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In vivo mutations of calmodulin: A mutant *Paramecium* with altered ion current regulation has an isoleucine-to-threonine change at residue 136 and an altered methylation state at lysine residue 115

(protein conformation/calcium-binding protein/amino acid sequence/cell behavior)

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ABSTRACT **The *Paramecium tetraurelia* mutants termed *pantophobiacs* have altered behavior due to perturbed calcium activation of ion channel activity. The calmodulin from pantophobiac A^1 (*pntA*^{1}) was shown in previous studies to have a single amino acid change at residue 101 that is selective in its effects on activity. This change has no effect on post-translational modifications. However, the calmodulin from the phenotypically related mutant pantophobiac A^2 (*pntA*^{2}) has a threonine residue at position 136, in the fourth calcium-binding domain, instead of an isoleucine or valine like all other calmodulins. This region of the calmodulin structure is within 4 Å of a complementary hydrophobic structure in the third calcium-binding domain, raising the possibility of a perturbation of interdomain interactions in the *pntA*^{2} mutant. This possibility is supported by the heterogenous methylation state of lysine-115 in the *pntA*^{2} calmodulin. This lysine residue, located in the peptide connecting calcium-binding domains three and four, is fully trimethylated in the wild-type and *pntA*^{1} calmodulins. The functional selectivity of these structural changes is demonstrated by the conservation of calmodulin activator activity with a calmodulin-regulated protein kinase that has been used as a standard of comparison. Overall, these results indicate the degree to which the calmodulin can be mutated *in vivo* without being lethal to the organism, and they provide genetic evidence suggesting that the post-translational methylation state of residue 115 requires the appropriate conformation in addition to the local amino acid sequence.**

Eukaryotic cells respond to a variety of drugs, hormones, neurotransmitters, toxins, and trophic stimuli through cellular mechanisms that involve transient increases in the concentration of intracellular ionized calcium. The molecular mechanisms by which these calcium signals are transduced into biological responses appear to be through the reversible interaction with a class of calcium-binding proteins that includes calmodulin, a ubiquitous representative with multiple biological roles (for reviews see refs. 1–4).

Current studies aimed at gaining insight into the exact molecular mechanisms involved in the selective transduction of calcium signals into biological responses through specific calmodulin-mediated pathways have benefited from mutant analysis approaches. In terms of calmodulin structure and function, mutant analyses have included (*i*) gene disruption experiments showing calmodulin to be vital in budding or fission yeast (5, 6); (*ii*) analysis of calmodulins isolated from mutant *Paramecium* with altered behavior (7–9); (*iii*) *in vitro* functional analysis of mutant calmodulin structures generated by site-specific mutagenesis (10–14); and (*iv*) *in vivo* functional analysis of mutant calmodulin structures generated by *in vitro* site-specific mutagenesis (15). The combination of these four mutant analysis approaches has the advantage of providing insight into *in vivo* roles while providing a firm biochemical basis for interpretation and extension of biological studies. For example, a previous report (9) on the characterization of a calmodulin isolated from a mutant organism demonstrated that *in vivo* mutations of calmodulin could be selective and nonlethal and supported the model of calmodulin involvement in the regulation of a class of potassium channels (16). Briefly, the behavioral and electrophysiological defects of a single-gene mutant (7) of *Paramecium tetraurelia*, called pantophobiac A^1 (*pntA*^{1}), was found to be temporarily cured upon microinjection of wild-type cytoplasm. Fractionation of this cytoplasm traced the curing activity to calmodulin (16). Purified *pntA*^{1} calmodulin was found to differ from wild-type calmodulin in electrophoretic migration and by other criteria (8). Amino acid sequence analysis showed that *pntA*^{1} calmodulin has a single serine-to-phenylalanine substitution at residue 101 (9, 17). Subsequent computational chemistry and *in vitro* mutagenesis combined with microinjection studies (16, 18) have supported the idea that the change in phenotype is probably due to the change in calmodulin residue 101 *per se* and that the mutational effect has implications beyond the *Paramecium* context.

