Influence of a mutation in the ATP-binding region of Ca^{2+}/calmodulin-dependent protein kinase II on its interaction with peptide substrates

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CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II) is expressed in high concentrations in the brain and is found enriched in the postsynaptic densities. The enzyme is activated by the binding of calmodulin to the autoregulatory domain in the presence of high levels of intracellular Ca^{2+}, which causes removal of autoinhibition from the N-terminal catalytic domain. Knowledge of the 3D (three-dimensional) structure of this enzyme at atomic resolution is restricted to the association domain, a region at the extreme C-terminus. The catalytic domain of CaMKII shares high sequence similarity with CaMKI. The 3D structure of the catalytic core of CaMKI comprises ATP- and substrate-binding regions in a cleft between two distinct lobes, similar to the structures of all protein kinases solved to date. Mutation of Glu-60, a residue in the ATP-binding region of CaMKII, to glycine exerts different effects on phosphorylation of two peptide substrates, syntide and NR2B (*N*-methyl-D-aspartate receptor subunit 2B) 17-mer. Although the mutation caused increases in the K_m values for phosphorylation for both the peptide substrates, the effect on the k_{cat} values for each was different. The k_{cat} value decreased in the case of syntide, whereas it increased in the case of the NR2B peptide as a result of the mutation. This resulted in a significant decrease in the apparent k_{cat}/K_m value for syntide, but the change was minimal for the NR2B peptide. These results indicate that different catalytic mechanisms are employed by the kinase for the two peptides. Molecular modelling suggests structural changes are likely to occur at the peptide-binding pocket in the active state of the enzyme as a consequence of the Glu-60 → Gly mutation.

Key words: Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), kinetics, *N*-methyl-D-aspartate receptor subunit 2B (NR2B), molecular modelling, phosphorylation, synapse.

INTRODUCTION

CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II) is a serine/threonine protein kinase involved in long-term potentiation in synapses, a process that underlies learning and memory [1]. The enzyme has an N-terminal kinase domain, followed by an autoinhibitory region that partially overlaps the calmodulin-binding domain and a C-terminal association domain, which is responsible for the unique oligomeric nature of the enzyme [2].

The 3D (three-dimensional) structures of the catalytic domains of kinases are highly conserved [3,4]. CaMKII shares highest sequence similarity with CaMKI, among the protein kinases of known structure. The catalytic domain comprises two lobes with the peptide substrate and ATP-binding regions located in the cleft between the two lobes. In the inhibited form of the enzyme, the auto-inhibitory domain binds to a pocket in the large lobe and extends through the catalytic site and the ATP-binding site, keeping the two lobes apart in an 'open' conformation inaccessible to substrates [4]. Based on structural studies on PKA (cAMP-dependent protein kinase), the two lobes are thought to move closer to each other to a 'closed conformation' upon activation [3,5]. A similar 3D structure is likely to exist in the case of CaMKII, as suggested by its sequence similarity to CaMKI and PKA [6,7]. Biochemical data indicate that upon binding of Ca^{2+}/calmodulin, the autoinhibitory domain is released from its binding interactions and the active site becomes accessible for substrates [2]. The C-terminal association domains of 14 CaMKII subunits bind to each other to form a core around which the N-terminal catalytic domains are tethered [2,8,9]. The crystal structure of the association domain of the enzyme reported recently [8] shows that the association domain not only mediates the formation of a multimer, but also seems to hold a binding site for other interacting proteins. However, the absence of crystal structure data on the catalytic domain of the enzyme still hampers attempts to relate the structure of the enzyme to its mechanism of regulation and catalysis.

Glu-60 of CaMKII is conserved in almost all protein kinases [10]. The equivalent residue in the C-subunit of mouse PKA, Glu-91, is present in the ATP-binding domain and is involved in a salt bridge with another conserved residue, Lys-72 (Lys-42 of CaMKII) that is essential for nucleotide binding [3]. This is also supported by mutational studies [11].

In the present study, we have investigated the mutagenesis of Glu-60 of CaMKII to glycine and its effect on the interaction of the enzyme with two peptide substrates (Figure 1), syntide, which has sequence similarity to the CaMKII phosphorylation site (site 2) on glycogen synthase [12], and NR2B (*N*-methyl-D-aspartate receptor subunit 2B) 17-mer, which is designed according to the CaMKII phosphorylation site (Ser-1303) on the NR2B subunit of the NMDAR (*N*-methyl-D-aspartate receptor) [13,14]. The NR2B subunit is one of the high-affinity binding partners of CaMKII present in the postsynaptic density [15–17]. Whereas syntide may bind only to the catalytic site of CaMKII, the interaction of CaMKII with NR2B is thought to involve binding in two modes, one by the catalytic site of CaMKII [13,14] and the other by a non-catalytic site on CaMKII [18,19]. We find that the mutation of Glu-60, a residue likely to be found some distance away from the peptide-binding pocket, has different effects on the kinetics of phosphorylation of the two peptide substrates.

