Structural Features of Ca^{2+}/Calmodulin-dependent Protein Kinase II Revealed by Electron Microscopy

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Abstract. The molecular conformation of Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) from the rat forebrain and cerebellum was studied by means of EM using a quick-freezing technique. Each molecule appeared to be composed of two kinds of particles, with one larger central particle and smaller peripheral particles and had shapes resembling that of a flower with 8 or 10 "petals." A favorable shadowing revealed that each peripheral particle had a thin link to the central particle. We predicted that the 8-petal molecules and 10-petal molecules were octamers and decamers of CaM kinase II subunits, respectively, each assembled with the association domains of subunits gathered in the center, and the catalytic domains in the peripheral particles. Binding of antibodies to the enzyme molecules suggested that molecules with 8 and 10 peripheral particles were homopolymers composed only of β subunit and of α subunit, respectively, specifying that CaM kinase II consists of homopolymer of either α or β subunits.

CALCIUM ions play important roles (Rubin, 1970) through the action of calmodulin-dependent protein kinases, by stimulating phosphorylation of a number of endogenous proteins in various tissues (Cohen, 1982; DeLorenzo et al., 1979; Schulman and Greengard, 1978; Yamauchi and Fujisawa, 1979). Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) is a multifunctional serine/threonine protein kinase and is one of the most abundant protein kinases in mammalian brain (Bennett et al., 1983; Goldenring et al., 1983; Kennedy and Greengard, 1981; Yamauchi and Fujisawa, 1980, 1983). Neuronal CaM kinase II plays a major part in the regulation of the synthesis and secretion of neurotransmitters, receptor function, structural modifications of the cytoskeleton, axonal transport, and in long term potentiation (for reviews see Colbran et al., 1989; Nairn et al., 1985; Soderling, 1990).

CaM kinase II purified from the rat forebrain has a native molecular mass of 550–650 kD, and is composed of two distinct but closely related protein subunits of 50 kD (α) and 60/58 kD (β/β'). Their cDNA sequences show that both subunits contain catalytic, regulatory (calmodulin-binding), and association domains (Bennett and Kennedy, 1987; Hanley et al., 1987; Lin et al., 1987). The subunits are the products of two highly homologous transcription units, with the larger size of the β subunit due to amino acid insertions at the carboxyl-terminal side of the calmodulin-binding domain. The composition of α and β subunits in CaM kinase II depends on the region of brain and on the stage of development. The ratios of α to β subunits are reported to be $\sim$3:1 and 1:4 in the adult forebrain and cerebellum, respectively (McGuinness et al., 1985; Miller and Kennedy, 1985). Retinal CaM kinase II is composed of the α subunit alone (Bronstein et al., 1988). The existence of two developmentally regulated isoforms of the kinase has been reported in the rat forebrain, with an α/β ratio for 10 d rat of 1:1, and for adult of 2.3:1 (Kelly et al., 1987; Rostas et al., 1988). CaM kinase II is known to undergo autophosphorylation in the presence of Ca^{2+} and calmodulin (Lai et al., 1986; Lou et al., 1986; Miller and Kennedy, 1986; Saitoh and Schwartz, 1985; Schworer et al., 1986), which results in the appearance of Ca^{2+}-independent enzyme activity. Recently, α and β subunits each showing enzymatic activity were expressed from their respective cDNAs in cultured mammalian cells and in *E. coli* (Ohsako et al., 1990; Yamauchi et al., 1989), and autophosphorylation was demonstrated to occur in both types of subunit, resulting in the appearance of Ca^{2+}-independent activity.

Despite a considerable body of knowledge on the biochemical nature of CaM kinase II, information about the structure of the enzyme is scant. One of the isoforms of CaM kinase II isolated from rabbit skeletal muscle has been studied by EM using negative staining techniques (Woodgett et al., 1983). Its structure was reported to be composed of two hexameric rings with a total of 12 subunits. Here, using a quick-freezing technique (Heuser, 1983), we describe a completely different "flower" structure in rat forebrain and cerebellar CaM kinase II, with either 8 or 10 peripheral particles surrounding a large central particle. The relative incidence of 8- and 10-petal molecules closely followed the proportions of β and α subunit, respectively, in both areas of brain. Furthermore, a mAb specific to α subunit recognized exclusively 10-petal molecules. We therefore propose that CaM kinase II exists principally in the form of homopolymers of 8 (β) or 10 (α) subunits. High magnification electron

