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Natively Unfolded C-Terminal Domain of Caldesmon Remains Substantially Unstructured After the Effective Binding to Calmodulin

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ABSTRACT **The structure of C-terminal domain (CaD136, C-terminal residues 636–771) of chicken gizzard caldesmon has been analyzed by a variety of physico-chemical methods. We are showing here that CaD136 does not have globular structure, has low secondary structure content, is essentially noncompact, as it follows from high R_g and R_S values, and is characterized by the absence of distinct heat absorption peaks, i.e. it belongs to the family of natively unfolded (or intrinsically unstructured) proteins. Surprisingly, effective binding of single calmodulin molecule (K_d = 1.4 ± 0.2 μM) leads only to a very moderate folding of this protein and CaD136 remains substantially unfolded within its tight complex with calmodulin. The biological significance of these observations is discussed. Proteins 2003;53:855–862.** © 2003 Wiley-Liss, Inc.

Key words: caldesmon; calmodulin; natively unfolded protein; intrinsically unstructured protein

INTRODUCTION

Caldesmon (CaD) is a ubiquitous actin-binding protein involved in the regulation of smooth muscle contraction, nonmuscle motility, and cytoskeleton formation.[1–4] CaD is thought to contribute to a thin-filament-linked regulation of smooth muscle contraction through its specific binding to F-actin and F-actin–tropomyosin with a concomitant inhibition of the actin-stimulated myosin ATPase.[2] The reversal of the CaD action is made possible by its additional interaction with calcium-dependent proteins such as calmodulin (CaM) and caltropin.[5] The functional activity of CaD is regulated by phosphorylation performed by a number of protein kinases[6] and by calcium via Ca^{2+}-binding proteins, with CaD being alternatively bound either to F-actin or CaM, depending upon the calcium concentration (so-called "flip-flop" binding). This thin filament-based modulatory effect is assumed to provide additional "fine-tuning" to the well-established, myosin light-chain phosphorylation-dependent, thick filament-based regulation of smooth muscle contraction.[7] The CaD regulatory role is further reinforced by the localization of the protein almost exclusively within the contractile domain of the smooth muscle cells.[8]

CaD shows broad variety in tightly bound partners, interacting specifically with myosin, actin, tropomyosin, CaM,[2] caltropin,[5] calcyclin,[9] $S100a_o$, S100a, and S100b proteins,[10] and nonmuscle tropomyosin[12] and possessing distinctive phospholipid-binding properties.[12–14] CaD consists of four independent functional domains. The first, N-terminal domain interacts with myosin and tropomyosin. The second domain characteristic of smooth muscle CaD participates in the binding of tropomyosin. The third domain is involved into the interaction of CaD with myosin, tropomyosin, and actin. The fourth C-terminal domain plays the most important role in the functioning of CaD, interacting with actin, Ca^{2+}-binding proteins, myosin, tropomyosin, and phospholipids.[11] Moreover, it has been established that interaction of CaD with actin, tropomyosin,[15] and CaM involves multiple sites.[11,16–18]

The features listed above (multiple binding partners and extended binding sites) might be typical of the natively unfolded (or intrinsically unstructured) proteins,[19–22] which constitute a class of polypeptides that exhibit an absence of globularity, low compactness, the absence of secondary structure, and high flexibility under the physiological conditions *in vitro*.[22–24] A disorder-order transition induced in intrinsically disordered proteins during the binding to their specific targets *in vivo* might represent a simple mechanism for regulation of numerous cellular processes, including transcriptional and translational regulation and cell cycle control.[19–23]

In this article we show that the C-terminal domain of chicken gizzard CaD, CaD136 (636–771 fragment), belongs to the family of natively unfolded proteins and binds

Abbreviations: CaD, caldesmon; CaD136, C-terminal domain (636–771) of CaD; CaM, calmodulin; CD, circular dichroism; UV, ultraviolet; SAXS, small angle X-ray scattering; DSC, differential scanning microcalorimetry; R_g, radius of gyration; R_S, Stokes radius; N, native; MG, molten globule; U, unfolded; NU(coil) natively unfolded coil-like; NU(PMG), natively unfolded premolten globule-like.

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effectively to the Ca^{2+}-loaded CaM. Importantly, CaD136 remains effectively unfolded within its complex with CaM. Biological significance of these observations is discussed.

