Subunit Dissociation, Unfolding, and Inactivation of Bothrojaracin, a C-Type Lectin-like Protein from Snake Venom[†]

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ABSTRACT: Snake venoms contain a large number of hemostatically active proteins that are structurally related to Ca^{2+}-dependent animal lectins. These proteins, called C-type lectin-like proteins (CLPs), are generally found as heterodimers composed of two homologous subunits linked by a disulfide bond. Here, bothrojaracin (BJC), a CLP from *Bothrops jararaca* venom that is also a thrombin inhibitor, has been used as a model to study the subunit dissociation and unfolding of CLPs from snake venom. Dithiothreitol (DTT) up to 10 mM produces minor effects on the tertiary structure and activity of BJC. On the other hand, chromatographic studies and fluorescence polarization measurements indicate that the interchain disulfide bond is disrupted by DTT, although the dimeric association is maintained. Treatment of BJC with urea produces a progressive red shift in the emission spectra of the tryptophan residues, and circular dichroism measurements show that BJC retains significant secondary structure in the presence of 8 M urea, suggesting only partial unfolding. The effects of urea are fully reversible, as there is complete recovery of BJC activity after removal of the denaturing agent. Addition of DTT to a protein sample previously treated with 8 M urea produces a slightly larger spectral shift than that observed with urea alone. Furthermore, in this condition BJC loses its secondary structure, and its subunits are dissociated. After removal of urea and DTT, BJC is inactive toward thrombin, suggesting the irreversibility of their combined action. Altogether, our data show that (i) BJC is highly resistant to urea or DTT effects, requiring the simultaneous action of both agents to fully denature the protein, and (ii) BJC monomers are tightly associated, and the presence of DTT combined with high urea concentrations is necessary to disrupt them. On the basis of these results we propose the first denaturation model for a CLP from snake venom.

Venoms from *Viperidae* snakes are a rich source of compounds that interfere with blood clotting and platelet function (*1*, *2*). Among these compounds is a group of nonenzymatic proteins that are structurally related to Ca^{2+}-dependent animal lectins (*3*, *4*) and are called C-type lectin-like proteins (CLPs).[1] In fact, most CLPs from snake venoms are not able to bind carbohydrates and thus cannot be considered true lectins. These proteins have molecular weights of ∼30000 and are commonly found as heterodimers composed of homologous subunits (α and β chains) linked by a single disulfide bond. The members of this family exhibit high primary sequence identity including well-conserved disulfide bond arrangements within each subunit (*5*−*8*). Recent crystallographic studies have shown that dimerization of CLPs also involves domain swapping, in which the projection of a central loop from each chain to the adjoining subunit results in a tight association (*9*−*12*). Despite the extensive structural similarities, CLPs from snake venoms have acquired completely diverse biological functions: botrocetin and bitiscetin bind to von Willebrand factor and induce its interaction with platelet glycoprotein Ib (*13*−*15*); IX/X-bp inhibits blood coagulation by forming noncovalent complexes with coagulation factors IX and IXa as well as factors X and Xa (*16*, *17*); alboaggregin-B (*18*), echicetin (*19*), and *Bothrops jararaca* glycoprotein Ib binding protein (*20*) as well as other CLPs interact with platelet glycoprotein Ib, thus promoting or inhibiting von Willebrand factor-mediated platelet agglutination (*21*); and bothrojaracin (BJC) from *B. jararaca* interacts with thrombin through the anion-binding exosites I and II, thus inhibiting platelet aggregation, fibrinogen clotting, and other thrombin functions (*22*, *23*). The cDNAs encoding the α and β chains of BJC have been cloned, and the deduced amino acid sequences show extensive similarity with botrocetin, IX/X-bp, and alboaggregin-B, with identities of 80%, 57%, and 54% for the α chains and 66%, 54%, and 56% for the β chains, respectively (*24*).

In the present study, we address questions related to the unfolding and dimerization processes of CLPs using BJC as

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[1] Abbreviations: BJC, bothrojaracin; CD, circular dichroism; CLP, C-type lectin-like protein; DTT, dithiothreitol; IX/X-bp, factor IX/factor X binding protein; TBS, Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl, pH 7.5).

