Ca(II)- and Tb(III)-induced stabilization and refolding of anticoagulation factor I from the venom of *Agkistrodon acutus*

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Abstract

Anticoagulation factor I (ACF I) isolated from the venom of *Agkistrodon acutus* is an activated coagulation factor X-binding protein in a Ca^{2+} -dependent fashion with marked anticoagulant activity. The equilibrium unfolding/refolding of apo-ACF I, holo-ACF I, and Tb^{3+} -reconstituted ACF I in guanidine hydrochloride (GdnHCl) solutions was studied by following the fluorescence and circular dichroism. Metal ions were found to increase the structural stability of ACF I against GdnHCl and thermal denaturation and, furthermore, influence its unfolding/refolding behavior. The GdnHCl-induced unfolding/refolding of both apo-ACF I and Tb^{3+} -ACF I is a two-state process with no detectable intermediate state(s), whereas the GdnHCl-induced unfolding/refolding of holo-ACF I in the presence of 1 mM Ca^{2+} follows a three-step transition, with intermediate state a (Ia) and intermediate state b (Ib). Ca^{2+} ions play an important role in the stabilization of the Ia and Ib states. The decalcification of holo-ACF I shifts the ending zone of unfolding/refolding curve toward lower GdnHCl concentration, whereas the reconstitution of apo-ACF I with Tb^{3+} ions shifts the initial zone of denaturation curve toward higher GdnHCl concentration. Therefore, it is possible to find a denaturant concentration (2.0 M GdnHCl) at which refolding from the fully denatured state of apo-ACF I to the Ib state of holo-ACF I or to the native state of Tb^{3+} -ACF I can be initiated merely by adding the 1 mM Ca^{2+} ions or 10 μM Tb^{3+} ions to the unfolded state of apo-ACF I, respectively, without changing the concentration of the denaturant. Using Tb^{3+} as a fluorescence probe of Ca^{2+} , the kinetic results of metal ions-induced refolding provide evidence that the compact Tb^{3+} -binding region forms first, and subsequently, the protein undergoes further conformational rearrangements to form the native structure.

Keywords: Fluorescence spectroscopy; circular dichroism; anticoagulation factor I; unfolding; refolding; intermediate state; calcium ion; terbium ion

Anticoagulation factor I (ACF I) is a nonenzymatic anticoagulant from the venom of *Agkistrodon acutus* that has marked anticoagulant activity with a unique anticoagulant mechanism, for it forms a 1:1 complex with activated co-

agulation factor X (FXa) in a Ca^{2+} -dependent fashion and thereby blocks the amplification of the coagulation cascade (Xu et al. 2000, 2001). ACF I is a member of coagulation factor IX/coagulation factor X-binding protein family. The proteins of this family have high homologous sequences and form 1:1 complexes with coagulation factor IX/activated coagulation factor IX or coagulation factor X/activated coagulation factor X (Sekiya et al. 1993; Atoda et al. 1995, 1998; Chen and Tsai 1996). All bindings are dependent on Ca^{2+} ions. In this family, the crystal structures of coagulation factor IX/factor X-binding protein (habu IX/X-bp) and coagulation factor IX-binding protein (habu IX-bp; Mizuno et al. 1999) purified from habu snake have been reported.

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Abbreviations: ACF I, anticoagulation factor I; FX, coagulation factor X; FXa, activated coagulation factor X; CD, circular dichroism; GdnHCl, guanidine hydrochloride; habu IX/X-bp, habu coagulation factor IX/factor X-binding protein; habu IX-bp, coagulation factor IX-binding protein.

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The ribbon model of habu IX/X-bp was shown in Figure 1 (Mizuno et al. 1997). Both structures of habu IX/X-bp and habu IX-bp are almost identical, with the same size of 7 nm × 3 nm × 3 nm. Each is a heterodimer protein consisting of two homologous chains with a similar topology structure linked with a disulphide-bond. Each chain has one Ca²⁺-binding site. One of them is formed by the oxygen atoms of Ser 41, Glu 43, Glu 47, and Glu 128 in A-chain, and the other site is formed by the oxygen atoms of Ser 41, Glu 43, Glu 47, and Glu 120 in B-chain, in habu IX/X-bp as well as in habu IX-bp. The two Ca²⁺ ions in habu IX/X-bp can be all replaced by trivalent lanthanide ions such as Lu³⁺ and Sm³⁺ (Mizuno et al. 1997), and the sites of Lu³⁺ and Sm³⁺ in heavy-atom soaked crystals of habu IX-bp are identical with that of Ca²⁺ (Mizuno et al. 1999).

ACF I has two chains with similar amino acid compositions (Xu et al. 2000) and N-terminal amino acid sequences to those of habu IX/X-bp (data not shown), and has the two Ca²⁺-binding sites with different association constant values $(1.8 \pm 0.5) \times 10^5 \text{ M}^{-1}$ and $(2.7 \pm 0.6) \times 10^4 \text{ M}^{-1}$, which are also similar to those of habu IX/X-bp, respectively (Xu et al. 2001). In addition, the size of ACF I was recently determined to be 7.4 nm × 3.6 nm × 3.1 nm by atomic force microscopy (data not shown), within experimental errors, which is similar to the sizes of habu IX/X-bp and habu IX-bp. It therefore is reasonable to assume that the fold does not differ much between ACF I and habu IX/X-bp or habu IX-bp. Thus, ACF I should contain α -helix and β -sheet conformations with similar structures of Ca²⁺-binding sites to habu IX/X-bp and habu IX-bp.

Fig. 1. Ribbon model of the heterodimer polypeptide chains of habu coagulation factor IX/factor X-binding protein (habu IX/X-bp). The Ca²⁺ ions and Trp residues are indicated as big balls and small balls, respectively. The picture was drawn with a Protein Data Bank file 1LXX (Mizuno et al. 1997).

ACF I is devoid of hemorrhagic and lethal activities, which may be useful both as a basis for designing anticoagulant drugs and as a convenient tool in exploration of the complex mechanisms of the coagulation cascade. ACF I has two Ca²⁺-binding sites with different affinities, and the occupation of both Ca²⁺-binding sites in ACF I with Ca²⁺ ions and subsequent conformational rearrangement may be essential for the binding of ACF I to FXa (Xu et al. 2001). We expected that analysis of the effect of Ca²⁺ ions on the structural stability of ACF I would be useful for improving our understanding of the function of Ca²⁺ ions in the binding of ACF I to FXa. Studies on guanidine hydrochloride (GdnHCl)- and thermal-induced denaturation and metal ion-induced refolding of ACF I were performed in this paper. Tb³⁺, as a fluorescence probe, has been widely used to study protein structural characteristics (Burroughs and Horrocks 1994; Mulqueen et al. 1985) and, therefore, is expected to be used as a fluorescence probe of Ca²⁺ to monitor the structural change of Ca²⁺-binding sites in ACF I during its unfolding/refolding.

