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Denaturation and Unfolding of Human Anaphylatoxin C3a:

An unusually low covalent stability of its native disulfide bonds

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Abstract

The complement C3a anaphylatoxin is a major molecular mediator of innate immunity. It is a potent activator of mast cells, basophils and eosinophils and causes smooth muscle contraction. Structurally, C3a is a relatively small protein (77 amino acids) comprising a N-terminal domain connected by 3 native disulfide bonds and a helical C-terminal segment. The structural stability of C3a has been investigated here using three different methods: Disulfide scrambling; Differential CD spectroscopy; and Reductive unfolding. Two uncommon features regarding the stability of C3a and the structure of denatured C3a have been observed in this study. **(a)** There is an unusual disconnection between the conformational stability of C3a and the covalent stability of its three native disulfide bonds that is not seen with other disulfide proteins. As measured by both methods of disulfide scrambling and differential CD spectroscopy, the native C3a exhibits a global conformational stability that is comparable to numerous proteins with similar size and disulfide content, all with mid-point denaturation of $[GdmCl]_{1/2}$ at 3.4-5M. These proteins include hirudin, tick anticoagulant protein and leech carboxypeptidase inhibitor. However, the native disulfide bonds of C3a is 150-1000 fold less stable than those proteins as evaluated by the method of reductive unfolding. The 3 native disulfide bonds of C3a can be collectively and quantitatively reduced with as low as 1 mM of dithiothreitol within 5 min. The fragility of the native disulfide bonds of C3a has not yet been observed with other native disulfide proteins. **(b)** Using the method of disulfide scrambling, denatured C3a was shown to consist of diverse isomers adopting varied extent of unfolding. Among them, the most extensively unfolded isomer of denatured C3a is found to assume beads-form disulfide pattern, comprising Cys^{36}-Cys^{49} and two disulfide bonds formed by two pair of consecutive cysteines, Cys^{22}-Cys^{23} and Cys^{56}-Cys^{57}, a unique disulfide structure of polypeptide that has not been documented previously.

Keywords

Conformational stability of C3a; Denaturation of C3a; Unfolding of C3a; Method of disulfide scrambling; Scrambled isomers of C3a; Reductive unfolding of native C3a

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INTRODUCTION

The complement system is a major arm of the innate immune system. One of the major biological consequences of complement activation is the generation of three small cationic peptides collectively referred to as complement anaphylatoxins. One of the anaphylatoxins, C3a, is a potent activator of mast cells, basophils, and eosinophils and causes smooth muscle contraction and increases vascular permeability *(1,2)*. The human C3a molecule is comprised of 77 amino acid residues *(3)*, is highly cationic, and contains six cysteine residues that form three intra-chain disulfide bonds. This latter feature has been thought to impart stability to the molecule. For example, extremes of pH (1 to 13 ranges) and heat (56°C for 30 min) do not destroy C3a biological activity. Circular dichroism (CD) spectra indicated that 40 to 45 % of the molecule is of regular helical structure and that very little beta-structure is present *(4)*. Confirmation of the high helical content was provided by crystallographic analysis, which indicated a 56 % alpha-helical content *(5)*. The crystal structure of C3a in combination with NMR studies *(6)* have indicated that C3a assumes a "drumstick" or "dagger" shape that contains a rigid disulfide-linked core portion (residues 17-66) comprised of three anti-parallel helical structures (helix 2, residues 17-28; helix 3, residues 35-43, and helix 4, residues 47-66) (Fig. 1). The N-terminal end of C3a (residues 1-15), which also contains a helical segment (helix 1, residues 8-15), exhibits a high degree of flexibility with helix 1 folding back against the core portion of the molecule. The C-terminal or effector region of C3a (residues 69-77) assumes no regular conformation and may either remain flexible or fold back onto the helical portion in a pseudo-beta turn conformation.

In this report, the structural stability of C3a has been investigated using three available techniques, including: **(a)** The conventional CD spectroscopy *(7)*; **(b)** The method of disulfide scrambling *(8,9)*, and **(c)** The method of reductive unfolding *(10-13)*. Results obtained from these studies allow us to attain the following goals: **(a)** To quantify the extent of unfolding of denatured C3a; **(b)** To compare the conformational stability of C3a in the absence and presence of thiol catalyst; **(c)** To evaluate the covalent stability of the native disulfide bonds of C3a in the absence of denaturant; and **(d)** To assess the interplay between the global conformational stability of C3a and the covalent stability of its native disulfide bonds.

MATERIALS and METHODS

Materials

Native C3a was isolated from normal human serum by activation of C3 with the natural alternative pathway C3 convertase in the presence of carboxypeptidase inhibitor. The C3a was isolated using cation exchange chromatography and gel filtration followed by high resolution cation exchange chromatography to separate C3a desArg from C3a as previously described *(14)*. Recombinant hirudin and tick anticoagulanrt peptide (TAP) were kindly provided by Novartis AG (Basel, Switzerland). The proteins were shown have purity of greater than 96%, as judged by the HPLC profiles and MALDI mass analysis. AspN endo-peptidase, dithiothreitol, 2-mercaptoethanol, urea, GdmCl and GdmSCN all with purities of greater than 99%, were purchased from Sigma.

Denaturation and unfolding of C3a analyzed using the method of disulfide scrambling

The native C3a (0.5 mg/ml) was dissolved in Tris-HCl buffer (0.1 M, pH 8.4) containing 0.2 mM of 2-mercaptoethanol and selected concentrations of denaturants (urea, GdmCl, or GdmSCN). The reaction was performed at 23°C for 20 h to ensure the equilibrium of the conversion between the native and denatured isomers. The denatured protein was quenched with an equal volume of 4% aqueous trifluoroacetic acid, and directly analyzed by reverse-phase HPLC using the following conditions. Solvent A was water containing 0.1%

trifluoroacetic acid. Solvent B was acetonitrile/water (9:1 v/v) containing 0.086% trifluoroacetic acid. The gradient was 25-50% B linear in 50 min. The flow rate was 0.5 ml/min. Column was Zorbax 300SB C18 for peptides and proteins, 4.6 mm × 5 μm. Column temperature was 23°C. The denatured isomers of C3a were collectively designated as X-C3a to distinguish them from native C3a (N-C3a).