Abbreviations used: CaMK, Ca^{2+}/calmodulin-dependent protein kinase; α-CaMKII, α-subunit of CaMKII; IPTG, isopropyl β-D-thiogalactoside; NMDAR, *N*-methyl-D-aspartate receptor; NR2B, NMDAR subunit 2B; PKA, cAMP-dependent protein kinase; 3D, three-dimensional; E60G, Glu-60 → Gly substitution.

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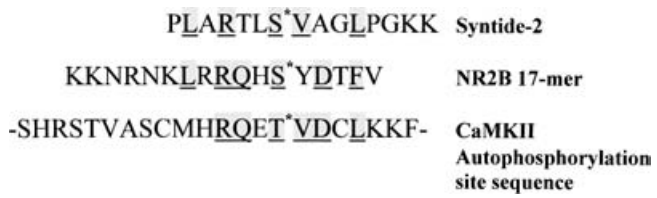

Figure 1 Sequences of the peptide substrates

The sequences of the peptide substrates used are aligned along with the sequence of the autoregulatory domain around Thr-286 of α-CaMKII. Homologous residues are highlighted and underlined. The phosphorylatable serine/threonine residues are indicated by asterisks.

Molecular modelling studies on CaMKII have been carried out to explore the possible structural changes at the peptide-binding pocket as a consequence of the E60G (Glu-60 → Gly) mutation.

EXPERIMENTAL

Materials

ATP, calmodulin, calmodulin–agarose, leupeptin, secondary antibody conjugates, fetal bovine serum, PMSF, dithiothreitol, nitrocellulose paper, insect cell culture media (TC100 insect cell medium) were from Sigma (St. Louis, MO, U.S.A.) or from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). Penicillin, streptomycin, fungizone and Bac-to-Bac baculovirus expression kit were from Gibco BRL (Gaithersburg, MD, U.S.A.). Anion exchange paper (p81) was from Whatman. Oligonucleotides were obtained from either Gibco BRL or from Sigma-GenoSys (Cambridge, U.K.). Restriction and modification enzymes and other molecular-biology-related chemicals were from Promega, New England Biolabs or Amersham Pharmacia Biotech. [γ-^{32}P]ATP was from Bhabha Atomic Research Centre, Mumbai, India. Monoclonal antibody against α-CaMKII (α-subunit of CaMKII) was from Affinity Bioreagents (Golden, CO, U.S.A.). Polyclonal antibody against the Thr-286 phosphorylated form of α-CaMKII was from Promega. The cDNA encoding α-CaMKII was a gift from Professor Mary B. Kennedy (Division of Biology, California Institute of Technology, Pasadena, CA, U.S.A.)

Construction of the expression vectors for α-CaMKII

The cDNA of the wild-type rat brain α-CaMKII was amplified by PCR from the vector pGEM-2α. The primers used were designed complimentary to the 5′ and 3′ ends of the cDNA of α-CaMKII in which the sites for the restriction enzymes *Bam*HI and *Kpn*I respectively were included. The amplified product obtained was then subcloned into the insect cell shuttle vector pFastBac1(Life Technologies), making use of the engineered restriction sites.

Sequencing of the cDNA coding for α-CaMKII

The full-length sequence of the amplified product was confirmed after cloning it into the expression vector pFastBac1. Sequencing was carried out by the dideoxy-chain-termination method using an automated DNA sequencer (ABI Prism).

Construction of E60G mutant of α-CaMKII

While subcloning the cDNA of α-CaMKII into the prokaryotic expression vector pET-32aα, one of the clones obtained after PCR amplification of the cDNA from the vector pGEM2α contained two amino acid substitutions in the CaMKII-coding sequence. The changes were E60G and E329G. The pET-32aα construct was digested to release a 1085-bp fragment containing the E329G mutation, using the unique *Sma*I site (310 bp) and *Bgl*II site (1405 bp) of the α-CaMKII cDNA. This region was replaced with the corresponding wild-type fragment. The E60G mutant of α-CaMKII thus constructed was subjected to full-length sequencing. The mutant was amplified by PCR from pET-32aα and was subcloned into the insect cell shuttle vector pFastBac1 in the manner described for the wild-type enzyme.

