Comparative Analyses of the Three-dimensional Structures and Enzymatic Properties of α, β, γ, and δ Isoforms of Ca²⁺-Calmodulin-dependent Protein Kinase II*

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Ca²⁺-calmodulin-dependent protein kinase II (CaM-kinase II) is a ubiquitous Ser/Thr-directed protein kinase that is expressed from a family of four genes (α, β, γ, and δ) in mammalian cells. We have documented the three-dimensional structures and the biological and enzymatic properties of the four gene products. Biochemical analyses showed that each isoform assembles into oligomeric forms and their three-dimensional structures at 21–25 Å revealed that all four isoforms were dodecamers with similar but highly unusual architecture. A gear-shaped core comprising the association domain has the catalytic domains tethered on appendages, six of which extend from both ends of the core. At this level of resolution, we can discern no isoform-dependent differences in ultrastructure of the holoenzymes. Enzymatic analyses showed that the isoforms were similar in their Km for ATP and the peptide substrate syntide, but showed significant differences in their interactions with Ca²⁺-calmodulin as assessed by binding, substrate phosphorylation, and autophosphorylation. Interestingly, the rank order of CaM binding affinity (γ > β > δ > α) does not directly correlate with the rank order of their CaM dependence for autophosphorylation (β > γ > δ > α). Simulations utilizing this data revealed that the measured differences in CaM binding affinities play a minor role in the autophosphorylation of the enzyme, which is largely dictated by the rate of autophosphorylation for each isoform.

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1 The abbreviations used are: CaM-kinase II, Ca²⁺/calmodulin-dependent protein kinase II; CaM, calmodulin; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; CaM-C75-IaEDANS, IaEDANS-labeled CaM(C75); IaEDANS, 5-((2-iodoacetyl)aminoethylamino)napthalene-1-sulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.

binding of Ca²⁺ ions to calmodulin (CaM), which binds to and activates CaM-kinase II. Upon activation, this enzyme has the ability to autophosphorylate, a process that confers Ca²⁺-independent activity upon the kinase (1) and greatly increases its affinity for CaM (2). Once activated, CaM-kinase II phospho-

rylates numerous target proteins and is involved in many cel-

lular functions, including synaptic plasticity, synaptic vesicle mobilization, regulation of gene expression, regulation of smooth muscle contractility, and modulation of ion channel function (3–7). The fact that CaM-kinase II has so many poten-
tial substrates raises the question of the relationship be-
tween its activation and a specific response to a particular Ca²⁺ signal. Possibly, the regulated expression of the multiple iso-

forms of CaM-kinase II confers these unique properties.

CaM-kinase II is expressed from a family of four closely related genes, α, β, γ, and δ, each of which produces mRNA that can be alternatively spliced, giving rise to at least 30 different proteins (8, 9). The overall organization of each of the four kinase isoforms is similar: an N-terminal catalytic domain is followed by a regulatory domain that contains an autoinhibitory region and a CaM-binding site, and a C-terminal association domain, through which the subunits interact to assemble into holoenzymes (10). Between the CaM-binding domain and the association domain is a region termed the variable domain, where the majority of the gene product and splice variant differences are found. Combinations of 10 possible sequences can be inserted in this region, leading to a large number of possible splice variants (8).

The four CaM-kinase II genes exhibit different expression profiles in mammalian tissues. All four are found in brain, with α and β mRNA expressed at higher levels than γ and δ (11). αCaM-kinase II is a neuron-specific isoform, and is present at high concentrations in postnatal forebrain neurons (12). βCaM-

kinase is primarily neuronal, although the B2 splice variant is found in skeletal muscle (13). The γ and δ isoforms are ex-

pressed throughout the body (13); δ plays an important role in cardiac muscle (14, 15), whereas both δ and γ variants are important in smooth muscle (16–18).

CaM-kinase II isoforms all assemble into oligomeric complexes. Estimates in the literature for the number of subunits within these complexes have ranged from 8 to 14 (15, 19–23), with some unusual exceptions (24, 25). We previously showed using three-dimensional reconstructions of single particle electron microscope images that the α isoform of CaM-kinase II is a dodecameric complex with overall dimensions of 200 × 220 Å. We further showed that the N-terminal catalytic domains reside in foot-like appendages extending away from a central core structure formed by the C-terminal association domains. Sev-
eral other analyses have been accomplished visualizing single particle images (23, 26, 27) and two-dimensional average images (28) and each of these studies identified that CaM-kinase II isoforms assemble into oligomeric complexes. However, inconsistencies remain regarding the subunit composition within the holoenzymes and to date, three-dimensional structural information is available only for the α isoform of CaM-kinase II. Many aspects of CaM-kinase II enzymatic function have been studied with respect to differences between pairs of isoforms or between sets of splice variants; however, there has not yet been a side-by-side comparison of members from each of the four distinct gene products of CaM-kinase II. In this study, we describe such a comparison, looking at potential differences in the structure and enzymatic properties between the α, β, γ, and δ isoforms.