MATERIALS AND METHODS

Materials

Samples of CaD136 and CaM were a kind gift of Dr. Yuji Kobajashi (Department of Physical Chemistry, Institute of Protein Research, Osaka University, Osaka 565, Japan).

All chemicals were of analytical grade from Fisher Chemicals. Concentrations of CaD and CaM were estimated spectrophotometrically. Molar extinction coefficient for CaM and CaD were calculated based on amino acid content according to the following[26]: ϵ_{280nm} = 2,980 M^{-1} cm^{-1}, and, ϵ_{280nm} = 18,400 M^{-1} cm^{-1}, respectively.

Circular Dichroism Measurements

CD spectra were obtained with an AVIV 60DS spectrophotometer (Lakewood, NJ) using CaD136 and CaM concentrations of 1 mg/mL. Near-UV CD spectra were recorded in a 1.0-cm pathlength cell and far-UV CD with a 0.01-cm pathlength cell. CD spectra of the appropriate buffers were recorded and subtracted from the protein spectra.

Fluorescence Measurements

Fluorescence measurements were carried out on a laboratory-made spectrofluorimeter, the main characteristics of which have been described earlier.[27] All spectra were corrected for spectral sensitivity of the instrument and fitted to log-normal curves[28] using nonlinear regression analysis.[29] The maximum positions of the spectra were obtained from the fits. The temperature inside the cell was monitored with a copper-constantan thermopile.

Small Angle X-Ray Scattering Experiments

Small angle X-ray scattering (SAXS) measurements were made using Beam Line 4-2 at Stanford Synchrotron Radiation Laboratory.[30] Experimental conditions and protocol were as described in Ref. 31. The radius of gyration (R_g) was calculated according to the Guinier approximation.[32]

Gel-Filtration Experiments

The hydrodynamic dimensions (Stokes radii, R_S) of CaD136 were measured by size-exclusion chromatography. Size-exclusion measurements were performed on a Superose-12 column using a Pharmacia FPLC chromatographic system. A set of globular proteins (Gel Filtration Chromatography Standards from Bio-Rad Laboratories) with known R_S values was used in order to create a calibration curve, $1000/V_{el}$ versus R_S.[33–37]

Hydrodynamic dimensions of native, completely unfolded, molten globule as well native coil and native premolten globule protein with known molecular mass M were calculated from the set of empirical equations[23]:

$$\log(R_S^N) = -(0.204 \pm 0.023) + (0.357 \pm 0.005) \cdot \log(M) \quad (1)$$

$$\log(R_S^{MG}) = -(0.053 \pm 0.094) + (0.334 \pm 0.021) \cdot \log(M) \quad (2)$$

$$\log(R_S^U) = -(0.723 \pm 0.033) + (0.543 \pm 0.007) \cdot \log(M) \quad (3)$$

$$\log(R_S^{NU(coil)}) = -(0.551 \pm 0.032) + (0.493 \pm 0.008) \cdot \log(M) \quad (4)$$

$$\log(R_S^{NU(PMG)}) = -(0.239 \pm 0.055) + (0.403 \pm 0.012) \cdot \log(M) \quad (5)$$

Here R_S^N, R_S^U, and R_S^{MG} are the Stokes radii of globular protein in its native (N), unfolded (U), and molten globule (MG) conformation, whereas $R_S^{NU(coil)}$ and $R_S^{NU(PMG)}$ correspond to the Stokes radii of natively unfolded coils and natively unfolded premolten globules.

Parameters of CaD136 Binding to CaM

The apparent binding constant for the formation of CaD136 complex with CaM was evaluated from a fit of the fluorescence titration data to the specific binding scheme using nonlinear regression analysis.[29] The binding scheme was chosen on the "simplest best fit" basis. The quality of the fit was judged by a randomness of distribution of residuals.

Differential Scanning Microcalorimetry

Scanning microcalorimetric measurements were performed on a DASM-4M differential scanning microcalorimeter (Institute for Biological Instrumentation of the Russian Academy of Sciences, Pushchino, Russia) in 0.45 mL cells at a 0.9 K/min heating rate. An extra pressure of 3 atm was maintained in order to prevent possible degassing of the solutions on heating and boiling at temperatures > 100°C. Protein concentration was about 1 mg/mL. The heat sorption curves were baseline corrected by heating the measurement cells filled by the solvent only. Protein specific heat capacity was calculated according to Refs. 38 and 39. Protein partial molar volume was calculated according to Ref. 40.

