His163	4.21 ± 0.20	6.3
HNGlu166...OSer-P2	3.30 ± 0.31	77.5
HGOGSer-P2...OE2Glu166	3.10 ± 0.39	61.7
HE2NE2Gln-P1...OE2Glu166	4.18 ± 0.35	21.2
HNGly143...OGln-P1	2.90 ± 0.14	99.5
HGOGSer144...OXTGln-P1	3.70 ± 0.35	70.7
HNSer144...OGln-P1	2.90 ± 0.12	99.7
HNCys145...OXTGln-P1	3.10 ± 0.22	89.8
HOHTyr161...OHis172	3.18 ± 0.30	60.4

Fig. 6. Separation between NE2(His163) and OE1(Gln-P1) for the simulation time from 0 to 10 ns of SARS M^{Pro} protomer A complex within dimer (black dash) and monomer complex (red dash). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

substrate analog P1site. The OE2 of Glu166 accepts a stable hydrogen bond from side-chain NE2 of glutamine at substrate analog P1site, with the average distance of 3.01 Å and occupancy of 65.0% (Fig. 9, Table I). The turn of the Glu166 has been monitored by the change in the dihedral angle CD-CG-CB-CA of Glu166. For the majority of the simulation time (see Fig. 10), the distribution of the angle concentrates mainly at the value around 180°, required for the OE2(Glu166)...NE2(Gln-P1) hydrogen bond. These two hydrogen bonds mentioned above determine the specificity for glutamine of the substrate at the P1 site.

The conformational properties for His163 and Glu166 specific recognition of glutamine at the substrate P1 site are partially characterized by a distance NE2(His163)...OE2(Glu166) of 6.5–8.7 Å and a dihedral angle NE2(His163)...NE2(His163)-CD2(His163)-CG(His-163) of (180 ± 30)° [Fig. 11(a)]. OE2 of Glu166 is nearly copla-

Fig. 7. Comparison of the principal interactions around the specific site for glutamine at the substrate P1 site of the MD averaged structure of (a) the SARS M^{Pro} protomer A complex within dimer and (b) monomer complex. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 8. Time-dependent variation of dihedral angle CA-CB-CG-CD2 of His163 in (a) the SARS M^{Pro} protomer A complex within dimer and (b) the monomer complex.

nar with the imidazole of His163. This structure is able to properly accommodate the carboxamide side chain of glutamine at substrate analog P1 site and forms a strong hydrogen bond with it. The dimer conformers are highly characterized that His163 and Glu166 form the “tooth” protruding into the “mouth” shaped void, the residues of S1 pocket of protomer A within the dimer. Throughout the entire MD simulation of dimer complex, the “mouth” is closed all the time, with the “tooth” fastening the glutamine residue. The properties of His163 in the MD simulation of the SARS M^{Pro} dimer complex are consistent with the fact that the interactions between histidine and glutamine

determine the coronavirus M^{Pro} specificity for glutamine at substrate P1 site.¹⁴

However, during the entire MD simulation of the SARS M^{Pro} monomer, the dihedral angle CA-CB-CG-CD2 of His163 is almost constantly around value 150°, compared with the dimer this amounts to a change of ~30° [Fig. 8(b)]. The imidazole ring of His163 rotates away from the carboxamide side chain of glutamine at P1 [Fig. 7(b)]. In this turned orientation, the distance between the imidazole moiety NE2 of His163 and OE1 of glutamine at P1 averages 7.90 Å (see Fig. 6), resulting in the disruption of the hydrogen bond. The NE2 of

Fig. 9. Separation between OE2(Glu166) and NE2(Gln-P1) for the simulation time from 0 to 10 ns of (a) SARS M^{pro} protomer A complex within dimer and (b) monomer complex.

Fig. 10. The distribution of the dihedral angle CD-CG-CB-CA of Glu166 during the simulation time from 0 to 10 ns of the SARS M^{pro} (a) protomer A complex within dimer (red column) and (b) monomer complex (blue column). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

imidazole cannot donate the proton to bind glutamine at substrate analog P1 site.

