Identification of Domains Essential for the Assembly of Calcium/Calmodulin-dependent Protein Kinase II Holoenzymes*

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Ca²⁺/calmodulin-dependent protein kinase II (CaM kinase II), as isolated from brain, is a multimeric complex composed predominantly of two subunits, α and β, products of unique genes. Little is known about how subunit composition influences holoenzyme structure or how the domain(s) of each subunit interact to form holoenzymes. We show here that holoenzymes composed of only α or only β subunits exhibit different biophysical properties. The S values of α and β are 17.2 and 14.5 S while the Stokes’s radii are 85 and 111 Å, respectively, indicating their structures are different. C-terminal truncations of the α subunit show that amino acids 382–478 are necessary for holoenzyme formation and that amino acids 427–478 contribute to holoenzyme stability. Additionally, the C-terminal domains of both the α subunit, a315–478, and β subunit, β314–542, formed oligomers indicating the sufficiency of the C-terminal domain for multimer formation. Using the yeast two-hybrid system we show, in vitro, that full-length subunits, α1–478 and β1–542, interact with themselves or each other interchangeably. Additionally, the C-terminal domains of the α subunit, α315–478 and β subunit, β314–542 associated with themselves in a manner indistinguishable from their association with full-length α or β subunits. Further studies revealed that the C-terminal domains of the α and β subunits contain information necessary for interaction with β but not α. These data are summarized into a model describing the assembly of CaM kinase II holoenzymes.

Ca²⁺/calmodulin-dependent protein kinase II (CaM kinase II) is a ubiquitous multifunctional serine/threonine kinase, highly enriched in neurons, that appears to mediate many effects stimulated by Ca²⁺ transients (for a review of CaM kinase II function, see Refs. 1 and 2). Much attention has focused on CaM kinase II autophosphorylation which occurs in a Ca²⁺/CaM-dependent manner and results in the expression of functional changes such as Ca²⁺/CaM-independent activity, Ca²⁺/CaM insensitivity, CaM-trapping, inactivation, and self-association (1–4). The generation of Ca²⁺/CaM-independent activity occurs via intersubunit reactions within holoenzymes (5, 6). However, little is known concerning how subunits interact to form the structure of CaM kinase II holoenzymes.

Four genes encode related but distinct isoforms of CaM kinase II (α, β, γ, and δ). Preparations of CaM kinase II purified from rat forebrain consist of the α (50 kDa) and β (60 kDa) subunits whose cDNAs have been cloned and sequenced (7–9). Amino acid differences reside predominantly in the C-terminal domain, and there is a stretch of amino acids termed the “unique” domain present in β, but absent in α. The function of the unique domain is entirely unknown. Other than results indicating that the two subunits exhibit slightly different $K_{act}$ for CaM (10, 11), no functional differences between α and β subunits have been identified and it is unclear how isoform heterogeneity contributes to CaM kinase II function.

CaM kinase II isolated from brain tissue exists predominantly as heteromultimers composed of α and β subunits (12, 13). In contrast, others have concluded that forebrain and cerebellum CaM kinase II preparations consist primarily of homomultimers of α and β subunits (14). These divergent conclusions are difficult to reconcile, while raising important questions concerning the ability of different CaM kinase II subunits to assemble with each other.

There also exists reasonably strong evidence that the C-terminal domain of CaM kinase II is necessary for holoenzyme formation. Proteolytic removal of the C-terminal domain from holoenzymes result in the isolation of catalytically active monomeric fragments (15–17). Additionally, truncation studies have demonstrated that removal of the C-terminal domain of the α subunit produces a monomeric form of CaM kinase II (10). This basic observation has also been confirmed in additional studies (5, 18, 19) and provides convincing evidence that the C-terminal domain of the α subunit of CaM kinase II is necessary for multimer formation.

The importance of intersubunit interactions for the autophosphorylation-dependent changes in CaM kinase II function has led us to investigate in detail the domain structure of α and β holoenzymes. In this paper, we describe the isolation, purification, and biophysical characterization of the α and β isoforms of CaM kinase II expressed in a baculovirus system. We show that both α and β subunits form holoenzymes of approximately the same number of subunits, however, β holoenzymes exhibit a significantly larger diameter in solution. Utilizing deleterious strategies, we show that amino acids 382–478, within the C-terminal domain, of the α subunit are necessary for the formation of holoenzymes and that the C-terminal domains of both α and β subunits are sufficient for oligomer
formation. Finally, the interactions between domains of α and β subunits were analyzed in vivo using the yeast two-hybrid system. The results indicate that full-length α and β subunits interact with themselves and each other and confirm that the C-terminal domain of each subunit is necessary for inter-subunit associations. Interestingly, these studies identify a specific domain in the C terminus of both α and β subunits that is necessary for interacting with β, but not α, subunits. We relate the potential implications of this latter finding on the structural difference identified between α and β holoenzymes expressed in the baculovirus system.

