Folding units in calcium vector protein of amphioxus: Structural and functional properties of its amino- and carboxy-terminal halves

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Abstract

Muscle of amphioxus contains large amounts of a four EF-hand Ca^{2+} -binding protein, CaVP, and its target, CaVPT. To study the domain structure of CaVP and assess the structurally important determinants for its interaction with CaVPT, we expressed CaVP and its amino- (N-CaVP) and carboxy-terminal halves (C-CaVP). The interactive properties of recombinant and wild-type CaVP are very similar, despite three post-translational modifications in the wild-type protein. N-CaVP does not bind Ca^{2+} , shows a well-formed hydrophobic core, and melts at 44°C. C-CaVP binds two Ca^{2+} with intrinsic dissociation constants of 0.22 and 140 μM (i.e., very similar to the entire CaVP). The metal-free domain in CaVP and C-CaVP shows no distinct melting transition, whereas its 1 Ca^{2+} and 2 Ca^{2+} forms melt in the 111°–123°C range, suggesting that C-CaVP and the carboxy- domain of CaVP are natively unfolded in the metal-free state and progressively gain structure upon binding of 1 Ca^{2+} and 2 Ca^{2+} . Thermal denaturation studies provide evidence for inter-domain interaction: the apo, 1 Ca^{2+} and 2 Ca^{2+} states of the carboxy-domain destabilize to different degrees the amino-domain. Only C-CaVP forms a Ca^{2+} -dependent 1:1 complex with CaVPT. Our results suggest that the carboxy-terminal domain of CaVP interacts with CaVPT and that the amino-terminal lobe modulates this interaction.

Key words: Calcium-binding protein; EF-hand; thermal stability; molten globule state; domain structure

The cephalochordate amphioxus is believed to constitute the evolutionary link between invertebrates and vertebrates. It contains a unique protein pair, Ca^{2+} vector protein (CaVP)

and its target Ca^{2+} vector protein target (CaVPT). These proteins have not yet been identified in any other phylum (Cox 1986). CaVP and CaVPT form a 1:1 complex and are abundant in the muscle, the spinal chord, and the gonads of amphioxus. The physiological role of the CaVP/CaVPT protein pair is still unknown.

Although CaVP (18.3 kD) shares ~35% of sequence identity with calmodulin (CaM) and troponin C (TnC) (Kobayashi et al. 1987), it does not substitute for the latter proteins in functional assays (Cox 1986). Bona fide CaM and TnC have been isolated from amphioxus (Cox 1986; Takagi et al. 1994). CaVP possesses four EF-hands, but due to mutations of Ca^{2+} -coordinating residues in sites I and II, it binds only two Ca^{2+} (Kobayashi et al. 1987). The protein contains an

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Abbreviations: CaVP, Ca^{2+} vector protein; CaVPT, Ca^{2+} vector protein target; N-CaVP, segment 1–86 of CaVP; C-CaVP, segment 81–161 of CaVP; CaM, calmodulin; TnC, troponin C; TML, trimethyllysine; DSS, disuccinimidyl suberate; CD, circular dichroism; DSC, differential scanning calorimetry; T_d , denaturation temperature; NMR, nuclear magnetic resonance.

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acetylated amino terminus, and two ϵ -trimethyllysines (TML) in the third EF-hand (Fig. 1). CaVPT (26.6 kD) is constituted of three distinct domains (Takagi and Cox 1990) with a Pro-Ala-Lys-rich motif, an IQ motif and two immunoglobulin II (IgII) folds. Preliminary data indicate that only free CaVPT, but not the complex CaVP/CaVPT, interacts with a 106-kD protein related to paramyosin (T.V. Petrova, unpubl. data).

In this study, we address questions related to the domain structure of CaVP and the interdomain interaction. On the basis of its structural homology with CaM and TnC, which are composed of two folding modules linked by a central α -helix, we hypothesized that the amino- and carboxy-terminal domains of CaVP constitute independent folding units (Cox et al. 1990). Therefore, CaVP, as well as its amino- and carboxy-terminal moieties were cloned, expressed in *Escherichia coli*, and purified. Because recombinant CaVP is not amino-acetylated and does not contain the two TML residues present in the wild-type protein, the interactive properties of the two forms were compared. We also analyzed the conformation and stability of the recombinant amino- and carboxy-terminal halves of CaVP and their interactions with ions, with each other and with CaVPT.