In the conformational change of Glu166, the dihedral angle CD-CG-CB-CA of Glu166 shows a bimodal distribution at around 180 and 265° in monomer [Fig. 10(b)], where the 265° modal is dominant. In the case of dimer, single modal around 175.6° is found. Associated conformational changes are that the OE2 of Glu166 averages 4.18 Å from the side-chain NE2 of glutamine at substrate analog P1 site (Fig. 9, Table I). The side-chain carbonyl of Glu166 cannot accept a hydrogen bond from side-chain NE2 of glutamine at substrate analog P1 site.

It is the flip of the imidazole ring of His163 and the turn of the carbonate group of Glu166 that destroy the “tooth” conformational properties of His163 and Glu166 required in the active form of SARS M^{pro}. As shown in Figure 11(b), the distance NE2(His163)...OE2(Glu166) becomes 8.0–10 Å

and the dihedral angle NE2(His163)...NE2(His163)-CD2(His163)-CG(His163) becomes disorder and unsystematic, which indicates that the destruction of the coplanarity between the side chain of Glu166 and the imidazole of His163. This induces the “mouth” open and “tooth” loosely packed, which fails to bind tightly with glutamine at substrate P1 site. This should be one possible reason to make the monomer inactive, and has been proven by the mutational experiment that any replacement of His163 completely abolished the proteolytic activities of the HCoV and feline infectious peritonitis virus (FIPV) main protease.^{30,31}

Conformational Variations of Substrate-Binding Pocket

In the dimer, the size of the substrate-binding pocket has been monitored by the distance between the Cα

Fig. 11. Conformations sampled by dihedral angle OE2(Glu166)...NE2-CD2-CG(His163) and distance OE2(Glu166)...NE2(His163) in (a) SARS M^{Pro} protomer A complex within dimer and (b) monomer complex during MD simulation. Structures within the red dashed lines have specific recognition of glutamine at substrate P1 site conformational criteria. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 12. Separation between CA(Asn142) and CA(Thr188) for the simulation time from 0 to 10 ns of SARS M^{Pro} protomer A complex within dimer (black dash) and monomer complex (red dash). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

atom of Asn142 in loop140–146 and the C α atom of Thr188 in loop184–197 (see Fig. 12), and the distance averages 17.75 Å, which means that the space of substrate-binding pocket is large enough to accommodate the substrate analog. As seen in Figure 13, the “oxyanion hole” at loop140–146, comprising Gly143, Ser144, and Cys145, which is generally accepted to stabilize the covalent tetrahedral intermediate resulting from substrate hydrolysis by enzyme,¹⁴ has proper space to accommodate the glutamine at substrate analog P1 site and form three stable hydrogen bonds with the OXT and O atom, HN(Gly143)...OXT(Gln-P1), HN(Ser144)...O(Gln-P1), and HN(Cys145)...O(Gln-P1) (occupancy of

Fig. 13. Superimposed average structures of the substrate-analog binding site of the SARS M^{Pro} protomer A complex (yellow) within dimer and monomer complex (blue). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

99.8, 92.3, and 97.8% respectively, Table I). The space is also large enough to accommodate the intermediate, oxyanion, and these three strong hydrogen bonds are important to stabilize the oxyanion. Comparing the partially solvent-accessible structure of leucine at substrate analog P2 site in crystal structure, the side chain of leucine at substrate analog P2 site is well accommodated in the hydrophobic pocket formed by Thr25, Leu27, Val42, and Ala44. The S3 pocket formed by the side chain of Asp187, Gln189, and the main chain of Arg188 at loop 184–197, which has been verified to be indispensable for proteolytic activity,¹⁴ can accommodate well the side chain of threonine at substrate analog P3 site.

