The severe acute respiratory syndrome (SARS) coronavirus (CoV) main protease represents an attractive target for the development of novel anti-SARS agents. The tertiary structure of the protease consists of two distinct folds. One is the N-terminal chymotrypsin-like fold that consists of two structural domains and constitutes the catalytic machinery; the other is the C-terminal helical domain, which has an unclear function and is not found in other RNA virus main proteases. In order to understand the functional roles of the two structural parts of the SARS-CoV main protease, we generated the full-length of this enzyme as well as several terminally truncated forms, differ from each other only by the number of amino-acid residues at the C-terminal or N-terminal regions. The quaternary structure and \( K_d \) value of the protease were analyzed by analytical ultracentrifugation. The results show that the N-terminal 1-3 amino acids truncated protease maintains 76 % of enzyme activity and that the major form is a dimer as in the wild type. However, the amino acids 1-4 truncated protease shows the major form to be a monomer and has little enzyme activity. As a result, the fourth amino acid seems to have a powerful effect on the quaternary structure and activity of this protease. The last C-terminal helical truncated protease also exhibits a greater tendency to form monomer and shows little activity. We conclude that both the C-terminal and the N-terminal regions influence the dimerization and enzyme activity of the SARS-CoV main protease.

Severe acute respiratory syndrome (SARS) is an acute respiratory illness, which causes an atypical and highly contagious pneumonia. It has spread in many countries and areas. The international outbreak of this disease resulted in great damages to public health (1-3). A novel form of coronavirus (CoV), SARS-CoV, has been identified to be the major cause of SARS (3-8).

Coronaviruses are positive-sense, single-strand RNA viruses. The genome of SARS-CoV is around 30,000 nucleotides in length and the organization is similar to that of other coronaviruses. The replicase gene encodes two overlapping polyproteins, polyprotein 1a (around 450 kDa) and polyprotein 1ab (around 750 kDa). The polyproteins are cleaved by the internally encoded main protease (\( M^{pro} \), 3CL), which is required for the production of new infectious viruses. The main protease represents an attractive target for the development of novel anti-viral agents, because of the functional importance of this enzyme in the viral life cycle (9-12).

The crystal structures of reported CoV including the SARS-CoV main proteases are homodimers (13-15). Each protomer of the main protease is composed of three structural domains (Fig. 1). The first two domains of SARS-CoV main protease have an antiparallel \( \beta \)-barrel structure, which is similar to the other CoV proteases and form a chymotrypsin-like fold responsible for catalytic reactions (15). The active site containing a catalytic dyad defined by His41 and Cys145 is located...
between domains I and II. The third domain contains five \( \alpha \)-helices with an unclear biological function. The domain III of one protomer and the domain II of another form a contacting region in the dimer. The N-terminus (N-finger containing amino acid residues 1-7) is seated at this region and plays an important role in dimerization (16).

We have demonstrated that the major quaternary structure of SARS-CoV main protease at neutral pH is a dimer, which is the catalytically competent form (17). It is ultimately important to understand the factors that control dimerization, since the dissociated monomer might be enzymatically inactive. The C-terminal helical domain might interact with the active site of another protomer in the dimer and switch the enzyme molecule from the inactive form to the active form (18). The structural and biochemical data also show that the N-terminal residues 1-7 might play an important role in the dimerization and formation of the active site of SARS main protease (15). N-terminal truncation of the whole N-finger \( \Delta(1-7) \) results in almost complete loss of enzymatic activity (19).

In this report, we study critically the functional role of N-terminal and C-terminal by serial truncations. We report the stability and structure-function relationship of the full-length SARS-CoV main protease in comparison with the various truncated forms. Our results demonstrate that both N-terminal and C-terminal regions are involved in the enzyme activity as well as in dimerization. We have narrowed down the critical amino acid residues to the fourth amino acid residue of the N-terminal and the last helical amino acids of the C-terminal region as those involved in dimerization to give a correct conformation of the active site.

