Functional Implications of Disulfide Bond, Cys206−Cys210, in Recombinant Prochymosin (Chymosin)†

Hongjie Chen,‡ Guobao Zhang,‡ Yuying Zhang,‡ Yicheng Dong,§ and Kaiyu Yang*,‡

Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, China, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China

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ABSTRACT: Prochymosin (chymosin) contains three disulfide bonds: Cys45−Cys50, Cys206−Cys210, and Cys250−Cys283. We have demonstrated that Cys250−Cys283 is indispensable for correct refolding of prochymosin, whereas Cys45−Cys50 is dispensable but has some contribution to the stability and substrate specificity of the enzyme. Here, we report the results about the functions of Cys206−Cys210 by site-directed mutagenesis studies. In a glutathione redox system C206A/C210A mutant exhibited oxidative refolding kinetics and efficiency (∼40% reactivation) similar to those of the wild-type prochymosin, indicating that Cys206−Cys210 is also dispensable for refolding. However, C206S/C210S and single-site mutants (C210A, C210S, and C206A) showed only about 3 and 0−0.4% reactivation, respectively. This is quite different from the Cys45−Cys50 deficient mutants (C45A, C50A, C45A/C50A, C45D, C50S, C45D/C50S, C45A/C50S), which have comparable refolding efficiencies, implying that the substituents at position 206 and 210 play more important role in determining correct refolding than those at position 45 and 50. Urea-induced denaturation and fluorescence quenching studies indicated that the prochymosin mutants C206A/C210A and C206S/C210S were 2.1 and 4.8 kJ/mol less stable than prochymosin and some tryptophan residue in the mutated molecules was less exposed. However, the wild-type and mutant prochymosins shared similar far-UV CD and fluorescence emission spectra and similar specific potential activity, suggesting that the overall conformation was maintained after mutation. Activity assay and kinetic analysis revealed that mutation did not change the specific milk-clotting activity significantly but resulted in an increase in K_m and k_{cat} toward a hexapeptide substrate. On the basis of the above-mentioned perturbance of tryptophanyl microenvironment and the three-dimensional structure of chymosin, we proposed that deletion of Cys206−Cys210 may induce a propagated conformational change, resulting in a perturbance of the local conformation around active-site cleft and in turn, an alteration of the substrate specificity.

Generally, disulfide bonds are essential for folding, stability, and tertiary structure of disulfide-containing proteins. In some cases, they are involved in determining biological functions of proteins. Therefore, studies on the functional implications of disulfide bonds will provide some basis to elucidate the mechanism of protein folding, to efficiently recover active recombinant proteins from inclusion bodies and to rationally design and engineer natural proteins.

Calf prochymosin is the zymogen of chymosin (EC 3.4.23.4), a commercially important enzyme used in the cheese-making industry. Chymosin belongs to the aspartic protease family containing essential catalytic residues Asp32 and Asp215 (pepsin numbering).[1] Prochymosin and its mature enzyme contain three disulfide bonds linking Cys45 to Cys50, Cys206 to Cys210 and Cys250 to Cys283 (pepsin numbering). Interestingly, among this family the vertebrate proteases have the same number and linkage pattern of disulfide, while their homologous proteases from *Mucor michei* and *Endothia parasitica* have two disulfide bridges from Cys45 to Cys50 and Cys250 to Cys283, and single bridge between Cys250 and Cys283, respectively (*1*). However, all these enzymes are bilobal with a deep, extended cleft, each lobe having a similar fold (*2*). These structural characteristics originating from evolution make them become an ideal model proteins for structure−function studies.

However, the function of disulfide bonds in aspartic proteases had never been addressed until our laboratory undertook relevant studies on recombinant prochymosin (chymosin) in the early 1990s. We have demonstrated that prochymosin is characterized for its two-stage refolding: formation and rearrangement of disulfide bonds at pH 11 followed by acquisition of native tertiary structure at pH 8 (*3*, *4*). This is quite different from other disulfide-containing proteins reported so far including its homologous protein, pepsinogen (*5*); for them oxidative refolding is accomplished during a single process at a given pH value. On the basis of this finding, 90% reactivation was achieved when PDI[2] was

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* To whom correspondence should be addressed. Fax: 8610-62560912. E-mail: yangky@sun.im.ac.cn. Phone: 8610-62566527.

‡ Institute of Microbiology.

§ Institute of Biophysics.

[1] To facilitate the comparison, the pepsin numbering system is used for all residues in prochymosin and chymosin (for alignments see ref *1*) except Figure 6 and corresponding discussion, where chymosin numbering is used.

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supplemented into the refolding system at the first stage of refolding.[3] All these results indicate that the formation of native disulfide bonds is a prerequisite for correct refolding of prochymosin. To further investigate the structural and functional roles of individual disulfide bond, chemical modification and site-directed mutagenesis were performed. It has been found that Cys250−Cys283 is not intimately involved in the catalytic mechanism of chymosin but is indispensable for correct refolding of prochymosin (*6*), whereas Cys45−Cys50 is dispensable for refolding but exhibits some contribution to the stability and substrate specificity of the enzyme (*7*). This paper is a continuation of our previous work in this regard and the effects of deletion of Cys206−Cys210 on the refolding, conformation and catalytic activity of prochymosin (chymosin) are reported. Comparison of the functions of different disulfide bonds in prochymosin (chymosin) is also discussed.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. A SGI station was used to perform molecular modeling. For energy minimization, the charm program of Quanta from Polygene Corporation was employed. For construction of the mutants oligonucleotides were synthesized with an ABI 381A DNA Synthesizer. *Eco*RI fragment containing codons for Cys206 and Cys210 from pTaAC 5 (*8*) was cloned into pBluescript II KS (+). The uracil-containing ssDNA was prepared from the resultant pBluescript II KS (+) replicated in *Escherichia coli* CJ 236 with a helper phage R408 (*9*) and used as a template for mutagenesis according to the method of Yuckenberg (*10*). All the mutations were confirmed by dideoxy chain termination method (*11*).

Expression of the Mutated cDNA. The mutated cDNAs were integrated to give expression plasmids with the same constitution as pBC4 (*12*), in which P_RP_L promoters and prochymosin B cDNA were harbored. They were transformed into *E. coli* BL21 (DE3) pLysS and cultured as described previously (*12*). Like the wild-type gene, expression of the mutated gene was induced by shifting the growth temperature from 30 to 42 °C. The expression product accumulated as a form of inclusion bodies.

