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Activity of the $E^{75}E^{76}$ mutant of the α subunit of casein kinase II from *Xenopus laevis*

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Abstract

The cDNA gene coding for the α subunit of *Xenopus laevis* casein kinase II was mutated using the overlap extension PCR method. The mutation substituted glutamic acids for Lys^{75} and Lys^{76}, changing the charge distribution of a very basic sequence found in the α subunit. Expression of the mutated cDNA in a pT7–7 vector in *E. coli* yielded an active mutant recombinant protein that was extensively purified. This mutant was not significantly affected in its app. K_m for casein or a model peptide substrate, nor in its interaction with the activating β subunit. Inhibition by quercetin and by 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole was also the same for mutant and wild type subunits. However, the CKII $\alpha E^{75}E^{76}$ mutant was at least one order of magnitude less sensitive to inhibition by polyanionic inhibitors such as heparin, poly U, copolyglutamic acid:tyrosine (4:1) and 2,3 diphosphoglycerate.

Key words: Casein kinase II; Mutated α subunit; *Xenopus laevis*; Heparin; Poly U; Quercetin

1. Introduction

Casein kinase II (CKII) is an ubiquitous eukaryotic protein kinase that plays an important role in the phosphorylation of many proteins that participate in nucleic acid synthesis and in cell proliferation [1–3]. The native CKII is a heterotetramer with an $\alpha_2\beta_2$ or $\alpha\alpha'\beta_2$ structure. The α and α′ subunits have an M_r of 38 to 44 kDa and possess the catalytic activity. The β subunits with M_r between 25–27 kDa serve a regulatory function, increasing 5- to 10-fold the activity of α.

The cDNAs coding for the α and β subunits of CKII in a number of species have been cloned and sequenced [4–8]. These studies have shown that both subunits have been highly conserved throughout evolution.

Studies dealing with the structure and function of CKII are now being carried out by a number of laboratories with the help of site directed mutagenesis and expression of the recombinant mutated proteins in *E. coli* [9,10]. Our laboratory has recently reported the expression of wild type and mutant subunits of the *Xenopus laevis* CKII [11].

Hu and Rubin [5] designed a mutant of the α subunit of CKII of *Caenorhabditis elegans* in which lysines 74 and 75 were substituted by glutamic acid. These authors found that this mutation was catalytically active, showing a very small increase in the apparent K_m for casein. However, they observed that substitution of these lysines for glutamic acids greatly reduced the capacity of heparin to inhibit its catalytic activity.

In this report we present the results obtained with a similar mutant prepared by substituting glutamic acid in the positions corresponding to lysines 75 and 76 in the α subunit of CKII from *X. laevis* (CKII $\alpha E^{75}E^{76}$). These results confirm and extend the previous observation of these authors. The double mutation significantly reduces the inhibition caused by other polyanions such as the copolymer of glutamic acid and tyrosine (ratio 4:1), by poly U and by 2,3-diphosphoglycerate. However, this mutation does not significantly affect the activation of the α subunit caused by the β subunit or the inhibition caused by quercetin or by 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB).

2. Materials and methods

2.1. Preparation of recombinant α and β subunits of CKII from X. laevis

Cloning and sequencing of the cDNA coding for the α and β subunits of CKII of *X. laevis* were described earlier [8]. The insertion of the α subunit cDNA into a pT7–7 expression vector and its expression and the purification of the resulting protein was described in detail by Hinrichs et al. [11]. This same publication reports the expression of the β subunit from a pGEX 2T vector, the purification of the fusion protein by glutatione-agarose beads and the release of the β subunit by thrombin treatment. The purified recombinant proteins prepared by this method are fully active and can reconstitute a functional holoenzyme.

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2.2. Preparation of the CKII $\alpha E^{75}E^{76}$ mutant

The mutant CK $\alpha E^{75}E^{76}$ was prepared from the starting p7-7 clone of the wild type α subunit by the method of 'overlap extension' using the polymerase chain reaction [12]. Two amplifications were made using the original clone as template. The first one used the primer 5'-CCCCTGCCAGATGTCAGGACCTGTG-3', which contains a *Pst*I site and the initial 5 codons of CKII α and the primer 5'-AAGCCTGTGAAG(G)AG(G)AGAAAATTAAGC-3' which contained the double mutant indicated by brackets where G's replaced A's.