RESULTS

Amino Acid Composition of CaD136 Predicts a Disordered Structure

It has been established that naturally folded and intrinsically unstructured proteins occupy nonoverlapping regions in the charge-hydrophobicity plots, with natively unfolded proteins being specifically localized within a particular region of charge-hydrophobicity phase space, satisfying the following relationship:[25]

$$\langle H \rangle \leq \langle H \rangle_b = \frac{\langle R \rangle + 1.151}{2.785}, \quad (6)$$

where $\langle H \rangle$ and $\langle R \rangle$ are the mean hydrophobicity and the mean net charge of the given protein, respectively, whereas $\langle H \rangle_b$ is the "boundary" mean hydrophobicity value, below which a polypeptide chain with a given $\langle R \rangle$ will be most

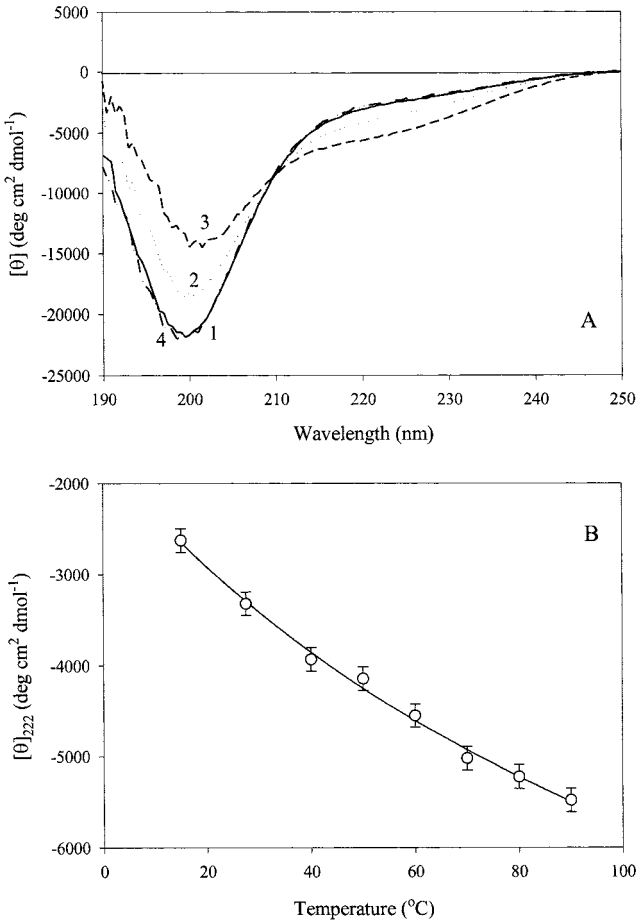

Fig. 1. (A) Far UV CD spectra of CaD136 measured at different temperatures: 15°C (1); 40°C (2), 90°C (3), and 15°C after the cooling (4). All measurements were carried out at a protein concentration of 0.8 mg/mL and cell pathlength 0.1 mm. (B) Effect of temperature on far UV CD spectra of CaD fragment depicted as $[\theta]_{222}$ vs. temperature dependence.

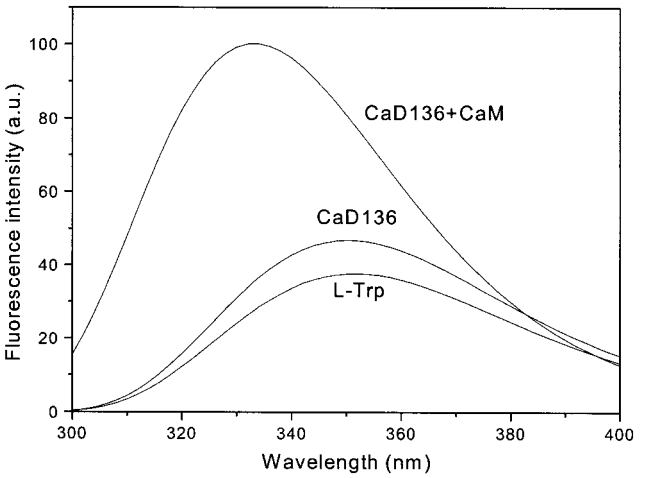

Fig. 2. Tryptophan fluorescence spectra of CaD136 in the free- and CaM-bound states at 20°C (pH 7.6; 10 mM HEPES-KOH buffer, 5 mM $CaCl_2$). Fluorescence was excited at 296.7 nm. Spectrum of L-Trp in water solution under the same absorption at 296.7 nm is shown for comparison.