Protein folding/unfolding is a highly cooperative process. It has recently been shown that the folding/unfolding of small globular proteins occurs via a two-state process, whereas the folding/unfolding of larger proteins (>100 amino acids) is complex and often involves the formation of intermediate(s) (Dobson and Karplus 1999; Englander 2000; Samuel et al. 2001). Protein folding/unfolding can be affected by factors such as pressure (Valente-Mesquita et al. 1998), temperature (Scalley and Baker 1997), pH (Jamin and Baldwin 1998; Bedell et al. 2000), salt (Mayr and Schmid 1993), and disulfide bond (Liu and Wang 2001). Recently, the effects of metal ions and anions on protein unfolding/refolding have received considerable attention. It has been shown that metal ion-induced conformation changes in several enzymes lead to stabilization of the proteins during protein folding/unfolding (Ahmad et al. 2001; Yuan et al. 2001). In addition, without changing the concentration of the denaturant, the refolding of several proteins can be induced simply by adding 0.2 to 1.0 M anions (Edwin and Jagannadham 2000; Muzammil et al. 2000).

The present investigation provides evidence that metal ions not only significantly increase the structural stability of ACF I against GdnHCl and thermal denaturation but also influence the unfolding/refolding behavior of ACF I, and furthermore, metal ions are able to induce the refolding of the unfolded apo-ACF I merely by adding very low concentration of metal ions (1 mM Ca²⁺ or 10 μ M Tb³⁺) to the unfolded apo-ACF I without changing the concentration of the denaturant. In addition, Tb³⁺ was found to be a useful fluorescence probe of Ca²⁺ for studying the mechanism of ACF I unfolding/refolding.

Results

Steady-state fluorescence of tryptophan

The intrinsic fluorescence of Trp residues in ACF I was used for studies of its unfolding/refolding behavior. As there are 14 Trps in ACF I (Xu et al. 2000), the overall changes in fluorescence reflect global changes in protein structure, and average microenvironments of Trps can be assessed. Figure 2A shows steady-state fluorescence emission spectra for apo-ACF I in the absence of Ca^{2+} by exciting the protein at 295 nm in the presence of increasing concentrations of GdnHCl. It is notable that increasing the GdnHCl concentration causes the fluorescence emission intensity to increase and the emission maximum λ_{max} to red shift (from 338 to 354 nm), which indicates that the native apo-ACF I assumes a compactly folded structure in which the most Trps and quenchers, such as the charged carboxyl and/or amino groups in the interior apo-ACF I, are adjacent as

observed for bovine β -lactoglobulin (Sakai et al. 2000). As shown in Figure 2C, GdnHCl-induced denaturation of apo-ACF I was found to be a single-step process with no detectable intermediate state(s). The transition starts at ~ 0.60 M GdnHCl and slopes off at 1.90 M GdnHCl. The refolding transition curve of apo-ACF I essentially superposes on its unfolding transition curve, indicating the GdnHCl-induced denaturation of apo-ACF I is reversible. Ca^{2+} ions (1 mM) were present during the unfolding/refolding of holo-ACF I, because occupation of its both Ca^{2+} -binding sites with Ca^{2+} requires at least 1 mM of Ca^{2+} ions (Xu et al. 2001). As shown in Figure 2, B and C, increasing the GdnHCl concentration causes the fluorescence emission of holo-ACF I in the presence of 1 mM Ca^{2+} to change in a different manner to apo-ACF I. GdnHCl-induced denaturation of holo-ACF I was found to be a three-step process with accumulation of two intermediate states. A similar transition curve was obtained for the refolding of holo-ACF I, indicating GdnHCl-induced denaturation of holo-ACF I is

Fig. 2. Guanidine hydrochloride (GdnHCl)-induced unfolding and refolding of anticoagulation factor I (ACF I) in 0.02 M Tris-HCl buffer (pH 7.6, 25°C). (A) Fluorescence spectra of apo-ACF I in the absence of Ca^{2+} and the presence of increasing GdnHCl concentrations of 0, 0.35, 0.88, 1.05, 1.23, 1.40, 1.58, 1.75, 2.10, 2.80, and 4.90 M for the curves from bottom to top, at an excitation wavelength of 295 nm. The concentration of apo-ACF I is 1 μM . (B) Fluorescence spectra of holo-ACF I in the presence of 1 mM Ca^{2+} and in the increasing GdnHCl concentrations of 0, 0.70, 1.05, 1.40, 1.75, 2.10, 2.45, 2.80, 3.15, 3.50, and 4.90 M for the curves from curve 1 to curve 11, at an excitation wavelength of 295 nm. The concentration of holo-ACF I is 1 μM . (C) Unfolding ($\square$) and refolding ($\circ$) transitions of apo-ACF I, and unfolding (∇) and refolding ($\triangle$) transitions of holo-ACF I monitored by measurement of fluorescence at 340 nm after exciting at 295 nm. (D) Dependence of free energy change on GdnHCl concentration for the unfolding transitions shown in C: (1) $\text{N} \leftrightarrow \text{Ia}$ (holo-ACF I), (2) $\text{N} \leftrightarrow \text{D}$ (apo-ACF I), (3) $\text{Ia} \leftrightarrow \text{Ib}$ (holo-ACF I), and (4) $\text{Ib} \leftrightarrow \text{D}$ (holo-ACF I).

also reversible. GdnHCl-induced denaturation of holo-ACF I may be approximated to a four-state transition, and the mechanism for unfolding/refolding of holo-ACF I may be represented as

where N, Ia, Ib, and D are the native, intermediate a, intermediate b, and denatured states, respectively. The first transition of holo-ACF I unfolding (Fig. 2C), which corresponded to the transformation of N state to the Ia state, starts at ~0.35 M GdnHCl and completes at 1.00 M GdnHCl concentration with a decrease of fluorescence emission intensity and a slight red shift of the $\lambda_{\max}$ (from 338 to 339 nm). This result indicates that the Ia state of holo-ACF I is a compact molten globule state with a similar structure to its native state and with only partially disturbed structures around the Trps. The Ia state is stable in the GdnHCl concentration range 1.00 to 1.10 M. The second transition, which corresponded to the transformation of the Ia state to the Ib state, starts at ~1.15 M GdnHCl and completes at 1.92 M GdnHCl concentration with the increase of fluorescence intensity and a significant red shift of the $\lambda_{\max}$ from 338 to 350 nm, which indicates that the unfolding Ib of holo-ACF I has extensively disordered structures. The Ib state is stable in the GdnHCl concentration range 1.95 to 2.10 M. The last transition, which corresponded to the unfolding of Ib state, starts at ~2.15 M GdnHCl and finally slopes off to the D state at 3.60 M GdnHCl concentration with a further increase of fluorescence intensity and a further slight red shift of the $\lambda_{\max}$ from 350 to 354 nm, indicating that a few folding conformation probably exists in the Ib state, which contains some local hydrophobic regions around some Trps.

The transitions of GdnHCl-induced unfolding and refolding of apo-ACF I in absence of Ca^{2+} follows a two-state mechanism, and the free energy of unfolding or refolding was calculated according to Equation 3. The free energy of unfolding or refolding in the absence of the denaturant (ΔG^0) can be obtained by extrapolation of ΔG to zero denaturant concentration using Equation 13.