EXPERIMENTAL PROCEDURES

Expression of Isoforms—CaM-kinase II isoforms were expressed in Sf21 cells and purified as previously described (29). A description of the expression of the α and δ isoforms can be found in Ref. 22. The cDNAs encoding the full-length rat δ, and human γC isoforms were obtained from Dr. Andy Hudmon and Dr. Howard Schulman and were cloned into the EcoRI site of the pFastbac-1 baculovirus expression vector. Bacmid, virus production, protein production, and protein purification were accomplished as described (29).

Expression, Purification, Mutagenesis, and Labeling of CaM—Calmodulin was expressed from the pET23 vector in the BL21 DE3 pLys-S strain of Escherichia coli. To purify CaM, the cell pellets were lysed by multiple cycles of freeze/thaw, followed by resuspension in 50 mM Tris, 10 mM EDTA, and sonication. The lysate was heated to 70 °C and then cell debris was pelleted by centrifugation at 100,000 × g for 1 h. The supernatant was brought to 2.5 M ammonium sulfate, the precipitate was spun out, and the resulting supernatant was then brought to saturation with ammonium sulfate. This solution was centrifuged, and the pellet was resuspended in 50 mM Tris, pH 7.5, 200 mM ammonium sulfate, and 1 mM EDTA and loaded onto a phenyl-Sepharose column. The flow-through from this column was brought to 2.5 mM CaCl₂, and then loaded on a second phenyl-Sepharose column. CaM was eluted with 50 mM Tris, pH 7.5, 1 mM NaCl, and 2.5 mM EGTA. CaM was mutat at Lys-75 and labeled with IAEADNS as described in Putkey and Washam (29).

Gel Filtration and Density Gradient Centrifugation—Gel filtration was accomplished using a Superose HR 10/30 column (Amersham Biosciences) on an AKTA FPLC system and sucrose gradient centrifugation on linear 5–24% gradients were performed as described in Kolb et al. (22).

Dynamic Light Scattering—To determine diffusion coefficients (D), the holoenzyme form of each subunit was first isolated by chromatography on a Superose HR6 10/30 column (Amersham Biosciences) in 40 mM Hepes, pH 7.5, 150 mM KCl, and 0.1 mM EGTA and then subjected to dynamic light scattering. The kinases, at concentrations of 0.089 to 0.25 mg/ml, were analyzed by the Fourier shell correlation with a Fourier ring criterion of 0.5 (32). The α, β, γ, and δ isoforms consisted of 3003, 3061, 5085, and 5313 images, and had resolution values of 21, 23, 22, and 25 Å, respectively. The solid-structured volumes were thresholded to a volume that corresponds to their approximate molecular weight, and the images were rendered using the Explorer software (NAG, Inc., Donner’s Grove, IL). A test for the 6-fold symmetry of the particles was accomplished by starting with the 6-fold symmetric model to generate reference projections at 5° increments setting the range of φ to 75 ° and 52.1°, to produce 5- and 7-fold models, respectively. During the backprojection to render the new volumes either 5- or 7-fold symmetry was imposed. These newly rendered structures were used as the reference volumes for the next set of alignments using projections at 3° increments. Three further sets of alignments were run at 2° increments using the previously generated structure as the model. The artificially generated model was also used to test the structural symmetry of the β and δ isoforms. In each case the refinements were evaluated by degree of convergence between subsequent steps, the value of the resolution estimation, and visual inspection of the final structures for obvious artifacts.

MALDI Mass Spectrometry—For analysis of isoform subunit mass, kinase samples were diluted 1:10 in H₂O with 10% acetonitrile and 0.01% formic acid. Dried droplet deposits were prepared by mixing the diluted samples 1:1 with sinapinic acid (10 mg/ml) dissolved in 100% acetonitrile with 0.1% formic acid. Positive ion MALDI mass spectra were acquired in linear mode on an Applied Biosystems Voyager DE-STR instrument using bovine serum albumin as an external calibrant. In some experiments, glutaraldehyde cross-linking was performed to produce covalent bonds between subunits. Glutaraldehyde (25%) was dissolved in deionized water and then subjected to linear prefocused grid (5085, 5108, and 5313 images, and had resolution values of 0.125 or 0.25% A). Aliquots were removed at 0.5, 1, 2, 5, and 10 min for analysis. Dried droplet deposits were prepared by mixing the protein solution 1:1 with saturated sinapinic acid or ferulic acid in 40% aqueous acetonitrile with 0.1% trifluoroacetic acid or 0.1% formic acid, respectively. After crystal formation, the samples were washed repeatedly with cold water to remove buffer salts and glycerol prior to MALDI mass spectrometry analysis. Spectra were obtained on the Voyager DE-STR instrument for positive ions in linear mode. Parameters were optimized for immunoglobulin G (25 kV accelerating voltage, 90% grid voltage, and 900–1500 ns delay time). The mass spectra are accumu-
The high level of expression it was straightforward to obtain highly purified protein preparations. A Coomassie-stained gel of the four isoforms is shown in Fig. 1, panel B. The expected molecular weights predicted by the sequence information are 54,110 (α), 60,397 (β), 58,361 (γ), and 60,076 (δ). These are largely consistent with the migration of the purified isoforms on SDS-PAGE, with the relative molecular weights of the expressed isoforms being calculated at ~52,200 (α), ~60,500 (β), ~61,000 (γ), and ~60,000 (δ). Note also that there is an additional protein band in the β preparation with a lesser amount in the δ preparation (arrowheads in Fig. 1) and none visible in either the α or γ preparations. The calculated mass of this additional band is ~64,700 and ~64,800, for β and δ, respectively, and this protein in the β preparation cross-reacted with β-specific monoclonal antibodies. The origin or nature of this higher molecular weight product is presently unknown.

