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Evidence for Domain Organization within the 61-kDa Calmodulin-Dependent Cyclic Nucleotide Phosphodiesterase from Bovine Brain†

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ABSTRACT: The complete amino acid sequence of the 61-kDa calmodulin-dependent, cyclic nucleotide phosphodiesterase (CaM-PDE) from bovine brain has been determined. The native protein is a homodimer of N^{α}-acetylated, 529-residue polypeptide chains, each of which has a calculated molecular weight of 60755. The structural organization of this CaM-PDE has been investigated with use of limited proteolysis and synthetic peptide analogues. A site capable of interacting with CaM has been identified, and the position of the catalytic domain has been mapped. A fully active, CaM-independent fragment (M_r = 36000), produced by limited tryptic cleavage in the absence of CaM, represents a functional catalytic domain. N-Terminal sequence and size indicate that this 36-kDa fragment is comprised of residues 136 to ≈450 of the CaM-PDE. This catalytic domain encompasses a ≈250 residue sequence that is conserved among PDE isozymes of diverse size, phylogeny, and function. CaM-PDE and its PDE homologues comprise a unique family of proteins, each having a catalytic domain that evolved from a common progenitor. A search of the sequence for potential CaM-binding sites revealed only one 15-residue segment with both a net positive charge and the ability to form an amphiphilic α-helix. Peptide analogues that include this amphiphilic segment were synthesized. Each was found to inhibit the CaM-dependent activation of the enzyme and to bind directly to CaM with high affinity in a calcium-dependent manner. This site is among the sequences cleaved from a 45-kDa chymotryptic fragment that has the complete catalytic domain but no longer binds CaM. These results indicate that residues located between position 23 and 41 of the native enzyme contribute significantly to the binding of CaM although the involvement of residues from additional sites is not excluded.

Changes in the intracellular concentration of free calcium ions can alter cyclic nucleotide metabolism through the action of the calcium/calmodulin complex (CaM)[1] on calmodulin-stimulated cyclic nucleotide phosphodiesterases (CaM-PDEs). CaM binds with high affinity and greatly increases the rate of cAMP or cGMP hydrolysis, thus providing a mechanism whereby stimuli that alter intracellular calcium concentrations (e.g., via phosphoinositide turnover) can modulate cellular responses that are mediated by changes in cyclic nucleotide concentrations.

CaM-PDEs constitute one of the five different families of cyclic nucleotide phosphodiesterase (PDE) isozymes [for reviews, see Beavo and Reifsnyder, 1990; Wang et al., 1990]. In mammals, there are at least five distinct isozymes within the CaM-PDE family. These are distinguished by apparent size, immunoreactivity, ability to be phosphorylated in vitro by protein kinases, affinity for CaM, and in some cases kinetic parameters (Beavo, 1988). The two major isozymes found in bovine brain are homodimers with subunit molecular weights of 61000 and 63000 (Sharma et al., 1984; Hansen & Beavo, 1986). The two brain isozymes can be readily resolved from one another and purified by procedures employing isozyme and conformation-specific monoclonal antibodies (Hansen et al., 1988). This study utilizes the 61-kDa isozyme since it is generally obtained in higher yield.

The present study provides the complete amino acid sequence of the 61-kDa CaM-PDE. The structural relationship among the mammalian CaM-PDE isozymes has not been elucidated, and it is not known whether these isozymes are

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[1] Abbreviations: PDE, cyclic nucleotide phosphodiesterase; BNPS-skatole, an acronym for the reagent described by Fontana et al. (1973); CaM, calcium/calmodulin complex; CaM-PDE, calmodulin-dependent cyclic nucleotide phosphodiesterase; CAP, catabolite activator protein, CM, S-carboxymethyl; HPLC, high-performance liquid chromatography; TPCK, N^{α}-*p*-tosyl-L-phenylalanine chloromethyl ketone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MOPS, 3-(*N*-Morpholino)propanesulfonic acid; Pth, phenylthiohydantoin; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.

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products of multiple genes or of a single gene that has undergone posttranslational (either biological or artifactual) or posttranscriptional processing. The 61-kDa CaM-PDE sequence is necessary for clarifying the structural relationship among the CaM–PDE isozymes as described in the following paper Novack et al. (1991). This primary structure also forms the basis for structure–function analyses and investigations of the mechanism of CaM activation. Toward these goals, this study provides evidence for the location of the CaM-binding site and catalytic domain of the enzyme.

Experimental Procedures

TPCK–trypsin, *Staphylococcus aureus* V-8 protease, and chymotrypsin were purchased from Cooper Biomedical, Miles, and Worthington, respectively. *Achromobacter* protease I was a gift of Dr. T. Masaki (Ibaraki University, Japan). Cyanogen bromide and citraconic anhydride were obtained from Eastman Kodak; trifluoroacetic acid was from Pierce Chemical Co. Iodoacetic acid from Sigma was twice recrystallized from chloroform. Poly(vinylidene difluoride) (PVDF) membranes (Immobilon) were purchased from Millipore.

Preparation of Reduced and Alkylated 61-kDa CaM-PDE. The 61-kDa CaM-PDE isozyme was purified from bovine brain with use of immobilized monoclonal antibodies (ACC-1 and ACAP-1) according to the large-scale protocol (procedure B) of Hansen et al. (1988). The quality and purity of enzyme preparations were evaluated by 12% SDS–PAGE (Laemmli, 1970) and specific activity (Hansen et al., 1988). Purified 61-kDa CaM-PDE was concentrated for S-carboxymethylation by precipitation with 10% (w/v) trichloroacetic acid or 70% ammonium sulfate (added as solid). The precipitate was dissolved in 0.5–2.5 mL of 6 M guanidine hydrochloride, 0.5 M Tris-HCl, 10 mM EDTA, 10 mM dithiothreitol, pH 8.2, reduced at 37 °C for 1 h, and carboxymethylated as described by Charbonneau (1989). SDS–PAGE revealed traces of low molecular weight contaminants that were removed by HPLC size-exclusion chromatography on tandem TSK-G4000SW columns equilibrated and eluted in 6 M guanidine hydrochloride, 10 mM sodium phosphate buffer (pH 6) at a flow rate of 0.5 mL min^{-1} or by conventional chromatography on a Sephacryl S-300 column. The overall yield from the preparation of CM-protein was low (10–40%), in part due to the artifactual formation of the low molecular weight fragments. CaM-PDE concentration was routinely determined by amino acid analysis.

Specific Cleavage of CM-61-kDa CaM-PDE. CM-protein was cleaved at methionyl and lysyl residues with use of CNBr and *Achromobacter* protease I, respectively, as described by Le Trong et al. (1990). Arginyl bonds were cleaved by trypsin following N-citraconylation of lysines according to procedures described by Le Trong et al. (1990). Intact CM-protein was cleaved at glutamate residues with staphyloccal V-8 protease in 4 M urea, 50 mM ammonium bicarbonate, pH 8. The protease was added in two equal aliquots to give a final protease:substrate ratio (w/w) of 1:25, and the mixture was incubated at 37 °C for 30 h. Chemical cleavage at aspartyl residues in the intact protein was performed in 2% formic acid for 4 h at 110 °C in tubes sealed under vacuum (Inglis, 1983). Subdigestion of large peptides at glutamyl bonds was carried out for 8–12 h as described above but in 2 M urea with a protease:substrate ratio of 1:50. Chymotryptic subdigestion conditions were the same as those for trypsin except that 2 mM *p*-aminobenzamidine was included.

