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ORIGINAL PAPER

Calcium Ion-Induced Stabilization and Refolding of Agkisacutacin from *Agkistrodon Acutus* Venom Studied by Fluorescent Spectroscopy

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Abstract Agkisacutacin isolated from the venom of *Agkistrodon acutus* is a coagulation factor IX / coagulation factor X-binding protein with marked anticoagulant- and platelet-modulating activities. Ca^{2+} ion-induced stabilization and refolding of Agkisacutacin have been studied by following fluorescent measurements. Ca^{2+} ions not only increase the structural stability of agkisacutacin against GdnHCl denaturation, but also induce its refolding. The GdnHCl-induced unfolding of the apo-agkisacutacin and the purified agkisacutacin is a single-step process with no detectable intermediate state. Ca^{2+} ions play an important role in the stabilization of the structure of agkisacutacin. Ca^{2+}-stabilized agkisacutacin exhibits higher resistance to GdnHCl denaturation than the apo-agkisacutacin. It is possible to induce refolding of the unfolded apo-agkisacutacin merely by adding 1 mM Ca^{2+} ions without changing the concentration of the denaturant. The kinetic result of Ca^{2+}-induced refolding provides evidences for that agkisacutacin consists of at least two refolding phases and the first phase of Ca^{2+}-induced refolding should involve the formation of the compact Ca^{2+}-binding site regions, and subsequently, the protein undergoes further conformational rearrangements to form the native structure.

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Introduction

Many C-type lectin-like proteins (CLPs) have been isolated from venoms of different snake species [1]. These CLPs are highly conservative in sequences and similar in three-dimensional structures. However, they possess very diverse functions for their abilities to recognize different ligands. Agkisacutacin alias FP, isolated from the venom of *Agkistrodon acutus* is a unique CLP with bifunctional activities, i.e. anticoagulant- and platelet-modulating activities [2–4]. Agkisacutacin has been identified as both platelet glycoprotein Ib-binding protein and coagulation factor IX (FIX)/coagulation factor X (FX)-binding protein [5]. Agkisacutacin is devoid of hemorrhagic and lethal activities, which can be used as a basis for designing anticoagulant drugs, as well as a useful tool for elucidating the complex mechanisms involved in clotting and platelet activation. Currently the detailed three-dimensional structure of agkisacutacin has remained undetermined. Agkisacutacin binds with one Ca^{2+} ion which is essential for the binding of agkisacutacin to FIX/FX [4, 5]. However the effect of Ca^{2+} ion on the conformational stabilization of agkisacutacin is still unclear. We expected that analysis of the effect of Ca^{2+} ions on the structural stability of agkisacutacin would be useful for improving our understanding of the function of Ca^{2+} ions in the binding of agkisacutacin to FIX/FX and platelet glycoprotein Ib. In order to examine the role of Ca^{2+} ion in the conformational stabilization of agkisacutacin, studies on guanidine hydrochloride (GdnHCl)-induced denaturation, and Ca^{2+} ion-induced refolding of agkisacutacin were carried out in this paper.

Fluorescence spectroscopy is an important analytical technique suitable for study of protein folding/unfolding due to its inherent sensitivity [6, 7]. Therefore it has been used to monitor the GdnHCl-induced unfolding/refolding of agkisacutacin. Protein folding/unfolding is *a highly cooperative process*. Protein folding/unfolding can be affected by factors such as pressure [8], temperature [9], pH [10], and disulfide bond [11]. Recently, the effects of metal ions on protein unfolding/refolding have received considerable attentions and it has been shown that metal ion-induced conformation changes in several enzymes lead to stabilization of the proteins during protein folding/unfolding [12, 13]. In addition, without changing the concentration of the denaturant, the refolding of several proteins can be induced simply by adding metal ions [14–16].

