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# High Level Expression and Preparation of Autonomous $Ca^{2+}$/Calmodulin-Dependent Protein Kinase II in *Escherichia coli*

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**The chymotryptic fragment of $Ca^{2+}$/calmodulin-dependent protein kinase II (30K-CaMKII) is a constitutively active enzyme that phosphorylates a variety of protein substrates *in vitro*. Although 30K-CaMKII is an often used and powerful tool for protein phosphorylation, the efficient production of catalytically active 30K-CaMKII in *Escherichia coli* has not yet been successfully realized, probably due to its toxicity in host cells. In this study, we found that a high-level expression of 30K-CaMKII as an insoluble form was attained when the N-terminal 43 amino acid residues of *Xenopus* CaMKI were fused to the N-terminal end of 30K-CaMKII (CX-30K-CaMKII). The inactive CX-30K-CaMKII thus expressed in *E. coli* was reactivated by simple denaturation/renaturation processes and purified on a $Ni^{2+}$-chelating column. The renatured CX-30K-CaMKII exhibited specific activity similar to that of rat brain CaMKII, and phosphorylated various proteins such as histones, myosin light chain, myelin basic protein, and synapsin I, as in case of 30K-CaMKII or purified CaMKII. Thus, CX-30K-CaMKII, an autonomous CaMKII, can be obtained with a simple procedure using *E. coli* expression system.**

**Key words: $Ca^{2+}$/calmodulin-dependent protein kinase II, catalytic fragment, phosphorylation, recombinant enzyme.**

Abbreviations: CaM, calmodulin; CaMK, $Ca^{2+}$/calmodulin-dependent protein kinase; CaMKP, $Ca^{2+}$/calmodulin-dependent protein kinase phosphatase; 30K-CaMKII, 30-kDa fragment of CaMKII; GST, glutathione S-transferase; MBP, myelin basic protein; MLC, myosin light chain.

$Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII) is a multifunctional Ser/Thr kinase with broad substrate specificity (*1*, *2*). The CaMKII family is composed of four different isoforms (α, β, γ, and δ) and their alternatively spliced variants (*3–5*). The γ and δ isoforms are expressed in most tissues, whereas the α and β isoforms are most prominent in neural tissues and comprise up to 2% of total protein in the hippocampus (*3*). Therefore, CaMKII is believed to play crucial roles in $Ca^{2+}$-responsive signal transduction including neuronal functions.

CaMKII is an oligomeric enzyme and requires $Ca^{2+}$/CaM for its activity, but becomes partially $Ca^{2+}$/CaM-independent after autophosphorylation at Thr286 (α)/Thr287 (β, γ, and δ) (*6*, *7*). Chymotryptic digestion of CaMKII after autophosphorylation produces a constitutively active 30-kDa fragment of CaMKII (30K-CaMKII) (*8–11*). Yoshimura *et al*. (*12*) obtained 30K-CaMKII by proteolytic digestion from the postsynaptic density fraction, where CaMKII exist abundantly. 30K-CaMKII has been utilized not only to study regulatory mechanisms of CaMKII (*8–13*) but also to explore physiological functions of CaMKII (*14*). 30K-CaMKII is also recognized to be a useful tool for studies on protein phosphorylation, because it phosphorylates a variety of protein substrates in the absence of $Ca^{2+}$/CaM (*15*). Another characteristic feature of 30K-CaMKII is that it phosphorylates substrates without showing autophosphorylation. Therefore, 30K-CaMKII is often used for the preparation of $^{32}P$-labeled protein substrates for protein phosphatase assays (*16*, *17*). Thus, 30K-CaMKII is a powerful tool for studies, not only on protein phosphorylation but also on protein dephosphorylation. However, the time-consuming and tedious procedures necessary for the preparation of 30K-CaMKII have hampered wide applicability of this useful reagent.

The N-terminal CaMKII fragment (α1-315), which shows $Ca^{2+}$/CaM-dependent activity, has been shown to be expressed at high levels in a baculovirus/insect cell expression system (*18*). In contrast, only low-level expression of catalytically active CaMKII (α1-282) was observed when an *E. coli* expression system was used (*19*). The fact that all attempts at high-level expression of 30K-CaMKII have so far been unsuccessful suggests that the expression of the catalytically active form of CaMKII is harmful for host cells.