Internal sequence from the lysyl peptide (K19/20) was obtained after N-acetylating the peptide and cleaving at a single tryptophan (Fontana et al., 1973). Cleavage was performed directly on the glass fiber filter used in the Model 470A gas-phase sequencer. The peptide was dried on the filter, treated with 25 μL of 12.5% (v/v) trimethylamine, and dried under vacuum. To the filter was added 15 μL of ice-cold 12.5% trimethylamine, followed immediately by 15 μL of acetic anhydride. The filter was covered, incubated for 30 min at room temperature, dried under vacuum, wet with 25 μL of water, dried, wet with 25 μL of 50% (v/v) acetic acid, and dried again. Cleavage was performed by wetting the filter with 2 mM BNPS-skatole in 50% acetic acid and incubating in a sealed vessel at room temperature for 12 h in the dark. This treatment was repeated for 36 h with 4 mM BNPS-skatole. The filter was dried, placed in the sequencer, and washed by manual delivery of butyl chloride (S3), ethyl acetate (S2), and heptane (S1) before the automated Edman degradation was initiated.

Peptide Purification. The purification of peptides from digests of the intact protein was facilitated by the use of a two-step procedure (Titani et al., 1986). Following-size exclusion HPLC chromatography, peptides of similar size were pooled together (Figure 1) and further purified by reversed-phase HPLC using C3 (Ultrapore RPSC; 4.6 × 75 mm) or C4 (Vydac, 4.6 × 250 mm) columns for large or moderate size peptides or a C18 column (Synchropak RP-P, 4.1 × 250 mm) for the smallest peptides. Size-exclusion columns were eluted with buffers containing 6M guanidine hydrochloride (Titani et al., 1986), and reversed-phase columns were eluted with linear gradients of 0.1% trifluoroacetic acid/acetonitrile using a Varian 5000 liquid chromatograph equipped with a variable-wavelength detector. With subdigests or low-level primary digests (<3 nmol of peptide), reversed-phase HPLC was performed with narrowbore columns (2.1 mm) with a Hewlett-Packard 1090 M chromatograph equipped with a diode array detector. For this purpose, 100-mm Brownlee Aquapore RP-300 (C8), RP-8 (C8), and RP-18 (C18) columns were eluted at a flow rate of 300 μL/min. Certain critical peptides of 10 or less residues (e.g., M9, K6) were lost in the guanidine-containing flow-through fractions of reversed-phase HPLC columns. To avoid this problem, size-exclusion chromatography was performed with two tandem TSK-3000 PW columns equilibrated in a volatile solvent (0.1% trifluoroacetic acid/40% acetonitrile) and eluted at a flow rate of 1.0 mL/min.

Automated Sequence Analysis and Amino Acid Analysis. Peptides available in quantities greater than 1 nmol were sequenced with a Beckman Model C spinning cup sequencer whereas those peptides available at 1 nmol or less were analyzed on an Applied Biosystems Model 470A gas-phase sequencer according to procedures described by Le Trong et al. (1990). Amino acid analyses were obtained by the reversed-phase separation of the phenyl thiocarbamoyl derivatives on a Waters Picotag system as described by Bidlingmeyer et al. (1984).

Sequence Analysis by Tandem Mass Spectrometry. Mass spectra were recorded on both a triple-quadrupole mass spectrometer (TSQ) (Hunt et al., 1986) and a quadrupole Fourier transform mass spectrometer (QFTMS) (Hunt et al., 1987a). The operation of these instruments and sample preparation for mass analysis have been described previously (Hunt et al., 1986, 1987a, 1989). Sequence analysis of peptides in the mass range up to 1800 Da was performed by collision-activated dissociation (CAD) on the triple-quadrupole instrument. Laser photodissociation on the quadrupole Fourier transform instrument (Hunt et al., 1987b) was employed to sequence peptides in the mass range above 1800 Da. The

peptide K1 was subdigested with *S. aureus* V-8 protease, and the resulting fragments were reanalyzed on one of the above instruments to confirm the amino acid sequence. The methyl ester derivatives of peptides were prepared as previously described (Hunt et al., 1989).

Limited Proteolysis. Limited tryptic or chymotryptic proteolysis of the 61-kDa CaM-PDE was performed according to procedures similar to those described by Kincaid et al. (1985). Prior to proteolysis, purified 61-kDa CaM-PDE was concentrated and trace CaM was removed by ion-exchange chromatography in EGTA-containing buffers as described below. For these experiments CaM-PDE concentration was estimated by the method of Bradford (1976) with bovine serum albumin as a standard. CaM-PDE (240 μg/mL) was incubated with trypsin (PDE/trypsin, 100:1 w/w) in 40 mM Tris, pH 8, 2 mM EGTA, 0.2 M NaCl at 30 °C. The reaction was terminated at 5, 10, 20, and 30 min by transferring aliquots containing 12 μg of CaM-PDE to an equal volume of 20% (w/v) trichloroacetic acid. After centrifugation, the precipitates were dissolved in SDS–PAGE sample buffer and electrophoresed with running gels of 10% (w/v) acrylamide (Laemmli, 1970). For enzymatic assays, aliquots of the digest (1 μg of CaM-PDE) were mixed with lima bean trypsin inhibitor to give a final ratio of inhibitor:trypsin of 1000:1. These samples were diluted 10-fold with assay buffer, and 2 μL was used to measure PDE activity with the colorimetric procedure described below. Chymotryptic proteolysis (PDE/protease, 100:1 w/w) was performed in a similar manner except that the reaction buffer also contained 10% glycerol. Limited proteolytic fragments from SDS–polyacrylamide gels were transferred to PVDF membranes in a Bio-Rad gel-transfer apparatus with CAPS buffer at pH 11 as described by Matsudaira (1987). The transfer was performed with a current of about 0.3 A for 2 h at 4 °C. After transfer membranes were stained with Coomassie Blue R-250 according to the protocol of Matsudaira (1987).

Peptide Synthesis. The Merrifield (Barany & Merrifield, 1979) solid-phase synthesis strategy was employed to prepare two peptides, P61C15 and P61C16, corresponding to residues 23–44 and 20–41 of the CaM-PDE sequence, respectively. The carboxy-terminal amide form of the peptide P61C15 was synthesized on a Biosearch peptide synthesizer, and the peptide P61C16 was prepared with a free carboxy terminus on an Applied Biosystems Model 430A peptide synthesizer. Both peptides were synthesized with use of t-Boc amino acids and were then side-chain deprotected and cleaved from the resin with anhydrous HF (Blumenthal & Krebs, 1987). Peptides were purified by preparative reversed-phase HPLC on a Waters Deltapak C18 column (7.8 × 300 mm) eluted with a trifluoroacetic acid/acetonitrile gradient at a flow rate of 3.0 mL/min with a Varian 5000 chromatograph. Purity was assessed by narrowbore reversed-phase chromatography on a Brownlee RP-300 column. The sequences of peptides were verified by gas-phase amino acid sequencing.