**EXPERIMENTAL PROCEDURES**

Construction of WT, N-terminal, and C-terminal Truncated SARS-CoV Main Proteases—The plasmids containing the full-length main protease were kindly provided by Dr. Shao-Hung Wang (Genome Research Center, National Yang-Ming University, Taipei, Taiwan). The genes of the full-length SARS-CoV main protease were amplified by polymerase chain reaction (PCR) with appropriate primers. The forward primer for the full-length WT SARS-CoV main protease was 5'-GGTGATCATATGAGTGGTTTAGG, and the reverse primer was 5'-AACTCGAGGTAAACACCAGAG. After digestion with BglII and XhoI, the PCR product was cut into two fragments, 168-bp and 747-bp. The 168-bp fragment was then digested with Ndel. Finally, the 168-bp Ndel-BglII and 747-bp BglII-XhoI fragments were co-ligated to the Ndel and XhoI sites of the vector pET-29a(+) (Novagen, Madison, WI).

The N-terminal truncated proteases were made by PCR. The forward primers were as follows,

\[ \Delta(1-3) \), 5'-GGAGATATACATATGAGGAA AATGGCATT; \]
\[ \Delta(1-4) \), 5'-GGAGATATACATATGAAAAAT GCATTCCCCG; \]
\[ \Delta(1-5) \), 5'-GGAGATATACATATGATGGCATTCCGTCAGG; \]
\[ \Delta(1-6) \), 5'-GGAGATATACATATGGCATT CCCGTGACCG; \]
\[ \Delta(1-7) \), 5'-GGAGATATACATATGTTCGATGCATATGAGGCAA; \]

The reverse mutagenic primers were as below,

\[ \Delta(1-3) \), 5'-GAATGCCATTTTCCTCATATG TATATCTCC; \]
\[ \Delta(1-4) \), 5'-CGGGGAATGCCATTTTCCTCATATG TATATCTCC; \]
\[ \Delta(1-5) \), 5'-TGACCGGGATGCATATGATGGCATTCCC; \]
\[ \Delta(1-6) \), 5'-GCCGCCAGGGAATGGCATTCCC; \]
\[ \Delta(1-7) \), 5'-TTTTGCCTGACGGGAACCATATGATGGCATTCCC; \]

The pET-SARS-CoV main protease vector was used as the template. The DNA polymerase Pfu (Promega, Madison, WI) extended and incorporated the mutagenic primers in the process of PCR. After 16-18 temperature cycles, the N-terminal truncated plasmid containing staggered nicks was generated. The PCR products were then treated with DpnI (New England Biolabs, Beverly, MA) to digest the template. Finally, the vector containing the protease cDNA with the desired mutation was transformed into *E. coli*. The C-terminal truncated proteases were
subsequently amplified by PCR, using the following sequence: The forward primer: 5’-TGAAGATCTGCTCATTCGCAA; The reverse primer of Δ(293-306), AACTCGACTGTAAACTCATCTTC; The reverse primer of Δ(201-306), AACTCGACTATGGTTGTGTCG. After digestion with BglII and XhoI, the PCR products were inserted into the BglII and XhoI sites of the pET-SARS-CoV main protease. The DNA sequences of the full-length, N-terminal, and C-terminal truncated SARS-CoV main proteases were checked by autosequencing. The recombinant SARS-CoV main protease has a His-tag at the C-terminus. This His-tag was not removed since earlier reports seemed to rule out the possible effect of His-tag on the dimeric structure or enzyme activity (17).