GdnHCl-induced denaturation of holo-ACF I follows a three-step mechanism represented as Equation 1. Assuming all three transitions to follow a two-state mechanism, we calculated the free energy of unfolding, ΔG_{Ia} , ΔG_{Ib} , and ΔG_D , respectively, as described in Equations 8, 10, and 12. A least squares analysis was used to fit the data to Equation 13 to determine ΔG_{Ia}^0 , ΔG_{Ib}^0 , and ΔG_D^0 . Value of ΔG_{Ia}^0 represents the value obtained from extrapolation of ΔG_{Ia} values up to zero denaturant concentration using Equation 13. Value of ΔG_{Ib}^0 represents the value obtained from extrapolation of ΔG_{Ib} values up to the starting of the process, $Ia \leftrightarrow Ib$. Value of ΔG_D^0 represents the value obtained from extrapolation of ΔG_D values up to the starting of the process, $Ib \leftrightarrow D$. Figure 2D shows the variation ΔG as a

function of GdnHCl concentration, and the equilibrium parameters (ΔG^0 , C_m , and m) for GdnHCl-induced structural alternations of apo-ACF I and holo-ACF I are compiled in Table 1. Free energy change of holo-ACF I in the presence of 1 mM Ca^{2+} associated with $N \leftrightarrow Ia \leftrightarrow Ib \leftrightarrow D$ transition can be obtained by summing the free energy change of the individual steps, that is, ΔG_{Ia}^0 , ΔG_{Ib}^0 , and ΔG_D^0 , because ΔG as a thermodynamic property does not depend on the path. The ΔG_{total}^0 of holo-ACF I in the presence of 1 mM Ca^{2+} , that is, the free energy change associated with the transformation of the N state to the Ia state and then to the Ib state and finally to the D state, was calculated to be 6.01 ± 0.09 kcal/mole. A comparison of the free energy changes of apo-ACF I and holo-ACF I during GdnHCl-induced unfolding clearly indicates that the ΔG_{total}^0 of holo-ACF I in the presence of 1 mM Ca^{2+} is greater than the ΔG^0 of apo-ACF I in the absence of Ca^{2+} , and the difference is found to be 1.51 ± 0.09 kcal/mole. These results show that Ca^{2+} ions in holo-ACF I markedly stabilize its conformation.

Similar results are obtained for equilibrium parameters of refolding transitions of apo-ACF I and holo-ACF I in GdnHCl solution compared to their unfolding transition as shown in Table 1. The difference of refolding free energy ($N \leftrightarrow D$) for holo-ACF I in the presence of 1 mM Ca^{2+} and apo-ACF I in the absence of Ca^{2+} is 1.62 ± 0.12 kcal/mole. The result also indicates that Ca^{2+} ions increase the conformational stability of holo-ACF I to the similar extent as determined from its unfolding transition.

Tb³⁺ steady-state fluorescence

Tb^{3+} ions can completely replace the two Ca^{2+} ions of holo-ACF I, and only two Tb^{3+} -binding sites are identified by equilibrium dialysis in the presence of 10 μM of Tb^{3+} (data not shown). Because high concentrations of Tb^{3+} will cause the precipitation of Tb^{3+} ions at pH 7.6, 10 μM of Tb^{3+} was present during the unfolding/refolding of Tb^{3+} -ACF I. The binding of Tb^{3+} to ACF I causes the intrinsic fluorescence of ACF I to decrease and results in the characteristic fluorescence emission of Tb^{3+} from its $^5\text{D}_4$ excited state to $^7\text{F}_j$ ($j = 6, 5, 4, 3$) state at 488, 545, 580, and 620 nm, respectively, by excitation at 295 nm. This characteristic emission of Tb^{3+} on its binding to ACF I is caused by the nonradiative energy transfer between Trps and the bound Tb^{3+} ions. Considering that the structural changes of the Tb^{3+} -binding sites should affect the characteristic emission of Tb^{3+} , we measured the changes in emission intensity both at 340 nm and at 545 nm during equilibrium unfolding of Tb^{3+} -ACF I to determine whether Tb^{3+} could be used as a fluorescence probe to monitor the structural change of the Ca^{2+} -binding sites during unfolding/refolding.

Figure 3 shows the GdnHCl-induced unfolding of Tb^{3+} -ACF I as monitored by the measurements of the intrinsic

Table 1. Thermodynamic parameters for unfolding and refolding equilibria of apo-ACF I, holo-ACF I, and Tb³⁺-ACF I by GdnHCl at 25°C, monitored by measurement of fluorescence at 340 nm

Species	C_m (M)	m (kcal·M ⁻¹ ·mole ⁻¹)	ΔG^o (kcal/mole)
Apo-ACF I (unfolding)	1.27 ± 0.04	3.55 ± 0.07	4.50 ± 0.08
Apo-ACF I (refolding)	1.29 ± 0.02	3.32 ± 0.04	4.45 ± 0.06
Tb ³⁺ -ACF I (unfolding)	2.52 ± 0.03	2.72 ± 0.05	6.86 ± 0.09
Tb ³⁺ -ACF I (unfolding)*	2.47 ± 0.05	2.72 ± 0.08	6.62 ± 0.13
Apo-ACF I (unfolding)**	1.26 ± 0.03	3.54 ± 0.06	4.37 ± 0.07
Holo-ACF I (unfolding)**	1.63 ± 0.07	3.12 ± 0.09	5.03 ± 0.13
Tb ³⁺ -ACF I (unfolding)**	2.41 ± 0.05	2.75 ± 0.05	6.58 ± 0.11

Species	C_m^{Ia} (N↔Ia) (M)	C_m^{Ib} (Ia↔Ib) (M)	C_m^D (Ib↔D) (M)	m_{Ia} (N↔Ia) (kcal·M ⁻¹ ·mole ⁻¹)	m_{Ib} (Ia↔Ib) (kcal·M ⁻¹ ·mole ⁻¹)	m_D (Ib↔D) (kcal·M ⁻¹ ·mole ⁻¹)
Holo-ACF I (unfolding)	0.66 ± 0.02	1.60 ± 0.04	2.52 ± 0.05	3.76 ± 0.09	3.63 ± 0.04	2.59 ± 0.06
Holo-ACF I (refolding)	0.67 ± 0.03	1.59 ± 0.02	2.55 ± 0.06	3.70 ± 0.05	3.79 ± 0.10	2.77 ± 0.08

Species	ΔG_{Ia}^o (N↔Ia) (kcal/mole)	ΔG_{Ib}^o (Ia↔Ib) (kcal/mole)	ΔG_D^o (Ib↔D) (kcal/mole)	ΔG_{total}^o (N↔Ia↔Ib↔D) (kcal/mole)
Holo-ACF I (unfolding)	2.51 ± 0.04	2.21 ± 0.02	1.29 ± 0.03	6.01 ± 0.09
Holo-ACF I (refolding)	2.60 ± 0.07	2.16 ± 0.03	1.31 ± 0.02	6.07 ± 0.12

All values represent the average of triplicate determinations.

* The values were monitored by measurement of the fluorescence of Tb³⁺ at 545 nm.

