Thermodynamic and Molecular Properties of the Interaction between Amphioxus Calcium Vector Protein and Its 26 kDa Target†

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ABSTRACT: Calcium vector protein (CaVP) of amphioxus shares some common structural features with Ca^{2+}-dependent activators such as troponin C and calmodulin, and is associated *in vivo* with a 26 kDa (CaVPT), a multidomain protein with one IQ- and two IgII-motifs. Isolated CaVP binds two Ca^{2+} ions with very different intrinsic affinity constants: $K'_{Ca1} = 4.9 \times 10^6\ M^{-1}$ and $K'_{Ca2} = 7.3 \times 10^3\ M^{-1}$, respectively. In the complex with CaVPT, CaVP also binds two Ca^{2+}, but with strong positive cooperativity ($n_H = 1.9$) and with distinctly higher affinity: $K'_{Ca1} = 2.4 \times 10^5\ M^{-1}$ and $K'_{Ca2} = 1.0 \times 10^8\ M^{-1}$. Since neither in the isolated CaVP nor in the complex Ca^{2+} binding is influenced by 2 mM $MgCl_2$, both sites can be considered as Ca^{2+}-specific. In the absence of Ca^{2+}, the complex is stable under physiogical conditions, but the interaction is governed by the principle of linked functions and Ca^{2+} binding to CaVP reinforces the affinity between CaVP and CaVPT 70-fold. Both proteins interact with the hydrophobic probe 2 *p*-toluidinylnaphthalene-6-sulfonate (TNS), but CaVPT enhances the fluoresence 45-fold, CaVP•Ca_2 and metal-free CaVP only 10- and 5-fold, respectively. Complex formation between CaVPT and CaVP leads to a 3-fold reduction of the fluorescence enhancement, suggesting that a strong solvent-shielded hydrophobic core is formed. CaVP contains two highly reactional thiols ($k_{SH} > 0.3\ s^{-1}$) for 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB); CaVPT contains three thiols, two of them also with $k_{SH} > 0.3\ s^{-1}$ in the native state. The buried thiol could be identified as Cys132. In the CaVP−CaVPT complex Cys132 remains inaccessible, but the reactivities of the other four thiols are dramatically reduced with $k_{SH} = 7.7 \times 10^{-3}\ s^{-1}$ for the two thiols of CaVP and $5.8 \times 10^{-4}\ s^{-1}$ for those of CaVPT. Interestingly, Ca^{2+} binding does not change the conformation of the complex, as probed with TNS and DTNB. In conclusion, the complex is strengthened by Ca^{2+} binding, but not strictly Ca^{2+}-dependent; whereas isolated CaVPT and CaVP show the characteristics of highly dynamic, interactive proteins, complex formation leads to a rigid structure with a very stable hydrophobic core, likely yielding a noninteractive end product.

In 1986, a new Ca^{2+}-binding protein, CaVP[1] (calcium vector protein), was described in the muscles of the higher invertebrate *Branchiostoma lanceolatum*, commonly called amphioxus or lancet fish (Cox, 1986). The protein is abundant in muscle (several hundred micromolar in terms of cellular volume) and occurs also in other organs such as the spinal chord and gonads (Valette-Talbi *et al.*, 1993). *In vitro*, nearly half of it is engaged in a 1 to 1 complex with a 26-kDa protein named CaVPT (Cox, 1986), whose cell concentrations are about half of those of CaVP (Valette-Talbi *et al.*, 1993). Sequence analysis reveals homology of CaVP with well-known Ca^{2+}-binding proteins, especially with calmodulin (CaM) and troponin C (TnC) (Kobayashi *et al.*, 1987), but functional assays indicate that this protein does not substitute for the latter proteins. Moreover, *bona fide* CaM and TnC (as well as the other subunits of a classical troponin) have been detected in amphioxus (Lehman & Szent-Györgyi, 1975; Takagi *et al.*, 1994). Sequence comparison revealed that CaVPT is a unique protein (Takagi & Cox, 1990), although it contains 1 IQ-motif for the binding of CaVP followed by 2 100-residue repeats similar to the C2 group of immunoglobulins (IgII-folds). The same type of repeat, often in numerous copies, is present in myosin light chain kinase (Olson *et al.*, 1990), telokin (Ito *et al.*, 1989), C-protein (Einheber & Fischman, 1990; Fürst *et al.*, 1992), twichin (Benian *et al.*, 1989), and titin (Labeit *et al.*, 1992). The IQ-motif, usually containing 23−25 residues with a consensus sequence IQxxxRGxxxR, has been detected in neuromodulin (LaBate & Skene, 1989) and neurogranin (Baudier *et al.*, 1990), where it was associated with Ca^{2+}-independent binding of calmodulin. Two less conserved IQ-motifs were found in scallop myosin heavy chain. They were identified as the binding sites for myosin light chains (Xie *et al.*, 1994). The screening of rat tissues with the antibodies against CaVP and CaVPT did not reveal a positive reaction, suggesting that these proteins are structurally not conserved or are absent in higher vertebrates. The function of CaVP−CaVPT is presently not known, but may be restricted to a regulatory role in invertebrates (unpublished data from our laboratory). Their abundance in amphioxus points to a major

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[1] Abbreviations: CaVP, calcium vector protein; CaVPT, calcium vector protein target; CaM, calmodulin; TnC, troponin C; TNS, probe 2-*p*-toluidinylnaphthalene-6-sulfonate; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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regulatory role, not in enzyme catalysis but in motility or cytoskeleton dynamics. Biophysical studies on CaM- and TnC-regulated systems have illustrated the complexity and high efficiency of these switch mechanisms. In contrast, very little is known on the nature of the Ca^{2+}−CaVP−CaVPT complex.