To provide a more accurate mass measurement of each purified preparation, MALDI-TOF mass spectrometry was performed. This analysis led to a molecular mass of 53,885 ± 97 kDa for α, 60,265 ± 66 kDa for β, 58,325 ± 15 kDa for γ, and 59,856 ± 63 kDa for δ (Table 1).

Hydrodynamic Analysis—Sucrose gradient centrifugation, dynamic light scattering, and gel filtration chromatography were used to determine the physical characteristics of each CaM-kinase II isoform. The sedimentation coefficient was determined by centrifugation of the preparations on a 5–24% sucrose gradient. The β isoform had the lowest sedimentation coefficient of 14.5 S, whereas the other three isoforms all had sedimentation coefficients of about 17 S (see Table I). Dynamic light scattering measures the diffusion coefficient of molecules, which is proportional to their hydrodynamic radius $R_h$, calculated through the Stokes-Einstein relationship. Using such an analysis, the $D$ and $R_h$ values were determined for each CaM-kinase II isoform. $\alpha$ has the smallest $R_h$ of 9.22 ± 0.13 nm, $\beta$ has an $R_h$ of 12.73 ± 0.07 nm, $\gamma$ has an $R_h$ of 11.35 ± 0.12 nm, and $\delta$ has an $R_h$ of 11.90 ± 0.17 nm. The 9.22 nm value for α-CaM-kinase II is very similar to that reported by Bradshaw et al. (34), who used dynamic light scattering to determine the $R_h$ of the α-CaM-kinase II enzyme complex to be 9.1 nm. The rank order of these $R_h$ values is the same as the rank order of the molecular weights of the subunits of each isoform ($\alpha < \gamma < \delta < \beta$). Similar results were found using gel filtration chromatography (data not shown), however, accurate estimates of $R_h$ values were difficult because of the fact that these large complexes elute near the void volume of the Superose HR6 column. We therefore utilized the dynamic light scattering data to calculate the overall mass of each holoenzyme complex. As shown in Table I, all four isoforms form large multisubunit complexes, and the rank order of the size of the holoenzymes is the same as for the individual subunits, $\beta > \gamma > \delta > \alpha$. The frictional ratios ($f/f_0$) suggest that $\alpha$ is somewhat less elongated than the other three isoforms, with $\alpha$ having an $f/f_0$ of 1.58, whereas the $f/f_0$ of the other three isoforms range from 1.83 to 1.95.

Three-dimensional Reconstructions of CaM-kinase II α, β, γ, and δ Isoforms—The high degree of amino acid sequence homology between the proteins and the consistent biophysical properties of each holoenzyme suggested that their overall architecture is similar. Using EM reconstructions of single particle images collected in methanolamine tungstate stain as described under “Experimental Procedures,” a three-dimensional structure was determined for each holoenzyme. These results reveal that the three-dimensional structures of the CaM-kinase II isoforms are very similar at the present resolution of 21, 23, 22, and 25 Å for the α, β, γ, and δ reconstructions, respectively (Fig. 2). Each is a dodecameric structure consisting of two morphologically distinct parts; the foot and
leg-shaped N-terminal functional domains that are attached to both ends of the gear-shaped core formed by the C-terminal association domains (Fig. 2). The gear-shaped core is 140 Å in diameter with a height of 100 Å. The six slanted cogs of the core have six of the foot and leg-like features attached at their outer edge at the top and bottom of the complex. The append-
ages increase the length of the complex to −200 Å and their diameter to −220 Å. The foot-like functional domain is tilted −30° away from the 6-fold axis of the structure and, consequently, the feet reside peripheral to the core and give rise to the ring of density about the core seen in the characteristic electron microscopy images of the end view of the molecule (Fig. 2). Previously, we showed that the subunits are arranged head to tail and that six dimers form twisted strands that traverse the core of the complex (10). As anticipated, at this level of resolution, it was not possible to identify features in the β, γ, or δ isoforms that could be attributed to the small (−6 kDa) unique domain or to the additional C-terminal protein domain present in δ (see Fig. 1). However, it is clear that each CaM-kinase II isoform assembles into a dodecameric complex with similar architectures. Attempts to align and refine the data sets to either 5- or 7-fold models (see “Experimental Procedures” for details) failed to converge on stable structures. Moreover, model free alignments of the end view projections of the structure consistently generated an image with 6-fold symmetry and principal component analysis and hierarchical clustering performed on the entire data set yielded only 6-fold group averages (data not shown). These results converge on 6-fold symmetry in the molecule supporting the dodecameric structure shown in Fig. 2.