Circular dichroism (CD) measurements of 'disulfide-intact' and 'disulfide-scrambling' denaturation

Native C3a (0.1 mg/ml in 0.1 M Tris-HCl, pH 8.4) was incubated for 16 h at 23°C with of different concentrations of GdmCl (1 M - 8 M). The experiments were carried out both in the absence and in the presence of 2-mercaptoethanol (0.2 mM). Control (blank) samples without C3a were also processed in parallel. Far UV CD spectra of the incubated samples were recorded from 260-200 nm. Each spectrum was an average of five scans. The spectra of controls were subtracted from the spectra of their respective C3a samples and the ellipticities at 222 nm were used to calculate the extent of C3a denaturation.

Plotting of denaturation curves of C3a

For the method of disulfide scrambling, the denaturation curves were drawn by plotting the fraction of C3a denatured at respective concentrations of denaturants. Calculation of denatured fraction was based on X-C3a/(XC3a + N-C3a) at each concentration of denaturant. Quantification of N-C3a and X-C3a was done by integration of the HPLC peak areas. For the method of CD measurements, fraction denatured (F_D) at each concentration of GdmCl was calculated by the following relation and plotted against respective GdmCl concentrations, $F_D = (\theta_N - \theta_i)/ (\theta_N - \theta_D)$, where θ_N and θ_D are the ellipticities at 222 nm for the native form (0M GdmCl) and fully denatured form (8M GdmCl) respectively, and θ_i is the observed ellipticity at 222 nm at a given concentration of GdmCl.

Plotting of unfolding curves of C3a

Unfolding curves of C3a were determined by the yield of X-C3a-a versus respective concentration of denaturant. The yield of X-C3a-a (%) was derived from the calculation of X-C3a-a/(X-C3a + N-C3a) at respective concentrations of denaturant.

Reductive unfolding in the absence of denaturant

N- and X-proteins (0.5 mg/ml) of C3a, hirudin, LCI and TAP were dissolved in the Tris-HCl buffer (0.1 M, pH 8.4) containing DTT (1 mM) in the absence of denaturant. The reduction was carried out at 23°C for up to 5 min. Intermediates of reduction were quenched with an equal volume of 4% aqueous trifluoroacetic at different time points and were directly analyzed by HPLC.

Characterization of disulfide structure of the most predominant isomer of denatured C3a

X-C3a-a was purified from HPLC and freeze-dried. The sample (10 μg) was treated with 0.5 μg of Asp-N endo-protease in 25 μl of ammonium bicarbonate solution (50 mM, pH 7.9). Digestion was carried out at 37°C for 16 h. Peptides were then isolated by HPLC and analyzed by both mass spectrometry and Edman sequencing in order to identify peptides that contain disulfide bonds.

Amino acid sequencing, mass spectrometry and CD measurement

Amino acid sequences of disulfide containing peptides were analyzed by automatic Edman degradation using a Perkin-Elmer Procise sequencer (Model 494) equipped with an online PTH-amino acid analyzer. The molecular mass of disulfide containing peptides were

determined by MALDI-TOF mass spectrometer (Perkin-Elmer Voyager-DE STR) using 2,5-dihydroxybenzoic acid as matrix. Molecular mass of analyzed peptides were calibrated by the following standards. Bradykinin fragment (residues 1-7) (MH^+ 757.3997); Synthetic peptide P14R (MH^+ 1533.8582); ACTH fragment (residues 18-39) (MH^+ 2465.1989); and Insulin oxidized B-chain (MH^+ 3494.6513). CD spectra of C3a were measured in a 200 μL cuvette (1 mm light pass) using a Jasco J-715 spectropolarimeter. A full scan was performed for each sample from 260 nm to 200 nm.

RESULTS

Denaturation and unfolding of native C3a analyzed by the method of disulfide scrambling

Denaturation of N-C3a was performed in the presence of 2-mercaptoethanol (0.2 mM) and increasing concentrations of urea, GdmCl and GdmSCN. The process of denaturation was carried out for 20h in order to allow the reaction to reach equilibrium. Denatured C3a was then acidified and analyzed by HPLC. The chromatograms are shown in Fig. 2. Denaturation converts N-C3a to a mixture of fully oxidized (three disulfides) scrambled X-C3a. This conclusion is supported by the observation that vinylpyridine modification affects neither the molecular mass nor the HPLC pattern of denatured X-C3a, an evidence for the absence of free cysteine in denatured X-C3a. The results also indicate that GdmSCN is roughly 1.5-fold more potent than GdmCl, which in turn is about 2-fold more effective than urea as a denaturant.

Denatured C3a consists of 5 fractions X-C3a (designated as “a” to “e”). Among them, fraction “e” overlaps extensively with N-C3a (see inset of Fig. 2). The most notable feature of the composition of X-C3a is the rising yield of fraction “a” at increasing concentrations of denaturant. This is particularly evident with GdmCl and GdmSCN induced denaturation. At 6M GdmSCN, fraction “a” constitutes 60% of the total denatured C3a. These results thus indicate that fraction “a” comprises a denatured isomer that represents the highest free energy among all 14 possible isomers of X-C3a.

The disulfide structure and CD spectrum of the most extensively unfolded isomer of denatured C3a

Fraction “a” of denatured C3a was isolated and digested with Asp-N. Four fractions of peptide fragments were purified by HPLC and analyzed by MALDI mass spectrometry and Edman sequencing. The results show that each fraction contains a single peptide and these four peptides encompass the entire sequence of C3a (Fig. 3A), with peptides 1, 3 and 4 correspond to residues 10-24 (Cys^{22}-Cys^{23}), 55-77 (Cys^{56}-Cys^{57}) and 25-54 (Cys^{36}-Cys^{49}), respectively. Thus, fraction “a” was shown to contain a single isomer, designated as X-C3a-a, that comprises three non-native disulfide bonds connected by 3 pairs of neighboring Cys, including 2 pairs of sequentially consecutive Cys (beads-form disulfide pattern) (Fig. 3C).

Native C3a comprises 56% of α-helical structure *(5)* and displays a strong CD signals with a double minimum at 208 and 222 nm, characteristics for α-helical rich proteins. In contrast, X-C3a-a exhibits a spectrum largely consistent with the structure of random coil (Fig. 3B). The CD spectrum of X-C3a-a also reveals residual α-helical structure, presumably contributed by the remaining structure of its C-terminal segment, which is not involved in disulfide bonding (Fig. 1).