As part of our project to elucidate the functional importance of this system, we here address the molecular mechanism of this regulatory process: we report the Ca^{2+}-binding properties of isolated CaVP and of the complex CaVP−CaVPT, as well as the changes in the microenvironment of the Cys residues in both proteins. Finally, we monitored the interaction of the fluorescent hydrophobic probe TNS with CaVPT, CaVP, and their complex, as well as the influence of Ca^{2+} on these conformational changes.

MATERIALS AND METHODS

Purification of the Complex CaVP−CaVPT, CaVP, and CaVPT. About 70 g of frozen amphioxus was homogenized with a Sorvall Omnimixer in 200 mL of 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, 5 μM $CaCl_2$, 0.1 mg/L pepstatin A, 5 mg/L leupeptin, 70 mg/L phenylmethanesulfonyl fluoride, and 0.1 mM diisopropyl fluorophosphate (buffer A). The pellet was separated by centrifugation for 40 min at 30000*g*, then the extraction procedure was repeated twice. The resulting extracts were combined and dialyzed overnight against buffer A without NaCl. The precipitate was removed by centrifugation at 6000*g*. The supernatant was loaded on a DEAE column (2.9 × 20 cm). Proteins were eluted with a linear gradient of 0−300 mM NaCl. The fractions containing the complex CaVP−CaVPT (according to the SDS−PAGE and Ca^{2+} content) were concentrated with Centriprep-10 (Amicon) and subjected to gel filtration on a Sephacryl S-200 column (2.7 × 150 cm) equilibrated in buffer A + 100 mM NaCl. Fractions containing the complex were collected and stored at −20 °C. To obtain the individual components, the complex was dissociated by the addition of 2 M urea and 1 mM EDTA and loaded on a DEAE column (2.7 × 13 cm) equilibrated in the same buffer. After removal of CaVPT, which did not bind to the resin, the column was washed with buffer A without urea. CaVP was eluted with a linear gradient of 50−350 mM NaCl. The concentration of CaVP, CaVPT, and CaVP−CaVPT complex was determined by UV spectrophotometry, using molar extinction coefficients of 13 700, 26 600, and 40 300 M^{-1} cm^{-1}, respectively.

Reconstitution of the Complex CaVP−CaVPT. Proteins (CaVP in buffer A, CaVPT in buffer A + 2 M urea) were mixed at a molar ratio of 1:1, dialyzed overnight against buffer A + 100 mM NaCl/1 mM $CaCl_2$, and concentrated with Centriprep-10. The complex was separated from the free components by gel filtration on a Sephacryl S-200 column (1.2 × 132 cm) equilibrated in buffer A + 100 mM NaCl. A similar experiment was done in the presence of 1 mM EDTA.

Calcium Determination and Calcium Removal. The Ca^{2+} concentration was determined with a Perkin-Elmer Cetus Instruments 2380 atomic absorption spectrophotometer. For removal of contaminating metals, CaVP and CaVP−CaVPT complex were dialyzed overnight against buffer A containing 1 mM EDTA, then concentrated with Centriprep-10, and passed through a 40 × 1 cm Sephadex G-25 column equilibrated in the assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM KCl). As an alternative method used for CaVP alone, the protein was precipitated with 3% trichloroacetic acid in the presence of 1 mM EDTA, followed by gel filtration on Sephadex G-25. The assay buffer was freed of contaminating metals by passage over an EDTA−Sepharose column (Haner *et al.*, 1984).

Calcium Binding. Ca^{2+}-binding to the complex and CaVP was measured at 25 °C by the flow dialysis method of Colowick and Womack (1969) using the automatic delivery station Microlab 2200 (Hamilton) and an improved computer program for data treatment (Dr. Schwendimann of our department). Protein concentration was 16−40 μM in 50 mM Tris-HCl (pH 7.5)/150 mM KCl (assay buffer). The Ca^{2+}-binding data were analyzed by means of the Adair equation (Adair, 1925) for two binding sites. Stoichiometric binding constants were calculated using the SigmaPlot (Jandel Scientific) curve-fitting program. Intrinsic binding constants, differing from the stoichiometric constants by the statistical factors 1/2 and 2 (Cornish-Bowden & Koshland, 1975), were used throughout the study. Some of the Ca^{2+}-binding parameters were occasionally confirmed by the equilibrium gel filtration method of Hummel and Dryer (1962). A Sephadex G-25 column (0.8 × 36 cm) was equilibrated in the assay buffer containing 5 μM $CaCl_2$; 0.8 mL of 40−70 mM metal-free CaVP or complex was applied to the column. In the eluant, Ca^{2+} and protein concentrations were determined by atomic and ultraviolet absorption, respectively.