Peptide Inhibition of the CaM-Dependent Activation of CaM-PDE. A preparation of 61-kDa CaM-PDE (containing less than 5% of the 63-kDa isozyme) was purified from bovine brain with use of ACC-1 immunoaffinity chromatography as described in procedure A of Hansen et al. (1988). To remove CaM and concentrate, the EGTA eluate of the ACC-1 column was loaded onto a DEAE-Sephacryl column (1–2 mL) equilibrated in 20 mM Tris, pH 8, 1 mM EGTA, 1 mM $MgCl_2$ and eluted with column buffer containing 0.2 M NaCl. Phosphodiesterase activity was assayed colorimetrically by following phosphate released from reactions conducted in the wells of a microtiter plate as described by Gillepsie and Beavo (1989). Reaction mixtures were incubated for 10 min at 30 °C with 1 mM cAMP in 100 μL of reaction buffer containing 20 mM Tris, pH 8, 20 mM imidazole, 3 mM $MgCl_2$, 0.2 mg/mL bovine serum albumin, 200 μM $CaCl_2$, 2.7 mM CaM, 0.3 nM enzyme, and variable quantities of synthetic peptide analogues. Peptide was added to premixed calmodulin and enzyme, and the samples were incubated at 30 °C for 20 min before the reaction was initiated by the addition of cAMP. In the absence of peptide, 2.7 nM calmodulin gave 75% of full CaM stimulation, which was about 8-fold greater than the rate measured in the presence of 2 mM EGTA.

CaM–Sepharose Chromatography of Peptide Analogues. Peptide analogues (10–20 nmol) were loaded on CaM-Sepharose columns (1.0 mg CaM/mL; 0.7 × 1.7 cm) equilibrated in buffer A (50 mM Tris, pH 8, 150 mM KCl, 0.5 M NaCl, 100 μM $CaCl_2$). After loading, columns were washed with ≈5 column volumes of buffer A and eluted with 5 column volumes of buffer A containing 2 mM EGTA instead of 100 μM $CaCl_2$. Fractions from each stage of chromatography were pooled, and the peptide present was quantitated by reversed-phase HPLC chromatography monitored at 206 nm.

Results

General Sequencing Strategy. The intact CM-protein was cleaved at arginyl, methionyl, and lysyl residues to produce three sets of unique peptides. Since a relatively large number of peptides was expected from these cleavages, all digests were initially fractionated by size-exclusion chromatography (Figure 1) in order to simplify subsequent reversed-phase purifications. Internal sequences within large peptides were determined by analysis of fragments produced by further enzymatic or chemical cleavage. Sequence analysis of the products from the three primary digests (Figure 1) and their subfragments identified 457 residues and provided the sequence of six large, nonoverlapping segments. The sequence analysis was completed by overlapping these six segments with peptides derived from the cleavage of intact protein at glutamyl or aspartyl residues. The reduced and alkylated intact protein was refractory to Edman degradation, and each of the three primary digests generated a peptide in good yield that gave no Pth-amino acids upon sequencing, indicating that the amino terminus of the protein was blocked. Tandem mass spectrometry was employed to determine the N-terminal sequence (residues 1–16) and identify the blocking group (see below).

Proof of Sequence for the 61-kDa CaM-PDE. The detailed proof of sequence for the 61-kDa CaM-PDE is shown in Figure 2 and is self-evident. The 61 kDa CaM-PDE from bovine brain comprises 529 residues with an N^{α}-acetylated Gly residue at the amino terminus. No evidence of additional post-translational modifications was found. The calculated monomeric molecular weight of 60755 is in good agreement with the values of 60000–61000 determined by SDS–PAGE (Hansen & Beavo, 1986; Sharma et al., 1984). Our sequence determination indicates that the dimeric native enzyme (Sharma et al., 1984; Kincaid et al., 1984) must be a homodimer since there was no evidence of sequence microheterogeneity.

In an earlier communication, partial sequence data (254 residues) were presented in a preliminary search for evolutionarily conserved segments (Charbonneau et al., 1986). The present sequence corrects four errors in that data, specifically where His 236 had been reported as Gly (44), Asn 320 as Trp (130), and Leu-His 375-376 as Lys-Leu (196-197).

All peptide sequences required for the proof (Figure 2) were directly established from Edman degradations. Over 96% of

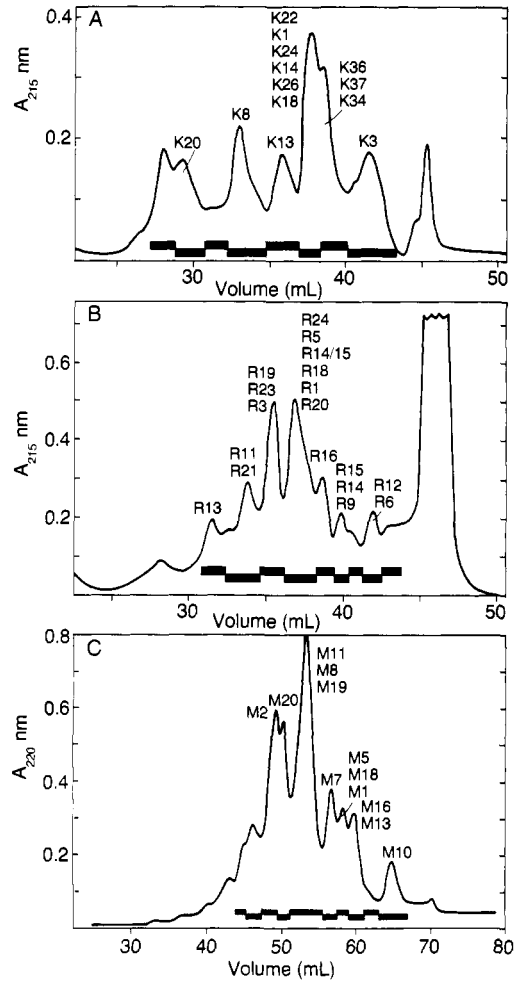

FIGURE 1: Size-exclusion chromatography (in 6 M guanidine hydrochloride, 10 mM sodium phosphate, pH 6), of peptides generated from the CM-protein by cleavage at lysine (A), arginine (B), and methionine (C). Panels A and B illustrate separations on tandem TSK-2000–2000SW columns at flow rates of 0.4 and 0.5 mL/min, respectively. In panel C the separation is on tandem TSK-3000–2000–2000SW columns at 0.4 mL/min. The names of the peptides in each pooled fraction are indicated in order (from the top) of elution from subsequent reversed-phase chromatography. Peptides are numbered in order from the amino terminus assuming complete and residue-specific cleavage. Many of these peptides are not illustrated in Figure 2.

the residues were identified in two or more distinct peptides; in most cases, overlaps included a minimum of three residues. Amino acid compositions were not required to establish the sequence and were only used as supporting evidence for the C-terminal sequence. This proof of structure is strengthened by the use of a number of redundant observations; nevertheless, several regions of the sequence presented special problems and were established with less certainty as described below.

The overlap of M2–D10 and M5 near residue 104 (Figure 2) presented a problem because of the difficult sequence KMGMMKKK. The overlap provided by peptide D10 was tentative because of a low initial yield, presumably due to partial modification and blocking of the N-terminal tryptophan (Mahoney, 1985). The one-residue overlap of R14 and R15 near position 284 is supported only by the tentative sequence of peptide R14/15 that was deduced from a peptide mixture. The contaminants (70% of the mixture) were products of incomplete digestion at Arg 72 with sequences corresponding to residues 72–93 and 73–93. Similarly, the overlap of M16–K3 and R20 includes only two residues and is supported only by the tentative sequence of K26, which degraded poorly, perhaps due to the His-His residues near its N-terminus. The critical overlap of M10 and M11 at residue 252 was difficult to obtain primarily because subfragments of K20 were obtained in low yield due to their tendency to adsorb to surfaces during purification. These problems were finally avoided by acetylating K20 and digesting it at its single Trp residue on a glass fiber filter (see Experimental Procedures). The single new sequence observed, K20-W2, provided the required overlap.