Results

Expression and purification of recombinant proteins

On the basis of the homology of CaVP with CaM and TnC, the amino-terminal half (N-CaVP) was designed to end at residue 86, which includes the two first EF-hand motifs; the carboxy-terminal half (C-CaVP) starts at position 81, so as to include a Trp (Fig. 1), which is convenient for detection and quantification of the polypeptide. Full-length CaVP, N-CaVP, and C-CaVP were overexpressed in *E. coli* and purified. All three proteins show electrophoretic homogeneity in native and SDS-PAGE. Their exact molecular masses, determined by electrospray mass spectrometry, are $18,223 \pm 1$ Da (vs. 18,224 calculated) for CaVP, $9,768 \pm 5$ Da (vs. 9,773) Da for N-CaVP and $9,402 \pm 2$ Da (vs. 9,269) for C-CaVP. The discrepancy observed for C-CaVP is due to a nonprocessed amino-terminal Met (133 Da), which was

confirmed by amino-terminal sequencing with the Edman method.

Comparative properties of recombinant and wild-type CaVP

Flow dialysis experiments demonstrated that recombinant CaVP binds two Ca^{2+} with the same affinity as the isolated wild-type protein (Fig. 2). Both proteins have similar Trp fluorescence spectra, with a red shift from 333 to 349 nm with denaturation, and denaturation curves, with half-maximal signal change at 1.4 M guanidine-HCl for the metal-free proteins.

To compare the binding affinity of recombinant and wild-type CaVP for CaVPT, the complex was reconstituted (Petrova et al. 1995) from the equimolar mixture of recombinant CaVP, wild-type CaVP, and CaVPT and submitted to gel filtration. Native gels (Durussel et al. 1993) allowed the discrimination between recombinant and wild-type CaVP, as the recombinant protein is more acidic. An equivalent distribution of the two CaVP forms was observed (data not shown). These data suggest that recombinant and wild-type CaVP possess similar conformations and are equipotent to form a complex with CaVPT.

Ca^{2+} -binding properties of N-CaVP and C-CaVP

Equilibrium gel filtration in 50 mM Tris-HCl, pH 7.5, and up to 200 μM free Ca^{2+} revealed that N-CaVP does not bind Ca^{2+} . The Ca^{2+} -binding isotherm (Fig. 2), measured by flow dialysis, indicated that C-CaVP binds two Ca^{2+} with intrinsic binding constants of $4.6 \times 10^6 \text{ M}^{-1}$ and $7.4 \times 10^3 \text{ M}^{-1}$. These values are similar to those of entire CaVP (Petrova et al. 1995). The difference in the Ca^{2+} affinity of the two sites underlines the abundance and importance of the 1Ca^{2+} form under physiological conditions: the Ca^{2+} occupancy is 1.0 ± 0.1 , from 2 to 25 μM free Ca^{2+} (Fig. 2).

Conformational properties of N-CaVP and C-CaVP monitored by circular dichroism

The far-UV circular dichroism (CD) spectrum of N-CaVP is not influenced by Ca^{2+} and corresponds to an α -helical

Fig. 1. Amino acid sequence of CaVP including N-CaVP (arrow points to the last residue) and C-CaVP (arrow points to the first residue). EF-hand motifs are indicated in bold. Overlapping of both segments is emphasized in box. Relevant residues for this study are highlighted. Underlined lysines stand for TML. X, Y, and Z are the residues positioned at the vertices of the Ca^{2+} -coordinating bipyramid.