Expression and Purification of WT, N-terminal, and C-terminal Truncated SARS-CoV Main Proteases—The modified plasmids of the recombinant proteases were transformed into the E. coli strain BL21 (DE3) competent cells. The cells were grown at 37 °C in Luria-Bertani medium with 50 μg/ml Kanamycin until the absorbance at 600 nm reached 0.8 and were then induced by 1 mM IPTG at 18 °C overnight. The cells were centrifuged at 5,000 x g, 4 °C for 10-min. The supernatant was removed and the pelleted cells were then suspended in binding buffer (20 mM Tris-HCl, 300 mM NaCl, and 2 mM BME, pH 7.6). The cells were sonicated for 10-min at 10-s burst cycle at 300 W with a 10-s cooling period between each burst. The cell debris was removed by centrifugation (10,000 x g at 4 °C for 25-min). One milliliter binding-buffer-equilibrated Ni-NTA slurry (Qiagen, Hilden, Germany) was then added to the soluble lysate and the solution was mixed gently at 4 °C for 50-min to equilibrium. The lysate-Ni-NTA mixture was then loaded into a column and washed with the washing buffer (20 mM imidazole, 20 mM Tris-HCl, 300 mM NaCl, and 2 mM BME, pH 7.6). Finally, the protease was eluted with elution buffer (400 mM imidazole, 20 mM Tris-HCl, 300 mM NaCl, and 2 mM BME, pH 7.6). The purified protein was then concentrated at 4 °C using Amicon Ultra-4 centrifugal filter units (Millipore, Bedford, MA) with molecular mass cutoff at 10 kDa. The purified protein was concentrated to 5-15 mg/ml and then diluted to 0.5-3 mg/ml by 10 mM PBS (containing 10 mM sodium phosphate buffer, 150 mM NaCl, 2 mM BME, pH 7.6), which was used to replace the elution buffer over six concentration-dilution cycles.

Sample from the purification step was separated on a 4-12% gradient sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) to check the homogeneity.

Circular Dichroism and Fluorescence Analyses—CD experiments were performed in a Jasco J-810 spectropolarimeter (Tokyo, Japan) equipped with a Neslab RTE-111 water-circulated thermal controller (Newington, NH). The samples were prepared in 10 mM PBS solution at pH 7.6 with a protein concentration of 0.5 mg/ml. Far-UV CD spectra from 250 to 190 nm were collected using a 0.01 cm path-length cuvette with a 0.1 nm spectral resolution at 25 °C. Ten independent scans were averaged for each sample. All spectra were corrected for buffer contributions and converted to mean residue ellipticity ([θ]). The [θ] at each wavelength was calculated from Equation 1,

\[
[\Theta] = \frac{MRW \cdot \theta_0}{10 \cdot l \cdot c}
\]

where MRW is the mean residue weight (a value of 111.3 was used for the WT), \( \theta_0 \) is the measured ellipticity in degree at wavelength \( \lambda \), \( l \) is the cuvette path length (0.01 cm), and \( c \) is the protein concentration in g/ml. The secondary structure analysis was performed by DICHROWEB (20,21), which provides an interactive web site server allowing the deconvolution of data from circular dichroism spectroscopy experiments (http://public-1.cryst.bbk.ac.uk/cdweb/html/).

DICHRWEB offers several important softwares, such as CDSSTR (22-24), CONTINLL (25,26), SELCON3 (27,28) and K2D (29).

Thermal stability of the WT and various truncated mutants were analyzed with spectropolarimeter by monitoring the 222 nm
circular dichroism at the temperature range between 30 to 90 °C and the temperature at which half of the protein molecules were unfolded was recorded (Tm).

Fluorescence experiments were performed in a Perkin-Elmer LS 50B luminescence spectrometer (Beaconsfield, Buckinghamshire, England). The sample was prepared in 10 mM PBS solution at pH 7.6 with a protein concentration of 8 μg/ml. The fluorescence emission spectra from 300 to 400 nm were collected after excitation at 280 nm. Fluorescence spectra of proteins were determined with a 1 cm-path quartz cuvette at 25 °C. The spectral bandwidth was 5 nm for excitation and 10 nm for emission. All spectra were corrected for the buffer contribution. The average emission wavelength (<λ>) was calculated from Equation 2 (30),

\[
<\lambda> = \frac{\sum_{i=1}^{N} (F_i \cdot \lambda_i)}{\sum_{i=1}^{N} F_i}
\]  

(2)

where \(F_i\) is the fluorescence intensity and \(\lambda_i\) is the wavelength.

