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Increasing the hydrolysis constant of the reactive site upon introduction of an engineered Cys^{14}–Cys^{39} bond into the ovomucoid third domain from silver pheasant

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P14C/N39C is the disulfide variant of the ovomucoid third domain from silver pheasant (OMSVP3) introducing an engineered Cys^{14}–Cys^{39} bond near the reactive site on the basis of the sequence homology between OMSVP3 and ascidian trypsin inhibitor. This variant exhibits a narrower inhibitory specificity. We have examined the effects of introducing a Cys^{14}–Cys^{39} bond into the flexible N-terminal loop of OMSVP3 on the thermodynamics of the reactive site peptide bond hydrolysis, as well as the thermal stability of reactive site intact inhibitors. P14C/N39C can be selectively cleaved by *Streptomyces griseus* protease B at the reactive site of OMSVP3 to form a reactive site modified inhibitor. The conversion rate of intact to modified P14C/N39C is much faster than that for wild type under any pH condition. The pH-independent hydrolysis constant (K_{hyd}°) is estimated to be approximately 5.5 for P14C/N39C, which is higher than the value of 1.6 for natural OMSVP3. The reactive site modified form of P14C/N39C is thermodynamically more stable than the intact one. Thermal denaturation experiments using intact inhibitors show that the temperature at the midpoint of unfolding at pH 2.0 is 59 °C for P14C/N39C and 58 °C for wild type. There have been no examples, except P14C/N39C, where introducing an engineered disulfide causes a significant increase in K_{hyd}°, but has no effect on the thermal stability. The site-specific disulfide introduction into the flexible N-terminal loop of natural Kazal-type inhibitors would be useful to further characterize the thermodynamics of the reactive site peptide bond hydrolysis.

Keywords: circular dichroism; engineered disulfide bond; hydrolysis constant of the reactive site; inhibitory specificity; serine protease inhibitors; thermal stability

Introduction

Protein inhibitors of proteases play a vital role in many physiological processes by regulating the proteolytic activity of their target proteases [1–6]. The majority of such inhibitors characterized so far are directed toward serine proteases. Most of them act by a standard mechanism [1,3,6]. The reaction between enzyme and inhibitor can be represented in simplified form as follows:

$$E + I \rightleftharpoons C \rightleftharpoons E + I^* \quad (1)$$

where E is an enzyme, I and I* are the intact and modified (reactive site cleaved) inhibitors, respectively, and C is the stable complex. The inhibitors tightly bind to the active sites of their target proteases in a manner similar to that of substrates but are remarkably resistant to hydrolysis. Both forms of inhibitor are still functional when equilibrium has been reached. The hydrolysis constant of the reactive site (K_{hyd}), which is defined as [I*]/[I], depends only upon the inhibitor structure but not upon the enzyme used to catalyze the reaction. The K_{hyd} values for most protein substrates are high, but those of inhibitors are low [7]. In this way K_{hyd} is one of the important thermodynamic parameters distinguishing protein inhibitors from substrates.

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Abbreviations used: *AEI, an elastase inhibitor from Anemonia sulcata; BPTI, bovine pancreatic trypsin inhibitor; CHT, α-chymotrypsin; CSH-motif, cystine-stabilized α-helical motif; ^{1}H NMR, proton nuclear magnetic resonance; Native PAGE, polyacrylamide gel electrophoresis under nondenaturing conditions; OMXXX3, ovomucoid third domain from species XXX; code of birds species; IPF, Indian peafowl; TKY, turkey; SVP, silver pheasant; PPE, porcine pancreatic elastase; SGPA and SGPB, Streptomyces griseus proteases A and B; T_m, the temperature at the midpoint of unfolding.*

