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Ca²⁺ induced linker transformation leads to compact and rigid collagen binding domain of *Clostridium histolyticum* collagenase

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Summary

Clostridium histolyticum—collagenase is responsible for extensive tissue destruction in gas gangrene, and its activity is enhanced by calcium ions. Collagen binding domain (CBD) is the minimal segment of the enzyme needed for binding to insoluble collagen fibrils and for subsequent collagenolysis. The linker undergoes secondary structural transformation from α -helix to β -strand and forms non-prolyl *cis* peptide in the presence of calcium ions. In this paper various biophysical methods were utilized to better understand the structure and functional role of the novel calcium activated linker. Two Ca²⁺ ions bind cooperatively with macroscopic association constants $K_1 = 5.01 \times 10^5 \text{ M}^{-1}$, $K_2 = 2.28 \times 10^5 \text{ M}^{-1}$. The chelation of second Ca²⁺ is enthalpically unfavorable which could be due to the non-prolyl *cis* peptide isomerization. *Holo* protein is more stable than *apo* against thermal denaturation ($\Delta T_m \sim 20^\circ \text{C}$) and chemical denaturation ($\Delta \Delta G_{\text{H}_2\text{O}} \sim 3 \text{ kcal/mol}$ for urea or guanidine HCl denaturation and $\Delta 20\% \text{ v/v}$ in TFE). The compact *holo* CBD is more resistant to proteolytic digestion than *apo* CBD. The orientation of linker appears to play crucial role in the stability and dynamics of CBD.

Keywords

Collagen binding domain; linker; Ca²⁺; structural transformation; stability

Introduction

Collagenase derived from an anaerobic Gram-positive bacterium *Clostridium histolyticum* is being used in non-surgical treatment of Dupuytren's disease [1,2]. It has been shown that *C. histolyticum* produces two classes of collagenases, ColG and ColH [3,4]. The segmental structure of ColG and ColH are S1, S2, S3a, S3b and S1, S2a, S2b, S3 respectively [5]. C-terminal domains, S3a and S3b of ColG and S3 of ColH, are responsible for the binding to insoluble collagen fibrils (a staggered array of triple-helical tropocollagen) [6,7]. These collagen binding domains (CBDs) are sequence homologs possessing approximately 120 amino acid residues. Truncation of CBDs from the bacterial collagenase makes it incapable to hydrolyze insoluble collagen but can hydrolyze non-triple helical and soluble gelatin [7]. For its collagen binding characteristics, CBD is being tested as a drug delivery vehicle for various growth factors. Nishi *et al.* have shown *in vivo* that the growth factors attached to

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CBD remained active at the injection site for up to 10 days whereas growth factors by themselves were rapidly degraded [5]. Homologous linker is found at N-terminus of CBDs, *i.e.*, between S2 and S3a, between S3a and S3b in ColG, and between S2b and S3 in ColH.

Depletion of calcium ion from CBDs deteriorates their affinity to insoluble collagen [7]. X-ray crystal structures solved at high resolution for calcium-bound (*holo*) and calcium-free (*apo*) forms of a CBD confirmed the presence of 14 amino acid linker at the N-terminus [8]. In the absence of Ca^{2+} , the linker coiled as α -helix (Fig. 1). However, the addition of Ca^{2+} unwinds the linker to form a new β -strand which staples two β -sheets in the β -sandwich of CBD (Fig. 1). The structural change is reversible upon titration with ion scavengers such as EGTA and the orientation of this linker could play a key role in domain rearrangement. Linker deletion or mutation of the chelating residues results in weakening of the collagen binding [8]. Linkers are usually not very well observed in crystal structures, they are too mobile to be able to be seen in NMR (nuclear magnetic resonance) time scale, and its relative length is too short to be monitored by other biophysical techniques like circular dichroism (CD). Therefore, their function other than to link domains has not been studied well. In order to understand the function and stability of bacterial collagenase on the event of structural transformation of the unique linker, variety of biophysical and biochemical experiments were performed.

Isothermal titration calorimetry (ITC) was performed to assess the Ca^{2+} binding affinity, binding mode and thermodynamic energy changes. An energetically unfavorable non-prolyl *cis* peptide is formed between E901 and N902 upon Ca^{2+} binding. Non-prolyl *cis* peptide bond is very rare and found only in functionally critical sites [9,10]. ITC results enabled us to put forth a hypothesis regarding structural transformation mechanism. It is well documented that conformational change triggered by the addition of Ca^{2+} stabilize proteins [11]. The free energy of structural stability is generally attributed to the gain in electrostatic interactions and hydrophobic interactions. By using various denaturants and heat in conjunction with the structural information, the relative contributors towards stability in *holo* and in *apo* were assessed. Unlike calmodulin and other Ca^{2+} binding proteins, little hydrophobic surface area change takes place between *apo* and *holo* in crystal. The transformation in solution was characterized by the fluorescent probe. The role of the linker in influencing the dynamics of CBD in the presence and absence of Ca^{2+} is addressed from limited proteolytic digestion.