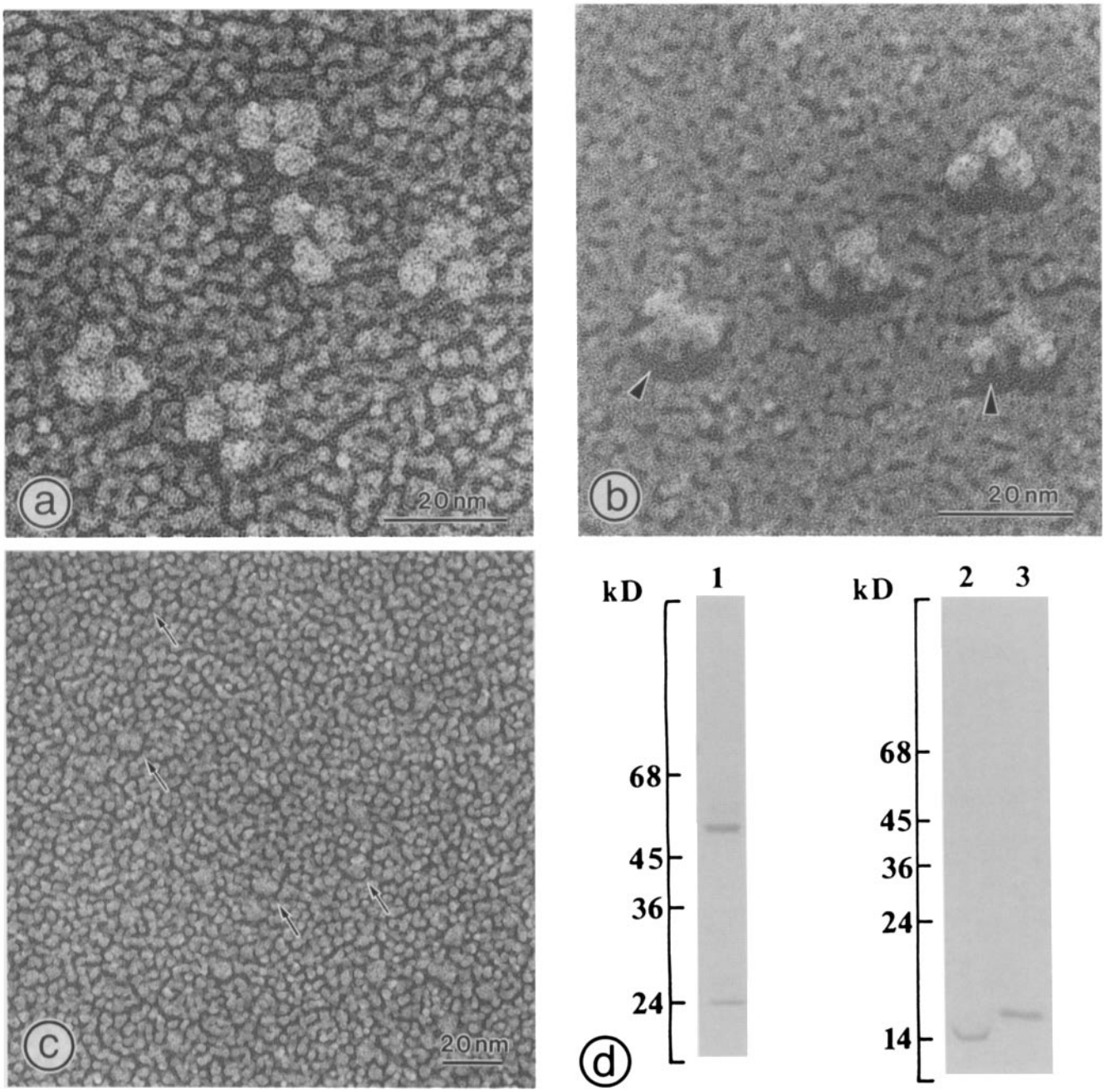


Figure 1. Morphological and biochemical demonstration of the purity of the monoclonal antibody and calmodulin preparations. (*a*) IgG molecules on replicas are seen as triangular aggregates of three 6 nm globules, as have been observed by Heuser (1983). (*b*) A favorable shadowing revealed what could be "pockets" in the variable regions of IgG molecules (*arrows*). (*c*) Calmodulin molecules on a replica. Although it is hard to distinguish the structure even with this technique, particles of ~5 nm are still observable (*arrows*). (*d*) SDS-PAGE of mAb and calmodulin. (lane *1*) mAb (1 μg protein). (lanes *2* and *3*) Calmodulin (1 μg protein) in the presence of 0.2 mM $CaCl_2$ and 1 mM EGTA, respectively.

micrographs suggested that the aggregated association domains of the subunits form the central particle, with catalytic and regulatory domains residing in the peripheral "petals."

Materials and Methods

Materials

CaM kinase II was purified from rat forebrain as described previously (Yamauchi and Fujisawa, 1983). Cerebellar CaM kinase II was purified from rat cerebellum by the same method with minor modifications. α-I protein, which is a deletion mutant of α polypeptide and is catalytically active, was prepared as described previously (Ohsako et al., 1990).

Hybridoma that secreted mAbs directed against α subunit of CaM kinase II were produced by fusion of SP2/0-Ag14 myeloma cells with spleen cells from mice that had been immunized with purified forebrain CaM kinase II essentially as described by Kohler and Milstein (1976). A mAb specific to the α subunit of forebrain CaM kinase II was purified from the culture medium of hybridoma cells by protein A-Sepharose 4B column. Protein A-Sepharose 4B was obtained from Pharmacia LKB Biotechnology, and [^{125}I]-labeled anti-mouse IgG(Fab')$_2$ from Amersham International (Amersham, UK).

Calmodulin was purified from rat testis (Dedman et al., 1977). The purity of the mAb (IgG) and calmodulin preparations used in this study is demonstrated in Fig. 1. Fig. 1 (*a* and *b*) shows replicas of quick-frozen and deep-etched mAbs on mica flakes. A replica of calmodulin molecules by the same technique is shown in fig. 1 *c*. Fig. 1 *d* shows SDS-PAGE of the IgG and calmodulin preparations. The mAb (lane *1*) and calmodulin (lanes *2* and *3*) are homogeneous on SDS gels. The migration rate of calmodulin in the presence of Ca^{2+} (lane *2*) is larger than in the absence of Ca^{2+} (lane *3*), which is consistent with a previous report by Autric et al. (1980).

EM

Almost all of the electron micrographs in this report were prepared using the mica flake cryotechniques described by Heuser (1983). Rotary replication with platinum-carbon was done using a Balzers' BAF 300, with the electron beam gun mounted 18° relative to the etched surface. The replicas were observed with a JEM 200 CX electron microscope operating at 200 kV. (The purified forebrain CaM kinase II shown in Fig. 2 was negatively stained with 1% uranyl acetate. Stained materials were observed with the same microscope at 80 kV.)

Binding to Calmodulin or Antibody

For calmodulin binding, the mixture was composed of 100 μg/ml calmodulin, 40 mM Tris/HCl buffer, pH 7.6, 1 mM DTT, 0.1 mM $CaCl_2$, 50 μM ATP, 8 mM $Mg(CH_3COO)_2$, and 50 μg/ml of forebrain CaM kinase II. The incubation was carried out at 0°C for 1 min before quick-freezing. For antibody binding, the mixture consisted of 3 μg/ml mAb, 40 mM Tris-HCl, pH 7.6, 1 mM DTT, and 50 μg/ml of forebrain CaM kinase II. The molar ratio of the antibody to the enzyme was ~2.0. The incubations were at 0°C for 1 min or at room temperature for 1 h before quick-freezing. The antibody binding experiment was controlled by mixing 50 μg/ml of forebrain CaM kinase II with 50 μg/ml of "purified mouse gamma globulin" (Cappel, Organon Teknika Corporation, 1230 Wilson Drive, West Chester, PA) in the presence of 40 mM Tris/HCl (pH 7.6) and 1 mM DTT. The incubation was carried out at 0°C for 10 min. The molar ratio of the gamma globulin to the enzyme was ~3.6.

Electrophoresis

SDS-PAGE was carried out by the method of Laemmli (1970). The densitometric scan of Coomassie blue-stained gels was carried out as follows. Images of the gel were sampled by a 16 bit linear frame grabber via a CCD video camera (Sony XC-77) and pixel values summated perpendicular to the direction of the gel.

Immunoblot Analysis

Immunoblot analysis followed the method of Towbin et al. (1979). Briefly, the samples were separated by 10% polyacrylamide SDS gel electrophoresis and electrophoretically transferred to nitrocellulose membrane. The transblots were incubated with the mAb followed by [^{125}I]-labeled anti-mouse IgG $(Fab')_2$. Radioactive bands were detected by autoradiography.

Results

Molecular Morphology

The molecules of purified forebrain CaM kinase II were negatively stained with uranyl acetate and are shown in Fig. 2. Each molecule appeared to be composed of a ring with a diameter of ~10 nm. With careful observation, it was possible to discern, on all of these rings, delicate material attached to their surfaces, surrounding the ring. The overall diameter of the molecule including the surrounding material is ~20 nm. When the staining was incomplete the rings were observed to have a hexameric structure (*arrows*). It was also found that when the molecules were over-stained the delicate material was embedded in the dense stain, with only the rings of hexameric structure remaining (data not shown).

An aliquot of purified CaM kinase II (50 μg/ml in 40 mM Tris-HCl, pH 7.6, and 1 mM DTT) isolated from the rat forebrain was adsorbed to mica flakes and quickly frozen by slamming against a liquid helium-cooled copper block. After freeze-fracturing, the fractured surface was etched for 3 min at −102°C and replicated.