Fig. 2. Ca^{2+} binding to recombinant (open circles) and wild-type CaVP (closed circles), as well as C-CaVP (triangles). Ca^{2+} binding was measured by flow dialysis at 25°C in buffer C. The solid line represents the theoretical isotherm generated with the intrinsic binding constants 4.6×10^6 and $7.4 \times 10^3 \text{ M}^{-1}$ for K'_{Ca1} and K'_{Ca2} , respectively.

content of 42% (data not shown). In contrast, the CD spectrum of C-CaVP shows a pronounced change with Ca^{2+} binding with an α -helical increase from 17% to 36% (Thret et al. 2000). In comparison, the α -helical structure of entire CaVP increases from 23% to 34%.

The near-UV CD spectra of C-CaVP, N-CaVP, and CaVP, in the presence of 5 mM Ca^{2+} , are shown in Fig. 3A–C. The negative peaks at 262 and 269 nm are characteristic of Phe residues. The positive peaks at 284 and 291 nm, observed for N-CaVP and CaVP, are attributed to Tyr-47, Trp-74, and Trp-81 (see Fig. 1). The spectrum of C-CaVP is characteristic of a Phe-only protein, which indicates that Trp-81 is free and highly mobile. Heating of C-CaVP to 90°C produces only a slight decrease of intensity, suggesting that this half is very stable. N-CaVP displays only the Trp/Tyr peaks, which disappear with heating and reappear with cooling. Whole CaVP behaves as the sum of the individual halves (Fig. 3D). None of these spectra is influenced by removal of Ca^{2+} .

Because some EF-hand proteins, such as parvalbumin or *Nereis* sarcoplasmic Ca^{2+} -binding protein (NSCP), display extensive interactions between their amino and carboxyl domains, even after proteolytic separation (Permyakov et al. 1991; Durussel et al. 1993), we examined the additivity of optical properties of N-CaVP and C-CaVP. The far-UV CD and fluorescence spectra of an equimolar mixture of N-CaVP and C-CaVP, as well as the near-UV CD spectrum of CaVP (Fig. 3D), coincide with the sum of the components, indicating that the isolated halves do not influence each others secondary and tertiary structure. In addition, a mixture of the two polypeptides failed to produce band shifts or new bands on a native gel (data not shown). Thus, the recombinant halves of CaVP do not interact together, al-

though an interdomain interaction exists in the entire CaVP (see below).

Thermal stability of CaVP and its halves

CaVP, N-CaVP, and C-CaVP solutions heated up to 90°C showed nearly complete reversibility in CD spectra (Fig. 3). At 222 nm, CaVP shows a single broad transition with denaturation temperatures, T_d of 47°C for the metal-free and Ca^{2+} -loaded forms (Fig. 4A; see also Table 1 for a synopsis of the thermodynamic parameters). At 291 nm CaVP shows much sharper transitions with T_d values of 37.2°C and 33.4°C for the apo and Ca^{2+} forms, respectively. The melting curves of isolated N-CaVP also show a broad far-UV CD transition and a sharp near-UV CD transition with a T_d value of ~44°C (Fig. 4B). The isolated amino domain is thus stable, but the metal-free carboxyl domain in CaVP destabilizes the amino-terminal hydrophobic core (7°C decrease in T_d) and Ca^{2+} binding to the carboxy-domain accentuates this (11°C decrease). The 222 nm melting curve of Ca^{2+} -loaded C-CaVP did not display any transition (data not shown), whereas metal-free C-CaVP displays two transitions of about equal importance at 38.4°C and 68°C, respectively (Fig. 4C).

Because the three-dimensional structures of apo-, 1Ca^{2+} -, and 2Ca^{2+} -C-CaVP are different (Thret et al. 2000), the stabilities of the three states were compared for CaVP and C-CaVP by differential scanning calorimetry (DSC). Figure 5A shows that apo-CaVP produces only one heat absorption peak with a T_d of 37.5°C (same as in CD experiments; see Table 1). If heating was stopped right after the peak, the reversibility was complete. The ratio of calorimetric versus effective enthalpy for this peak approaches one, indicating a