The results from the study of the *pntA*^{1} *Paramecium* mutant demonstrated the utility and insights that can be derived from the analysis of viable *Paramecium* mutants. However, this represents only one example. Another mutant *Paramecium*, pantophobiac A^2 (*pntA*^{2}), with a cellular phenotype similar to but distinct from the *pntA*^{2} mutant, has been characterized preliminarily (19–24). These two mutants are allelic, indicating that the *pntA*^{2} mutation also resides in the primary sequence of calmodulin. However, calmodulin from *pntA*^{2}, but not *pntA*^{1}, is a substrate for N-methylation *in vitro*, suggesting that it is undermethylated *in vivo*. The purification and characterization of calmodulins from multiple mutant organisms with related phenotypic defects has the potential of providing additional insight into how calmodulin structure is related to *in vivo* function and how calcium modulation of distinct ion channels might be coupled at a molecular level. In this context, we summarize here the properties of a calmodulin purified from the *pntA*^{2} mutant *Paramecium* that has altered calcium regulation of ion channel activity (19–25).

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METHODS

Cells of *Paramecium tetraurelia* strain *pntA*^{2} were grown and calmodulin was isolated as previously described (20–22). Briefly, calmodulin was isolated from late-logarithmic growth phase cells harvested with a continuous flow rotor and disrupted with a Teflon–glass homogenizer. Homogenates were pelleted and the supernatant fraction was processed through Ca^{2+}-dependent phenyl-Sepharose chromatography and DEAE-cellulose ion-exchange chromatography. Two separate preparations of *pntA*^{2} calmodulin were used in the studies described here. The final step of calmodulin purification was reversed-phase chromatography on a 2.1 × 30 mm column of RP300 C8 silica (Brownlee/Applied Biosystems). This last step was found to be necessary to remove traces of proteolytic activity which slowly degrade the calmodulin and can affect activity analyses. A single major peak of absorbance at 220 nm was detected during this final purification step. This peak had two leading shoulders. These two shoulders and the main peak were collected separately. By amino acid compositional analysis, the major peak represented 88% of the recoverable mass of calmodulin and was used for the subsequent amino acid sequence analyses.

Amino acid compositions, amino acid sequences, and peptide purifications were exactly as previously described (9, 17). The approach used was a comparative one, designed to detect differences between wild-type *Paramecium* calmodulin and the calmodulin isolated from the mutant organism. Peptides were generated by digestion of 25–50 μg of calmodulin with *Staphylococcus aureus* V8 protease (Miles) as described previously (17). Amino acid analyses, including the detection of methylated amino acids, were done by two complementary methods. On a routine basis, analyses were done by using a Picotag system as described by Bidlingmeyer *et al.* (26) with the modifications described previously (17). Further analysis of methylated peptides was done using precolumn derivatization with *o*-phthalaldehyde. Briefly, the procedure was similar to that described in ref. 27 except that the thiol was 3-mercaptopropionic acid and the procedure was automated by using a Varian 9000 Microbot autosampler. Chromatography was done in an Applied Biosystems 130A microbore chromatograph with Hewlett–Packard amino acid analysis column HP 79916AAA-512. Buffers and separation conditions were based on those reported in Hewlett–Packard technical literature. Standards of ε-amino methylated lysines were obtained from Calbiochem/Boehringer Mannheim. With the approach used previously (9, 17) for wild-type and *pntA*^{1} *Paramecium* calmodulins, peptides which resulted from the V8 protease digestion were separated by reversed-phase chromatography on a 1.0 mm × 250 mm column of RP300 C8 silica (Brownlee/Applied Biosystems) eluted with a gradient of 0.1% trifluoroacetic acid/water and 60% (vol/vol) acetonitrile/water. Recovery of peptides was 22–66% based on the amount of digest applied to the column. Some peptides were rechromatographed on the same column with a different gradient to achieve homogeneity (see discussion in refs. 9 and 17). Automated Edman degradation of peptides was done with an Applied Biosystems 475A sequencer equipped with a 900A data system-controller.

RESULTS

The amino acid composition of purified *pntA*^{2} calmodulin is shown in Table 1 along with a comparison to those of wild-type and *pntA*^{1} *Paramecium* calmodulins. The values for *pntA*^{2} calmodulin have not been corrected for losses during hydrolysis and analysis. The uncorrected values match well with the composition calculated from the subsequently determined amino acid sequence (Fig. 1). Although the absence of an isoleucine and the presence of an additional threonine were indicated, these changes cannot be reliably determined at the level of compositional analyses of the entire protein due to the variable loss of threonine during acid hydrolysis. In contrast to *pntA*^{1} calmodulin, the phenylalanine content of *pntA*^{2} calmodulin is similar to that of the wild-type protein.