Glutaraldehyde Cross-linking and Mass Spectrometry—The subunit composition of αCaM-kinase II was also assessed by glutaraldehyde cross-linking subunits within holoenzyme complexes and collecting MALDI mass spectra at increasing times of reaction. The mass spectrum for the enzyme prior to cross-linking is shown in the top panel of Fig. 3. The appearance of dimers, trimers, and tetramers is an artifact of the MALDI process and is observed in the un-cross-linked mass spectrum of both αCaM-kinase II (top panel, Fig. 3) and bovine serum albumin, which was used as a negative control (data not shown). The amplitude of the signals of these multiply charged protein species follows an exponential decay and is straightforward to distinguish from cross-linked intermediates. After 2 min of cross-linking, ion signals are observed for monomer, dimer, trimer, tetramer, and hexamer forms of αCaM-kinase II (Fig. 3, middle and lower panels). These species have masses (mean ± S.D.; n = 4) of 53,343 ± 260, 106,874 ± 427, 160,604 ± 979, 213,997 ± 1538, and 321,917 ± 2568 Da, respectively. At this 2-min time point, the amplitude of the signal from the multimeric forms does not follow an exponential decay as it does in the non-cross-linked sample (compare Fig. 3, top and middle panels, respectively). The peaks in the cross-linked sample are also somewhat broadened indicative of microheterogeneity likely because of a variable number of glutaraldehyde molecules reacting with the protein. This is more evident when the spectrum is background corrected (Fig. 3, bottom panel). There was no evidence in the cross-linking spectra of heptameric (7-mer) intermediates. Under these reaction and analysis conditions, we did not see consistent formation of higher order structures. Occasionally, a small peak was observed at a dodecameric mass, but was not of significant magnitude for a proper mass assignment. Overall, this pattern of ion signals is consistent with a hexameric structure (and a 6-fold axis of symmetry) in native αCaM-kinase II holoenzymes.
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and supports the results that CaM-kinase II forms dodecameric holoenzyme complexes (Fig. 2).

**CaM Binding**—The affinity of each isoform for CaM was determined using a fluorescently labeled CaM, CaM-C75-IAE-DANS, which exhibits an increase in fluorescence intensity upon binding to a target protein. Titration of CaM-C75-IAE-DANS with increasing concentrations of each CaM-kinase II isoform resulted in the sigmoidal binding curves shown in Fig. 4. The $K_D$ values and Hill coefficients from fitting the data to the Hill equation are summarized in Table II. $\alpha$ exhibits the lowest affinity for CaM with a $K_D$ of $62.4 \pm 25.1 \text{ nM}$, and $\gamma$ has the highest affinity with a $K_D$ of $6.9 \pm 1.4 \text{ nM}$, while $\delta$ and $\beta$ lie in between with $K_D$ values of $25.7 \pm 7.9$ and $33.5 \pm 13.2 \text{ nM}$, respectively. The $K_D$ for CaM binding to $\gamma$ was significantly different from the $K_D$ values for $\alpha$, $\beta$, and $\delta$ ($p < 0.02$, $p < 0.01$, and $p < 0.05$, respectively, Student’s $t$ test). The $K_D$ values for $\alpha$ and $\beta$ were also significantly different from each other ($p < 0.02$). Whereas the Hill coefficients for each curve were somewhat variable, they were all greater than 1.5, mostly clustering around 2, indicating positive cooperativity of the binding reaction between CaM and each CaM-kinase II isoform.

**CaM Dependence of Substrate Phosphorylation**—Fig. 5 shows the results from kinase activity assays that measure phosphate incorporation into the peptide substrate syntide, at varying concentrations of CaM. Plots of the enzyme specific activity versus CaM concentration were fit with the Hill equation and the half-maximal activation concentration ($K_{0.5}$) and Hill coefficients are summarized in Table II. As for binding to CaM, $\alpha$ has the highest $K_{0.5}$ ($68.1 \pm 40.3 \text{ nM}$), $\gamma$ has the smallest ($10.6 \pm 5.4 \text{ nM}$), and $\beta$ and $\delta$ fall in between ($22.4 \pm 6.5$ and $19.1 \pm 12.1 \text{ nM}$, respectively). The rank order for both CaM-dependent enzyme activation and CaM binding to each isoform is the same, and the overall values are remarkably similar to each other for the 4 isoforms. Interestingly, the Hill coefficients for enzyme activation for exogenous substrate phosphorylation are all about 1 as compared with 2 for CaM binding.

**CaM Dependence of Autophosphorylation**—The calmodulin dependence of autophosphorylation was determined by performing autophosphorylation assays for each isoform at varying levels of calmodulin. Plots of the resulting data are shown in Fig. 6. The half-maximal activation ($K_{0.5}$) and Hill coefficients were determined by fitting the data to the Hill equation. Note that these parameters are dependent on the reaction conditions, including the length of the reaction, and so the information gained from this experiment is most relevant for comparisons between the isoforms. $\alpha$ has the highest $K_{0.5}$ of $222.5 \pm 14.3 \text{ nM}$, whereas $\beta$ has the lowest ($90.4 \pm 17.4 \text{ nM}$). $\gamma$ and $\delta$ fall in between, with $K_{0.5}$ values of $117.5 \pm 47.8$ and $123.7 \pm 31.3 \text{ nM}$, respectively. $T$ tests show that the value for $\alpha$ is significantly different from those for $\beta$ ($p < 0.005$) and $\delta$ ($p < 0.05$). The Hill coefficients are all greater than 1.7, similar to those for CaM binding, but different from CaM activation of the enzyme for substrate phosphorylation.