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a model. By use of intrinsic fluorescence, circular dichroism, and HPLC chromatography we show that dissociation of BJC subunits as well as their complete unfolding requires the combined use of urea and dithiothreitol. On the basis of these results we propose the first denaturation model for a CLP from snake venom, which may help to further clarify the folding mechanism of this intriguing protein family.

EXPERIMENTAL PROCEDURES

Materials. BJC was purified from lyophilized crude *B. jararaca* venom purchased from Instituto Butantan (São Paulo, Brazil) as previously described by Arocas et al. (*23*). Human α-thrombin was purified from frozen human plasma samples following the method described by Ngai and Chang (*25*). Protein purity was assessed by SDS−PAGE, and the concentration of proteins was estimated by the 280 nm absorbance with the following molecular masses and absorption coefficients [$(mg/mL)^{-1}\cdot cm^{-1}$], respectively: BJC (27000), 2.00; human α-thrombin (36600), 1.83 (*26*). All other reagents were of analytical grade. Urea, DTT, and human fibrinogen were from Sigma Chemical Co. (St. Louis, MO).

Schematic Disulfide Bridge Arrangement of BJC. A scheme for the inter- and intrachain disulfide bonds of BJC was developed by aligning the complete primary sequences deduced from the cloned cDNAs encoding α and β chains of BJC (*24*) with the sequences reported for IX/X-bp (*4*) and botrocetin (*6*). Since all cysteine residues are conserved among these proteins, the disulfide bridge arrangements determined for IX/X-bp (*5*) and botrocetin (*6*) were used to predict the disulfide bond pattern of BJC.

Fluorescence Assays. Fluorescence experiments were performed on a F-4500 spectrofluorometer (Hitachi, Japan). Samples were prepared by incubating BJC (0.5 μM) overnight with urea and/or DTT in 20 mM Tris-HCl and 150 mM NaCl, pH 7.5 (TBS). Intrinsic fluorescence emission was followed by exciting the samples at 280 nm and measuring the emission between 300 and 400 nm. Fluorescence spectra were quantified by the center of spectral mass $\langle\nu\rangle$ according to the previously described equation (*27*):

$$\langle\nu\rangle = \sum \nu_i F_i / \sum F_i \quad (1)$$

where F_i is the fluorescence emitted at wavelength ν_i and the summation is carried out over the range cited above.

Fluorescence polarization assays were performed on an ISS-PC1 spectrofluorometer (ISS Inc., Champaign, IL). BJC (10 μM) was treated overnight with urea and/or DTT in TBS. Samples were excited at 280 nm, and emission was collected through WG320 and 7-54 filters. Polarization was calculated from

$$P = (I_{||} - I_{\perp})/(I_{||} + I_{\perp}) \quad (2)$$

where $I_{||}$ and $I_{\perp}$ are the fluorescence intensities detected through a polarizer oriented parallel ($I_{||}$) or perpendicular ($I_{\perp}$) to the direction of polarization plane of the excitation beam. Results were expressed as polarization units (*P*). The errors in polarization measurements did not exceed ±0.001 *P*.

Circular Dichroism Studies. CD spectra were collected on a Jasco J-715 spectropolarimeter (Japan). BJC (10 μM) was incubated overnight in the presence of urea and/or DTT in TBS before measurements. The spectra were obtained at 25 °C in a 1.0 mm optical path cell by scanning from 260 to 195 nm at 50 nm/min with a band-pass of 0.5 nm. CD spectra for native BJC were analyzed independently by CONTIN, SELCON3, and CDSSTR computer programs (for detailed descriptions of each method, see ref *28*). The secondary structure content is expressed as the means of the analyses obtained by these three methods.