Solubilization of Inclusion Bodies and Preparation of Fully Reduced Prochymosin Species. Inclusion bodies prepared as described previously (*3*) were solubilized in buffer A (50 mM KH_2PO_4, 50 mM NaCl, 1 mM EDTA, pH 11) containing 8 M urea at 30 °C for 2 h followed by centrifugation at 10000*g* for 10 min. The supernatant was recovered as inclusion-body solution. The prochymosin molecules present in the inclusion-body solution were demonstrated to contain both free thiols and disulfide bonds (*4*) and designated as partially reduced prochymosin. To obtain fully reduced prochymosin species inclusion bodies were solubilized in the presence of 1 mM DTT. The reduced protein was separated from the excess DTT by gel filtration on a Sephadex G25 column. Full reduction was confirmed by thiol titration (*13*).

Refolding. The refolding process consisted of two steps: first, the inclusion-body solution (1−2 mg/mL of partially reduced or fully reduced proteins) was diluted with 10 vol buffer A and left at 4 °C for 24 h unless otherwise indicated; second, the pH 11 refolding solution was adjusted to pH 8 with 1 M HCl, maintained at 4 °C for 1 h and dialyzed against buffer B (20 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, pH 8) at 4 °C for 24 h unless otherwise indicated. When refolding was performed in the presence of oxidized and reduced glutathione 160 μM GSH and 80 μM GSSG were supplemented into the refolding system at the beginning of the first stage.

Activation. The refolded prochymosin and its mutants were activated at pH 2 and 15−20 °C for 1−10 h to produce pseudochymosin as described by Pederson et al. (*14*). Pseudochymosin was further converted into chymosin by dialysis against 0.1 M acetate buffer, pH 5.5 at 25 °C overnight (*14*). The autocatalytic activation products were separated by SDS−PAGE (*15*). The activability of the species of prochymosin was expressed as a percentage of pseudochymosin produced after activation in relation to the prochymosin input, as judged by gel scanning on a Shimadzu CS-9000 densitometer, and used to evaluate refolding efficiency.

Assay for Milk-Clotting Activity. The milk-clotting activity was measured at pH 6.3 based on the microtiter plate assay method of Emtage et al. (*16*). The authentic calf chymosin was used as a standard. Prochymosin itself did not exhibit milk-clotting activity, but the activity could be detected after activation due to the production of pseudochymosin and designated as potential activity for prochymosin. The activity measured after refolding relative to the prochymosin input was expressed as reactivation or renaturation, which was also used to evaluate refolding efficiency.

Kinetic Analysis. The hexapeptide Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe (Sigma) was used as a substrate to perform kinetic analysis. Kinetic measurements were performed in 0.1 M acetate buffer, pH 3.7, 30 °C, and the change in absorbance at 310 nm was monitored on a Shimadzu UV−vis recording spectrophotometer according to the method of Martin et al. (*17*). The k_{cat} values were calculated based on the concentration of enzyme estimated by either Lowry assay (*18*) or a $\epsilon_{1cm}^{1mg/mL}$ at 277.5 nm value of 1.53 (*17*).

Purification of Prochymosin and Chymosin. The refolded prochymosin mutants with 60−70% purity was applied onto a DEAE-Sepharose Fast Flow column equilibrated with buffer B containing 50 mM NaCl. After the column had been washed with buffer B containing 0.1 M NaCl, the adsorbed proteins were eluted with a linear gradient of 0.1 to 0.5 M NaCl in buffer B. The fractions showing homogeneity as judged by SDS−PAGE were collected and used for further analysis.

To obtain chymosin and its mutants, the refolded protein was activated and the produced chymosin was purified by DEAE-Sepharose Fast Flow column chromatography as described above, except that pH 6.3 phosphate buffer was used.

Spectroscopic Analyses. CD spectra were determined on a Jasco-720 spectropolarimeter (cuvette path length 0.01 cm). Each scan was measured at 50 nm/min with sensitivity of 2 mdeg. A total of four scans were made for each sample. Protein fluorescence was recorded on a Hitachi 850 fluorescence spectrophotometer (cuvette path length 1 cm). For

[2] Abbreviations: PDI, protein disulfide isomerase; EDTA, ethylenediamine tetraacetic acid; DTT, dithiothreitol; GSH, reduced glutathione; GSSG, oxidized glutathione; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CD, circular dichroism.

[3] Wei, C., Zhang, Y., and Yang, K., submitted manucript.

fixed wavelength measurements, the excitation monochromator was set at 281 nm (295 nm for quenching studies), and the emission monochromator was set at 333 nm. Emission spectra were recorded at a speed of 60 nm/min. The excitation and emission slit widths were adjusted to 5.0 nm band-pass.

Quenching Studies. Aliquots of 8 M acrylamide were diluted into 2 mL of protein solution (0.2 mg/mL) to give different concentrations of acrylamide ranging from 0−0.21 M and the fluorescence change was recorded after each addition. Fluorescence data were analyzed by the modified Stern−Volmer equation (*19*):

$$F_0/\Delta F = 1/f_a + 1/K_{sv}[Q]$$

where $\Delta F = F_0 - F_Q$, F_0 and F_Q are fluorescence emission intensities in the absence of quencher and in the presence of quencher at concentration [Q]. K_{sv} is a constant indicating the accessibility of the fluorophore to the quencher. f_a is a fraction of fluorescence from residue accessible to the quencher.

Equilibrium Denaturation Studies. Portion of stock protein solution was diluted into various concentrations of recrystallized ultrapure urea (Sigma) in buffer B containing 50 mM NaCl. The final protein concentration was 20 μg/mL. After incubation for 30 min at 25 °C the intrinsic fluorescence was recorded.

RESULTS

Mutation and Expression of Prochymosin Mutants. Generally, the length of a disulfide bond is estimated to be 2.02 Å and the distance between two C_βs of cysteine residues is 3.50 to 4.50 Å. The space for paired cysteine residues can only accommodate smaller amino acid residues and the preferential substitutes reported are pairwise Ala or Ser. Ala is usually used to substitute for buried Cys, while Ser for exposed Cys. The three-dimensional structure of recombinant chymosin (*20*) indicates that Cys206−Cys210 (pepsin numbering) is buried in the molecule of chymosin, therefore, hydrophobic Ala was preferentially chosen to replace the cysteine residues. Computer graphics modeling reveals that Ser substitution provides possibility to form hydrogen bond and to compensate in part for the loss of this disulfide. To explore the possibility Ser was also employed. Considering that individual cysteine residues may play different roles in oxidative refolding of protein, single-site mutation was performed too. By site-directed mutagenesis five mutated cDNAs for prochymosin B were generated and confirmed by DNA sequencing. They are C206A, C210A, C206A/C210A, C206S/C210S, and C210S. The mutated cDNAs were integrated to give expression plasmids and transformed into *E. coli* BL21(DE3)pLysS. All the five variants were expressed and accumulated in the cells in a form of inclusion bodies. The expression levels of the mutant proteins except C206A are comparable with that of the wild-type prochymosin, amounting to about 50% of the total cellular proteins (data not shown).