** The values were monitored by the measurements of ellipticity at 222 nm

fluorescence at 340 nm and the fluorescence of Tb³⁺ at 545 nm, respectively, by exciting the protein at 295 nm. Increasing the GdnHCl concentration induces an increase in the fluorescence intensity of Trps and decreases in the intensities of the four fluorescence peaks of Tb³⁺ at 488, 545, 580, and 620 nm (Fig. 3A). As shown in Figure 3B, GdnHCl-induced denaturation of Tb³⁺-ACF I was found to be a single-step process with no detectable intermediate state(s) when studied by both intrinsic fluorescence and Tb³⁺ fluorescence. The transition monitored by the measurements of intrinsic fluorescence at 340 nm shows that the abrupt unfolding of Tb³⁺-ACF I starts at ~1.90 M GdnHCl and completes at ~3.10 M GdnHCl with a red shift of the λ_{em} (340→352 nm), whereas the transition monitored by the measurements of Tb³⁺ fluorescence at 545 nm shows that the abrupt unfolding of Tb³⁺-ACF I starts at ~1.91 M GdnHCl and completes at ~3.08 M GdnHCl. The midpoint of the red shift of the λ_{em} of Trps occurs at ~2.47 M GdnHCl, which is identical to the midpoint (2.47 M GdnHCl) for the change of the Tb³⁺ emission intensity. These results clearly show that the increasing of the fluorescence of Trps at 340 nm and the decreasing of the fluorescence of Tb³⁺ at 545 nm are simultaneous, which shows that the unfolding of Tb³⁺-ACF I leads to the disruption of the compact Tb³⁺-binding region and breaks the nonradiative energy transfer from Trp residues in ACF I to the bound Tb³⁺ ions.

Using a two-state (N↔D) mechanism, the ΔG^o , C_m , and m values for the GdnHCl-induced unfolding transition of Tb³⁺-ACF I were obtained (Fig. 3C) and compiled in Table 1. These data obtained by the measurements of the intrinsic fluorescence and Tb³⁺ fluorescence are in good agreement with each other. A comparison of the free energy changes of Tb³⁺-ACF I and holo-ACF I during GdnHCl-induced unfolding clearly indicates that the ΔG^o of Tb³⁺-ACF I is greater than the ΔG_{total}^o of holo-ACF I, and the difference is found to be 0.85 ± 0.09 kcal/mole monitored by the measurements of intrinsic fluorescence at 340 nm. The result indicates that Tb³⁺-stabilized ACF I shows higher resistance to GdnHCl denaturation than the holo-ACF I.

Circular dichroism measurements

To test if the unfolding transition monitored by fluorescence reflects a disruption of the overall structure of the protein or just a local unfolding, we analyzed the chemical denaturation of ACF I induced by GdnHCl using far-ultraviolet (UV) circular dichroism (CD) spectroscopy. As shown in Figure 4 (A–C), all native apo-ACF I, holo-ACF I, and Tb³⁺-ACF I have a similar far-UV CD spectrum, which indicates both Ca²⁺ and Tb³⁺ have no obvious effect on the secondary structure of ACF I. The changes in ellipticity at 222 nm for the protein incubated in various concentrations

Fig. 3. Guanidine hydrochloride (GdnHCl)-induced unfolding of Tb^{3+} -reconstituted anticoagulation factor I (ACF I) in $10 \mu\text{M}$ Tb^{3+} and 0.02 M Tris-HCl buffer (pH 7.6, 25°C). (A) Fluorescence spectra of Tb^{3+} -ACF I in the presence of increasing GdnHCl concentrations of 0, 0.35, 0.70, 1.05, 1.40, 1.75, 2.10, 2.45, 2.80, 3.15, 3.50, and 4.90 M for the curves 1 to 12, at an excitation wavelength of 295 nm. The concentration of Tb^{3+} -ACF I is $1 \mu\text{M}$. (B) Unfolding transitions of Tb^{3+} -ACF I monitored by measurement of Trps fluorescence at 340 nm (squares) and Tb^{3+} fluorescence at 545 nm (circles), respectively, after exciting at 295 nm. (C) Dependence of free energy change of Tb^{3+} -ACF I on GdnHCl concentration monitored by measurement of Trps fluorescence at 340 nm (squares) and Tb^{3+} fluorescence at 545 nm (circles), respectively.

of GdnHCl were used to construct the stability profiles (Fig. 4D). All transition profiles for apo-ACF I, holo-ACF I, and Tb^{3+} -ACF I show single-step with no apparent intermediate state(s). Using a two-state mechanism (Fig. 4E), the ΔG^0 , C_m , and m values for the GdnHCl-induced unfolding tran-

sition of apo-ACF I, holo-ACF I, and Tb^{3+} -ACF I, monitored by the measurements of ellipticity at 222 nm, were calculated and compiled in Table 1.

The transition of apo-ACF I monitored by the measurements of ellipticity at 222 nm shows that the abrupt unfolding of apo-ACF I starts at $\sim 0.60 \text{ M}$ GdnHCl and completes at $\sim 1.90 \text{ M}$ GdnHCl. The ΔG^0 ($4.37 \pm 0.07 \text{ kcal/mole}$) estimated from the far-UV ellipticity is similar to that based on fluorescence spectroscopy ($4.50 \pm 0.08 \text{ kcal/mole}$). Using Equation 2, the normalized transition curves for GdnHCl-induced unfolding of apo-ACF I monitored by the measurements of ellipticity at 222 nm and fluorescence at 340 nm are nearly superimposable (data not shown). These results further indicated that GdnHCl-induced unfolding process of apo-ACF I follows a two-state ($\text{N} \leftrightarrow \text{D}$) without the accumulation of stable equilibrium intermediate(s).

The transition of holo-ACF I monitored by the measurements of ellipticity at 222 nm shows that the abrupt unfolding of holo-ACF I starts at $\sim 1.05 \text{ M}$ GdnHCl and completes at $\sim 2.20 \text{ M}$ GdnHCl (Fig. 4D). The normalized transition curve for GdnHCl-induced unfolding of holo-ACF I monitored by the measurements of ellipticity at 222 nm and the normalized transition curve for the GdnHCl-induced second transition ($\text{Ia} \leftrightarrow \text{Ib}$) of holo-ACF I monitored by the fluorescence at 340 nm are nearly superimposable (data not shown), indicating that the two-state transition of holo-ACF I monitored by the measurements of ellipticity at 222 nm corresponds to the second transition ($\text{Ia} \leftrightarrow \text{Ib}$) of holo-ACF I monitored by the fluorescence at 340 nm. The unfolding ΔG^0 ($5.03 \pm 0.13 \text{ kcal/mole}$) estimated from the far-UV ellipticity is similar to that of the $\text{N} \leftrightarrow \text{Ia} \leftrightarrow \text{Ib}$ transition based on fluorescence spectroscopy ($4.72 \pm 0.06 \text{ kcal/mole}$). However, the existence of the first and third unfolding transitions monitored by the fluorescence measurements, that is, $\text{N} \leftrightarrow \text{Ia}$ and $\text{Ib} \leftrightarrow \text{D}$, were not detected by far-UV CD measurements. These results further show that the Ia state of holo-ACF I is a compact molten globule state with a similar secondary structure to its native state, whereas the unfolding Ib of holo-ACF I is extensive disordering of the native structures only with retaining a few secondary structures.