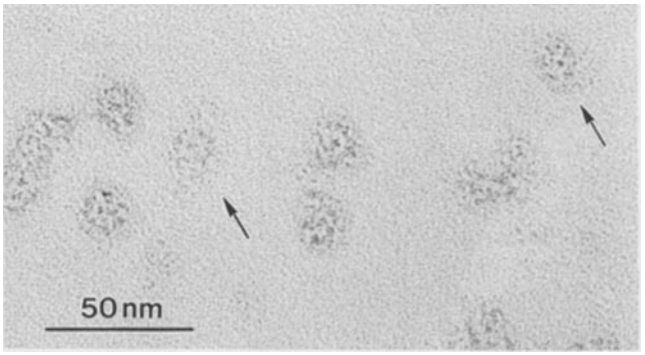
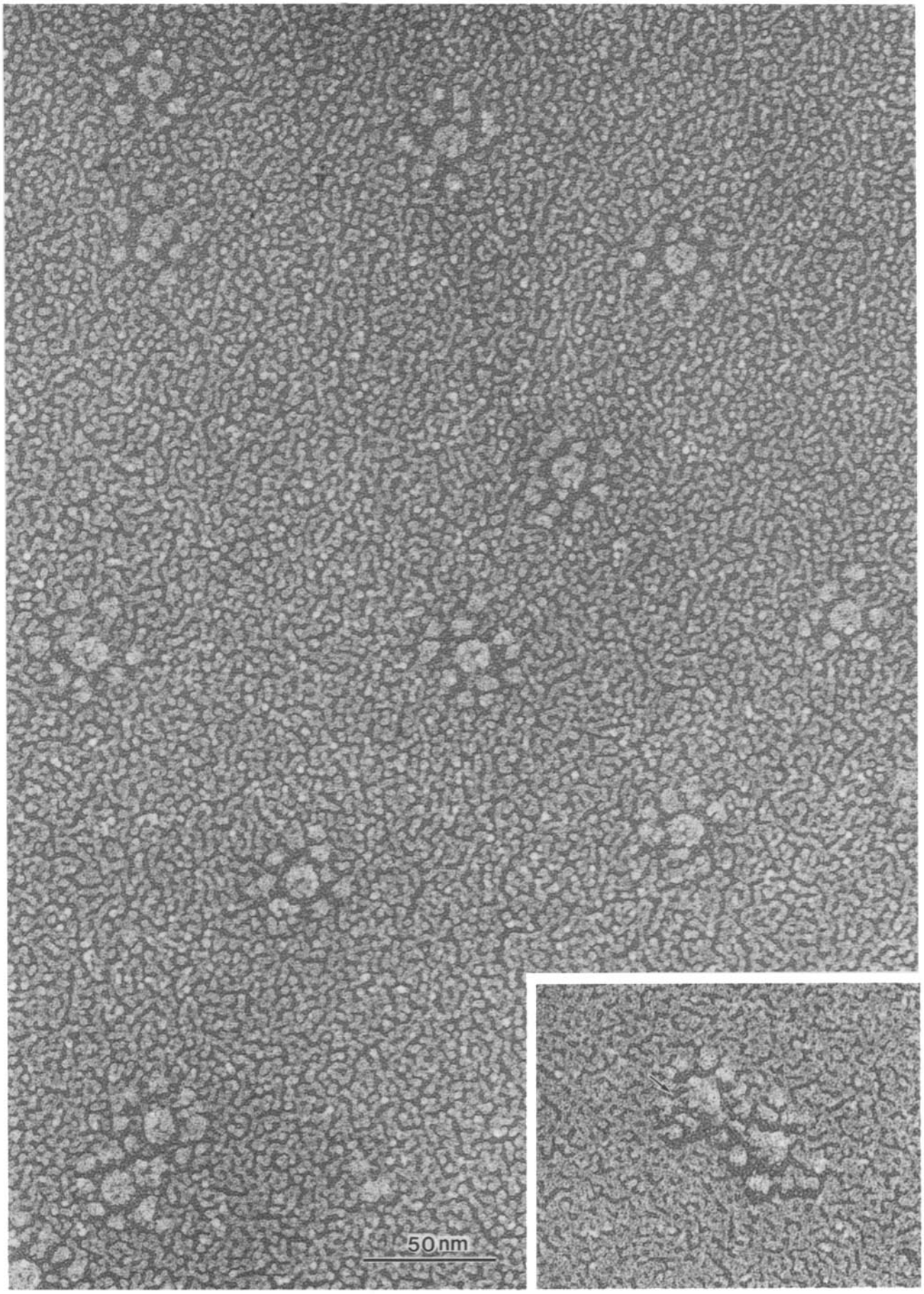


Figure 2. Negatively stained forebrain CaM kinase II. Each molecule appeared to be composed of a ring with a diameter of ~10 nm. With close observation, it is possible to discern, on all of these rings, delicate material attached to their surfaces, surrounding the ring. The overall diameter of the molecule including the surrounding material is ~20 nm. When the staining was incomplete the rings were observed to have a hexameric structure (*arrows*).

The appearance of individual molecules of CaM kinase II on replicas is shown in Fig. 3. Each molecule appeared to be composed of two kinds of particles, with one larger central particle and smaller peripheral particles, with the entire configuration resembling a flower. A favorable shadowing revealed that each peripheral particle has a thin link to the central particle (Fig. 3, *inset*). After counting the number of the peripheral particles of more than 2,000 "flowers," it was established that each central large particle has 6 to 12 peripheral particles. However, the "flowers" can clearly be divided into two main groups; molecules with 8 peripheral particles and molecules with 10 peripheral particles (see Fig. 13 *a*). In forebrain enzyme, the majority of molecules possessed 10 petals. The outer diameter of the 8-petal flower was measured to be slightly smaller than ~33 nm, while that of the 10-petal flower was slightly larger than ~33 nm. These sizes are plausible for the native form of CaM kinase II, which has a molecular mass of 500–600 kD.

High magnification observation of the peripheral particles, the central particle, and the thin links revealed the following points (Figs, 3, 4, and 6). (*a*) The average diameter of the peripheral particles in 8-petal flowers was slightly larger (~7 nm) than that of peripheral particles in 10-petal flowers (~6 nm). (*b*) The diameter of the central particle was measured to be ~10 nm. A hollow of complex shape is always observed in the center of the central particle, and appears as if dividing the central particle into several parts. (*c*) The thin link between small and large particles was estimated to be <2 nm thick and 3–5 nm in length.

These observations suggest that each peripheral particle with its intercalated link has another globular ending, which aggregates with those of the other peripheral particles, to compose the large central particle. We surmised that the 8-petal and 10-petal molecules are octamers and decamers of CaM kinase II, respectively, each assembled with the association domains of subunits gathered in the center, and the catalytic domains in the peripheral particles.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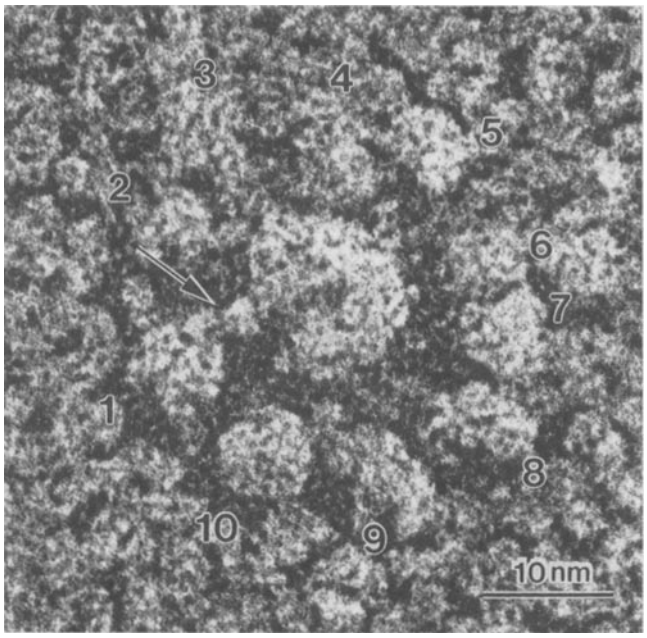

Figure 4. A high magnification electron micrograph of a CaM kinase II molecule having 10 peripheral particles. An arrow indicates a thin projection linking peripheral and central particles. The material was isolated from the rat forebrain.

