Activity of Recombinant α and β Subunits of Casein Kinase II from
*Xenopus laevis*

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**ABSTRACT:** Casein kinase II (CKII) is a ubiquitous protein kinase, found predominantly in cell nuclei, which has two subunits in a tetrameric αβ;2 or αα'ββ' configuration. The catalytic center is present in the α subunit which is active by itself while β is a regulatory subunit that can greatly enhance the activity of α. The cDNA genes of *Xenopus laevis* coding for the α and β subunits of CKII have been expressed in *Escherichia coli* and extensively purified. The recombinant subunits reconstitute a fully active holoenzyme when incubated in stoichiometric amounts. Mutations that change serines in positions 2 and 3 of the β subunit for glycines completely eliminate the autophosphorylation site present in this subunit but do not significantly affect the capacity of β to activate α. A fusion protein composed of glutathione transferase linked to the *X. laevis* CKII β subunit can also activate α. This fusion protein binds to glutathione–agarose beads and can mediate the binding of the α subunit to this matrix. Conversely, the α subunit was found to bind to glass fiber filters in an active form that can still be activated by β to an extent similar to that seen in solution. Using peptides containing tyrosine and glutamic acid as inhibitors of the activity of the isolated α subunit and of the holoenzyme, the effect of β on the specificity of inhibition was studied. The results obtained demonstrate that the β subunit participates in the specific recognition of tyrosine by the holoenzyme and in the discrimination in the position of the tyrosyl residues with respect to the acidic amino acids.

Casein kinase II (CKII) is a protein kinase that is present in all eukaryotic cells and is predominantly localized in the cell nucleus [reviewed in Pinna (1990) and Tuazon and Traugh (1991)]. This enzyme has been found to be a tetramer of an αβ;2 or αα'ββ' configuration. The α or α' subunits contain the catalytic center and are active by themselves while β is a regulatory subunit that greatly stimulates the activity of α (Cochet & Chambaz, 1983).

CKII phosphorylates serines or threonines that are present in acidic regions of proteins. The study of sequences phosphorylated by CKII and the use of model peptides has defined a minimum recognized substrate sequence with the structure Ser/Thr-X-X- acidic residue. The acidic residue can be an aspartic acid, glutamic acid, or phosphorylated serine, threonine, or tyrosine. The X residues are commonly also acidic in nature (Meggio et al., 1984; Kuenzel & Krebs, 1985).

The physiological mechanism for the regulation of CKII activity is not known; however, there are several reports that indicate that its activity varies in cells stimulated to divide by various mitogenic stimuli (Klarlund & Czech, 1988; Ackerman & Osheroff, 1989). *In vitro*, it has been found that CKII activity can be significantly stimulated by polyamines such as spermine and spermidine and by polylysine (Cochet et al., 1980; Meggio et al., 1987).

Heparin has been found to be a very potent inhibitor (Hathaway et al., 1980); however, the strength of the inhibition depends on the nature of the substrate protein (Taylor et al., 1987). Other polyanions such as polyglutamic and polyaspartic acid and some nucleic acids have also been found to be inhibitory (Meggio et al., 1983; Gatica et al., 1989). Interestingly, the presence of tyrosine in acidic clusters greatly enhances the inhibitory capacity of these polymers (Meggio & Pinna, 1984; Tellez et al., 1990).

CKII has been found to phosphorylate a large number of different proteins both *in vivo* and *in vitro*. Special importance has been given to the fact that CKII phosphorylates several enzymes and factors involved in the synthesis of nucleic acids and in the regulation of transcription. In the case of topoisomerase II (Corbett et al., 1992) and the protein products of the protooncogenes myb (Lüscher et al., 1990), jun (Lin et al., 1992), and max (Berberich & Cole, 1992), it has been reported that CKII phosphorylation modifies the physiological activity of these proteins.