Fig. 3. Near-UV CD spectra of C-CaVP (A), N-CaVP (B), and CaVP (C) at pH 7.5 in the presence of 5 mM $CaCl_2$ at different temperatures: 10°C (solid line), 90°C (dashed line), after heating to 90°C and cooling down to 10°C (dotted line). (D) Near-UV CD spectra of C-CaVP (dotted line), N-CaVP (dashed line), CaVP (solid line), and of the calculated sum of both segments (chain line).

simple two-state transition. $2Ca^{2+}$ -CaVP melts in two very distant steps with T_d of 31.5° and 117.9°C, respectively. $1Ca^{2+}$ -CaVP also melts in two steps; the low temperature peak is composed of two components with T_d of 28°C and 37°C, whereas the second step displays a unique T_d of 111.7°C. Isolated N-CaVP displays a single two-state transition with a T_d of 44.3°C (Fig. 5B), a value also found in CD experiments. The profile of apo-C-CaVP was flat without any clear melting transition (Fig. 5C). In contrast, $2Ca^{2+}$ -C-CaVP and $1Ca^{2+}$ -C-CaVP display single transitions with peaks at 123°C and 111.2°C, respectively. The denaturation peak of $1Ca^{2+}$ -C-CaVP is well described by a

two-state irreversible transition (data not shown) according to the model proposed in Sanchez-Ruiz et al. (1988). These data suggest that the low temperature transition for CaVP represents melting of the amino domain and that the high temperature transition corresponds to the carboxyl domain. The latter increases the amino domain melting sensitivity, which is progressively enhanced with binding of one and two Ca^{2+} . In the high temperature range, the T_d for CaVP and C-CaVP are similar: 111°C for the $1Ca^{2+}$ forms and 118°C and 123°C for $2Ca^{2+}$ -CaVP and $2Ca^{2+}$ -C-CaVP, respectively.

Fig. 4. Temperature dependence of the CD intensity for CaVP, N-CaVP, and C-CaVP at pH 7.5. (A) CaVP in the presence of 1 mM EGTA at 291 nm (thin dotted line) and 222 nm (thick dotted line) and in the presence of 2 mM $CaCl_2$ at 291 nm (thin solid line) and 222 nm (thick solid line). (B) N-CaVP at 222 nm (thick line) and 291 nm (thin line). (C) C-CaVP at 222 nm in the presence of 1 mM EGTA. The inset shows the first derivative of the CD melting curve.

Table 1. Thermal denaturation parameters of CaVP, C-CaVP, and N-CaVP at pH 7.5

Protein	DSC				CD	
	T _d ^a (°C)	ΔH _{cal} ^a (kcal/mol)	T _d ^b (°C)	ΔH _{cal} ^b (kcal/mol)	T _d at 222 nm (°C)	T _d at 291 nm (°C)
Apo CaVP	37.5	45	—	—	47	37.2
1Ca ²⁺ · CaVP	28/37 ^c	26	111.7	na	nd	nd
2Ca ²⁺ · CaVP	31.5	18	117.9	na	47	33.4
Apo C-CaVP	—	—	—	—	38.4/68	na
1Ca ²⁺ · C-CaVP	—	—	111.2	53	nd	na
2Ca ²⁺ · C-CaVP	—	—	123.0	72 ^d	—	na
N-CaVP	44.3	53	—	—	43.9	44.6

(—) no transition; (nd) not done; (na) not applicable.

^a N-domain.^b C-domain.^c Temperatures were obtained from computer deconvolution of the transition excess heat capacity into two non-two-state transitions.^d Enthalpy value was calculated assuming that the extent of asymmetry of peaks for irreversible two-state transitions is near to 1.7 (Potekhin et al. 1999).