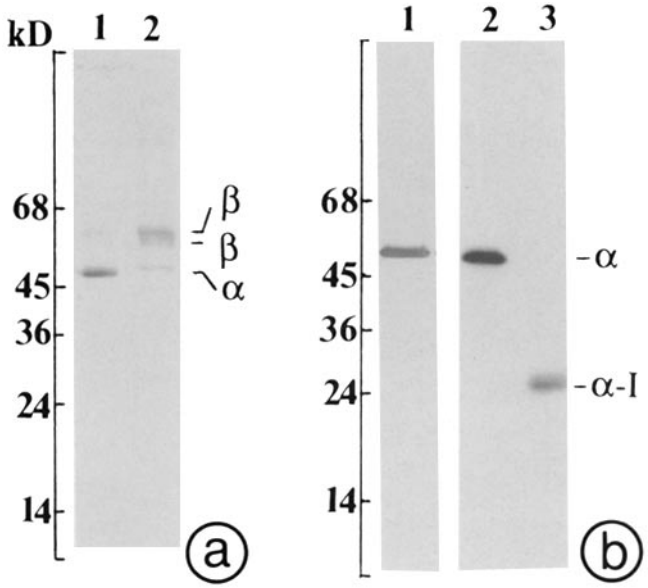

Figure 5. (*a*): SDS-PAGE of the forebrain (lane *1*) and cerebellar (lane *2*) CaM kinase II used in EM. The forebrain CaM kinase II showed a major 50-kD (α) band and a minor 60-kD (β) band, while cerebellar CaM kinase II showed a minor 50-kD (α) band and a major 60/58-kD (β/β') band, though the preparation was slightly contaminated. In particular, a 59-kD band observable between β and β' seemed also to be a contaminating fraction. (*b*) Immunoblot of CaM kinase II by a mAb specific to the α subunit of the enzyme. (lane *1*) 100 ng CaM kinase II of the forebrain. (lane *2*) Crude extracts of the rat forebrain (50 μg protein). (lane *3*) A crude supernatant of transfected *E. coli* expressing α-I protein (50 μg protein). α-I protein is a deletion mutant of α polypeptide, and is catalytically active (Ohsako et al., 1990).

The appearance of rat cerebellar CaM kinase II (50 μg/ml in 40 mM Tris-HCl, pH 7.6, and 1 mM DTT) was also observed using the same cryotechniques (Fig. 6). Although the preparation was slightly contaminated, the flowers were easily identified. In contrast to the forebrain enzyme, 8-petal molecules predominated (see Fig. 13 *a*). The ratios of numbers of 8-petal and 10-petal molecules in forebrain and cerebellum appeared to follow the respective ratios of α and β subunits as assessed by staining with Coomassie blue (Fig. 5 *a*).

The CaM kinase II molecules had a tendency to associate with each other to form a two-dimensional crystalline configuration (Figs. 7, *a* and *b*). The failure to observe piled-up molecules on replicas is a further indication that the three-dimensional structure of the molecule is not spherical but disc-like, as we observed.

Specific Binding

As shown in Fig. 5 *b*, a mAb raised against the α subunit of the rat forebrain CaM kinase II bound specifically to the α subunit of the enzyme. In the purified forebrain CaM kinase II (lane *1*) as well as in homogenates of the forebrain (lane *2*), the antibody recognized a single band of 50 kD. In addition, the antibody identified a catalytically active polypeptide (α-I), a deletion mutant of the α subunit (lane *3*), which had been expressed in *E. coli*. These results implied that we could use the antibody to attempt to locate catalytic domains in the native enzyme. Thus, binding experiments between the antibody (IgG) and purified forebrain enzyme were carried out, both to assess whether the catalytic domain resides in the peripheral particles or in the central particle, as well as to clarify whether α and β subunit proteins aggregate in homopolymers.

In these experiments, a fixed quantity (50 μg/ml) of forebrain CaM kinase II was incubated with the 37 μg/ml of mAb for 1 min at 0°C (Fig. 8 *a–c*) or for 1 h at room temperature (Figs. 8 *d* and 10). IgG antibodies on replicas are observed as triangular aggregates of three 6-nm globules (Heuser, 1983). Antigen–antibody complexes can be observed in Figs. 8 and 10. High magnification electron micrographs of the complex showed that the recognition occurred on the peripheral particles of the CaM kinase II molecule. This result indicates that the catalytic domains of the molecule are located on the peripheral particles.

This fact was confirmed by another experiment, where we mixed purified calmodulin molecule (100 μg/ml) with the purified forebrain CaM kinase II (50 μg/ml), in the presence

Figure 3. Flower-shaped molecules of CaM kinase II on a replica. The material was isolated from the rat forebrain. Molecules are composed of a large central particle and a number of peripheral particles. 12 molecules may be observed, with two possessing 8 peripheral particles, nine with 10 particles, and one with 11. (*inset*) A favorable shadowing over the molecule revealing a thin projection (*arrow*) between peripheral and central particles.