The cDNAs coding for the α, α', and β subunits of CKII from humans, drosophila, yeast, and several other species have been cloned and sequenced (Saxena et al., 1987; Meisner et al., 1989; Grankowski et al., 1991; Hu & Rubin, 1990a). In our laboratory, we have reported the cloning and sequence of the α and β subunits of the *Xenopus laevis* CKII (Jedlicki et al., 1992). Analysis of these sequences shows a remarkable degree of conservation in evolutionarily distant species; for instance, the sequences from *Xenopus* and humans of the α subunit differ only in 1 amino acid in the first 300 amino acids, and the 2 β subunits of the same species differ only in 2 amino acids over the whole length of the polypeptide.

Expression and mutagenesis of cloned subunits of CKII are presently being used to determine the structure–function relationship in the mechanism of action of this enzyme. There have been studies that indicate that the β subunit, in addition to its stimulatory capacity, also influences the specificity in the interaction of the enzyme with its substrates (Meggio et al., 1990).
Expression and Purification of the α and β Subunits of CKII. Cloning and sequencing of the CKII α and β subunits were described earlier (Jedlicki et al., 1992). A clone containing a 2000 bp insert with 1050 bp corresponding to the α subunit coding region was amplified by PCR using oligonucleotide primers containing PsiI restriction sites complementary to the sequence bordering the coding region. The sequence of the upstream primer was 5′CCCTGCGATGTGACAACTGTTG3′ and of the downstream primer 5′CCCTGCGATCATCTGCGCTAC3′. The amplified and PsiI-digested fragment was subcloned into the expression vector PT7-7 (Tabor & Richardson, 1985) and introduced into the E. coli expression strain BL21(DE3). Expression was induced with 1 mM isopropyl-β-D-thiogalactoside. Cells were harvested after 2-h incubation and resuspended in 20 mM Tris-HCl, pH 7.5, buffer containing 50 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 µg/mL leupeptin, and 0.7 µg/mL pepstatin. Cells were sonicated and centrifuged at 12000g for 30 min. The α subunit of CKII present in the supernatant solution was purified on DEAE-Sephadex and phosphocellulose as described for the holoenzyme (Hathaway & Traugh, 1983). The average yield of α subunit was 3 mg of protein/L of bacterial culture. The protein was stored in 25 mM potassium phosphate buffer, pH 7.5, in 0.7 M KCl at 4 °C. The specific activity obtained in different preparations ranged from 65 to 90 units/mg, assayed under standard conditions described above for the holoenzyme.

The expression of the β subunit was achieved by use of a pGEX vector to produce a fusion protein with glutathione S-transferase (Smith & Johnson, 1988). A clone containing the entire coding region of the CKII β subunit was subcloned in the EcoRI site of the expression vector pGEX-2T after introducing EcoRI sites in both extremes of the β-coding region and amplification by PCR using synthetic oligonucleotide primers. The sequence of the upstream primer was 5′TACG GAATTCTAAAATGATGATGCTG3′ and the downstream primer 5′GGGGAATCTTCAACGATGGTCT3′. The ligation product was used to transform E. coli AG1, and transformants were selected on LB/ampicillin plates. The fusion protein was purified on glutathione–agarose beads exactly as described (Smith & Johnson, 1988). Removal of the glutathione transferase moiety was achieved by incubation with 30 µg of human thrombin in 2.5 mM CaCl2 for 1 h at 30 °C. The glutathione transferase was separated from the β subunit by a second passage of dialyzed fusion protein through the glutathione–agarose column. The purified β subunit was stored in 50 mM Tris-HCl, pH 7.5, in 150 mM NaCl at 4 °C.

Mutation of the Autophosphorylation Site of Subunit β. Series in positions 2 and 3 of the β subunit were mutated through PCR amplification of the cloned cDNA gene (Jedlicki et al., 1992). The appropriate base changes were introduced in the upstream primer which had the sequence 5′CCCGAAT TCAGGCTTGGTCGGA3′ (the underlined bases are the ones changed from the wild-type sequence). The downstream primer was the same as described previously for cloning into the pGEX-2T expression vector. The amplified DNA was again introduced into that vector, and an identical procedure was followed to express and purify the mutated β subunit. The entire nucleotide sequence of the mutated expression clone was corroborated by sequencing (Sanger et al., 1977).