Interaction with 2-p-Toluidinylnaphthalene-6-sulfonate (TNS). Fluorescence spectra were recorded with a Perkin-Elmer LS-5B spectrofluorimeter interfaced to a computer. Measurements were carried out as described by McClure and Edelman (1966) on 5−10 μM protein solutions, containing 1 μM TNS, with the excitation wavelength of 328 nm and slits set at 5 nm; 500 μM EDTA and 1 mM $CaCl_2$ were added to obtain metal-free and Ca^{2+}-loaded forms, respectively.

Thiol Titration. The reactivity of thiol groups was monitored with 5,5′-dithiobis(2-nitrobenzoic acid) as described by Riddles *et al.* (1983). Beforehand, protective DTT was removed and proteins were equilibrated in the assay buffer [50 mM Tris-HCl (pH 7.5)/150 mM KCl] by Sephadex G25 chromatography. All solutions were freed of dissolved oxygen by flushing them with pure nitrogen; 1 mM $CaCl_2$, 1 mM EDTA, or 4 M guanidine hydrochloride was added to obtain Ca^{2+}-loaded, Ca^{2+}-free, or the denaturated form, respectively. The protein concentration was 5−15 μM, and that of DTNB 1 mM. The concentration of free thiol groups was determined using molar extinction coefficients of 14 200 and 13 700 M^{-1} cm^{-1} (in guanidine hydrochloride containing solutions) at 412 nm. The reaction rate constants were calculated using the Guggenheim equation (Cornish-Bowden, 1976) for pseudo-first-order reactions and a SigmaPlot curve-fitting program (Jandel Scientific).

To identify the location of reactive thiols, DTNB was added to the CaVP−CaVPT complex (25 μM in assay buffer) to a final concentration of 1 mM and allowed to react for 5 min. The pH was adjusted to 3.5, the precipitated DTNB was removed by centrifugation for 3 min, and the supernatant was chromatographed on a Sephadex G-25 column equilibrated in 20 mM acetic acid. The protein-containing fraction was loaded on a column of DEAE-cellulose (0.8 × 2 cm)

equilibrated in 20 mM Tris-HCl, pH 7.5, 6 M urea, and 1 mM EDTA. The column was washed by several volumes of the same buffer; then CaVP and CaVPT were differentially eluted with a linear 0−300 mM gradient of NaCl, as monitored by SDS−PAGE. In the CaVP- and CaVPT-containing fractions, covalently bound TNB was dissociated from the protein by the addition of 1 mM DTT, and, from the resulting absorbance at 412 nm, the total concentration of modified thiols was calculated. The protein concentration was determined by the method of Bradford (1976) using standard solutions of nonmodified CaVP and CaVPT, respectively.

Identification of the Buried Cysteine Residue in CaVPT. To localize the buried cysteine residue, native CaVPT was allowed to react with 1 mM DTNB for 20 min. After removal of excess reagent by gel filtration, 10 mM iodoacetamide and 4 M guanidine hydrochloride were added to the protein solution in order to block the unmasked cysteine residue. The alkylation was allowed to proceed for 20 min and was stopped by the addition of 1 M DTT, which also displaced the TNB group from the cysteine residues previously substituted with DTNB in the native protein. After dialysis against water and lyophilization, 0.2 μg of the covalently modified CaVPT was dissolved in 0.2 mL of 6 M guanidine hydrochloride containing 0.5 M Tris-HCl, pH 8.5, and 10 mM EDTA. It was reduced with 5 μL of 1 M DTT and then pyridylethylated with 2 μL of 4-vinylpyridine. Excess reagents were removed by dialysis against water, then the sample was dried with a Speedvac. It was dissolved in 50 μL of 0.1 M ammonium bicarbonate, pH 8.5, and digested with 4 mg of TPCK-treated trypsin at 37 °C for 18 h. Peptides were separated with HPLC on a column of Toso ODS 80TM (4.6 × 100 mm) equilibrated in 0.1% trifluoroacetic acid with a linear gradient of acetonitrile. Absorbances were monitored at 215 and 280 nm. Amino acid sequences of cysteine-containing peptides were determined with an automated sequencer (Applied Biosystem Model 477 A on-line with Model 120A PTH-analyzer).

RESULTS AND DISCUSSION

Sequence similarity and common physical properties with TnC and CaM, such as asymmetric shape, interaction with hydrophobic matrices, and amphiphilic peptides (Cox, 1986), indicate that CaVP belongs to the family of Ca^{2+}-dependent activators. However, this protein does not activate target enzymes of CaM or actomyosin ATPase. *In vivo*, the role of CaVP seems to be confined to its association with CaVPT, although *in vitro* the interaction with troponin I has been observed (T.V.P. and J.A.C., manuscript in preparation). CaVPT is a multidomain protein which can also interact with other protein components, but not in the presence of CaVP (unpublished observations). The description of the molecular functioning of the very selective interaction between CaVP and CaVPT can help to delineate the role of these two abundant proteins in amphioxus tissues and may provide us clues as to the regulation of this protein complex.