Oxidative Refolding of Prochymosin Mutants. Refolding of Prochymosin Mutants by Air Oxidation. Prochymosin is characterized for its two-stage refolding: renaturation is initiated by diluting the prochymosin solubilized from inclusion bodies into pH 11 buffer and followed by neutralization at pH 8. To investigate the effect of deletion of Cys206−Cys210 on the refolding of prochymosin the two-stage renaturation procedure was adopted. After refolding in air the refolded products were activated at pH 2 followed by SDS−PAGE analysis and assay for milk-clotting activity to evaluate refolding efficiency. Pederson et al. (*14*) demonstrated that activation of prochymosin at pH 2 resulted in the formation of the active enzyme, pseudochymosin, by proteolytic cleavage at the bond of Phe27−Leu28 of prochymosin. Activation is initiated by an autocatalytic process; under acidic conditions prochymosin molecule assume an active conformation with its propeptide situated in the active cleft, ready for intramolecular cleavage (*1*). In other words, the activity of autocatalytic activation is determined by the correct refolding of prochymosin polypeptide, leading to the formation of an active cleft and positioning the propeptide correctly. On the basis of this mechanism, we consider the activable molecule to be a correctly refolded prochymosin and use activability of the refolding products as judged by SDS−PAGE and gel scanning after acid treatment to evaluate refolding efficiency, expressed as conversion percentage. Meanwhile, pseudochymosin exhibits milk-clotting activity comparable to that of chymosin; the activity of activation products was also used to evaluate refolding efficiency, expressed as reactivation percentage. Figure 1A indicates that after refolding at first stage and second stage for 24 h, respectively, and 10 h activation, 90.4, 75.4, and 27.7% conversion of prochymosin into pseudochymosin are achieved for wild-type prochymosin, C206A/C210A and C206S/C210S proteins respectively; conversion is barely observed for C206A, C210A, and C210S. However, when the second stage was prolonged from 24 to 240 h, conversion is detected for C210A and C210S, but still not for C206A (Figure 1B). Activity assay supports these observations; the reactivation efficiency is correlated to the conversion efficiency, although the former is much lower than the latter (Table 1). Taking together, it is evident that the mutants with double-site mutation and single-site mutation at position 210

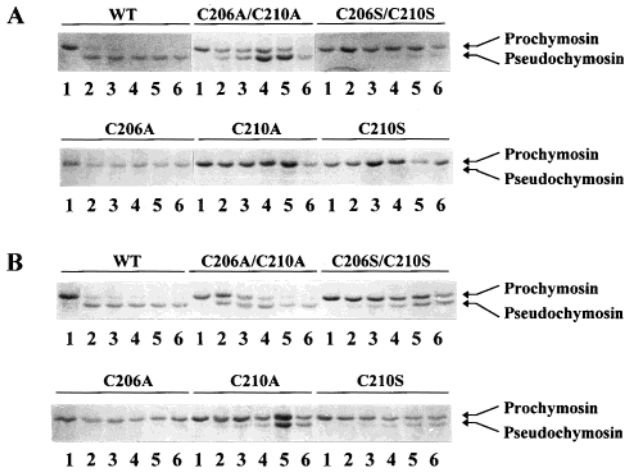

FIGURE 1: SDS−PAGE analysis of the autocatalytic activation of the wild-type (WT) and mutant prochymosins activated at pH 2 after refolding at the second stage for 24 h (A) or 240 h (B). Inclusion bodies containing wild-type or mutant prochymosins were solubilized and subjected to refolding at pH 11 (first stage) for 24 h then at pH 8 (second stage) for 24 or 240 h. The refolded proteins were activated at pH 2 and 15−20 °C for 1−10 h. Aliquots were analyzed by SDS−PAGE. Lanes 1−6 represent activation for 0, 1, 2, 4, 7, and 10 h, respectively.

Table 1: Refolding Efficiency of Wild-type (WT) and Mutant Prochymosins[a]

	refolding at first stage for 24 h		refolding at second stage for 240 h	
protein	conversion (%)	reactivation (%)	conversion (%)	reactivation (%)
WT	90.4 ± 1.9	28.2 ± 1.5	95.1 ± 1.8	26.9 ± 1.5
C206A/C210A	75.4 ± 2.2	13.8± 1.0	86.3 ± 1.6	14.5 ± 1.0
C206S/C210S	27.7 ± 2.1	1.06 ± 0.95	51.1 ± 1.6	3.26± 0.50
C210A	12.4 ± 2.0	0.35 ± 0.24	45.3 ± 3.5	0.72 ± 0.42
C210S	less than 5%	0	40.7 ± 2.6	0.45 ± 0.39
C206A	0	0	0	0

[a] After renaturation at first stage for 24 h and at second stage for 24 or 240 h the refolded products were activated at pH 2 for 10 h followed by SDS−PAGE analysis and assay for milk-clotting activity to evaluate conversion and reactivation. The results were given as the means of triplicate assays ±SD.

can proceed oxidative refolding to form correctly refolded molecules capable of undergoing autocatalytic activation leading to the production of active pseudochymosin analogues, indicating that disulfide Cys206−Cys210 is dispensable for correct refolding of prochymosin. However, among the activable mutants the refolding efficiencies are quite different. Pairwise Ser-substitution is inferior to pairwise Ala-substitution; the former shows about two-thirds of the conversion efficiency and one-fourth of the reactivation efficiency of the later. Single-site mutation at position 210 results in even lower refolding efficiency, showing one order less reactivation relative to that of C206A/C210A. As for C206A no correct refolding occurs. All these results demonstrate that amino acid residues at position 206 and 210 play an important role in determining the correct refolding of prochymosin, although Cys206−Cys210 is dispensable.