In the monomer, the distance between the C α atom of Asn142 and the C α atom of Thr188 decreases largely relative to dimer (see Fig. 12), with a maximum value of more than 4 Å, showing the size of substrate-binding

pocket is reduced largely. As shown in Figure 13, the loop 140–146 and loop 184–197 all move toward the active site, which has the consequences for the residues of the “oxyanion hole” and the S3 pocket, particularly for Gly143, with the movement of 2.74 Å toward active site contrast to the counterpart in dimer. The collapse of the “oxyanion hole” and the S3 pocket leaves no space to accommodate the glutamine and threonine of the substrate analog, squeezing glutamine at the substrate analog P1 site away from the S1 pocket of enzyme and threonine at substrate analog P3 site into bulk solvent, respectively. Furthermore, the collapse of the “oxyanion hole” leaves no space for a tetrahedral intermediate.

As a consequence, the threonine at the substrate analog P2 site switches from the S2 hydrophobic pocket and is oriented toward bulk solvent, moreover, serine at the substrate analog P4 site and asparagine at the substrate analog P5 site become disorder. It can be seen that the reduced substrate-binding pocket in monomer cannot bind the substrate because of the collapses of “oxyanion hole” and S3 pocket. This is verified by the fact that the substrate cannot bind to the N-terminus deleted proteinase.³² According to our proceeding analysis, it can be deduced that the reduction of substrate-binding pocket of monomer is caused by the loss of N-terminus of counter partner.

We conclude that in the SARS M^{pro} monomer, due to the longer distance between Cys145 and His41, the destruction of the “tooth” conformational properties of His163 and Glu166 and the reduction of the substrate-binding pocket, the process of substrate specific recognition, combination and hydrolysis is destroyed, which results in the inactivation of monomer.

Analysis of Intermolecular Interactions between the Two Monomers within Dimer

To further investigate the reasons that cause the conformational variations between monomer and dimer, we superimpose the MD averaged structure of SARS M^{pro} monomer with dimer. Domains II and III of one monomer interact with the same domains and the N-terminus of the other monomer, the latter plays dominant role because N-terminus is close to substrate-binding pocket of the other monomer. These intermolecular interactions may be probably the key factors that cause the differences between monomer and dimer.

Stabilization of His163 by N-terminus

To interact with the glutamine at substrate P1 site, the imidazole ring of His163 has to maintain the conformational stability. This is achieved by two interactions: (i) stacking onto the phenyl ring of Phe140 and (ii) accepting a hydrogen bond from the Tyr161 hydroxyl group which has no other hydrogen-bonding partner.¹⁴

Two strong hydrogen bonds are observed between Ser1B of N-terminus of protomer B and Phe140 of protomer A during the entire MD simulation of SARS M^{pro} dimer complex [Fig. 7(a)]. The main chain N atom of

Ser1B donates a 2.76 Å hydrogen bond to the main chain O atom of Phe140, and the main chain O atom of Ser1B accepts a 2.90 Å hydrogen bond from the main chain N atom of Phe140 (Table I), which results in a stable conformation of the phenyl ring of Phe140. As shown in Figure 14, during the whole MD simulation of the SARS M^{pro} dimer complex, the dihedral angle CD1-CG-CB-CA of Phe140 remains at a value for π - π stacking interaction with imidazole ring of His163, ensuring that His163 can maintain a neutral state at physiological pH.

Analysis of the intermolecular interactions between N-terminus of protomer B and protomer A shows that a special water molecule is always sandwiched between the two protomer during the entire MD simulation of SARS M^{pro} dimer complex, which plays an important role in binding of the N-terminus of protomer B with protomer A. Water 829 forms a stable water bridge, linking the main chain N atom of Leu2B with the main chain O atom of Gly170 (Table I). Through the water-bridge, the conformation of the β -strand 163–172 is stabilized, avoiding the movement of His172 toward Tyr161 and the formation of hydrogen bond between these two entities. As a consequence, the hydrogen bond between the OH atom of Tyr161 and the ND1 atom of His163 is stable during the entire MD simulation of the SARS M^{pro} dimer complex (see Fig. 15), with an average distance of 3.07 Å and an occupancy of 95.9% (Table I).