**EXPERIMENTAL PROCEDURES**

**Preparation and Purification of Recombinant CaM Kinase II**—The cDNA clone containing the entire coding sequence of the β subunit was isolated from a rat brain cDNA library. A fragment containing the complete coding sequence was cloned into the EcoRI site of the baculovirus shuttle vector pBakPak-9, and recombinant baculovirus was prepared as described by the manufacturer (CLONTECH, Palo Alto). Sf21 insect cells were utilized for construction, selection, and amplification of viral stocks. The production of αCaM kinase II in baculovirus was reported previously (20). Infection with virus at a multiplicity of infection (MOI) of 5–15 produced maximal protein expression approximately 72–96 h post-infection at which point the cells were harvested. Recombinant α and β enzymes were purified from Sf21 as described by Brickey et al. (21) with modifications as described in Putkey and Waxman (20).

The purification protocol was basically a 3-step procedure involving affinity purification of soluble cell extracts on CaM-Sepharose followed by fractionation in sucrose gradients and finally concentration on Macro-S cation exchange resin. The sucrose gradient step was omitted in some preparations to avoid a size selection bias before the analyses on gel filtration columns. Multiple preparations of baculovirus-infected cells expressing α and β subunits generated similar results throughout this study.

**Production of a Subunit Truncations**—The CaM kinase II α subunit truncations, 1–382 and 1–427, were created by placing in-frame stop codons at amino acids 382 and 427, respectively, using site-directed mutagenesis as described previously (22). The sequence of these mutations was verified, and the cDNAs were first cloned into pGBT-9 (CLONTECH), previously engineered to contain the entire coding sequence of the α subunit. This was accomplished using the Smal/BlgII restriction enzymes internal to the α subunit cDNA. The EcoRI/SalI fragments, containing the coding sequence of the α subunit with each of the internal stop codons, were then individually subcloned into the HTA version of the Bac-to-Bac vector (Life Technologies, Inc.). In so doing, a fusion protein was produced, containing a N-terminal His-tag that permitted affinity purification on Ni²⁺-NTA resin. The N-terminal fusion protein contributed a total of 29 amino acids to each fragment cloned in the EcoRI site. Recombinant bacmid DNA was prepared by transforming HTA plasmid DNA into competent DH10Bac cells (CLONTECH). pGBT-9 encodes the N-terminal GAL4 activation domain and a LEU2 marker (amino acids 1–147). pGAD424 contains the activation domain and a TRP1 marker (amino acids 768–881). The CaM kinase II α subunit cDNA encoding the full-length 50-kDa (α) protein (described in Ref. 26) was digested with NcoI, blunt-ended, and cloned into the Smal site of pGBT9 producing pGβTα. This places the α subunit coding sequence in-frame with the GAL4 DNA-binding domain. An EcoRI/SalI digest was then used to place the α subunit cDNA into the pGAD424 vector producing pGADα which then encodes a GAL4 activation domain-α subunit fusion protein. To clone the CaM kinase II β subunit cDNA into the pGβT9 vector, an NcoI site was created in the DNA at the start codon of the protein using Kunkel’s method of oligonucleotide primed site-directed mutagenesis (22). A partial digest of this cDNA with NcoI and HindIII was then blunt-ended cloned into the Smal site of pGBT9 producing pGβTβ. This cDNA was then subcloned into pGAD424 as described for the α subunit producing pGADβ. The remaining truncations were produced by polymerase chain reaction (PCR) and were constructed to contain amino acids that flanked the desired coding region of the α or β subunit. The polymerase chain reaction amplified fragment was first cloned into pGβT9 and when necessary, then cloned into pGAD424.

For some experiments, cDNA encoding a 27-amino acid glycine-rich linker was appended in-frame between the GAL4 DNA-binding domain and a TRP1 marker (amino acids 1–147). The C-terminal domain of each subunit was encoded in one pGBK-based vector expressing the amino acids encoded in the newly generated linker. This linker was constructed from two overlapping primers containing EcoRI sites at the 5′ and 3′ ends. After polymerase chain reaction amplification, the insert was cloned into the EcoRI site of pGBK1 (382–478). Orientation was determined by a HpaI site engineered into the linker sequence. The 27 amino acids were 5′-GGGGGGSSGGAGGSGGGSSGGGGVNG-3′.

**Yeast Transformation and Screening for Interactions**—Saccharomyces cerevisiae reporter strain SY526 (MATa, ura3-52, his3–200 ade2–101, lys2–801, trp1–901 leu2–3, 112, can–1, gal4–542, gal80–538 and URA3::GAL1-inA2 (27)) was used for all transformations. After confirming the phenotype of the reporter strain, a single colony was grown overnight in YPD medium and transformed by a lithium acetate method described in the two-hybrid kit (CLONTECH) using 2 μg of plasmid DNA for each sample. Transformants were plated on dropout selection media appropriate for the deletion and were grown for 10–12 h at 30 °C.