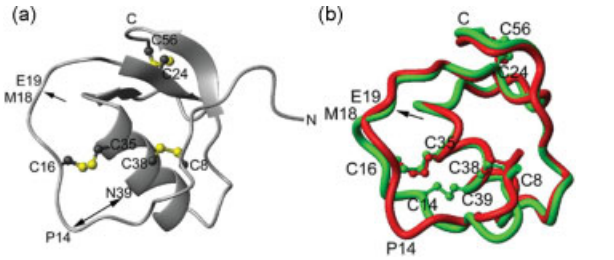

Figure 1. Molecular structure of OMSVP3 (A) in a crystal state [10]. The reactive site residues (Met^{18} and Glu^{19}) and amino and carboxyl termini (N and C, respectively) are shown. Disulfide bonds (Cys^{8}–Cys^{38}, Cys^{16}–Cys^{35}, and Cys^{24}–Cys^{56}) are ball-and-stick representations. Double-directed arrow points out two residues forming an engineered disulfide bond in P14C/N39C. Schematic representation of main chain folding of wild type OMSVP3 (red) overlaid with that of P14C/N39C (green) (B) [17]. Note that the six N-terminal residues of wild type and P14C/N39C have been omitted from the models. These figures are generated by MOLMOL [30].

Some of the best studied protein inhibitors obeying the standard mechanism are the ovomucoid third domains, which are classified in the Kazal-type inhibitor family [8,9]. Structures of ovomucoid third domains and their complexes with target proteases have been determined [10–13]. The molecular structure of OMSVP3 is shown in Figure 1A. The remarkable feature of this type of inhibitors is that some conformational flexibility in the reactive site loop of the free inhibitor might be related to the broad specificity [14]. OMSVP3 can potently inhibit chymotrypsin, subtilisin, and elastase [8]. The K_{hyd} value for OMSVP3 is reported to be 1.60 at pH 6 [15]. As the value is near unity, both intact and reactive site modified forms are approximately equally effective as inhibitors. The effects of a single residue substitution in ovomucoid third domain on the K_{hyd} value have been rationalized in terms of residue–residue interactions that are affected by the reactive site hydrolysis [8,15]. Song and Markley [16] reported the NMR study on the role of backbone structure and dynamics in controlling the K_{hyd} value of OMTKY3 from turkey. However, the structural basis of the lower values of K_{hyd} has only been studied by using naturally occurring variants. Thus, the comparative study using many designed variants, including the non-natural disulfide-introducing ones, would be worthwhile in elucidating the structural basis of inhibitory activity.

P14C/N39C is the disulfide variant of OMSVP3 introducing an engineered Cys^{14}–Cys^{39} bond near the reactive site based on the sequence homology between OMSVP3 and ascidian trypsin inhibitor with the CSH-motif [17,18]. Introduction of the non-natural disulfide into the reactive site loop has been applied to serpins [19], but not to a small-sized and compact inhibitor such as the ovomucoid third domains other than P14C/N39C. In the study elucidating the structural basis of the broad specificity of ovomucoid, we have unexpectedly found that P14C/N39C loses almost all inhibitory activity toward PPE, while it retains the potent inhibitory activities toward CHT, SGPA and SGPB [17]. Schematic representation of main chain folding of wild type OMSVP3 overlaid with that of P14C/N39C are shown in Figure 1B. Similar functional change has been also observed in the CSH-motif-introducing AEI analog [20], where AEI is a 'non-classical' Kazal-type elastase inhibitor from *Anemonia sulcata*. Such dramatic inhibitory change upon introduction of an engineered disulfide bond has not been reported before in the naturally occurring ovomucoid inhibitors. Thus, we would like to further characterize the thermodynamic properties of the variant.

In the present study, we first examine its susceptibility to proteases and then determine the K_{hyd} value. Second, the effect of introduction of an engineered Cys^{14}–Cys^{39} bond on the thermal stability is examined. The results show that the K_{hyd} value of P14C/N39C is significantly higher than that of natural OMSVP3, even though both inhibitors have almost the same thermal stability. Finally, the structural basis of the increased K_{hyd} value of P14C/N39C is discussed.

Materials and Methods

Materials

Recombinant wild type OMSVP3, P14C/N39C and P14A were obtained, as previously described [17]. Serine proteases, including CHT, PPE, SGPA, and SGPB were also obtained, as described in the previous paper [17]. The numbering system used in this study is according to Laskowski *et al*. [9].