Analysis of the Association of CKIIα with CKIIβ Fusion Protein. The fusion protein glutathione transferase–β subunit
(50–60 μg in a typical experiment) obtained as described above was incubated 15 min at room temperature with 100 μL of a 50% slurry of GSH–agarose in a 200-μL final volume. The GSH–agarose was washed by microcentrifugation (the supernatant was designated the S-1 fraction) with 5 × 1 mL of PBS. Subunit α (45 μg) was added to the wash agarose containing the bound fusion protein in a final volume of 0.8 mL in 20 mM potassium phosphate buffer, pH 6.8, and 0.5 M NaCl. Incubation of α subunit with the bound fusion protein was for 1 h at 4 °C with gentle mixing. The slurry was microcentrifuged for 15 s, and the supernatant solution (designated the S-2 fraction) was assayed for kinase activity and analyzed by 10% SDS–polyacrylamide gel electrophoresis. The agarose beads were then washed by centrifugation with 5 × 1 mL of PBS and resuspended in 300 μL of PBS. Aliquots of the slurry were then used for kinase assays, and for gel analysis after the addition of a 2× SDS gel sample buffer.

Adsorption of α to Glass Fiber Filters. The retention of α, or α and β, subunits of CKII on glass fiber filters was as follows: α or α plus β subunits containing between 0.5 and 10 μg of protein in 100 μL of 25 mM Hepes, pH 7.5, and 0.7 M KCl were incubated with an 0.8 cm² GF/C glass fiber filter for 15 min at 4 °C. The filters were washed free of unbound enzyme by incubating 3 times consequently in 100 mL of 25 mM Tris-HCl, pH 7.5, at 4 °C for 5 min. Each filter was blotted quickly just after Whatman no. 3 filter paper and transferred to a 1.5-mL Eppendorf tube for protein kinase assay. The standard protein kinase assay with casein as substrate was used except with a final volume of 100 μL. An aliquot of 60 μL was removed for measurement of [32P]-phosphate incorporation. Controls were performed using filters without protein or filters with α or α + β bound subunits subjected to 100 °C for 3 min prior to assay. In assays using 1.5 × 10⁶ cpm, these blanks yielded less than 1500 cpm for both boiled filters and filters not exposed to enzyme. The incorporation of [32P]phosphate is proportional to the amount of enzyme protein used during the adsorption step.

The possibility that α subunit was released from the filter during the assay period was tested by the incubation of 8.5 μg of α subunit bound to a filter as described above but without substrates. After centrifugation at 12,000 rpm for 3 min, the supernatant solution was found not to contain kinase activity.

Microsequencing of Recombinant β Subunit. Amino-terminal amino acid sequencing of the CKII β subunit produced after thrombin treatment of the fusion protein was performed by the Peptide and Protein Service LANAI-PRO, Buenos Aires, Argentina, and the sequence GSPGIQME... was determined. The first six amino acids of the amino terminal originate from the fusion protein.

Peptide Synthesis. The peptides YYEEEEEEE (Y5E5) and EEEEEEYEE (E6E6) were synthesized using a commercial peptide synthesizer (Model 950 Pep Synthesizer, Milligen/Biorsearch). Their purity was checked by HPLC, and their composition was corroborated by amino acid analysis.

Oligonucleotide Synthesis. Oligonucleotides for PCR primers and for sequencing reactions were synthesized in an Applied Biosystems DNA synthesizer, Model 381A.

Other Procedures. Protein determinations were performed by the method of Bradford (1976) using bovine serum albumin as standard. SDS–polyacrylamide gel electrophoresis was performed essentially as described by Laemmli (1970). Autoradiography was performed using Kodak X-Omat AR film.