probably unfolded. The mean hydrophobicity, $\langle H \rangle$, is defined as the sum of the normalized hydrophobicities of all residues divided by the number of residues in the polypeptide. The mean net charge, $\langle R \rangle$, is defined as the net charge at pH 7.0, divided by the total number of residues. Analysis of the CaD136 amino acid sequence shows that this protein is characterized by $\langle H \rangle = 0.4038$, $\langle R \rangle = 0.0657$, and $\langle H \rangle_b = 0.4369$, thus fulfilling the requirement for the natively unfolded proteins, namely that $\langle H \rangle < \langle H \rangle_b$ ($0.4038 < 0.4369$).

Furthermore, amino acid sequence-based calculation of the secondary structure content using HNN predictor[41] revealed that only 16 and 20 residues of CaD136 may be involved in the formation of α-helices and β-structure, respectively, thus, predicting a very low content of the potentially ordered secondary structure, ~25%. These predictions were confirmed by experimental results.

Secondary Structure (Far-UV CD Spectrum)

Figure 1(A) represents far-UV CD spectra of CaD136 measured at different temperatures. At low temperature this protein is characterized by a far-UV CD spectrum typical of an essentially unfolded polypeptide chain. On the other hand, more detailed analysis of far UV CD spectrum shows that CaD136, being considerably distorted, is still far from to be completely unfolded and preserves some residual structure [e.g., $[\theta]_{222} \sim -2500°$ cm^2 $dmol^{-1}$, the minimum is located at 200, rather than at 196–198 nm; see Fig. 1(A)]. Furthermore, Figure 1(A) shows that the shape and intensity of CaD136 spectrum undergoes considerable changes with temperature, reflecting the temperature-induced formation of ordered secondary structure. Figure 1(B) represents corresponding $[\theta]_{222}$ vs. temperature dependence and shows that the temperature increase is accompanied by the monotonous increase in the negative ellipticity at 222 nm. This behavior is totally different from that of a normal globular protein, which shows temperature-induced reduction in the content of ordered secondary structure. It has been noted that such structure forming effect of heating, being typical of the intrinsically unstructured proteins, might be ascribed to the heat-induced intensification of the hydrophobic interactions.[22,23]

Tryptophan Fluorescence

Figure 2 represents tryptophan fluorescence spectra of CD136. It is clearly seen that the spectrum of free CaD136 has maximum at about 350.2 nm; i.e., it is close to that of tryptophan in water (351.5 nm). This indicates that the CaD136 tryptophan residues are almost totally exposed to water.

DSC Analysis of CaD136

The effect of temperature on CaD136 structure was further analyzed by DSC. We have established that CaD136 is characterized by the absence of distinct heat sorption peaks within the temperature region from 10°C to 110°C, indicative of the absence of rigid tertiary structure for the protein. Comparison of temperature dependence of the

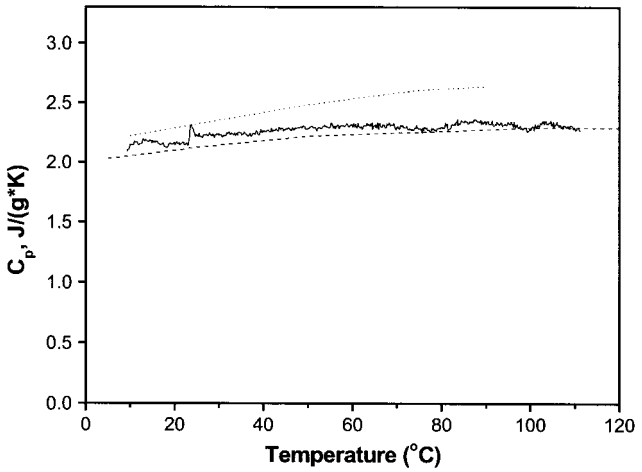

Fig. 3. Temperature dependence of the partial heat capacity of CaD136 at pH 8.0 (50 mM borate). Protein concentration was 1.5 mg/mL. The dashed and dotted lines represent the heat capacity functions calculated for the completely unfolded protein according to Makhatadze and Privalov[43] and Hackel et al.,[42] respectively.