The transition of Tb^{3+} -ACF I monitored by the measurements of ellipticity at 222 nm shows that the abrupt unfolding of Tb^{3+} -ACF I starts at $\sim 1.76 \text{ M}$ GdnHCl and completes at $\sim 3.16 \text{ M}$ GdnHCl (Fig. 4D). The normalized transition curves for GdnHCl-induced unfolding of Tb^{3+} -ACF I monitored by the measurements of ellipticity at 222 nm and fluorescence at 340 nm or at 545 nm are nearly superimposable (data not shown). These results further indicate that GdnHCl-induced unfolding process of Tb^{3+} -ACF I is single-step without the accumulation of detectable equilibrium intermediate(s). The ΔG^0 ($6.58 \pm 0.11 \text{ kcal/mole}$) estimated from the far-UV ellipticity during GdnHCl-induced unfolding is similar to that based on fluorescence spectroscopy (6.86 ± 0.09 and $6.62 \pm 0.13 \text{ kcal/mole}$ monitored by

Fig. 4. Guanidine hydrochloride (GdnHCl)-induced changes of the far-UV circular dichroism (CD) spectra of anticoagulation factor I (ACF I) in 0.02 M Tris-HCl buffer (pH 7.6, 25°C). The protein concentration is 0.10 mg/mL. (A) Far-UV CD spectra of apo-ACF I in the presence of increasing GdnHCl concentrations of 0, 0.35, 0.53, 0.70, 0.88, 1.05, 1.23, 1.40, 1.58, 1.93, 2.45, and 4.90 M for the curves from bottom to top. (B) Far-UV CD spectra of holo-ACF I in the presence of 1 mM Ca^{2+} and in the presence of increasing GdnHCl concentrations of 0, 0.35, 0.53, 0.88, 1.23, 1.40, 1.75, 1.93, 2.80, 3.50, and 4.90 M for the curves from bottom to top. (C) Far-UV CD spectra of Tb^{3+} -ACF I in the presence of 10 μM Tb^{3+} and in the presence of increasing GdnHCl concentrations of 0, 0.70, 1.40, 1.75, 2.10, 2.45, 2.63, 2.80, 3.15, 4.20, and 4.90 M for the curves from bottom to top. (D) Changes in far-UV CD ellipticity at 222 nm of apo-ACF I (circles), holo-ACF I (squares), and Tb^{3+} -ACF I (triangles). (E) Dependence of free energy changes of apo-ACF I (circles), holo-ACF I (squares), and Tb^{3+} -ACF I (triangles) on GdnHCl concentration for the unfolding transitions shown in D.

the measurements of fluorescence at 340 and 545 nm, respectively), and is also greater than the $\Delta G_{\text{total}}^0$ of holo-ACF I based on fluorescence spectroscopy (6.01 ± 0.09 kcal/mole). These results further prove that Tb^{3+} -stabilized ACF I shows higher resistance to GdnHCl denaturation than the holo-ACF I.

Metal ions-induced refolding of ACF I

Because Ca^{2+} and Tb^{3+} ions can increase the structural stability of ACF I, higher concentrations of denaturant are

required to induce it to unfold for holo-ACF I and Tb^{3+} -ACF I than for apo-ACF I. It is possible to find a denaturant concentration at which refolding from the fully denatured state to intermediate state or to the native state could be initiated by adding the metal ion to the unfolded state. It is obvious from Figures 2C and 3B that at 2.0 M GdnHCl, apo-ACF I is in the denatured state, whereas holo-ACF I and Tb^{3+} -ACF I are in the Ib state and the native state, respectively. Therefore, it might be possible to perform a refolding jump from the unfolded state of apo-ACF I to the Ib state of holo-ACF I by adding the Ca^{2+} ions, or from the

unfolded state of apo-ACF I to the native state of Tb^{3+} -ACF I by adding the Tb^{3+} ions to the unfolded state of apo-ACF I. Such transitions could be monitored by fluorescence measurements. It was found that unfolded apo-ACF I showed no change in intrinsic fluorescence spectrum after 1 h at 2.0 M GdnHCl concentration without adding metal ions. The intrinsic fluorescence intensity of apo-ACF I began to decrease after addition of 1 mM Ca^{2+} to the unfolded apo-ACF I, and the fluorescence intensity of Tb^{3+} began to increase with the decrease of the intrinsic fluorescence intensity of apo-ACF I after addition of 10 μM Tb^{3+} to the unfolded apo-ACF I. The quenching of Trp fluorescence by Ca^{2+} and Tb^{3+} and the enhancing of Tb^{3+} reflect the formation of a compact metal-binding region, indicating metal ions-induced refolding of the protein. There were no further changes of the fluorescence spectra observed after 40 min of refolding, indicating that the refolding process was completed within this time.

The refolding kinetics was monitored by Trp fluorescence at 340 nm and by Tb^{3+} fluorescence at 545 nm. Figure 5 shows the representative kinetic traces. The kinetics of Ca^{2+} -induced refolding monitored by Trp fluorescence at 340 nm could not be satisfactorily fit to a single exponential function. A sum of two-exponential terms best fits the refolding curve, yielding refolding rate constant values of 5.49 ± 0.07 and $0.90 \pm 0.01 \text{ min}^{-1}$ for the faster and slower phases, respectively. The kinetics of Tb^{3+} -induced refolding monitored by Trp fluorescence at 340 nm were also best fitted to the sum of two-exponential terms, yielding refolding rate constant values of 2.96 ± 0.04 and 0.112 ± 0.003

Fig. 5. Metal ions-induced refolding of apo-anticoagulation factor I (ACF I) in 0.02 M Tris-HCl buffer (pH 7.6), monitored by measurement of fluorescence by exciting at 295 nm. Refolding was initiated by adding 1 mM Ca^{2+} (squares, quenching of Trps fluorescence at 340 nm) or by adding 10 μM Tb^{3+} (circles, quenching of Trps fluorescence at 340 nm; triangles, enhancing of Tb^{3+} fluorescence at 545 nm) to 1 μM apo-ACF I in 2.0 M guanidine hydrochloride (GdnHCl). The curves were obtained after fitting to a single exponential term (triangles) and the sum of two-exponential terms (circles and squares), respectively.

Fig. 6. Thermal denaturation of apo-anticoagulation factor I (ACF I) and holo-ACF I in 0.02 M Tris-HCl buffer (pH 7.6). The thermal unfolding transition of apo-ACF I (squares) in the absence of Ca^{2+} and holo-ACF I (circles) in the presence of 1 mM Ca^{2+} was determined by monitoring the loss in their anticoagulant activity associated with the increase in temperature. The protein concentration was 1.0 $\mu\text{g/mL}$. Data are expressed in terms of relative activity using the activity of native holo-ACF I as references (100%).

min^{-1} for the faster and slower phases, respectively, whereas the kinetics of Tb^{3+} -induced refolding monitored by Tb^{3+} fluorescence at 545 nm were best fitted to a single exponential equation, yielding a refolding rate constant of $2.82 \pm 0.06 \text{ min}^{-1}$.