Denaturation curves and unfolding curves of C3a measured by the method of disulfide scrambling

Using the method of disulfide scrambling *(8,9)*, it is possible to distinguish and quantify the process of denaturation and unfolding of C3a. The extent of denaturation is determined by the simple conversion of N-C3a to isomers of X-C3a. The extent of unfolding is defined by the

structure of denatured X-C3a and is quantified by the recovery of X-C3a-a, which represents the most extensively unfolded isomer and possesses the highest free energy among all denatured isomers of X-C3a.

Based on the definition described above and the HPLC data presented in Fig. 2, the two-state denaturation curves of C3a and the close to linear unfolding curves of C3a against urea, GdmCl and GdmSCN are given in Fig. 4. Mid-point denaturation of native C3a requires 5.7M urea, 3.4M GdmCl, and 1.8M GdmSCN. Mid-point unfolding of C3a occurs at about 5.2M GdmSCN and greater than 8M of GdmCl.

Denaturation curves of C3a measured by the conventional spectroscopic technique

The denaturation curve of C3a was also analyzed by the conventional spectroscopic method using the CD spectra. The intensity of signal at 222 nm under different concentrations of GdmCl (1-8M) was used to construct the denaturation curves of C3a (Fig. 5). The experiments were performed both in the absence and presence of 2-mercaptoethanol (0.2 mM) (Fig. 5). In the absence of 2-mercaptoethanol, C3a undergoes ***disulfide-intact*** denaturation and exhibits a mid-point denaturation at 4.6M of GdmCl. In the presence of 2-mercaptoethanol, the conformational stability of C3a is lower, because the native disulfide bonds of C3a are permitted to reshuffle. The process of denaturation is accompanied by ***disulfide-scrambling*** and the mid-point $[GdmCl]_{½}$ denaturation is decreased to 3.2 M, which is comparable to 3.4M GdmCl determined by HPLC quantification of N- and X-isomers of C3a (Fig. 4).

Stability of the native disulfide bonds of C3a measured by the method of reductive unfolding

The stability of native disulfide bonds of C3a was evaluated by the method of reductive unfolding and by their ability to withstand the reduction against DTT. Reduction of the three native disulfide bonds of C3a (Cys^{22}-Cys^{49}, Cys^{23}-Cys^{56}, and Cys^{36}-Cys^{57}) was shown to undergo an *all-or-none* mechanism without accumulation of significant concentration of partially reduced intermediate (Fig. 6A), similar to the mode of reductive unfolding of many 3-disulfide proteins *(10,15,16)*. However, the concentration of DTT needed to reduce native C3a is surprisingly low. At 1 mM of DTT, pH 8.4 and 22°C, the 3 native disulfide bonds of C3a were collectively reduced with a rate constant of 0.25±0.03 min^{-1} (Fig. 6A). Under identical reducing conditions (1 mM DTT), disulfide bonds of most native proteins remain totally intact (Fig. 7A). These proteins include hirudin *(10)*, tick anticoagulant peptide (TAP) *(10)* and leech Carboxypeptidase inhibitor (LCI) *(16)*. Reduction of native disulfide bonds of these proteins requires at least 20 -100 mM of DTT (Table 1). Complete reduction of C3a disulfide bonds resulted in a concurrent loss of most of its α-helical structure (Fig. 6B), similar to the CD spectrum of reduced-carboxymethylated C3a *(4)*. Our result and those observed by Hugli et al. *(4)* together indicate that content of C-terminal α-helical structure is also dependent on the intactness of three native disulfide bonds of C3a.

The stability of native disulfide bonds of C3a was further compared to that of non-native disulfide bonds of scrambled X-isomers of C3a, hirudin and TAP. X-isomers were produced by incubating native proteins in the buffer (pH 8.4) containing GdmSCN (6M) and 2-mercaptoethanol (0.2 mM). They comprise heterogeneous species that adopt mostly non-native disulfide bonds. X-C3a, X-TAP and X-Hir were reduced with DTT (1 mM) at 23°C for different time periods up to 5 min. Reduced samples were quenched by acidification and analyzed by HPLC. The rate of reduction was evaluated by the time course recovery of fully reduced proteins and was compared to that of N-C3a. The native disulfide bonds of N-C3a are slightly more stable than non-native disulfide bonds of X-C3a, but significantly weaker than that of X-Hir and X-TAP (Fig. 7B).

DISCUSSION

The methods of disulfide scrambling versus conventional spectroscopic techniques

The conformational stability of disulfide proteins has been traditionally investigated by the **disulfide-intact** denaturation, in which differential CD and fluorescence signals observed at the absence and presence of denaturants are used to determine the extent of denaturation (unfolding) *(7)*. Despite its simplicity and reliability, this conventional method has limitations. One inadequacy is its inability to discern the state of unfolding of a denatured protein. This drawback can be overcome by the technique of **disulfide scrambling** *(8,9)*, in which denaturation of protein is analyzed by the reversible conversion between the native N-isomer and denatured X-isomers. The method of disulfide scrambling exhibits several unique features. **(a)** The denatured protein comprises heterogeneous X-isomers that are stabilized by non-native disulfide bonds and amenable to separation by reversed phase HPLC. This allows further structural characterization of a denatured protein. **(b)** The process of denaturation and disulfide scrambling can be quenched by sample acidification at any time point of denaturation. This permits structural characterization of partially as well as extensively denatured proteins. **(c)** The method allows quantification of the extent of unfolding of a denatured protein. This is achieved by analysis of heterogeneous isomers of a denatured protein and identification of a unique isomer that represents the most extensively unfolded state. Criteria for identifying this most extensively unfolded isomer are based on two experimental data. During the unfolding experiment, it is the only denatured isomer with increase of recovery that is constantly proportional to the increasing strength of denaturant. During the refolding experiment, it is the only isomer that flows through all other identified isomers, but not *vice versa*. In another word, it is the isomer that exhibits the highest free energy *(17)*.

In this report, the conformational stability of native C3a was first investigated by the method of disulfide scrambling. The denaturation curves of C3a were determined by the yield of collective X-C3a as percentage of total C3a (N+X) (Fig. 4A). Unfolding curves of C3a are determined by the yield of isomer X-C3a-a (Fig. 4B). The ability of this method to differentiate the state of unfolding of denatured C3a is evident. Despite the completion of denaturation at 4M GdmSCN and ~5.5M GdmCl, denatured C3a continues to unfold as the concentration of denaturant increases. In between 4 and 6 M GdmSCN, the extent of unfolding of C3a rises from 35% to 60%, based on the recovery of X-C3a-a. Extrapolation of the unfolding curves implies that complete unfolding of C3a can be in theory achieved at about 9.6M GdmSCN and 14.6M GdmCl; both are experimentally unfeasible due to the limited solubility of denaturants.