The autophosphorylation curves are functions of both the affinity of CaM-kinase II for CaM and the rate of autophosphorylation of the kinase, and reveal information about both. Because we have explicitly measured the affinity of each isoform for CaM, differences between the curves that clearly do not depend on CaM affinity must be because of different rates of autophosphorylation. The $\gamma$ isoform, which has the lowest $K_{0.5}$ for CaM, would be expected to have the lowest $K_{0.5}$ for autophosphorylation. Because it does not, the result implies that the rate for $\gamma$ of autophosphorylation is slower than the rates of the other isoforms.

$K_m$ for ATP and Syntide—Table III compares the kinetic variables for the phosphorylation of syntide by the four isoforms. The $K_m$ for ATP was $28.77 \pm 5.29 \text{ \mu M}$ for $\alpha$, $24.33 \pm 2.85 \text{ \mu M}$ for $\beta$, $16.85 \pm 0.81 \text{ \mu M}$ for $\gamma$, and $19.06 \pm 4.24 \text{ \mu M}$ for $\delta$. Of these, the only significant difference was that the value for $\gamma$ was less than those for $\alpha$ and $\beta$ ($p < 0.02$). The $K_m$ for syntide was $13.80 \pm 2.86 \text{ \mu M}$ for $\alpha$, $8.90 \pm 1.05 \text{ \mu M}$ for $\beta$, $13.28 \pm 6.54 \text{ \mu M}$ for $\gamma$, and $11.84 \pm 3.23 \text{ \mu M}$ for $\delta$. The only significant difference in these values was between those for $\alpha$ and $\beta$ ($p < 0.05$). The $V_{\max}$ values ranged between 14.9 and 22.4 \mu mol/min and were not significantly different between the isoforms.

**Computer Simulations of Autophosphorylation**—To gain insight into how the measured biochemical differences between the four isoforms might affect their function, we ran simulations of the response of CaM-kinase II to 1-\muM pulses of Ca$^{2+}$. We held the association rate constant for CaM binding to each isoform constant at $2.1 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$, and varied the off-rate to match the measured $K_{0.5}$ for each isoform. Fig. 7A shows the percentage of kinase bound to CaM during and immediately following a 100-ms pulse of Ca$^{2+}$. Surprisingly, the four isoforms behave very similarly despite the differences in their CaM dissociation rates. However, the amount of autophosphorylation of each isoform in response to the same 100-ms pulse of Ca$^{2+}$ is remarkably different (Fig. 7B). This is because of the

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3T. R. Gaertner and M. N. Waxham, unpublished observation.
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Fig. 4. CaM-C75-IAEDANS binding to CaM-kinase II isoforms. CaM-kinase II isoforms were titrated into a fluorimetry cuvette containing 25 or 100 nM CaM-C75-IAEDANS in 25 mM MOPS, pH 7.0, 150 mM KCl, 0.5 mM CaCl₂, and 0.1 mg/ml bovine serum albumin. After each addition of CaM-kinase II, fluorescence was measured in a PTI fluorimeter, with excitation at 345 nm and emission monitored at 465 nm (5 nm band widths). For each point, fluorescence was averaged over a 1-min period. The fraction of CaM bound was calculated using the equation, CaM bound = (1 - I free)W(I bound - I free), where I is the measured fluorescence intensity, I free is the fluorescence intensity in the absence of CaM-kinase II, and I bound is the fluorescence intensity of the fully bound CaM. The x axis in each graph is the free concentration of CaM-kinase II at each point, calculated by subtracting the concentration of bound CaM from the total concentration of CaM-kinase II subunits. Each titration was performed at least three times for each isoform, and the data was fit to the Hill equation, y = ax^h(KD + x^h), where h is the Hill coefficient). Shown are representative curves for each isoform: A, α; B, β; C; and D, δ.

TABLE II

Calmodulin interactions with CaM-kinase II isoforms

Experimental data such as that shown in Figs. 4 (for binding), 5 (for activity), and 6 (for autophosphorylation) were fitted with the Hill equation (y = (ax^h)/(b^h + x^h)), where b is the KD or KA and h is the Hill coefficient). The values reported are mean ± S.D. of at least three curves.