Reduction of BJC Disulfide Bonds by DTT. Accessibility of BJC disulfide bridges to DTT was evaluated as previously described (*29*). This method is based on the increase in the absorbance at 310 nm as a function of the oxidized DTT formed during the reduction process. Samples of BJC (20 μM) were incubated overnight in the absence or in the presence of urea in TBS. After incubation, the reaction was started by adding 10 μL of a 200 mM DTT stock solution (10 mM final concentration), and A_{310} was recorded for 60 min using a GBC Cintra 20 spectrophotometer (GBC Scientific Equipment, Victoria, Australia). The concentration of oxidized DTT formed was determined by the extinction coefficient of 110 M^{-1} cm^{-1} at 310 nm, and the result was converted to the number of BJC disulfide bonds reduced under each condition.

Chromatographic Studies. Gel filtration chromatography was performed using a Superdex G-75 (Pharmacia, Uppsala, Sweden) column attached to a high-performance liquid chromatography (HPLC) system (Shimadzu, Tokyo, Japan). Protein elution was monitored by the absorbance at 280 nm, and experiments were performed at a flow rate of 0.5 mL/min. Samples were prepared by incubating the protein (50 μg) overnight with TBS in the absence or in the presence of DTT and/or urea. Prior to injection, the column was equilibrated with TBS or TBS containing 10 mM DTT. For each condition, column calibration was performed using carbonic anhydrase (29 kDa) and cytochrome *c* (14 kDa) as markers. Hydrophobic chromatography was performed on a Sephasyl C8 reverse-phase column (Pharmacia, Uppsala, Sweden) attached to the HPLC system. Elution was performed at a flow rate of 0.5 mL/min using an acetonitrile gradient (0−100% containing 1% trifluoroacetic acid), with absorbance monitored at 214 nm. BJC samples (50 μg) were incubated overnight in the absence or in the presence of 10 mM DTT in TBS. Samples were further treated for 60 min with a 100-fold molar excess of iodoacetic acid and applied onto the column.

Functional Studies of BJC. The ability of BJC to inhibit thrombin-induced fibrinogen clotting was measured on a Thermomax Microplate Elisa Reader (Molecular Devices, Menlo Park, CA) following a previously described method (*30*). BJC (10 μM) was incubated overnight in the absence or in the presence of DTT and/or urea in TBS. Samples were then extensively dialyzed against TBS. Thrombin inhibition was assayed by incubating BJC (2.5−0.010 μM) with 8 nM human α-thrombin in TBS (50 μL) for 2 min at 37 °C, followed by the addition of 50 μL of human fibrinogen (4 mg/mL). Fibrinogen clotting was followed for 10 min at 405 nm, and the IC_{50} was calculated from the dose−response curves obtained.

RESULTS

Structural Features of BJC. BJC is a heterodimer composed of two polypeptide chains, α and β, containing 132

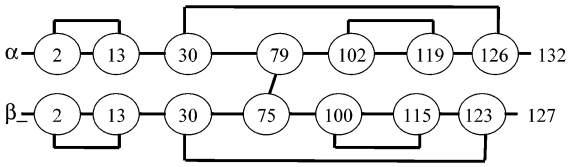

FIGURE 1: Schematic disulfide bridge arrangement of BJC. This model was constructed as described in Experimental Procedures.

and 127 amino acids, respectively (*24*). The α and *β* chains of BJC present 47% identity and also a high degree of identity with botrocetin and IX/X-bp (*24*). All cysteine residues are conserved in these proteins, and the disulfide bond positions have been demonstrated explicitly for botrocetin (*6*) and IX/X-bp (*5*). Comparison of the BJC sequence deduced from the cDNAs encoding α and *β* chains (*24*) with the sequences obtained for botrocetin (*6*) and IX/X-bp (*4*) allows us to propose a model for the arrangement of the intra- and interchain disulfide bonds of BJC (Figure 1). In this model, cysteine 79 in the α chain is covalently linked to cysteine 75 in the *β* chain. Figure 1 also shows the six intrachain disulfide bonds proposed for BJC. It is worth emphasizing that botrocetin and IX/X-bp are found in *B. jararaca* venom and together with BJC may derive from a common ancestor gene.