Transformants were screened for expression of β-galactosidase (β-gal) by replica plating colonies onto filters; the filters were dipped in liquid nitrogen, soaked in buffer containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside, and incubated for 12 h at 30 °C. A blue colony indicated β-gal expression. The time taken for the filter to turn blue was used as an indicator of the strength of the protein-protein interaction. (+ + +) =

\[
M_s = 6 \pi n \rho \omega^2 \frac{s_{20,w}}{R_w} - N/1 \quad (\text{Eq. 1})
\]

\[
\rho_s \omega^2 R_w = \frac{M_s (4 \pi N \eta)^{1/3}}{1} \quad (\text{Eq. 2})
\]

where \( R_w \) = Stoke’s radius (cm), \( s_{20,w} \) = sedimentation velocity (S × 10⁻¹⁵ s), \( n \) = Avogadro’s number (6.022 × 10²³ mol⁻¹), \( \eta_s \omega^2 R_w \) = viscosity of water at 20 °C (0.01002 g cm⁻¹ s⁻¹), \( \rho_s \omega^2 R_w \) = density of water at 20 °C (0.9931 g cm⁻³), and \( v \) = partial specific volume where a value of 0.725 cm³ g⁻¹ was used.
The domain structure of the α and β subunits of CaM kinase II—Fig. 1 compares the domain structure of rat α and β subunits of CaM kinase II. The two subunits are highly homologous with the exception of a 63-amino acid segment, absent from the α subunit just C-terminal to the regulatory domain (9). The N-terminal end of the molecule, α1–280 and β1–281, contains the catalytic domain, which comprises the Mg\(^{2+}\)/ATP and protein substrate-binding sites (>90% identity). The central region, α281–314 and β282–315, contains the regulatory domain, further separable into the autoinhibitory domain and the calmodulin-binding domain (Fig. 1A). The amino acid segment in the β subunit that forms the unique domain is defined as amino acids 314–387 (Fig. 1, A and B). The 164 amino acids comprising the association domain of the α and β subunits are highly homologous (Fig. 1B). This region exhibits 76% identity at the amino acid level, 96% similarity if conservative amino acid replacements are permitted.

Recombinant α and β Isozymes Form Holoenzymes of Different Size—The α and β isoforms of rat CaM kinase II were expressed in the baculovirus system and purified as described under “Experimental Procedures.” Both preparations were purified to near homogeneity as demonstrated by SDS-PAGE (Fig. 2). The α subunit migrated as a single band on SDS-PAGE (~50 kDa; Fig. 2, lane c) and had a molecular mass of 54,296 Da as determined by matrix-assisted laser desorption/mass spectrometry (Table I). This is similar to the molecular mass predicted from the amino acid sequence (54,110 Da). The β preparation migrated as a doublet (Fig. 2, lane b), and both bands of the doublet were immunoreactive with a monoclonal antibody specific to the β subunit (data not shown). The major species of

RESULTS

Domain Structure of the α and β Subunits of CaM Kinase II—Fig. 1 compares the domain structure of rat α and β subunits of CaM kinase II. The two subunits are highly homologous with the exception of a 63-amino acid segment, absent from the α subunit just C-terminal to the regulatory domain (9). The N-terminal end of the molecule, α1–280 and β1–281, contains the catalytic domain, which comprises the Mg\(^{2+}\)/ATP and protein substrate-binding sites (>90% identity). The central region, α281–314 and β282–315, contains the regulatory domain, further separable into the autoinhibitory domain and the calmodulin-binding domain (Fig. 1A). The amino acid segment in the β subunit that forms the unique domain is defined as amino acids 314–387 (Fig. 1, A and B). The 164 amino acids comprising the association domain of the α and β subunits are highly homologous (Fig. 1B). This region exhibits 76% identity at the amino acid level, 96% similarity if conservative amino acid replacements are permitted.

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CaM Kinase II Subunit Interactions

To provide analogous preparations for comparison, cells expressing full-length \( \alpha \) and \( \beta \) subunits were processed in an identical manner. The \( \alpha 1-478 \) from this crude preparation exhibited a retention time similar (24 min) to that of the purified enzyme (compare Figs. 3 and 4, main protein peak in fractions 9–11). Note also that there are no detectable monomer forms of \( \alpha 1-478 \) expressed in these cells (monomers would appear at approximately fractions 18 and 19). The \( \beta 1-542 \) preparation exhibited a retention time of approximately 20.5 min (main protein peak in fractions 6–8) and again, no detectable monomeric forms were observed. The \( \beta 1-542 \) construct was expressed at much lower levels than the other constructs utilized in this study. The most abundant protein evident in the starting material of this preparation (first lane, panel labeled

The full-length \( \alpha \) and \( \beta \) subunits of CaM kinase II were purified from baculovirus-infected SF21 cells as described under “Experimental Procedures” and analyzed on SDS-PAGE followed by Coomassie Blue staining. Lanes contained molecular weight standards (a), \( 5 \mu g \) of the \( \beta \) subunit preparation (b), and \( 8 \mu g \) of the \( \alpha \) subunit preparation (c). The gel was purposely overloaded in attempts to expose protein contaminants in the preparations. Expression of the cDNAs encoding the \( \alpha \) subunit truncated at amino acids 427 and 382, purified from baculovirus-infected cells, are shown in lanes d and e, respectively. Note that due to the addition of the His-tag and additional linker sequence, the fragments migrate at larger amino acids than the full-length subunit. These truncated molecules were purified using Ni\(^{2+}\)/NTA affinity chromatography, and the protein patterns in lanes d and e represent \( 5 \mu g \) of each preparation.