Analytical Ultracentrifugation Analysis—The molar mass and sedimentation coefficient of the proteases were analyzed by sedimentation velocity experiment. It was performed on a Beckman Optima XL-A analytical ultracentrifuge (Fullerton, CA). Prior to the experiments, the sample was diluted to various protein concentrations with 10 mM PBS buffer at pH 7.6. Sample (400 μl) and buffer (440 μl) solutions were loaded into the double-sector centerpiece separately. All experiments were carried out at 20 °C with an An50 rotor at speed of 42,000 rpm. The proteins were measured by UV absorbance at 280 nm in a continuous mode with time interval of 480-s. The recorded scans at different time points were collected and fitted to a continuous size distribution model by the SEDFIT program (31-34). The observed sedimentation profiles of a continuous size distribution \(c(s)\) can be calculated from Equation 3,

\[
a(r,t) = \int c(s)L(s,D,r,t)ds + \varepsilon
\]

(3)

where \(a(r,t)\) denotes the experimental observed signal, \(L(s,D,r,t)\) denotes the solution of the Lamm equation for a single species (35) and \(\varepsilon\) is the noise component.

For a precise determination of the monomer-dimer equilibrium of the SARS-CoV main protease, the sedimentation velocity experiment was performed at three different protein concentrations and all sedimentation data were subjected to the monomer-dimer equilibrium model fitting. The partial specific volume of the protease, solvent density, and viscosity were calculated by the software SEDNTERP (36). The dissociation constant \((K_d)\) was calculated by the global modeling of SEDPHAT program (33).

Enzymatic Activity Assay of the SARS-CoV Main Protease Using a Fluorogenic Substrate—The kinetic measurements of the SARS-CoV main protease activity were performed in 10 mM PBS with 2 mM BME at 30 °C. The reaction was initiated by adding 12 μg WT and 1.5-2.0 mg mutants in 1 ml reaction mixture. Enhanced fluorescence due to cleavage of the internally quenched fluorogenic substrate peptides (ABZ-TSAVLQSGFRK-DNP) by protease was monitored at 420 nm with excitation at 362 nm using a Perkin-Elmer LS-50B luminescence spectrometer.

The mixture containing N-terminal peptides (ABZ-TSAVLQ) and C-terminal peptides (SQFRK-DNP) of different concentrations were prepared to monitor the specific fluorogenic intensity. All intensities were corrected for the buffer contributions. The serial intensities at 420 nm were used to construct a standard curve for quantifying the product. In this way, the enzyme activity can be precisely determined.
purified by a single affinity column. All the recombinant SARS-CoV main proteases were found in the soluble fraction of the cell lysate. The expressed SARS-CoV main proteases bound to the nickel column, but other proteins flowed through the column to be a refuse. SDS-PAGE analysis indicated that the recombinant proteins are almost homogeneous in solution. All purified proteins have $M_r$ in agreement with the theoretical values. After concentration, 5-15 mg/ml recombinant main protease could be obtained from 200 ml cells. Unfortunately, the other C-terminal truncated mutants were not successfully expressed probably due to their instabilities.

To determine the secondary structure of the successfully expressed and purified recombinant main proteases, far UV CD spectra were recorded. The overall CD spectra were shown in Figure 2. The spectra of all recombinant SARS-CoV main proteases seemed to be similar, except that of $\Delta(201-306)$ as anticipated (Fig. 1A). These results indicated that the proteins have well-defined secondary structure. This is reflected in the secondary structural estimation by the DICHROWEB server (20,21). Here, we showed the CDSSTR analysis in Table I. The normalized root mean square deviation (NRMSD) values of the data fitting for WT and various truncations were all below 0.2 and thus showed excellent goodness-of-fit parameters (37). The analysis of the secondary structure of the full-length SARS-CoV main protease is consistent with the data derived from the crystal structure, 1UK3 (Table I). The helical contents of the recombinant WT and crystal structure (1UK3) were 0.21 and 0.22, respectively. All truncations show similar results with full-length main protease, except that $\Delta(201-306)$ has significant low $\alpha$-helix content, which is in agreement with the structure that the whole helical domain III was deleted. The thermal stability of the recombinant SARS-CoV main proteases was also examined (Table I). Among the truncated SARS-CoV main proteases, the C-terminal truncated protease $\Delta(293-306)$ has significantly lower $T_m$ than WT.