The present investigation provides evidence that Ca^{2+} ion not only significantly increases the structural stability of Agkisacutacin against GdnHCl denaturation, but also induce the refolding of the unfolded apo-Agkisacutacin merely by adding 1 mM Ca^{2+} to the unfolded apo-Agkisacutacin without changing the concentration of the denaturant. Ca^{2+} ion-induced refolding kinetic results suggest that Agkisacutacin consists of at least two refolding domains and the first phase of Ca^{2+} ion-induced refolding should involve the formation of the compact metal-binding site regions.

Materials and methods

Materials

Lyophilized venom powder was provided by the TUN-XI Snakebite Institute (Anhui, P.R. China). Guanidine hydrochloride (GdnHCl; ultrapure) and $CaCl_2$ were obtained from Sigma Chemical Company (St Louis, MO, USA). Chelex-100 was purchased from Bio-Rad Laboratories (Richmond, Calif. USA). All other reagents were of analytical reagent grade. Milli-Q purified water was used throughout.

Protein purification and preparation

Agkisacutacin was purified by the method described previously [4]. The apo-agkisacutacin was prepared by dialysis of purified agkisacutacin against a suspension of Chelex-100 (Bio-Rad) (1 g/L) in 0.01 M Tris-HCl (pH 7.6). The solution of Ca^{2+} ions was prepared from $CaCl_2$ in Milli-Q water and standardized by titration with standard EDTA solution. Tris-HCl buffer used was freed from any possible contamination of multivalent cations by passage through a column (25 $\times$ 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 25.0°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.

Unfolding experiments

Solutions for the unfolding experiments were prepared from stock solutions of protein and GdnHCl prepared in 20 mM Tris-HCl buffer, pH 7.6. The concentration of concentrated stock solution of GdnHCl was determined refractometrically [17]. According to the method described by Muzammil et al. [18], in unfolding experiments, to a stock protein solution, different volumes of the buffer were added first and the denaturant was added last *to get the desired concentration of denaturant*.

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 337 nm for intrinsic fluorescence with a response time of 1.0 S. 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+} ions and continued for at least 40 min. The kinetic experiment was carried out three times to ensure that the results were reproducible.

Data analysis

Unfolding curves were analyzed using two state mechanism [15, 16, 18]. The two-state model is represented as:

$$N \leftrightarrow D \tag{1}$$

where N and D are the native and denatured states, respectively. The equilibrium constant is defined *as follows*:

$$K = [D]/[N] \tag{2}$$

The fraction of each species is

$$f_N = 1/(1 + K);\ f_D = K/(1 + K) \tag{3}$$

The difference in free energy between the native and the unfolded states, ΔG, was calculated by the following equation:

$$\Delta G = -RT \ln K \tag{4}$$

where R is the gas constant and T is the absolute temperature.

The measured signal, Y, is assumed to contain contributions from each species

$$Y = f_{\rm N} Y_{\rm N} + f_{\rm D} Y_{\rm D} \tag{5}$$

where f_j represents the fraction of species j (as defined in Eq. (3)) and Y_j is the molar signal of species j. The signals corresponding to the native and the unfolded state are considered as local fitting parameters that have a linear dependence on denaturant:

$$Y_j = Y_j^0 + \alpha_j [\text{GdnHCl}] \tag{6}$$

where Y_j^0 is the molar signal of species j at zero denaturant and α_j is a "slope" describing the dependence of signal Y_j on denaturant concentration. In the denaturant range where the unfolding process does not make a significant contribution to changes *in the spectrum, Eq. (6)* describes the "baseline" of species j.

ΔG corresponding to equilibrium constant is assumed to be linearly dependent on [GdnHCl] denoted here by C, essentially as described in detail previously [15, 16, 18]:

$$\Delta G = \Delta G^0 - mC = m(C_m - C) \tag{7}$$

in which ΔG^0 and ΔG represent the free energy of unfolding in the absence and presence of GdnHCl, respectively, C_m is the midpoint concentration of GdnHCl required for unfolding, and m stands for the slope of the unfolding curve at C_m and is a measure of the dependence of ΔG on denaturant concentration.