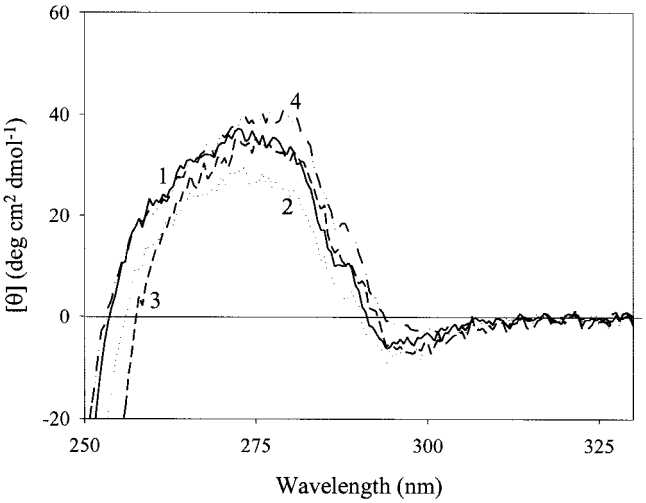

Fig. 4. Near UV CD spectra of CaD136 measured at different temperatures: 15°C (1); 40°C (2), 90°C (3), and 15°C after the cooling (4). All measurements were carried out at a protein concentration of 0.8 mg/mL and cell pathlength 10 mm.

partial heat capacity of CaD136 with the predictions of two models for the completely unfolded polypeptide chain (Hackel-Hinz-Hedwig model[42] or Makhatadze-Privalov model[43]) shows that absolute heat capacity of the protein lays between these two estimations in the whole temperature range (see Fig. 3). Furthermore, high values of CaD136 heat capacity are typical for random coil, suggesting that CaD136 possesses a substantially unfolded structure.

Residual Tertiary Structure by Near-UV CD Spectrum

Figure 4 represents the near-UV CD spectra of CaD136 measured at different temperatures. Contrary to the previous observations, CaD136 shows rather intensive and pronounced near UV CD spectrum. This means that tryptophan residues of this protein are in the relatively asymmetric environment; i.e., protein possesses some residual structure. Figure 4 shows also that heating does not affect this residual structure to the significant extent.

Hydrodynamic Properties of CaD136 from Gel-Filtration Experiments

To obtain information about the hydrodynamic dimensions of CaD136, the gel-filtration behavior of this protein at neutral pH in the absence or in the presence of 6 M GdmCl was studied. Chromatographic analysis confirmed the results of other techniques and showed that CaD136 was essentially unfolded under the conditions of neutral pH. In fact, we have established that the hydrodynamic dimensions of CaD136 are relatively close to those measured in the presence of 6 M GdmCl (R_S = 28.1 ± 0.8 and 35.3 ± 0.8 Å, respectively), confirming the fact that CaD136 is essentially unfolded even in the absence of denaturant. Comparison of these measured values with R_S calculated using Equations 1–5 for the different conformations of a protein with a molecular mass of 14,514 Da [19.1, 21.7, 34.4, 31.7, and 27.4 Å for N, MG, U, NU(coil), and NU(PMG), respectively] suggests that CaD136 belongs to the class of native premolten globules.[22,23]

Hydrodynamic Properties of CaD136 from SAXS

SAXS is a very powerful method for the analysis of conformation, shape, and dimensions of biopolymers in solution. Analysis of the scattering curves using the Guinier approximation provides the radius of gyration, R_g, whereas scattering data in the form of Kratky plots provides information about the globularity (packing density) and conformation of a protein,[32,44] as for a native globular protein this plot has a characteristic maximum, whereas unfolded and partially folded polypeptides have significantly different-shaped Kratky plots.

Figure 5(A) represents the results of Guinier analysis of the scattering data for CaD136. The linear Guinier plot indicates that the solution of this protein was homogeneous. The radius of gyration of a random coil, R_g^U, may be estimated from the corresponding Stokes radius, R_S^U, using the relation R_g^U/R_S^U = 1.51.[45] The observed R_g value for CaD136 (40.8 ± 0.8 Å) is smaller than that estimated for a random coil conformation for a protein of this size (51.9 Å), indicating that the natively unfolded conformation of this protein is in fact more compact than that of a random coil. Finally, analysis of the X-ray scattering data in a form of the Kratky plot shows that CaD136 does not have any well-developed globular structure [Fig. 5(B)].