The conformational stability of native C3a was also investigated by the conventional spectroscopic method using the helical signal of native C3a (Fig. 5). Two remarkable aspects of the results are elaborated here. **(a)** In the presence of thiol catalyst, the denaturation curve of C3a (GdmCl) measured by the decrease of CD signal (Fig. 5) is almost superimposable with that quantified by the yield of denatured X-isomers (Fig. 4). These results validate the technique of disulfide scrambling for measuring the conformational stability of proteins. **(b)** Conformational stability of C3a differs in the absence and presence of catalytic thiol. In the presence of thiol catalyst, the conformational stability C3a is significantly lower. This is because in the presence of thiol catalyst, the rigid network of native disulfide bonds is rendered flexible and denaturation of the protein is accompanied by disulfide scrambling according to the conformation induced by the denaturant. A similar result was found in the case of phospholipase A_2 *(18)*.

The fact that conformational stability of a disulfide protein may decrease in the presence of catalytic thiol has important implications. It raises the question as to whether the factual *in vivo* stability of a disulfide protein can be deduced from the *in vitro* analysis performed in the absence of thiol catalyst. There are complex endogenous thiols present in the biological milieu.

For instance, in human serum, there is a total of 10-15 μM of thiol compounds (Cys and GSH) *(19)*. They may act as thiol catalyst to facilitate scrambling of native proteins that are under stressful conditions. We have recently shown that insulin may irreversibly breakdown in the presence of 10-15 μM of thiol at mildly elevated temperature in an *in vitro* experiment *(20)*.

The most extensively unfolded isomer of denatured C3a adopts a unique disulfide structure

The most extensively unfolded isomer has been detected in several 3- and 4-disulfide proteins denatured by the method of disulfide scrambling. With the exception of α-lactalbumin *(9)*, this isomer has been invariably found to adopt beads-form disulfide pairing, in which disulfide bonds are formed by 3- and 4-pairs of neighboring cysteines These proteins include hirudin *(21)*, TAP *(8)*, potato carboxypeptidase inhibitor *(22)*, Insulin-like growth factor *(23)*, BPTI *(24)*, LCI *(25)*, and lysozyme *(26)*. In term of configuration, the beads-form disulfide pattern represents the most linear structure of the polypeptide among all possible configurations of scrambled isomers.

In the case of C3a, the most extensively unfolded denatured isomer (X-C3a-a) was also found to adopt the beads-form disulfide pairing. However, two of the three non-native disulfide bonds of X-C3a-a are paired by consecutive cysteines, Cys^{22}-Cys^{23} and Cys^{56}-Cys^{57} (Fig. 3). Such disulfide pairing is thought to be thermodynamically unfavorable. It rarely occurs naturally due to the steric constraints of bending the side chain of two adjacent cysteines *(27)*. Although disulfide bond connecting two consecutive Cys has been documented in other native and denatured proteins *(28,29)*, X-C3a-a represents to our best knowledge the first reported case in which one single polypeptide comprises two such unusual disulfide bonds. The unique disulfide connectivity of X-C3a-a further demonstrates the structural elasticity of polypeptide bonds.

The stability of disulfide bonds of native C3a is unusually low – implications of biological functions

An intriguing result of this study is the unusually low covalent stability of the three native disulfide bonds of C3a, measured by the method of reductive unfolding. The technique of **reductive unfolding** is applied to analyze the stability of various native disulfide bonds (in the absence of denaturant) to withstand the reduction by a reducing agent (e.g. DTT) *(10-13)*. Because covalent stability of a disulfide bond is generally reinforced by its adjacent structures, its strength to resist the reduction therefore should somehow reflect the stability of a domain structure surrounding this disulfide bond. In this notion, one may expect that there ought to be a positive correlation between the global conformational stability of a native protein and the covalent stability of its native disulfide bonds. High conformational stability of a protein should in general translate into more stable covalent stability of its disulfide bonds. However, the results obtained from C3a and other disulfide proteins suggest that this is not the case.

Similar to many disulfide proteins, the three native disulfide bonds of C3a are reduced in a collective manner without accumulation of significant concentration of intermediates (Fig. 6). These proteins include hirudin *(10)*, TAP *(10)* and LCI *(16)*, which all have similar size and comparable conformational stability as C3a (Table 1). However, the concentration of DTT required to reduce C3a is drastically lower than those needed for hirudin, TAP and LCI. The native disulfide bonds of C3a can be readily reduced with 1 mM DTT within 6 min, a condition and kinetic that are typically observed with the reduction of non-native disulfide bonds of scrambled proteins *(10)*. Under the same condition, the native disulfide bonds of hirudin, TAP and LCI remain completely intact. The relative covalent stability of their disulfide bonds is shown in Table 1. The difference of stability between C3a and other three proteins ranges from 156-fold (TAP) to 840-fold (LCI) and 1000-fold (hirudin). These disparities are determined

by multiplying the differences of both the concentration of DTT required and the kinetics of N⇨R conversion.

There are few plausible explanation for the unusually low covalent stability of the native disulfide bond of C3a. **(a)** The native disulfide bonds of C3a might be highly exposed and easily accessible to DTT. However, this is not evident from the structure of C3a *(5,6)*. The three native disulfide bonds of C3a are embedded at the N-terminal domain. **(b)** One of the native disulfide bonds of C3a might be "strained" and the reduction of this hyper-reactive disulfide bond by DTT leads to a rapid collapse of the remaining disulfide bonds. Such hyper-reactive disulfide bonds were observed in secretory leucocyte protease inhibitor *(31)*, α-lactalbumin *(32,33)* and bovine pancreatic trypsin inhibitor *(34-37)*. However, reductive unfolding of these three proteins *(31-36)* all proceeds via a sequential manner with significant accumulation of partially reduced intermediates. This is not seen in the case of C3a (Fig. 6A). **(c)** The most likely scenario is that all 3 disulfide bonds of native C3a are "strained". This would explain the rapid and collective reduction of native C3a disulfide bonds.

These data (Table 1) also reveal a general disconnection between the global conformational stability and the covalent stability of native disulfide bonds. A notable case is demonstrated by LCI. Both native C3a and LCI exhibit comparable conformational stability as judged by their mid-point denaturation $[GdmCl]_{½}$ of 3.4 M. However, the covalent stability of their native disulfide bonds differs by a factor of 840-fold. An even more remarkable case is RNase A. The conformational stability of RNase A appears to be lower than that of C3a with $[GdmCl]_{½}$ of 2.25 M *(30)*. However, collective reduction of the four native disulfide bonds of RNase A requires 100 mM of DTT with observed first order kinetics of 0.004 min^{-1} *(10)*. This translates into a 6250-fold disparity of the covalent stability of the native disulfide bonds between C3a and RNase A.