The \( \beta \) subunit had a molecular mass, determined by matrix-assisted laser desorption/mass spectroscopy, of 60,234 Da, again similar to its predicted molecular mass (60,397 Da). The minor species had a molecular mass of 63,350 Da (Table I). Reasons for the two species of the \( \beta \) subunit on SDS-PAGE is not known, however, given the mass difference detected by matrix-assisted laser desorption/mass spectroscopy, it is unlikely to involve small covalent modifications like, for example, differential protein phosphorylation. We are currently identifying the chemical nature of this mass difference. The \( \alpha (34 \pm 5.2 \mu mol/min/mg) \) and \( \beta (31 \pm 1.5 \mu mol/min/mg) \) enzymes utilized in this study possessed similar specific activities.

The \( \alpha \) subunit expressed in eucaryotic cells is multimeric and it is the principle isoform that has been characterized biochemically (5, 10). Much less is established concerning the assembly of monomers into holoenzymes, although one report indicated that \( \beta \) remains monomeric when expressed in the absence of the \( \alpha \) subunit (10). Gel-filtration chromatography and sucrose gradient centrifugation were utilized to probe the hydrodynamic properties of \( \alpha \) and \( \beta \) enzymes purified as described. Table I shows that the Stokes radii of the \( \alpha \) and \( \beta \) holoenzymes determined by gel-filtration chromatography were 85 \( \pm \) 3 and 111 \( \pm \) 5 \( \AA \), respectively. Thus, both enzymes are multimeric and the \( \beta \) enzyme possessed a larger diameter in solution than the \( \alpha \) holoenzyme. The sedimentation velocity of the \( \beta \) holoenzyme (14.5 \( \pm \) 1.1 S) was smaller than that of the \( \alpha \) holoenzyme (17.2 \( \pm \) 0.4 S). The \( S \) values and Stokes’ radii were utilized to calculate the molecular mass of each holoenzyme as described under “Experimental Procedures” and these values are 599 and 663 kDa, respectively, for the \( \alpha \) and \( \beta \) holoenzymes (Table I). By dividing the holoenzyme mass by the mass of each subunit, the number of subunits in \( \alpha \) and \( \beta \) holoenzymes were calculated to be 11.1 and 11.0, respectively.

The C-terminal Domain of the Purified \( \alpha \) Subunit Is Essential for Holoenzyme Formation—The consequence of removing amino acids from the C terminus of the \( \alpha \) subunit on the assembly of monomers into holoenzymes was analyzed. To aid in purification, the truncated \( \alpha \) subunits were prepared as fusion proteins with a 29-amino acid tag on the N terminus including 6 His residues and a linker sequence. The preparations were highly enriched for the appropriately sized CaM kinase II fragment when purified using Ni\(^{2+}\)/NTA resin and analyzed by SDS-PAGE. Fig. 2, lanes d and e, show the Coomassie Blue-stained SDS-PAGE analysis of the \( \alpha 1-427 \) and \( \alpha 1-382 \) preparations, respectively.

As noted, the full-length \( \alpha \) holoenzymes (1–478) exhibited an average retention time (\( n = 3 \)) of 24 min and had a Stoke’s radius of 85 \( \pm \) 3 \( \AA \) (Table I). Fig. 3 shows a SDS-PAGE of the fractions eluting from the Superose 6 column. A sharp peak of protein was detected with \( \alpha 1-478 \) (top panel, fractions 9–12). When a stop codon was placed at amino acid 427, to make \( \alpha 1-427 \), the resulting protein exhibited a broad elution profile with two main peaks of protein at retention times of 26.2 and 32.8 min. This profile was consistent in 3 independent analyses of two different protein preparations and shows that \( \alpha 1-427 \) was distributed across each of the fractions (middle panel, Fig. 3). However, there were two peaks of protein centered around fractions 12–14 and 18 and 19 that were consistent with the retention times of peaks measured by \( A_{280} \) of the column effluent. The molecular mass of the \( \alpha 1-427 \) protein on SDS-PAGE was constant, indicating degradation was not the cause of the broad protein distribution on the gel-filtration column. We conclude that amino acids 427–478 are required for efficient assembly of \( \alpha \) subunit holoenzymes, however, sufficient contacts remain to produce heterogeneous multimers. Due to the heterogeneous nature of \( \alpha 1-427 \), it was not possible to define a Stoke’s radius and additional analyses were not pursued with this construct. Further truncation of the \( \alpha \) subunit to amino acid 382 produced a protein with a single well resolved peak at a retention time on the gel-filtration column of 33 min (\( n = 4 \)) which provides a calculated Stoke’s radius of 27 \( \pm \) 0.6 \( \AA \) (Table I). SDS-PAGE of the fractions from the gel-filtration column showed that the peak of protein for \( \alpha 1-382 \) was in fractions 19 and 20 and that the protein preparation had not undergone degradation (lower panel, Fig. 3). When analyzed by sucrose gradient centrifugation, \( \alpha 1-382 \) exhibited a \( S \) value of 3.1 \( \pm \) 0.5 (Table I). From the Stoke’s radius and \( S \) value, we calculated a mass for \( \alpha 1-382 \) of 34 kDa indicating that removing amino acids 383–478 produced a monomeric species of the \( \alpha \) subunit.