Susceptibility to Protease

SGPB can specifically convert intact inhibitor of ovomucoid third domain to modified inhibitor at pH values as low as pH 1 [15]. Thus, SGPB was mainly used as a target protease in this study. The unusual pH-dependency of the conversion rate is thought to be an index for discriminating potent inhibitors from mere substrates. Thus, wild type, P14C/N39C and P14A (60 μM) were separately incubated at 25 °C with SGPB (3 μM) in 0.1 M Tris-HCl, pH 7.8 or in 0.1 M sodium formate buffer, pH 3.5 The inhibitors were also incubated with CHT and SGPA for comparison. For determination of a pH-independent K_{hyd} value (K_{hyd}°) inhibitors were incubated with SGPB in 0.1 M 2-(*N*-morpholino)ethansulfonic acid–NaOH, pH 6.0, according to Ardelt and Laskowski [15]. The digests were analyzed at 4 °C on Native PAGE with 5% concentration gel and 15% separation gel using Laemmli's buffer system [21]. The PAGE method used in this study is not accurate as the HPLC method using a Mono Q column [15], although the former method has an advantage of simplicity. The protein band was stained with Coomassie Brilliant Blue R-250. The density of stained band on the dried gel was scanned, and the image data, which had been processed by Scion Image for Windows, were used to estimate the concentrations of I and I*. For determination of the cleaved site, the protein band on a gel was electrotransferred onto polyvinylidenedifluoride membrane and the N-terminal amino acid sequence analysis was carried out, as previously described [17].

CD Spectroscopy and Thermal Stability

P14C/N39C and wild type OMSVP3, both of which have the reactive site intact peptide bonds, were used as samples in this section. The CD spectra were measured on a Jasco J-720 CD spectropolarimeter equipped with a thermoelectric temperature controller. It has been reported by Swint and Robertson that OMTKY3, a highly analogous inhibitor to OMSVP3, is highly stable over a wide range of pH and temperature [22]. Therefore, far-UV CD spectra were collected at pH 2.0 on unbuffered samples containing 0.1 mg/ml using a cell with 1 mm pathlength to obtain more complete denaturation transitions. To obtain T_m, temperature was increased at a rate of 2 °C/min between 20 and 80 °C while monitoring the ellipticity at 222 nm. After the raw data of mean residue ellipticity were plotted against temperature (T) in Kelvin, a fraction of denatured form (f_d) *versus* temperature curves were generated, considering slopes of

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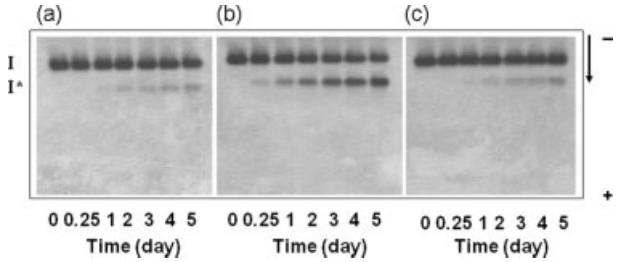

Figure 2. Limited proteolysis of OMSVP3 with SGPB at pH 7.8. Wild type OMSVP3 (A), P14C/N39C (B) and P14A (C) (60 μM each) were incubated with SGPB (3 μM) at 25 °C and pH 7.8 for up to 5 day and aliquots (each containing 3 μg of the inhibitor) were withdrawn at intervals and subjected to Native PAGE at 4 °C. Bands I and I* represent intact and modified inhibitor, respectively. A faint band was observed in the concentration gel (data not shown) under any digestion condition, which may correspond to either EI or EI* or both.

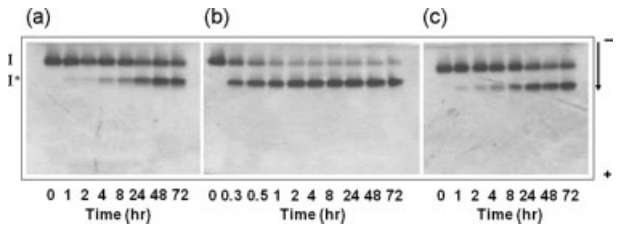

Figure 3. Limited proteolysis of OMSVP3 with SGPB at pH 3.5. Wild type OMSVP3 (A), P14C/N39C (A) and P14A (C) were incubated with SGPB at 25 °C and pH 3.5 for up to 72 h, and aliquots were subjected to Native PAGE, as described in legend of Figure 2.

the native and denatured baselines [23]. The data were fitted to the equation:

$$f_d = \frac{\exp\{(\Delta H_m/R)((1/T_m) - (1/T))\}}{1 + \exp\{(\Delta H_m/R)((1/T_m) - (1/T))\}} \quad (2)$$

where R is the gas constant, and ΔH_m is the enthalpy of unfolding at T_m. To obtain the T_m and ΔH_m values curve fitting was performed using nonlinear regression analysis (KaleidaGraph for Windows, Synergy Software).