Recently, we isolated the cDNA clone for a novel CaMKI from *Xenopus laevis* (CaMKIx). This enzyme could be expressed in large amounts in a soluble form using *E. coli* expression systems (*20*). Therefore, we attempted to express 30K-CaMKII as a fusion protein with N-terminal 43 amino acid residues of CaMKIx, which we designated as CX-30K-CaMKII. In this paper, we report that CX-30K-CaMKII could be expressed at high levels in an

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*E. coli* expression system in an insoluble form, and that the insoluble enzyme could be effectively reactivated through simple denaturation/renaturation processes. The reactivated enzyme exhibited high specific activity, comparable to that of brain CaMKII, and efficiently phosphorylated various substrate proteins, like 30K-CaMKII prepared from rat brain.

## MATERIALS AND METHODS

*Materials*—ATP, bovine serum albumin, histone type IIA, and myelin basic protein (MBP) were purchased from Sigma Chemicals. [$\gamma$-$^{32}$P]ATP (5,000 Ci/mmol) and the HiTrap Chelating HP column were from Amersham Biosciences. Syntide-2, a synthetic peptide substrate for CaMKII (*21*), was synthesized using a Shimadzu PSSM-8 automated peptide synthesizer and was purified by reverse-phase HPLC on a C18 column (ODS-80Tm, Tosoh Co.). Smooth muscle myosin light chain (MLC) and synapsin I were prepared as described previously (*22*). Calmodulin was purified from rat testis as described previously (*23*). CaMKII was purified from rat brain (*24*), and constitutively active 30K-CaMKII was prepared as described previously (*8*, *13*).

*Construction of a Plasmid of 30K-CaMKII*—For PCR of 30K-CaMKII$\gamma$, the following primers were used: 30K-CaMKII$\gamma$-5′ upstream primer (5′-AAA GCT AGC ATG GAG ACC ACC GCC ACC TG-3′) and 30K-CaMKII$\gamma$-3′ downstream primer (5′-TTT CTC GAG GAC CCA TGG GTG CTT GAG AGC-3′). The 30K-CaMKII$\gamma$-5′ upstream primer contained an *Nhe*I site (underlined) and the 30K-CaMKII$\gamma$-3′ downstream primer contained an *Xho*I site (double underlined). For PCR of CX-30K-CaMKII$\gamma$, the following primers were used: CX-30K-CaMKII$\gamma$-5′ upstream primer (5′-AAA GAA TTC GAT GGC CAC CAC CGC CAC CTG-3′) and 30K-CaMKII$\gamma$-3′ downstream primer. The CX-30K-CaMKII$\gamma$-5′ upstream primer contained an *Eco*RI site (underlined). PCR was performed with a GeneAmp PCR System 2700 (Applied Biosystems) for 30 cycles (for 30K-CaMKII$\gamma$, 96°C, 10 s/60°C, 5 s/72°C, 2 min; for CX-30K-CaMKII$\gamma$, 96°C, 10 s/72°C, 2 min) using Pyrobest DNA Polymerase (TaKaRa), and a rat brain 5′-RACE ready cDNA library or pET-30K-CaMKII$\gamma$ as a template. After gel purification, the amplified product was digested with *Nhe*I and *Xho*I (or *Eco*RI and *Xho*I). To generate the $His_6$-tagged fusion protein (or the N-terminal 43 amino acid residues of CaMKIx and $His_6$-tagged fusion protein), the *Nhe*I/*Xho*I fragment was cloned into *Nhe*I/*Xho*I-digested pET-23a(+) (Novagen) [or the *Eco*RI/*Xho*I fragment was cloned into *Eco*RI/*Xho*I-digested pETCaMKIx (*20*)], sequenced, and designated as pET-30K-CaMKII$\gamma$ or pET-CX-30K-CaMKII$\gamma$, respectively.

*Expression, Renaturation, and Purification of the 30K-CaMKII Fusion Protein—E. coli* strain BL21(DE3) (Novagen) cells transformed with pET-30K-CaMKII$\gamma$ (or pET-CX-30K-CaMKII$\gamma$) were grown at 37°C for 16 h in 3 ml of medium A (LB medium supplemented with 100 μg/ml ampicillin) with shaking. The culture was then transferred to 100 ml of medium A, followed by incubation at 37°C for 24 h with shaking. The cells were harvested by centrifugation and suspended in 10 ml of buffer A [20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 0.05% Tween 40]. After sonication, the *E. coli* homogenate was centrifuged (20,000 × *g*) at 4°C for 10 min, and the supernatant was removed. To the pellet, 10 ml of denaturation buffer consisting of 50 mM Tris-HCl (pH 7.5), 6 M guanidine-HCl, and 10 mM 2-mercaptoethanol was added, followed by solubilization by sonication. After sonication, the solubilized sample was centrifuged (20,000 × *g*) at 4°C for 10 min, and then the solubilized CX-30K-CaMKII was used for the renaturation experiments.