**RESULTS**

Expression and Purification of the Recombinant α and β Subunits of CKII from X. laevis. The cloned cDNA gene coding for the α subunit of Xenopus laevis CKII was introduced into a PT7-7 expression vector and expressed in E. coli as detailed under Materials and Methods. Purification of the X. laevis α subunit from the bacterial extracts by conventional column chromatography on DEAE-Sephadex and phosphocellulose yielded a highly purified enzyme preparation with a main electrophoretic band of approximately M, 42,000 (Figure 1A). This enzyme had a specific activity of 65 units/mg of protein. This specific activity is similar to that of other purified preparations of CKII α subunits reported in the literature (Grankowski et al., 1991; Lin et al., 1991).

Likewise, the cDNA gene coding for the β subunit of X. laevis was expressed in E. coli as a fusion protein using a pGEX-2T expression vector. The results of the purification on GSH–agarose and cleavage of this fusion protein (M, 52,000) by thrombin are shown in Figure 1B. The β subunit hydrolyzed from the fusion protein (M, 26) has six additional amino acids at its amino-terminal end with the sequence GSPGIQMESDEEV... (the amino acids numbered belong to the normal β sequence). The correctness of the predicted sequence and the purity of this subunit preparation was verified by microsequencing the first seven amino acids of the amino-terminal end. The expression and purification procedure yielded approximately 2 mg of β subunit per liter of culture of the transformed E. coli.

Stimulation of α by Wild-Type β and by a Mutant β Lacking the Autophosphorylation Site. Addition of wild-type β subunit to the purified recombinant α increases its catalytic activity 5-fold. As seen in Figure 2, stoichiometric amounts of β are required to yield maximum activation of α.

It has long been known that the β subunit of CKII is autophosphorylated (Hathaway & Traugh, 1979). Recent
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Figure 2: Activation of the α subunit of CKII by subunit β. Activation of CKII α-subunit activity was assayed as described under Materials and Methods, using 8 pmol of the α subunit and varying amounts of β subunit. The basal activity without β (19 pmol/min) has been subtracted from all values. Data analysis was performed using a program based on the algorithm of Marquardt (1963) for nonlinear fitting. The wild-type recombinant β (●) or the mutant β (○), in which serines in positions 2 and 3 have been replaced by glycines, was used.

Figure 3: Autophosphorylation of the CKII α and β subunits and the effect of polylysine. The α (M, 42 000) and β (M, 26 000) subunits of CKII that had been incubated with [γ-32P]ATP in the standard assay mixture but in the absence of casein were analyzed by autoradiography on 10% SDS-polyacrylamide gels; 40 pmol of α subunit or both α and β subunits were used. (A) Incubations contained α subunit (lanes 1 and 2) or α and β subunits (lanes 3 and 4); without additions (lanes 1 and 3) or with 40 μg/mL polylysine (lanes 2 and 4). (B) Incubations contained α subunit and either the mutant β with glycines in positions 2 and 3 (lane 5) or the wild-type β subunit (lane 6). The autoradiography shown in (A) was exposed for 28 h while that for (B) was exposed for 12 h.

work from the group of Krebs (Lichfield et al., 1991) has established that the autophosphorylation site resides in the sequence MS*SS*SEE which is at the amino-terminal end of β. In this sequence, serines-2 and -3 (with asparagines) fit the minimum requirements for CKII phosphorylation. Using PCR mutagenesis, a mutant β-subunit cDNA has been prepared in which the triplets for these two serines have been changed to code for glycines. The mutant β subunit expressed and purified exactly as the wild-type β was tested for its capacity to activate α. The results obtained are shown in Figure 2 and demonstrate that the mutant β is capable of activating α to an extent similar to the wild-type subunit. Variations in the degree of the stimulatory capacity of different preparations of β were between 4- and 5.5-fold activation.