\( \alpha \) and \( \beta \) C-terminal Domains Are Sufficient for Oligomer Formation—The above results indicated that the C-terminal domain of the \( \alpha \) subunit was necessary for oligomerization. To test whether the C-terminal domain was, by itself, sufficient for oligomerization, amino acids 315–478 of the \( \alpha \) subunit and 314–542 of the \( \beta \) subunit were expressed in the baculovirus system and subjected to gel-filtration and sedimentation velocity analyses. Although each fragment was expressed as a fusion protein with the His-tag at its N terminus, attempts at purification utilizing a Ni\(^{2+}\)/NTA resin met with poor success. Fortunately, the yield of each protein was high enough in the baculovirus-infected cells that an enrichment step utilizing ammonium sulfate precipitation of cytosolic extracts was sufficient to provide unambiguous identification of the expressed protein fragments by SDS-PAGE.
Hydrodynamic properties of CaM kinase II isoforms and truncated fragments

| Construct | Subunit mass (theoretical) | Sedimentation coefficient | Stokes radius | Frictional ratio | Total mass | Number of subunits |
|-----------|---------------------------|---------------------------|---------------|------------------|------------|-------------------|
| α         | 54,296                    | 17.2 ± 0.4                | 85 ± 3        | 1.52             | 599        | 11.1              |
| β         | 60,234                    | 14.5 ± 1.1                | 111 ± 5       | 1.93             | 663        | 11.0              |
| His-a315–478 | 22,212                  | 10.1 ± 1.2               | 49 ± 1        | 1.26             | 204        | 9.2               |
| His-b314–542 | 28,580                  | 9.0 ± 0.8                | 99 ± 7        | 2.10             | 367        | 12.8              |
| His-a1–382  | 46,744                    | 3.1 ± 0.5                | 27 ± 6        | 1.25             | 34         | 0.7               |

β1–542 is not the β subunit. The β subunit migrates just below this protein and is obscured in the Coomassie-stained lane of the starting material. This protein is evident in fractions 18–23 and was present in each preparation (for example, compare fractions 18–23 for α1–478 and β1–542) and likely represents a cytoskeletal protein resident in SF21 cells. It should not be confused as a monomeric form of the β subunit.

Hydrodynamic analyses of the C-terminal domains of the α and β subunits indicated that both were oligomeric. His-a315–478 exhibited a retention time on the gel-filtration column of 28.3 min with a calculated Stoke’s radius of 49 ± 1 Å. The main protein peak eluted in fractions 14–16 and again, no monomeric forms were evident in the later fractions (Fig. 4). His-a315–478 resolved as a relatively homogeneous preparation of oligomeric molecules. Note that His-a1–427 is evident in most every fraction indicating that the preparation is composed of a heterogeneous population of holoenzymes composed of various numbers of subunits. His-a1–382 resolves as a relatively homogeneous mixture of monomeric molecules.

β1–542 was appended to the AD (a1–478AD) and co-transformed with a1–478 appended to the BD (a1–478BD) a strong positive result was detected (Fig. 5A). In fact, the rate at which the co-transformed colonies turned blue, a crude measure of the strength of protein-protein interactions (27), was similar to that in yeast co-transformed with pTD1 (the SV-40 T antigen/AD hybrid) and pVA3 (the p53/BD hybrid) constructs provided as a positive control with the two-hybrid kit (CLONTECH; data not shown).

A similar approach with the β subunits showed that when β1–542AD and β1–542BD were co-transformed into yeast a positive β-gal signal was produced (Fig. 5B). The signal was indistinguishable from the α-α interactions. Yeast were then co-transformed with either a1–478AD and β1–542BD (Fig. 5B) or a1–478BD and β1–542AD (see Fig. 5A) and assayed for β-gal activity. Again positive interactions were detected with either plasmid pair indicating α and β subunits interact. From this data, we conclude the full-length α and β subunits of CaM kinase II interact interchangeably.

**The C-terminal Domains of α and β Subunits Are Sufficient for Subunit Interactions in Vivo**—We found that removal of sequences from the C terminus of the α subunit initially decreased, a1–427BD, and eventually abolished, a1–382BD, the interaction with full-length α subunits (Fig. 5A). These truncations also behaved in a similar fashion when tested against the full-length β subunit or themselves (Fig. 5A). To establish that the absence of interactions with a1–382BD was not due to an absence of fusion protein expression, we performed Western blots of yeast extracts with an antibody specific for the GAL4 DNA-binding domain. Full-length subunits and all truncations were visualized as the appropriately sized fusion proteins (data not shown). Thus, the negative interactions reported with a1–382BD, and the other truncations described below, could not be ascribed to a lack of production of the fusion proteins. We conclude that if other domains of CaM kinase II are involved in subunit interactions they are either too weak to detect in the system, or they require that the C-terminal domains associate first.

To define the domain(s) of the α subunit sufficient for subunit interactions, a series of N-terminal deletions were constructed and tested for interactions. The C-terminal domain of

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Table I

| Fraction Number | α1–478 |
|-----------------|--------|
| 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 |
| α1–427 | (His)x1–427 |
| α1–382 | (His)x1–382 |

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2 M. N. Waxham, unpublished observations.
the $\alpha$ subunit, $\alpha_{315-478BD}$, was found to interact with full-length $\alpha$ and $\beta$ subunits and to self-associate (Fig. 5A). This data confirms the importance and sufficiency of the C-terminal portion of the $\alpha$ subunit in producing subunit interactions. In fact, for all the associations reported in the two-hybrid analysis, the $\alpha_{315-478BD}$ and the $\alpha_{1-478BD}$ provided identical