This apparent disconnection of conformational and disulfide stability may imply the existence of a yet to be explored diversity regarding the structure-function of disulfide proteins. For C3a, this unusually low stability of native disulfide bonds may facilitate its core domain to undergo conformational change under mild reducing conditions, which in turn would affect the helical structure of its C-terminal domain and regulate the biological function of C3a *(38)*.

Concluding remarks

As a final note, we wish to point out that this presentation is intended to highlight two remarkable structural properties of human C3a which, in our best knowledge, have not been observed previously by other laboratories. One is the unique disulfide structure of a denatured C3a isomer present under strong denaturing conditions (Fig. 3). The second is the unusually low covalent stability of native disulfide bonds of C3a (Figs. 6 and 7). We have not attempted to demonstrate or elaborate the relevance of current findings to the biological function of C3a. Indeed, a 21-residue synthetic C-terminal fragment of C3a (residue 57-77) was shown to be nearly as active as the intact C3a *(38)*. This led to the suggestion that the N-terminal portion (residues 1-21) and the disulfide-linked core region (residues 22-57) in intact C3a serve primarily to stabilize ordered conformation in the C-terminal region (residues 58-77). This proposal *(38)* appears to be consistent with our data which show that reduction of the C3a disulfide bonds leads to instantaneous disruption of the helical structures of C3a (Fig. 6).

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Abbreviations

SLPI, secretory leucocyte protease inhibitor; BPTI, bovine pancreatic trypsin inhibitor; TAP, tick anticoagulant peptide; LCI, leech Carboxypeptidase inhibitor; DTT, reduced dithiothreitol; HPLC, high pressure liquid chromatography; MALDI-TOF, matrix assisted laser desorption ionization/time-of flight; N-isomer, native protein; X-isomer, fully oxidized scrambled isomer.

REFERENCE

1. Hugli TE, Mueller-Eberhard HJ. H.J. Adv. Immunol 1978;26:1–53.
2. Wetsel, RA.; Kildsgaard, J.; Haviland, DL. Therapeutic Interventions in the Complement System. Lambris, JD.; Holers, VM., editors. Humana Press; Totowa, New Jersey, USA: 2000. p. 113-153.
3. Hugli TE. J. Biol. Chem 1975;250:8293–8301. [PubMed: 1238393]
4. Hugli TE, Morgan WT, Mueller-Eberhard HJ. J. Biol. Chem 1975;250:1479–1483. [PubMed: 234458]
5. Huber R, Scholze H, Paques EP, Deisenhofer J. Hoppe Seylers Z. Physiol. Chem 1980;361:1389–1399. [PubMed: 7439885]
6. Chazin WJ, Hugli TE, Wright PE. Biochemistry 1988;27:9139–9148. [PubMed: 3266557]
7. Pace CN. Methods Enzymol 1986;131:266–280. [PubMed: 3773761]
8. Chang J-Y. J. Biol. Chem 1999;274:123–128. [PubMed: 9867819]
9. Chang J-Y, Li L. J. Biol. Chem 2001;276:9705–9712. [PubMed: 11118458]
10. Chang J-Y. J. Biol. Chem 1997;272:69–75. [PubMed: 8995229]
11. Li YJ, Rothwarf DM, Scheraga HA. Nat. Struct. Biol 1995;2:489–494. [PubMed: 7664112]
12. Mendoza JA, Jarstfer MB, Goldenberg DP. Biochemistry 1994;33:1143–1138. [PubMed: 7509189]
13. Xu G, Narayan M, Welker E, Scheraga HA. Biochemistry 2004;43:3246–3254. [PubMed: 15023075]
14. Hugli TE, Gerard C, Kawahara M, Scheetz ME II, Barton R, Briggs S, Koppel G, Russell S. Molecular and Cellular Biochemistry 1981;41:59–66. [PubMed: 6977086]
15. Arias-Moreno X, Arolas JL, Aviles FX, Sancho J, Ventura S. J. Biol. Chem 2008;283:13627–13637. [PubMed: 18343813]
16. Arolas JL, Bronsoms S, Lorenzo J, Aviles FX, Chang JY, Ventura S. J. Biol. Chem 2004;279:37261–37270. [PubMed: 15226306]
17. Chang J-Y. J. Biol. Chem 2002;277:120–126. [PubMed: 11560938]
18. Singh RR, Chang J-Y. Biochem. J 2004;377:685–692. [PubMed: 14533980]
19. Williams RH, Maggiore JA, Reynolds RD, Helgason CM. Clinical Chem 2001;47:1031–1039. [PubMed: 11375288]
20. Jiang C, Chang J-Y. FEBS Lett 2005;579:3927–3931. [PubMed: 15990096]
21. Bulychev A, Chang J-Y. J. Protein. Chem 1999;18:771–777. [PubMed: 10691187]
22. Chang J-Y, Li L, Canals F, Aviles FX. J. Biol. Chem 2000;275:14205–14211. [PubMed: 10799497]
23. Chang J-Y, Maerki W, Lai PH. Protein. Sci 1999;8:1463–1468. [PubMed: 10422834]
24. Chang J-Y, Ballotore A. FEBS Lett 2000;473:183–187. [PubMed: 10812071]
25. Salamanca S, Villegas V, Vendrell J, Aviles FX, Chang J-Y. J. Biol.Chem 2002;277:17538–17543. [PubMed: 11893741]
26. Chang J-Y, Li L. FEBS Lett 2002;511:73–78. [PubMed: 11821052]
27. Henschen, A. Advanced Methods in Protein Microsequence Analysis. WittmannLiebold, B.; Salnikow, J.; Erdmann, VA., editors. Springer-Verlag; Berlin-Heidelberg: 1986. p. 244-255.
28. Marti T, Rösselet SJ, Titani K, Walsh KA. Biochemistry 1987;26:8099–8109. [PubMed: 3502076]
29. Lengweiler S, Schaller J, Rickli EE. FEBS Lett 1996;380:8–12. [PubMed: 8603752]
30. Chang J-Y. Anal. Biochem 1999;268:147–150. [PubMed: 10036174]
31. Lin C-J, Chang J-Y. Biochemistry 2006;45:6231–6240. [PubMed: 16681396]
32. Ikeguchi M, Sugai S, Fujino M, Sugawara T, Kuwajima K. Biochemistry 1992;31:12695–12700. [PubMed: 1472507]