Complete cleavage of the intact CM-protein at glutamyl bonds with staphyloccal V-8 protease proved to be impossible despite the use of 4 M urea, long digestion times, and up to a 1:15 (w/w) protease:substrate ratio. Fragments of molecular weight 20 000–45 000 were observed in the digest, and no peptides derived from residues 80–288 were detected. It is not clear why the intact CaM-PDE was resistant to cleavage by V8 protease.

Sequence Analysis of the N- and *C-Termini.* Since the N-terminus of the protein is blocked, mass spectrometry was used to determine the sequence of the blocked peptide K1. A spectrum of K1 recorded on a quadrupole Fourier transform (QFTMS) instrument showed an abundant $(M + H)^+$ ion at m/z = 1800.3, average mass. Laser photodissociation of the $(M + H)^+$ ion (m/z 1870.2, average mass) observed for the corresponding methyl ester generated the fragments required to deduce the amino acid sequence of K1 and demonstrate the presence of an *N*-acetyl blocking group (Figure 3). Predicted masses for fragment ions of type b and y (Biemann, 1988) are shown, respectively, above and below the structure in Figure 3. Those observed in the spectrum are underlined. Confirmation of the above sequence was obtained by subdigesting K1 with *S. aureus* V-8 protease and by sequencing each of the resulting five peptides by the technique of collision-activated dissociation on a triple-quadrupole instrument (see Experimental Procedures). The amino acid sequences for all five peptides were in complete agreement with the results obtained from the above laser photodissociation experiment. Peptide K1 contains a five-residue overlap with peptide E6 (Figure 2).

The identification of the C-terminus is somewhat tentative since it relies principally on termination of the Edman degradation. R24 was the only isolated product of cleavage at arginyl bonds that lacked Arg; similarly only M20 in the CNBr digest lacked homoserine, suggesting that both peptides are derived from the C-terminus. The amino acid composition (data not shown) of R24 is in good agreement with that predicted from the sequence of residues 500–529, suggesting that no additional amino acids are present at the C-terminus.

Structural Relationship to PDEs and Other Proteins. A search of the Protein Identification Resource protein sequence database (PIR release 21.0) using the SEARCH program (Dayhoff et al., 1983) and a scan (GENEPRO software) of the GenBank database (release 61.0) for cognate nucleotide sequences revealed *no* proteins other than PDEs with sequence similarity to the 61-kDa isozyme. A single 250-residue segment (residues 193–446) from the 61-kDa CaM-PDE is homologous to corresponding regions of 12 other PDE isozymes with diverse substrate specificity, regulatory properties, and phylogenetic origins (Charbonneau et al., 1986; Beavo & Reifsynder, 1990). From these structural relationships, it is clear that the CaM-PDE is a member of a unique family of PDEs each of which bears one segment derived from a common progenitor. However, a low-affinity cAMP PDE encoded by the yeast PDE1 gene (Nikawa et al., 1987) and an extracellular PDE from *Dictyostelium* (Lacombe et al., 1986) are

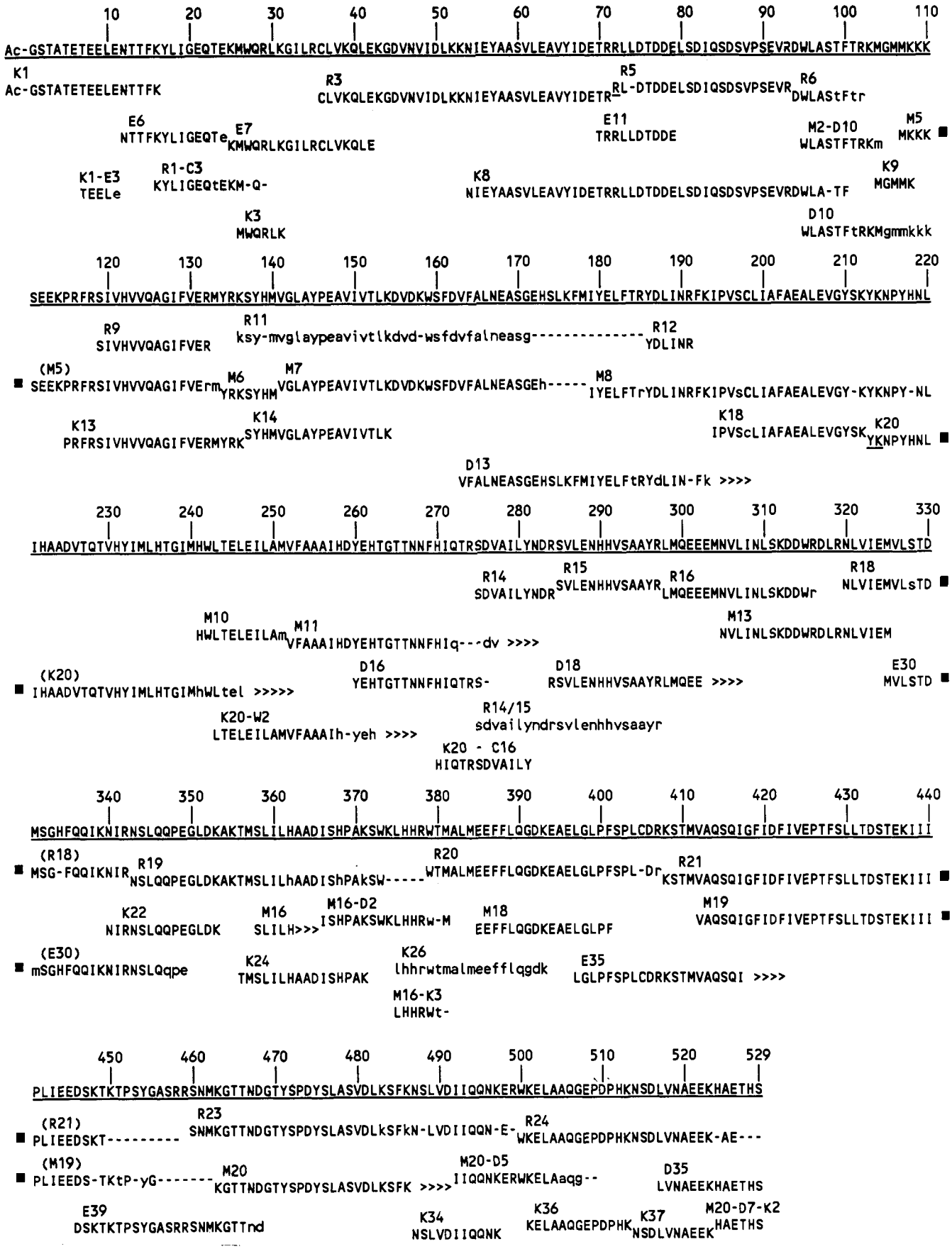

FIGURE 2: Summary of the proof of sequence for the 61-kDa CaM-PDE. The sequences of specific peptides are given in single-letter code below the summary sequence (underlined). Peptide names given above the sequences use the prefixes M, K, R, E, and D to designate peptides generated by cleavage at methionyl, lysyl, arginyl, glutamyl, and aspartyl bonds, respectively. Subpeptides have hyphenated suffixes, where C indicates products of cleavage with chymotrypsin. Peptide sequences in capital letters were proven by Edman degradation (except for that of K1, which was determined by mass spectrometry) while those in lower case letters were tentative identifications. A dash indicates positions where no identification was made, while >>> indicates a large peptide where the C-terminal sequence was not obtained. The ■ at the right end of a line indicates that the sequence of a peptide continues from the same symbol on the left of the line below.