Expression in baculovirus/Sf21 cell system

The Sf21 strain of insect cells were cultured as a monolayer in TC100 medium containing 10% fetal bovine serum and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin and 25 μg/ml fungizone) at 27 °C. The recombinant virus containing α-CaMKII cDNA was prepared according to the manufacturer's instructions using the Bac-to-Bac baculovirus expression system. For purification of the enzyme, monolayer cultures of Sf21 cells in 182 cm^2 flasks were infected with viral stocks and were harvested 60–72 h post infection.

Purification of α-CaMKII expressed in baculovirus/Sf21 cell system

The infected cells were resuspended in PBS and were pelleted at 800 ***g*** for 10 min. They were stored at − 70 °C until purification. For purification, the cells from three or more flasks were pooled and were resuspended in buffer containing 50 mM Pipes (pH 7.0), 5.0% betaine, 1 mM EGTA, 1 mM EDTA and 1 × complete protease inhibitors cocktail (Sigma). The resuspended cells were lysed by homogenization using a Potter-Elvejham homogenizer (5 strokes) and sonication (3 × 10 s with 10 s intervals). The homogenate was clarified by centrifugation at 100000 ***g*** for 30 min. The pellet obtained was washed in the lysis buffer by rehomogenization and centrifugation, and the wash was pooled with the first supernatant. $CaCl_2$ was added to a final concentration of 2 mM in the supernatant. This was loaded on to calmodulin–Sepharose column (2–4 ml) pre-equilibrated with loading buffer [40 mM Hepes (pH 7.3)/2 mM $CaCl_2$/10% glycerol/0.1 M NaCl/0.5 mM dithiothreitol /0.1 mM PMSF]. The flow-through was reloaded and the column was washed with loading buffer containing 1 M NaCl. CaMKII was then eluted with buffer containing 40 mM Hepes (pH 7.3), 10% glycerol, 0.1 M NaCl and 2.5 mM EGTA. The peak activity fractions were pooled and were used as the purified recombinant α-CaMKII [20]. Protein estimation [21], SDS/PAGE [22] and Western blotting [23] using monoclonal anti-α-CaMKII antibody were carried out on the enzyme preparation. A single species of α-CaMKII subunit with no major degradation product was observed in the enzyme purified after expression in insect cells (see Figures 2A and 2B).

Assay of CaMKII activity

Assay of CaMKII activity was carried out by measuring the incorporation of ^{32}P-labelled P_i from [γ-^{32}P]ATP into the peptide substrate, as described by Mayadevi et al. [14]. The concentration of calmodulin was 3 μM and either 0.4 mM EGTA (minus Ca^{2+} to act as a control) or 0.4 mM EGTA/1.3 mM $CaCl_2$ (plus Ca^{2+}) was present. The reaction was initiated by addition of the enzyme and was terminated 30 s later. The Ca^{2+}-dependent activity obtained by subtracting the activity of the control (without Ca^{2+}) from the activity measured for the reaction with Ca^{2+} was used for

kinetic analysis. Activity of CaMKII was expressed as μmol of P_i incorporated to the peptide/min under the above conditions.

Kinetic analysis

Assays of CaMKII activity were done at different concentrations of the respective peptide substrate or ATP. Approx. 0.022–0.033 μg of purified wild-type CaMKII or 0.033–0.075 μg of CaMKII(E60G) was used in each reaction. The kinetic parameters, apparent k_{cat} (catalytic constant) and apparent K_m (Michaelis–Menten constant) for peptide and ATP were determined from Eadie–Hofstee plots [24].

Autophosphorylation of CaMKII

Autophosphorylation of CaMKII at Thr-286 was carried out as described by Miller and Kennedy [25]. The reaction mixture comprised 50 mM Tris (pH 8), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 5.8 μM calmodulin and 0.2 mg/ml BSA with 500 μM and 1 mM ATP for the wild-type and CaMKII(E60G) respectively, and either 0.003–0.008 mg/ml of wild-type enzyme or 0.004 mg/ml of the mutant enzyme. The reaction was initiated by addition of ATP after preincubation of the reaction system at 30 °C for 1 min. The reaction was stopped after 30 s by addition of 2.5 mM EGTA and 10 mM EDTA. This was used as the autophosphorylated enzyme sample for activity assays. Assay of the autophosphorylated enzyme was carried out using 300 μM ATP and 50 μM peptide substrate. A non-autophosphorylated control was prepared in a similar manner in which ATP was added after addition of EGTA/EDTA mix. For Western blotting using anti-(phospho-Thr-286 α-CaMKII) antibody, the autophosphorylation reaction was terminated by the addition of trichloroacetic acid (16% final concentration). The precipitated proteins were washed with acetone to remove trichloroacetic acid, dried and were resuspended in 1 × SDS sample buffer.