Interaction between CaVP moieties and CaVPT

Complex formation between the CaVP halves and CaVPT was analyzed using three methods. Native PAGE of equimolar mixtures of CaVPT with either N-CaVP, C-CaVP, or CaVP in the presence of Ca²⁺ demonstrated the appearance of a single new band in the C-CaVP/CaVPT and CaVP/CaVPT mixtures, which likely corresponds to a 1:1 complex (arrows in Fig. 6A, top). Complex formation was confirmed by cross-linking experiments as described by Durussel et al. (1993) (data not shown). Both in the presence and absence of Ca²⁺, a 1:1 complex was formed between CaVPT and C-CaVP or CaVP (of 40 and 50 kD, respectively), whereas no covalent adduct was formed with N-CaVP. The band intensities were stronger in the presence of Ca²⁺, suggestive of more efficient cross-linking. Finally, the gel filtration profile of an equimolar mixture of C-CaVP and CaVPT displays a single peak; SDS-PAGE shows that a 1:1 complex is formed (Fig. 6B, a). Addition of EDTA leads to the complex dissociation (Fig. 6B, b). A competition experiment with an equimolar mixture of CaVP, C-CaVP, and CaVPT suggests that the affinity of C-CaVP for CaVPT is weaker than the one of CaVP (Fig. 6B, d). In the presence of Ca²⁺, C-CaVP alone elutes at two elution volumes, corresponding to apparent molecular masses of 15 and 30 kD (Fig. 6B, c). Nevertheless, the comparison of the profiles of Figure 6, a and 6, c demonstrates the formation of a complex between C-CaVP and CaVPT. Under the same conditions, N-CaVP elutes alone with an apparent molecular mass of 10 kD (data not shown).

Discussion

This study provides a detailed analysis of the domain structure of CaVP, that is the Ca²⁺- and CaVPT-binding domain,

as well as the domain cross talk are characterized. For this purpose, recombinant CaVP was expressed and purified. We demonstrate here that two TML groups, which are present on wild-type CaVP, but absent on the recombinant protein, do not affect Ca²⁺ binding, conformational changes or interaction with CaVPT. A similar conclusion was reached previously in the case of CaM, which possesses one TML that may protect CaM against ubiquitin-dependent proteolysis (Gregori et al. 1985).

CaVP binds only two Ca²⁺ with its carboxyl domain and the isolated carboxy-terminal half binds two Ca²⁺ with the same binding pattern as whole CaVP with a high and a low affinity site, indicating that the amino-domain does not modify cation binding. Our DSC results also demonstrate

Fig. 5. Temperature dependence of the partial molar heat capacity of CaVP, N-CaVP, and C-CaVP at pH 7.5. (A) apo form of CaVP in the presence of 0.1 mM EGTA (dotted line), 1Ca²⁺ form in 0.1 mM CaCl₂ (solid line), and 2Ca²⁺ form in 2 mM CaCl₂ (dashed line). The inset shows the temperature dependence of the excess heat capacity for CaVP low temperature peak. (B) N-CaVP in the presence of 5 mM CaCl₂. (C) C-CaVP in the presence of 0.1 mM EGTA (dotted line), 0.1 mM CaCl₂ (solid line), and 2 mM CaCl₂ (dashed line).

Fig. 6. Interaction analysis of N-CaVP, C-CaVP, and CaVP with CaVPT. (A) N-CaVP, C-CaVP, recombinant CaVP, and an equimolar mixture of N-CaVP, C-CaVP, or recombinant CaVP with CaVPT, in the presence of 2 M urea, were microdialyzed 60 min against buffer A containing 100 mM NaCl plus 1 mM CaCl_2 , before loading on a native gel (12.5% acrylamide) in the presence of 10% glycerol. All samples contain 2.5 μg of each protein. Arrows indicate the mobility of the complex formed in the presence of Ca^{2+} . Recombinant CaVP represents the positive control (same results with wild-type CaVP). This protein migrates in three bands. (B) Samples were chromatographed on a size-exclusion S-200 column under the same conditions as standards of known molecular masses (top), and analyzed by SDS-PAGE (15% acrylamide). An equimolar mixture of either C-CaVP and CaVPT (a), or of C-CaVP, CaVP, and CaVPT (d) was dialyzed against buffer A plus 100 mM NaCl and 1 mM CaCl_2 . Addition of 2 mM EDTA dissociates the C-CaVP/CaVPT complex (b). C-CaVP alone has a tendency to dimerize in 1 mM CaCl_2 (c). At left, the positions of SDS-PAGE molecular markers are indicated (kD).