Results and Discussion

Energetic changes and structural changes accompanied by Ca^{2+} binding

Isothermal calorimetric titrations were performed to assess the binding affinity and binding mode of CBD towards Ca^{2+} . Our previous results using size exclusion chromatography and fluorescence spectroscopy were not able to give dissociation constants for individual binding sites [8]. Best fit using non-linear least squares yielded a sigmoidal binding isotherm with the stoichiometry of Ca^{2+} binding to CBD being two (Fig. 2). The macroscopic binding constants were $K_1 = 5.01 \times 10^5 \pm 5.5 \times 10^4 \text{ M}^{-1}$ and $K_2 = 2.28 \times 10^5 \pm 5.5 \times 10^4 \text{ M}^{-1}$. Normally K_1 and K_2 refer to the binding of the first and second calcium ion to the protein irrespective of the occupied site. An average calcium binding affinity $K = \sqrt{K_1 K_2} = 3.38 \times 10^5 \text{ M}^{-1}$ corresponds well with binding affinity $K = 2.72 \times 10^5 \text{ M}^{-1}$ indirectly obtained earlier [8]. The Hill coefficient obtained by fitting the experimental binding data was 1.35 which reflects cooperative binding mode. As seen from our crystal structure two Ca^{2+} sites are only 3.7 Å apart and three residues E901, D927 and D930 chelate with both Ca1 and Ca2 and thus binding of one Ca^{2+} can influence the other. Mutation of those three residues diminished Ca^{2+} binding [8]. However, with such an extent of coupling between two sites, $\Delta\Delta G$ of interaction calculated is only -0.5 kcal/mol using the equation as described by Linse

et al [12]. For cooperative calcium binding proteins, $\Delta\Delta G$ typically lies between $-1.2 \sim -3.8$ kcal/mol [13].

For this novel dual-calcium motif, it is possible that cooperative but stepwise (or sequential) binding of calcium takes place. The special situation has been explained by Linse and Forsen, and the relationship between macroscopic binding constants K_1 and K_2 to microscopic binding constants K_I and $K_{II,I}$ have been worked out [14]. K_I is the binding constant for the first site with tighter affinity. $K_{II,I}$ is the binding constant for the second site when the first site is already occupied. When $K_I > K_{II,I}$, most calcium should be constrained to the first site. The scheme of Ca^{2+} binding to CBD adopted from Linse *et al* [14] is shown in Fig. 3. Structurally it seems that Ca1 should bind first as only a partial unwinding of the helical linker would be necessary to bind the first site while the binding to Ca2 site requires a complete unwinding of the linker (Fig. 4A and 4B). Mutation of a residue that interacts with Ca1 (N903A) alone lost ability to bind not only to Ca1 but also to Ca2 and resembled *apo*; whereas mutation to a residue that only interacts with Ca2 (E899A) retained some *holo-like* structure [8].

Attempts are made to correlate thermodynamic entities related to the binding of each Ca^{2+} . The first site with $\Delta H_1 = -4.8$ (kcal/mol) could correspond to the enthalpic energy change for Ca1 binding site in frequently observed pentagonal bipyrimidal geometry with coordination number (CN) = 7. In order to bind the first site, 907-909 section of the linker would have to unwind a little to form π -helix, and 902-906 would have to unwind completely (Fig. 4A and 4B). The linker section 903-905 sterically forces Tyr931 to adopt a strained χ_1 angle (-54° in *apo* to -118° in *holo*) [8]. The χ_1 change may cost ~ 1 kcal/mol [15]. The entropy change for the first binding site is $\Delta S_1 = 10.1$ kcal/mol/T. Observed ΔS per bound Ca^{2+} in various proteins is around 24 kcal/mol/T stemming from the release of water molecules from the solvated metal ion [14].