The fluorescence emission spectra of the recombinant SARS-CoV main proteases were shown in Figure 3. Only $\Delta(201-306)$ had significant low fluorescence intensity. The average emission wavelengths were calculated by the method of del Pino and Fersht (30), which accounts for both wavelength shift and the fluorescence intensity attenuation. The average emission wavelength of full-length SARS-CoV main protease is 342 nm. With the only exception of $\Delta(201-306)$, which has an emission wavelength 11 nm lower than WT, other truncations show minor difference (Table I).

**Analytical Ultracentrifugation Analysis**—
Analytical ultracentrifugation was performed to investigate the association states of WT and truncated SARS-CoV main proteases. This method was successfully used to demonstrate the dimerization of SARS-CoV main protease under various conditions (17). The data were analyzed by continuous size distribution, which implemented a highly reliable model as indicated by the homogeneous bitmap picture (Fig. 4 and 5, Insets). All these data were derived from excellent matching curve of the original raw sedimentation data and the randomly distributed residual values (data not shown). WT protease shows a monomer-dimer equilibrium in solution. The sedimentation coefficients of 2.4 S and 4.2 S were monomer and dimer, respectively, corresponding to species with molar mass of 34 kDa and 68 kDa (17). WT, N-terminal, and C-terminal truncated proteases also displayed a mixture of monomer and dimer (Fig. 4 and 5). Deletion of three residues from the N-terminus showed a similar monomer-dimer distribution with WT. Monomer became the major species when the fourth amino acid residue was deleted from the N-terminus (Fig. 4). Further deletion of more residues [$\Delta(1-5)$, $\Delta(1-6)$ and $\Delta(1-7)$] from the N-terminus showed similar pattern with $\Delta(1-4)$. In addition, we have also examined the involvement of domain III in the subunit association. Truncation of the last helix, the $\Delta(293-306)$ mutant, caused the SARS-CoV main protease to become a monomer (Fig. 5).
These results clearly indicated that residues 4 and 293-306 are critically involved in stabilizing the dimer structure.

The influence of individual residues or domains in the subunit interaction was further quantified by comparing the monomer-dimer dissociation constants. As mentioned above, the global analysis was employed to determine the $K_d$ value of WT and various truncated mutant proteases (Fig. 6). The $K_d$ value for monomer-dimer equilibrium of WT was measured to be 0.28 $\mu$M (Table II). Sequential deletion of residues from N-terminus increased the $K_d$ value. The C-terminal truncated proteases have much higher $K_d$ value than the others.

Kinetic Properties of the Protease—The deletion mutant of the related TGEV main protease that lacks residues 1-5 is almost enzymatically inactive (13). Crystal structure of SARS-CoV main protease reveals that the N-terminal residues 1-7 from subunit A are directly inserted into the active site of subunit B (15). We determined the enzyme activity of the full-length, sequentially N-terminal-, and C-terminal-truncated proteases. The enzyme activity was measured by the peptide cleavage assay (17). The internally quenched fluorescent substrate is cleaved specifically at the Q-S peptide bond (38). The apparent $K_m$ value of WT measured was 17 ± 1 $\mu$M and the apparent $k_{cat}$ value was 198 ± 22 s$^{-1}$. The mutant main protease $\Delta$(1-3) still possesses 76 % of enzyme activity as compared with WT (Table II). However, the enzyme activity of other truncated mutants was decreased to only 0.2-1.3 % of WT activity.