To more accurately characterize the differences between *pntA*^{2} and wild-type calmodulin, peptides encompassing the entire protein were purified from *S. aureus* V8 protease digests of *pntA*^{2} calmodulin as previously done for the *pntA*^{1} calmodulin mutant (9). The amino acid compositions (Table 2) and amino acid sequences (Fig. 1) of these peptides were determined. The amino acid sequence of peptide SP8, the amino-terminal-blocked peptide, was not determined but its composition is identical to that for the corresponding peptide from the wild-type calmodulin (17). Complete amino acid sequence data through the apparent carboxyl terminus were obtained for each of the other peptides except SP12 and SP9b, where the last three and two amino acids, respectively, were not placed. However, the amino acid compositions of these peptides are in excellent agreement with those of the corresponding wild-type calmodulin peptides, which were characterized in detail by using a combination of Edman chemistry and fast atom bombardment mass spectral analyses (17). These peptides from *pntA*^{2} calmodulin were, therefore, not characterized further. Placement of the remaining peptide amino acid sequences is shown in Fig. 1.

The only differences in amino acid composition (Table 2) or amino acid sequence (Fig. 1) of peptides derived from *pntA*^{2} calmodulin compared to the wild-type *Paramecium* calmodulin sequence were found in peptides SP5 and SP11. The change in methyllysine content of the *pntA*^{2} calmodulin indicated potential changes in peptides SP1 or SP11 because these are the peptides that contain the single residues of dimethyllysine (residue 13) and trimethyllysine (residue 115). As shown in Table 2 and Fig. 1, the amino acid composition

Table 1. Amino acid compositions of wild-type and mutant calmodulins

Amino acid	Residues per molecule		
	Wild type	*pntA*^{1}	*pntA*^{2}
Asp	22	22	22.7 (22)
Glu	27	27	28.1 (27)
Ser	5	4	4.3 (5)
Gly	11	11	11.8 (11)
His	2	2	1.8 (2)
Lys(Me_3)	1	1	— (<1)
Arg	6	6	6.1 (6)
Lys(Me_2)	1	1	2.0 (>1)
Thr	9	9	9.8 (10)
Ala	11	11	11.1 (11)
Pro	2	2	3.1 (2)
Tyr	1	1	1.3 (1)
Val	7	7	6.9 (7)
Met	8	8	6.3 (8)
Ile	9	9	7.8 (8)
Leu	12	12	12.0 (12)
Phe	8	9	8.0 (8)
Lys	6	6	6.2 (6)

Values for the wild-type and *pntA*^{1} calmodulins are calculated from their published amino acid sequences (9, 17). Values for *pntA*^{2} calmodulin were determined as described in the text and are normalized to a molecular weight of 17,000; numbers in parentheses are residues determined by amino acid sequence determination (see Fig. 1). Lys(Me_2), N^{ϵ},N^{ϵ}-dimethyllysine; Lys(Me_3), N^{ϵ},N^{ϵ},N^{ϵ}-trimethyllysine. The amino acid composition of *pntA*^{2} calmodulin included a mixture of methylated lysines due to the partial methylation state of residue 115 (see Table 2 and Fig. 1).

Table 2. Amino acid compositions of peptides from *S. aureus* V8 protease digestion of *pntA*^{2} calmodulin