Structural Consequences of CaM Binding to CaD136

Chicken gizzard CaD contains five tryptophan residues per protein molecule; three of them, Trp674, Trp707, and Trp737, are located within the CaD136 domain. On the other hand, CaM does not have any Trp residues. This implies changes in the CaD136 intrinsic tryptophan fluorescence as fast and easy method to detect CaD–CaM

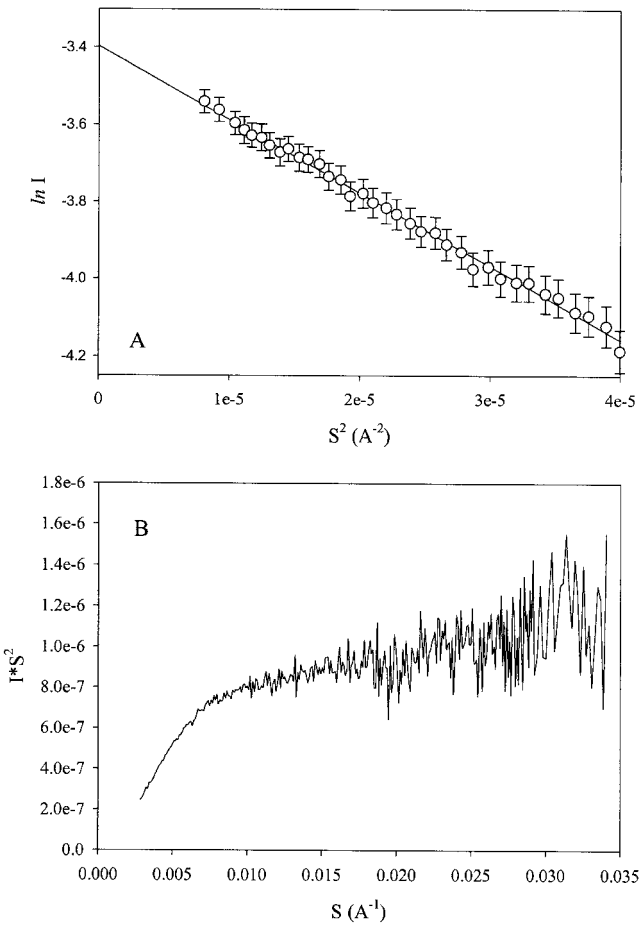

Fig. 5. SAXS analysis of CaD136 solutions. (A) Guinier plot; (B) Kratky plot. All measurements were performed at a protein concentration of 3.5 mg/mL

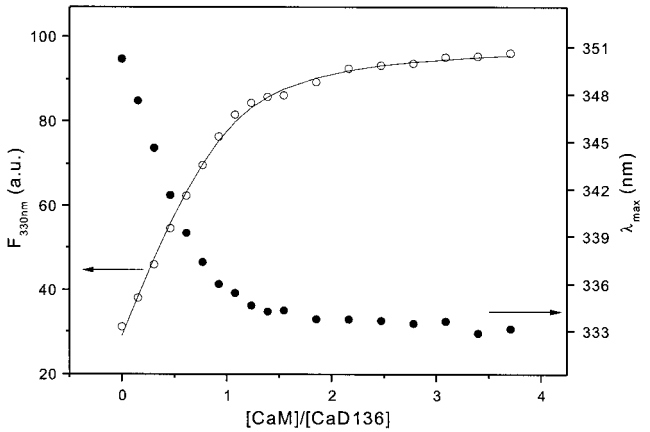

Fig. 6. Spectrofluorimetric titration of caldesmon fragment CaD136 (9.5 μM) by CaM at 20°C (pH 7.6; 10 mM HEPES-KOH buffer, 5 mM $CaCl_2$). Fluorescence was excited at 296.7 nm. □, tryptophan fluorescence intensity at 330 nm, F_{330nm}, arbitrary units; ●, fluorescence spectrum maximum position, λ_{max}, nm. Points are experimental and the curve is theoretically fitted to the experimental points according to the single site binding scheme.[7]