The thermodynamic and spectroscopic parameters were optimized in global fits by nonlinear least-square analysis using the Marquardt-Levenburg algorithm in a routine of software. Global analysis is a powerful tool for revealing thermodynamic properties of protein unfolding/refolding [19].

Results

The effect of Ca^{2+} ions on the intrinsic fluorescence of agkisacutacin

The intrinsic fluorescence of Trp residues in agkisacutacin was used for studies of its unfolding behavior. As there are

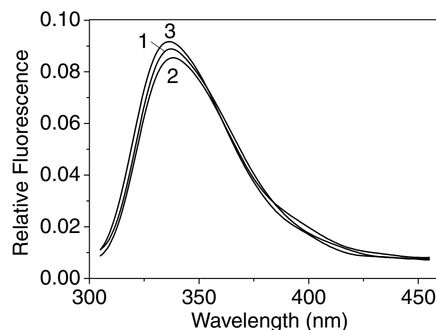

Fig. 1 The intrinsic fluorescence emission spectrum of agkisacutacin excited at 295 nm in 0.02 M Tris-HCl buffer (pH 7.6, 25°C). (1) The purified agkisacutacin in the absence of Ca^{2+}. (2) The apo-agkisacutacin in the absence of Ca^{2+}. (3) The purified agkisacutacin in the presence of 1 mM Ca^{2+}

14 Trps in agkisacutacin [5], the overall changes in fluorescence reflect global changes in protein structure, and average microenvironments of Trps can be assessed. Agkisacutacin contains both Trp and Tyr residues. The fluorescence of Trp residues can be selectively investigated by exciting the protein *at wavelength 295 nm* because at this wavelength, the fluorescence emission is essentially due to Trp residue [20]. The wavelength of the emission maximum $\lambda_{\max}$ for Trp depends on its microenvironment. Specifically, a low polarity, hydrophobic microenvironment is characterized by $\lambda_{\max} \approx$ 331 nm, while for Trp in an aqueous phase $\lambda_{\max}$ is 350–353 nm [21]. As shown in Fig. 1 (curve 1), the maximum excitation and emission of purified agkisacutacin are at 280 nm and 337.5 nm respectively. The about 13 nm blue shift of $\lambda_{\max}$ of the purified agkisacutacin relative to that of Trp in an aqueous phase suggests that some Trp residues in the purified agkisacutacin are located in some hydrophobic environment. Removal of Ca^{2+} from the purified agkisacutacin causes its fluorescence emission intensity to slightly decrease with 0.5 nm red-shift of emission maximum $\lambda_{\max}$ (Fig. 1, curve 2). As shown in Fig. 1 (curve 3), the fluorescence emission intensity of the purified agkisacutacin slightly increases with 0.5 nm blue-shift of emission maximum $\lambda_{\max}$ after addition of 1 mM Ca^{2+}. *These results suggest that* Ca^{2+} *has no obvious effects on the microenvironments of the Trps in agkisacutacin.*

GdnHCl-induced unfolding

Figure 2 shows the effects of GdnHCl on the fluorescence of the apo-agkisacutacin and the purified agkisacutacin in the absence or presence of 1 mM Ca^{2+}. It is notable that the GdnHCl-induced unfolding causes the fluorescence emission intensities of both apo-agkisacutacin and the purified agkisacutacin in the absence or presence of 1 mM Ca^{2+} to increase with the red-shift of emission maximum $\lambda_{\max}$, suggesting that both apo-agkisacutacin and the purified