	Residues per molecule of peptide												
Amino acid	SP1	SP2	SP5	SP6	SP7	SP8	SP9A	SP9B	SP10	SP11*	SP12	SP13	SP14
Asp	—	—	3.7 (4)	1.8 (2)	—	—	2.0 (1)	3.2 (4)	1.1 (1)	3.9 (4)	2.9 (3)	3.4 (3)	1.0 (1)
Glu	1.2 (1)	1.2 (1)	2.1 (2)	2.7 (2)	—	5.5 (6)	2.6 (3)	1.2 (1)	4.3 (4)	3.5 (3)	1.0 (1)	1.2 (1)	6.1 (6)
Ser	—	—	—	—	0.8 (1)	—	0.8 (1)	0.5 (0)	1.3 (1)		—	1.0 (1)	1.6 (2)
Gly	—	—	1.9 (2)	—	—	—	2.3 (2)	2.4 (2)	—	1.4 (1)	2.0 (2)	2.4 (2)	—
His	—	—	0.9 (1)	—	—	—	—	—	—	0.8 (1)	—	—	—
Lys(Me_3)	—	—	—	—	—	—	—	—	—	0.3	—	—	—
Arg	—	1.0 (1)	—	—	1.0 (1)	—	1.1 (1)	—	1.1 (1)	0.8 (1)	—	1.1 (1)	1.2 (1)
Lys(Me_2)	1.0 (1)	—	—	—	—	—	—	—	—	0.2	—	—	—
Thr	—	—	0.9 (1)	—	—	1.0 (1)	2.0 (2)	0.9 (1)	—	1.8 (2)	2.6 (3)	—	—
Ala	—	—	1.0 (1)	—	—	2.0 (2)	1.2 (1)	1.0 (1)	0.8 (1)		1.9 (2)	3.2 (3)	1.0 (1)
Pro	—	—	—	0.9 (0)	—	—	1.2 (1)	1.0 (1)	—		—	—	—
Tyr	—	—	0.6 (1)	—	—	—	—	—	—		—	—	—
Val	—	—	—	0.6 (0)	1.7 (2)	—	1.3 (1)	1.0 (1)	—	2.0 (2)	—	1.0 (1)	—
Met	—	0.5 (1)	—	0.0 (1)	1.0 (1)	—	0.5 (1)	—	1.4 (2)	0.8 (1)	—	—	2.0 (2)
Cys	—	—	—	0.5 (0)	—	—	—	—	—		—	—	0.6 (0)
Ile	—	1.2 (1)	1.0 (1)	1.0 (1)	—	1.0 (1)	—	0.9 (1)	—		0.9 (1)	1.0 (1)	0.9 (1)
Leu	—	—	—	1.4 (1)	—	1.1 (1)	1.7 (2)	—	2.0 (2)	2.9 (3)	1.1 (1)	1.1 (1)	3.3 (3)
Phe	1.0 (1)	—	—	—	0.8 (1)	—	—	0.9 (1)	0.7 (1)		1.8 (2)	2.1 (2)	0.8 (1)
Lys	—	—	—	—	0.8 (1)	—	—	—	1.7 (2)	0.3	1.9 (2)	1.2 (1)	2.1 (2)
% recovery	36.6	60	65.5	51	32.3	38.3	29.9	22.1	50	26	30	40	37

Molar ratios greater than 0.5 are shown. Values in parentheses are the number of residues based on amino acid sequence determination. *This amino acid analysis was done by the precolumn *o*-phthalaldehyde method. A mixture of methylated lysines was detected. N^{ϵ}-monomethyllysine could not be accurately quantitated because it was not well resolved from the arginine derivative. The estimated molar ratio of this derivative is 0.2 mol/mol.

(Table 2) and sequence of SP1 (Fig. 1) are identical to those of the wild-type *Paramecium* calmodulin, including a fully dimethylated lysine at residue 13. In contrast, the amino acid composition of SP11 (Table 2) showed a mixture of methylated and unmethylated lysines. During the automated Edman degradation of SP11 from wild-type *Paramecium* calmodulin (17), no phenylthiohydantoin derivative of lysine (PTH-lysine) was detected at the cycle corresponding to residue 115, but a stoichiometric amount of a phenylthiohydantoin derivative that migrated with the PTH-trimethyllysine standard was detected (the value approaches 1 mole per mole of peptide analyzed but is never 1 due to the incomplete recovery inherent in the analytical chemistry method). In contrast, during the Edman degradation of SP11 from *pntA*^{2} calmodulin (Fig. 2), PTH-lysine was detected at this same cycle although in low amounts. PTH-trimethyllysine was not identified. Although the amino acid composition indicated the presence of methylated lysines in the peptide (Table 2),