complex formation.[46,47] Figure 2 compares the tryptophan fluorescence spectra (excitation at 296.7 nm) of CaD136 measured in the absence or presence of CaM. Notably, excess of Ca^{2+} (5 mM $CaCl_2$) was used to saturate CaM by calcium, which is a necessary condition of effective binding of CaM by CaD.[11] The temperature was kept at 20°C in order to distance from thermal transition of Ca^{2+}-loaded CaM. It can be seen that CaM binding to CaD136 led to the considerable (1.9-fold) increase in the fluorescence quantum yield and an essential (17 nm) blue shift of the CaD136 fluorescence spectrum, reflecting the transfer of tryptophans into the less mobile and polar environment. This most likely reflects some CaM-induced compaction of a polypeptide chain at least in the vicinity of tryptophans or these changes in fluorescence are due to the insertion of Trp into a hydrophobic binding pocket on the CaM. These observations have been used to evaluate the binding parameters of CaD136-CaM complex (see Fig. 6). It should be noted that the characteristic bend of the CaM-titration curve in Figure 6, corresponding to saturation of CaD136 by CaM, takes place around CaM to CaD136 ratio about 1, which is an evidence of the binding of a single CaM molecule per CaD136 molecule:

$$\mathrm{CaD136} + \mathrm{CaM} \overset{K}{\Leftrightarrow} \mathrm{CaD136-CaM}. \qquad (7)$$

The experimental data for the changes in fluorescence intensity were fitted by a theoretical curve computed according to the one-site binding scheme. The best fit was achieved with $K_d = (1.4 \pm 0.2)$ μM. Comparable dissociation constant values have been earlier reported for the formation of CaD–CaM complexes.[16–18,46–48]

Figure 7 represents far-UV CD spectra measured for the CaD136 and CaM along as well as for the CaD136–CaM complex and a spectrum calculated as a simple weighted sum of spectra of CaD136 and CaM. The idea behind the comparison of measured and calculated spectra was as following: if an interaction between CaD136 and CaM does not induce any structural changes in proteins, then the measured spectrum would be equal to the calculated one. However, if the CaD136–CaM complex formation does induce structural changes, then the difference between the observed and calculated spectra must be seen. Figure 7 shows that that the experimentally measured spectrum of CaD136–CaM complex is notably different from the spectrum calculated as (0.5 × CaM + 0.5 × CaD136). Most likely this reflects partial folding of CaD136 rather than the partial unfolding of its binding partner, CaM. Based on this assumption, the apparent spectrum of CaD136 within its complex with CaM has been calculated from CaD136–CaM and CaM spectra as [(CaD136–CaM − 0.5 × CaM)/0.5]. This assumes that the efficiency of CaD136–CaM complex formation is 100&%. In fact, calculations based on the measured K_d value [$(1.4 \pm 0.2) \times 10^{-6}$ M] revealed that the actual population of complex was 77.8% under conditions studied (i.e., when [CaD136]/[CaM] = 1 and concentration of each protein was of $\sim 10^{-4}$ M). Importantly, Figure 7 shows that the correction of the measured CaD136–CaM spectrum for the presence of free proteins (11.1%) does not affect significantly the resulting spectrum of bound CaD136.

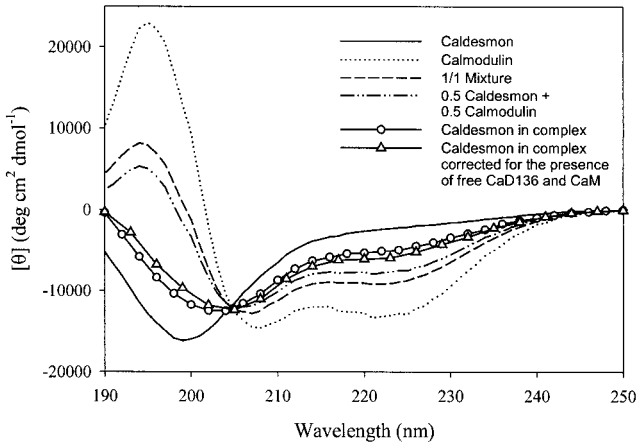

Fig. 7. Far UV CD spectra of CaD136 (solid line), CaM (dotted line), and CaD136-CaM complex (dashed line). All measurements were carried out a cell pathlength of 0.1 mm and at concentrations of Cad136 and CaM of 1.45 and 1.67 mg/mL, respectively. Spectrum calculated as a simple weighted sum of CaD136 and CaM spectra is shown as dashed-dotted line. Spectrum of CaD136 in its complex with CaM is shown by line with open circles. This spectrum was calculated assuming that both proteins are completely involved into the complex formation and a difference between the measured CaD136-CaM and CaM spectra are due to the partial folding of CaD136 rather than a partial unfolding of CaM; i.e., as [(CaD136-CaM − 0.5 ÷ CaM)/0.5]. Spectrum of bound CaD136 corrected for the existence of unbound CaD136 and CaM (see the text) is shown by line with open triangles. This spectrum was computed as: [((CaD136-CaM − 0.111 × (CaM + CaD136))/0.778 − 0.5 × CaM)/0.5], where CaD136, CaM, and CaD136-CaM correspond to far-UV CD spectra measured for caldesmon, calmodulin, and their complex, respectively.