Renaturation of CX-30K-CaMKII$\gamma$ was examined by means of two different procedures. For the first method, solubilized CX-30K-CaMKII$\gamma$ (1 ml) was dialyzed against a renaturation buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 40, 10% ethylene glycol, and 1 mM dithiothreitol] at 4°C for appropriate times to remove 6 M guanidine-HCl. For the second method, the solubilized enzyme was rapidly diluted; the solubilized CX-30K-CaMKII$\gamma$ was diluted with varying volumes of ice-cold renaturation buffer containing 10 mM 2-mercaptoethanol, followed by incubation at 4°C or 16°C. Renaturation of CX-30K-CaMKII$\gamma$ was monitored by means of the standard protein kinase assay.

$His_6$-tagged CX-30K-CaMKII$\gamma$ could be purified on a HiTrap Chelating HP column. Solubilized CX-30K-CaMKII$\gamma$ in 6 M guanidine-HCl (1 ml) was diluted with 199 ml of renaturation buffer and then kept at 4°C for 24 h. After centrifugation (20,000 × *g*) at 4°C for 10 min, the reactivated CX-30K-CaMKII$\gamma$ was loaded on a HiTrap Chelating HP column (1 ml) pre-equilibrated with buffer A containing 10 mM 2-mercaptoethanol. The column was washed successively with 10 ml of buffer A containing 20 mM imidazole and buffer A containing 50 mM imidazole, and then CX-30K-CaMKII$\gamma$ was eluted with buffer A containing 200 mM imidazole. The purified CX-30K-CaMKII$\gamma$ was dialyzed against renaturation buffer containing 5 mM dithiothreitol and stored at –80°C until use.

*Protein Kinase Assay and Phosphorylation of Proteins*—The protein kinase activity of CX-30K-CaMKII$\gamma$ was determined essentially according to a method described previously (*25*). The protein kinase assay was carried out at 30°C in a reaction mixture (20 μl) consisting of 40 mM Hepes-NaOH (pH 8.0), 2 mM dithiothreitol, 0.1 mM EGTA, 5 mM $Mg(CH_3COO)_2$, 40 μM syntide-2, 50 μM [$\gamma$-$^{32}$P]ATP (300–500 cpm/pmol), and the indicated amount of CX-30K-CaMKII$\gamma$. After incubation at 30°C for 1 min, 15 μl-aliquots were withdrawn and $^{32}$P-phosphate incorporation into syntide-2 was measured essentially according to the method of Roskoski (*26*).

Protein phosphorylation was carried out in a reaction mixture (10 μl) consisting of 40 mM Hepes-NaOH (pH 8.0), 2 mM dithiothreitol, 0.1 mM EGTA, 5 mM $Mg(CH_3COO)_2$, 100 μM [$\gamma$-$^{32}$P]ATP, and 0.1 mg/ml protein substrate. In the case of phosphorylation by CaMKII, 0.5 mM $CaCl_2$ and 1 μM CaM were added to the reaction mixture. After 30 min incubation at 30°C, the reaction was stopped by the addition of 10 μl of 2× SDS sample buffer. Phosphorylated proteins thus obtained were subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and detected by autoradiography.

*Protein Determination and SDS-PAGE*—Protein concentrations were determined by the method of Bensadoun and Weinstein using bovine serum albumin as a standard (*27*). SDS-PAGE was carried out essentially according to the