Results and Discussion

Susceptibility to Protease

One of the striking features of protein inhibitors is that the hydrolysis of the reactive site by a target protease proceeds extremely slowly at neutral pH and does not proceed to completion [7]. Wild type, P14C/N39C and P14A were separately incubated with SGPB at pH 7.8 and at pH 3.5. Figures 2 and 3 show the respective proteolysis patterns of the three inhibitors on Native PAGE. A gradual increase in the relative intensity of a fast moving band could be observed for all. N-terminal sequence analysis of a faster moving band in a SGPB digest of P14C/N39C confirmed that this component corresponds to the modified inhibitor (I*) cleaved at the reactive site (Met18-Glu19) for OMSVP3. The reactive sites of three inhibitors were hydrolyzed more rapidly at pH 3.5 than at pH 7.8, as expected [15]. Further, the conversion rate of I to I* for P14C/N39C at pH 7.8, which is evaluated from the initial slope of the reaction, is approximately seven times faster than that for wild type. Similar cleavage patterns were obtained at pH 3.5 (Figure 3),

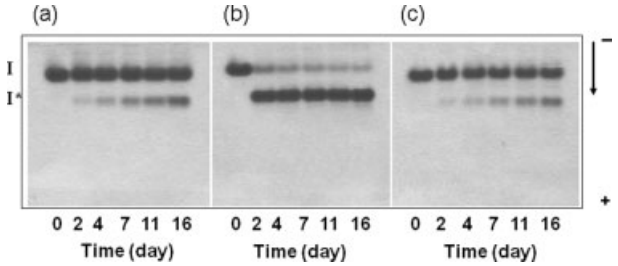

Figure 4. Limited proteolysis of OMSVP3 with SGPB at pH 6.0. Wild type OMSVP3 (A), P14C/N39C (B) and P14A (C) were incubated with SGPB at 25 °C and pH 6.0 for up to 16 day, and aliquots were subjected to Native PAGE, as described in legend of Figure 2. The K_{hyd}° value for P14C/N39C was calculated by measuring the densities of bands for the modified and intact inhibitor after the 16 day-incubation. The K_{hyd}° value is obtained from a single experiment. Thus, a standard deviation value is not given.

where the rate of P14C/N39C is approximately 50 times faster than that for wild type. P14A exhibits essentially the same susceptibility toward proteases as wild type. The above results suggest that P14C/N39C may still have some specific barrier against hydrolysis under neutral pH conditions. It should be noted further that the hydrolysis of the reactive site of P14C/N39C does not proceed to completion under the digestion conditions used.

Hydrolysis Constant (K_{hyd}) of the Reactive Site for the Inhibitor

As expected from Eqn (1), most protein inhibitors are still functional when equilibrium has been reached. K_{hyd} is one of the important thermodynamic parameters describing equilibrium. K_{hyd} depends on pH [15], which can be explained in terms of a pH-independent K_{hyd} term (K_{hyd}°) plus the proton dissociation constants of the newly formed amino and carboxylate groups at the cleavage site. For estimation of the K_{hyd}° value of P14C/N39C we followed the time course of the SGPB digestion of inhibitors at pH 6.0 (Figure 4). Equilibrium seems to be reached after a 7 day-incubation for P14C/N39C under the conditions used. The K_{hyd}° value for P14C/N39C was calculated by measuring the densities of bands for the reactive site modified inhibitor and intact inhibitor after the 16 day-incubation. Then we could obtain the composition values of 84.5% reactive site modified inhibitor and 15.5% intact inhibitor at equilibrium. The K_{hyd}° value thus calculated is approximately 5.5 for P14C/N39C, which is significantly higher than the value of 1.6 for natural OMSVP3 [15]. Thus, the reactive site modified form is thermodynamically more stable than the intact form for P14C/N39C. In this study we could not determine the respective K_{hyd}° values for wild type OMSVP3 and P14A exactly, because equilibrium would not have been reached even after a 16 day-incubation (Figure 4).