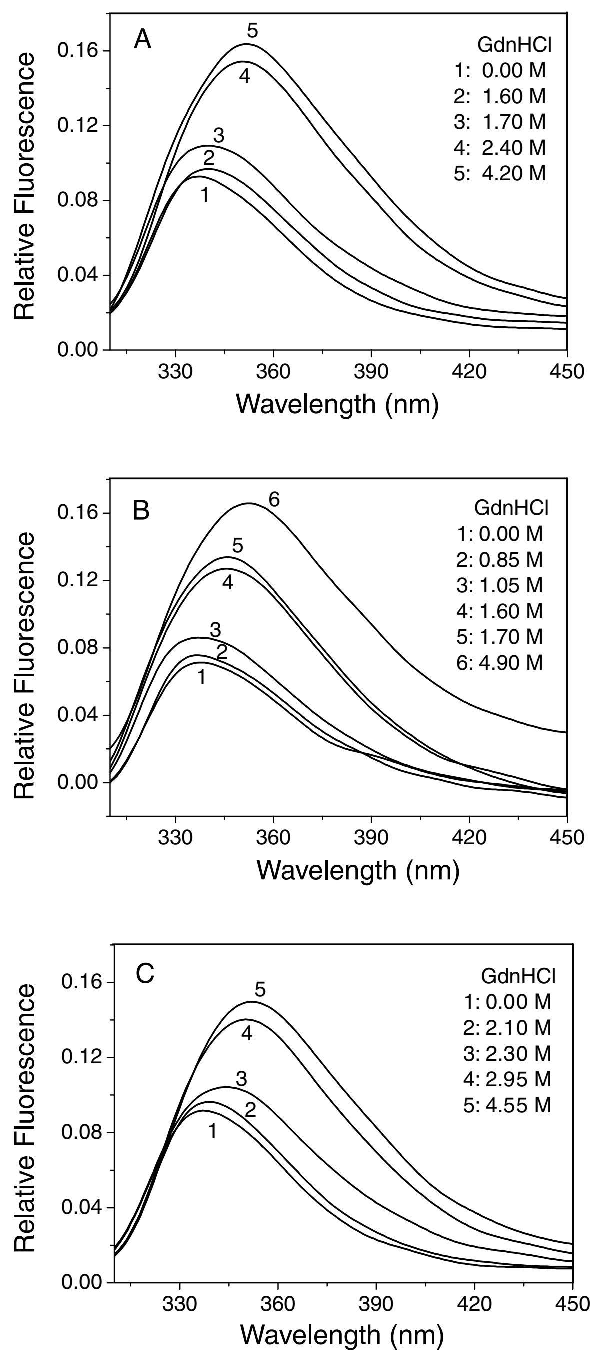

Fig. 2 The intrinsic fluorescence emission spectrum of agkisacutacin excited at 295 nm in the presence of increasing GdnHCl concentration in 0.02 M Tris-HCl buffer (pH 7.6, 25°C). (A) The purified agkisacutacin in the absence of Ca^{2+}. (B) The apo-agkisacutacin in the absence of Ca^{2+}. (C) The purified agkisacutacin in the presence of 1 mM Ca^{2+}

agkisacutacin assume a compactly folded structure in which the most Trps and quenchers, such as the charged carboxyl and/or amino groups in the interior protein, are adjacent as observed for bovine β-lactoglobulin [22].

As shown in Fig. 3 (curve 1, 2), GdnHCl-induced denaturation of both apo-agkisacutacin and the purified agkisacutacin was found to be a single-step process with no detectable intermediate state(s). The transition starts at around 1.0 M GdnHCl and slopes off at 2.4 M GdnHCl with a red shift of the λ_{max} (from 338 to 352 nm) for apo-agkisacutacin. The transition starts at around 1.5 M GdnHCl and slopes off at 2.8 M GdnHCl with a red shift of the λ_{max} (from 337.5 to 352 nm) for the purified agkisacutacin. As shown in Fig. 3 (curves 3), GdnHCl-induced denaturation of purified agkisacutacin was still a single-step process in the presence of 1 mM Ca^{2+}. The transition starts at around 2.1 M GdnHCl and slopes off at 3.3 M GdnHCl with a red shift of the λ_{max} (from 337 to 352 nm) in the presence of 1 mM Ca^{2+}.