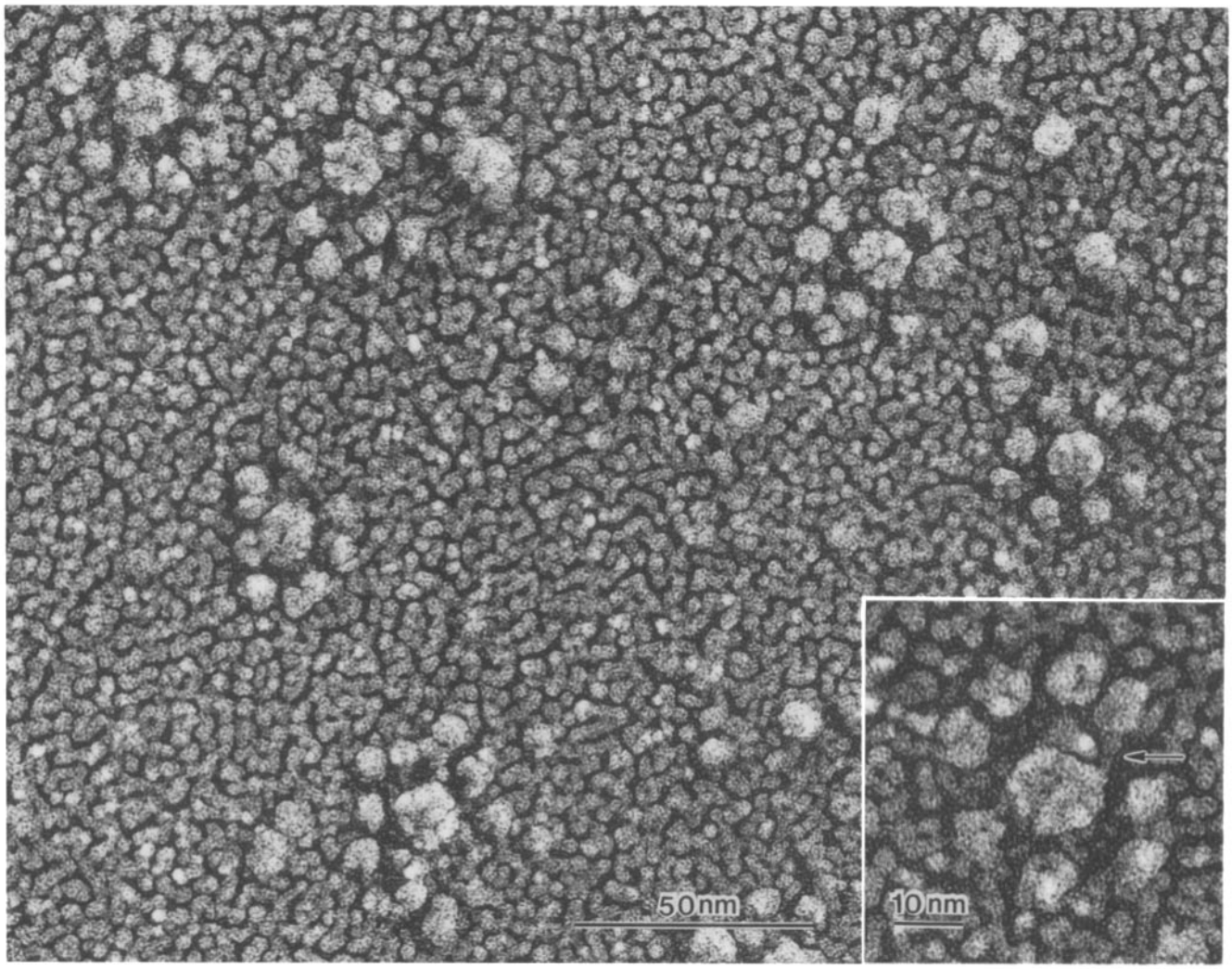

Figure 6. CaM kinase II isolated from the rat cerebellum. Although the cerebellar preparation had a small amount of contamination (Fig. 5 *a*, lane 2), the flower-shaped molecules can clearly be identified on this replica. Three of the molecules have eight peripheral particles. The average diameter of the peripheral particles is larger than that of the particles shown in Fig. 3. The central particle appears to be separated into several parts. (*inset*) A high magnification electron micrograph of a molecule having eight peripheral particles. An arrow indicates a thin link to the central particle.

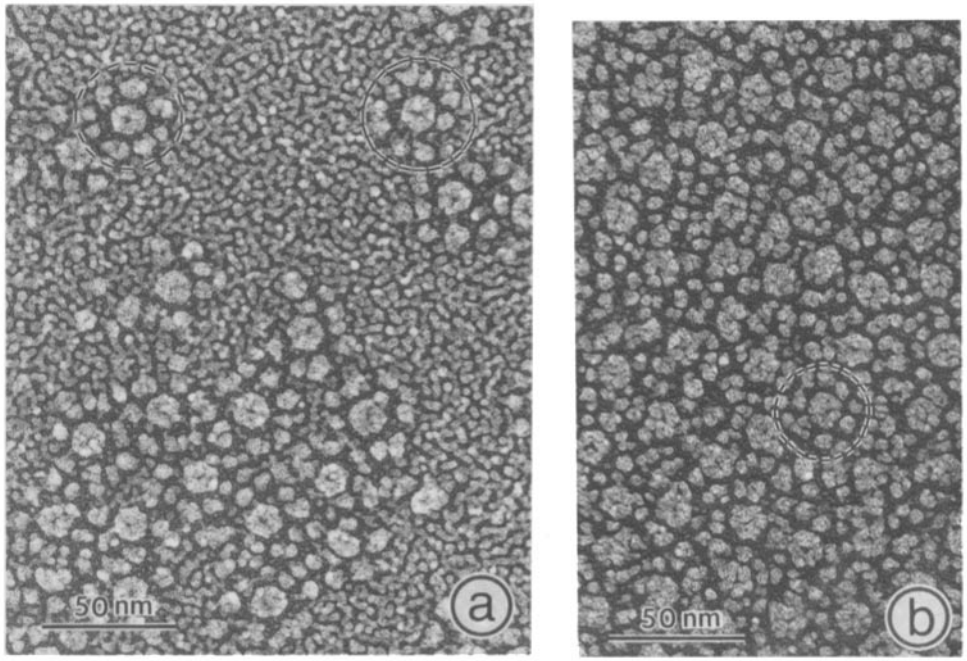

Figure 7. CaM kinase II isolated from the rat forebrain. (*a*) Molecules show a tendency to associate to take on a two-dimensional crystalline appearance. (*b*) The lower magnification view in this figure is reminiscent of a cross section of a myofibril A-band. The overall shape of the molecule is apparently disc-like. Encircled molecules in Fig. 7 (a and b); the molecules with 8 and 10 peripheral particles. Note that every central particle has in its center a complex-shaped hollow.

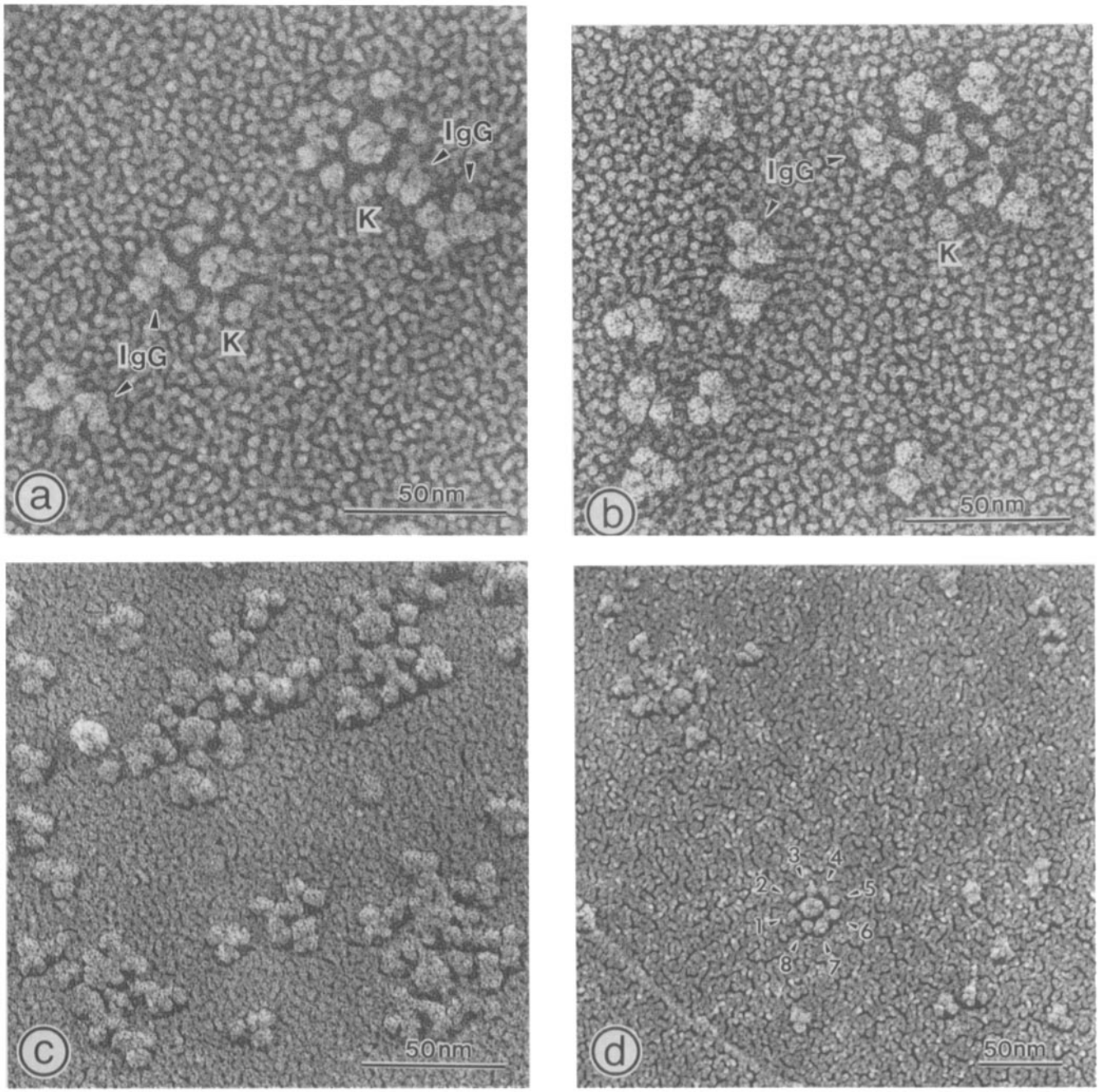

Figure 8. Binding of forebrain CaM kinase II with specific mAb. (*a* and *b*) The mAbs (IgG) that recognized α-subunit (and α-I protein) of CaM kinase II in Fig. 5 *b* are observed associated with peripheral particles of the enzyme molecule (*K*). (*c*) It was difficult to establish the maximum number of antibodies that could bind to one CaM kinase II molecule, but it appeared to be 5 or 6. (*d*) Even when the antigen-antibody mixture was established 1 hr before quick-freezing, CaM kinase II molecules without associated antibody are observed. Antibody-free CaM kinase II molecules were always of the eight-petal variety.

of Ca^{2+} and ATP (Fig. 9). The calmodulin molecules, each of diameter of less than 6 nm, associate with the peripheral particles of both 8-petal and 10-petal flowers from the outside.