Complex Reconstitution. Whereas the complex can be purified under nondenaturing conditions, the purification of isolated CaVP and CaVPT necessitates dissociating conditions such as treatment with 2 M urea. The question then arises whether the latter protocol leads to correct protein refolding. Therefore, reconstitution of the complex was assayed by mixing equimolar amounts of CaVP and CaVPT in the presence of urea and removal of urea by dialysis. Upon gel-filtration in the presence of Ca^{2+} (Figure 1), the complex migrates with an apparent molecular mass of 63−67 kDa, a value also obtained for the native complex and indicative of its asymmetric shape (the calculated molecular mass is 44 kDa). The recovery of the reconstituted complex, as judged from the amount of nonreacting CaVP in Figure 1, is 80−90%. The fraction of CaVPT which did not react with CaVP likely represents the disulfide bridge-linked homodimer of CaVPT (void fractions in Figure 1). A CaVP−CaVPT complex with the same apparent molecular mass is also formed in the absence of Ca^{2+} (data not shown). Since also no differences were observed in Ca^{2+} binding, thiol reactivity, and interaction with TNS (see below) between the reconstituted and native complex, it can be concluded that our dissociation protocol involving urea treatment does not irreversibly alter the interactive properties of the two proteins.

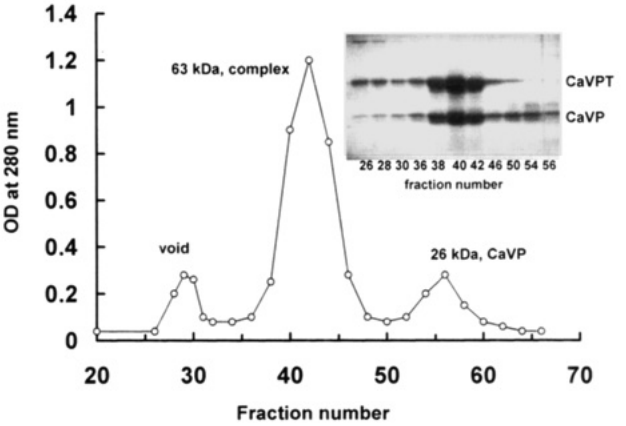

FIGURE 1: Reconstitution of the complex CaVP−CaVPT. An equimolar mixture of CaVP and CaVPT containing 2 M urea was dialyzed overnight against buffer A + 100 mM NaCl. The sample was submitted to gel filtration on Sephacryl S-200, and the proteins in the indicated fractions were identified by SDS−PAGE on 12.5% acrylamide gels (inset).

Calcium Binding. CaVP contains four putative EF-hand motifs (Kretsinger, 1987), but binds only 2 mol of Ca^{2+}/mol of protein. It was predicted earlier that sites I and II, situated in the N-terminal part of the molecule, would be nonfunctional due to deleterious mutations in the Ca^{2+}-coordinating residues (Kobayashi *et al.*, 1987). Detailed Ca^{2+}-binding studies by flow dialysis (Figure 2) show that the two sites have very different affinities. Analysis according to the Adair equation (Adair, 1925) yields intrinsic binding constants (K') of 4.9×10^6 and 7.3×10^3 M^{-1}. The presence of 2 mM $MgCl_2$ does not influence Ca^{2+} binding to CaVP, confirming that the sites are Ca^{2+}-specific. CaVP thus differs markedly from the corresponding C-terminal sites in TnC, which were described as mixed Ca^{2+}/Mg^{2+} ones with equally high affinty for Ca^{2+} (Potter & Gergely, 1975), and from the corresponding sites of CaM, which are of the low-affinity, Ca^{2+}-specific type (Milos *et al.*, 1985). Since, according to their sequence, the EF-hands III and IV of CaVP possess full Ca^{2+}-binding potency and display a high degree of homology to the corresponding sites in CaM—about 50% for site III and 35−40% for site IV [see Cox *et al.* (1990)]—it is difficult to identify directly which site binds Ca^{2+} with lower affinity. Nevertheless, one can argue that site IV bears more nonconservative replacements than site III. The most unusual of them is a proline residue at the beginning of

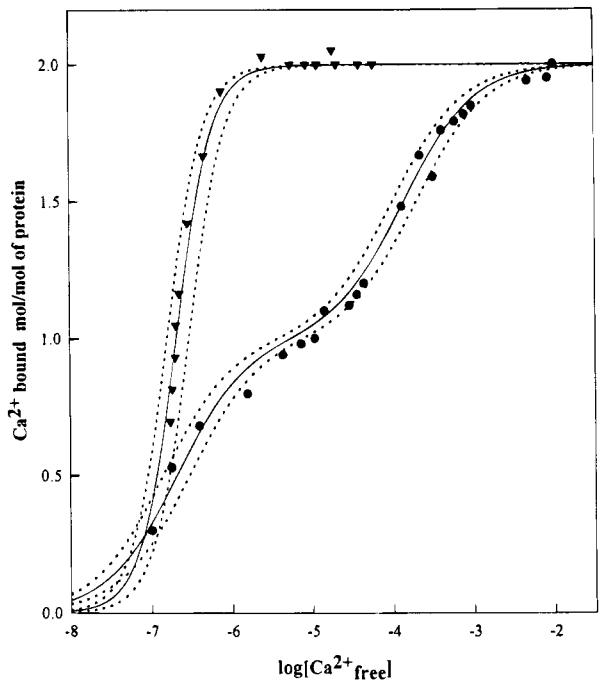

FIGURE 2: Ca^{2+} binding to CaVP (●) and CaVP−CaVPT (▼). Solid lines represent theoretical isotherms generated with the following intrinsic binding constants (K'_{Ca1} and K'_{Ca2}): for CaVP, $(4.9 \pm 0.7) \times 10^6$ and $(7.3 \pm 1.2) \times 10^3$ M^{-1}; for CaVP−CaVPT, $(2.4 \pm 0.4) \times 10^5$ and $(1.0 \pm 0.2) \times 10^8$ M^{-1}. The dotted lines delineate the confidence zone calculated with the maximal and minimal K' values, respectively.