The results presented in Figure 3 are autoradiographs of the CKII subunits incubated with [γ-32P]ATP and subsequently analyzed on SDS-polyacrylamide gels. Figure 3A demonstrates the pronounced phosphorylation of β (M, 26) by the α subunit. It can also be seen that the α subunit (M, 42) is also slightly autophosphorylated and that this phosphorylation is stimulated by the presence of β. Polylysine, a known activator of CKII activity, which apparently operates through β, does not stimulate the autophosphorylation of α when incubated in the absence of β but stimulates moderately the autophosphorylation of β and of α when β is present. In a separate experiment presented in Figure 3B, it is observed that the Gly2-Gly3 mutant of β is not phosphorylated by α, confirming the finding that Ser2 and Ser4 are the only autophosphorylation sites of this subunit.

Interactions of Subunits Attached to a Solid Matrix. Studies of protein-protein interaction can be facilitated by the attachment of one of the protein components to a solid matrix. One of the byproducts of the expression of the β subunit is the fusion protein containing glutathione transferase in its amino-terminal half and the CKII β subunit in its carboxy-terminal end. This fusion protein can interact in solution with α as shown by the fact that it can stimulate its activity almost as much as the free β subunit resulting from thrombin treatment of the fusion chimera. For instance, in experiments in which free β yielded a 4.4-fold stimulation of α, an equimolar amount of fusion protein yielded a 3.2-fold stimulation.

The fusion protein has the useful property of binding with high affinity and specificity to glutathione-Sepharose beads. The possibility was explored, therefore, of using the fusion protein to mediate the attachment of the catalytically active α to these beads. The binding of CKIIα subunit to GSH-agarose—fusion protein is confirmed by detection of the 42-kDa protein together with the fusion protein of 52 kDa upon polyacrylamide gel analysis of the GSH-agarose preparation. These bands are observed despite the thorough washing of the GSH-agarose beads in buffered saline. Figure 4 (lanes 5-8) shows the increment in the 42-kDa protein band obtained upon analysis of increasing amounts of the GSH-agarose preparation. Parallel analysis of aliquots of the same preparation showed proportional amounts of CKII activity; for example, 5 and 10 μL of the bead slurry (as used in Figure 4, lanes 5 and 6) had an activity of 5 and 12 pmol/min, respectively.

The supernatant obtained after incubation of the α subunit and the GSH-agarose fusion protein (S-2 fraction) contained less than 10% of the total kinase activity used, and no protein band was detectable upon gel analysis (lane 2).
Table I: Activity of the α Subunit of CKII Immobilized on Glass Fiber Filters# 

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#The experimental procedures and assay are described under Materials and Methods.

Conversely, it has been observed that the α subunit can bind tightly to glass fiber filters. As shown in Table I, the CKII activity bound to these filters increases proportionately with the concentration of the α subunit originally incubated with the filters.

It is also apparent from this table that the holoenzyme is also tightly bound to the glass fiber matrix and that both the immobilized holoenzyme and α subunit retain the property of being strongly inhibited by heparin. The most important observation presented in this table, however, is the stimulation of the bound α by β subunit. The activation observed is at least as high as that measured with α in solution. Separate experiments have shown that the capacity of β to activate α does not depend on the original concentration of the immobilized α. The activity of the α subunit bound to glass fiber is labile at 37 °C but can be greatly stabilized by the addition of β subunit.

Influence of the β Subunit in the Recognition of Peptide Inhibitors Containing Tyrosine and Glutamic Acid. It has been shown previously that the CKII holoenzyme can be strongly inhibited by random copolymers of glutamic acid and tyrosine (Meggio & Pinna, 1989; Tellez et al., 1990). In Figure 5A, it can be observed that copoly(Glu:Tyr) (4:1) is more than 2 orders of magnitude more potent than polyclutamic acid) or copoly(Glu:Phe) (4:1) in inhibiting the holoenzyme. A similar experiment carried out with the isolated recombinant α subunit is shown in Figure 5B. Although copoly(Glu:Tyr) (4:1) is still considerably stronger than poly-

(glutamic acid), the difference between the potency of the two inhibitors is reduced to 1 order of magnitude.