that the carboxy-terminal domain in intact CaVP has similar Ca^{2+} -dependent behavior as C-CaVP. Analysis of 1Ca^{2+} -C-CaVP by nuclear magnetic resonance (NMR) indicates that site III binds the first Ca^{2+} ion and adopts a well-defined structure, whereas site IV remains highly fluctuating (Thret et al. 2000). In 2Ca^{2+} -C-CaVP, both sites are well-structured and show a similar folding as the Ca^{2+} -saturated carboxy-terminal half of CaM. Our data show that the isolated amino- and carboxy-terminal moieties of CaVP are independent folding units with well-defined secondary and tertiary structures and that only the carboxy-terminal segment displays pronounced Ca^{2+} -dependent conformational changes. N-CaVP contains as much α -helical structure

(~50%) as any functional EF-hand motifs, and possesses a well-formed hydrophobic core. The difference in the thermal stabilities of N-CaVP and C-CaVP (melting at 44C and 123C, respectively) is also valid for entire CaVP, which allowed us to identify the unfolding of each domain. These characteristics have not been observed in CaM or TnC and may impart a unique physiological significance.

Whereas the Ca^{2+} -loaded forms of CaVP and C-CaVP are well structured, the carboxyl domain in both apo-CaVP and apo-C-CaVP displays the characteristics of a molten globule state (Ptitsyn et al. 1990): (1) the lack of a tertiary structure, as DSC experiments do not yield any partial molar heat capacity (C_p) signal change for this domain in the 10–120C range; (2) the presence of an α -helical structure (but with a content much lower than in the Ca^{2+} -loaded forms). This α -helical structure is likely fluctuating along the polypeptide sequence, as it is not observed for C-CaVP by NMR (Thret et al. 2000). The far-UV CD signal shows a biphasic melting curve, suggesting that two groups of helical conformations with different stabilities are dominant in the apo-C-CaVP equilibria. Because NMR of apo-C-CaVP indicated no persistent dipolar interactions (Thret et al. 2000), we assume that its side chains are highly mobile and average out the nuclear Overhauser effect (NOE) signals, as in a state close to the molten globule (Ptitsyn et al. 1990). Recently, an increasing number of structurally disordered proteins in nondenaturing conditions have been described and the biological advantages for induced folding in molecular recognition discussed (for review, see Wright and Dyson 1999).

The absence of interaction between isolated CaVP halves suggests that they can interact with targets, as do CaM or TnC. However, the carboxyl domain of CaVP affects the thermal stability of the amino domain, which implies a crosstalk. Interestingly, the interdomain interaction is at the level of the tertiary structure. The melting temperatures for apo-CaVP and 2Ca^{2+} -CaVP in far-UV CD coincide, whereas near UV-CD and DSC melting temperatures for the amino domain in these two states are different. N-CaVP has a T_d of 44C. The first transition in CaVP, which is attributed to the amino domain, occurs at 37.5C in the metal-free form and at 31.5C in the 2Ca^{2+} form. For the 1Ca^{2+} form, the amino domain shows biphasic thermal stability with one component similar to the metal-free state, and one close to the value of the 2Ca^{2+} state. These data clearly show that the carboxyl domain, and its degree of Ca^{2+} occupancy, modulates the stability of the amino domain. Because our data provide evidence that in intact apo-CaVP, the carboxy-terminal half also possesses a molten globule-like conformation, it may be postulated that this fluctuating structure has a lower destabilizing capacity than a well-formed Ca^{2+} saturated EF-hand pair. A crosstalk also occurs from the amino to the carboxyl domain, as the Ca^{2+} -saturated carboxyl domain of CaVP melts 5C earlier than C-CaVP. The thermal

destabilization of an independent folding unit has also been described for CaM: the interdomain interaction is more pronounced in the metal-free than in the Ca^{2+} -bound state (Protasevich et al. 1997; Sorensen and Shea 1998).