DISCUSSION

The biophysical analyses of SARS-CoV main protease and its truncated mutants performed here allowed a detailed structural characterization of the mutants compared with the full-length protease. In our present data, except the domain III truncated protease, $\Delta$(201-306), which has lower mean residue ellipticity and fluorescence intensity, the CD and fluorescence emission spectra of other truncated proteases are similar to the full-length protease. With the secondary and tertiary structures as anticipated, we then try to study the structure and function relationships of N-terminal and C-terminal regions of the SARS-CoV main protease, especially on the correlation between dimerization and enzyme activity. We used the CASTp program (39) to analyze the pockets and cavities of the enzyme. Only three of the cavities with mouths are big enough to be significant (Fig. 1C). One of these pockets is located at the interface between domains II and III with a surface area of 2.82 nm$^2$ and has no contact with protomer B (Fig. 1C, yellow pocket). The two largest pockets are located at the subunit interfacial region. The N-terminal finger and C-terminal tip are sited at the pocket with a solvent accessible surface area of 18.41 nm$^2$ and a volume of 2.173 nm$^3$ (Fig. 1C, purple pocket). The amino-acid residue contacts between subunits A and B were analyzed with the CSU software (40). Phe-3 in subunit A contacts with subunit B with only one destabilizing hydrophobic-hydrophilic contact. However, Arg-4(A) extends deeply into subunit B (Fig. 1 and 7) involving six destabilizing contacts. It is then clear that removal of Arg-4 has great impact on the structure of the enzyme molecule.

All coronavirus conserved an extra large C-terminal $\alpha$-helical domain that is not found in other RNA virus 3C-like proteases (13,15). The precise biological role of the $\alpha$-helical domain is very interesting but still not completely understood. Our experimental data indicate that the C-terminal domain plays an important role in the dimerization and enzyme activity. These results are consistent with the previous report that loss of the extra domain III of SARS-CoV main protease induced monomer formation and loss of enzyme activity. A further step forward, we have narrowed down the region to a single $\alpha$-helix.

Similar thermal stability curve was observed for most truncations except for the last $\alpha$-helical truncated protease, $\Delta$(293-306), which showed significantly lower Tm than WT (Table I). Deletion of the whole domain III, on the other hand, restores the stability of
the molecule. These results imply the functional role of domain III of the protease. The deleted last helical segment is located at a large pocket between domains II and III (Fig. 1C, yellow pocket). This helix is essential for protein stability of the whole molecule. Without this helix, the remaining domain III becomes a burden for domains I and II that renders the protease unstable.

With a complete domain III, the full-length SARS main protease was in monomer-dimer equilibrium with dimer as the major form even in very low protein concentration (0.1 mg/ml) (17). This result is different from some of the recent reports (18, 41), which showed a major monomer in a protein concentration of less than 0.2 mg/ml. This discrepancy is due to technical differences used in characterizing the quaternary structure of the protein. It is extremely important to obtain an unequivocal answer to this question since the dissociated monomer might be enzymatically inactive. To study the protein self-association, we used the rebirth state-of-the-art analytical ultracentrifugation technique. The $K_d$ value determined by AUC (0.28 $\mu$M), however, is approximately 360 times and 810 times smaller than that previously estimated from analytical gel filtration experiment and isothermal titration calorimeter, respectively (19, 42). Our $K_d$ value for the monomer-dimer equilibrium of SARS-CoV main protease was obtained from global analysis of three different protein concentrations. Under the stringent conditions in AUC, we believe that the $K_d$ obtained is more reliable (33).

Our data showed that the N-finger of SARS-CoV main protease was indispensable for proteolytic activity, which was consistent with previous findings (19). The contribution of the N-finger in dimerization was clearly demonstrated by AUC analysis (Fig. 4). As shown in Figure 7, the N-finger from protomer A is completely buried into protomer B (Fig. 7, A and B). We have further narrowed down to a single residue Arg-4 that plays a pivotal role in building the molecular interaction (Fig. 4). The correlation between enzymatic activity and dimerization is also demonstrated. The fourth residue plays a critical role in dimerization of SARS-CoV main protease, which is essential for the enzymatic activity.