Unfolding of BJC. The effects of urea and DTT on the tertiary structure of BJC were evaluated by following changes in its intrinsic fluorescence emission, taking advantage of the presence of five and six tryptophan residues in the α and *β* subunits, respectively. The tryptophan residues are located in positions 6, 23, 67, 83, and 114 in the α chain and positions 6, 23, 65, 72, 79, and 110 in the *β* chain. The center of spectral mass of the tryptophan fluorescence emission of native BJC is shifted to the red (350 nm), suggesting that the residues with the highest quantum yields are located close to the protein surface. The addition of DTT up to 10 mM induced a negligible change in the center of spectral mass (Figure 2, empty circles), suggesting that even if disulfide bonds are being disrupted, there are no major changes in the tertiary structure of the protein. On the other hand, incubation of BJC with urea (up to 8 M) produced a significant red shift in the center of spectral mass (from 350 to 354 nm) (Figure 2, filled circles). The urea-unfolding curve showed a single transition from 3 to 8 M, with a $U_{1/2}$ value of 6 M urea. The red shift is consistent with the exposure of tryptophan(s) to a more hydrophilic environment, as expected for an unfolding process. No protein concentration dependence was found (data not shown). Calculation of the free energy change based on a single transition from the native to the urea-denatured state (*31*) showed that $\Delta G° = 3.69 \pm 0.72$ kcal mol^{-1} ($m = 0.60 \pm 0.14$ kcal mol^{-1} M^{-1}).

The combined effects of urea and DTT were evaluated by adding the reducing agent to a protein sample previously incubated with 4 M (Figure 2, squares) or 8 M urea (Figure 2, triangles). In 4 M urea alone, there was no major change in tryptophan emission, but titration with DTT up to 2 mM caused an almost complete shift to the red (from 350.5 to 353 nm). Addition of DTT to BJC in 8 M urea, where tryptophan emission was already red shifted, led to a small additional shift to the red (from 354 to 354.5 nm). These data suggest that the tryptophan residues in the denatured, dimeric BJC (in the presence of 8 M urea) experience a similar environment regardless of whether the disulfide bonds are intact or not.

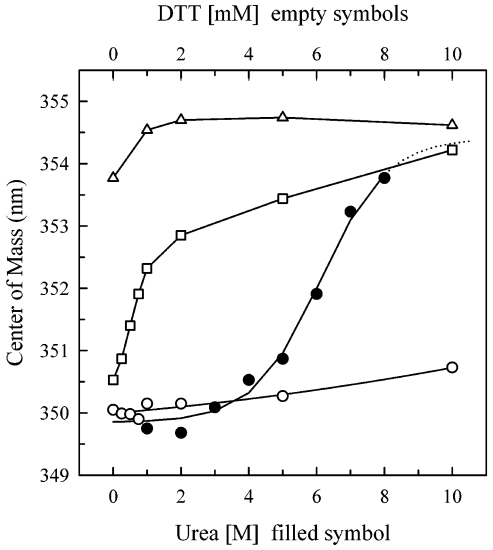

FIGURE 2: Effects of DTT and urea on the tertiary structure of BJC. Filled symbols show the effect of urea on the center of mass of the intrinsic fluorescence spectra of BJC (0.5 *μ*M). Empty symbols show the effect of DTT without urea (○), with 4 M urea (□), and with 8 M urea (△). Experiments were performed in TBS at 25 °C. Samples were excited at 280 nm, and fluorescence emission was measured between 300 and 400 nm.