Failure of the antibody to bind to flower configuration molecules was also observed. Strikingly, antibody was never observed to bind to well-resolved 8-petal molecules (Figs. 8 *d* and 10). This is a strong indication that molecules with eight peripheral particles are homopolymers composed only of β subunits. On the other hand, the maximum number of antibodies bound to one 10-petal molecule was only five or six (Fig. 8 *c*). This could result from steric hindrance or simply reflect the equilibrium reached with antibody binding, but the possibility that 10-petal molecules could contain β subunit cannot be ruled out. However, it seems likely, from the similarity between the ratios between numbers of 8- and

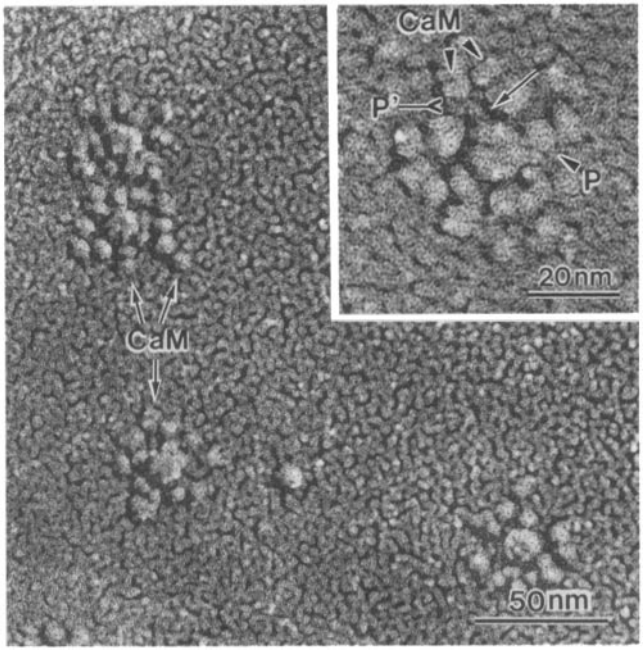

Figure 9. Binding of forebrain CaM kinase II with calmodulin. Calmodulin molecules (*CaM*), with diameter of <6 nm, associated with the peripheral particles (*P*) from the outside. (*inset*) Two molecules of calmodulin are observed covering a peripheral particle (*P'*) which has a clear thin link (*arrow*).

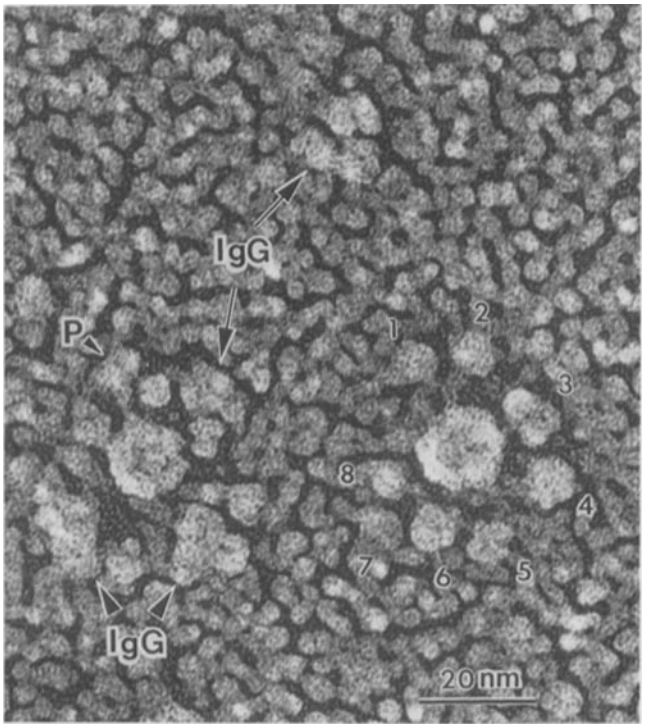

Figure 10. A high magnification electron micrograph showing a free IgG molecule and two CaM kinase II molecules. The CaM kinase II on the left side has three IgGs associated with its peripheral particles. As judged by the size of the labeled particles (*P*) with those seven countable peripheral particles, the left molecule appears to have 10 petals. The molecule on the right has eight peripheral particles, and no antibodies can be observed associated with it.

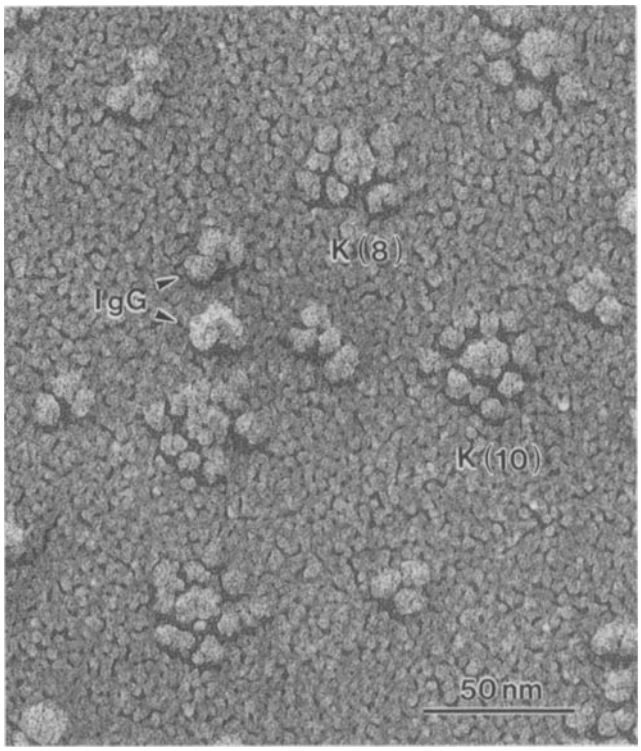

Figure 11. A control experiment for the antibody binding. 50 μg/ml of forebrain CaM kinase II was mixed with 50 μg/ml of "purified mouse gamma globulin" purchased from Cappel (Organon Teknika Corporation) in the presence of 40 mM Tris-HCl (pH 7.6) and 1 mM DTT. The incubation was carried out at 0°C for 10 min before quick-freezing. Neither the molecules with 8 peripheral particles [K(8)] nor these with 10 peripheral particles [K(10)] were attached to the gamma globulin (*IgG*).

10-petal molecules and the ratios of α and β subunits, that the 10-petal molecule is also a homopolymer, composed exclusively of α subunits.