**Fig. 5.** Interactions between full-length and truncations of the $\alpha$ and $\beta$ subunits of CaM kinase II in the yeast two-hybrid system. A, a map of the $\alpha$ subunit includes the catalytic domain (striped), the regulatory domain (white), and the C-terminal domain (black). C-terminal truncations of the $\alpha$ subunit appended to the BD domain are specified by the amino acid numbers on the left. The ability of these fusion proteins to activate the reporter LacZ upon co-transfection with the full-length $\alpha$ and $\beta$ subunits or to the same truncation in the AD (self) is shown on the right. B, a map of the $\beta$ subunit includes the catalytic domain (striped), the regulatory domain (white), the unique domain (roped), and the C-terminal domain (black). C-terminal fragments of the $\beta$ subunit appended to the BD are specified by the amino acid numbers on the left. The ability of these fusion proteins to activate the reporter LacZ upon co-transfection with the full-length $\alpha$ and $\beta$ subunits or to the same truncation in the AD (self) is shown on the right. Strength of interaction was measured as the time taken for colonies to turn blue in 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactoside filter lift assays: ++, <30 min; +, >30 min < 4 h; -, no detectable $\beta$-gal activity in 24 h; and ND, not determined.
results when assayed against the panel of truncations.

As the C-terminal domain was further shortened to make α355–478BD, there were again positive interactions with the α and β subunits (Fig. 5A), however, the self-association of α355–478BD could not be tested (see “Experimental Procedures”). Further truncation, to make α382–478BD, produced a loss of interaction with β1–542AD, but not with α1–478AD and additional truncation, to make α427–478BD, resulted in loss of interaction with both full-length subunits (Fig. 5A). These results indicate that the minimal domain of α subunits sufficient for association with the full-length α subunit is α382–478, while the minimal domain sufficient for association with the full-length β subunit is α355–478.

To test whether the C-terminal domain of the β subunit provided an analogous function for association to that in the α subunit, a comparable C-terminal fragment of the β subunit was constructed. β314–542 contains the amino acids equivalent to the C-terminal fragment of the α subunit, α315–478, and additionally, includes the unique domain of the β subunit, β314–387. β314–542 associated with both α and β subunits and self-associated (Fig. 5B). In fact, in all reported associations, β314–542 behaved identically to β1–542. N-terminal truncations, β329–542, β351–542, and β387–542 behaved similarly to β314–542, associating with both the α and β subunits.

We also tested whether β446–542BD, which is homologous to α382–478BD, would interact with the α subunit but not the β subunit. β446–542BD was positive for interaction with α but negative for β (Fig. 5B). This result provides further evidence that amino acids 355–382 in the α subunit (and presumably the homologous amino acids 419–446 in the β subunit) are essential for interaction with β subunits but not with α subunits. We conclude that the failure of β to interact with either α382 or α382–478 is due to the fact that domains within both fragments are necessary for binding.

The Failure of α382–478 to Interact with the β Subunit Is Not Due to a Restricted Conformation Associated with Fusion to the GAL4 Promoter Domain—To address the possibility that the failure of α382–478 to interact with the β subunit is due to some conformational constraint as a result of forming the hybrid protein, we designed a 26-mer glycine-rich linker sequence and inserted it between the GAL4 DNA-binding domain and α382–478. Glycine was chosen due to its small size and high relative mobility. The ω-linker 382–478BD protein was able to associate with α1–478AD as before but failed to associate with β1–542AD (Fig. 6). The linker expressed by itself as a BD fusion protein failed to interact with either α or β subunits (Fig. 6) as predicted. Thus, the amino acids in the linker were unable to mimic the property of the amino acids in α355–382 domain that converts an “α-only” associating fragment, α382–478, into an α and β associating domain, α355–478, despite containing the same number of amino acids. This indicates that the association between α and β subunits requires additional interactions between CaM kinase II C-terminal domains that are sequence-specific and located within amino acids 355–382 of the α subunit.

The Failure of α382–478 and β446–542 to Interact with the β Subunit Is Not Due to the Unique Domain of the β Subunit—Our results demonstrated that α382–478 and β446–542 did not interact with the β subunit but did interact with the α subunit. To investigate whether the unique domain of the β subunit was responsible for the lack of association, we produced a chimeric protein where the unique domain of the β subunit (amino acids 314 to 387) was fused onto the C terminus of the α subunit (amino acids 315–478). This chimeric construct is shown in Fig. 7. The chimeric molecule maintained its capacity to associate with both α355–478 or α382–478 (Fig. 7).

The unique domain of the β subunit by itself showed no evidence of association with any of the fragments (Fig. 7). For comparison, the results are also shown for β314–542 which associated with α355–478 and not with α382–478. These results indicate that the unique domain does not cause the difference detected in association of the β subunit with α382–478. We predict that it must be amino acid differences in the remainder of the β C-terminal domain that are responsible for the differences in subunit association.

**DISCUSSION**

Miller and Kennedy (12) characterized in detail the biochemical and biophysical properties of CaM kinase II isolated from both forebrain and cerebellum. The forebrain enzyme has an α:β subunit ratio of 3:1, a Stoke’s radius of 94.7 Å, and a sedimentation velocity of 16.4 S. The cerebellar enzyme has an α:β ratio of 1:4, a Stoke’s radius of 88 Å, and a sedimentation velocity of 14 S. Using both S values and Stoke’s radii, they calculated a mass of 673 and 543 kDa for the forebrain and cerebellar enzymes, respectively, and concluded that the forebrain and cerebellar enzymes are composed of 12 and 8 subunits, respectively.