| Isoform | Binding KD (nM) | Hill coefficient | Activity KD (nM) | Hill coefficient | Auto phosphorylation KD (nM) | Hill coefficient |
|---------|----------------|------------------|------------------|------------------|-------------------------------|-----------------|
| α       | 62.4 ± 25.1    | 1.9 ± 0.7        | 68.1 ± 40.3      | 0.8 ± 0.1        | 222.6 ± 14.3                 | 1.9 ± 0.1       |
| β       | 25.7 ± 7.9     | 2.4 ± 0.9        | 22.4 ± 6.5       | 1.0 ± 0.1        | 90.4 ± 17.4                  | 2.4 ± 0.3       |
| γ       | 6.9 ± 1.4      | 2.1 ± 0.3        | 10.6 ± 5.4       | 1.0 ± 0.3        | 117.5 ± 47.8                 | 1.7 ± 0.5       |
| δ       | 33.5 ± 13.2    | 1.6 ± 0.3        | 19.1 ± 12.2      | 1.0 ± 0.1        | 123.7 ± 31.3                 | 1.8 ± 0.3       |

Differences in the rates of autophosphorylation of the isoforms. These rates were empirically fitted to the data measuring the CaM dependence of autophosphorylation (Fig. 6). As discussed above, these data had revealed that the γ isoform had a slow rate of autophosphorylation compared with the other three isoforms, but did not provide an intuitively obvious description of the rates of autophosphorylation of the other isoforms. Fitting our model to the CaM-dependent autophosphorylation data suggests that the rank order of rates of autophosphorylation is δ > β > α > γ.

In response to repetitive pulses of Ca²⁺, the autophosphorylation of each isoform shows distinct frequency dependence (Fig. 7C). The relationship between the amount of autophosphorylation and frequency of Ca²⁺ pulses is exponential, as has been seen experimentally (35, 36). The autophosphorylation of the δ isoform has the fastest exponential increase with respect to frequency, followed by β, α, and then γ. The differences between the curves are because of the differences in the rates of autophosphorylation, as described above. This demonstrates that whereas the biochemical differences reported in this paper appear fairly subtle, they can have a non-linear and significant effect on the functional properties of the isoforms.

DISCUSSION

We describe in this report a direct comparison of the structural and biochemical properties of each of the four mammalian gene products that compose the CaM-kinase II isoform family. The deduced protein sequences of the α, β, γ, and δ isoforms are largely identical with the greatest differences between them residing in the area just C-terminal to the calmodulin-binding domain. The δ isoform also has an additional protein domain at its C terminus. This high degree of protein sequence similarity suggests that the overall architecture of holoenzymes formed from each isoform would be similar at the low resolution three-dimensional EM structural level and this is the case. Comparison of the three-dimensional EM reconstructions for all four isoforms at 21–25 Å reveals a great degree of architectural similarity. All have catalytic domains that reside on foot-like appendages extending out from a central barrel-shaped core and all are dodecameric complexes with similar overall dimensions of ~200 x 220 Å. Apparently because of the unique architecture of this multisubunit complex, biophysical analysis of the enzyme does not lead to an accurate estimate of the subunit composition. As visualized for all four isoforms, the 12
catalytic domains extend away from the core on thin stalks that produce a significant inhomogeneity in the protein distribution within a holoenzyme complex.

Our conclusion that CaM-kinase II forms dodecameric complexes is consistent with early biophysical and EM characterization of CaM-kinase II. Miller and Kennedy (19) isolated CaM-kinase II from forebrain (α to β ratios of ~3:1) and Woodgett et al. (27) isolated the enzyme from skeletal muscle. Both used biophysical techniques (similar to those applied in the present study) and arrived at the conclusion that CaM-kinase II was a 12-subunit complex. Woodgett et al. (27) further showed that negative stained skeletal muscle CaM-kinase II appeared to be two stacked hexameric rings. From that point, there has been ambiguity in the literature concerning the subunit composition of CaM-kinase II. The cerebellar form of CaM-kinase II was thought to be a 10-mer by similar biophysical analyses (37) and Kanaseki et al. (23), using rotary shadowing and EM visualization of CaM-kinase II isolated from forebrain and cerebellum, arrived at the conclusion that forebrain enzyme was a 10-mer and the cerebellar enzyme was an 8-mer. Many of these previous results were accomplished by using CaM-kinase II isolated from tissue (e.g. brain, muscle, etc.). These preparations are often a mixture of different subunits that combine in a stochastic fashion inside cells into an array of different CaM-kinase II oligomeric complexes that might further complicate interpretations of the number of subunits within each holoenzyme complex.

Others have taken an approach similar to ours and expressed the cloned cDNA for different isoforms to minimize complications associated with the mixed subunit nature of the tissue-isolated CaM-kinase II holoenzymes. Using expression of the cloned cDNAs for α and β in eukaryotic cells, Yamauchi et al. (38) reached the conclusion that β was likely monomeric, whereas α was oligomeric. Similarly, Shen et al. (24) suggested that βCaM-kinase II expressed in cells (with a GFP tag) was significantly smaller than α, with the prediction of about 4 subunits in a complex, although the sizing technique utilized (intracellular fluorescence photobleaching recovery) was largely indirect. Similar efforts analyzing individual isoforms expressed from cloned cDNAs indicated that each formed holoenzymes composed of 8–12 subunits of α, β (22), γC (21), γC, γB, δA, δB, and δC. Doosemecri et al. (26) provided single particle analysis of pure α holoenzymes observed by rotary shadowing and like Kanaseki et al. (23) described αCaM-kinase II holoenzymes as being composed of flower-shaped molecules with 8–10 peripheral petals.