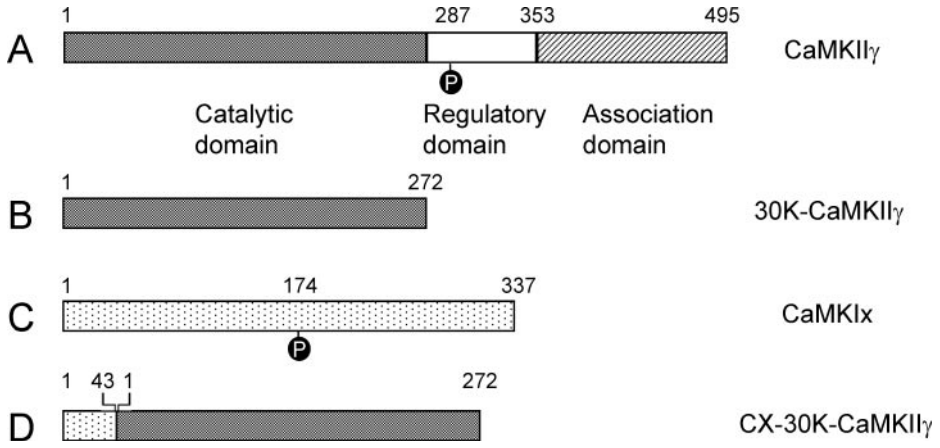


Fig. 1. **Structures of CaMKII, 30K-CaMKII, CaMKIx, and CX-30K-CaMKII.** The domain structure of CaMKIIγ is represented in (A). All the isoforms and alternatively spliced variants of CaMKII have essentially the same domain structure. Thr287 represents the autophosphorylation site of CaMKIIγ. 30K-CaMKIIγ consists of only the catalytic domain of CaMKIIγ (B). CaMKIx is composed of 337 amino acid residues (C). CX-30K-CaMKIIγ is a fusion protein consisting of amino acid residues 1–43 of CaMKIx and amino acid residues 1–272 of 30K-CaMKIIγ (D).

method of Laemmli (*28*) on slab gels consisting of a 12% acrylamide separation gel and a 3% stacking gel.

## RESULTS

*Expression of 30K-CaMKII and CX-30K-CaMKII*—CaMKII is composed of an N-terminal catalytic domain, a central regulatory domain containing an autoinhibitory domain and a CaM binding region, and a C-terminal association domain (*6*, *7*). 30K-CaMKII is a constitutively active enzyme that lacks the regulatory and association domains of CaMKII (*8*) (Fig. 1, A and B). Since 30K-CaMKII phosphorylates a variety of protein substrates without the addition of $Ca^{2+}$/CaM, it is a useful tool for protein phosphorylation. To obtain catalytically active 30K-CaMKII in *E. coli*, expression vectors for the α- and γ-isoforms of 30K-CaMKII were constructed. The coding sequences of 30K-CaMKIIα(1-271) and 30K-CaMKIIγ(1-272) were subcloned into the pET-23a(+) bacterial expression vector to construct pET-30K-CaMKIIα and pET-30K-CaMKIIγ. These expression vectors were transfected into *E. coli* strain BL21(DE3), and then whether or not they could each be expressed as an active form in *E. coli* was examined. Transfected *E. coli* cells were disrupted by sonication, and the soluble fraction was separated from the insoluble fraction by centrifugation. Both the soluble and insoluble fractions were electrophoresed, and then the expression levels of the recombinant proteins were assessed based on the protein staining patterns with Coomassie Brilliant Blue. As shown in Fig. 2A, a protein staining band corresponding to 30K-CaMKIIγ was only slightly observed in the insoluble fraction, and not at all in the soluble fraction. Essentially, no expression of 30K-CaMKIIα was detected in either fraction as judged from protein staining (data not shown). To improve the expression levels of these recombinant proteins, expression vectors for glutathione S-transferase (GST) fusion proteins for 30K-CaMKIIα and 30K-CaMKIIγ were constructed. Although the expression of GST-30K-CaMKIIα/γ was examined under various conditions, expression of the recombinant proteins was not detected by protein staining with Coomassie Brilliant Blue (data not shown).

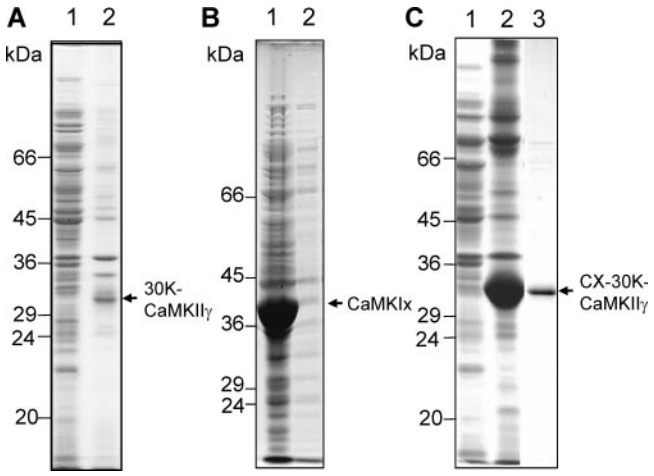


Fig. 2. **Expression of recombinant 30K-CaMKIIγ, CaMKIx, and CX-30K-CaMKIIγ.** (A) Expression of recombinant 30K-CaMKIIγ in *E. coli*. The supernatant (20 μg protein, lane 1) and precipitate (7 μg protein, lane 2) of a lysate of *E. coli* cells transformed with pET-30K-CaMKIIγ were subjected to SDS-PAGE and then staining with Coomassie Brilliant Blue. (B) Expression of recombinant CaMKIx. The supernatant (36 μg protein, lane 1) and precipitate (5 μg protein, lane 2) of a lysate of *E. coli* cells transformed with pETCaMKIx were analyzed by SDS-PAGE. (C) Expression and purification of CX-30K-CaMKIIγ. The supernatant (8 μg protein, lane 1) and precipitate (19 μg protein, lane 2) of a lysate of *E. coli* cells transformed with pET-CX-30K-CaMKIIγ were analyzed by SDS-PAGE. CX-30K-CaMKIIγ (0.3 μg, lane 3) purified on a HiTrap Chelating HP column was analyzed by SDS-PAGE. Arrows on the right show the respective recombinant proteins.