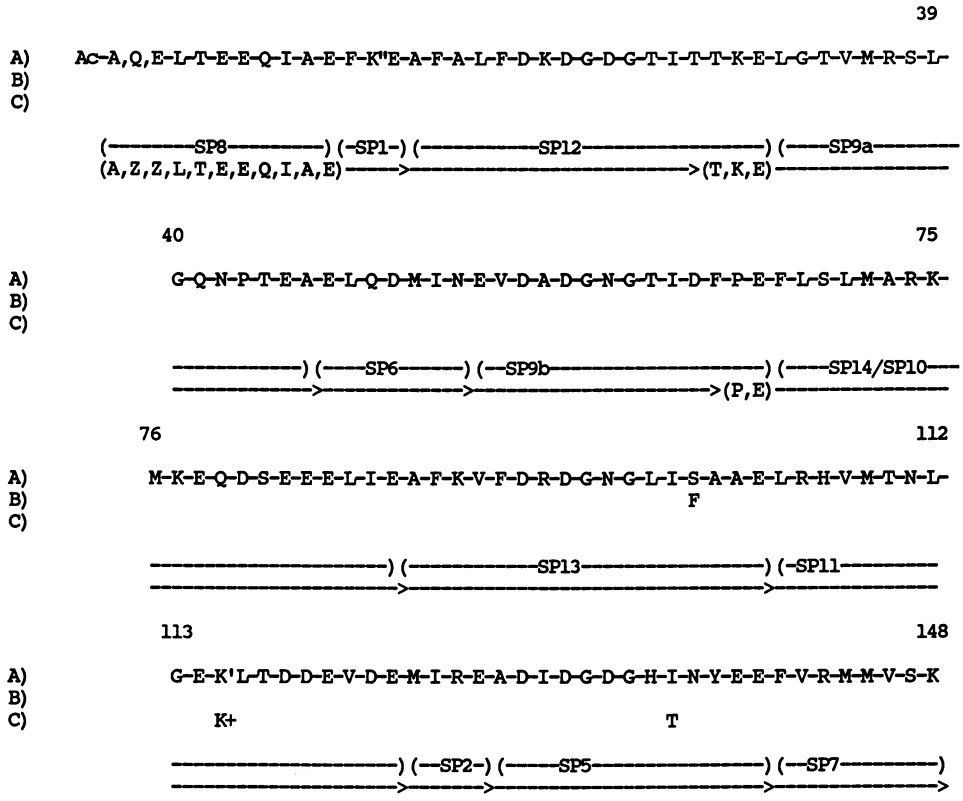

FIG. 1. Sequence comparisons and strategy. The amino acid sequence of wild-type *Paramecium* calmodulin (17) is given in line A. The differences from the wild-type sequence are given in line B for the *pntA*^{1} mutant calmodulin (9) and in line C for the *pntA*^{2} mutant calmodulin (this report). The only differences detected between wild-type and *pntA*^{2} mutant calmodulin were a difference in the methylation state of residue 115 and threonine instead of an isoleucine at residue 136. Standard single-letter codes for amino acids are used except for K′ = trimethyllysine and K″ = dimethyllysine. K+ on line C denotes that a mixture of lysine and methylated lysines was detected at this position for the *pntA*^{2} calmodulin as described in the text. Arrows denote residues identified by Edman degradation. Amino acids listed in parentheses were placed on the basis of compositional analysis of peptides with retention times equivalent to those from the wild-type protein. Amino acid compositions of the purified peptides are given in Table 2.

A. Peptide SP5 (325 pmol analyzed):

Degradation Cycle No.:	1	2	3	4	5	6	7	8	9*	10	11	12	13
Amino Acid Identified:	Ala	Asp	Ile	Asp	Gly	Asp	Gly	His	Thr	Asn	Tyr	Glu	Glu
Amount, pmol:	293	189	218	116	154	124	110	10	73	12	70	16	51

B. Peptide SP11 (100 pmol analyzed):

Degradation Cycle No.:	1	2	3	4	5	6	7	8	9	10	11*	12	13	14	15	16	17	18	19
Amino Acid Identified:	Leu	Arg	His	Val	Met	Thr	Asn	Leu	Gly	Glu	Lys	Leu	Thr	Asp	Asp	Glu	Val	Asp	Glu
Amount, pmol:	64	7	4	35	42	40	46	49	41	42	2	33	15	21	28	15	12	13	9