H-helix next to the glutamate at position -Z. The disruption of this α-helix can influence the Ca^{2+}-coordination properties of this ligand and decrease the affinity of site IV.

The complex CaVP−CaVPT binds two Ca^{2+} ions with log $[Ca^{2+}]_{0.5} = -6.85$ (Figure 2). The binding isotherm shows almost complete positive cooperativity with a Hill coefficient (n_H) of 1.91 and with intrinsic binding constants of 2.4×10^5 and 1.0×10^8 M^{-1}. The profound change in the Ca^{2+}-binding characteristics of CaVP upon complex formation with CaVPT was confirmed in equilibrium gel-filtration experiments: in the presence of 5 μM $CaCl_2$, isolated CaVP fixed 1 mol of Ca^{2+}, whereas two sites were occupied in the complex. Our data highlight two particular features: (1) While isolated CaVP possesses high- and low-affinity sites, which seem to be independent, in the complex the first Ca^{2+} binds to a low-affinity site, followed by an allosteric rearrangement with a strong increase of affinity for the second Ca^{2+}. At present, these data do not allow us to determine if a sequential binding mechanism (Fletcher *et al.*, 1970) occurs. Strong positive cooperativity in Ca^{2+} bindng results in narrowing of the Ca^{2+} gradient necessary to provoke the transition from the Ca^{2+}-free of the Ca^{2+}-loaded protein and creates, therefore, a highly sensitive way of regulating the interactions between the proteins. (2) According to the principle of linked functions (Weber, 1975), the affinity of CaVP for CaVPT is different in the absence of Ca^{2+}, as deduced from Scheme 1 where K'_{Ca1}, K'_{Ca2} and $K'_{Ca1,compl}$, $K'_{Ca2,compl}$ are the intrinsic Ca^{2+}-binding constants of CaVP in the absence and presence of CaVPT, respectively, and K_{compl}, K_{comp2}, and K_{comp3} are the affinity constants of the different CaVP•Ca_n species for CaVPT. In our system, $K_{comp3}/K_{compl} = K'_{ca1,compl}K'_{Ca2,compl}/K'_{Ca1}K'_{Ca2} = 68$.

The complexation-induced increase in affinity was previously observed for the CaM−target enzyme complexes (Cox, 1989), myosin light chain−myosin complex (Xie *et al.*, 1994), and TnC−TnI complex (Potter & Gergely, 1975), where protein association also resulted in a 10−3000-fold increase of affinity for Ca^{2+}. However, the absence of protein dissociation and moderate increase of affinity place the CaVP−CaVPT complex closer to the TnC−troponin I complex than to CaM−target enzyme complexes.

Scheme 1

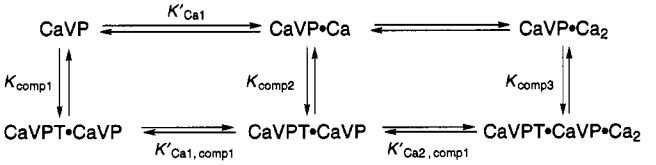

Table 1: Thiol Reactivity in CaVP, CaVPT, and the Complex

	total Cys/mol	accessible thiols/mol	$t_{1/2}$ (for accessible Cys)
CaVP	2	2	<2 s
CaVPT	3	2	<2 s
CaVP−CaVPT	5	4 → 2	1.5 min
		2	20 min

Table 2: Identification of the Buried Thiol in Native CaVPT

amino acid sequence	type of modification
-TYT*CQAT-	iodoacetamide
-PAK*CTKQ-	vinylpyridine
-AY*CTV	vinylpyridine

Thiol Reactivity in CaVP, CaVPT, and the Complex. CaVPT contains three Cys residues in positions 132, 155, and 238. Two of them react very rapidly with DTNB (Table 1), whereas the third is not accessible to this reagent unless guanidine hydrochloride is added. We first addressed the question of which thiol is buried in CaVPT. For this purpose, the two accessible thiols in CaVPT were blocked by DTNB; then the hidden cysteine residue was modified covalently by iodoacetamide in the presence of 4 M guanidine hydrochloride (see Materials and Methods). After removal of TNB groups with an excess of DTT, liberated thiols were modified by vinylpyridine, and the protein was subjected to digestion with trypsin followed by HPLC and sequencing of the peptides containing cysteines, modified either by iodoacetamide or by vinylpyridine. The results, summarized in Table 2, indicate that the buried thiol is Cys132. This is in very good agreement with the predicted tertiary structure of CaVPT. Indeed, the three Cys residues of CaVPT are located in the two IgII-folds, which display marked sequence similarity to the single IgII-fold of telokin, whose three-dimensional structure has recently been solved. On the basis of this sequence similarity, we have created energy-minimized models of the IgII-folds I (residues 57−150) and II (residues 151−243) of CaVPT (Figure 3), which differed in fact very little from the telokin template model. One observes that Cys132 is completely buried within the hydrophobic core of the protein and that Cys155 and Cys238 are solvent-exposed. The correct prediction of the reactivities of the thiols gives more credit to the three-dimensional model of the Ig folds in CaVPT.