33. Chang J-Y, Li L. Biochemistry 2002;41:8405–8413. [PubMed: 12081489]J.-Y.
34. Chang J-Y, Li L, Bulychev A. J. Biol. Chem 2000;275:8287–8289. [PubMed: 10722657]J.-Y.
35. Creighton TE. Science 1992;256:111–114. [PubMed: 1373519]
36. Goldenberg DP. Trends Biochem. Sci 1992;17:257–261. [PubMed: 1380192]
37. Weissman JS, Kim PS. Science 1991;253:1386–1393. [PubMed: 1716783]J. S.
38. Lu ZX, Fok KF, Erikson BW, Hugli TE. J. Biol. Chem 1984;259:7367–7370. [PubMed: 6610676]

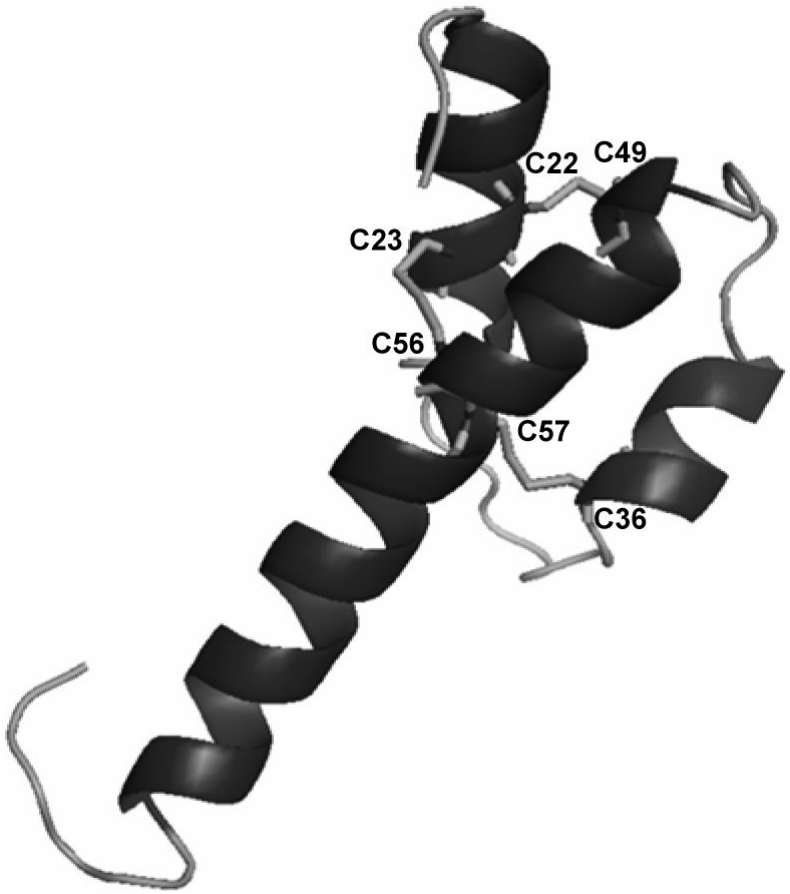

Fig. 1. Structure of C3a

The native C3a comprises an N-terminal domain intra-connected by three native disulfide bonds (Cys^{22}-Cys^{49}, Cys^{23}-Cys^{56}, and Cys^{36}-Cys^{57}) and a helical C-terminal domain *(5)*.

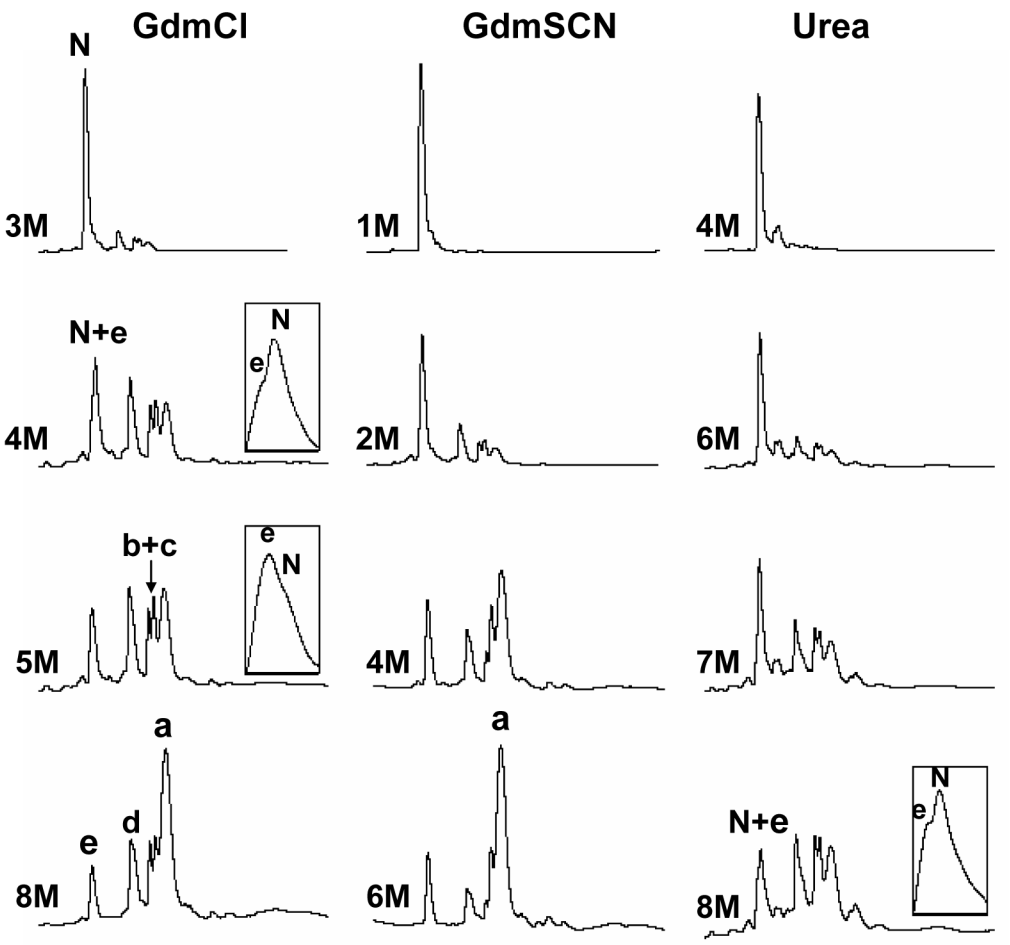

Fig. 2. Denaturation and unfolding of C3a by different concentrations of GdmCl, GdmSCN and urea

Denaturation was carried out at 23°C for 20 hours in the Tris-HCl buffer (pH 8.4) containing 2-mercaptoethanol (0.2 mM). Denatured samples were acidified with 4% aqueous trifluoroacetic acid and directly analyzed by reversed-phase HPLC using the conditions described in the section of methods. “N” indicates native C3a. Five fractions of scrambled X-C3a are marked by lower case (a-e). N-C3a and X-C3a-e overlap extensively (see inset).