FIG. 2. Identification of amino acid sequence differences by automated Edman degradation. (*A*) The only peptide with a coding change was peptide SP5. The results from one of the Edman degradation analyses done on the purified peptide are shown. The amino acid composition of the peptide is given in Table 2. The mutation is the presence of a threonine at cycle 9, indicated by an *, instead of an isoleucine as determined for the wild-type protein (17). (*B*) The results from one of the analyses done on peptide SP11 are shown. Cycle 11 released the only identified lysine in this peptide.

the phenylthiohydantoin derivatives of these modified amino acids elute as comparatively low, broad peaks that often overlap with other amino acids. This severely impedes accurate quantification of each of these modified amino acids at the level of material analyzed. Therefore, quantification of the different methylated amino acids during the Edman degradation was not attempted. Altogether, the data are indicative of a mixture of methylation states of lysine-115 in *pntA*^{2} calmodulin but with no changes in the genetically encoded amino acid sequence.

Peptide SP5 was found to have a single amino acid change (Table 2): a threonine residue was detected at cycle 9 of the Edman degradation (residue 136) instead of isoleucine (Fig. 2). This change is the only genetically encoded amino acid sequence change found in *pntA*^{2} calmodulin compared to the wild-type *Paramecium* calmodulin and is one of only two amino acid sequence changes detected.

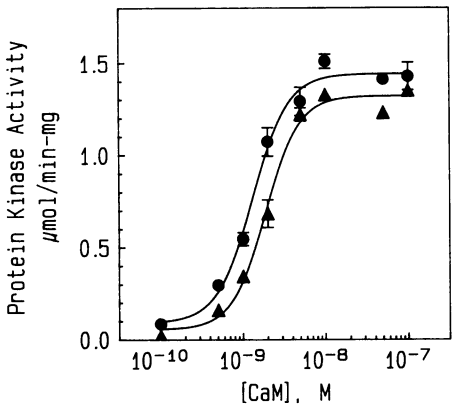

FIG. 3. Activation of myosin light chain kinase by calmodulins. Chicken gizzard myosin light chain kinase activity was measured in a buffer containing 50 mM Hepes at pH 7.5, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM dithiothreitol, and various concentrations of calmodulin (CaM): SYNCAM-1 standard (●) or *pntA*^{2} (▲) calmodulin. Curves were generated by fitting the data points for each calmodulin to a nonlinear regression routine for dose–response data (GraphPad program, Version 2.0: H. Motulsky, distributed by ISI Software). Error bars are indicated where the deviation is larger than the size of the symbol. The ATP concentration was 200 μM; [γ-^{32}P]ATP specific activity was 200–400 cpm/pmol. The peptide substrate (50 μM) was a synthetic peptide analog (K K R P Q R A T S N V F A M) of the myosin light chain phosphorylation site. Protein kinase reactions took place in a final volume of 50 μl and were initiated by adding kinase (5 μl) previously diluted in a buffer containing bovine serum albumin at 1 mg/ml to a final concentration of 1.3 nM. Tubes were incubated for 20 min at 25°C, and aliquots were withdrawn, applied to phosphocellulose paper, and processed as described previously (12).

The ability of the *pntA*^{2} *Paramecium* to survive this mutation of calmodulin demonstrates the selectivity of this amino acid sequence change. This was confirmed by a quantitative *in vitro* study of the *pntA*^{2} calmodulin's ability to activate a well-characterized calmodulin-regulated enzyme that has been used in comparative studies of calmodulins, including mutant calmodulins (12, 15, 18, 28). As shown in Fig. 3, *pntA*^{2} calmodulin is able to activate quantitatively smooth muscle myosin light chain kinase.

DISCUSSION

The studies summarized here demonstrate that the *pntA*^{2} mutant *Paramecium* has a molecular defect that is distinct from that found in the *pntA*^{1} organism (9) and further demonstrate the utility of the mutant *Paramecium* system (25). The structural basis of the activity changes observed with the *in vivo* mutant *pntA*^{2} is complex, may involve a protein folding problem, and is not as localized as that found with the *pntA*^{1} mutant calmodulin. Altogether, the results (19–24) suggest that the altered phenotype of *pntA*^{2} *Paramecium* may involve a mixture of effects, including possible alterations of protein interactions and protein turnover.