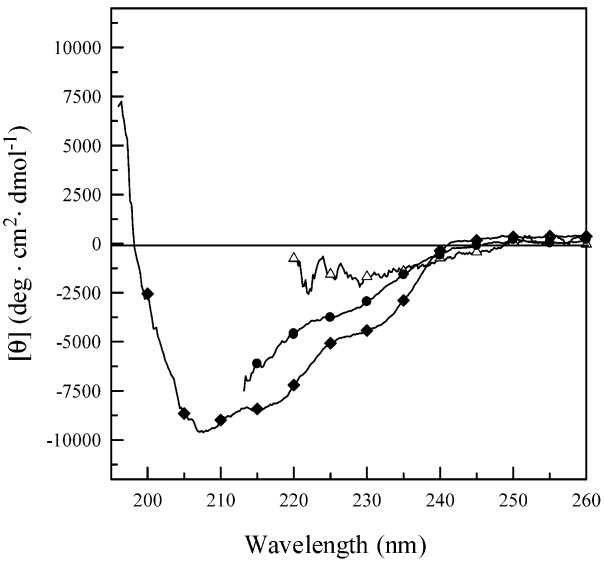

FIGURE 3: Effects of DTT and urea on the secondary structure of BJC. CD spectra of BJC (10 *μ*M) in TBS were measured under native conditions (◆), in the presence of 8 M urea (●), or in 8 M urea combined with 10 mM DTT (△).

The effects of DTT and urea on the secondary structure of BJC were evaluated from circular dichroism (CD) measurements. Figure 3 shows a typical CD spectrum obtained for native BJC (diamonds). Analysis of the secondary structure content (described under Experimental Procedures) revealed that BJC contains 24% α-helix, 38% *β*-sheet, 26% *β*-turn, and 12% nonstructured regions. Surprisingly, despite the fact that the tertiary structure of BJC was severely perturbed by the presence of 8 M urea (Figure 2), the secondary structure was preserved to some extent (Figure 3, circles). In contrast, the CD spectrum of BJC treated with 8 M urea containing 10 mM DTT was consistent with a

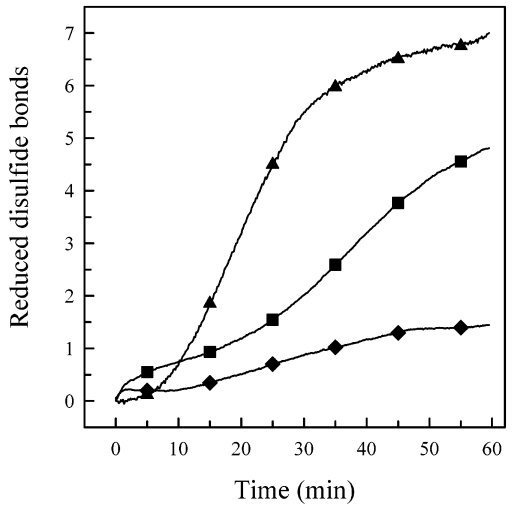

FIGURE 4: Accessibility of BJC disulfide bonds to DTT. Oxidized DTT formed by reduction of BJC disulfide bonds was evaluated by changes in absorbance at 310 nm. This figure shows the number of disulfide bonds reduced during 60 min, as calculated from the molar extinction coefficient. Assays were started by addition of DTT (10 mM final concentration) to solutions containing BJC (20 μM) under native conditions (◆), in the presence of 4 M urea (■), or in 8 M urea (▲).

completely unfolded protein (Figure 3, triangles). These data indicate that even though the treatment of BJC with urea or urea combined with DTT produces similar changes in the tertiary structure of the protein, only the combination of urea with DTT leads to complete unfolding.

Effect of DTT on BJC Disulfide Bonds. The accessibility of BJC disulfide bonds to DTT under native or perturbing conditions was evaluated from the change in absorbance at 310 nm (Figure 4). When added to the folded BJC, DTT (10 mM) reduced a single disulfide bridge during the 60 min of reaction (Figure 4, diamonds). In contrast, prior treatment of BJC with 4 or 8 M urea increased the number of bonds accessible to DTT to five (Figure 4, squares) and seven (Figure 4, triangles), respectively. These results indicate that although the tertiary structure of BJC is perturbed similarly by DTT combined with 4 or 8 M urea, the accessibility of its disulfide bonds under these distinct conditions is different.