Ca^{2+} binds to the second site with an unfavorable enthalpy, $\Delta H_2 = 3.1$ (kcal/mol). The local structure suggests that Ca2 may not bind as tightly as Ca1 ($K_I > K_{II}$). Ca2 in *holo* CBD interacts with 8 oxygen atoms (CN= 8), and is in a rarely seen square antiprismatic geometry. Out of the 8 oxygen atoms coordinating to Ca2, six of them are partially negatively charged. Electrostatic repulsion could reduce the affinity. To bind the second ion, the segment 896-901 would have to unwind so that E899 be positioned by N903; 897-899 segment makes parallel β -sheet interaction to seal the site (Fig. 4B). The parallel β -sheet interaction and side-chain H-bonds could partly offset the enthalpic cost of unwinding the α -helix. The second binding site initiates the non-prolyl *cis*-peptide bond isomerization between E901 and N902 (Fig. 4B). The isomerization will require Ca1 and Ca2 to cooperatively pull E901 by rotating the peptide bond. The enthalpic cost for the isomerization is difficult to estimate accurately due to strong thermodynamic advantage of *trans* over *cis*, however, it should be greater than ~ 5 kcal/mol [16]. Greater entropic gain for the second site ($\Delta S_2 = 34.7$ kcal/mol/T) than average ΔS per bound Ca^{2+} could compensate for less than expected change for the first binding site. Precise breakdown of the free energy change (-15.0 kcal/mol) for *apo* to *holo* transformation towards contributions from various other factors is not possible; however, it is clear that the transformation is largely entropically driven (positive ΔS). In summary it appears that the enthalpically costly linker transformation is largely driven by desolvation of calcium ions.

Holo gains electrostatic contribution

As urea and heat denaturation predominantly disrupt electrostatic interactions [17], their effects on CBD due to Ca^{2+} binding was analyzed. The unfolding transitions of CBD in urea are cooperative and a two-state model fits the experimental data satisfactorily (Fig. 5A). The concentration of denaturant at which the proteins are half unfolded (C_m) provides an

estimate of the protein stability. The C_m for *apo* and *holo* in urea were 5.68 ± 0.02 M and 6.89 ± 0.04 M respectively. Separate extrapolatable lines for plots of free energy of unfolding (ΔG_u) versus concentration of urea (equation 5) were used to estimate the Gibbs free energy of unfolding (ΔG_{H_2O}) at zero denaturant concentration. The ΔG_{H_2O} for *apo* and *holo* were calculated to be 6.49 ± 0.2 kcal/mol and 9.73 ± 0.2 kcal/mol respectively. The denaturant sensitivity factor (m) showed a Ca^{2+} dependent increase, from 1.14 ± 0.03 kcal/mol/M to 1.41 ± 0.04 kcal/mol/M. In summary the *holo* form is 1.5 fold more stable than *apo* CBD (Table-1).

The thermal unfolding monitored by fluorescence spectroscopy indicates that unfolding takes place in single cooperative transition (Fig. 5B). The temperature at which *apo* is half unfolded (T_m) is 82 °C. T_m for *holo* could not be calculated as even at 100 °C as the protein is not denatured completely. Differential scanning calorimetry (DSC) showed that T_m measured for *apo* was 74 °C and *holo* was 93 °C (supplementary Fig. S1). Discrepancies in T_m values between fluorescence spectroscopy and DSC methods have been well documented [18]. Higher T_m values obtained using fluorescence spectroscopy could be due to a locally stable core around W956. ΔT_m agrees well in two methods.

High-resolution structures allowed us to scrutinize potential contributors for electrostatic interaction like (i) Ca-O interactions, (ii) hydrogen bonds and salt-bridges, (iii) void cavities and (iv) accessible surface area. *Holo* (PDB code-1NQD) and *apo* (PDB code-1NQJ) crystal structures are virtually identical (RMSD = 0.3 Å) except for the linker. Two molecules found in an asymmetric unit (molecule A and molecule B) are extremely alike (RMSD = 0.4 Å for *holo* 0.36 Å for *apo*) providing us with two independent data sets per state. (i) Five negatively charged side chains chelate with two Ca^{2+} ions. The imbalance of charges could be a compromise that was achieved during the evolution for affinity and kinetics. It supports the observation that the linker and CBD would bind and dissociate Ca^{2+} (ii) Conventional H-bonds (2.4-3.4 Å) and CH...O hydrogen bonds (3.0 - 3.5 Å) [19] were archived using the program CCP4 [20]. As seen from the table-2, the numbers of hydrogen bonds are similar for all molecules except *apo* A. *Apo* A lacks the linker domain in the crystal structure and only *apo* B should be considered for analyses of *apo* protein structure. There is a loss of a few hydrogen bonds on the event of Ca^{2+} binding. Different distance cut off for H-bonds resulted in the same trend. In addition there is no salt-bridge found in either *holo* or *apo* molecules. Thus hydrogen bonding or salt bridges do not seem to contribute for the increased stability in *holo*. (iii) Void cavities in proteins are expression of defective side chain packing, and a correlation exists between the solvent inaccessible cavities and the stability of the proteins due to van der Waals interaction [21,22]. The presence of these cavities in the *apo* and *holo* structures of CBD was located using the program CASTp [23]. Structures of *apo* and *holo* CBD show significant differences in the number of cavities and their sizes. Cavities with surface area >30 Å² in both structures were taken into account (Table- 3). As seen from the table, the surface area and volume of cavities found in *apo* are larger than its *holo* counterpart. Stability is known to decrease significantly with the increase in the volume of void cavities [24,25]. In addition to the common cavities, *apo* has four more cavities that are not found in *holo*, and *holo* has 2 uncommon cavities (Table-3). Residues L958, P960, N965, R967, I968, T970, K983, L984, R985, Y989 in *apo* contribute to a large cavity with surface area 145.3 Å² and volume 141.5 Å³ and is adjacent to another cavity contributed by P960, E961, S962, N965, R985, P986, G987, K988, Y989 (Supplementary Fig. S2) which possibly provide access to solvent molecules to penetrate and thereby weakening the interactions in the core of the CBD. *Holo* is more compact and has fewer cavities than *apo* (Table-3). (iv) Accessible surface area calculations were performed for both *apo* and *holo* CBD using the program NACCESS [26]. *Apo* CBD has 6828 Å² and *holo* CBD has 6132 Å² of accessible surface area. On the whole gained compactness and Ca-O interactions in *holo* could contribute towards its stability of CBD.