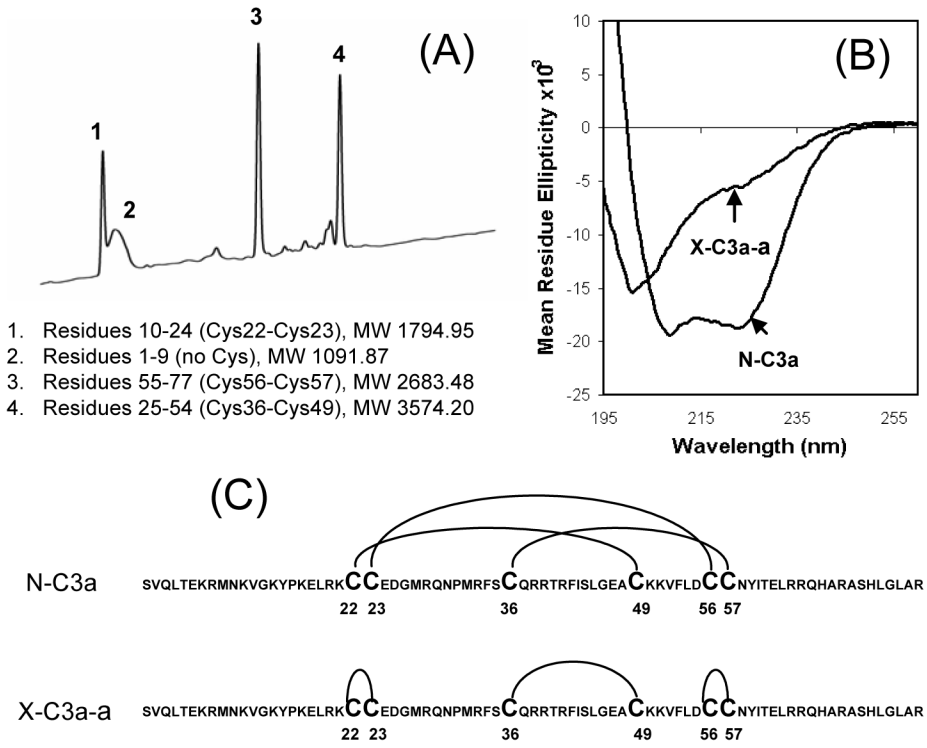

Fig. 3. The disulfide structure and CD spectrum of X-C3a-a

(A) Peptide mapping of Asp-N digested of X-C3a-a. Peptides were separated by HPLC using the conditions described for the separation of C3a isomers, except for using a different gradient system which was 0-70% solvent B in 60 min. Mass and sequence analysis reveal that the four peptide fragments encompass the entire sequence of C3a. **(B)** Far UV CD spectra of N-C3a and X-C3a-a. **(C)** The disulfide connectivity of N-C3a and X-C3a-a.

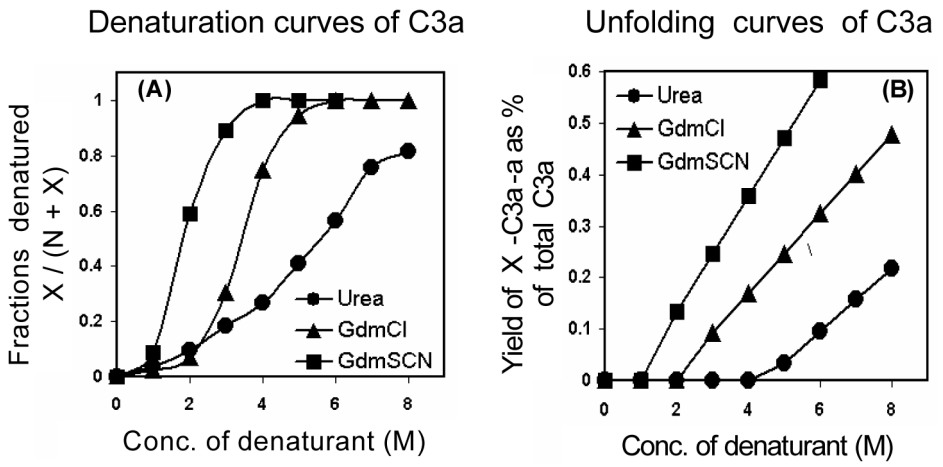

Fig. 4. Denaturation curves and unfolding curves of C3a

These figures are derived from HPLC data presented in Fig. 2. The denaturation curves are obtained by plotting fractions of denatured C3a against the concentrations of denaturants. Fractions denatured are calculated as percentages of total C3a converted to X-C3a. The unfolding curves are obtained by plotting the yield of X-C3a-a as percentage of total C3a against the concentrations of denaturants.