In conclusion, both the N-terminal and C-terminal regions play pivotal roles in controlling the enzyme’s dimerization and enzymatic activity. Our study provides fundamental information for the novel design of inhibitors of the SARS-CoV main protease activity by disrupting its dimerization interface.

REFERENCES


FOOTNOTES

*This work was supported by the National Science Council, ROC.

1 The abbreviations used are: SARS, severe acute respiratory syndrome; CoV, coronavirus; WT, wild type; Δ(n1-n2), deletion mutant of the SARS-CoV main protease with the amino acid residues from n1 to n2 deleted; CD, circular dichroism; IPTG, isopropyl-1-thio-β-D-galactoside; PCR, polymerase chain reaction; BME, 2-mercaptoethanol; PBS, phosphate-buffer saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel; ABZ, ortho-aminobenzoic acid; DNP, 2,4-dinitrophenylamide; AUC, analytical ultracentrifugation.

FIGURE LEGENDS

FIG. 1. Structural features of the dimeric SARS-CoV main protease. (A) Secondary structure of the enzyme and the deleted regions of the various deletion mutants. (B) The enzyme structure (PDB code: 1uk3) is presented as ribbons and labeled with stick model. Protomer A is colored with the catalytic domains I and II in blue and the helical domain III in purple. The N-finger residues and the last α-helix of protomer A are shown with stick model and in blue and CPK, respectively. The catalytic dyad His-41 and Cys-145 are in red. The surface of protomer B is shown in mesh model. This figure was generated with Spock. (C) The backbone of the enzyme is shown as stick model in blue and green and the major pockets are shown as space filling model. This figure was generated with CASTp (39).

FIG. 2. CD spectra of the full-length WT and truncated SARS-CoV main proteases. Far-UV CD spectra of all recombinant SARS-CoV main proteases were monitored at a 0.8 mg/ml concentration in 10 mM PBS buffer (pH 7.6) at 25 °C.

FIG. 3. Fluorescence spectra of the full-length WT and truncated SARS-CoV main proteases. Fluorescence emission spectra of all recombinant proteases were monitored at 8 μg/ml concentration in 10 mM PBS buffer (pH 7.6) at 25 °C.

FIG. 4. Continuous sedimentation coefficient distribution of the full-length WT and N-terminal truncated SARS-CoV main protease. The residuals bitmap of various main proteases were shown in the inset. All enzyme preparations used a concentration of 1 mg/ml in 10 mM PBS buffer (pH 7.6) at 20 °C. A, WT; B, Δ(1-3); C, Δ(1-4); D, Δ(1-5); E, Δ(1-6); F, Δ(1-7). The left dotted line indicated the monomer, and the right one is the dimer form.
FIG. 5. **Continuous sedimentation coefficient distribution of the full-length WT and C-terminal truncated SARS-CoV main protease.** The residuals bitmap of various main proteases were shown in the inset. All enzyme preparations used a concentration of 1 mg/ml in 10 mM PBS buffer (pH 7.6) at 20 °C. A, WT; B, Δ(293-306); C, Δ(201-306). The left dotted line indicated the monomer, and the right one is the dimer form.

FIG. 6. **Global analysis of the sedimentation velocity data of full-length WT SARS-CoV main protease at three protein concentrations.** Sedimentation was performed at 20 °C with An50 rotor and at rotor speeds of 42,000 rpm. (A, B, and C) The concentrations of the protein were 0.1 mg/ml, 0.5 mg/ml and 1 mg/ml, respectively. The sedimentation profiles were from the absorbance optical system at wavelength of 280 nm. The symbols were the raw sedimentation data and the lines were the theoretical fitted data to the Lamm equation implemented in the software SEDPHAT. (D, E, and F) The randomly distributed residuals of the fitting model from the upper panel provide a credible analysis result for a dissociation constant (Kd) of the monomer-dimer equilibrium.