***Holo* gains hydrophobic contribution**

The burial of hydrophobic side-chains is a major stabilizing factor in protein folding. Since guanidine hydrochloride (Gu. HCl) predominately disrupt hydrophobic interactions [17], unfolding pathway of CBD was studied using Gu. HCl (Fig. 6). The C_m for *apo* and *holo* in Gu.HCl are 0.84 ± 0.01 M and 1.55 ± 0.02 M respectively (Table-1). The Gibbs free energy changes ΔG_{H_2O} for *apo* and *holo* CBD calculated from the extrapolated lines on plots of free energy of unfolding (ΔG_u) versus concentration of Gu.HCl according to Equation 5 are 2.9 ± 0.2 kcal/mol and 6.0 ± 0.4 kcal/mol, respectively. The denaturant sensitivity factor (m) for *apo* and *holo* are 3.46 ± 0.2 kcal/mol/M and 3.89 ± 0.2 kcal/mol/M, respectively. *Holo* CBD is 2.0 folds more stable than *apo* CBD.

2,2,2-Trifluoroethanol (TFE) disrupts hydrophobic interactions in proteins at low concentration, and it promotes formation of non-native α -helix in protein/peptide by stabilizing localized hydrogen bonding at high concentration [27,28]. Stabilization of the hydrophobic interactions in CBD by Ca^{2+} against the denaturing effects of TFE was monitored using CD spectroscopy. Far UV-CD spectrum monitored for both *apo* and *holo* CBD showed positive ellipticity maxima at 236 nm and negative ellipticity minima at 220 nm a characteristic spectrum for proteins with extended β -sheet structure. TFE was added to *apo* and *holo* CBD at an increasing concentration, 0-80% (v/v). The changes in the secondary structures of both the CBDs upon addition of TFE could be classified into 3 phases (Fig. 7A). The first phase, in *apo* CBD lies in the concentration of 0-30% (v/v) of TFE where no significant change in the secondary structure is observed. The second phase occurred at 30-40% (v/v) of TFE; the CD spectra of *apo* show a transition between the native state and the formation of non-native helices. Spectra obtained for *apo* beyond 40% (v/v) of TFE (third phase) exhibit predominately α -helical characteristics. We analyzed the CD spectrum for *holo* in the same manner. The initial native phase upon the addition of TFE is extended up to 50% (v/v), the transition phase occurs between 50-70% (v/v) and beyond 70% (v/v) the non-native helix induction occurs (Fig. 7A, open circle). The native state structure is stabilized against TFE by 20% (v/v) in the presence of Ca^{2+} . The linker by adopting a new β -strand staples two sheets in the β -sandwich motif could exclude solvent molecule from the core, thereby strengthening hydrophobic interactions.

Ca^{2+} delays the onset of aggregation

In addition to inducing a non-native helical structure, TFE is also known to accelerate aggregation [29,30]. To explore the extent of intermolecular interaction, *apo* and *holo* samples were incubated with 0-80% (v/v) TFE and turbidity was measured. The turbidity changes in the *apo* follow three phases (Fig. 7B, solid circle). The first phase at 0 - 20% (v/v) of TFE exhibited native fold with no aggregates. The aggregation phase (second phase) occurs at 25-50% (v/v). The maximum aggregation was observed at 30% (v/v). When more than 50% (v/v) TFE was added the solution turns clear. At the third phase, the secondary structure is primarily α -helical. *Holo* protein also exhibits 3 phases (Fig. 7B, open circle). The initial phase occurs between 0% and 55% (v/v) of TFE, and the protein adopts native fold. The second phase occurs at 60-70% (v/v) with the aggregation maximum at 65% (v/v). Higher than 75% (v/v) the solution turns clear and non-native helix is induced. Careful examination of the raw data indicates that aggregation triggered by TFE in *holo* is 10 folds less than that triggered in *apo*. Folded proteins have a propensity to aggregate with organic solvents which promote partial unfolding [31]. Proteins with well stabilized three dimensional fold do not unfold to initiate aggregation [32]. Our studies show that the *holo* CBD resists forming aggregates and resists developing non-native helices.