Because of the strong OH (Tyr161)...ND1(His163) hydrogen bond and the stable π - π stacking interaction between the phenyl ring of Phe140 and the imidazole ring of His163, the conformation of the imidazole ring of His163 can be kept stable at the request for the binding glutamine of substrate at P1 site. Either of these two interactions is destroyed, the conformation of the imidazole ring of His163 will be caused unstable, which will decrease the enzyme activity or make it inactive. A good evidence for this is that the mutation of Tyr161 makes the enzyme almost inactive.³¹

In the monomer, because of the absence of the interactions with the N-terminus of protomer B, the conformation of Phe140 is instable. As shown in Figure 14, the phenyl ring of Phe140 has a $\sim 40^\circ$ increment in the average dihedral angle CD1-CG-CB-CA of Phe140. In this turned orientation [Fig. 7(b)], the phenyl ring of Phe140 rotates away from the imidazole ring of His163 and cannot stack onto the imidazole ring of His163. Furthermore, without the interaction of the N-terminus through the water-bridge, β -strand 163–172 becomes partly disordered and moves towards Tyr161. The main chain O atom of His172 competes with ND1 atom of His163 by forming stable hydrogen bond with the OH atom of Tyr161 (Table II), resulting in the disruption of the OH(Tyr161)...ND1(His163) hydrogen bond (Fig. 15, Table II). In the monomer, since the two interactions that maintain the stable conformation of the imidazole ring of His163 are destroyed, the conformation of the imidazole ring of His163 is not stable, making the turn mentioned above.

Fig. 14. Separation dihedral angle CD1-CG-CB-CA of Phe140 for the simulation time from 0 to 10 ns of (a) the SARS M^{Pro} protomer A complex within dimer and (b) the monomer complex.

Fig. 15. Separation between OH(Tyr161) and ND1(His163) for the simulation time from 0 to 10 ns of (a) SARS M^{Pro} protomer A complex within dimer and (b) monomer complex.

Stabilization of Glu166 by N-Terminus

The main chain O atom of SerB1 is also found to approach the hydrogen atom of Glu166 and maintain a stable hydrogen bond during the MD simulation of the SARS M^{Pro} dimer complex, with an occupancy of 61.3% [Fig. 7(a), Table I]. The hydrogen bond is indispensable for the stability of side chain carbonyl of Glu166 at request for accepting a hydrogen bond from side-chain NE2 of glutamine at substrate analog P1site. In the monomer, for the absence of this hydrogen, the conformation of side chain carbonyl of Glu166 is unstable, making the turn mentioned above.

Thus it can be concluded that through stabilizing the conformation of the imidazole ring of His163 and the side chain carbonyl of Gly166, the N-terminus of protomer B plays an important role in stabilizing the “tooth”

conformation between His163 and Glu166 required at discriminating specific glutamine at substrate P1site.

Effect on Conformation at Variations of Substrate-Binding Pocket by N-Terminus and Domain III

The substrate-binding pocket is reduced by the movements of loop 140–146 and loop 184–197 towards the active site. In the dimer, the hydrogen bond between the main chain of Ser1B and the main chain of Phe140 can stabilize not only the conformation of the phenyl ring of Phe140, but also that of loop 140–146, which makes the “oxyanion hole” in this loop keep proper conformation to accommodate glutamine at substrate analog P1 site and a tetrahedral intermediate. In the monomer, because of the absence of these two hydrogen bonds, the whole

140–146 loop moves a lot toward the active site, resulting in the collapse of the “oxyanion hole”.