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             60        70        80        90        100       110       120       130       140       150
CaVPT 1   SIPKFSQVLKDMFVMEGSAVTFFARVWGIPDPVIKWFKDGQEVKQGPKHEIKFDDKDGTFLIIRNADGDDVGTYTCQATNSFGEAFDSARLALE

Telokin   VKPYFTKTILDMEVVEGSAARFDCKIEGYPDPEVMWYKDDQPVKESRHFQIDYDEEGNCSLTISEVCGDDDAKYTCKAVNS-GEATCTAELLVE

CaVPT 2   VPAKCTKQPDNITVKAGGNFEIKVEIAGEPEPDVIWTKGKKEIDEGGRFSYEDSP-DYSILRVEKAQAGDAGKYEIEIVNDLGKARAYCTVKVN
                 160       170       180       190       200       210       220       230       240
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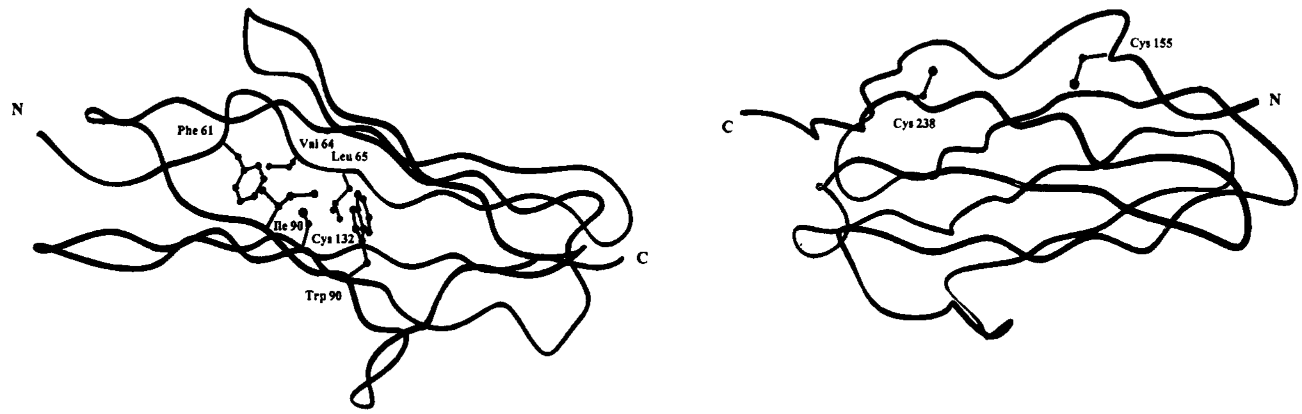

FIGURE 3: (Top) Sequence similarity between C2-type Ig-folds in CaVPT (residues 57−150 and 151−243) and telokin (residues 44−135). The first digit corresponds to the right number. Identical residues and conservative replacements are shown in boldface letters. (Bottom left) Position of cysteine residue 132 in a three-dimensional model of Ig-fold 57−150 of CaVPT. Hydrophobic residues, surrounding Cys132, are shown. (Bottom right) Cysteines 155 and 238 in the three-dimensional model of Ig-fold 151−243 of CaVPT. The program package Brugel, version 10, has been used to substitute amino acid residues in the three-dimensional structure of telokin (Holden *et al.*, 1992) by those of CaVPT. The mutations were followed by a conformational energy search in order to obtain a locally minimized conformation. Further minimization was performed with the steepest descent (Levitt & Lifson, 1969) and the conjugate gradient method (Fletcher & Lifson, 1964). The RMS difference for the α carbons was less than 1.5 Å.

CaVP contains two cysteine residues in its N-terminal half. The reaction of these thiols with DTNB is very fast (Table 1) and Ca^{2+}-independent, which can be explained by their remote position from the Ca^{2+}-binding sites. In the three-dimensional model of CaVP (Cox *et al.*, 1990), the cysteine residues are situated close to each other and easily form a disulfide bridge. Disulfide bridging in CaVP can be monitored by SDS−PAGE on photopolymerized gels in the absence of oxidoreducing agents, since oxidized CaVP shows a higher mobility than reduced CaVP. Using this method, we observed that CaVP, freshly dissociated with CaVPT, did not contain a disulfide linkage, whereas upon storage the isolated protein shows a clear tendency for intramolecular disulfide bridge formation.