Refolding of Prochymosin Mutants in the Presence of Oxidized and Reduced Glutathione. Generally, the oxidative refolding efficiency is determined by the formation of native disulfide bonds. At the initial stage of refolding incorrect pairing of cysteine residues is inevitable. This mispaired disulfide bond can only be corrected via thiol/disulfide interchange. During refolding in air the thiol/disulfide exchange proceeds within the polypeptide, once all the free thiols are paired, the exchange reaction stops leaving the scrambled molecules unchangeable. When exogenous reductant and oxidant are included into the refolding system, disulfide oxidation, reduction, and rearrangement can be initiated by the redox couple; different refolding pathways and efficiencies may be observed. To further investigate how the mutation influences prochymosin oxidative refolding, a mixture of oxidized and reduced glutathione was used. Experiments were performed with two kinds of molecules: partially reduced form and fully reduced form. As shown in Figure 2B, GSH/GSSG can accelerate the refolding of C206A/C210A and boost the overall yield of the active product; compared with the spontaneous refolding in air the refolding efficiencies of the partially reduced and fully reduced form are increased by 2.3- and 8.8-fold respectively, amounting to about 40%, comparable with that of the wild-type prochymosin (Figure 2A). In a sharp contrast, the redox couple fails to assist refolding of the partially reduced C206S/C210S and C210A and exhibits favorable effect on the fully reduced ones, but showing only about 3 and 0.4% reactivation, respectively (Figure 2C and D). Moreover, no reactivation was observed with C210S and C206A, no matter which oxidation form was employed. The effect of GSH/GSSG varying with the variant proteins and with their oxidation states reveals that the unfolded states of the start molecules and the polypeptide conformations adopted during refolding play an important role in determining the function of GSH/GSSG. This difference supports our above proposal: for prochymosin refolding, disulfide Cys206−Cys210 is dispensable, but the residue substitutes are critical.

Conformation and Conformational Stability of Prochymosin Mutants. Conformation of Prochymosin Mutants. To test the effect of deletion of Cys206−Cys210 on the conformation of prochymosin the refolded mutants C206A/C210A and C206S/C210S were purified to homogeneity and subjected to spectroscopic analyses. Figure 3 indicates that the mutants and wild-type prochymosin exhibit similar far-UV CD spectra characteristic of a predominance of β-sheet with a small fraction of α-helix, revealing that they assume similar secondary structure.

Fluorescence analysis indicates that the wild-type and mutant proteins display similar fluorescence emission spectra with an identical wavelength of maximum emission (Figure 4A). However, mutation tends to increase the fluorescence intensity, reflecting a difference in the environment of the fluorophor. Since the fluorescence at around 333 nm is predominantly from tryptophanyl residues, determining the accessibility of these residues by quenching studies may provide further insights into the conformational change due to mutation. Acrylamide, as a fluorescence quencher, is very sensitive to the exposure of tryptophans in proteins and can sense a perturbance of the structure in the proximity of these groups (*21*). The Stern−Volmer plots (Figure 4B) indicates that after incubation with different concentrations of acrylamide almost the same line is observed with the denatured proteins, whereas downward curves are observed with native proteins. The curvature deviation is more apparent for the mutants. This observation reveals that in the native proteins tryptophan residues are heterogeneous with respect to accessibility to quencher and mutation results in the increase of the heterogeneity. According to the modified Stern−Volmer equation (*19*), the fraction of fluorescence from residues accessible to quencher (f_a) was determined to be 0.87, 0.78, and 0.75 for wild-type, C206A/C210A, and C206S/C210S proteins, respectively, and the quenching constants of the mutants are lower than that of the wild-type prochymosin (data not shown), suggesting that certain tryptophan residue shifts to a more apolar microenvironment upon mutation. This is consistent with the increase of the fluorescence intensity. To determine whether the conformational alteration affects biological activity, the purified prochymosin and its analogues were activated at acidic conditions followed by milk-clotting activity assay. It was found that the specific potential activities of wild-type prochymosin, C206A/C210A, and C206S/C210S were 1.34 ± 0.03, 1.52 ± 0.12, and 1.41 ± 0.05, respectively, indicating that mutations does not reduce the specific potential activity of prochymosin. Taking all the results mentioned above into consideration it is reasonable to conclude that after deletion of Cys206−Cys210 only a minor perturbance around some tryptophan residue(s) occurs and the integrity of the active conformation is still maintained.