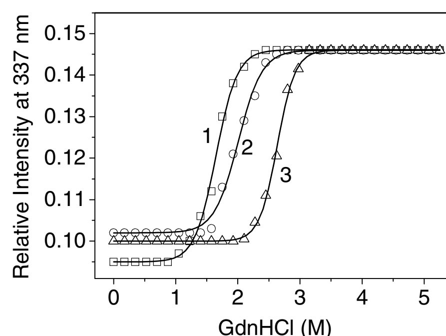

Fig. 3 GdnHCl-induced unfolding of agkisacutacin in 0.02 M Tris-HCl buffer, pH 7.6, 25°C. The protein concentration of 1 μM was used in the study. Unfolding transitions of agkisacutacin were monitored by measurement of fluorescence at 337 nm after exciting at 295 nm. The curves are the fitting curves based on experimental points by the global analysis according to Eq. (5). (1) The apo-agkisacutacin in the absence of Ca^{2+}. (2) The purified agkisacutacin in the absence of Ca^{2+}. (3) The purified agkisacutacin in the presence of 1 mM Ca^{2+}

The transition of GdnHCl-induced unfolding of apo-agkisacutacin and the purified agkisacutacin in the absence or presence of 1 mM Ca^{2+} follows two-state mechanism. The thermodynamic parameters were obtained by the global analysis for two-state transition according to Eq. (5). The ΔG°, C_m and m values obtained for the GdnHCl-induced unfolding transition of apo-agkisacutacin are 5.01 $\pm$ 0.08 kcal mol^{-1}, 1.59 $\pm$ 0.02 M and 3.15 $\pm$ 0.06 kcal M^{-1} mol^{-1} respectively. Similarly, the ΔG°, C_m and m values obtained for the GdnHCl-induced unfolding transition of the purified agkisacutacin in absence of Ca^{2+} are 5.97 $\pm$ 0.07 kcal mol^{-1}, 1.99 $\pm$ 0.03 M and 3.00 $\pm$ 0.04 kcal M^{-1} mol^{-1} respectively; while the ΔG°, C_m and m values obtained for the GdnHCl-induced unfolding transition of purified agkisacutacin in the presence of 1 mM Ca^{2+} are 10.80 $\pm$ 0.11 kcal mol^{-1}, 2.63 $\pm$ 0.03 M and 4.10 $\pm$ 0.07 kcal M^{-1} mol^{-1} respectively.

A comparison of the free energy changes of apo-agkisacutacin and the purified agkisacutacin in absence of Ca^{2+} and in the presence of 1 mM Ca^{2+} during GdnHCl-induced unfolding clearly indicates that the ΔG of the purified agkisacutacin decreases 0.96 $\pm$ 0.13 kcal mol^{-1} after removal of Ca^{2+} and increases 4.83 $\pm$ 0.18 kcal mol^{-1} in the presence of 1 mM Ca^{2+}, i.e. decalcified agkisacutacin

exhibits lower resistance to GdnHCl denaturation than the purified agkisacutacin, and the purified agkisacutacin exhibits higher resistance to GdnHCl denaturation in the presence of 1 mM Ca^{2+} than in the absence of Ca^{2+}. These results demonstrate that Ca^{2+} ion in agkisacutacin markedly stabilizes its conformation.

Ca^{2+}-induced refolding of agkisacutacin

Because Ca^{2+} ions can increase the structural stability of agkisacutacin, higher concentrations of denaturant are required to induce it to unfold for the purified agkisacutacin in the presence of 1 mM Ca^{2+} than for apo-agkisacutacin in the absence of Ca^{2+}. It is possible to find a denaturant concentration at which refolding from the denatured state to the native state could be initiated by adding the Ca^{2+} ion to the unfolded state. It is obvious from Fig. 3 that at 2.2 M GdnHCl, apo-agkisacutacin is in the denatured state, while the purified agkisacutacin in the presence of Ca^{2+} is in the native state. Therefore, it might be possible to perform a refolding jump from the unfolded state of apo-agkisacutacin to the native state of the purified agkisacutacin by adding the Ca^{2+} ions. Such transitions could be monitored by fluorescence measurements. It was found that without adding Ca^{2+} ions, unfolded apo-agkisacutacin exhibited no change in intrinsic fluorescence spectrum after 1 h at 2.2 M GdnHCl concentration. The intrinsic fluorescence intensity of apo-agkisacutacin began to decrease with a blue shift of the λ_{max} (from 352 to 337 nm) after addition of 1 mM Ca^{2+} to the unfolded apo-agkisacutacin.