Holo* is less dynamic than *apo

X-ray crystallography can provide the amplitudes of displacements of atoms (B-factors) from average positions [33]. Dynamics of *apo* and *holo* based on B-factors differ. *Holo* is considerably less mobile than its *apo* (normalized B-factors; *Holo* = 0.348105 vs. *Apo* = 0.561361). A plot of deviation from mean of the B-factors in both the conformers indicates that *holo* deviate less (Fig. 8). It should be noted that high B-factors seen in *holo* than *apo* in the regions containing residues 934-940 and 987-990 are likely due to the slight crystallographic packing differences. Limited proteolysis can also monitor structural dynamics [34,35]. The peptide region that is either very mobile, highly solvent accessible or devoid of interactions is vulnerable to proteolytic digestion [36,37]. Bacterial collagenases are secreted into animal tissues where proteases are abundant. The ability of collagenase to resist proteolytic digestion may indicate its survivability *in vivo* as well. Both linker and CBD are rich in lysine and arginine residues. Hence trypsin is an apt choice to monitor the dynamics of the protein. Digested products of CBD in the presence and the absence of Ca^{2+} were analyzed at different time points based on the decrease in the intensity of the intact band on SDS-polyacrylamide gels stained with Coomassie brilliant blue (Fig. 9A & 9B). Cleavage of *apo* occurs instantaneously upon addition of trypsin, and the native band completely vanishes in two hours (Fig. 9A). Trypsin appears to have not only cleaved off the linker but also cleaved accessible sites within CBD. In contrast, Ca^{2+} offers tremendous resistance to trypsin cleavage (Fig. 9B). *Holo* remains intact even after 3.5 hours. In order to rule out the possibility that Ca^{2+} may alter the activity of trypsin, cleavage experiments were also conducted on bovine serum albumin (BSA) in the presence and absence of Ca^{2+} (supplementary Fig. S3). The results illustrated that the tryptic activity against BSA is slightly elevated in the presence of Ca^{2+} . Reduced flexibility and dynamics due to formation of a new parallel β -strand by the linker and additional contacts in the core of CBD, explains why *holo* is less prone to proteolytic degradation. Increased sensitivity to proteolysis may play a regulatory role. Collagenase secreted to extracellular matrix with physiological Ca^{2+} concentration which stabilize the collagen binding domain and thus will be able to cause maximal destruction to the insoluble tropocollagen fibril at the onset of infection.

Collagen binding does not require a hydrophobic exposure

Ca^{2+} binding induces conformational changes that expose hydrophobic surface in calmodulin and other EF-hand containing proteins that stabilize protein-protein interaction [38,39]. CBD binds to collagen only in the presence of Ca^{2+} and thus it is important to analyze if there is any exposure of hydrophobic surface in CBD upon Ca^{2+} binding. Little change in hydrophobic surface between *holo* and *apo* was seen in crystal. To confirm the 'in crystal' observation 'in solution' 8-anilinonaphthalene-1-sulfonate (ANS) binding experiment was performed. ANS is a sensitive fluorescent probe for detecting the solvent exposed hydrophobic pockets. ANS is non-fluorescent in water however upon binding to hydrophobic pockets it emits a bright blue fluorescence [40]. ANS binding assay were performed for *apo* and *holo* CBD. Lack of blue shift by Ca^{2+} -binding confirms that hydrophobic residues are kept buried deep inside the structure (supplementary Fig. S4). Therefore unlike calmodulin, increased protein-protein interaction in the presence of Ca^{2+} is likely due to the immobilization of the linker and not due to hydrophobic surface change. It should be noted that the CBD is the C-terminal segment of the bacterial collagenase and all other domains including the catalytic domain are present in the N-terminal. The highly mobile linker which links the other domains to CBD probably hinders collagen binding in the absence of Ca^{2+} by steric interference.

Conclusion

The linker transformation could involve cooperative binding of two calcium ions. The evolutionary choice for built-in unfavorable torsional strains could be to achieve a correct affinity for calcium. The K_d measured in this study closely matches the calcium concentration estimated inside a bacterial cell [41]. The 14 amino acid long linker glued to the surface of CBD by two calcium atoms had an impact in the core of CBD. The *holo* CBD has fewer cavities and residues surrounding the cavities in *holo* are less dynamic than *apo*. Increased flexibility and dynamics of the linker and CBD could be evolved to alter their vulnerability to extracellular proteases and thus could regulate the truncation of collagenase to gelatinase. Tremendous stability of CBD could explain the longevity of clostridial collagenase [42] and growth factors fused to CBD in vivo [5].