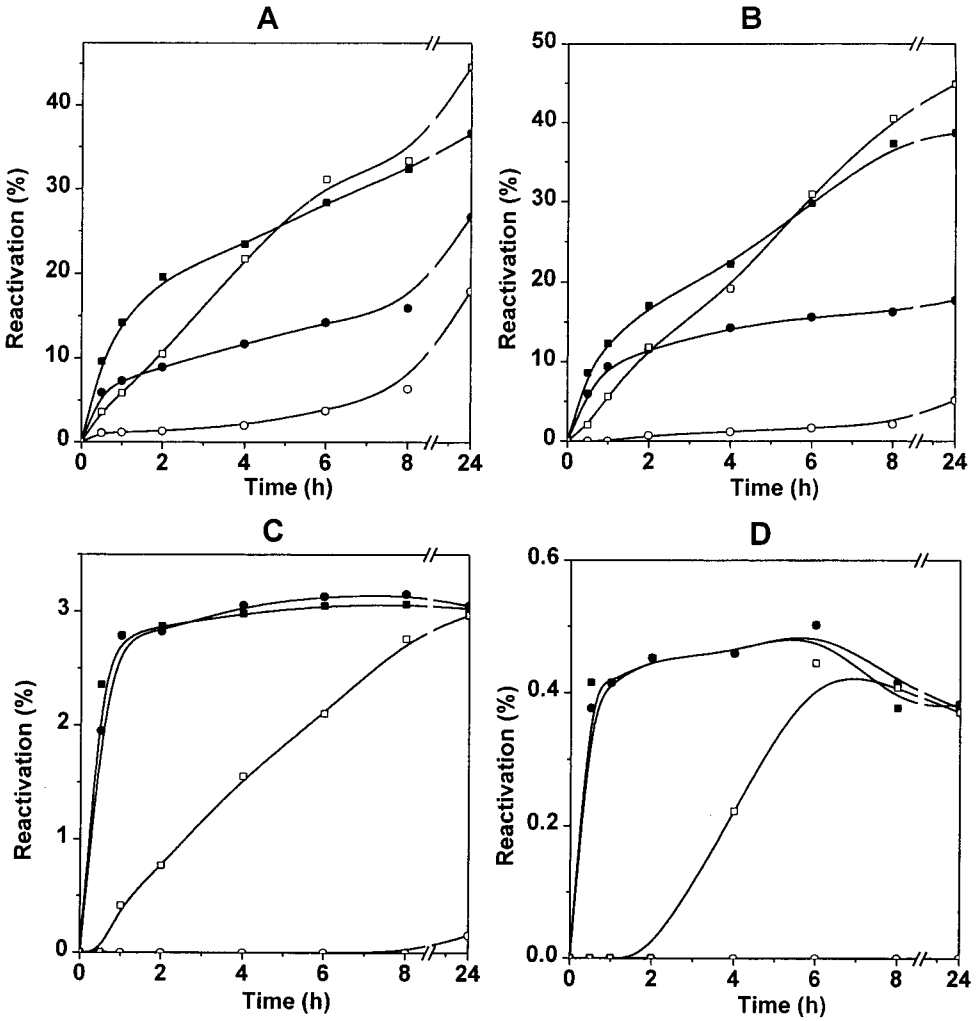

FIGURE 2: Renaturation progress of the partially and the fully reduced wild-type and mutant prochymosins following the time course of the first stage of refolding in air or in the presence of GSH/GSSG. (A) Wild-type prochymosin. (B) C206A/C210A mutant. (C) C206S/C210S mutant. (D) C210A mutant. (●) Partially reduced form. (○) Fully reduced form. (■) Partially reduced + GSH/GSSG. (□) Fully reduced + GSH/GSSG.

Urea Equilibrium Unfolding. The conformational stabilities of prochymosin variants were compared by urea equilibrium unfolding. The unfolding transition curves were measured for wild-type, C206A/C210A, and C206S/C210S prochymosins in 20 mM Tris buffer at pH 8 and 25 °C by monitoring the change of intrinsic fluorescence at 333 nm. The data were analyzed by assuming a two-state unfolding transition and fitted to the equation of Santoro and Bolen (*22*):

$$y = \{(y_N + m_N[D]) + (y_U + m_U[D]) \times (\exp[-(\Delta G(H_2O)/RT + m[D]/RT)])\}/\{1 + \exp[-(\Delta G(H_2O)/RT + m[D]/RT)]\}$$

where y_N and m_N and y_U and m_U are the slope and intercept of the pre- and posttransition baselines, respectively. [D] is the urea molarity; $\Delta G(H_2O)$ is the value of ΔG in the absence of urea and m is a measure of the dependence of ΔG on urea concentration. The urea transition curves are given in Figure 5, the $\Delta G(H_2O)$, m values and transition midpoints ($[urea]_{0.5}$) are presented in Table 2. The standard free-energy differences, $\Delta G(H_2O)$, between the folded and unfolded state of C206A/C210A and C206S/C210S are 14.2 and 11.5 kJ mol^{-1}, respectively. Both are less than 16.3 kJ mol^{-1}, the

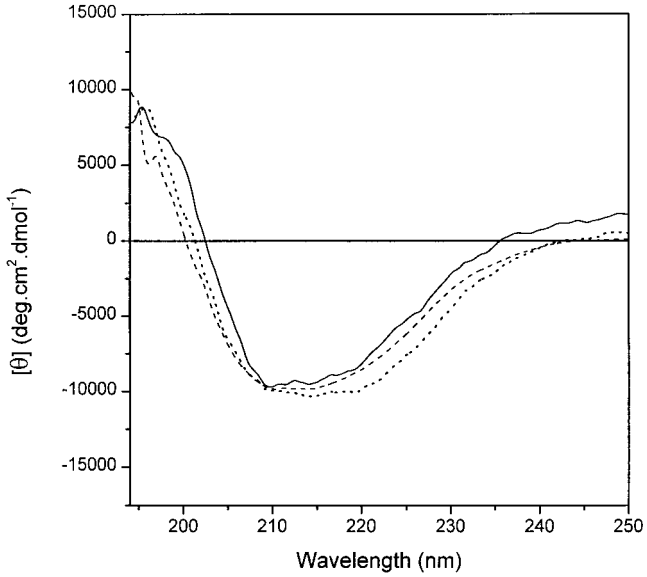

FIGURE 3: CD spectra of wild-type and mutant prochymosins in the far UV region. Measurements were made in buffer B (20 mM Tris-HCl, pH 8, 1 mM EDTA) containing 50 mM NaCl at a protein concentration of 0.2 mg/mL. (—) Wild-type prochymosin. (---) C206A/C210A. (···) C206S/C210S.

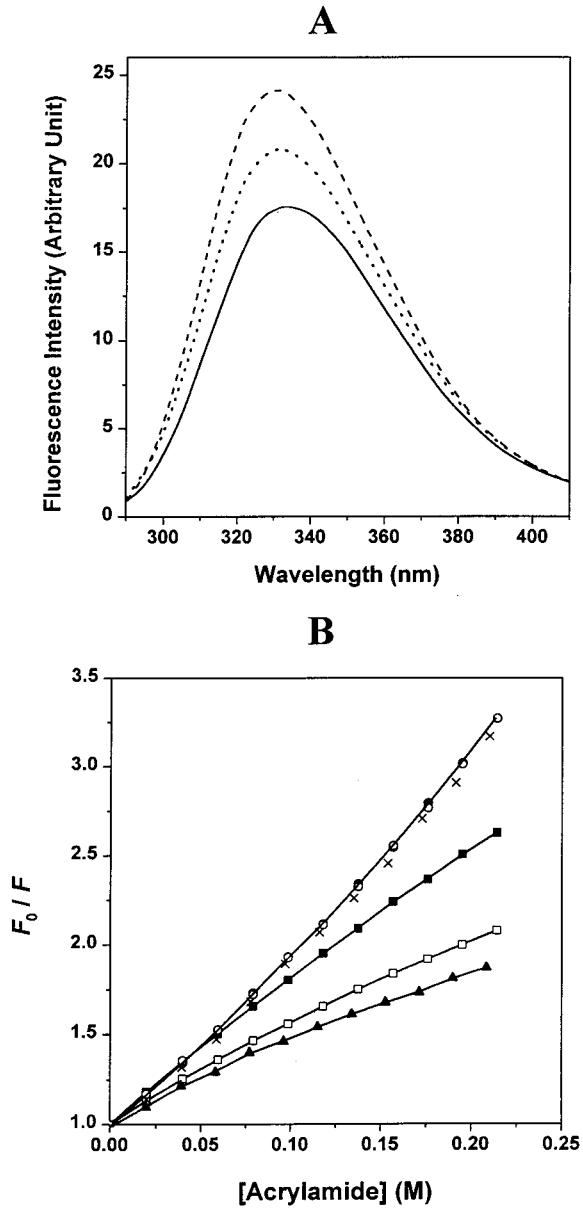

FIGURE 4: Fluorescence analysis of wild-type and mutant prochymosins. (A) Fluorescence emission spectra of wild-type and mutant prochymosins. Measurements were made in buffer B (pH 8) containing 50 mM NaCl at 25 °C with an excitation wavelength of 281 nm. The protein concentration was 20 μg/mL. (—) Wild-type prochymosin. (---) C206A/C210A. (···) C206S/C210S. (B) Fluorescence quenching of wild-type (WT) and mutant prochymosins. Portions of 8 M acrylamide were added to prochymosin (0.2 mg/mL) in buffer B (pH 8) containing 50 mM NaCl or in 8 M urea−buffer B containing 50 mM NaCl. The fluorescence intensity at 333 nm (the excitation wavelength was 295 nm) was recorded after each addition and the data was displayed by a Stern−Volmer plot, where F_0 and F are, respectively, the fluorescence intensity in the absence and in the presence of acrylamide. (●) WT in 8 M urea. (○) C206A/C210A in 8 M urea. (×) C206S/C210S in 8 M urea. (■) Native WT. (□) Native C206A/C210A. (▲) Native C206S/C210S.