Recently, we cloned and characterized a novel CaMKI homologue of *Xenopus laevis*, and designated it as CaMKIx (*20*). A large amount of CaMKIx could be expressed in a soluble form in *E. coli* as shown in Fig. 2B. Indeed, as much as 20 mg of purified CaMKIx was obtained from transfected *E. coli* cells grown in 50-ml of culture medium under the

experimental conditions. These observations led us to examine whether or not recombinant 30K-CaMKIIγ could be expressed when the N-terminal amino acid sequence of CaMKIx was introduced to the N-terminal end of 30K-CaMKIIγ as illustrated in Fig. 1D. The length of the N-terminal peptide (43 amino acid residues) of CaMKIx added to 30K-CaMKII was determined on the basis of the restriction enzyme (*Eco*RI) site of the CaMKIx gene. To obtain a fusion protein, CX-30K-CaMKIIγ, which was composed of 30K-CaMKIIγ and the N-terminal region of CaMKIx, the coding sequence of 30K-CaMKIIγ was subcloned into pETCaMKIx bacterial expression vector to construct pET-CX-30K-CaMKIIγ. The pET-CX-30K-CaMKIIγ expression vector was transfected into *E. coli* strain BL21(DE3), and expression of the fusion protein was examined under various conditions. When transfected *E. coli* cells were cultured at 37°C for 24 h, high-level expression of recombinant enzyme was observed. Although a significant amount of CX-30K-CaMKIIγ was expressed, the recombinant fusion protein was exclusively obtained in an insoluble fraction as shown in Fig. 2C.

*Renaturation of CX-30K-CaMKIIγ*—Although a large amount of CX-30K-CaMKIIγ could be expressed (Fig. 2C), all attempts to express the recombinant fusion protein in a soluble form were unsuccessful. Since the insoluble CX-30K-CaMKIIγ exhibited no kinase activity (data not shown), we examined whether or not it could be renatured by means of denaturation–renaturation procedures. Insoluble CX-30K-CaMKIIγ obtained by centrifugation could be solubilized in a denaturation buffer consisting of 50 mM Tris-HCl (pH 7.5), 6 M guanidine-HCl, and 10 mM 2-mercaptoethanol. To renature CX-30K-CaMKIIγ, the solubilized recombinant protein in the denaturation buffer was dialyzed against a renaturation buffer consisting of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 40, 10% ethylene glycol, and 1 mM dithiothreitol. However, the solubilized protein again became an insoluble form during the dialysis process (data not shown). Although dialysis was carried out under various conditions, the solubility of the recombinant protein could not be improved (data not shown).

Next, we examined whether or not CX-30K-CaMKIIγ could be renatured by dilution of the solubilized recombinant protein with the renaturation buffer. As shown in Fig. 3A, when the solubilized CX-30K-CaMKIIγ was diluted 50–800 fold with the renaturation buffer and kept at 4°C for 24 h, the enzyme activity was significantly restored. The most efficient reactivation was attained with 200-fold dilution of the solubilized enzyme, while higher dilutions resulted in lower specific activity of the enzyme. Stepwise dilution of the solubilized CX-30K-CaMKIIγ did not improve the recovery of the enzyme activity (data not shown). The time course of the restoration of enzyme activity is shown in Fig. 3B. When the diluted enzyme solution was incubated at 16°C, kinase activity reached a maximal level at 4–8 h after dilution, but the activity gradually declined thereafter. When renaturation was carried out at 4°C, enzyme activity was gradually restored and still continued to recover at 24 h after dilution (Fig. 3B). These results, taken together, suggest that a relatively low protein concentration and low temperature may facilitate the efficient refolding of CX-30K-CaMKIIγ.