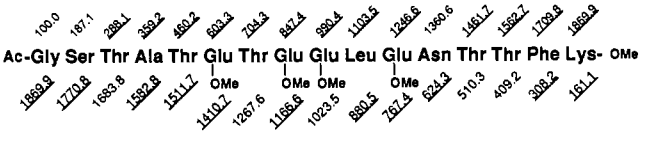

FIGURE 3: Amino acid sequence deduced from a laser photodissociation mass spectrum recorded on the $(M + H)^+$ ion (m/z 1870.2, average mass) derived from the methyl ester of N-blocked lysyl peptide K1. Predicted masses for fragment ions of type b and y are shown above and below the structure, respectively. Those observed in the spectrum are underlined.

homologous to one another but *not* to the conserved segment of the 61-kDa CaM-PDE or its homologues.

Mapping of the 36- and 45-kDa CaM-Independent Fragments. In the absence of Ca^{2+}/CaM, limited tryptic and chymotryptic proteolysis of CaM-PDE produces fragments (36 and 45-kDa, respectively) that are fully activated, unaffected by CaM, and unable to bind to CaM–Sepharose (Krinks et al., 1984; Kincaid et al., 1985). The position of the 36- and 45-kDa fragments was mapped by N-terminal sequencing in order to delimit boundaries for the functional domains of the 61-kDa CaM-PDE. The 36- and 45-kDa fragments were generated by limited tryptic or chymotryptic cleavage of the PDE in the presence of EGTA with the use of conditions similar to those of Kincaid et al. (1985). The proteolysis reactions were monitored at several time points between 5 and 30 min by both SDS–PAGE and enzymatic assays (data not shown). In agreement with previous reports (Krinks et al., 1984; Kincaid et al., 1985), the appearance of the 36- and 45-kDa fragments was accompanied by an activation of the enzyme and a complete loss of CaM-dependence. By 5 min, the stable 36- and 45-kDa fragments were the major species observed upon SDS–PAGE analysis. Other investigators (Krinks et al., 1984; Kincaid et al., 1985) measured activities for these CaM-independent fragments that were equal to those of the maximally stimulated native PDE; however, in our experiments both fragments were about 2–3-fold more active than the CaM-stimulated enzyme. The reason for these differences is not understood at present.

Each of the fragments was resolved from other minor proteolytic products by SDS–PAGE, electroblotted onto PVDF membranes (Matsudaira, 1987), and subjected to the Edman degradation. Sequence analysis of the 36-kDa fragment yielded two related sequences in a 3:2 ratio. The predominant sequence observed was Lys-Ser-Tyr-Xaa-Met-Val-Gly-Leu-Ala-Tyr, which corresponds to residues 136–145 of the 61-kDa CaM-PDE (Figure 2). The minor sequence was identical except that it was one residue shorter at its N-terminus beginning with a Ser indicating that trypsin cleaves after both Arg 135 and Lys 136 (Figure 2). On the basis of its M_r (derived from SDS–PAGE), the C-terminus of the 36-kDa fragment is predicted to correspond to either Lys 437, 448, or 450 or Arg 458 of the native enzyme (Figure 2). These data indicate that trypsin severs the parent molecule at a minimum of two protease-sensitive sites leading to the loss of about 135 residues from the N-terminus and about 90 residues from the C-terminus. The amino-terminal sequence of the major chymotryptic fragment (45 kDa) was identified as His-Xaa-Val-Gly-Leu-Ala-Tyr, which indicates cleavage after Tyr 138. A minor fragment (about 20% of the total signal) possessed four additional N-terminal residues and was derived from cleavage after Tyr 134. An apparent M_r of 45 000 (determined by SDS–PAGE) suggests that the 45-kDa fragment is comprised of residues 139 to $\approx$ 529. It should be noted that molecular weights derived from SDS–PAGE can be erroneous and that the identity of the C-termini of these fragments must be confirmed by more direct procedures.

Identification of Potential CaM-Binding Sites. Sequences that bind CaM with high affinity in a calcium-dependent manner typically have the potential to form basic, amphiphilic α-helices (O'Neil & DeGrado, 1990). Potential CaM-binding sites from the 61-kDa CaM-PDE were identified with use of a search procedure similar to that originally described by Erickson-Viitanen & Degrado (1987), who used three parameters, the mean helical hydrophobic moment ($\langle\mu_h\rangle$), the average hydrophobicity ($\langle$Hb$\rangle$), and the net charge, as indicators of a segment's ability to bind CaM. As Erickson-Viitanen & Degrado (1987) have demonstrated, peptides or protein segments that bind CaM with high affinity have a net charge greater than +4, $\langle\mu_h\rangle$ values greater than 0.4, and $\langle$Hb$\rangle$ within the range of −0.2 to +0.3 kcal/mol. Initially, 15-residue segments with a net charge of +4 or greater were identified by use of the CHARGE routine from the GENEPRO software package (Riverside Scientific, Seattle) that assigns a value of +1 to Arg and Lys, −1 to Asp and Glu, and +0.5 to His. Each segment with a net positive charge of +4 or greater was further screened for amphiphilic character by calculating its $\langle\mu_h\rangle$ and $\langle$Hb$\rangle$ values. The *only* segments of the CaM-PDE that fulfilled the criteria given above were centered between residues 32 and 36. A segment spanning residues 27–41 (Figure 2) was considered the best candidate since its net charge, $\langle\mu_h\rangle$, and $\langle$Hb$\rangle$ values of +4, +0.62, and −0.1 kcal/mol, respectively, were most similar to those of known high-affinity CaM-binding sequences.

Peptide analogues corresponding to two distinct 22-residue segments surrounding the potential CaM-binding region (residues 27–41) were chemically synthesized and tested for their ability to bind CaM in a calcium-dependent manner. P61C15, an analogue of residues 23–44 (TEKMWQRLK-GILRCLVKQLEKG-NH_2) was synthesized with an isosteric Nle residue substituted for Met-26 (to avoid oxidation) and a C-terminal α-amide. A second peptide, P61C16, corresponding to residues 20–41 (GEQTEKMWQRLK-GILRCLVKQL), was synthesized with Met and an unmodified C-terminus. Peptide analogues P61C15 and P61C16 each possess two negatively charged residues within sequences adjacent to the basic, amphiphilic core segment (residues 27–41) but retain an overall net charge of +3 or greater. It should be noted that CaM-binding domains from several other target enzymes (e.g., adenylate cyclase, calcineurin, phosphofructokinase, and caldesmon) contain at least one negatively charged residue [see Figure 2 in O'Neil and DeGrado (1990)].