Figure 7 shows that the formation of CaD136–CaM complex led to a partial folding of the natively unfolded CaD136. As it was already pointed out, in the absence of CaM CaD136 possesses a far-UV CD spectrum typical of an essentially unfolded polypeptide chain; i.e., a spectrum with an intense minimum in the vicinity of 200 nm, and the absence of characteristic bands in the 210–230 nm region. However, as the CaD136–CaM complex was formed, the minimum at 200 nm became less intense and shifted toward the longer wavelength, whereas the negative intensity of the spectrum around 222 nm increased, reflecting the formation of ordered secondary structure. Importantly, the extent of this folding was relatively low and CaD136 remained substantially unstructured even after the effective binding to CaM. Potentially, there is an alternative scenario of CaD136 binding to CaM, where CaD136 gains some sort of rigid but nonregular structure as a result of complex formation. However, the existing far-UV CD data are nonsufficient to verify the validity of this assumption and application of other techniques, such as multidimensional NMR, is required.

DISCUSSION

We are showing in this article that the functionally important C-terminal domain of CaD, CaD136, is an intrinsically unstructured. In fact, it shows large hydrodynamic dimensions (gel-filtration and SAXS), does not have cooperatively melted tertiary structure (DSC), possesses absence of globular structure (SAXS), and is characterized by low content of ordered secondary structure (far-UV CD). Moreover, our data are consistent with the conclusion that CaD136 is not completely unfolded, belonging to the subclass of the native premolten globules, according to its hydrodynamic properties and far-UV CD spectrum. Interestingly, the residual structure of CaD136 might be relatively well organized, because this protein has distinctive near-UV CD spectrum. The ability of the CaD and its C-terminal fragments to interact specifically with CaM has been established long ago,[47] and several models of this complex have been suggested (reviewed in Ref. 11). Importantly, our data suggest that the formation of the tight CaD136–CaM complex (K_d = 1.4 ± 0.2 μM) leads only to the marginal folding of the natively unfolded CaD136. These facts might shed some light on the mechanism of CaD and CaM interaction.

It has been suggested that the lack of rigid globular structure under physiological conditions might represent a considerable functional advantage of natively unfolded proteins, because their large plasticity allows them to interact efficiently with several different targets.[19–22] In agreement with this observation, CaD was shown to possess high affinity to at least 30 different target proteins,[49,50] including myosin, actin, tropomyosin, CaM,[2] caltropin,[5] calcyclin,[9] S100a$_o$, S100a and S100b proteins,[10] nonmuscle tropomyosin,[11] to several rather short amphiphilic peptides (mastoparan, melittin, dynorphin)[51] as well as to phospholipids[12–14] and some hydrophobic drugs (chloropromazine). Importantly, large portion of binding sites being localized within the C-terminal domain of CaD, which can interact with actin, Ca^{2+}-binding proteins, myosin, tropomyosin and phospholipids.[11] Another important feature of the natively unfolded proteins is their capability to undergo function-related disorder-order transition.[19–24] Accordingly, we are showing that CaD136 folds to a partially folded conformation as a result of its interaction with CaM.

It is known that the C-terminal domain of CaD contains three CaM-binding sites, centers A (close to Trp674), B (close to Trp707), and B′ (close to Trp737). It has been shown that sites A and B′ interact with C-terminal lobe of CaM (this protein has dumbbell shape with two α-helical Ca^{2+}-binding globular domains, separated by an extended "handle" formed by a seven-turn α-helix), whereas center B forms complex with the N-terminal globular domain.[11] We assume that the natively unfolded conformation of CaD136 permits this domain to fulfill its function. The possibility exists that CaM binds at a hydrophobic patch of CaD136 made by Trp residue(s), leading to the burial of the indole group(s), as given by the blue shift of the maximum wavelength of fluorescence emission from 350 to 333 nm (see Fig. 6). In this case, the binding of CaD136 to CaM can be viewed as a local binding encompassing a rather short part of the polypeptide chain of CaD136 and not as an overall binding of CaD136 (which has to be accompanied by a major conformational change of the protein).

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