CD Spectra and Thermal Stability

Disulfide bridges can contribute to the stability of proteins [24], and many attempts have been made to increase protein stability by introducing novel disulfide bridges [25,26]. It is thus interesting to examine the effect of introducing an engineered Cys^{14}–Cys^{39} bond into a small-sized and compact OMSVP3 molecule on the thermal stability. A series of CD spectra of both inhibitors were measured at temperatures in the thermal transition zone in the far- and near-UV range (Figure 5). The spectrum of P14C/N39C is slightly red-shifted in the region of 220–260 nm when compared with that of wild type. Isodichroic points are clearly observed at

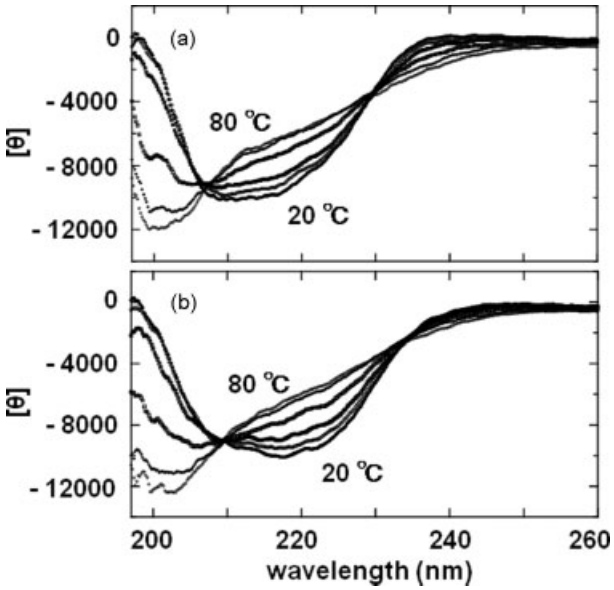

Figure 5. Thermal denaturation of wild type OMSVP3 (A) and P14C/N39C (B). CD spectra were measured at 20 °C (●), 40 °C (○), 50 °C (■), 60 °C (□), 70 °C (◊), and 80 °C (⧫) on 0.1 mg/ml of samples using 1 mm-pass length cell at pH 2.0. The units of [θ] are deg·cm^2·dmol^{-1}.

210 and 235 nm for P14C/N39C, while those for wild type are at 206 and 230 nm. Thus, the thermal denaturation is consistent with a two-state process in the temperature range used. It is easily estimated that both inhibitors have very similar thermal stability. The CD intensity at 222 nm was then monitored continuously as a function of temperature up to 80 °C. A fraction of denatured form (f_d) *versus* temperature curves were generated (data not shown). The T_m values obtained after curve fitting are 58 °C for wild type and 59 °C for P14C/N39C, both of which are nearly equal to the reported value of 58.6 °C for natural OMTKY3 [22]. The enthalpies of unfolding at T_m are 17.2 and 16.7 kcal/mol for wild type and P14C/N39C, respectively.

A notable conclusion in this section is that the introduction of an engineered Cys^{14}–Cys^{39} bond into a small-sized and compact OMSVP3 molecule has almost no effect on the thermal stability.

Comparison of P14C/N39C with the Naturally Occurring Ovomucoid Variants and Other Serine Protease Inhibitors

A summary of the parameters characterizing wild type and P14C/N39C is shown in Table 1. Introduction of an engineered Cys^{14}–Cys^{39} bond into OMSVP3 caused an inhibitory specificity change and a significant increase in K_{hyd}°, while it had almost no effect on the thermal stability. The thermally stabilizing effect expected by the introduction of the non-natural disulfide seems to have been cancelled either by the loss of the hydrogen bonds between Asn^{39} and Lys^{13} in the natural inhibitor [13] or by the possible strain in the intact loop ranging from Cys^8 to Cys^{16} associated with the closure of the novel disulfide bridge [17], or by both.