FIG. 7. **Interfacial regions of the dimeric SARS-CoV main protease.** The interfacial contact surface regions within 1 nm for both protomer A and protomer B are shown in mesh model. The same surface areas were turned 90° around the x-axis to give C and D, respectively, from A and B. The C-terminal helical region (red) and the N-terminal finger (blue) are shown in stick mode. This figure was generated with Spock.
### TABLE I

*Structural characteristics of the recombinant SARS-CoV main proteases in crystal and solution*

<table>
<thead>
<tr>
<th>Protease</th>
<th>α-Helix</th>
<th>β-Strand</th>
<th>Turns</th>
<th>Unordered</th>
<th>Normalized NRMSD&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Tm&lt;sup&gt;c&lt;/sup&gt; (°C)</th>
<th>&lt;λ&gt; &lt;sup&gt;d&lt;/sup&gt; (nm)</th>
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<tr>
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<td>43</td>
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<sup>a</sup> Crystal structure determined by X-ray crystallography (pdb code: 1uk3).

<sup>b</sup> Normalized root mean standard deviation of the secondary structure fitting results.

<sup>c</sup> Melting temperature determined by the CD spectropolarimeter.

<sup>d</sup> Average fluorescence emission wavelength shift.

<sup>e</sup> Not determined.
<table>
<thead>
<tr>
<th>Protease</th>
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<th>Fold</th>
<th>RMSD$^b$</th>
<th>Relative Enzyme Activity (%)$^c$</th>
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<tbody>
<tr>
<td>WT</td>
<td>0.28 ± 0.01</td>
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<td>0.003-0.005</td>
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<tr>
<td>Δ(1-3)</td>
<td>3.4 ± 0.1</td>
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<td>0.007-0.010</td>
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<tr>
<td>Δ(1-4)</td>
<td>57.5 ± 6.3</td>
<td>205</td>
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<tr>
<td>Δ(1-5)</td>
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<td>Δ(1-6)</td>
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<td>539</td>
<td>0.003-0.007</td>
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<tr>
<td>Δ(1-7)</td>
<td>357 ± 19</td>
<td>1275</td>
<td>0.006-0.010</td>
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<tr>
<td>Δ(293-306)</td>
<td>163 ± 3.2</td>
<td>582</td>
<td>0.005-0.009</td>
<td>1.1</td>
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<tr>
<td>Δ(201-306)</td>
<td>7870 ± 302</td>
<td>28107</td>
<td>0.005-0.008</td>
<td>0.2</td>
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</tbody>
</table>

$^a$ Dissociation constant of the dimeric SARS-CoV main proteases.
$^b$ Root mean square deviation of the fitted sedimentation velocity data.
$^c$ Enzyme activity assay performed with the internally quenched substrate ABZ-TSAVLQSGFRK-DNP. The enzyme activities of the various truncated mutants were too small to allow a precise determination of the kinetic parameters. Only the relative enzyme activity was reported.
### Figure 1

#### A

<table>
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<th>N-finger</th>
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<th>Domain III</th>
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</tbody>
</table>

#### B

![Diagram of protein structures](image)

#### C

![Diagram of protein structures](image)
Figure 4

Continuous Sedimentation Distribution

Sedimentation Coefficient (S)

Δt(1-7)  Δt(1-9)  Δt(1-3)  Δt(1-4)  Δt(1-5)
Figure 5
Critical assessment of important regions in the subunit association and catalytic action of the severe acute respiratory syndrome coronavirus main protease

Wen-Chi Hsu, Hui-Chuan Chang, Chi-Yuan Chou, Fu-Jen Tsai, Pei-In Lin and Gu-Gang Chang

J. Biol. Chem. published online April 14, 2005

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