Synthesis of peptides

Peptide synthesis was carried out according to Kumar et al. [26] or Leena and Kumar [27], or by substituting commercial Merrifield resin in one of the above methods. After synthesis, the peptides were purified by gel filtration using Sephadex G25. Reverse-phase HPLC analysis of each peptide on C_{18} columns showed only one major peak. The peptides were subjected to matrix-assisted laser-desorption ionization–time-of-flight MS in a Kratos Analytical Compact Discovery MALDI-TOF mass spectrometer. A match between the calculated and measured masses was accepted as confirmation of their identity.

Comparative modelling of the catalytic region of CaMKII

Since CaMKI is nearest to CaMKII in terms of the extent of sequence similarity, the structure of CaMKII has been modelled on the basis of the crystal structure of CaMKI [4]. However, we used the crystal structure of PKA bound to MnATP and protein kinase inhibitor [28] in modelling the interactions of substrates with CaMKII. The sequences of PKA and CaMKI were aligned on the basis of their structural features using the program COMPARER [29,30]. The sequence of CaMKII was then aligned with PKA and CaMKI (results not shown).

The suite of programs encoded in COMPOSER [31] and incorporated in SYBYL (Tripos, St Louis, MO, U.S.A.) was used to generate a 3D model of CaMKII. The structures of the conserved regions of CaMKI have been extrapolated to the equivalent regions of CaMKII. The variable regions and side chains are modelled based on the rules derived previously by analysis of protein structures [32,33].

Energy minimization

The COMPOSER generated models were energy minimized in SYBYL using the AMBER force-field [34], as described previously in comparative modelling studies of protein kinase C-α and protein kinase CK2 (also known as casein kinase II) [35,36].

Modelling of complexes

The binding of a pseudo-substrate sequence at the catalytic site of CaMKII was modelled using the structure of PKA in complex with the pseudo-peptide. Positions of side chains at the recognition site in CaMKII, as well as in the peptide, were manually adjusted using SYBYL to optimize the interactions between the peptide and the enzyme. MnATP in the catalytic domain was modelled in a similar way. The modelled structures have been analysed using the graphics program SETOR [37].

RESULTS AND DISCUSSION

We investigated the effect of mutation of Glu-60 of CaMKII to glycine on the kinetics of phosphorylation of the peptide substrates, syntide and NR2B 17-mer (Figure 1). CaMKII was expressed in Sf21 insect cells using a baculovirus vector. The wild-type and the E60G mutant enzymes purified by calmodulin-affinity chromatography showed a major band of approx. 50 kDa corresponding to α-CaMKII (Figure 2A). Western blotting using monoclonal anti-α-CaMKII antibody also showed that the purified enzyme preparations had a single species of α-CaMKII corresponding to the full-length subunit without any degradation (Figure 2B). Upon Ca^{2+}/calmodulin-dependent autophosphorylation, both wild-type and the E60G mutant were autophosphorylated at Thr-286, as indicated by Western blotting using an antibody specific for the Thr-286 phosphorylated form of α-CaMKII. However, the E60G mutant was autophosphorylated to a lower level compared with the wild-type (Figure 2C), even at much higher concentration of ATP that was provided to compensate for the high K_m value of the E60G mutant for ATP (see Table 1a). Similar results were obtained when incorporation of $^{32}P[P_i]$ to wild-type and E60G mutant enzymes were compared after autophosphorylation using [γ-^{32}P]ATP (results not shown). The autophosphorylated wild-type enzyme exhibited Ca^{2+}-independent activity (Figure 2D) consistent with the finding that autophosphorylation occurred at Thr-286. The Ca^{2+}-independent activity of the autophosphorylated enzyme was higher for syntide compared with NR2B 17-mer, which is very similar to the Ca^{2+}-dependent activities of the autophosphorylated, as well as the non-autophosphorylated enzymes (Figure 2D). It has not been possible to measure autophosphorylation-dependent autonomous activity for the E60G mutant, since the extent of autophosphorylation was low for this mutant. Both the enzymes showed similar calmodulin saturation patterns (Figure 2E).