In this study, we showed that C-CaVP binds to CaVPT, whereas N-CaVP does not form a complex. Similar to full-length CaVP (Petrova et al. 1995), the C-CaVP/CaVPT complex is more stable in the presence of Ca^{2+} . Although no complex was detected in dissociating methods such as native PAGE and gel filtration chromatography, some cross-linked complex can be detected even in the absence of Ca^{2+} . A comparison can be made with the complex formation between CaM and its target peptide in myosin light chain kinase. In the absence of Ca^{2+} , the carboxy-terminal lobe of CaM interacts with the amino-terminal part of the target peptide (K_d in the micromolar range; Tsvetkov et al. 1999). In the presence of Ca^{2+} , the complex is strengthened by additional interaction of the amino-terminal lobe of CaM with the carboxyl-end of the peptide. Likewise, the carboxy-terminal domain of CaVP binds to CaVPT in vitro even in the presence of EDTA and our data suggest that the binding affinity of C-CaVP for CaVPT is weaker than the one of CaVP. We suggest a model where CaVP binds to CaVPT in a sequential process. In a first step the carboxy-terminal domain binds to CaVPT. This brings the amino-terminal domain close enough to also bind CaVPT. This last step could lead to a subsequent increase in the binding affinity.

Materials and methods

Cloning and expression of whole CaVP and its moieties

Total RNA of adult *Branchiostoma lanceolatum* was prepared according to the acid guanidium thiocyanate method (Chomczynski and Sacchi 1987), and the mRNA was purified with an Oligotex dT-30 Super (Japan Roche). The single-stranded cDNA was synthesized using a First-Strand cDNA Synthesis Kit (Pharmacia). The redundant oligomer used for cDNA amplification by polymerase chain reaction (PCR) was 5'-GARGARAARGAYGAR-TGY-ATGAAR-3' (where R represents A or G, and Y represents C or T), a design based on the amino acid sequence EEKDECMK (residues 11–18) of *B. lanceolatum* CaVP. The oligo(dT) adaptor, 5'-GGGATCCGAATTCT₁₇-3', was used as another primer. The 5' upstream of cDNA was determined as follows: the *Eco*RI-ended double-stranded cDNA was synthesized from mRNA using Time-Saver cDNA Synthesis Kit (Pharmacia). The *Eco*RI Cassette (Takara) was ligated at each end of cDNA. The 5' upstream region was amplified with PCR using the cassette-specific primer C1, 5'-GTACATATTGTCGTTAGAACGCG-3' and 5'-CGAAGACAACACAGACTTTATTAC-3' (complementary to the nucleotides position 1054–1078).

Recombinant proteins were overproduced using a vector under the control of T7 RNA polymerase. Open reading frames were inserted into the vector pET24a (Novagen) using both *Nde*I (with the 5'-CATATG-3' sequence at the 5' end) and *Hind*III (with 5'-AAGCTTA-3' at the 3' end) restriction sites. The vector-borne Shine-Dalgarno sequence was used for proper mRNA translation

in *E. coli*. Cloning was done in strain NM554/pDIA17 (Raleigh et al. 1988). BL21(DE3)/pDIA17 strains were transformed with the plasmids pHSP312, pHSP272, pHSP315, coding for entire CaVP, N-CaVP, or C-CaVP, respectively. Cells were grown at 37°C in a twofold concentrated 2YT medium containing appropriated antibiotics, and induced with IPTG for 3 h.