stabilization energy value for wild-type prochymosin, demonstrating that Cys206−Cys210 has some contribution to the stability of prochymosin.

Catalytic Activity of Chymosin Mutants. Chymosin exhibits specific milk-clotting activity (*C*) and general proteolytic activity (*P*). The former is achieved by the specific cleavage of κ-casein between the Phe105−Met106 bond leading to the coagulation of milk micelles and the latter is a nonspecific proteolysis. Among aspartic proteases chymosin is the best milk coagulant for its high ratio of *C*/*P*. To probe the effect of mutation on the catalytic property of chymosin the milk-clotting activity of the purified chymosin analogues were assayed and their specific activities were determined. It should be noted that milk-clotting process consists of enzymatic cleavage stage and nonenzymatic coagulation stage rather than a simple enzymatic reaction; therefore, it is impossible to determine kinetic parameters by using milk as a substrate. Instead, a synthetic hexapeptide, Leu-Ser-Phe-(NO_2)-Nle-Ala-Leu-OMe was used to perform kinetic analysis. This peptide is an analogue of the sequence Leu-Ser-Phe-Met-Ala-Ile covering the chymosin-sensitive site Phe105-Met106 bond of κ-casein and is cleaved at the bond of Phe(NO_2)−Nle by the enzyme. As shown in Table 3, the specific milk-clotting activity of the mutants seems to increase slightly. While comparison of the kinetics characteristics of the mutants and wild-type chymosin toward the synthetic substrate showed that mutation reduced the substance binding ability (K_m) but increased the catalytic efficiency (k_{cat}), resulting in a reduction in the specificity constant (k_{cat}/K_m). These results reveal that elimination of Cys206−Cys210 tends to increase the specific activity toward κ-casein in milk slightly but changes the substrate specificity toward the hexapeptide significantly. Similar observation that mutation of chymosin had different effects on the enzymatic action toward different substrates has been reported by Williams et al. (*23*). They found that the G243D mutation remarkably reduced the specificity constant of the enzyme toward the hexapeptide but had somewhat favorable effect on the enzymatic activity toward κ-casein and proposed that the increase in enzymatic activity of G243D for casein could be the result of favorable interactions with large peptide. Aspartic proteases have extended active-site cleft, which can accommodate at least seven amino acids of a substrate in the S_4−S_3' subsites (*24*). Differences between the action of some aspartic proteases on the hexapeptide and on κ-casein were also observed. Martin et al. (*17*) proposed that these differences might partly be ascribed to whether the whole active site of the enzyme, and especially the binding site, participated in the reaction; with κ-casein, the amino acid residues far away from the sensitive bond Phe-Met may play an important role in the interactions between the enzyme and the substrate. These points of view can be used to explain our results. Removal of Cys206−Cys210 may lead to a perturbance around the active-site cleft of the enzyme. Nevertheless, compared with the short substrate, hexapeptide, the large substrate, κ-casein, may occupy the whole active cleft and additional interactions between the enzyme and the substrate may take place, which appears favorable for maintaining the catalytic characteristics of the enzyme with respect to casein.

DISCUSSION

Comparison of the Functions of Different Disulfide Bonds in Prochymosin (Chymosin). Contribution to the refolding of prochymosin. Previous site-directed mutagenesis studies revealed that Cys250−Cys283 deficient prochymosin analogues (C250D, C283S, C250D/C283S, C250A, C283A, and C250A/C283A) failed to undergo autocatalytic activation (ref

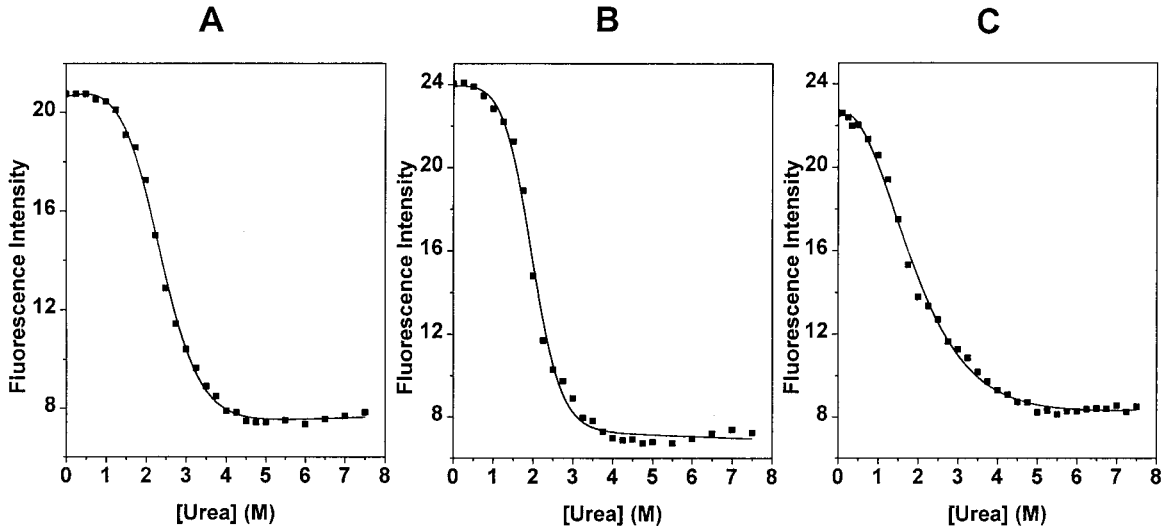

FIGURE 5: Urea-induced unfolding of wild-type (WT) and mutant prochymosins. The wild-type and mutant prochymosins (20 μg/mL) were incubated in solutions of various concentrations of urea−buffer B (pH 8) containing 50 mM NaCl. After incubation for 30 min at 25 °C the fluorescence intensities at 333 nm were recorded with an excitation wavelength of 281 nm. (A) Wild-type prochymosin. (B) C206A/C210A. (C) C206S/C210S.