As a control for the antibody binding, 50 μg/ml of forebrain CaM kinase II was mixed with 50 μg/ml of "purified mouse gamma globulin" (Cappel, Organon Teknika Corporation, West Chester, PA) and incubated at 0°C for 10 min before quick-freezing. The resultant replica is shown in Fig. 11. Neither the molecules with 8 peripheral particles [K(8)] nor those with 10 peripheral particles [K(10)] were attached to the gamma globulin (IgG).

Structural Composition

From the evidence presented above we propose that CaM kinase II enzyme is a mixture of two distinct homopolymers α or of β polypeptide, which we term CaM kinase II type α and CaM kinase II type β, respectively.

A schematic drawing of the two proposed types of CaM kinase II isoform is presented in Fig. 12, with the approximate dimensions of the homopolymers, peripheral particles, central particles and intercalated thin links. We examined the published primary structures of the α and β subunits of neuronal CaM kinase II (Bennett and Kennedy, 1987; Lin et al., 1987) to look for features which might explain the observed molecular architecture. A schematic representation of the

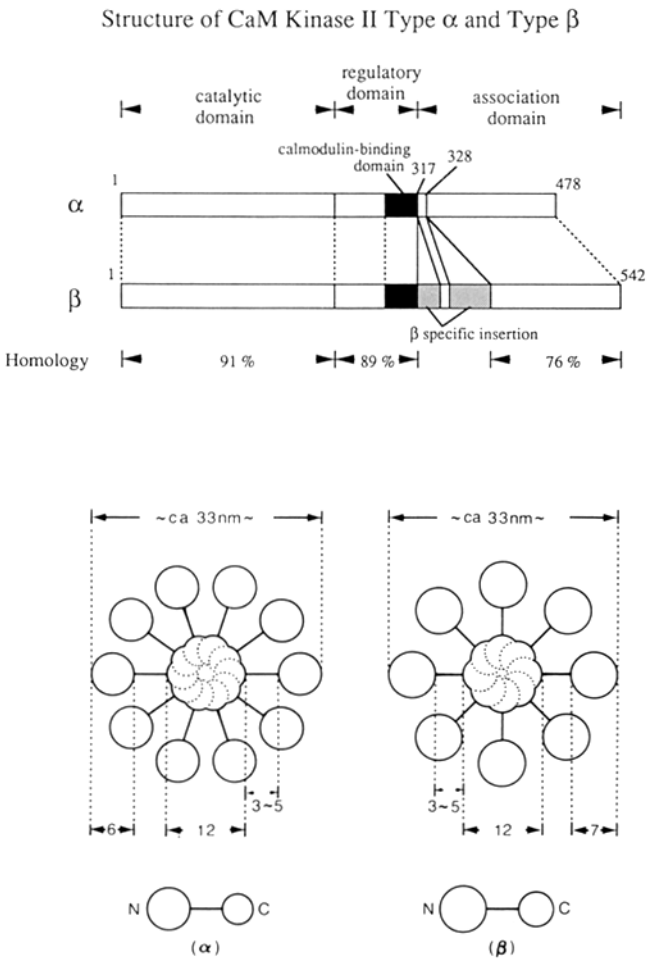

Figure 12. Schematic representations of the primary structures of α and β subunits of CaM kinase II, and of the proposed subunit structure. See text for interpretation.

primary structures of α and β subunits is shown at the top of Fig. 12.

Both subunits have been considered to be divided into three domains; catalytic, regulatory and association (Bennett and Kennedy, 1987; Hanley et al., 1987; Lin et al., 1987). The amino acid homology between corresponding domains of α and β subunits is indicated in Fig. 12. The black boxes denote putative calmodulin binding domains. The two shaded boxes adjacent to the black box in the β subunit have been explained as specific insertions of ~70 amino acids which are responsible for the larger size of β subunit (Bennett and Kennedy, 1987). In between the two shaded boxes a small white box is shown, representing a sequence of 12 amino acids. A similar sequence (80% homology) is observed in the α subunit from amino acids 317–328. A standard technique for predicting secondary from primary structure (Chou and Fasman, 1974) suggested that this region is unlikely to be α-helical but is likely to be an almost fully extended β-turn (our unpublished observation).

The thin link when observed with high power micrographs had a thickness of <2 nm and a length of 3–5 nm. There are several compelling reasons to suppose that this link corresponds to the 12-amino acid segment. (*a*) The length of a fully-extended chain of 12 amino acids should lie between 3 and 5 nm and its thickness should be measured as <2 nm. (*b*) The diameter of the peripheral particles of the octamer always appeared slightly larger than that of the peripheral particles of the decamer. This would be accounted for by the additional shaded insertion region to the left of the 12 amino acid "link", in the β subunit. (*c*) Since calmodulin molecules associate with the peripheral particles of both octamer or decamer, the peripheral globules on replicas should contain both catalytic and regulatory domains. In the case of the octamer, the peripheral particle should contain a part of the association domain as defined by Bennett and Kennedy (1987), shown as a shaded region in the β subunit.

The association domains at the COOH terminals, excluding the proposed "link" segments should also be globular. This view is supported by the fact that the central particles of both octamers and decamers on replicas always appeared separated into smaller lumps. Each subunit may thus be considered to have a dumbbell-like appearance, as shown at the bottom of Fig. 12.

The stained bands of α and β subunits shown on the gels of Fig. 5 *a* were scanned and displayed densitometrically in Fig. 13 *b*. The calculated ratio between α and β subunits in forebrain enzyme was 3.4:1. The ratio of decamers to octamers from the same source counted on replicas was 3.2:1 (Fig. 13 *a*), which predicts an α/β ratio of 4.0:1 close to the observed ratio of 3.4:1. The ratio of numbers of decamers to octamers on replicas from cerebellar material was 1:2.6 (Fig. 13 *a*), predicting an α:β ratio of 1:2.1, which is very similar to the 1:2.0 actually measured (Fig. 13 *b*). This result is good support for the view that the native form of CaM kinase II (at least in the rat forebrain and cerebellum) consists of distinct homopolymers of α or β subunits.

Discussion

CaM kinase II has generally been considered to be a heteropolymer of α and β subunit proteins (Bennett et al., 1983; Goldenring et al., 1983), which would be consistent with the difficulty in separating respective subunits through the applied purification methods so far available. However, we have recently succeeded in expressing, in cultured mammalian cells and *E. coli.*, pure α and β subunits from their corresponding cDNAs, both of which show kinase activity, though judging from their sizes, not all subunits were polymerized. On the other hand, almost all of the isoforms of the native CaM kinase II in the variety of tissues so far investigated seem to be in polymerized form, including those of the retina where the enzyme is composed only of α subunit (Bronstein et al., 1988). The existence of an isoform of the enzyme predominantly composed of β polypeptide has also been suggested in the cerebellum of the Purkinje cell degeneration mice and nervous mice (Walaas et al., 1988). A homopolymer structure for CaM kinase II provides a natural explanation for the well-known variations in α/β ratio between different regions of brain and during development, as simple differences in proportion of type α and type β homopolymers.