Synthetic Peptide Analogues Bind CaM with High Affinity. The data of Figure 4 shows that nanomolar concentrations of P61C15 inhibited the CaM-dependent activation (2.7 nM CaM) of CaM-PDE with an IC_{50} of 27 nM. Control experiments demonstrated that 27–540 nM peptide did not affect basal PDE activity measured in the presence of 2.0 mM EGTA (data not shown). Furthermore, addition of excess CaM (4.7 μM) overcame the inhibition from 270 nM peptide and gave maximal activation of the PDE. These results suggest that P61C15 inhibits the activation of CaM-PDE by binding directly to Ca^{2+}/CaM and preventing interaction of CaM with the corresponding residues 23–44 of PDE. In similar experiments, the other peptide analogue, P61C16, also inhibited CaM activation with an IC_{50} of 37 nM, indicating that substitution of Nle for Met has little effect on the ability of these peptides to inhibit activation of CaM-PDE.

CaM–Sepharose chromatography was used to demonstrate a direct interaction between CaM and these peptide analogues. When 18 nmol of the P61C16 peptide was applied to the

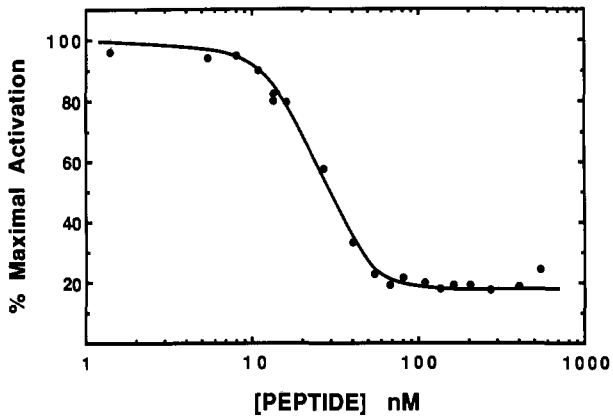

FIGURE 4: Inhibition of the CaM-dependent activation of the 61-kDa CaM-PDE by the synthetic peptide P61C15. PDE assays were performed by use of the colorimetric procedure described in the Experimental Procedures section. Activity was measured in the presence of calcium and a fixed subsaturating concentration of CaM (2.7 nM) with the indicated concentrations of peptide. The percent of maximal activation is defined as follows: $(R_o - R_e/R_m - R_e) \times 100$, where *Ro* is the rate observed in the presence of added peptide, R_e is the basal rate in the presence of 2 mM EGTA, and R_m is the rate measured without added peptide. Each data point is the mean of three determinations. The curve was drawn by simple visual inspection of the data. Peptide concentration was determined by amino acid analysis. The concentrations of CaM and synthetic peptide (P61C15) were determined by UV absorbance measurements using an extinction coefficient at 276 nm of 3300 M^{-1} cm^{-1} and at 280 nm of 5555 M^{-1} cm^{-1}, respectively. Concentrations determined spectrophotometrically were in good agreement with values obtained by amino acid analysis.

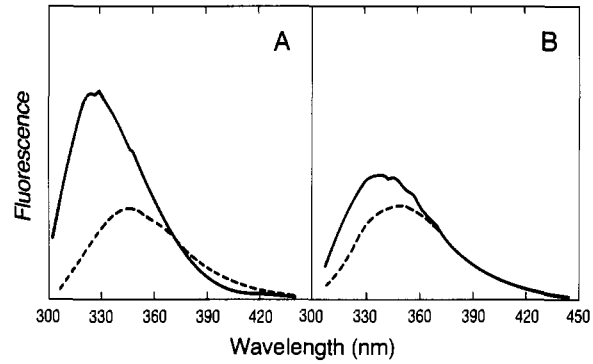

FIGURE 5: Effect of Ca^{2+}/CaM on the intrinsic Trp fluorescence of P61C15. Before spectra were obtained, P61C15 (2.1 μM) was dissolved in 2.0 mL of buffer (50 mM MOPS, pH 7.5, 0.1 M KCl, 0.1 mM $CaCl_2$, 1 mM DTT) and incubated at 37 °C for 2 h to ensure complete reduction of peptide thiol. Panel A shows the uncorrected spectra of P61C15 in buffer containing 0.2 mM Ca^{2+} before (---) and after (—) the addition of 6.2 μM CaM. Panel B shows the fluorescence of P61C15 (2.1 μM) in buffer with 2 mM EGTA before (---) and after (—) the addition of 6.2 μM CaM. Fluorescence spectra were obtained with a Perkin-Elmer MPF 44A fluorometer. An excitation wavelength of 295 nm was used in order to reduce Tyr emission from CaM that was present in most experiments. Excitation and emission bandwidths were 10 and 5 nm, respectively. Spectra were not corrected for the wavelength dependence of the monochromator and detector.

column in the presence of 0.5 M NaCl as described in the Experimental Procedures section, 94% of the peptide loaded was retained on the column after washing with Ca^{2+} buffer. Subsequent treatment of the column with EGTA-containing buffer released 93% of the bound peptide. In similar experiments with 1.0 M instead of 0.5 M NaCl, all of the P61C15 peptide loaded was retained in the presence of Ca^{2+} and about 50% was eluted with EGTA. In contrast, when low ionic strength buffers (no added NaCl) were used, P61C16 was bound to the column but was not eluted with EGTA; only 2% was released by 2 M urea plus EGTA. These experiments demonstrate a direct interaction between immobilized Ca^{2+}/CaM that was stable in high ionic strength buffers (up to 1.0 M NaCl). If Ca^{2+} was removed, the peptide/apo-CaM complex persisted both in low ionic strength buffers and in 2 M urea but was disrupted by high ionic strength (0.5–1.0 M NaCl or 6M guanidine hydrochloride). Thus reversible, Ca^{2+}-dependent binding of peptides on CaM–Sepharose was observed only in the presence of buffers of high ionic strength.

Additional evidence for a direct interaction between CaM and P61C15 was also provided by examining the effect of CaM on the intrinsic tryptophan fluorescence of the peptide (bovine CaM has no tryptophan). As shown in Figure 5 (panel A), the addition of CaM to peptide produces a 21-nm blue shift in the λ_{max} and a 2.3-fold enhancement in the maximum fluorescence intensity. The observed perturbation of fluorescence suggests that the tryptophan residue is exposed to a less polar environment as it is bound and/or that its mobility is restricted. Spectra obtained in the presence of 1 mM EGTA (Figure 5, panel B) demonstrate that there is an interaction between peptide and CaM in the absence of Ca^{2+}. However, the extent of fluorescence perturbation is much less pronounced with a 1.3-fold increase in intensity at the λ_{max} and a 12-nm blue shift. The difference in the extent of the fluorescence perturbation suggests that the Trp residue is bound in a different mode in the absence of Ca^{2+}. This observation is consistent with the results of CaM–Sepharose chromatography, which also shows a Ca^{2+}-independent interaction between peptide and CaM at low ionic strength.

Both CaM–Sepharose chromatography and Trp fluorescence studies suggest that in the absence of Ca^{2+} and in low ionic strength buffers a complex is formed between apo-CaM and the model peptides. Fluorescence spectra (Figure 5) suggest that the apo-CaM/peptide and Ca^{2+}-CaM/peptide complexes have distinct structural features since the Trp residue appears to reside in different local environments within each complex. The complex formed in the absence of Ca^{2+} is disrupted by increased ionic strength, indicating a significant electrostatic component. Synthetic peptide analogues of two other target enzymes display similar behavior since they interact with apo-CaM to form complexes that are structurally distinct from those formed with Ca^{2+}/CaM (DeGrado et al., 1987; Klevit et al., 1985). Although no such dependence on ionic strength has been reported, apo-CaM binds native CaM-PDE with an estimated K_d of 80 mM (Olwin & Storm, 1985).