Table 2: Equilibrium Studies on Unfolding of Wild-Type (WT) and Mutant Prochymosins[a]

protein	$[\text{urea}]_{0.5}$[b] (M)	$\Delta G(H_2O)$[c] (kJ/mol)	m[d] (kJ mol^{-1} M^{-1})
WT	2.5	16.3 ± 0.1	6.4 ± 0.2
C206A/C210A	2.1	14.2 ± 0.1	6.8 ± 0.2
C206S/C210S	1.8	11.5 ± 0.4	6.3 ± 0.3

[a] The equilibrium data were analyzed according to ΔG−[urea] plots and the results were given as the means of triplicate assays ± SD. [b] $[\text{urea}]_{0.5}$ is the molar concentration of urea corresponding to the midpoint of the unfolding transition. [c] $\Delta G(H_2O)$ is the stabilization energy of the native state to unfolding in the absence of denaturant. [d] m is a measure of the dependence of symbol ΔG on urea concentration.

Table 3: Kinetic Parameters of Wild-Type (WT) and Mutant Chymosins Using Leu-Ser-Phe(NO2)-Nle-Ala-Leu-OMe Substrate[a]

enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	specific activity
WT	0.90 ± 0.04	17.0 ± 1.0	18.9 ± 0.3	1.45 ± 0.05
C206A/C210A	2.71 ± 0.07	48.2 ± 1.0	17.8 ± 1.0	1.67 ± 0.06
C206S/C210S	1.52 ± 0.02	19.3 ± 0.2	12.7 ± 0.3	1.56 ± 0.07

[a] Kinetic measurements were performed in 0.1 M acetate buffer, pH 3.7, 30 °C, and the change in absorbance at 310 nm was monitored. The K_m and k_{cat} values were based on Lineweaver−Burke plot ($1/v$ versus 1/[S]). The enzyme concentration were 30, 32, and 30 nM for WT, C206A/C210A, and C206S/C210S, respectively. Specific activity was the milk-clotting activity of purified enzyme. The results were given as the means of triplicate assays ± SD.

6 and unpublished observations),[4] whereas Cys45−Cys50 deficient prochymosin analogues (C45D, C50S, C45D/C50S, C45A, C50A, C45A/C50A, and C45A/C50S) were activable to produce active mature enzyme (*7*), demonstrating that Cys250−Cys283 is indispensable for correct refolding of recombinant prochymosin, while Cys45−Cys50 is dispensable. This paper shows that both C206A/C210A and C206S/C210S can refold into a form capable of undergoing autocatalytic activation and assuming the secondary and tertiary structures similar to those of the wild-type protein and that in a glutathione redox system C206A/C210A exhibits oxidation refolding kinetics and efficiency similar to those of the wild-type prochymosin, indicating that Cys206−Cys210 is also dispensable for prochymosin refolding. However, by comparison it was found that of the two unessential disulfide bonds, amino acid residues at position 206 and 210 play more important role in determining correct refolding than those at position 45 and 50. (1) Single-site mutation at either site of Cys206−Cys210 leads to a dramatic reduction in refolding efficiency. For C206A, even neither autocatalytic-activation activity nor milk-clotting activity could be detected. This is quite different from the variants with single-site mutation at Cys45 or Cys50. In that case, all four single-site mutants (C45A, C45D, C50A, C50S) are active (*7*). These results imply that not only the individual disulfide bonds of prochymosin have different functions in refolding but the roles of each cysteine residues of a disulfide bond may also be different. Similar observation was reported with human lysozyme (*25*). Lysozyme has four disulfide bonds. When the cysteine residues of Cys65−Cys81 and Cys77−Cys95 were replaced by alanine one by one, of the four mutants lacking one cysteine (C65A, C81A, C77A, C95A), only C65A could not fold correctly in vivo, indicating that Cys65 is a requisite for correct folding. (2) For double-site mutants it was shown that the reactivation efficiency of C45D/C50S was comparable to that of C45A/C50A (*7*), whereas C206S/C210S and C206A/C210A had 4−10-fold differences in reactivation efficiency, depending on the oxidation conditions. Considering that the purified, refolded mutants C206A/C210A and C206S/C210S share similar conformation and specific activity, it is presumed that this difference may originate from the effect of the substituents on the refolding pathways. This is supported by the following observations. First, during refolding, prolongation of the second stage at pH 8 had little effect on the reactivation of the wild-type and C206A/C210A prochymosin but brought about an enhancement of reactivation for C206S/C210S. Previously, we have demonstrated that the formation and adjustment of native tertiary structure of prochymosin mainly occurs at the second stage of refolding (*4*). It follows that, compared with Ala, Ser appears less favorable for correct

[4] Guo, R., Zhang, Y., and Yang, K., unpublished observations.

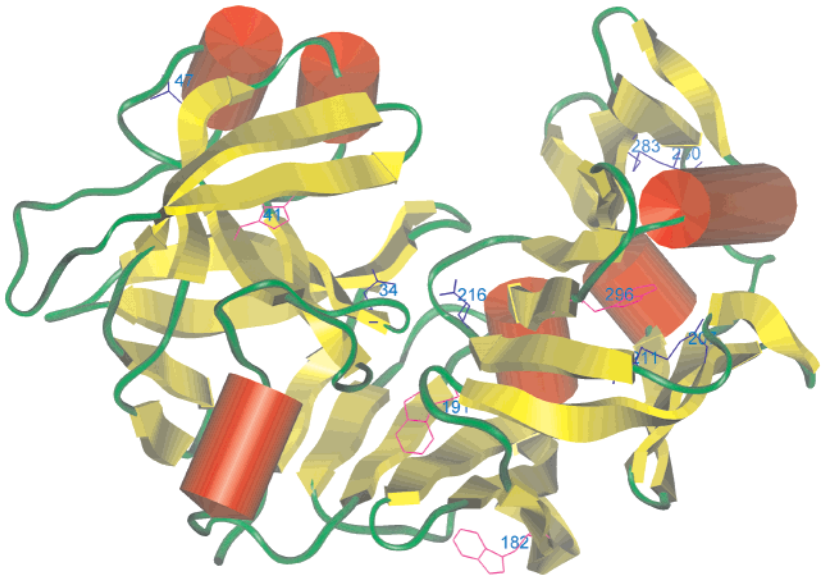

FIGURE 6: Schematic representation of recombinant bovine chymosin. The figure was generated using the program Insight II based on PDB code 1cms. Three disulfide bonds Cys47(corresponding to position 45 in pepsin numbering, the same below)−Cys52(50), Cys207-(206)−Cys211(210), Cys250(250)-Cys283(283), four tryptophan residues Trp41(39), Trp182(181), Trp192(190), Trp296(300), and two active site aspartates Asp34(32) and Asp216(215) are shown.

interresidue interactions to acquire native conformation during refolding. Second, GSH/GSSG can boost the reactivation of C206A/C210A dramatically, but fails to do so for C206S/C210S. Considering that the nonrandom conformation of a polypeptide has a great effect on the efficiency of thiol/disulfide exchange and on the formation of particular disulfide bonds, it is reasonable to assume that the failure is likely due to the improper conformations adopted during C206S/C210S refolding, which may make some SH groups and/or disulfide bonds inaccessible to GSH/GSSG or disfavor the formation of native disulfide bonds.