The novel shape of the molecule could also explain its unusual autophosphorylation behavior. The autophosphorylation reaction of CaM kinase II is intramolecular (Kuret and Schulman, 1985), and the generation of Ca^{2+}-independent

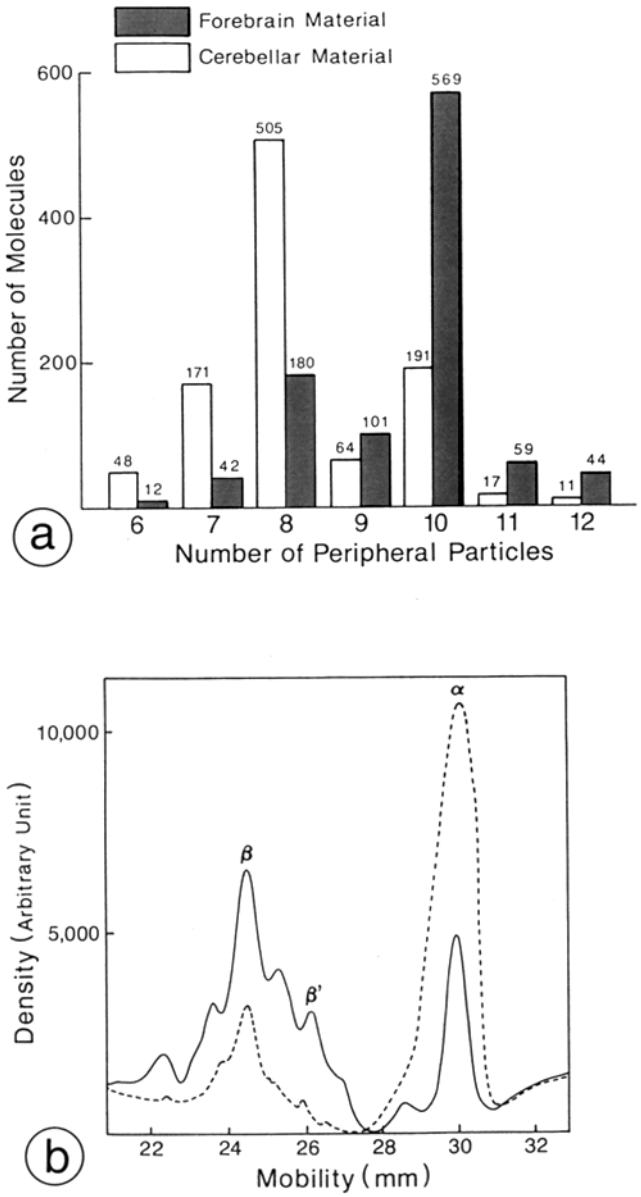

Figure 13. (*a*) Frequency distribution of the number of peripheral particles per CaM kinase II molecule. (*Black bars*) forebrain enzyme. (*white bars*) Cerebellar enzyme. In total, 1,007 well-resolved CaM kinase II molecules on replicas were counted from each source. (*b*) A comparison of the densitometric scan of Coomassie blue–stained gels of Fig. 5 *a*. (*dotted curve*) The forebrain CaM kinase II shown in lane 1 of Fig. 5 *a*. (*solid curve*) The cerebellar CaM kinase II shown in lane 2 of Fig. 5 *a*. The ratios between the densities of peaks corresponding to α and β' subunits yielded α/β subunit ratios of 3.4:1 (forebrain) and 1:2.0 (cerebellum).

CaM kinase II can be induced by autophosphorylation of only one subunit per holoenzyme in the presence of Ca^{2+} and calmodulin (Lickteig et al., 1988). In our structural model, the link to peripheral particles, each of which contains a catalytic domain, would be flexible. When one peripheral particle is autophosphorylated, the activated particle may phosphorylate adjacent particles which in turn would phosphorylate others of the same molecule until the Ca^{2+}-independent activity of holoenzyme is fully induced.

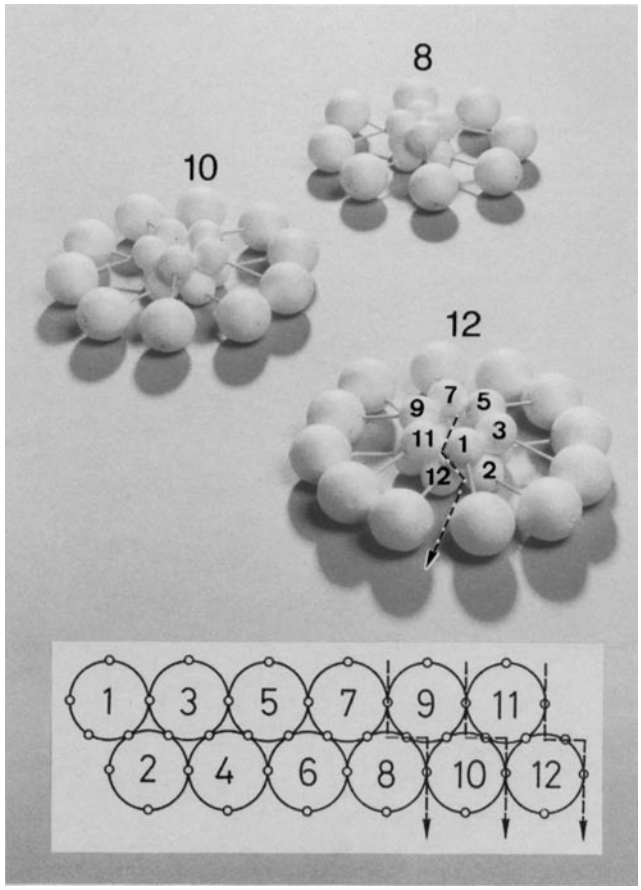

Figure 14. A three-dimensional model of CaM kinase II molecules. Large numerals indicate the number of subunits. The particles labeled with small numerals making up the central particle are shown cut along the arrowed line and spread two dimensionally in the inset at the bottom. See interpretation in the Discussion.

A flower-shaped enzyme is also well-suited for simultaneous phosphorylation of multiple substrate sites, for example along a single microtubule-associated protein 2 (MAP 2) molecule, with catalytic domains spread over a large area.

In conclusion, we note the following points. (*a*) We postulate that the thin links to the peripheral particles are composed of amino acids 317–328 of the α polypeptide and 341–353 of the β polypeptide, based on a prediction of an extended β-turn, using a standard method of secondary structure prediction (Chou and Fasman, 1974). However, a similar β-turn structure was also predicted in the conserved region of amino acids around 331–340 in the α polypeptide and around 393–400 in the β polypeptide. We cannot rule out the possibility that the thin link corresponds to these regions. (*b*) Recently, the existence of additional γ and δ polypeptides of CaM kinase II has been suggested from cDNA cloning in the rat forebrain (Tobimatsu et al., 1988; Tobimatsu and Fujisawa, 1989). Although isolation of these polypeptides has not yet been reported, the putative γ and δ polypeptides show remarkable homology to α and β subunit sequences. The possibility that some 8-petal molecules could incorporate γ and δ subunits cannot be ruled out. (*c*) We did not measure the thickness of the replica film, so that feature sizes on replicas may be only relative. However,

we note that IgG molecules had the same dimensions as described by Heuser (1983). (*d*) The antibody binding experiments were not carried out at saturation for the antibody. When the experiment was performed with the molar ratio of the antibody to the enzyme exceeding 2.0, many of the 10-petaled flowers were collapsed or apparently breaking up, while 8-petaled flowers were intact. An example of a flower that seemed to be fragmenting may be observed in the upper left corner in Fig. 8 *d*. At present, we have no explanation for this collapsing phenomenon at high antibody concentrations. (*e*) Although 8-petal molecules were easily resolvable, there may have been some inaccuracy in counting molecules with higher number of particles, separated by correspondingly shorter distances. We reconstructed an approximate three-dimensional model of the enzyme molecules on the basis of the highest resolution micrographs, to attempt to understand the possible packing of central particles. Judging from the elevated appearance of the surface of the central particle, the organization of subunits may resemble that shown in Fig. 14. At the bottom of Fig. 14, the arrangement of globules constituting the central particle is shown spread two dimensionally, with small circles on each globule indicating points of interaction with other globules or with the mica surface. Interestingly, this type of packing of central particles strongly favours molecules with an even number of peripheral particles.

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