The quenching of Trp fluorescence with a blue shift of the λ_{max} (from 352 to 337 nm) by Ca^{2+} reflects the formation of compact metal-binding regions, suggesting Ca^{2+}-induced refolding of the protein. There were no further changes of the fluorescence spectra observed after 30 min of refolding, indicating that the refolding process was completed within this time. The refolding kinetics was monitored by Trp fluorescence at 337 nm after addition of Ca^{2+} ions to the unfolded apo protein. Figure 4 shows the representative kinetic trace. The kinetics of Ca^{2+}-induced refolding monitored by Trp fluorescence at 337 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 3.632 $\pm$ 0.023 and 0.746 $\pm$ 0.008 min^{-1}, for the faster and slower phases, respectively.

Discussion

The present study aims to investigate the effects of Ca^{2+} on the conformational stability of agkisacutacin. The result indicates that Ca^{2+} ions not only increase the structural stability of agkisacutacin but also induce the refolding of

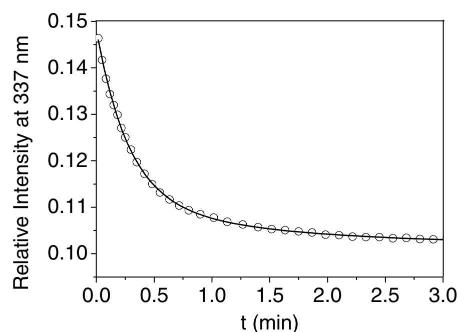

Fig. 4 Ca^{2+} ions-induced refolding of apo-agkisacutacin from unfolded state at 2.2 M GdnHCl in 0.02 M Tris-HCl buffer, pH 7.6, monitored by measurement of fluorescence at 337 nm by exciting at 295 nm. Refolding was initiated by adding 1 mM Ca^{2+} to 1 μM apo-agkisacutacin in 2.2 M GdnHCl solution. The curve was obtained after fitting to a sum of two-exponential terms

unfolded apo-agkisacutacin simply by adding 1 mM Ca^{2+}. As shown in Fig. 3, the GdnHCl-induced unfolding of apo-agkisacutacin and the purified agkisacutacin in the absence of Ca^{2+} or in the presence of Ca^{2+} is a two-state process with no detectable intermediate(s). The decalcification of the purified agkisacutacin shifts both the initial and ending zones of unfolding curve towards lower GdnHCl concentrations. Addition of 1 mM Ca^{2+} to the purified agkisacutacin significantly shifts both the initial and ending zones of unfolding curve towards higher GdnHCl concentrations. It can be deduced from these results, that Ca^{2+} ions should play an important role in the stabilization of the structure of the purified agkisacutacin.

The previous study shows that one purified agkisacutacin molecule binds with one Ca^{2+} ion [4]. The shift of the unfolding transition to higher GdnHCl concentration for the purified agkisacutacin caused by addition of 1 mM Ca^{2+} suggests more than one Ca^{2+}-binding site in agkisacutacin. This speculation is confirmed by our recent result that one agkisacutacin molecule binds with two Ca^{2+} ions in the presence of 1 mM Ca^{2+} determined by equilibrium dialysis (unpublished data). The ΔG of the purified agkisacutacin increases 4.83 $\pm$ 0.18 kcal mol^{-1} after addition of 1 mM Ca^{2+}, indicating that the binding of the protein with another Ca^{2+} ion further increases its structural stability.