On the basis of the above analysis it is reasonable to consider position 206 and 210 as key sites for prochymosin refolding. To further determine the refolding pathways of Cys206−Cys210 deficient analogues and to characterize their refolding intermediates will facilitate understanding of the mechanism of prochymosin refolding.

Contribution to the Conformation of Prochymosin. Spectroscopic analyses indicated that removal of Cys45−Cys50 and Cys206−Cys210 individually did not change prochymosin secondary structure but cause a decrease and an increase of fluorescence intensity, respectively. Prochymosin has four tryptophan residues, Trp39 (pepsin numbering) is located in the N-terminal domain and close to Cys45−Cys50 and the others are in C-terminal domain, in which Cys206−Cys210 resides. It follows that deletion of Cys45−Cys50 may make Trp39 more exposed, while deletion of Cys206−Cys210 make at least one of the other tryptophan residues less exposed.

Urea-induced unfolding equilibrium studies revealed that Cys45−Cys50 and Cys206−Cys210 stabilized the conformation of prochymosin to almost the same extent. The $\Delta\Delta G(H_2O)$, the differences between the stabilization energy of wild-type prochymosin and that of C45A/C50A or that of C206A/C210A were estimated to be 2.8 and 2.1 kJ/mol, respectively. Destabilization of protein due to deletion of disulfide bonds has been attributed in part to a stabilization of its unfolded state resulting from the increase of loop entropy. The change in the free energy of the unfolded state is related to the size of the loop between the relevant cysteine residues (*26*). Since there is only one residue difference between the Cys45−Cys50 loop and the Cys206−Cys210 loop, the fact that they have almost the same contribution to prochymosin stability is reasonable.

Computer graphics modeling has indicated that there is some possibility to form hydrogen bond between 45D and 50S and between 206S and 210S and in turn to compensate for the reduction of the stabilization energy of prochymosin due to deletion of corresponding disulfide linkage. However, experiment data revealed that the stabilization energies of C45D/C50S and C206S/C210S were 1.3 and 2.7 kJ/mol lower than their counterpart C45A/C50A and C206A/C210A, respectively. Assuming by analogy with chymosin that the disulfide bridges between Cys45 and Cys50 and between Cys206 and Cys210 are buried in prochymosin molecule, the further destabilization by the polar residue substituents should be not surprising. It is generally believed that the hydrophobic environment in a protein is favorable for the hydrophobic interaction between pairwise Ala and unfavorable for the side-chain interactions between polar residues.

Contribution to the Catalytic Activity of Chymosin. Chemical modification studies indicated that the disulfide Cys250−Cys283 of bovine chymosin was more sensitive to chemical modification and could be selectively reduced and mercurated and that the reduced and mercurated chymosin remained 74.3 and 78.6% activity relative to that of the unmodified enzyme, respectively (*6*), demonstrating that this disulfide is not directly involved in the catalytic function of chymosin. On the other hand, site-directed mutagenesis studies revealed that deletion of Cys45−Cys50 or Cys206−Cys210 did not influence the specific milk-clotting activity significantly but affected the kinetic parameters of general proteolysis. For example, C45A/C50A mutation resulted in 3-fold decrease in both k_{cat} and K_m (*7*), whereas C206A/C210A mutation caused an opposite effect, showing higher

k_{cat} and K_m values compared with those of the wild-type chymosin.

Interestingly, by examining the structure of chymosin some clues to understanding the mechanism of different contributions of the disulfide bonds could be found. First, both the preferential modification (*6*) and X-ray analysis (*20*) demonstrate that Cys250−Cys283 is located on the surface of the molecule, far away from the active site, therefore, no direct contribution to the catalytic function is understandable. Second, as described above, deletion of Cys45−Cys50 resulted in a reduction of fluorescence intensity. Three-dimensional structure of chymosin (Figure 6) reveals that Trp39 (pepsin numbering, corresponding to Trp41 of chymosin numbering as indicated in Figure 6) is close to Cys45−Cys50 (pepsin numbering or Cys47−Cys52 of chymosin numbering) and partially buried in the molecule, therefore, it is conceivable that deletion of this disulfide bond may make Trp39 even more exposed. On the other hand, Groves et al. (*27*) reported that when chymosin was complexed with an inhibitor a hydrogen bond from Trp39 to Tyr75 was formed which appeared to stabilize the flap above the active-site aspartate residues. It is well accepted that this flap plays an important role in determining the enzymatic activity of aspartic proteases. It follows that Trp39 is related to the catalytic function. Taking these two aspects into consideration it is reasonable to assume that mutation at Cys45 and Cys50 induces a propagated conformational change in the enzyme, resulting in a perturbance of the local conformation around active-site cleft and in turn an alteration of the substrate specificity. Third, according to the chymosin conformation (Figure 6), among the three tryptophan residues located in the C-terminal domain, Trp181 and Trp190 (pepsin numbering, corresponding to 182 and 191 of chymosin numbering) are 17 and 18 Å away from the C_α of Cys210 (or Cys211 of chymosin numbering), respectively, and the former is the most exposed and the latter is the most buried, whereas Trp300 (pepsin numbering, corresponding to 296 of chymosin numbering) is partially exposed and the distances between two C_αs of Cys210 and Trp300 and between S_GS of Cys210 and NEI of Trp300 are estimated to be 7.42 and 3.78 Å, respectively. Meanwhile, Trp300 is adjacent to the 294−298 loop (pepsin numbering) which forms part of S_1' pocket (*28*). Consequently, Trp300 is much more likely than the other two tryptophan residues responsible for the increase of the fluorescence intensity due to mutation, indicative of the structural change around the substrate binding cleft.

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