Thermal denaturation

Thermal denaturation studies on apo-ACF I and holo-ACF I have been conducted by incubating the protein at variable temperatures for 30 min and evaluating the denaturation of the protein by monitoring the loss in their anticoagulant activity associated with the increase in temperature. Figure 6 shows the effect of increasing in temperature on the loss of the anticoagulant activity in the temperature range of 30°C to 90°C. For both apo-ACF I and holo-ACF I, a sigmoidal decrease in activity with increasing temperature was observed. However, a difference in the temperature corresponding to half-denaturation (T_m) of the protein under these conditions was observed between apo-ACF I and holo-ACF I. For the apo-ACF I in the absence of Ca^{2+} ions, a T_m of $56.1 \pm 0.2^\circ\text{C}$ was observed under these conditions, whereas for the holo-ACF I in the presence of 1 mM Ca^{2+} ions, an enhancement of $5.5 \pm 0.5^\circ\text{C}$ in T_m ($61.6 \pm 0.3^\circ\text{C}$) was observed. These results indicate that Ca^{2+} -stabilized holo-ACF I shows higher resistance against thermal denaturation than the apo-ACF I.

Discussion

One of the most intriguing observations in the present study is that Ca^{2+} ions not only increase the structural stability of ACF I but also influence its unfolding/refolding behavior.

The GdnHCl-induced unfolding/refolding of apo-ACF I in the absence of Ca^{2+} is a single-step two-state process with no detectable intermediate(s), whereas the GdnHCl-induced unfolding/refolding of holo-ACF I in the presence of 1 mM Ca^{2+} follows a three-step transition with two intermediate states. As shown in Figure 2C, the GdnHCl concentrations required for the initial unfolding of apo-ACF I and holo-ACF I are similar to each other, but the GdnHCl concentrations for the complete unfolding are very different. The decalcification of holo-ACF I shifts the ending zone of unfolding/refolding curve toward lower GdnHCl concentration, thus abolishing the formation of intermediates in the absence of Ca^{2+} ions. It is deduced from these results that Ca^{2+} ions should play an important role in the stabilization of the Ia and Ib states. The Ia and Ib states should be unstable at higher GdnHCl concentration in the absence of Ca^{2+} ions; as a result, the GdnHCl-induced unfolding/refolding of apo-ACF I in the absence of Ca^{2+} is a two-state process with no intermediate(s).

The Ia state of holo-ACF I at 1.05 M GdnHCl seems to have following characteristics: (1) a molten globule state with the incomplete native structure, at least around part of Trp residues, according to the change of intrinsic fluorescence intensity from the N state to the Ia state; (2) intact secondary structure, as judged from little change of far-UV CD spectra from the N state to the Ia state; and (3) the presence of a compact dehydrated structure with only a minor increase in the level of solvent exposure of the Trp residues, as indicated by the little shift of the intrinsic fluorescence from the N state to the Ia state. The properties of the unfolding Ib state at 2.05 M GdnHCl seems to be as follows: (1) the lost of native structure, according to the significant increase of intrinsic fluorescence intensity from the Ia state to the Ib state; (2) the lost of most secondary structure, as indicated by markedly change of far-UV CD spectra from the Ia state to the Ib state; (3) the retention of a few secondary structures within local regions, as judged from the increase of intrinsic fluorescence intensity from the Ib state to the D state; and (4) the exposure of the most Trp residues, based on the significant red shift of the intrinsic fluorescence from the Ia state to the Ib state.

It is obvious from Figures 2C and 3B that the GdnHCl concentrations required for the complete unfolding of holo-ACF I and Tb^{3+} -ACF I are almost identical, but the binding of Tb^{3+} ions to ACF I shifts the initial zone of denaturation curve toward higher GdnHCl concentration, thus abolishing the formation of intermediate(s) in the presence of 10 μM Tb^{3+} ions. In other words, Tb^{3+} ions are able to more strongly stabilize the native conformation of ACF I than Ca^{2+} ions, which can be inferred from the greater free energy change ΔG^0 of Tb^{3+} -ACF I than the ΔG_{total}^0 of holo-ACF I. Therefore, a higher concentration of GdnHCl is required to induce the protein unfolding, at which the Ia and

Ib states should be unstable; as a result, Tb^{3+} -ACF I has undergone single-step transition with no detectable intermediate(s) in the presence of 10 μM Tb^{3+} ions (Fig. 3B).

As indicated in the introduction, it is reasonable to assume that ACF I should have a similar backbone structure and similar structures of Ca^{2+} -binding sites to habu IX/X-bp and habu IX-bp. All atoms coordinating with both Ca^{2+} ions in habu IX/X-bp and habu IX-bp are oxygen atoms (Mizuno et al. 1999). The sites of Lu^{3+} and Sm^{3+} in heavy-atom soaked crystals of habu IX-bp are identical with that of Ca^{2+} . The structures of Tb^{3+} -binding sites in ACF I should be also identical with that of Ca^{2+} -binding sites. Based on this assumption, all atoms coordinating with both Ca^{2+} or Tb^{3+} ions in ACF I are also oxygen atoms. Oxygen atom is a hard base, whereas Ca^{2+} and Tb^{3+} are hard acids. Tb^{3+} has higher charge and smaller ionic radius than Ca^{2+} and, therefore, has stronger ionic potential and harder acidity. Thus, it has higher affinity to oxygen atom than Ca^{2+} according to the hard-soft acid-base principle (Chattaraj et al. 2001). It might be the reason why Tb^{3+} -ACF I is more stable than Ca^{2+} -ACF I.

Interestingly enough, by comparing the denaturation profiles of apo-ACF I, holo-ACF I, and Tb^{3+} -ACF I, we found that under appropriate denaturing condition (2.0 M GdnHCl), a refolding jump could be initiated. Indeed, we were able to initiate refolding of unfolded apo-ACF I simply by adding 1 mM Ca^{2+} or 10 μM Tb^{3+} . Trps fluorescence measurements show that both refolding processes from the unfolded state of apo-ACF I to the Ib state of holo-ACF I induced by 1 mM Ca^{2+} and from the unfolded state of apo-ACF I to the native state of Tb^{3+} -ACF I induced by 10 μM Tb^{3+} are best fit a sum of two exponential terms, indicating a fast and a slow folding population in both processes (Fig. 5). It is interesting to note that the kinetics of Tb^{3+} -induced refolding from the unfolded state of apo-ACF I to the native state of Tb^{3+} -ACF I monitored by Tb^{3+} fluorescence at 545 nm is best fitted to a single exponential equation, yielding a refolding rate constant of $2.82 \pm 0.06 \text{ min}^{-1}$, which is similar to the refolding rate constant value of $2.96 \pm 0.04 \text{ min}^{-1}$ for the faster phase of Tb^{3+} -induced refolding obtained from Trps fluorescence measurements. This indicates that the increasing of Tb^{3+} fluorescence reflects the faster step in the refolding reaction, which involves formation of a compact metal-binding site region. It can be deduced from these results that the compact Tb^{3+} -binding regions form first, and subsequently, the protein undergoes further conformational rearrangements, as observed from the decrease of the intrinsic fluorescence corresponding to the slower step in the refolding reaction. Although we can not infer the detailed picture of the pathway of the metal ions-induced refolding from present data, it is certain that the metal ions-induced refolding of apo-ACF I could be performed without changing the concentration of the denaturant. Further investigation is necessary to elucidate the structures of intermediate

states and the mechanism of the metal ions-induced refolding.