A titration of 2.1 μM P61C15 with CaM (Figure 6) shows that the fluorescence enhancement increases linearly with increasing CaM and plateaus when the CaM:P61C15 ratio is approximately 0.5. The bimodal linearity in the plot (Figure 6) and the sharp transition indicate that CaM binds 2 mol of peptide with a submicromolar dissociation constant. Although the experiments were not designed for a quantitative determination of the dissociation constant, both the inhibition and fluorescence studies are consistent with a $K_d \leq 50$ nM. Taken together these experiments demonstrate that in the presence of Ca^{2+} these peptide analogues are capable of binding CaM with high affinity. Thus, the linear sequence comprising residues 23–41 of the CaM-PDE may form a major recognition site for CaM.

Discussion

The primary sequence of the 61-kDa CaM-PDE presented here provides the molecular basis for describing the domain organization of the enzyme and elucidating the mechanism by which CaM activates the enzyme. The domain substructure

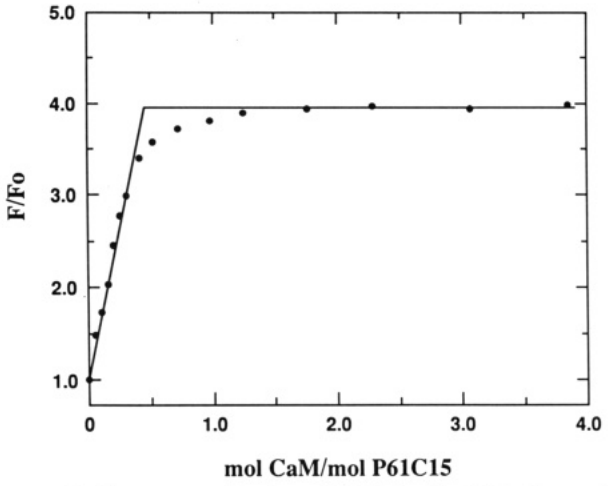

FIGURE 6: Fluorescence titration of P61C15 with CaM. Successive aliquots of a CaM stock solution were added to P61C15 (2.1 μM) in 2.0 mL of 50 mM MOPS, pH 7.5, 0.1 M KCl, 0.1 mM $CaCl_2$, 1 mM DTT buffer. The peptide solution was incubated at 37 °C for 3 h before initiation of the titration in order to achieve complete reduction. Tryptophan fluorescence was monitored at 325 nm with use of an excitation wavelength of 295 nm. The titration was conducted at room temperature without temperature control. Fluorescence signals are expressed as the ratio of observed fluorescence (F) to peptide fluorescence in the absence of CaM (F_0). The two lines were drawn by visual inspection of the data. The point of intersection was used to estimate the ratio of CaM:Peptide at which saturation occurs. Peptide and CaM concentrations were determined by amino acid analysis.

of this PDE was examined by mapping the position of several well-characterized limited proteolytic fragments (Kincaid, et al., 1985) and by using synthetic peptide analogues to provide evidence for the location of the CaM-binding region. As described in the following paper (Novack et al., 1991), the primary structure is also important for sequence comparisons that provide an understanding of the structural relationship among CaM-PDE isozymes.

Identification of the Catalytic Domain. Limited proteolysis studies have indicated that the CaM-binding and active sites of the 61-kDa CaM-PDE are located within separate domains. Limited tryptic proteolysis, performed in the absence of Ca^{2+}/CaM, generates a 36-kDa fragment that is active and CaM-independent. Of the active proteolytic fragments studied (Krinks et al., 1984; Kincaid et al., 1985), the 36 kDa is the smallest and as such best represents the catalytic domain. The N-terminus of the 36-kDa fragment was unequivocally placed at residue 136 by direct sequencing whereas the C-terminus was estimated to be residue 450, but only on the basis of size. Thus, the catalytic domain of the 61-kDa CaM-PDE is generated by the loss of 135 residues from the N-terminus and about 80 residues from the C-terminus. A fully activated and CaM-independent, 45-kDa chymotryptic fragment is also derived from cleavage within the same N-terminal, protease-sensitive region. However, its larger size indicates that the C-terminal region has not been excised.

As described above, residues 193–446 of the CaM-PDE comprise a segment that is conserved within several other PDE isozymes. This conserved segment is totally encompassed within the catalytic domain (see Figure 7) in support of previous suggestions that this region of homology contains the active site (Charbonneau et al., 1986). Recent limited proteolysis and direct photolabeling studies with a cGMP-stimulated PDE have identified a 36-kDa fragment that has properties of a fully functional catalytic domain (Stroop et al., 1989). This fragment also encompasses the 250-residue conserved segment found in the various PDE isozymes. These

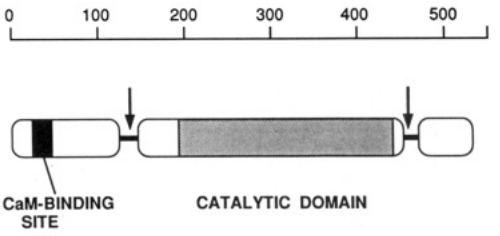

FIGURE 7: Model of the proposed domain organization within a single chain of the homodimeric 61-kDa CaM-PDE. The catalytic and other postulated domains are illustrated as boxes with narrow lines between them representing protease-sensitive interconnecting segments. The shaded area within the catalytic domain indicates the position of sequences that are homologous to other PDE isozymes. The arrows designate the sites of tryptic cleavage that generate the 36-kDa fragment. The C-terminal tryptic site is placed on the basis of M_r determined by SDS–PAGE. Residue numbers are indicated by the scale given at the top.

findings from two different isozymes clearly demonstrate that the segments conserved among this family of PDEs comprise residues that form the core structure of the catalytic domain.

Identification of a CaM-Binding Site. A systematic screen (Erickson-Viitanen & DeGrado, 1987) of the 61-kDa CaM-PDE sequence for segments having the ability to form basic, amphiphilic α-helices identified one potential binding site at residues 27–41. Two peptide analogues of the putative binding site displayed the following properties that provide evidence that residues 23–41 comprise at least a part of the CaM-binding site: (i) both peptide analogues inhibit the activation of PDE by CaM, (ii) both bind directly to CaM with high affinity (estimated $K_d \approx 30$ nM), (iii) at high ionic strength ($\geq$0.5 M NaCl), this binding is reversible and calcium-dependent, and (iv) the proposed site is contained within the only segment lost (residues 1–138) when the enzyme is converted to a 45-kDa fragment that no longer binds CaM.