Most recently, the crystal structure of a bacterially expressed C-terminal association domain of αCaM-kinase II was determined to be a 14-mer with a 7-fold axis of rotation, a conclusion supported by physicochemical analysis (39). These authors suggest the intriguing possibility that the oligomerization state of various CaM-kinase II assemblies may be different. However, our results do not support this possibility as we find that all four full-length and catalytically active isoforms of CaM-kinase II assembled into dodecameric complexes. The finding that the truncated core is a 14-mer is also in contrast to the data presented in this paper and to our original three-dimensional EM reconstructions of a similar truncated form of the core of αCaM-kinase II (10). At present it is difficult to reconcile these findings. Cell type-specific factors (prokaryotic versus eukaryotic) might regulate the assembly of CaM-kinase II complexes, or perhaps variations in the cellular milieu such as pH, protein concentration, temperature, and ionic strength might also impact the final structures formed. Our conclusion about the
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Fig. 6. Dependence of CaM-kinase II autophosphorylation on CaM. CaM-kinase II autophosphorylation was measured at varying concentrations of CaM. Each reaction contained 50 mM HEPES, 70 mM KCl, 0.5 mM CaCl₂, 0.4 mM dithiothreitol, 10 mM MgCl₂, 100 μM [γ-32P]ATP (2 μCi/μl reaction), 100 ng reaction kinase, and 3.0 mM to 3.2 μM CaM. Reactions were started by the addition of kinase, incubated on ice for 15 s, and then stopped by the addition of trichloroacetic acid. Each reaction was performed in duplicate, and the entire set of reactions was completed three times. The amount of autophosphorylation, as a percentage of the maximal autophosphorylation for each isoform, was plotted against the concentration of CaM in the reaction, and the curves were fitted with the Hill equation. Shown are representative curves: A, α; B, β; C, γ; and D, δ.

dodecameric form of the kinase was further supported by glutaraldehyde cross-linking and MALDI-TOF mass spectrometry. We found clear evidence of hexameric but not heptameric intermediates in the data. Furthermore, attempts to force 7-fold (or 5-fold) symmetry on our single particle data sets did not produce convergence onto reliable structures. All of the present results support the conclusion that full-length CaM-kinase II subunits assemble into dodecameric holoenzymes. We conclude that differences in the amino acid sequence between the isoforms (see Fig. 1) do not play roles in determining the basic dodecameric architecture of CaM-kinase II holoenzymes. Higher resolution EM reconstructions will be needed to identify the unique differences between the isoforms.

The disposition of the catalytic domains in foot-like appendages extending away from the central core is a unique feature of CaM-kinase II. The data indicate that the 12 catalytic domains are arranged into two clusters of 6 that extend out and away from the central core when viewed from the side (see Fig. 2). Others have proposed hypothetical models where the catalytic domains form a ring around the equator of the central core (28, 39); however, such models are incongruent with the three-dimensional structures of the 4 isoforms presented herein. We also find no evidence in the raw data (Fig. 2) for such an equatorial ring of high protein density that would manifest itself as a band in side views of the particles. Because of the large distance (~200 Å) separating the two clusters of 6 catalytic domains it appears that each cluster functions as an independent entity, assuming that there is not a significant collapse of the holoenzyme upon activation. However, the 6 catalytic domains within each ring lie within a constrained area of ~200 Å in diameter that increases their effective concentration to millimolar levels. Presumably this high effective concentration provides a strong driving force for the autophosphorylation reaction between neighboring subunits and may be one of the primary reasons for CaM-kinase II to assemble into an oligomeric complex.

In studying the interactions of CaM-kinase II with CaM, we have found quantitative differences in CaM binding to and activation of the four isoforms. γ has a lower $K_D$ for CaM and is half-maximally activated at a lower CaM concentration than the other isoforms. α has a higher $K_D$ for CaM and is half-maximally activated at a higher CaM concentration than the other isoforms. β and δ have intermediate $K_D$ values for CaM and are half-maximally activated at intermediate concentrations compared with the other isoforms. Therefore, in the cellular environment, γ is predicted to be more sensitive and α is less sensitive to changes in Ca²⁺ concentration than the other isoforms.

Previous studies on CaM-dependent activation between isoforms fall into two categories: those comparing α versus β isoforms or forebrain versus cerebellar isozymes, and those comparing splice variants of a given isoform. Several groups have found that the α isoform of CaM-kinase II isolated from forebrain has a higher $K_D$ for activation by CaM and autophosphorylation than the β isoform or the cerebellar enzyme (19, 35, 40). Our findings are consistent with these results. Studies comparing CaM activation of splice variants of a given isoform of CaM-kinase II lead to no obvious conclusions about the role of the variable domain in CaM sensitivity. Bayer et al. (36) suggest that a shorter insert in the variable domain may cause a decreased affinity for CaM, which is supported by the comparison between α (with no insert in the variable domain and low CaM affinity) and β (with a large insert and higher CaM affinity). However, other studies have shown no correlation between insert length and CaM activation (15, 41). As reported here, we also do not find a correlation between insert length and affinity for CaM. The α isoform lacks an insert in the variable domain and does indeed have the lowest affinity for CaM. However, the β isoform, which has the longest insert in the variable domain, would be expected to have the highest affinity for CaM and this is not the case. The γ isoform exhibited the highest CaM binding affinity of the four isoforms.