Protein activity can be regarded as the most sensitive probe for studying the changes in the protein conformation during denaturation, as it reflects subtle readjustments at the active site. However, it is very difficult to trace the change of the anticoagulant activity of ACF I during its unfolding and refolding. Anticoagulant activity was determined using a modification of the plasma prothrombin time assay as described previously (Xu et al. 2000). When we measured the anticoagulant activity of ACF I during its unfolding and refolding, we found that GdnHCl had a marked effect on its plasma prothrombin time. Even though in the absence of ACF I, rabbit plasma did not clot in 0.30 M GdnHCl after 12-h incubation of the plasma with thromboplastin and 5 mM Ca^{2+} . A plausible explanation is that GdnHCl is a competitive inhibitor of the trypsin-like serine proteases in the coagulation cascade. It is also difficult to trace the change of the binding ability of ACF I to FXa in the denaturation process for a possible reason that FXa, as a protein, may be also affected by GdnHCl. After extensive dialysis of 6.0 M GdnHCl denatured holo-ACF I and apo-ACF I against 0.02 M Tris-HCl buffer (pH 7.6) containing 1 mM Ca^{2+} to remove GdnHCl, respectively, both almost recover the full anticoagulant activities in their native states, indicating that both GdnHCl-induced denaturations of holo-ACF I and apo-ACF I are reversible.

It has been reported that habu IX/X-bp undergoes a conformational change on binding of Ca^{2+} ions and forms a crystal only when 3 mM Ca^{2+} ions are present, indicating that it adopts a loose amorphous conformation and a rigid ordered conformation in the absence and presence of Ca^{2+} ions, respectively (Mizuno et al. 1991). Similarly, holo-ACF I and Tb^{3+} -ACF I show higher resistance to GdnHCl denaturation than the apo-ACF I, indicating they should have a significantly more compact conformation than the apo protein. The stabilizing effect of Ca^{2+} ions on the overall structure of holo-ACF I was also confirmed by studying the thermal denaturation of apo-ACF I and holo-ACF I. From these results, we speculate that Ca^{2+} ions may play similar roles in keeping protein structure and function for ACF I as well as for habu IX/X-bp.

The difference in folding of apo-ACF I, Ca^{2+} -ACF I, and Tb^{3+} -ACF I is probably caused by differences in metal binding properties. ACF I consists of two chains. Each chain should function as individual domain against unfolding and have one Ca^{2+} -binding site based on the assumption that ACF I has a similar structure to habu IX/X-bp. Without Ca^{2+} -induced stabilization of ACF I, two domains of apo-ACF I might have a similar stability against unfolding, with a similar unfolding transition. Tb^{3+} binds to both domains with very similar affinity (unpublished data), thus two domains of Tb^{3+} -ACF I might also have similar stability against unfolding with a similar unfolding transition. As a

result, it is possible that the unfolding of both apo-ACF I and Tb^{3+} -ACF I has a two-state transition. Because the both domains show different calcium affinities, each domain might follow a two-state model with different C_m , and the unfolding of Ca^{2+} -ACF I should follow a three-state transition. However the above results show that Ca^{2+} -ACF I shows a four-state transition. Therefore, further investigation is necessary to clarify the effects of metal ions on the folding.

Although Tb^{3+} has stronger stabilization of the conformation of ACF I than Ca^{2+} , as inferred from the higher initial zone of denaturation curve of Tb^{3+} -ACF I than that of holo-ACF I, the binding of ACF I with FXa is dependent on Ca^{2+} ions, whereas trivalent lanthanide ions, such as Nd^{3+} , Sm^{3+} , Eu^{3+} , Gd^{3+} , and Tb^{3+} , are all ineffective to induce the binding (data not shown). The particular GdnHCl-induced transition profile of holo-ACF I, compared with that of apo-ACF I and Tb^{3+} -ACF I, reveals that Ca^{2+} ions play unique roles in stabilizing the specific conformation of holo-ACF I. This specific conformation should be helpful to its recognition of the structure of FXa, thereby promoting the association of two proteins. However, trivalent lanthanide ions have stronger ionic potentials and harder acidity than Ca^{2+} ; thus, the Tb^{3+} ions-stabilized conformation of Tb^{3+} -ACF I may be unsuitable for its recognition of the conformation of FXa and cannot support the binding of Tb^{3+} -ACF I with FXa. Another possible reason for the dependence of the binding of ACF I with FXa on Ca^{2+} ions may be that FXa itself is a Ca^{2+} -binding protein with multiple Ca^{2+} -binding sites, and the binding of Ca^{2+} ions to FXa also induces the conformational changes of FXa (Jackson 1984; Stenflo 1991; Sunnerhagen et al. 1995, 1996a,b). Thus, the binding of Ca^{2+} ions to ACF I and FXa not only induces the conformational change of ACF I but also induces the conformational change of FXa, and both conformational changes might be essential for the recognition of each other.

Metal ions not only increase the structural stability of ACF I against GdnHCl denaturation and thermal denaturation but also influence its unfolding/refolding behavior. The GdnHCl-induced unfolding/refolding of apo-ACF I and Tb^{3+} -ACF I is a single-step two-state process with no detectable intermediate state(s), whereas the GdnHCl-induced unfolding/refolding of holo-ACF I in the presence of 1 mM Ca^{2+} follows a three-step transition with two intermediate states. Ca^{2+} ions play an important role in the stabilization of both Ia and Ib states. Tb^{3+} -stabilized ACF I shows higher resistance to GdnHCl denaturation than does the holo-ACF I. It is possible to induce refolding of the unfolded apo-ACF I merely by adding 1 mM Ca^{2+} ions or 10 μM Tb^{3+} ions without changing the concentration of the denaturant. The kinetic results of metal ions-induced refolding provide evidence that the compact Tb^{3+} -binding regions form first, and subsequently, the protein undergoes further conformational rearrangements to form the native structure.

Materials and methods

Materials

Lyophilized venom powder was provided by the TUN-XI Snakebite Institute (P.R. Anhui, China). Guanidine hydrochloride (GdnHCl; ultrapure), Tb_4O_7 (99.9%), was obtained from Sigma Chemical Company. Chelex-100 was purchased from Bio-Rad Laboratories. All other reagents were of analytical reagent grade. Milli-Q purified water was used throughout.