Using synthetic undecapetides containing two tyrosines and nine glutamic acids, the importance of the position of the tyrosines with respect to the polyglutamic cluster was tested. Figure 6A shows the inhibitory capacity of two such peptides, containing the tyrosines either in the amino end or in the carboxyl end, toward the activity of the CKII holoenzyme. It is evident that the Y2E6 peptide is a significantly better inhibitor than the one with the sequence E2Y6. However, using these same two peptides with the isolated recombinant α subunit, the potency of both peptides is the same (Figure 6B).

DISCUSSION

The bacterial expression and purification of the recombinant α and β subunits of the X. laevis cAMP kinase II are described. Both subunits obtained by this procedure are functional and can reconstitute a fully active holoenzyme when they are incubated in a standard assay mixture in stoichiometric amounts. The β subunit can stimulate about 5-fold the activity of α, which has a basal specific activity which is similar to that reported for recombinant α from human and drosophila (Grankowski et al., 1991; Lin et al., 1991).

The availability of these purified recombinant subunits of CKII opens up the possibility of studying the interaction of these two polypeptides and of exploring the structural features that are important for their biological activity.

It has long been known that the β subunit of CKII was autophosphorylated (Hathaway & Traugh, 1979; Meggio & Pinna, 1984). However, the lack of knowledge of the amino acid sequence of β and the low amounts of the subunit available made it difficult to define the precise autophosphorylation sites and to determine whether this reaction was important for enzyme activity. More recently, the laboratory of Krebs reported experiments that indicated that the principal autophosphorylation sites were serines in positions 2 and 3 of the β subunit and that this site was also phosphorylated in vivo (Litchfield et al., 1991). However, no evidence was presented as to the possible functional effect of this phosphorylation. Using recombinant β subunit, Boldyreff et al (1992) have made deletion mutants that eliminate different parts of the molecule, including an amino-terminal peptide containing the autophosphorylation site. This deleted protein was found to be as active as the wild-type peptide in activating α. Using a different approach, we have specifically mutated serines-2
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and -3 to glycines in the recombinant beta subunit. This mutant completely lacked the capacity to be phosphorylated by alpha, but was active in stimulating the catalytic activity of alpha. These results confirm the identification of the autophosphorylation site and establish that autophosphorylation of beta is not necessary for the stimulatory capacity of this protein.

There have been reports that indicate that phosphorylation of the beta subunit can cause activation of CKII. Ackerman et al. (1990) reported that EGF stimulation of CKII was correlated to the phosphorylation state of the beta subunit. Alkaline phosphatase treatment decreased the CKII level of activity to the basal levels found in untreated cells. However, this work did not identify the sites of phosphorylation in the beta subunit nor the kinases responsible for their phosphorylation. Munler-Lorillon et al. (1990) published the observation that p34<sup>cdc2</sup> kinase from starfish eggs could phosphorylate the beta subunit of CKII from <i>X. laevis in vitro</i> and that this phosphorylation caused an activation of CKII. Lichtfield et al. (1992) have confirmed the phosphorylation of CKII beta by p34<sup>cdc2</sup> and have shown that, both in <i>vivo</i> and <i>in vitro</i>, the site phosphorylated is serine-209 in the human CKII enzyme. This work, however, did not define any effect of this phosphorylation on enzyme activity.

The availability of the recombinant beta subunit will now provide an opportunity for systematic testing of the possible regulatory effects of phosphorylation by other kinases of the other serine, threonine, and tyrosine residues present in this protein.

In this report, data are presented for two methods for the study of the interaction of the CKII alpha and beta subunits. One approach uses a byproduct of the expression of the beta subunit: the fusion protein that contains at its amino end the sequence of glutathione transferase and at its carboxy end the beta subunit of CKII. We observe that this fusion protein has the capacity to interact with alpha and to activate it. Obviously, this fusion protein can use its glutathione transferase moiety to bind to glutathione-Dephasorose beads. These beads, therefore, make it possible to immobilize the chimeric protein and, subsequently, to bind the alpha subunit to this solid matrix. This method could permit the study of the effects of mutations in alpha and beta subunits on the interaction of the two subunits, independent of activity measurements.