Table 1 shows the results of kinetic analysis. The K_m value of α-CaMKII for ATP increased significantly upon mutation (Table 1a), indicating that E60 plays a role in ATP binding in CaMKII which is consistent with previous studies on mutation of the equivalent residue in yeast PKA, Glu-135 [11]. Wild-type CaMKII phosphorylated the NR2B 17-mer peptide with lower K_m and k_{cat} values compared with syntide (Table 1b). However,

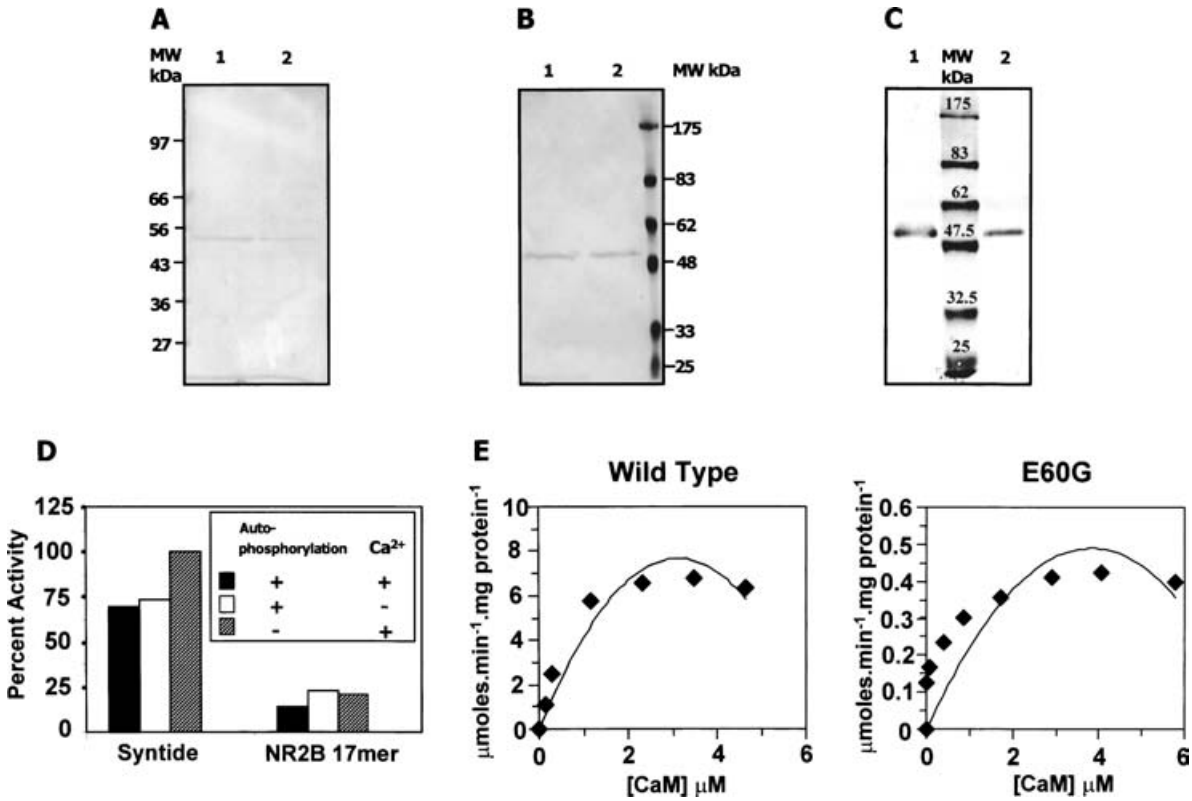

Figure 2 Expression and characterization of wild-type and E60G mutant of α-CaMKII

The wild-type and the E60G mutant of α-CaMKII were expressed in baculovirus/Sf21 cell system and were purified. MW indicates the lane with molecular mass markers. (**A**) SDS/PAGE of the purified enzymes: lane 1, wild-type (0.4 μg); lane 2, E60G mutant (0.8 μg) of α-CaMKII. (**B**) Western blot of 2 μg each of purified wild-type (lane 1) and E60G mutant (lane 2) of α-CaMKII using monoclonal anti-α-CaMKII antibody. (**C**) Western blot of 2 μg each of purified wild-type (lane 1) and E60G mutant (lane 2) of α-CaMKII that were autophosphorylated *in vitro*. Anti-(phospho-Thr-286) α-CaMKII polyclonal antibody was used for immunodetection. (**D**) Autonomous activity of autophosphorylated wild-type α-CaMKII. Data are presented as the percentage of the Ca^{2+}-dependent activity of non-autophosphorylated wild-type enzyme for phosphorylation of syntide. The open bars represent autonomous activity of the autophosphorylated enzyme in the absence of Ca^{2+} and the filled bars indicate activity of the autophosphorylated enzyme in the presence of calcium. The hatched bars indicate Ca^{2+}-dependent activity of the non-autophosphorylated enzyme. The activity measured for the non-autophosphorylated enzyme in the absence of Ca^{2+} was indistinguishable from the background. The respective substrate peptides used for measurement of activity are indicated. (**E**) Calmodulin (CaM) saturation pattern of wild-type and E60G mutant. Activities were measured under saturating concentrations of ATP (250 μM for wild-type and 800 μM for E60G) and syntide (100 μM for wild-type and 300 μM for E60G).