Protein purification

E. coli cultures were resuspended with 20 mM Tris-HCl at pH 7.5, 1 mM dithiothreitol (DTT), 40 μM phenylmethane-sulfonyl fluoride (PMSF), and 0.2 g/mL pepstatin (buffer A), containing 10 μM CaCl_2 and 0.1 mM of diisopropyl fluorophosphate. After sonication, the suspension was centrifuged and the pellet was reextracted. The supernatants of CaVP or C-CaVP were loaded on an ion exchange DEAE-cellulose column and eluted with a 0–350 mM NaCl gradient. CaVP- or C-CaVP-containing fractions were applied on a second DEAE-cellulose column equilibrated with buffer A plus 1 mM EDTA and 50 mM NaCl, and eluted with a 50–300 mM NaCl gradient. Proteins were chromatographed through Sephadex G-75 equilibrated in 20 mM HEPES at pH 7.5, 1 mM DTT, 40 μM PMSF, 0.2 $\mu\text{g/mL}$ pepstatin (buffer B) plus 100 μM CaCl_2 . N-CaVP was purified by QAE ion exchange chromatography with a 0–250 mM NaCl gradient. Wild-type CaVP and CaVPT were purified as previously described (Petrova et al. 1995). The concentrations of CaVP, N-CaVP, C-CaVP, and CaVPT were determined by UV spectrophotometry, using extinction coefficients at 278 nm of 13,700, 12,660, 5,690 and 26,600 $\text{M}^{-1}\text{cm}^{-1}$, respectively.

Complex formation and analysis

Complexes between CaVPT and either N-CaVP, C-CaVP, or CaVP were formed and analyzed as previously described (Petrova et al. 1995).

Ca^{2+} removal and Ca^{2+} binding

Contaminating Ca^{2+} was removed as previously described (Cox 1996). The contamination was <0.05 Ca^{2+} /protein. Ca^{2+} -binding measurements were carried out by flow dialysis as previously described (Cox 1996).

Circular dichroism

CD spectra were acquired with a Jasco J-715 spectropolarimeter equipped with thermostated water-jacketed cells and Neslab RTE-111 programmable bath. Far-UV CD was performed in a 1-mm cell on 0.25 mg/mL protein in 5 mM Tris-HCl at pH 7.5 and in a 0.2-mm cell on 0.3–0.4 mg/mL protein in 50 mM cacodylate at pH 7.5; near-UV CD was done in a 10-mm cell on 1–1.3 mg/mL protein in 50 mM cacodylate at pH 7.5. Ellipticities were normalized to residue concentration using $\theta_{\text{MRW}} = \theta_o M_r / l c$ (θ_o = observed ellipticity in millidegrees; M_r = average molecular weight of an amino acid in the protein [i.e., 113.2, 113.6, 114.7 for CaVP, N-CaVP, and C-CaVP, respectively]; l = path length in millimeters; c = protein concentration in grams per liter). The results were also expressed as molar circular dichroic absorption using $\Delta\epsilon = \theta_o / 3,300 l c$ (c = molar protein concentration). The secondary structure content was evaluated according to Johnson (1999). Continuous CD melting experiments were performed at a heating rate of 1 K/min. Denaturation temperatures were determined from

the peak in the first derivatives of the melting profiles (accuracy 0.5°C).

Differential scanning calorimetry

Microcalorimetric measurements were carried out on a MicroCal VP-DSC instrument in 0.51 mL of cells and on a DACM-4 microcalorimeter (NPO Biopribor, Pushchino, Russia) in 0.48 mL of cells at a heating rate of 1 K/min on 1.3 mg/mL CaVP, 0.6–1.7 mg/mL N-CaVP and 0.9 mg/mL C-CaVP in 50 mM cacodylate at pH 7.5. The apo, 1Ca^{2+} , and 2Ca^{2+} states were obtained by dialysis of CaVP and C-CaVP against the buffer containing 0.1 mM EGTA, 0.1 mM Ca^{2+} or 2 mM Ca^{2+} , respectively. Curves were corrected for the instrumental baseline obtained by heating the solvent used for protein solution. The reversibility of denaturation was checked routinely by sample reheating after cooling in the calorimetric cell. The partial molar heat capacity of the protein (C_p), denaturation temperature (T_d), calorimetric denaturation enthalpy (ΔH_{cal}) and effective or van't Hoff denaturation enthalpy (ΔH_{eff}) were determined as described elsewhere (Privalov and Potekhin 1986), with the partial specific volume of $0.73\text{ cm}^3\text{g}^{-1}$ calculated according to Makhatadze et al. (1990). To analyze functions of excess heat capacity, the MicroCal Origin (4.1) software was used. The accuracy of the calorimetric and effective enthalpies was 8%, that of T_d within 0.2°C.

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