To determine the effect of DTT on the interchain disulfide bond of BJC, gel filtration and hydrophobic chromatography were performed (Figure 5). Figure 5A shows the elution profile of native BJC on a Superdex G-75 column, where it elutes as a single peak at ~11 mL. The DTT-treated protein elutes at the same position as the native BJC (Figure 5B), suggesting that the dimer remains associated. To determine whether the interchain disulfide bond is the one being disrupted by DTT in the absence of urea, hydrophobic chromatography after cysteine alkylation was performed. Figure 5C shows the elution profile of BJC treated with iodoacetic acid in the absence of DTT, where a single peak corresponding to the dimeric BJC is eluted from the column. On the other hand, BJC treated with 10 mM DTT and further alkylated with iodoacetic acid gives rise to two peaks that, according to previous observations (*24*), correspond to the α and β chains of BJC (Figure 5D). A small peak corresponding to dimeric BJC is also observed.

Additional evidence regarding the interchain disulfide bond of BJC was obtained from intrinsic fluorescence polarization

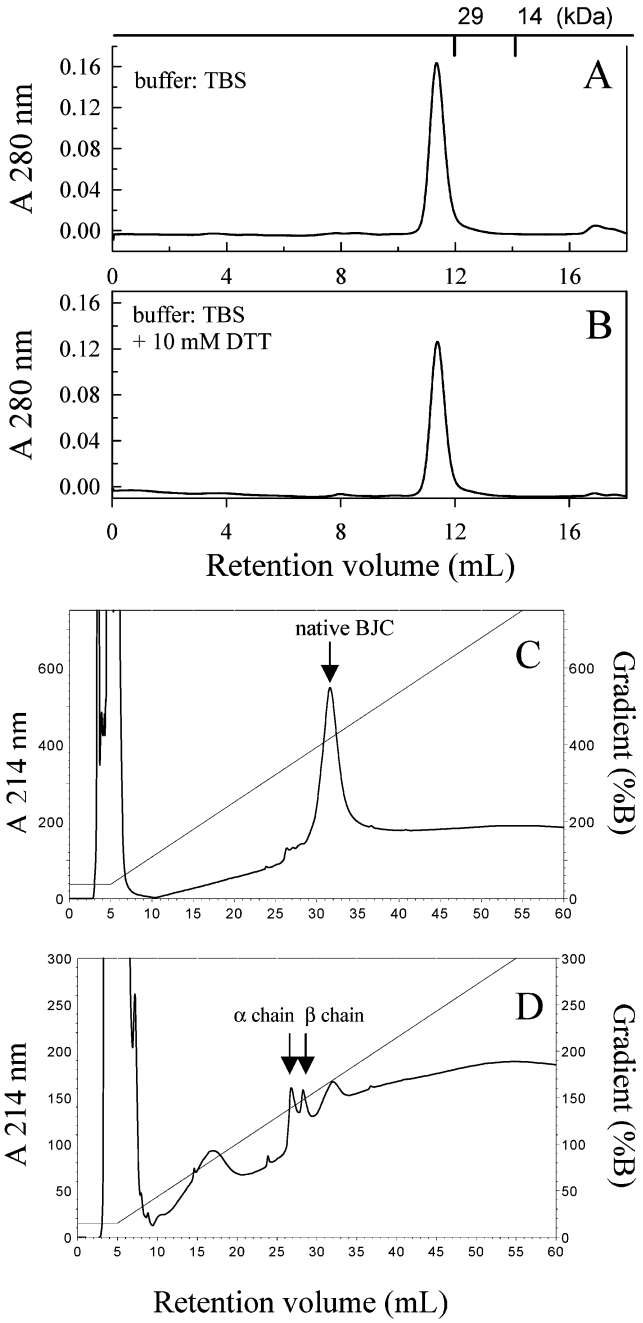

FIGURE 5: Effect of DTT on the interchain disulfide bond of BJC. BJC samples (50 μg) were incubated overnight with TBS (A) or TBS containing 10 mM DTT (B), followed by gel filtration chromatography on Superdex G-75. The retention volumes for carbonic anhydrase (29 kDa) and cytochrome *c* (14 kDa) are indicated above the figure. Reduction of the interchain disulfide bond by DTT was evaluated as follows: BJC samples (50 μg) were incubated overnight in the absence (C) or in the presence (D) of 10 mM DTT. Samples were further treated for 60 min with a 100-fold molar excess of iodoacetic acid and subjected to hydrophobic chromatography on Sephasyl C8. Elution was performed using an acetonitrile gradient (0−100% acetonitrile containing 1% trifluoroacetic acid).