In attempts to define the relative contributions of α and β subunits to the biophysical properties of CaM kinase II holoenzymes, we utilized a similar analysis to establish the properties of holoenzymes composed of only α or β subunits. We find that the α holoenzyme has a Stoke’s radius of 85 Å and a sedimentation velocity of 17.2 S, while the β holoenzyme has a Stoke’s radius of 111 Å and a sedimentation velocity of 14.5 S. Utilizing this combined data a total mass of 599 and 663 kDa was calculated for the pure α and β holoenzymes, respectively, that leads to a calculated subunit number for each of approximately 11. Based on Miller and Kennedy’s results (12), we initially hypothesized that sequence differences between α and β subunits might lead to holoenzymes composed of different subunit number, β containing fewer than α. The dominance of the β subunit in the cerebellar isoform would explain why it appeared to contain fewer subunits. However, our results do not support such a model. Our data suggest that both α and β holoenzymes are composed of a similar number of subunits. It is possible that when α and β subunits are mixed within a holoenzyme, different constraints are placed on the number of subunits incorporated into the final structure. Alternatively, as yet undefined cellular factors may regulate the assembly of CaM kinase II holoenzymes in the brain. Experiments to address the biophysical consequences of having both subunits assembled in the same holoenzyme are currently underway.

From these data, it appears that the main difference in
The larger diameter in solution of the β holoenzyme. Frictional ratios calculated for the α and β holoenzymes (Table I) further emphasize this point and additionally suggest that the β holoenzyme assumes a more elongated structure in solution than α holoenzymes (for discussion of frictional ratios, see Ref. 28).

Previous studies showed that deletion of the C-terminal domain of the α subunit (for example, ending at amino acid 355) produced a monomeric form of the enzyme (5, 10, 18, 19) indicating that C-terminal residues were necessary for oligomer formation. Bulleit et al. (9) noted that the C termini of CaM kinase II subunits are largely hydrophilic and contain alternating clusters of positively and negatively charged residues. They suggested that these hydrophilic clusters might be important for stabilizing subunit-subunit interactions. A re-investigation of these domains by hydrophobicity analysis indicated the presence of three hydrophilic stretches in the α subunit, amino acids 362–374, 393–413, and 438–467, separated by spans of uncharged residues. We produced C-terminal truncations of the α subunit terminated at amino acids 382 and 427 to investigate the contribution of the two C-terminal hydrophilic domains on oligomer formation. Our results indicate that removal of amino acids 382–478 eliminates the capacity for subunits to form oligomers while removal of amino acids 427–478 compromises, but does not eliminate, oligomer formation. Therefore, it appears that the majority of the C-terminal domain extending from amino acid 382 of the α subunit is necessary for efficient oligomer formation.

We additionally showed that the C-terminal domain of both the α and β subunits are, by themselves, sufficient for oligomer formation. These studies specifically identify that amino acids 315–478 of the α subunit and 314–542 of the β subunit contain the necessary amino acids to form oligomers. Calculations based on hydrodynamic data (Table I) indicated that His-α315–478 was approximately 204 kDa while His-β314–542 was 367 kDa. From these mass values we estimated that the α and β C-terminal domain oligomers were composed of ~9 and 13 subunits, respectively. One striking finding was the unexpectedly large Stoke’s radius for His-β314–542. Calculations of frictional ratios (Table I) suggest that His-β314–542 exists in a more elongated structure than His-α315–478 which is interesting based on the findings in the two-hybrid system discussed in more detail below. The main sequence difference in the two C termini is the unique domain present in His-β314–542 although other significant differences do exist (see below). It is possible that the unique domain sequence extends out from the central core producing a more elongated structure. As noted, the frictional ratio of β1–542 is also larger than that of α1–478, although the difference is smaller than the difference in the C-terminal domains. This suggests that the β holoenzyme also exists as a more elongated structure than the α holoenzyme. While these types of calculations are open to alternative interpretations, what can be unambiguously concluded is that the C-terminal domains of the α and β subunits are sufficient for oligomerization and that the structures of the C-terminal domains are different.

Our results in the two-hybrid system support and extend the conclusions above based on the hydrodynamic studies of protein expressed in the baculovirus system. They indicate that the full-length α and β subunits interact with themselves and each other interchangeably in intact cells. Moreover, the C-terminal domains of either subunit, α(315–478) and β(314–542), interact in a manner that is not detectably different from the interactions detected using full-length subunits, supporting that the C termini contain the information sufficient for oligomerization. We conclude that residues comprising the interface(s) between CaM kinase II α and β subunits responsible for holoenzyme formation are contained within the C-terminal residues.