In the complex, Cys132 remains completely inaccessible to DTNB unless the protein is denatured. Whereas in isolated CaVP and CaVPT the thiol reactivities are high (see above), in the complex almost 1 h is needed for the reaction to be completed. The kinetic data fit best to the model of two pairs of independently reacting thiols with the following pseudo-first-order rate constants (Figure 4): $k_1 = k_2 = 7.7 \times 10^{-3}\ s^{-1}$ ($t_{1/2}$ = 1.5 min) and $k_3 = k_4 = 5.8 \times 10^{-4}\ s^{-1}$ ($t_{1/2}$ = 20 min). Interestingly, the kinetics of these reactions were exactly the same in the presence and absence of 1 mM Ca^{2+}, indicating that Ca^{2+} binding does not change the environment of any of the four thiols. To identify the location of the two pairs of thiols in the complex, only the highly reactive thiols in the complex were allowed to react with DTNB (see Materials and Methods). Then the two proteins were separated by DEAE-cellulose chromatography in the presence of 6 M urea (Figure 5), and the degree of thiol modification was determined in each protein. The DTNB-modified thiols were mainly found in CaVP (73% reaction), whereas the cysteine residues of CaVPT were only

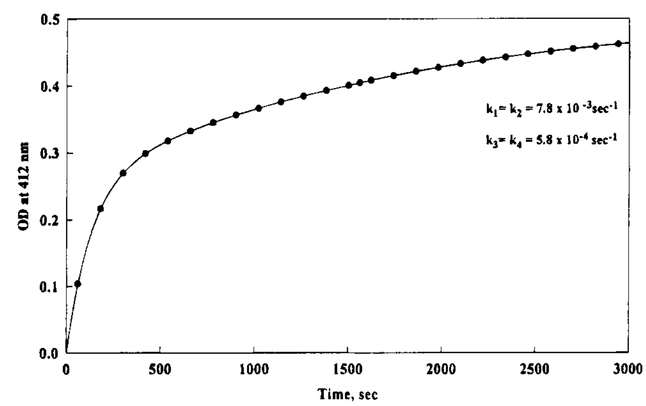

FIGURE 4: Spectrophotometric titration of the CaVP−CaVPT complex with DTNB (●). DTNB (500 μM) was added at time zero in the reference cell, containing assay buffer, and the sample cell, containing protein solution (5 μM). The absorbance at 412 nm was recorded as a function of time. The solid line is a theoretical curve, calculated with the Guggenheim equation with the constants indicated in Table 1.

slightly affected (less than 15% reaction). These data suggest that the constant of $7.7 \times 10^{-3}\ s^{-1}$ can be attributed to the thiols in CaVP, and those of $5.8 \times 10^{-4}\ s^{-1}$ to CaVPT. The remarkable decrease (600-fold in CaVPT, 45-fold in CaVP) in thiol reactivity upon complex formation suggests that the complex is much more compact and rigid than the individual proteins and that thiols of both subunits become buried due to either allosteric refolding or direct hiding in the interface CaVP−CaVPT. According to structural predictions and proteolytic data (Takagi & Cox, 1990) as well as studies with the synthetic peptide (T.V.P. and J.A.C., unpublished data), CaVP binds to segment 33−50 (IQ-motif) preceding the first Ig-fold. Therefore, the question arises how CaVP binding can modify the reactivity of the thiols in the second

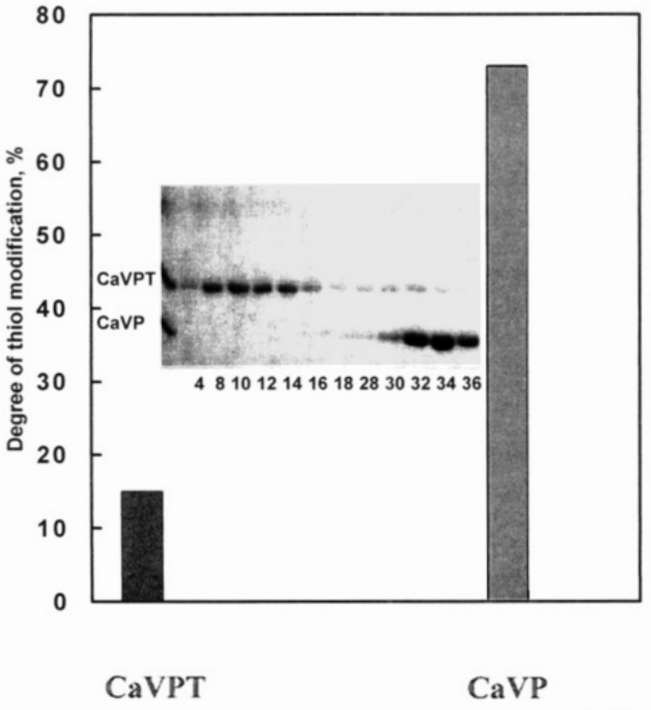

FIGURE 5: Distribution of the reactive thiols in the complex CaVP−CaVPT. An insert shows separation of the components on the DEAE column in the presence of 6 M urea as monitored by SDS−PAGE on 12.5% acrylamide gels. Pure CaVP and CaVPT-containing fractions were pooled, and the degree of thiol modification measured was as described under Materials and Methods.

Ig-fold domain, especially since there is no linker between Ig-folds I and II to provide a bend. Futher studies, including crystallization of the complex, are necessary to resolve this intriguing question.