This location for the proposed CaM-binding site is in accord with the known structural and functional differences between the 59- and 61-kDa isozymes. The 59-kDa isozyme has a 10–20-fold greater affinity for CaM. The only structural changes that can explain this difference in affinity occur within a 34-residue segment where eight positions within the proposed 61-kDa CaM-binding site have been replaced in the 59-kDa isozyme (Novack et al., 1991). In preliminary sequencing studies,[2] a 47-kDa chymotryptic fragment of the 61-kDa CaM-PDE, prepared in the presence of Ca^{2+}/CaM (Kincaid et al., 1985), appeared to be N-terminally blocked, suggesting that CaM-binding may prevent the proteolytic release of residues 1–138. This protection of the N-terminus is also consistent with the location of the proposed CaM-binding site. Finally, Nibhanupudy et al. (1988) reported that the treatment of CaM-PDE with an arginine-specific reagent decreased the affinity for CaM by about 100-fold without significantly altering basal activity. Kincaid (1984) described the preparation of a fully activated, disulfide-bonded complex between CaM-PDE and thiolated CaM. The presence of Arg 29, Arg 35, and Cys 36 within the proposed CaM-binding site can account for the results of these chemical modification studies.

The estimated K_d (30 nM) for peptide binding to Ca^{2+}/CaM is about 30-fold greater than that for binding native enzyme to CaM (Hansen & Beavo, 1986); this corresponds to a difference in binding energy (25 °C) of about 2 kcal/mol, which is roughly equivalent to the free energy of a single hydrogen bond. Thus, the linear sequence including residues 23–41 can provide most but not all of the contacts or inter-

[2] H.C., J.P.N., J.A.B., and K.A.W., unpublished results.

actions needed to account for the CaM-binding energy of the native PDE. Clearly, these studies can not exclude the possibility that other unidentified sites in the molecule may also participate in the interaction with CaM. For example, two distinct regions comprise the CaM-binding site of the γ-subunit of phosphorylase *b* kinase (Dasgupta et al., 1989) and only one of these is predicted to form a positive, amphiphilic α-helix. It should be noted that some differences between the peptide and native PDE may arise from limitations inherent in the use of short peptide analogues that may be unable to assume a conformation identical with that of the native protein because of the absence of appropriate long-range interactions.

Several studies indicate that one molecule of CaM is bound per subunit of the dimeric enzyme (Krinks et al., 1984; Wang et al., 1980). However, a titration of the peptide analogues with CaM (Figure 5) shows that the CaM:peptide stoichiometry is 1:2. This discrepancy in stoichiometry may be due to the tendency of the P61C15 peptide to aggregate. A salt-induced increase in Trp fluorescence intensity and an accompanying blue shift in emission maxima was noted during the course of our studies,[3] a phenomenon suggesting that the peptide may undergo a self-association reaction as observed for another high-affinity CaM-binding peptide, mellittin (Talbot et al., 1979). Thus, it is possible that there is a single site on CaM for PDE docking but that the peptide analogue is bound as a dimer due to its tendency to aggregate. Alternatively, since CaM is bimodal in structure with potential binding domains at each end (Babu et al., 1988), it is possible that each end of CaM binds one peptide or possibly one PDE subunit. These considerations indicate the need to carefully reassess the CaM-binding stoichiometry for CaM-PDE.

A model for the domain organization of a 61-kDa subunit of the dimeric CaM-PDE is illustrated in Figure 7. Limited proteolysis shows that this enzyme is comprised of multiple domains since the CaM-binding and catalytic functions can be separated (Kincaid et al., 1984). We have demonstrated that the catalytic domain represented by the 36-kDa tryptic fragment extends from residue 136 to $\approx$ 450 and completely encompasses the 250-residue segment conserved in all metazoan PDEs. On the basis of peptide analogue studies demonstrating that residues 23–41 form a major site of interaction with CaM, we postulate the existence of a distinct domain that may span from residue 1 to 135 and that has CaM-binding as one of its major functions. It should be emphasized that the proposed CaM-binding and the 80–90-residue, C-terminal domain illustrated in Figure 7 are hypothetical since no corresponding fragments have been detected in this study or that of Kincaid et al. (1984). Apparently, these proposed domains are completely destroyed under the proteolytic conditions employed here; but it is possible that they could be demonstrated with use of milder conditions or different proteases. The catalytic and proposed CaM-binding sites are separated by a linking segment of $\approx$100 residues that has at least one exposed, protease-sensitive region located near residue 135. It is not clear how the binding of CaM at a distant site can alter enzyme activity, although it has been suggested that when bound, CaM releases the suppression of activity imposed by a separate inhibitory domain (Kincaid et al., 1985; Krinks et al., 1984). Further studies are needed to confirm the presence of such an inhibitory domain and, if it exists, to locate its position within the molecule. With the complete sequence and a general view of the domain organization of the PDE, it should now be possible to explore the CaM-activation mechanism in detail.

[3] H.C. and K.A.W., unpublished results.

Acknowledgments

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Sequence Comparison of the 63-, 61-, and 59-kDa Calmodulin-Dependent Cyclic Nucleotide Phosphodiesterases†

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ABSTRACT: Partial protein sequences from the 59-kDa bovine heart and the 63-kDa bovine brain calmodulin-dependent phosphodiesterases (CaM-PDEs) were determined and compared to the sequence of the 61-kDa isozyme reported by Charbonneau et al. [Charbonneau, H., Kumar, S., Novack, J. P., Blumenthal, D. K., Griffin, P. R., Shabanowitz, J., Hunt, D. F., Beavo, J. A. & Walsh, K. A. (1991) *Biochemistry* (preceding paper in this issue)]. Only a single segment (34 residues) at the N-terminus of the 59-kDa isozyme lacks identity with the 61-kDa isozyme; all other assigned sequence is identical in the two isozymes. Peptides from the 59-kDa isozyme that correspond to residues 23-41 of the 61-kDa protein bind calmodulin with high affinity. The C-terminal halves of these calmodulin-binding peptides are identical to the corresponding 59-kDa sequence; the N-terminal halves differ. The localization of sequence differences within this single segment suggests that the 61- and 59-kDa isozymes are generated from a single gene by tissue-specific alternative RNA splicing. In contrast, partial sequence from the 63-kDa bovine brain CaM-PDE isozyme displays only 67% identity with the 61-kDa isozyme. The differences are dispersed throughout the sequence, suggesting that the 63- and 61-kDa isozymes are encoded by separate but homologous genes.

Cyclic nucleotide phosphodiesterases (PDEs)[1] catalyze the hydrolysis of cAMP and/or cGMP to their corresponding 5′-nucleoside monophosphates. At least five different enzyme families have been identified, and most of these families contain multiple forms of closely related PDEs. One large family, the CaM-dependent PDEs (CaM-PDEs) is particularly critical for intracellular signaling in that they respond to calcium by decreasing the concentration of cAMP and cGMP [for review see Wang et al. (1990)]. Although a number of members of the CaM-PDE family have been described (Beavo, 1988; Beavo & Reifsnyder, 1990), three well-characterized forms are the 59-kDa isozyme isolated from bovine heart and the 61- and 63-kDa isozymes isolated from bovine brain (LaPorte & Storm, 1979; Hansen & Beavo, 1982; Sharma & Wang, 1986, 1987). Sharma and Wang (1986) also described a 58-kDa CaM-PDE from bovine lung that bound

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[1] Abbreviations: PDE, cyclic nucleotide phosphodiesterase; CaM, calcium/calmodulin complex, CaM-PDE, calmodulin-dependent cyclic nucleotide phosphodiesterase; CM, *S*-carboxymethyl; BNPS-skatole, an acronym for the reagent described by Fontana et al. (1973); HPLC, high-performance liquid chromatography; TPCK, N^{α}-*p*-tosyl-L-phenylalanine chloromethyl ketone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Pth, phenylthiohydantoin.

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