One may also consider the roles of particular sequences within the inserts in conferring sensitivity to CaM. Kwiatkowski and McGill (42) compared the CaM activation of two γ splice variants, γβ and γγ, which differ by a 21-residue insert present in γγ. They found that the $K_D$ for γγ is less than half of that for γβ. They conclude that this region modulates CaM sensitivity, and, using site-directed mutagenesis, show that a charged residue in this sequence is responsible for part of the difference in affinity for CaM. A sequence homologous to this insert is found in the β isoform, which, as we show here, has a
lower affinity for CaM than \( \gamma \). Therefore, one cannot make simple predictions based on inserts found in the variable domain as to their impact on CaM binding and activation of CaM-kinase II and other determinants must also play a role in defining the interactions of each isoform with CaM.

Outside of the variable domain, the differences between the isoforms are quite few. The CaM-binding domain is identical for the \( \alpha \), \( \beta \), and \( \delta \) isoforms, whereas the \( \gamma \) isoform has three conservative substitutions. The catalytic and association domains of the four isoforms are also very similar, with only a few mostly conservative differences. The large differences between the isoforms are clearly in the variable domain and this is the most likely candidate site for effects on CaM binding, because the variable region follows directly after the CaM-binding domain. However, as described above, it is difficult to infer direct correlations between length of linker or even presence or absence of a specific insert and the interactions of CaM with CaM-kinase II. The enzymatic differences between the isoforms are likely because of a combination of influences, including the size of the variable domain, the specific residues that are present, and differences in sequence in other regions of the protein. The impacts of residues in both the core CaM-binding domain, and the catalytic domain that govern CaM binding affinity are highlighted by the study of Zhi et al. (43). Production of chimeric constructs between CaM-kinase II and skeletal muscle myosin light chain kinase demonstrated that the CaM activation of the chimeras was dictated largely by the catalytic domain and not the core CaM-binding domain. The biochemical differences that we observed between the isoforms are relatively small, but they may result in large functional differences (i.e. in activation or autophosphorylation) under conditions in which Ca\(^{2+}\) levels are changing rapidly, such as spikes or oscillations in Ca\(^{2+}\). This idea was put forth by De Koninck and Schulman (35), who described the dependence of CaM-kinase II autophosphorylation on the frequency of Ca\(^{2+}\) input into an in vitro system, and showed that \( \alpha \) and \( \beta \) differed in the dependence of autophosphorylation on Ca\(^{2+}\) frequency. Our simulations indicate that the dependence on Ca\(^{2+}\) frequency is affected more by the rate of autophosphorylation than by the affinity of the isoform for CaM. The underlying mechanism is that isoforms with faster rate of autophosphorylation will undergo more autophosphorylation during a given Ca\(^{2+}\) pulse. Autophosphorylation at Thr-286 leads to trapping of CaM on the kinase (2), so that at the next pulse of Ca\(^{2+}\), the occupancy of CaM on the enzyme is greatly increased, and this leads to even more autophosphorylation. This non-linear effect leads to large differences in autophosphorylation for subtle differences in autophosphorylation rate. This idea was also put forth by Bayer et al. (36) who suggested that differences in both rate of autophosphorylation and affinity for CaM could alter the frequency dependence of autophosphorylation. They experimentally compared the frequency dependence of autophosphorylation between three splice variants of \( \beta \)CaM-kinase II and found that the \( \beta \)M isoform had higher autophosphorylation in response to Ca\(^{2+}\) pulses than did \( \beta \), which in turn had a higher response than \( \beta \)M. They attributed these differences to the fact that \( \beta \)M had a lower affinity for CaM, and \( \beta \)M had a faster rate of autophosphorylation than \( \beta \). Our simulations suggest that the difference in CaM affinity alone would not cause a significant difference in the frequency dependence curves for these splice variants.

Why do some cell types express one particular isoform of CaM-kinase II rather than another and when multiple isoforms are expressed in the same cell type, as in neurons, are they activated by different stimuli? Our data demonstrate that the enzymatic properties of CaM-kinase II isoforms allow them to respond differentially to a given Ca\(^{2+}\) signal. For instance, in response to 30 s of Ca\(^{2+}\) pulses at 8 Hz, there is 6-fold more autophosphorylation of the \( \delta \) isoform compared with the \( \gamma \) isoform. The differences between the isoforms in CaM dependence of activation and rate of autophosphorylation are relatively subtle but are likely to be significant in the cellular context. It is important to realize that, although our studies compared homo-oligomers of the four isoforms, in the cell CaM-
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kinase II subunits additionally assemble into hetero-oligomers. The functional properties of the holoenzymes will therefore be based on the relative levels of expression of the isoforms. By altering the level of expression of each isoform, the cell can tune the properties of CaM-kinase II to respond appropriately to incoming Ca\textsuperscript{2+} signals.

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