ACF I was purified by the method described previously (Xu et al. 2000). The apo-ACF I was prepared by dialysis of purified ACF I against a suspension of Chelex-100 (1 g/L; Bio-Rad) in 0.01 M Tris-HCl (pH 8.0). ACF I concentration were calculated from the absorption coefficient ($A_{1\text{cm}}^{1\%} = 30$) at 280 nm and the relative molecular weight ($M_r = 29,604$). Tb_4O_7 was dissolved in concentrated HCl by gentle heating to dryness and then dissolved in Milli-Q super-pure water, and the pH value was adjusted to 6.0 with HCl or NaOH. The solutions of Tb^{3+} and Ca^{2+} were standardized by titration with standard EDTA solution. Tris-HCl buffer used was freed from any possible contamination of multi-valent cations by passage through a column (25×3 cm) of Chelex-100. GdnHCl was determined to be metal-free by extraction with dithizone (6 mg/L) in carbon tetrachloride. All utensils used during the experiments were made metal-free by soaking in 2 M HNO_3 for 24 h and then by extensively rinsing with Milli-Q purified water.

Steady-state fluorescence measurements

All fluorescence measurements were performed on a Shimadzu RF-5000 spectrofluorometer using a 10-mm quartz cuvette. The sample temperature was kept at 25°C with a circulating water bath. In all experiments, the samples were excited at 295 nm, and the bandwidths for excitation and emission were both set to 5 nm. Each spectrum is the average of three consecutively acquired spectra. All spectra were corrected by subtracting the spectrum of the blank, lacking the protein but otherwise identical to the sample.

CD measurements

CD measurements were performed with a Jasco J-720 spectropolarimeter. All the CD measurements were made at 25°C with a thermostatically controlled cell holder. Far-UV CD spectra were collected with a scan speed of 20 nm/min and a response time of 1 sec, at a protein concentration of 0.10 mg/mL in quartz cells of 1-mm path length. The obtained values were normalized by subtracting the baseline recorded for the buffer having the same concentration of salts under similar conditions. The data were expressed in mean residue ellipticity (θ) in $\text{deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$, which is defined as $[\theta] = 100\theta_{\text{obs}}(lc)^{-1}$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in residue moles per liter, and l is the length of the light path in centimeters.

Denaturation and renaturation experiments

Solutions for the denaturation and renaturation experiments were prepared from stock solutions of protein and GdnHCl prepared in 20 mM Tris-HCl buffer (pH 7.6). According to the method described by Muzammil et al. (2000), in denaturation experiments to a stock protein solution, different volumes of the buffer were added first and the denaturant was added last so as to get the

desired concentration of denaturant. On the other hand, in renaturation experiments to a stock protein solution, different volumes of concentrated denaturant solution were added first, the mixture was incubated for 4 h, and finally buffer was added to get desired denaturant concentration. The final solution mixture for both the denaturation and renaturation experiment was incubated for 12 h at 25°C before fluorescence measurements.

Refolding kinetic measurements

For the refolding kinetic measurements, 1 mL of the sample solution was continuously excited at 295 nm, and the emission intensity was collected at 340 nm for intrinsic fluorescence or at 545 nm for Tb^{3+} fluorescence with a response time of 2.0 sec. The data were collected in the time scan mode after the refolding process was initiated by adding 2 μL of 0.5 M Ca^{2+} or 2 μL of 5 mM Tb^{3+} ions and continued for at least 40 min. The kinetic experiment was performed three times to ensure that the results were reproducible.

Thermal denaturation

Apo-ACF I (1 $\mu\text{g/mL}$) and holo-ACF I (1 $\mu\text{g/mL}$) in 20 mM Tris-HCl buffer (pH 7.6) were incubated in the absence and presence of 1 mM CaCl_2 , respectively, at the desired temperature for 30 min followed by quenching in ice for 5 min, and finally, the anticoagulant activity of the protein was measured using a modification of the plasma prothrombin time assay as described previously (Xu et al. 2000).

Data analysis

Unfolding and refolding curves were analyzed using either two-state or four-state mechanisms.

Two-state mechanism

Unfolding curves for the $\text{N} \leftrightarrow \text{D}$ transition were normalized to the apparent fraction of the unfolding form, F_D , using the following equation (Tanford 1968):

$$F_D = (Y - Y_N)/(Y_D - Y_N) \quad (2)$$

where Y is the observed variable parameter, and Y_N and Y_D are the values of the characteristic of the native and fully unfolded conformations, respectively. The difference in free energy between the folded and the unfolded states, ΔG , was calculated by the following equation:

$$\Delta G = -RT \ln K = -RT \ln[F_D/(1 - F_D)] \quad (3)$$

where K is the equilibrium constant, R is the gas constant, and T is the absolute temperature.

Four-state mechanism

For the unfolding transition, $\text{N} \leftrightarrow \text{Ia} \leftrightarrow \text{Ib} \leftrightarrow \text{D}$, where Ia and Ib are the intermediate state a and the intermediate state b, respectively, and each step may be assumed to follow a two-state mechanism. The fraction of the Ia, F_{Ia} , in the transition $\text{N} \leftrightarrow \text{Ia}$ can be obtained from the relation:

$$F_{Ia} = (Y - Y_N)/(Y_{Ia} - Y_N) \quad (4)$$

where $F_{Ia} + F_N = 1$. The fraction of the Ib, F_{Ib} in the transition $Ia \leftrightarrow Ib$ can be calculated from the relation:

$$F_{Ib} = (Y - Y_{Ia})/(Y_{Ib} - Y_{Ia}) \quad (5)$$

where $F_{Ib} + F_{Ia} = 1$. Similarly, the fraction of the D state, F_D in the transition $Ib \leftrightarrow D$ can be calculated from the relation:

$$F_D = (Y - Y_{Ib})/(Y_D - Y_{Ib}) \quad (6)$$

where $F_D + F_{Ib} = 1$. The equilibrium constant and the free energy for the above transitions may be calculated from the following relationships:

For $N \leftrightarrow Ia$ transition,

$$K_{Ia} = F_{Ia}/(1 - F_{Ia}) \quad (7)$$

and

$$\Delta G_{Ia} = -RT \ln K_{Ia} \quad (8)$$

For $Ia \leftrightarrow Ib$ transition,

$$K_{Ib} = F_{Ib}/(1 - F_{Ib}) \quad (9)$$

and

$$\Delta G_{Ib} = -RT \ln K_{Ib} \quad (10)$$

For $Ib \leftrightarrow D$ transition,

$$K_D = F_D/(1 - F_D) \quad (11)$$

and

$$\Delta G_D = -RT \ln K_D \quad (12)$$

These data were analyzed assuming the free energy of unfolding or refolding, ΔG to be linearly dependent on $[GdnHCl]$ denoted here by C essentially, as described in detail previously (Pace, 1990):

$$\Delta G = \Delta G^0 - mC = m(C_m - C) \quad (13)$$

in which ΔG^0 and ΔG represent the free energy of unfolding or refolding in the absence and presence of GdnHCl, respectively; C_m is the midpoint concentration of GdnHCl required for unfolding or refolding; and m stands for the slope of the unfolding or refolding curve at C_m . A least-squares curve fitting analysis was used to calculate the values of ΔG^0 , m , and C_m by a routine of software.

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