measurements (Table 1). If the subunits dissociate under the influence of DTT or urea, the rate of rotation should increase, causing a fall in the fluorescence polarization. As expected, samples prepared in 8 M urea showed no change in the polarization value. In the same way, BJC in the presence of 10 mM DTT or 10 mM DTT combined with 4 M urea displayed polarization values identical to those found for the

Table 1: Effects of DTT and Urea on BJC Fluorescence Polarization[a]

incubation with	polarization value
buffer	0.161 ± 0.001
10 mM DTT	0.160 ± 0.002
8 M urea	0.159 ± 0.001
4 M urea + 10 mM DTT	0.161 ± 0.001
8 M urea + 10 mM DTT	0.105 ± 0.001

[a] BJC (10 μM) was treated overnight with urea and/or DTT in TBS. Samples were excited at 280 nm, and emission was collected through WG320 and 7-54 filters. Polarization was calculated as described in Experimental Procedures and is shown as the average ± SD of five measurements.

Table 2: Effects of DTT and Urea on BJC Activity[a]

incubation with	IC_{50} (nM) for inhibitory activity of BJC upon α-thrombin
buffer	3.0 ± 0.2
10 mM DTT	8.0 ± 1
8 M urea	3.0 ± 0.3
4 M urea + 10 mM DTT	330 ± 14
8 M urea + 10 mM DTT	>2500

[a] BJC was incubated overnight with urea and/or DTT as indicated. After extensive dialysis, BJC was assayed for inhibition of α-thrombin-induced fibrinogen clotting (as described in Experimental Procedures). Data represent the mean ± SD of two independent determinations.

native protein, suggesting that the subunit association is preserved under these conditions. However, when BJC was treated with 10 mM DTT combined with 8 M urea, there was a significant decrease in the fluorescence polarization, thus indicating its dissociation into unfolded monomers.

Altogether, these results indicate that the interchain disulfide bond of BJC is accessible to DTT under native conditions, although the hydrophobic interactions between α and β chains sustain its dimeric association. Even after DTT treatment, high urea concentrations are required to disrupt these noncovalent interactions.

Functional Studies of BJC. The reversibility of DTT and urea effects on BJC structure were evaluated by assaying its inhibitory activity toward thrombin. Table 2 shows the IC_{50} values found for inhibition of thrombin-induced fibrinogen clotting by BJC. Samples were first treated overnight as indicated, and then the perturbing agents were removed by extensive dialysis. In agreement with the minor effects of DTT on BJC tertiary structure, treatment with the reducing agent caused a negligible increase in the IC_{50} for thrombin inhibition. On the other hand, the effect of 8 M urea on BJC activity was fully reversible. BJC treated with 10 mM DTT combined with 4 M urea showed an ~100-fold decrease in the inhibitory activity while samples treated with DTT combined with 8 M urea were inactive. In addition, isolated BJC subunits obtained after reduction and alkylation procedures were also inactive toward thrombin (data not shown). Taken together, these results suggest that the isolated effects of urea or DTT on BJC structure are fully reversible, while their combined action is irreversible and BJC does not refold to its native, active conformation upon their removal.

DISCUSSION

In this study we have characterized for the first time the subunit dissociation and unfolding of a CLP from snake venom. We used the thrombin inhibitor BJC as a model since it exhibits the typical heterodimeric structure of this protein family (Figure 1). On the basis of the data presented here we propose a denaturation model for BJC (Figure 6).