Materials and Methods

Expression and purification of CBD

Expression and purification of collagen binding domain (CBD) as a glutathione *S*-transferase (GST) fusion protein was achieved using methods described by Matsushita *et al.*, [7]. Purified CBD was dialyzed four times against one liter of 10 mM HEPES, 150 mM NaCl and 0.2 mM EGTA, pH 7.5. The chelating agent was then removed by dialyzing four times against 1 l of 50 mM Tris. HCl, pH 7.5 and then applied to a Q-Sepharose column (bed volume, 2 ml: Pharmacia) equilibrated with same buffer. The flow-through fractions were pooled and concentrated with Centriprep 3 device, and stored at -80 °C till use.

Experimental condition

All the experiments were done at 25°C, the solutions were prepared in 50 mM TrisHCl (pH 7.5) containing 100 mM NaCl for *apo* and solutions were prepared in 50 mM TrisHCl (pH 7.5) containing 100 mM NaCl and 2 mM CaCl_2 for *holo* protein.

Circular dichroism

All CD measurements were made on a Jasco-720 spectropolarimeter (Tokyo, Japan), and carried out at 100 $\mu\text{g/mL}$ protein concentration. A 0.2 cm quartz cell was used for all the far UV experiments and the signal was monitored in the wavelength range of 195-250 nm. An average of 10 scans was used to generate the data. The far UV CD spectra were smoothed using the noise reducing option in the software supplied by JASCO.

Turbidity measurements

Turbidity measurements were performed on a HITACHI U-3310 spectrophotometer (Tokyo, Japan). Both the *apo* and *holo* CBD protein used for the measurements were of at 100 $\mu\text{g/mL}$ concentration. These protein samples were incubated for 5 hrs with increasing concentrations of TFE in different sample tubes. Turbidity was assured by measuring the absorbance at 350 nm. The path length of the cell used for measurement was 1 cm.

Steady state fluorescence measurements

The thermodynamic stability of the protein was assessed from the equilibrium denaturation experiments using fluorescence spectroscopy. Fluorescence spectra were measured on a Hitachi F-2500 fluorimeter (Tokyo, Japan) with excitation and emission bandwidths at 2.5 or 10 nm respectively. The excitation wavelength used was 280 nm and the emissions were monitored between 300 nm and 450 nm. Unless otherwise mentioned, all fluorescence measurements were made using a protein concentration of 50 $\mu\text{g/mL}$. For its size, CBD itself possess many fluorophores, specifically there is one tryptophan and eight surface tyrosine

residues. The native spectrum of CBD has only one-emission maxima at 318 nm (inset in Fig. 5A) due to tyrosine residues and the only tryptophan (W956) in CBD buried deep inside the hydrophobic core [43]. The denatured spectrum of CBD shows two maxima at 318 nm and 350 nm respectively. In the denatured state the intensity of tyrosine emission at 318 nm is dropped by half. The fraction of unfolded species was estimated from the ratio of the emission intensities at 318 nm and 350 nm. Denaturation profiles were followed with urea and guanidine hydrochloride (Gu. HCl) at 25 °C, the temperature was maintained with a Neslab RTE-110 (Haverhill, MA) circulating water bath. The required temperature(s) for the thermal denaturation experiments were also attained with the same circulating water bath.

Equilibrium Unfolding and Data Analysis

Equilibrium unfolding data were analyzed using the method developed by Pace [44] for a two state transition process:

Equilibrium unfolding data obtained were converted to plots of F_u , the fraction of the protein in the unfolded state, *versus* denaturant concentration using Eq. 1,

$$F_u = (y_i - y) / (y_u - y) \quad (2)$$

Where y is the observable spectroscopic property at a particular denaturant concentration and y_i and y_u represent the values of y at folded and unfolded states of the protein respectively.

Fraction unfolded F_u is related to fraction folded F_f by the relation

$$F_f = 1 / (1 - F_u) \quad (3)$$

For a two state (Native $\leftrightarrow$ Denatured) unfolding pathway, the free energy of unfolding by denaturant (ΔG_u) is related to F_f by transformation of the Gibbs-Helmholtz equation.

$$\Delta G_u = -RT \ln F_f \quad (4)$$

The ΔG_u values were linearly extrapolated to zero denaturant concentration to obtain conformational stability ΔG_{H_2O} using the relation:

$$\Delta G_u = \Delta G_{H_2O} + m[D] \quad (5)$$

Where, ΔG_{H_2O} and m are the intercept and slope, respectively of the plot of ΔG_u *versus* the concentration of the denaturant. m is the measure of the cooperativity of the unfolding reaction, and ΔG_{H_2O} is the change in free energy for a transition from folded to unfolded state(s) in the absence of the denaturant.