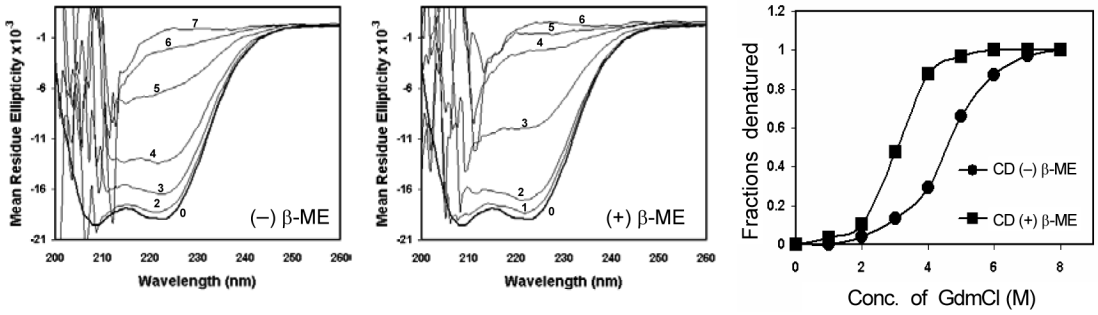

Fig. 5. Denaturation of C3a analyzed by the method of differential CD spectroscopy

C3a was denatured with different concentrations of GdmCl (1-8M) in the absence and presence of 2-mercaptoethanol (0.2 mM). Far UV CD spectra of the denatured samples were recorded from 260-200 nm. Each spectrum was an average of five scans. The spectra of controls were subtracted from the spectra of their respective C3a samples. The ellipticities at 222 nm were used to calculate the extent of C3a denaturation (fraction denatured) and the denaturation curves of C3a (right panel). In the absence of 2-mercaptoethanol, C3a undergoes "**disulfide intact**" denaturation (left panel). In the presence of 2-mercaptoethanol, C3a undergoes "**disulfide scrambling**" denaturation (middle panel).

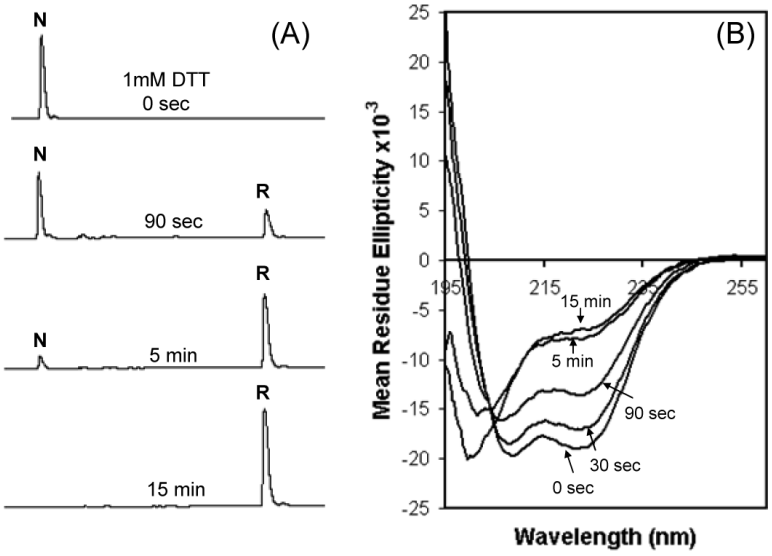

Fig. 6. Reductive unfolding of the native C3a

The native C3a (0.5 mg/ml) was dissolved in the Tris-HCl buffer (0.1 M, pH 8.4) containing 1 mM DTT. The reaction was carried out at 23°C. Aliquots of unfolding samples (40 μl) were quenched at different time points with 160 μl of 0.5% aqueous TFA (final protein concentration is 0.1 mg/ml). These samples were directly measured with a Jasco J-715 spectropolarimeter to obtain their CD spectra **(B)**, followed by HPLC analysis **(A)**. A full CD scan was performed for each sample from 260 nm to 195 nm. Samples of C3a reduced by DTT but without acidification exhibit similar extent of decrease of α-helical structure.

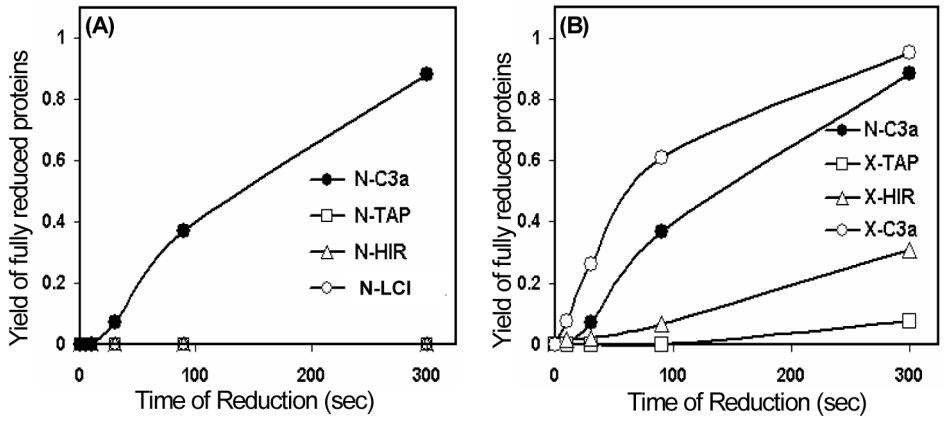

Fig. 7. Covalent stability of native and non-native disulfide bonds determined by the kinetics of reductive unfolding

(A) Comparison between native C3a, TAP, hirudin and LCI. **(B)** Comparison between native C3a and scrambled X-isomers of C3a, TAP and hirudin. Proteins (0.5 mg/ml) were incubated in the Trs-HCl buffer containing DTT (1 mM) in the absence of denaturant. The reduction was carried out at 23°C for up to 15 min. Intermediates of reduction were quenched with an equal volume of 4% aqueous trifluoroacetic. Yield of fully reduced protein was quantified by HPLC analysis.

Table 1

Comparison of the stability of disulfide proteins

Proteins[a]		***[GdmCl]***$_{1/2}$[b]	***[DTT]***[c]	***(K***$_{N⇨R}$***)***[d]
C3a	(77 a.a. 3SS)	3.4 M	1 mM	(0.25 min^{-1})
Hirudin	(65 a.a. 3SS)	5 M	100 mM	(0.024 min^{-1})
TAP	(60 a.a. 3SS)	4.2 M	12.5 mM	(0.02 min^{-1})
LCI	(66 a.a. 4SS)	3.4M	100 mM	(0.03 min^{-1})
RNase A	(124 a.a. 4SS)	2.25M	100 mM	(0.004 min^{-1})

[a] Hirudin is a leech derived thrombin specific inhibitor. TAP is tick derived factor Xa inhibitor. LCI is leech derived carboxypeptidase inhibitor.

[b] Midpoint of the GdmCl unfolding curve in [M], determined by the method of disulfide scrambling. The data are allowed a deviation of ± 3-4%.

[c] Concentration of DTT applied for reductive unfolding. Experiments were performed in the Tris-HCl buffer (0.1M, pH 8.4) in the absence of denaturant.

[d] First-order kinetic of N(native) ⇨ R(fully reduced) conversion at indicated concentrations of DTT. The data are allowed a deviation of ± 5%.