Hydrophobic Interactions in CaVP, VaVPT, and the Complex. TNS is a fluorescent probe which exhibits a remarkable fluorescent enhancement provided it is bound to a hydrophobic matrix at the surface (Madan *et al.*, 1994) or in the core of a protein (Ptitsyn *et al.*, 1990; Shi *et al.*, 1994). For instance, Ca^{2+}-loaded CaM enhances the fluorescence of TNS 10−30-fold, whereas only a 2-fold increase is obtained with metal-free CaM (Tanaka & Hidaka, 1980). CaVP, belonging to the same family of Ca^{2+}-dependent activators as CaM, also interacts with TNS (Figure 6), provoking a 5-fold and 10-fold increase of the fluorescence intensity in the Ca^{2+}-free and Ca^{2+}-loaded forms, respectively. Nevertheless, the fact that CaVP is poorly retained on phenyl-Sepharose (the presence of 1 M ammonium sulfate in the buffer is necessary for binding) and eluted independently of the presence of Ca^{2+} by lowering the ammonium sulfate concentration to 0.5 M (Valette-Talbi and J.A.C., unpublished observations) suggests that the fluorescence enhancements merely reflect the accessibility of TNS to the hydrophobic core rather than the exposure of the hydrophobicity at the surface.

Surprisingly, CaVPT enhances TNS fluorescence 45-fold and provokes a blue shift from 432 to 425 nm. On the other side, when CaVPT was passed through a column of phenyl-Sepharose equilibrated in 20 mM Tris-HCl, pH 7.5, 1 mM DTT, and 1 M NaCl, nearly 90% of the protein passed straight through, and the small portion retained could be eluted with 6 M urea, but not by low-salt buffer. These experiments strongly suggest that CaVPT does not possess an extended solvent-exposed hydrophobic area and that the

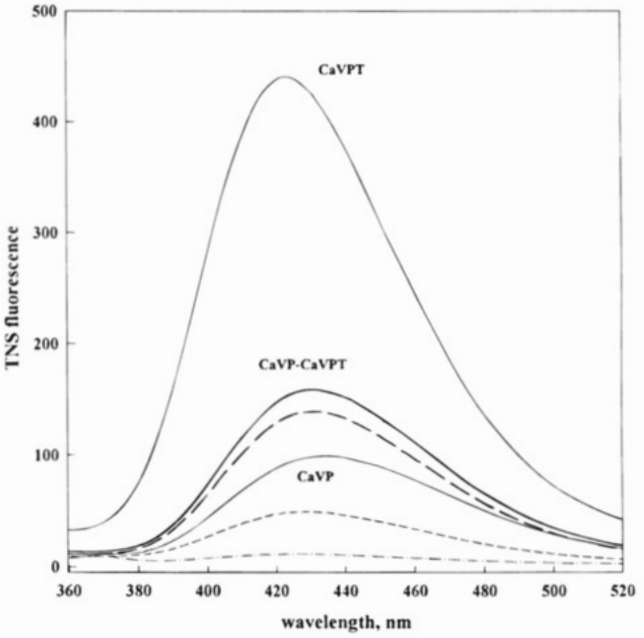

FIGURE 6: Fluorescence enhancement of TNS in the presence of CaVP, CaVPT, and the complex. 500 μM EDTA and 1 mM $CaCl_2$ were added to obtain Ca^{2+}-free (dashed lines) and Ca^{2+}-loaded forms (solid lines). The dashed−dotted line represents the fluorescence of TNS in the absence of protein.

binding of TNS to CaVPT is related to its accessibility to the hydrophobic core.

In the CaVP−CaVPT complex, the TNS fluorescence enhancement is 3-fold lower as compared to the calculated value for the sum of the components. Moreover, this lower enhancement is nearly identical in the presence or absence of Ca^{2+}. This confirms that the complex does not dissociate in the absence of Ca^{2+} and, more importantly, indicates that Ca^{2+}-induced conformational changes in the complex are small, as was already observed in the thiol reactivity experiments. Similarly, no significant changes were observed in the intrinsic fluorescence and far-UV circular dichroic spectra of Ca^{2+}-free and Ca^{2+}-bound forms of the complex (data not shown). The decreased TNS fluorescence upon complex formation reflects a tightening or rearrangement of the CaVPT structure, so that access of the probe to the core is reduced. Extensive solvent-shielding of hydrophobic residues is the driving force of the interaction of CaM with target peptides (Ikura *et al.*, 1992; Meador *et al.*, 1992) and likely enzymes. However, the question arises if in the CaVP−CaVPT complex the cause for decreased TNS fluorescence is not fundamentally different. The decreased thiol reactivity in Ig-fold II and hydrophobicity point to a profound structural rearrangement in CaVPT upon complex formation with CaVP.

CONCLUSIONS

In conclusion, CaVP clearly resembles TnC and CaM not only as an isolated protein but also in its interactive properties. The increase of the sensitivity to the calcium signal evokes the regulatory mechanism of calmodulin, but, as in the case of the troponin complex, the CaVP−CaVPT complex seems to be more permanent. In the complex, the conformational changes seem to be completely independent of Ca^{2+}, suggesting that the complex does not play the activator role, but rather one of its components. Since *in*

vivo CaVP seems only to interact with CaVPT, the latter is the best candidate to regulate a target: recent studies demonstrate that almost all intracellular Ig-fold-containing proteins and isolated segments are able to interact with the rod of myosin (Shirinsky *et al.*, 1993; Okagaki *et al.*, 1993). Work is in progress to identify the target(s) of CaVPT.

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