In the crystal structure of vertebrate calmodulin (29), residue 136 is a valine and is present in a three-residue β-sheet structure that is antiparallel to a similar structure in the third calcium-binding loop. The carbonyl oxygen of residue 136 is within potential hydrogen bonding range of the amide at residue 100 in the third calcium-binding site, and the amide of 136 is a similar distance from the carbonyl of residue 100. Thus, there appears to be an interaction between the loop of the third calcium-binding site and the loop of the fourth calcium-binding site. Such interactions might be part of the structural features that contribute to the functional coupling between calcium-binding sites in calmodulin (14, 30). In all calmodulins characterized, residue 136 is either a valine or an isoleucine (for a review see ref. 2). This indicates some structural variability is allowed, although the absence of leucine at position 136 suggests a requirement for a β branched-chain hydrophobe at this position. The complementary region of the third calcium-binding loop always has an isoleucine at residue 100. In fact, the same relative position in domains one, two, and three have an isoleucine invariant across phylogenetic groups. Only the isoleucine in the fourth domain (residue 136) is allowed to vary, and this variation is only between isoleucine and valine. Therefore, on

the basis of the available patterns in the vertebrate crystal structure and the primary structure across broad phylogenetic classes, it is reasonable to speculate that the isoleucine-to-threonine change found in *pntA*^{2} calmodulin may perturb the structure due to the disruption of key hydrophobic interactions, possibly resulting in alteration of the calcium-binding properties of both domain three and domain four. This possibility is supported by the observations that mutation of a calcium-ligating residue, glutamic acid-140, in the fourth domain of calmodulin results in the loss of two calcium-binding sites (31). Clearly, calcium-binding studies on a calmodulin with threonine 136 should directly address this possibility.

Although the serine-to-phenylalanine change at residue 101 in *pntA*^{1} is closer to residue 115 in the primary and tertiary structure than that described here for *pntA*^{2} calmodulin, there was no effect on the methylation state of residue 115 in *pntA*^{1} *Paramecium* calmodulin (9, 17). Residue 115 is solvent exposed (29) and is part of a structure that joins the last helix of domain three with the first helix of domain four. Previous studies (11) of the methylation of residue 115 have shown that amino acid sequence variability in the region of residue 115 can be accommodated, indicating that simply any change in this region of the molecule does not alter the ability of the protein to be stoichiometrically methylated. Based on the tertiary structure interactions of calmodulin that are found in the crystallized vertebrate protein (29), the change of an isoleucine to a threonine at residue 136 might disturb the relative orientations of the helix–loop–helix structures of the fourth and third domains, with a resultant perturbation of the connecting peptide structure that contains residue 115. Thus, the molecular phenotype of *pntA*^{2} provides genetic evidence suggesting that the methylation state of lysine 115 requires the presence of a correctly folded polypeptide chain. Studies that employ site-specific mutagenesis of several positions in this region, combined with conformational and functional analyses, might provide some insight into how the features of the side-chain atoms present at these positions can affect structure and activity.

The observations that the methylation of residue 115 can affect enzyme activator activity (11) and decrease the susceptibility of the peptide bond linking residues 115 and 116 to proteolytic cleavage (32) logically raise the question of whether the mixed methylation state of *pntA*^{2} calmodulin alters the turnover or effector regulatory activities of this protein in *Paramecium*. These are possibilities that can be investigated further with the availability of this viable mutant *Paramecium*. Clearly, the available data demonstrate that the change in the regulatory activities of *pntA*^{2} calmodulin is selective. First, the ability to activate a calmodulin-regulated enzyme that has been used as a standard of comparison in a variety of studies provides biochemical evidence in support of this concept. Second, and more significant physiologically, the ability of the *pntA*^{2} *Paramecium* to survive and replicate shows the *in vivo* selectivity.

In summary, the *pntA*^{2} defect described here furthers our understanding of how the structure of calmodulin is related to its function *in vivo*. At present, *Paramecium* behavioral genetics (25) is the only system described that has generated, *in vivo*, viable mutants with defective calmodulins. This system demands that the calmodulin defects be strong enough to affect ion channels but subtle enough to allow growth and division. Therefore, further analysis of *pntA*^{1}, *pntA*^{2}, and other *Paramecium* calmodulin mutants should enrich our understanding of the structure, functions, and regulation of this molecule, which is so pivotal in sensory transductions through calcium.

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