Here, we consider a significant increase in K_{hyd}° comparing it with several naturally occurring ovomucoid variants. Ardelt and Laskowski [15] have measured the K_{hyd} values at pH 6.0 in 42 variants of avian ovomucoid third domains. The reported values range from 0.4 to approximately 35. The effects of a single residue substitution on K_{hyd}°, as well as the denaturation equilibrium constant (K_{den}), have been rationalized in terms of residue–residue interactions that are affected by the reactive site hydrolysis [15]. There are notable examples where a single amino acid substitution could cause a significant increase in K_{hyd}°. Among them chestnut belied scaled quail (OMSCQ3N) and blue scaled quail (OMSCQ3S) are noted here, because they not only exhibit an inhibitory specificity similar to P14C/N39C, but they also have mutation site(s) at the unique position(s) [8]. OMSCQ3N ($K_{hyd}^{\circ} = 2.5$) has a substitution of P_4 (Ala^{15}) by Asp on the N-terminal loop (Cys^8-Thr^{17}) of OMTKY3 ($K_{hyd}^{\circ} = 1.0$), and OMSCQ3S ($K_{hyd}^{\circ} = 6.0$) has an additional substitution of P_{15}' (Asn^{33}) by Ser on the central helix [9,15]. Unfortunately, the tertiary structures of the two inhibitors have not yet been analyzed. Thus, we performed a model-building study in order to elucidate the structural basis of the increase in K_{hyd}° by using molecular operational environment (MOE) software from Chemical Computing Group. Briefly, the putative complex between OMSCQ3N and SGPB was first constructed by substitution of Ala^{15} in OMTKY3 by Asp on the basis of the X-ray crystal structure of OMTKY3–SGPB (PDB : 3SGB). The complex between OMSCQ3S and the enzyme was then constructed by sequential substitution of Asn^{33} by Ser. Energy minimization of the enzyme–inhibitor complex was accomplished by using the energy minimization protocol of MOE with CHARMM27 force-field. The results show that the substitution of Ala^{15} in OMTKY3 to Asp in OMSCQ3N does not break the hydrogen bonds between the side chain of Asn^{33} on the central helix and the backbone oxygen of Thr^{17} and that of Glu^{19}, which greatly contribute to the stability of the reactive site of OMTKY3. At the present time, we are unable to discuss the reason for a 2.5-fold increase in K_{hyd}° of OMSCQ3N. Contrary to this, the respective hydrogen bonds are broken in OMSCQ3S. The side chain of Ser^{33} could not interact with the backbone oxygen of Thr^{17} and that of Glu [19]. This may cause the additional enhanced K_{hyd}° value of OMSCQ3S, as previously suggested by Ardelt and Laskowski [15]. Interestingly, in the context of the present study, P14C/N39C ($K_{hyd}^{\circ} = 5.5$) has an engineered disulfide bond linking the N-terminal loop and the central helix. Furthermore, several NMR structures of P14C/N39C (PDB: 1iy6) showed the existence of two hydrogen bonds between the side chain of Asn^{33} and the backbone oxygen of Cys^{16} (instead of Thr^{17} in the case of natural OMSVP3) and that of Glu^{19} near the reactive site [17]. The upfield and downfield shift (6.26 and 8.19 ppm, respectively) of the side chain $H^{N\delta}$ protons of Asn^{33} confirmed the existence of the hydrogen bonds described above (BMRB accession No. 5470). It is noteworthy that the reason for the significant increase in K_{hyd}° may be different between P14C/N39C and OMSCQ3S. Thus, P14C/N39C is a very unique variant of ovomucoid third domains. There have been no examples, except P14C/N39C, where an engineered disulfide introduction causes a significant increase in K_{hyd}°, but no effect on the thermal stability.

Next, we consider the significant increase in K_{hyd}° from the viewpoint of the role of backbone structure and dynamics. The role of backbone structure and dynamics in controlling the K_{hyd} value of ovomucoid third domains has been reported by NMR [16]. In the study special attention has focused on the difference between OMTKY3 ($K_{hyd}^{\circ} = 1.0$) and OMIPF3 from Indian peafowl ($K_{hyd}^{\circ} = 5.15$), which differs from OMTKY3 by the substitution of P_2'-Tyr^{20} by His. Replacing Tyr by His further leads to an approximately fourfold increase in K_{den} [15], indicating a significant decrease in thermal stability. The NMR results using the reactive site intact and cleaved inhibitors indicated that the difference in K_{hyd}° arises from different intramolecular interactions within the reactive site intact inhibitors in a region of each protein that becomes

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Table 1. Summary of the parameters characterizing wild type OMSVP3 and P14C/N39C