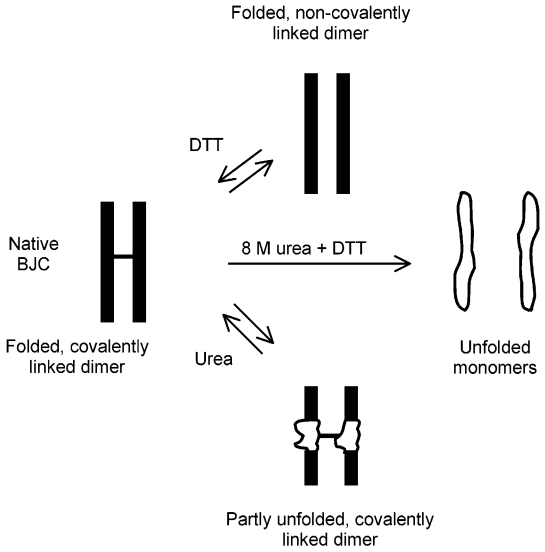

FIGURE 6: Denaturation model proposed for BJC.

The treatment of BJC with DTT alone has only a modest effect on its tertiary structure and activity. However, BJC behaves as a noncovalently linked dimer in the presence of DTT, based on (i) gel filtration chromatography, where BJC migrates as a dimer in the presence of 10 mM DTT (Figure 5B), (ii) cysteine alkylation experiments, where BJC samples prepared in DTT generate isolated α and β subunits (Figure 5D), and (iii) fluorescence polarization measurements, showing no difference between BJC in the presence of 10 mM DTT and the native, dimeric protein (Table 1). Rhodocetin, a CLP from *Calloselasma rhodostoma* venom, exhibits a dimeric conformation despite the absence of the cysteine residues that form the interchain disulfide bond present in other CLPs (*32*). Thus, BJC treated with DTT resembles native rhodocetin in that it maintains its dimeric structure despite the absence of an interchain disulfide bond. On the other hand, crystallographic data show that the orientation of the α subunits in relation to the β subunits differs significantly from one CLP to another (*9*−*12*). Thus it may be that the interchain disulfide bridge is more important for maintaining the relative orientation between the CLP subunits than for stabilizing the dimer.

The effects of urea on the tertiary and secondary structures of BJC are consistent with an incomplete unfolding process. In a high urea concentration, it seems that BJC loses its tertiary structure while the secondary structure remains almost unchanged. At this point, we suggest that BJC behaves as a partly denatured, covalently linked dimer in 8 M urea. This proposal is consistent with models of other proteins that retain residual structures at high urea concentrations (*33*, *34*).

Analysis of the intrinsic fluorescence changes and CD spectra showed that BJC can be completely unfolded only by the combined action of DTT with high urea concentrations. This observation indicates that, as expected, the intrachain disulfide bonds of BJC play a central role in stabilization of the protein structure. Accordingly, dissociation of BJC subunits occurred only in the presence of DTT

and 8 M urea (Table 1). These results suggest that the noncovalent interactions produced by domain swapping promote a tight association between CLP subunits (*9*−*12*). Comparison of the crystal structure of the IX/X-bp with a monomeric C-type lectin suggests that dimerization of CLPs from snake venoms may be critical for the acquisition of distinct biological functions (*9*, *10*).

Determination of the crystal structure of a complex formed from factor X binding protein and the γ-carboxyglutamic domain of factor X showed that the binding region on the snake protein is a concave surface involving residues from both α and β chains (*35*). Crystallographic studies of other CLPs suggest the existence of similar regions that may be involved in their biological activities (*11*, *12*). However, the relative importance of α or β subunits for the activity of CLPs is still controversial. It has been observed that isolated subunits of rhodocetin do not inhibit collagen-induced platelet aggregation (*31*), indicating that the synergistic action of rhodocetin subunits is essential for its biological activity. On the other hand, Peng et al. suggested that the β subunit of echicetin is responsible for its ability to bind the platelet glycoprotein Ib (*36*). Pólgar et al. further argued that these data probably resulted from contamination with native echicetin, as they were not able to obtain active echicetin subunits (*8*). Accordingly, isolated BJC monomers obtained by denaturation and alkylation procedures are not well recognized by polyclonal antibodies raised against native BJC (data not shown). In fact, even in the absence of alkylating agents, the unfolding of BJC produced by the combined action of DTT and urea is irreversible (Table 2). The irreversibility of the denaturation process may be the main impediment to obtaining active CLP monomers. In addition, the in vivo folding of CLPs may depend on the action of chaperone proteins in order to facilitate the correct folding pathway.

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