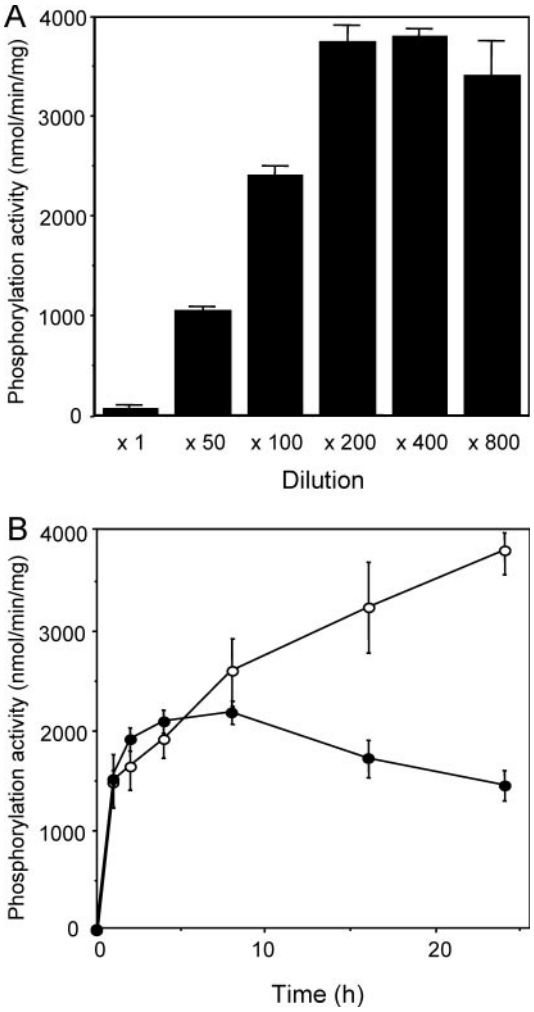


Fig. 3. **Renaturation of CX-30K-CaMKIIγ.** (A) Effect of dilution on restoration of the catalytic activity of CX-30K-CaMKIIγ. CX-30K-CaMKIIγ was expressed in *E. coli* and then lysed by sonication. The precipitate was solubilized in the denaturation buffer containing 6 M guanidine-HCl at a final concentration of approximately 3 mg/ml. To the CX-30K-CaMKIIγ solution (5 μl), ice cold renaturation buffer, 245 μl (×50), 495 μl (×100), 995 μl (×200), 1,995 μl (×400), or 3,995 μl (×800), was added, followed by quick mixing. The diluted mixtures were kept at 4°C and protein kinase activities were determined after 24 h as described under "MATERIALS AND METHODS." (B) The time course of renaturation of CX-30K-CaMKIIγ. Solubilized CX-30K-CaMKIIγ was diluted with a 200-fold volume of the renaturation buffer as in (A), and then kept at 4°C (open circles) or 16°C (solid circles). At the indicated times (0, 1, 2, 4, 8, 16, and 24 h), renaturation of the enzyme was monitored by determining protein kinase activity using syntide-2 as a substrate. Data are means ± SD values for three independent experiments.

*Purification of CX-30K-CaMKIIγ*—Since CX-30K-CaMKIIγ is a C-terminal $His_6$-tagged protein, the renatured enzyme could be purified on a HiTrap Chelating HP column. Using standard purification processes, consisting of solubilization of the recombinant protein with 6 M guanidine-HCl, dilution with a renaturation buffer and $Ni^{2+}$-chelating column purification, approximately 2.14 mg of purified CX-30K-CaMKIIγ could be obtained from a 100-ml *E. coli* culture. The purified CX-30K-CaMKIIγ showed a single protein band on SDS-PAGE