ANS binding experiment

The 8-anilino-1-naphthalenesulfonic acid (ANS) emission experiments were performed using a Hitachi F-2500 spectrofluorometer (Tokyo, Japan) at 25 °C. Fluorescence spectra were collected using an excitation wavelength of 390 nm and an emission wavelength ranging from 400 to 600 nm. Control experiments with ANS were carried out under the same buffer conditions, in the absence of the protein. The final concentrations of the protein and ANS used were 5 μ M and 50 μ M, respectively

Isothermal Calorimetry

Binding of Ca^{2+} to collagen-binding domain was analyzed by measuring the heat changes upon titrating CaCl_2 into protein solution using a VP-ITC Microcalorimeter (Microcal, Northampton, MA). All solutions were micro filtered, degassed and equilibrated at 25 °C before data collection and were stirred at 300 rpm during the experiment. The sample cell (1.4 mL) contained 0.1 mM of CBD in 20 mM Tris HCl at pH 7.5 containing 100 mM NaCl and the reference cell contained distilled water purified using a MilliQ apparatus. 2 mM CaCl_2 prepared in the same buffer as used in the sample cell was drawn in the syringe and injected in 48×6 μ L aliquots with 480 s spacing between each injection to allow the sample to return to baseline. A blank titration was also done with Ca^{2+} free buffer taken in the syringe. The CBD versus Ca^{2+} titration curve was corrected with the blank control and analyzed using the Origin for ITC software supplied by the manufacturer. Care was taken to properly clean the ITC sample cell and syringe before each titration. This was done by incubating the sample cell with 5% contrad detergent solution (Decon Laboratories, Inc., Bryn Mawr, PA) at 55 °C for 1h and washed excessively with the same detergent solution. After this the cell was rinsed with deionized water with 0.5 M EDTA for 5 min and then flushed with >300 mL of metal-free deionized water before loading the sample. The loading syringe was also washed in the same way.

Differential scanning Calorimetry

Calorimetry was performed using a Nano-Differential Scanning Calorimeter (NDSC) model CSC 6300 from Calorimetry Sciences Corporation. Purified CBD 0.5 mg/mL dissolved in 20 mM Tris (pH=7.5), 100 mM NaCl for *apo* and CBD 0.5 mg/mL dissolved in 20 mM Tris (pH=7.5), 100 mM NaCl with 2 mM CaCl_2 for *holo* were degassed prior to the run. A heating scan from 5 °C to 105 °C at a scan rate of 1 °C/min was done, followed by a cooling scan from 105 °C to 5 °C at the same rate. Analysis was performed using DSC analysis software from the manufacturer to calculate the transition temperatures of the folding and refolding reactions after subtraction of the baseline.

Limited proteolytic digestion

Apo and *holo* CBD were subjected to limited proteolytic digestion with trypsin (Sigma Co., St. Louis, MO). The protein was kept 0.8 mg/mL in both the cases and incubated with 0.05 mg/mL trypsin in 20 mM Tris HCl (pH 7.5) containing 100 mM NaCl. The hydrolysis was stopped at particular time points by precipitating the mixture with trichloroacetic acid. After precipitating the protein the mixture was spanned and rinsed with acetone to remove excess TCA. The resulting product was heated on a heating block <90 °C for 10 mins. The products from the proteolytic digestion were analyzed by SDS-PAGE. The intensity of intact CBD band estimated by densitometry, not subjected to any protease action, was considered as control with 100% protection against trypsin cleavage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CBD	Collagen binding domain
ITC	Isothermal titration calorimetry

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Fig 1. Overlay of *apo* and *holo* CBD with a 14-amino-acid long linker. The linker undergoes structural change from *apo* (green) to *holo* (yellow). Only significant change in the core of CBD is the shift in χ_1 angle of Y931. The Ca²⁺ ions are shown as red spheres. This figure was made using MOLMOL [45].

Fig 2. Isothermal titration calorimetric curve for CBD titrated against Ca^{2+} . Application of a two binding site model fits well. The upper panel shows the raw data of the titration, and the bottom panel shows the integrated form of the titration. All the data points were corrected for the heat of dilution.

Fig 3. Schematic representation of Ca^{2+} binding and structural transformation of CBD. K_I , K_{II} , $K_{I,II}$, $K_{II,I}$ are definitions of microscopic equilibrium binding constants for a two-site system [14]. Ca^{2+} binding sites are shown as *open circles* and Ca^{2+} ions are shown in *solid circles*.

Fig 4.