Deletion of the final hydrophilic domain, His-α1–427, leads to instability in the formation of holoenzymes when analyzed using the baculovirus system and this conclusion was also supported by results in the two-hybrid system. This indicates that the stretch of hydrophilic residues (438–467) contributes to the stability of subunit binding. However, the fragment containing the C-terminal hydrophilic domain, α427–478, does not form a detectable association with α or β subunits in the two-hybrid system suggesting this domain alone is not sufficient to produce stable interactions. In addition, since α382–478 exhibits associations indistinguishable from the full-length α subunit, it appears that the first hydrophilic stretch in the α subunit, 362–374, is not required for α subunit interactions.
Additionally, α1–382 produces a monomeric form of CaM kinase II when expressed in the baculovirus system. Together, this data indicates that the structural information necessary and sufficient for stable α subunit associations is present in α382–478 and may utilize the two hydrophilic amino acid regions 393–413 and 438–467.

Recently, Shen and Meyer (19) published a study that also analyzed the role of the C terminus of the α subunit in oligomerization. They showed that a fragment containing amino acids 344–478 of the α subunit was necessary and sufficient for oligomerization utilizing both in vitro and in situ criteria in close agreement with our findings which showed oligomerization of a fragment composed of amino acids 315–478 and associations with a fragment encompassing amino acids 382–478. One subtle exception between the studies (even though different fragments were analyzed) is that we observed that a mutant containing amino acids 1–427 maintained a weak ability to interact with itself in both the two-hybrid analysis and in the baculovirus system. We concluded that oligomer formation is maintained but is abnormal in this mutant, possibly due to lower affinity associations. Shen and Meyer (19) showed that a fragment composed of amino acids 315–432 did not oligomerize and they concluded that amino acids 315–432 were insufficient for oligomerization.

While it appears there are significant similarities between the association of α and β subunits, important differences were also found. The fragments α382–478 and β446–542 interacted selectively with the α subunit and not the β subunit in the two-hybrid system. In contrast, no subunit selectivity was observed with α355–478 which indicates that amino acids 355–382 are required for stable β subunit interactions. Apparently, amino acids within α355–382, or the analogous residues in β, are required, along with the remainder of the β C terminus to produce stable β subunit interactions. Interestingly, the domains of the β subunit analogous to 362–374 and 393–413 of the α subunit, amino acids 426–438 and 456–476, respectively, are weaker in their predicted hydrophilicity. If these domains contribute to subunit interactions, it is possible that the reduced hydrophilicity of 456–476 of the β subunit weakens interactions to the point that, in the absence of 355–382 of the α subunit or 419–446 of the β subunit, stable interactions between the β C-terminal domain cannot form. Based on hydrodynamic data we concluded that His-β314–542 appears to have a more elongated structure than His-α315–478. It is possible that in utilizing the three hydrophilic domains in the C terminus to make stable contacts, oligomers composed of the β subunit adopt a more elongated structure than those of the α subunit which only appears to require two of these domains for oligomerization. As noted, the unique domain of the β subunit could also contribute to this more elongated structure. Utilizing electron microscopy, Kanaseki et al. (14) described that holoenzymes they believed were composed of β subunits exhibited slightly more elongated “stems” linking the catalytic domains to the core structures than in holoenzymes composed of α subunits. One or both of the above mentioned possibilities could be the cause of these elongated stems.

The selective requirement of the β subunit for interacting with α382–478 cannot be attributed to the presence of the unique domain in β. When the unique domain was deleted from the β C terminus, α382–478 still did not interact. Furthermore, when the β unique domain was fused onto the N terminus of α382–478 it continued to interact with the α C terminus. A functional role for the β subunit unique domain remains unknown but from this approach, we could not find evidence that it contributes to intersubunit interactions.

Recently, a nonkinase product of the α CaM kinase II gene (AKAP) that contains the C-terminal domain of α CaM kinase II was described in skeletal muscle (29). AKAP was suggested to function as an anchoring protein to localize CaM kinase II to particular subcellular sites (29). Our results predict that this protein would interact with both the α and β subunits, because it contains the sequence 355–478 of the α subunit. One could envision a neuronal AKAP, or a protein such as this which contains a portion of the CaM kinase II association domain, selectively targeting CaM kinase II to specific subcellular sites, such as post-synaptic densities. Additionally, AKAP-like proteins could potentially disrupt subunit assembly by competing for domains essential for holoenzyme formation. A model whereby the subunit composition of holoenzymes, the monomeric or multimeric state, and the subcellular distribution of CaM kinase II is determined by associations with distinct cellular proteins has been proposed by Rostas and Dunkley (30).

What is clear from the present work is that the α and β subunits of CaM kinase II assemble into holoenzymes with unique structures due to differences in their C-terminal domains. Specifically, the results indicate that the α subunit utilizes its C-terminal 96 amino acids to form stable interactions, while the β subunit utilizes its C-terminal 155 amino acids to form stable interactions. Additionally, the diameter of the β holoenzyme as measured in solution is significantly larger than that of the α holoenzyme and this difference seems to involve largely the C-terminal domain. We predict that these unique structures may provide the basis for explaining important aspects of the biology of CaM kinase II, such as the tissue specific differences in subcellular localization that have been correlated with holoenzymes enriched in either α or β subunits (12). Ultimately, a full appreciation of the functional differences related to each holoenzyme’s structure awaits the resolution of the three-dimensional structures of α and β holoenzymes. We are currently attempting these studies in the hopes of defining both the relative orientation of subunits within holoenzymes, as well as defining, in precise terms, the differences in structure between α and β holoenzymes.

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