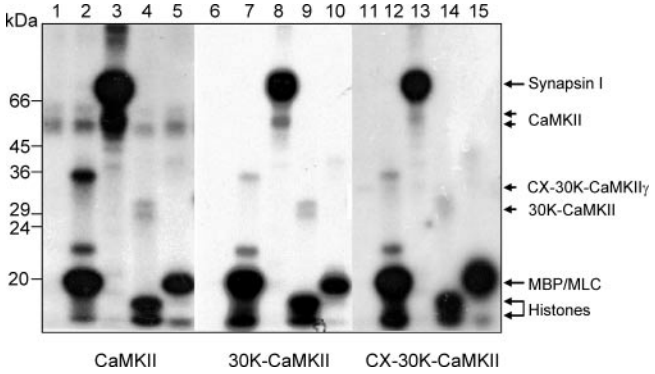


Fig. 4. **Phosphorylation of various proteins by CX-30K-CaMKIIγ, 30K-CaMKII, and CaMKII.** MBP (lanes 2, 7, and 12), synapsin I (lanes 3, 8, and 13), histones (lanes 4, 9, and 14), and MLC (lanes 5, 10, and 15) were phosphorylated with CaMKII (lanes 1–5), 30K-CaMKII (lanes 6–10), or CX-30K-CaMKIIγ (lanes 11–15). Phosphorylation was also carried out in the absence of a protein substrate (lanes 1, 6, and 11). Phosphorylation by CaMKII was carried out in the standard phosphorylation mixture in the presence of $Ca^{2+}$/CaM, but phosphorylation by 30K-CaMKII and CX-30K-CaMKIIγ was carried out in the absence of $Ca^{2+}$/CaM. After incubation at 30°C for 30 min, the reactions were stopped by the addition of the same volume of 2× SDS sample buffer, followed by SDS-PAGE. The phosphorylation of the proteins was visualized by autoradiography. The migration positions of CaMKII, 30K-CaMKII, CX-30K-CaMKIIγ, and the respective protein substrates are shown on the right of the autoradiograms.

(Fig. 2C, lane 3). The maximal specific activity of the purified CX-30K-CaMKIIγ was calculated to be 7,074 nmol/min/mg, which is comparable to that of the purified CaMKII preparation from rat brain (8,372 nmol/min/mg). The specific activity of the purified CX-30K-CaMKIIγ did not decrease even after four freeze-thaw cycles, indicating that this recombinant 30K-CaMKII is a relatively stable enzyme.

*Phosphorylation of Substrate Proteins by CX-30K-CaMKIIγ*—CaMKII is known to be a multifunctional protein kinase that phosphorylates various protein substrates (*1*, *2*). To compare the substrate specificity of CX-30K-CaMKIIγ with those of CaMKII and 30K-CaMKII, four typical substrates for CaMKII; MBP, synapsin I, histones, and MLC, were used for phosphorylation. As shown in Fig. 4 (lanes 1–5), CaMKII phosphorylated all of these substrates, but only in the presence of $Ca^{2+}$/CaM. In addition, autophosphorylation of the 50-kDa and 60-kDa isoforms of CaMKII was observed. In contrast, CX-30K-CaMKIIγ, as well as purified 30K-CaMKII, phosphorylated MBP, synapsin I, histones, and MLC efficiently even in the absence of $Ca^{2+}$/CaM (Fig. 4, lanes 6–15). In these cases, no significant autophosphorylation of CX-30K-CaMKIIγ or 30K-CaMKII was observed. These results indicate that CX-30K-CaMKIIγ exhibited essentially the same substrate specificity as those of 30K-CaMKII and CaMKII.

## DISCUSSION

Catalytically active 30K-CaMKII has been used as a useful tool for the functional analysis of CaMKII. For example, the regulatory roles of the autoinhibitory domain of CaMKII have been clarified using 30K-CaMKII as a model (*8*, *13*). Involvement of CaMKII in cADP-ribose mediated $Ca^{2+}$ release from rat islet microsomes has also been demonstrated with 30K-CaMKII (*14*). In other cases, 30K-CaMKII was used for the preparation of phosphorylated substrates for protein phosphatase assays, especially for CaMK phosphatase (CaMKP) (*16*, *17*, *29*). One of the characteristic features of 30K-CaMKII is that it shows essentially the same substrate specificity as the original CaMKII without exhibiting autophosphorylating activity (Fig. 4). Since CaMKII is efficiently autophosphorylated, this could be a significant obstacle when analyzing the phosphorylation of proteins with molecular weights comparable to those of CaMKII isoforms ranging from 50 kDa to 60 kDa. For example, when CaMKP was phosphorylated with CaMKII in the presence of $Ca^{2+}$/CaM, autophosphorylated CaMKII and the phosphorylated CaMKP showed similar migration on SDS-PAGE, and therefore these protein bands were difficult to distinguish. This confusion could be overcome by the use of 30K-CaMKII instead of CaMKII (*15*). In this case only phosphorylated CaMKP was clearly observed, as it was not disturbed by autophosphorylated CaMKII. Since 30K-CaMKII is a convenient enzyme, as described above, it would be useful if we could obtain the catalytically active enzyme with a simple *E. coli* expression system.