(**A**) Close up view of the linker in *apo*-state. Hydrogen bonds are shown as dashes. Residues 899-908 form α -helix. Alternate side-chain conformations are shown for K896, E899, and S906. Peptide bond between 901-902 is in *trans* conformation. (**B**) Close up view of the linker in *holo*-state. Peptide bond between 901 and 902 adopts *cis* conformation. Residues 897-899 forms β -strand, and 907-909 form π -helix. The hydrogen bonds are shown as dashes. Ca^{2+} ions are indicated as Ca1 and Ca2. This figure was generated using Pymol [46].

Fig 5.

(**A**) Urea unfolding curves of CBD in 20 mM Tris buffer (pH 7.5) with 100 mM NaCl are shown in solid circles for *apo* and open circles for *holo*. (**B**) Thermal unfolding curves of CBD in 20 mM Tris buffer (pH 7.5) with 100 mM NaCl are shown in solid circles for *apo* and open circles for *holo*.

Fig 6. Gu. HCl unfolding curves of CBD in 20 mM Tris buffer (pH 7.5) with 100 mM NaCl are shown in solid circles for *apo* and open circles for *holo*.

Fig 7.

(A) Molar ellipticity changes at 222 nm in far UV CD plotted against the increasing concentrations of TFE. Solid circles show the ellipticity changes monitored on *apo* CBD and the open circles show the ellipticity changes monitored on *holo* CBD. (B) Turbidity measured from UV absorbance at 350 nm plotted against the increasing concentrations of TFE. Solid circles show the aggregation monitored on *apo* CBD and the open circles show the aggregation monitored on *holo* CBD.

Fig 8.

A plot of deviation from mean of B-factors with residue number. B-factors for *apo* CBD is shown in gray and *holo* CBD is shown in black.

Fig 9.

SDS-PAGE analysis of time course trypsin digestion on CBD in the absence (A) and the presence (B) of Ca^{2+} . The trypsin band remains constant throughout the gels. Lane-M shows the protein marker in kDa. The other lanes are: 1- Control, 2-0.5h, 3-1h, 4-1.5h, 5-2h, 6-2.5h, 7-3h, 8-3.5h. The gels were stained in Coomassie brilliant blue. The difference in the cleavage pattern clearly indicates that Ca^{2+} stabilizes CBD against protease. Undigested CBD was loaded in both cases for control show that the native CBD remains intact in the absence of trypsin.

Table 1Parameters characterizing the denaturation of *apo* and *holo* CBD at pH = 7.5, 25°C

	<i>Apo</i> CBD		<i>Holo</i> CBD	
Parameters	Urea	Gu.HCl	Urea	Gu.HCl
ΔG_{H_2O} (kcal/mol) ^a	6.49 ± 0.2	2.89 ± 0.2	9.73 ± 0.2	6.0 ± 0.4
m (kcal/mol/M) ^a	1.14 ± 0.03	3.46 ± 0.2	1.41 ± 0.04	3.89 ± 0.2
C_m (M) ^b	5.68±0.02	0.84 ± 0.01	6.89±0.04	1.55±0.02

^(a) Values obtained from Equation 5^(a) Midpoint of the denaturation curves shown in Figures 5 and 7.

Table 2Hydrogen bonds in *apo* and *holo* CBD

Molecule	Contacts		
	N...O between 2.4-3.0 Å	O...O between 2.4-3.0 Å	CH...O between 3.0-3.5 Å
<i>Apo</i> A	61	10	18
<i>Apo</i> B	70	16	17
<i>Holo</i> A	68	9	20
<i>Holo</i> B	70	13	20

Table 3

Cavities for *apo* (1NQJ) and *holo* CBD (1NQD)

Cavity	Contributing residues	<i>Apo</i>		<i>CBD</i>		<i>Holo</i>		<i>CBD</i>
		Surface Area Å ²	Volume Å ³	Surface Area Å ²	Volume Å ³	Surface Area Å ²	Volume Å ³	
1	L956-P958, R967-T969, V982-R985, Y989	145.3	141.5	85.7	84.6			
2	I912-F915, F934, E935, K937, V1006	119.7	142	93	109			
3	T918, E945, D947, E1003-R1005	61.5	57.8	30.9	25.9			
4	I922, T927, M929, L1004-V1006	41.5	22	36.9	18.5			
5	I922, N924, T927, V1006	33.9	16.5	31.1	15			
6	S905, K908, A909, Y931, Y932, T957, H959, Y990-L992	139.4	111.8					
7	P960-S962, N965, R985-Y989	66.9	71.8					
8	S907, A910, V912, S933, K988	32.3	23.8					
9	K898, D901, N902, S905, H959	37.2	29.1					
10	K898, Q920-S922, K949, N1002, Y1003			61.6	69.4			
11	V942, I944, N980, V982			39.5	22.5			