Table 1 Apparent kinetic constants of the wild-type and E60G mutant of α-CaMKII expressed in insect cells

Each value is the mean $\pm$ S.D. for three (a) or four (b) separate determinations. Each determination had six or more data points in the Eadie–Hofstee plot. (a) The concentrations of the constant substrate, syntide, were 100 μM and 300 μM for wild-type (WT) and E60G respectively. (b) The concentrations of the constant substrate, ATP, were as follows: 250 μM for WT for both the peptide substrates; 800 μM and 250 μM when syntide or NR2B 17-mer respectively was used as the peptide substrate for E60G.

(a)

Enzyme	Variable substrate	K_m (μM)	k_{cat} (μmoles · min^{-1} · mg of protein^{-1})	k_{cat}/K_m ($\times 10^2$)
WT	ATP	127 $\pm$ 29	4.13 $\pm$ 0.58	3.33 $\pm$ 0.7
E60G	ATP	316 $\pm$ 66	0.43 $\pm$ 0.13	0.15 $\pm$ 0.08

(b)

Enzyme	Variable substrate	K_m (μM)	k_{cat} (μmoles · min^{-1} · mg of protein^{-1})	k_{cat}/K_m ($\times 10^2$)
WT	Syntide	13.4 $\pm$ 1.8	2.0 $\pm$ 0.25	15.4 $\pm$ 4.1
E60G	Syntide	132 $\pm$ 19	0.51 $\pm$ 0.18	0.39 $\pm$ 0.13
WT	NR2B 17-mer	0.29 $\pm$0.1	0.62 $\pm$ 0.16	225 $\pm$ 29
E60G	NR2B 17-mer	4.56 $\pm$ 0.25	2.26 $\pm$ 0.31	49 $\pm$ 6

the net catalytic efficiency of phosphorylation by CaMKII was higher for NR2B 17-mer. The comparatively low K_m value for the NR2B peptide is anticipated, since it has more specificity determinants preferred by CaMKII [38–40] than syntide. Indeed, it has previously been shown that the phosphorylation site on NR2B, Ser-1303, is a high-affinity substrate sequence for CaMKII compared with its other substrates [13]. However, the nature of the interactions that may contribute to a greater affinity, as well as the residue environment, of the substrate-binding site has not been previously explored.