The loss of the activity of the coronavirus main protease deleted domain III might be caused by the conformational variations of loop 184–197,¹⁴ which means that domain III is important to the stability of loop 184–197. In the dimer, there exist two type of intermolecular interactions between the two monomers to fix the domain III: (i) the salt-bridge between Arg4 at the N-terminus of protomer B and Glu290 at domain III in protomer A¹⁹, which in this MD simulation of the SARS M^{pro} dimer, possesses the occupancy of 100% and average distance of 2.71 Å (Table I) and (ii) the specific electrostatic and hydrophobic interactions between the domains III of the monomers.¹³ By these intermolecular interactions, the domain III is fixed in a proper conformation required for stabilization of loop 184–197, which makes the S3 pocket in this loop keep the proper conformation to accommodate threonine at the substrate analog P3 site. In the monomer, because of the absence of these intermolecular interactions, the whole 184–197 loop moves toward the active site, resulting in the collapse of the S3 pocket. A good evidence for this is that any mutant of Glu290 abolishes completely the activity of the SARS main protease.¹⁹

From the analysis, a reasonable conclusion can be got that the intermolecular interactions between the two monomers in the dimer, especially for the interactions around N-terminus and domain III of one monomer, are key to stabilizing the residues of the active pocket of the other monomer in the active form, therefore insuring the successful proceeding of the combination and hydrolysis of the substrate in the dimer. This is well verified by the experiments that a deletion mutant of $\Delta 1-5$ residues or domain III of the related TGEV M^{pro} are almost completely inactive.¹⁴ Thus, in SARS M^{pro} dimer, the enzyme is switched from the inactive form (monomer) to the active form (dimer) by the intermolecular interactions between the two monomers within the dimer. The interface of the SARS M^{pro} dimer can be treated as a new target for the design of specific protease inhibitors.

CONCLUSIONS

In the dimer, through the water-bridge, the N-terminus of one monomer stabilizes the conformation of the β -strand 163–172 of the other. This prevents His170 competing with His163 and forming H-bond with Tyr161 and maintains the stable hydrogen bond between Tyr161 and His163. Furthermore, the N-terminus of the counter monomer interacts directly with Phe140, making the conformation of the phenyl ring of Phe140 stable and stack onto the imidazole ring of His163. Just for these two interactions, the imidazole ring of His163 can maintain the conformation required for donating the proton to bind glutamine at the substrate analog P1 site. The N-terminus of the counter monomer also forms a stable hydrogen bond with Glu166, which stabilizes the proper conformation of the side chain carbonate

group of Glu166 required for accepting a hydrogen bond from glutamine at the substrate analog P1 site. His163 and Glu166 form the “tooth” conformation characteristics and result in the specificity for glutamine at the substrate P1 site. While in the monomer, because of the lack of the stabilization of the N-terminus of the counter monomer to His163 and Gly166, the “tooth” conformation is destroyed, resulting in a destruction of the specific recognition of the glutamine of the substrate at the P1 site and the inactivation of monomer. Furthermore, it is the lack of the stabilization of the N-terminus of the counter monomer to Phe140 that makes the loop 140–146 move a lot toward the active site, which causes the collapse of the “oxyanion hole” and leaves no space to accommodate glutamine at the substrate analog P1 site and a tetrahedral intermediate. Because of the loss of the salt-bridge between the N-terminus of counter monomer and domain III and the specific electrostatic and hydrophobic interactions between domain III of monomers within the dimer, the conformation of domain III cannot be fixed. As a consequence, the loop 184–197 becomes disorder and the S3 pocket collapses, leaving no space to accommodate the threonine at the substrate analog P3 site. The movements of loop 140–146 and loop 184–197 toward the active site induce the reduction of substrate-binding pocket. As a consequence, the SARS M^{pro} monomer cannot bind the substrate. In the dimer, the distance between NE2 of His41 and SG of Cys145 averages 3.72 Å, which agrees well with the experimental observations made by X-ray crystallography. While in the monomer, the longer distance between the two entities deviates a lot from the crystal structure and might be unfavorable to substrate hydrolysis by the monomer by a general-base mechanism.

In this study, by comparing MD simulation of dimer and monomer, the indirect reasons for the inactivation of the monomer have been found, that is the conformational variations of the active site in the monomer relative to dimer. Furthermore, the ultimate reasons that cause the conformational differences between dimer and monomer, the intermolecular interactions between the two monomers within the dimer and their important roles in how to stabilize the active pocket, have been investigated, which provides useful insight for the design of specific protease inhibitors using the interface of the SARS M^{pro} dimer as a new target.

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