Agkisacutacin is a member of coagulation factor IX/coagulation factor X-binding protein family [5]. The proteins of this family have very similar amino acid compositions and high homologous sequences, and form 1:1 complexes with coagulation factor IX or coagulation factor X [1, 23–26]. 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) [25] and coagulation factor IX-binding protein (habu IX-bp) [26] purified from habu snake have been reported. Both proteins have a very similar structure with two Ca^{2+}-binding sites. Agkisacutacin has

a high homologous sequence compared with habu IX/X-bp and habu IX-bp: a 75.1% identity for habu IX/X-bp, and a 74.6% identity for habu IX-bp [2]. Like habu IX/X-bp and habu IX-bp, agkisacutacin also has two Ca^{2+}-binding sites. Agkisacutacin probably has a similar structure to that of habu IX/X-bp and habu IX-bp. Further investigation is necessary to determine the structure of agkisacutacin.

It has been reported that habu IX/X-bp undergoes a conformational change upon 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 [27]. Similarly, agkisacutacin bound with two Ca^{2+} ions exhibits much higher resistance to GdnHCl denaturation than the apo-agkisacutacin, indicating it should have a significantly more compact conformation than the apo protein. From these results, we speculate that Ca^{2+} ions may play a similar role in keeping a rigid structure for agkisacutacin as well as for habu IX/X-bp. Ca^{2+} ions play unique roles in stabilizing the specific conformation of holo-agkisacutacin. The Ca^{2+}-stabilized specific conformation of agkisacutacin should be helpful to its recognition of the structure of coagulation factor IX/coagulation factor X or platelet glycoprotein Ib, thereby promoting the association of two proteins.

Interestingly, by comparing the denaturation profiles of apo-agkisacutacin in the absence of Ca^{2+} and the purified agkisacutacin in the presence of 1 mM Ca^{2+}, we found that under appropriate denaturing condition (2.2 M GdnHCl), a refolding jump could be initiated. Indeed, we were able to initiate refolding of unfolded apo-agkisacutacin simply by adding 1 mM Ca^{2+}. Fluorescence measurements show that *the refolding process* from the unfolded state of apo-agkisacutacin to the folded state of the purified agkisacutacin induced by 1 mM Ca^{2+} is *best fit to* a sum of two exponential terms, suggesting a faster and a slower folding populations in the process (Fig. 4). Because Ca^{2+} ions have no direct quenching (such as collisional quenching and energy transfer quenching) effect on the fluorescence of free tryptophan [28], Ca^{2+}-induced fluorescence quenching of unfolded apo-agkisacutacin should be attributed to the complex formation between protein and Ca^{2+}, and this binding then induces the formation of folded structure and perturbs the microenvironment around the relevant tryptophan residue(s) and therefore *causes* the fluorescence quenching of the protein. Ca^{2+} ions probably are rapidly bound to the protein initially and the fastest step should involve the formation of the compact metal-binding site regions. Subsequently, the protein undergoes further conformational rearrangements, which corresponds to the second step to form its native structure as observed from the further decrease of the intrinsic fluorescence. Although we can not infer the detailed picture of the pathway of the Ca^{2+} ions-induced refolding from present data, it is certain that the Ca^{2+}-induced refolding of apo-agkisacutacin could be performed without changing the concentration of the denaturant. Further investigation is necessary to elucidate the mechanism of the Ca^{2+}-induced refolding.

Conclusions

Ca^{2+} ions not only increase the structural stability of agkisacutacin against GdnHCl denaturation, but also induce its refolding. The GdnHCl-induced unfolding of apo-agkisacutacin and the purified agkisacutacin is a single-step process with no detectable intermediate state. Ca^{2+} ions play an important role in the stabilization of the structure of agkisacutacin. Ca^{2+}-stabilized agkisacutacin exhibits higher resistance to GdnHCl denaturation than the apo-agkisacutacin. It is possible to induce refolding of the unfolded apo-agkisacutacin merely by adding 1 mM Ca^{2+} ions without changing the concentration of the denaturant. The kinetic result of Ca^{2+}-induced refolding provides evidences for that agkisacutacin consists of at least two refolding phases and the first phase of Ca^{2+}-induced refolding should involve the formation of the compact Ca^{2+}-binding site regions, and subsequently, the protein undergoes further conformational rearrangements to form the native structure.

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