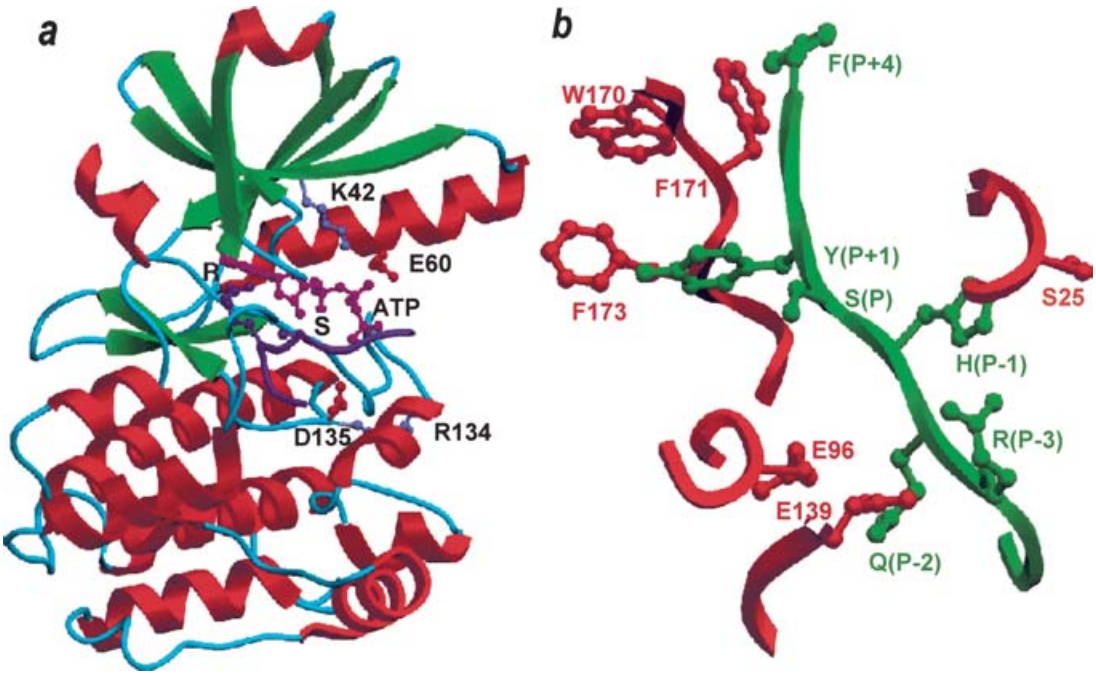

Figure 3 Structural model of CaMKII

(**a**) Overall fold of CaMKII model bound to ATP and NR2B substrate peptide. The α-helices are shown in red, β-sheets in green and ATP in pink. The substrate peptide with the arginine and serine residues in the key consensus motif of CaMKII substrates is shown in purple. Functional residues suggested to be involved in catalysis (R134 and D135) and ATP binding (E60 and K42) are shown. (**b**) The key interactions of the kinase domain with the NR2B substrate. The aromatic cluster is formed by the kinase residues (W170, F171 and F173) and the peptide residues [Y(P + 1) and F(P + 4)]. Other residues involved in the kinase–substrate interactions, E96–R(P − 3), E139–Q(P − 2) and S25–H(P − 1), are also shown.

A structure-based alignment of CaMKII with PKA and with CaMKI was generated (results not shown) and 3D models of CaMKII (Figure 3) and its complexes with substrate peptides, syntide and NR2B 17-mer were constructed. The interactions of the autoregulatory domain with the catalytic domain of CaMKII have previously been explored using molecular modelling based on the 3D structures of CaMKI and PKA [7]. The present analysis investigates the nature of interactions of two peptide substrates, syntide and NR2B 17-mer with the catalytic domain using 3D models of CaMKII–substrate-peptide complexes. The residue environment at the active site for the two peptides was analysed by identifying the residue pairs of the enzyme and peptide capable of potential interactions. A higher affinity of wild-type CaMKII for the NR2B peptide could be accounted for by more favourable interactions of the residues in -1, -2, $+1$ and $+4$ positions of the substrate peptide with CaMKII (Figure 3). A histidine residue at the -1 site of NR2B peptide that interacts with the side chain of Ser-25 is exposed and is in a favourable polar environment near the γ-phosphate of ATP. A leucine residue at the -1 site in syntide is less preferred in such a polar environment. Secondly, a glutamine residue at -2 position of the NR2B peptide has polar interactions with the side chain of Glu-139 in the conserved motif (RDLKPEN) of CaMKII. The corresponding residue in syntide, a threonine residue with a shorter side chain may not be able to interact with Glu-139, unless substantial conformational changes occur on peptide binding. A tyrosine residue at the $+1$ site of NR2B peptide is accommodated in an aromatic pocket constituting Trp-170, Phe-173 and Phe-171 and has extensive hydrophobic interactions with all the three residues. The corresponding residue in syntide, a valine residue in the $+1$ site, despite its hydrophobic nature interacts only with Ala-174 of CaMKII, as it is much shorter than a tyrosine residue. The occurrence of a valine residue at a similar position in the autophosphorylation site (Figure 1) may result in fewer apolar interactions with the substrate-binding site, similar to the binding mode of syntide, during autophosphorylation of Thr-286 by a neighboring subunit. The $+4$ site phenylalanine residue of the NR2B peptide is also in an aromatic environment, with Phe-171 and Trp-170 of CaMKII in its close proximity. The corresponding residue in syntide, a leucine residue at the $+4$ site, however, lacks such interactions, being aliphatic and short. These differences could lead to difference in the affinities of the two peptides, which might be reflected in their K_m values.

As reported for PKA [11], mutation of Glu-60 caused the K_m values for both the peptide substrates to increase, the increase being marginally higher for NR2B 17-mer (approx. 14-fold) than for syntide (approx. 10-fold) (Table 1b). Glu-60 is far away from the substrate-binding site and is unlikely to be directly involved in binding the peptide substrate, as inferred from the present model of the enzyme–substrate complex of CaMKII and the 3D structure of PKA complexed with its peptide inhibitor [5]. In CaMKII, Glu-60 is likely to form a salt-bridge with Lys-42 in the active state of the enzyme [28]. Glu-60 is partially buried and is located close to the ATP-binding site in an α-helix (C-helix in PKA [28]) whose conformation depends on the state of activity of the enzyme. In the active state, the N-terminal and the C-terminal lobes of protein kinases are close to each other (in a 'closed' state), bringing the C-helix nearer to the activation loop situated in the cleft between the two lobes.

Mutation of Glu-60 to a glycine could probably result in the creation of a void, which may be compensated by changes in packing of residues in the vicinity coupled with conformational changes in the C-helix. This is likely to cause the C-helix to drift away from the cleft. As the substrate-binding residues are located in the cleft, the catalytic core may tend towards an inactive conformation or an 'open' state, although not an entirely inactive

state. Such conformational changes may affect the easy access of the peptide substrate to the cleft and, consequently, result in a lower affinity of CaMKII for the peptide substrates.