	K_i (M)				
	CHT	PPE	K_{hyd}°	T_m	Susceptibility
Wild type OMSVP3	6.5×10^{-12}[a]	4.3×10^{-11}[a]	1.6[b]	58 °C	low
P14C/N39C	6.6×10^{-11}[a]	4.8×10^{-6}[a]	5.5	59 °C	High

[a] Data from the literature [17].
[b] Data from the literature [15].

disordered upon reactive site cleavage. Further internal dynamics have also been examined by NMR using *Cucurbita maxima* trypsin inhibitor (CMTI)-III of the squash family ($K_{hyd} = 2.4$) and CMTI-V of the potato I family ($K_{hyd} = 9$), both of which have different binding loop-scaffold interactions [27]. The results suggested that the difference in K_{hyd} may, in part, be attributed to different internal dynamics of newly formed C- and N-terminal in the reactive site cleaved inhibitors. Resynthesis of the P_1–P_1' bond between Arg^5 and Ile^6 of CMTI-III seems to be better favored by a more ordered P_1 residue of the newly formed C-terminal gaining a smaller magnitude of flexibility due to the Cys^3–Cys^{20} crosslink and by a more flexible P_1' residue of the newly formed N-terminal most likely due to lack of an interaction between the P_1' residue and the scaffold in the reactive site cleaved form of the inhibitor, leading to a lower K_{hyd} value. Recently, a high-resolution crystal structure of a complex formed between a reactive site cleaved form of BPTI (I*) and a catalytically inactive trypsin variant with the catalytic Ser residue (Ser^{195}) replaced with Ala has been resolved, and it has been compared with the structure of a complex formed between a reactive site intact BPTI and an active trypsin in detail [28]. The P_1' residue of the reactive site cleaved inhibitor lies in just the position expected for the product of the first step in the catalytic reaction and is poised to reverse this step by attacking the acyl-enzyme carbonyl. The result is coincident with the previous findings that BPTI is highly resistant to tryptic attack and the K_{hyd} value is very small [7,29].

In case of P14C/N39C, the dramatic conformational changes of the N-terminal loop (Cys^8 - Thr^{17}) have been observed upon introduction of the engineered Cys^{14}–Cys^{39} bond (Figure 1B). Thus, the loop of P14C/N39C is expected to reduce flexibility compared with that of wild type. We have also observed a very similar conformational change of the N-terminal loop (Pro^5-Cys^8) in the CSH-motif-introducing AEI analog, which is chemically synthesized when a Cys^4–Cys^{34} bond was changed to a Cys^6–Cys^{31} bond [20]. The heteronuclear single quantum correlation spectra of wild type AEI and the AEI analog suggested that a conformational exchange on time scales of milliseconds to microseconds may occur in the N-terminal loop region of wild type, while it may be suppressed in the analog. The tertiary structure of the reactive site modified form for P14C/N39C has not been analyzed yet, but the higher K_{hyd}° value suggests that the conformational rigidity of the N-terminal loop and some unfavorable strain produced in the reactive site with the closure of the novel disulfide bond could have been possibly released upon hydrolysis of the reactive site. Probably this may not be poised to advance the I* to I reaction. It is interesting to know the structure of a complex formed between a reactive site cleaved form of P14C/N39C and a catalytic inactive CHT variant.

Conclusions

P14C/N39C, like wild type, is selectively hydrolyzed by SGPB at the reactive site for OMSVP3. The disulfide variant exhibits significant enhanced susceptibility to protease under the pH conditions used. P14C/N39C causes an increase by a factor of 3.4 in K_{hyd}°, while it has almost the same thermal stability. The stabilizing effect expected from the novel disulfide bond seems to have been cancelled either by the loss of the hydrogen bonds between Asn^{39} and Lys^{13} in the natural inhibitor or by the possible strain associated with the closure of the novel disulfide bridge, or by both. In P14C/N39C some unfavorable strain produced in the reactive site loop with the closure of the novel disulfide bridge may have been released upon hydrolysis of the reactive site. There have been no examples, except P14C/N39C, where an engineered disulfide causes a significant increase in K_{hyd}°, but has no effect on the thermal stability. The site-specific introduction of an engineered disulfide bond into the flexible N-terminal loop of natural inhibitor would be useful to further characterize the structure-function relationship of the natural inhibitor such as Kazal-type inhibitors.

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