To date, recombinant CaMKII has been prepared using various expression systems. An *E. coli* expression system is convenient and economic, and so has often been used for the expression of recombinant CaMKII. However, the specific activities of recombinant CaMKII expressed in *E. coli* were much lower (1,000–1,525 nmol/min/mg) than those of CaMKII purified from rat brain (9,248–17,582 nmol/min/mg) (*30*, *31*). When a baculovirus expression system was used for preparation of recombinant CaMKII (*32*) and CaMKII fragment (α1-315) (*18*), a significantly high level of expression was observed. These recombinant enzymes were highly active only in the presence of $Ca^{2+}$/CaM. However, there has been no report concerning the production of catalytically active 30K-CaMKII using a baculovirus expression system. Ohsako *et al*. (*19*) reported that low-level expression of CaMKII(α1-282) was observed when an *E. coli* expression system was used. However, our attempts at large scale production of catalytically active 30K-CaMKII with an *E. coli* expression system have so far all been unsuccessful. These results, taken together, suggest that the expression of a considerable amount of the active 30K-CaMKII exhibiting broad substrate specificity may lead to death of the host cells.

In the present study, we found that CX-30K-CaMKIIγ could be expressed as an insoluble form at a significant level in *E. coli*. The inactive CX-30K-CaMKIIγ could then readily be reactivated by the simple denaturation/renaturation processes. The characteristic properties of the purified CX-30K-CaMKIIγ were compared with those of CaMKII and 30K-CaMKII, and are summarized in Table 1. The intact CaMKII used in the present study was purified from rat cerebrum (*24*), and 30K-CaMKII was prepared from the purified CaMKII (*8*, *13*). The preparation of these enzymes required time-consuming multiple steps and many rats were sacrificed. For the preparation of 1 mg of purified CaMKII or 30K-CaMKII, 42–108 g or 146–376 g of rat cerebrum, respectively, was required as the starting material under our experimental conditions.

Table 1. **Characteristic properties of CX-30K-CaMKIIγ compared with those of CaMKII and 30K-CaMKII.**

| | CaMKII | 30K-CaMKII | CX-30K-CaMKIIγ |
|---|---|---|---|
| Source of enzyme | Rat brain | Rat brain | *E. coli* |
| Starting material[a] (g) | 42–108 | 146–376 | 0.25 |
| Specific activity[b] (nmol/min/mg) | 8,372 | 19,632 | 7,074 |
| Autophosphorylation | + | – | – |
| Phosphorylation of | | | |
| MBP | + | + | + |
| MLC | + | + | + |
| Synapsin I | + | + | + |
| Histones | + | + | + |

[a] Wet weight of starting material required for the preparation of 1 mg of the purified kinases, [b]Determined by means of the standard assay with syntide-2 as a substrate.

Similar values have been reported for the preparation of 30K-CaMKII from the post-synaptic density fraction of rat forebrain (*12*). In contrast, only about 0.25 g of *E. coli* cell pellet, which could be obtained from 45-ml of *E. coli* culture medium, was sufficient for the preparation of 1 mg of purified CX-30K-CaMKIIγ. The highest specific activity obtained for CX-30K-CaMKIIγ was 7,074 nmol/min/mg, which was almost one-third of that for 30K-CaMKII (19,632 nmol/min/mg), and was comparable to that for purified rat brain CaMKII (8,372 nmol/min/mg).

The CX-30K-CaMKIIγ, thus obtained, exhibited essentially the same catalytic properties as those of 30K-CaMKII prepared from rat brain, and could be used in place of 30K-CaMKII. Since it is constitutively active without $Ca^{2+}$/CaM, it may substitute for auto(thio)phosphorylated CaMKII, which cannot be readily prepared but has been used to prove the involvement of CaMKII in various physiological systems such as neuronal transmission (*33*, *34*). Therefore, CX-30K-CaMKIIγ will serve as a powerful tool for studies on protein phosphorylation/dephosphorylation, as in the case of the catalytic subunit of cAMP-dependent protein kinase that is widely used experimentally.

Of particular interest is the finding that the addition of the N-terminal 43 amino acid residues of CaMKIx to the N-terminal of 30K-CaMKII drastically increased the expression of the protein in *E. coli* (Fig. 2, A and C). Although the minimum length of the additional peptide required for high level expression of 30K-CaMKII has not been clarified yet, the addition of a short peptide comprising the N-terminal 5 amino acid residues of CaMKIx was found to be ineffective (data not shown). We are now investigating how many amino acid residues are necessary for the maximal expression of the fusion proteins. To date, many recombinant proteins have been expressed as fusion proteins with GST; however, the addition of GST sometimes results in changes in the functional properties of the original proteins because of the more than 200 additional amino acids derived from GST. The addition of the 43 amino acid residues on the N-terminal side of 30K-CaMKII appears to cause no significant changes in the catalytic properties, such as the substrate specificity of the enzyme (Fig. 4). Therefore, the addition of this sequence to various proteins may improve the expression of proteins that have not been obtainable with *E. coli* expression systems without losing their original properties. Experiments along these lines are now in progress in our laboratory.

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