Based on modelling studies of the catalytic domains tethered to the association domains of CaMKII [8], substrate-bound complexes of CaMKII in the 'closed' or active state would differ in their relative orientation with the core of the associated domain, from the auto-inhibited conformations observed for twitchin kinase and titin kinase. The oligomeric assembly of such substrate-bound enzymes would hence be loosely packed to the central core.

A defect in binding of ATP could also lead to defective binding of peptide substrates, since an ordered substrate-binding mechanism has been proposed in the case of CaMKII in which ATP binds the enzyme first and then the peptide substrate [41]. Due to the binding reciprocity between ATP and calmodulin, as reported by Torok et al. [42], under subsaturating concentrations of ATP it is possible that calmodulin binding may also be subsaturating. This could also contribute to lowered activity of the enzyme under such conditions. All the measurements of kinetic parameters of peptides were carried out at saturating ATP concentrations and under these conditions both the wild-type and the E60G mutant were saturated by 3 μM calmodulin (Figure 2E), the concentration used in all the kinetic analysis experiments.

Interestingly, the E60G mutation had strikingly different effects on the k_{cat} values of the two peptide substrates. The k_{cat} value when syntide is used as the substrate showed an almost 4-fold decrease, whereas the NR2B peptide showed an increase in k_{cat} value (about 3-fold) as a result of the mutation (Table 1b). Consequently, the catalytic efficiency for phosphorylation of syntide decreased dramatically (39-fold), whereas the change was much less (5-fold) for the NR2B peptide. These observations indicate either differences in the binding modes of the two substrates or differences in the rate constants of some intermediate step(s) in the catalytic mechanisms. Similar results were obtained when the wild-type and E60G mutant forms of α-CaMKII, purified after expression in *Escherichia coli*, were subjected to kinetic analysis with the two peptides (results not shown).

It is possible that the conformational changes that may arise due to mutation E60G are responsible for the decrease in k_{cat} value of the mutant when syntide is the substrate. The NR2B 17-mer peptide on the other hand, is known to be involved in interactions with regions outside the catalytic site of the enzyme [18,19]. These interactions may induce additional conformational changes in the enzyme, which might be responsible for the differential effect of Glu-60 mutation on the k_{cat} for the NR2B peptide compared with syntide. It is interesting to note that the NR2B 17-mer peptide shows significant sequence similarity to the autoregulatory domain around Thr-286 of CaMKII (Figure 1). The Gln residue at the -2 position and Asp residue at the $+2$ position are conserved between NR2B 17-mer and the auto-inhibitory domain, but not in syntide. This raises the possibility that the mechanism of autophosphorylation of CaMKII at Thr-286, as well as phosphorylation of similar peptide substrates such as autocamtide-2, may also follow the same kinetic mechanism as that of NR2B 17-mer, which is different from that for syntide and other similar phosphorylation sites.

The mechanism by which mutation of Glu-60, a residue previously thought to be involved in ATP binding, differentially affects the catalysis of different peptide substrates needs further investigation. It would be interesting to see if this mutation would affect the co-localization of CaMKII with NR2B observed in studies carried out in hippocampal neurons, as well as in heterologous expression systems such as HEK-293 cells, by co-expression of CaMKII with NR2B [18,19].

This work was funded by the Department of Biotechnology, Rajiv Gandhi Centre for Biotechnology, and Department of Atomic Energy of the Government of India. N.S. thanks the Wellcome Trust, U.K., for the support in the form of International Senior Fellowship. M.P., A.K., S.L., K.K.P and R.R.K. are recipients of fellowships from the Council of Scientific and Industrial Research, India. J.C. is a recipient of Junior Research Fellowship from the University Grants Commision, India. We gratefully acknowledge Dr K. Santhosh Kumar for help with the synthesis of peptides. We thank Professor Mary B. Kennedy, Division of Biology, California Institute of Technology, U.S.A., for the cDNA of α-CaMKII. We thank Dr R.V. Thampan, Professor M.R.N. Murthy, Dr M.R. Das, Professor K.P. Gopinathan, Dr T.J. Rasool, Dr D. Karunagaran, Dr Suparna Sengupta, Dr T.R. Santhosh Kumar and Dr. Moinak Banerji for help and suggestions during the course of this work.

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Received 20 May 2003/30 September 2003; accepted 15 October 2003
Published as BJ Immediate Publication 15 October 2003, DOI 10.1042/BJ20030741