Similarly, the stable adsorption of the alpha subunit to glass fiber filters can be useful since the bound subunit is active. Although no information was obtained on the nature of the interaction of this protein with the glass fibers, it was observed that the activity retained on the filters was a function of the concentration of the enzyme used in the incubation with the filters. It was also observed that the binding occurred at 4°C and high concentration of salt (0.7 M NaCl). The activity of immobilized alpha retained its characteristic property of being inhibited by heparin. The most interesting observation made with the alpha subunit bound to glass fiber filters, however, was the fact that it could be activated by beta to a degree similar to that observed in solution.

Conclusive evidence has been presented by several workers that the isolated alpha subunit of CKII is a monomer at salt concentrations ranging from 0.6 to 2 M (Hu & Rubin, 1990b; Lin et al., 1991). When beta is added to alpha, however, the alpha beta tetramer is formed. Presumably, therefore, alpha is immobilized on the glass fiber matrix as a monomer, and it that state, the formation of the tetramer would be impeded. The fact, therefore, that beta can activate immobilized alpha would suggest that tetramer formation is not required for the stimulus of the catalytic activity by beta and that an alpha beta heterodimer might be fully active.

The availability of isolated recombinant alpha subunits permits one to study the effect of the beta subunit on the recognition of protein and peptide substrates and inhibitors. Several reports have recently shown that the relative affinity and specificity of the alpha subunit for protein and peptide substrates are different from that of the tetrameric holoenzyme (Meggio et al., 1992; Grankowski et al., 1991). A striking difference was described in the case of calmodulin which can only be phosphorylated by the holoenzyme in the presence of polylysine; however, calmodulin is readily phosphorylated by isolated alpha in the absence of this polycation.

In this report, the recognition of peptides containing tyrosine and glutamic acid has been studied with the recombinant subunits. As previously shown using the naturally occurring holoenzyme, the presence of tyrosine residues in glutamic acid rich copolymers enhances the inhibitory capacity of the acidic peptide by 2 orders of magnitude. As presented above, the enhancement of the inhibition caused by tyrosine is reduced by 1 order of magnitude when the isolated alpha subunit is used.

It is also interesting that the position of the tyrosine residues within the poly(glutamic acid) cluster is recognized by the holoenzyme. Thus, using synthetic peptides and the holoenzyme, it was demonstrated that an undecapeptide that has two tyrosine residues in the amino-terminal end is a stronger inhibitor than a peptide of the same composition but with the aromatic amino acids at its carboxy end. Similar positional effects were noted for acidic hexapeptides containing tyrosine in relation to their efficiency in inhibiting the CKII holoenzyme from rat liver (Marin et al., 1986); again, the N-terminal position of tyrosine was most effective. The isolated alpha subunit, on the other hand, fails to discriminate between these two structural conformations.

In addition to the interest of this finding for the beta subunit in the recognition of substrate and inhibitory sequences, the result obtained with the holoenzyme is important in itself. The favored position of the tyrosine residues in the amino-terminal side of the polyglutamic cluster is similar to the preferred position of the seryl and threonyl residues in substrate sequences (Meggio et al., 1984). It seems possible, therefore, to postulate that Tyr-X-X-X sequences (where X is an acidic amino acid or a phosphorylated amino acid) may act as pseudosubstrate inhibitors. This would imply that CKII is recognizing tyrosyl residues as related to serine and threonine. The recent finding of dual-specificity protein kinases capable of phosphorylating tyrosine residues as well as serine/threonine (Kosako et al., 1992) may be pertinent to this observation. However, CKII and the dual-specificity kinases do not share sequence similarities other than those sequences common to all protein kinases. The fact that CKII, like tyrosine kinases, can efficiently use Mn<sup>2+</sup> or Co<sup>2+</sup> as well as Mg<sup>2+</sup> (Gatica et al., 1993) also supports the speculation that CKII might be a protein kinase on the evolutionary path that leads to these ambivalent or dual-specificity protein kinases.

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