For the second amplification the primers used were 5'-GCTTAATTTTCT(C)CT(C)CTTCACAGGCTT-3' which contained the complementary double mutant primer where the bracketed C's have replaced the original T's and 5'-CCCCTGCAGTCATACTGGCGCT-ACT-3' containing a *Pst*I sequence, the stop codon and the last carboxyl end triplets.

The overlapping products of these amplifications were mixed and a third amplification run using the two terminal primers with *Pst*I sites previously described. The resulting mutated full-length cDNA was purified in a 1% agarose gel, treated with *Pst*I and subcloned in the expression vector pT7-7 [13] and introduced into *E. coli* BL 21 (DE3) for preparation of the mutant protein. The full-length cDNA obtained after the mutation was completely sequenced to ascertain that no other sequence alterations had occurred during the process. Purification of the recombinant mutant protein was carried out as described for the wild type subunit [11].

2.3. Assay of CKII activity

Reactions (50 μl) contained 50 mM HEPES, pH 7.8, 150 mM KCl, 7 mM $MgCl_2$, 0.5 mM dithiothreitol, and 100 μM [γ-^{32}P]ATP (500–1000 cpm/pmol). Assays also contained recombinant subunits and 5.0 mg/ml of desphosphocasein or the peptide RRREEETEEE [14] as the protein substrate. The reaction was started by the addition of the enzyme and the incubation was for 10 min at 30°C. It has been determined that under these conditions the reaction is linear for at least 30 min. An aliquot was spotted on a 2 × 1 cm Whatman P81 phophocellulose paper which was then immersed in 75 mM phosphoric acid. The paper was washed 3 times in the same acid, dried and counted. Values reported have been corrected for controls run in the presence of heat denatured enzyme. All assays were performed in duplicate and are representative of two to four experiments.

2.4. Materials

Oligonucleotides were purchased from the Centro de Síntesis y Secuenciación de Biomoléculas of the University of Chile.

Copoly (Glu:Tyr) (4:1) (M_r 46,000), quercetin, DRB, human thrombin, glutathione-agarose beads, polyuridylic acid, 2,3-diphosphoglycerate were obtained from Sigma Chemical Co.

3. Results

The *X. laevis* CKII $\alpha E^{75}E^{76}$ subunit expressed in *E. coli* is catalytically active. Its apparent K_m for casein was calculated to be 4.5 mg/ml, in comparison with an apparent K_m value of 2.5 mg/ml for the wild type subunit (CK α_{WT}). The app. K_m for the model peptide RRREEETEEE was 0.23 mM for the CK α_{WT}, while for CK $\alpha E^{75}E^{76}$ the value was 0.54 mM (not shown).

Fig. 1 shows the effect of adding increasing concentrations of β on the activity of the wild type and mutant α subunits. It is clear that there is no major difference in the concentration of β subunit required to interact with the wild type and mutant α subunits and that the degree of activation attained is also similar.

As reported by Hu and Rubin [5], these mutations greatly reduce the capacity of heparin to inhibit its catalytic activity. In the case of *X. laevis* enzyme, the I_{50} value

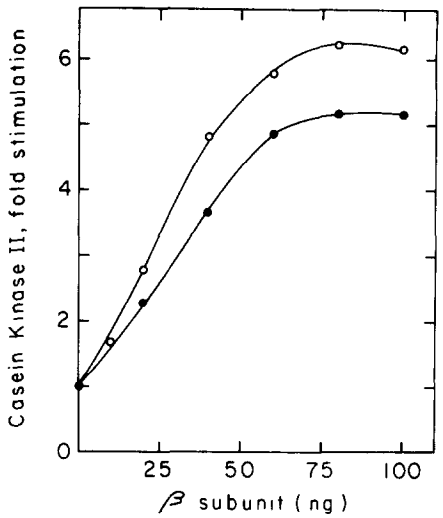

Fig. 1. The activation of the wild type and mutant α subunits by different concentrations of recombinant β. Different amounts of recombinant β subunit were added to the reaction mixture containing 48 ng of CKII α wild type (●) and to 50 ng of CKII $\alpha E^{75}E^{76}$ (○) and the kinase activity with casein as substrate was assayed as detailed in section 2. The activity of CKII α_{WT} without β was 21.0 pmol of ^{32}P incorporated into casein and for CKII $\alpha E^{75}E^{76}$ without β it was 28.5 pmol.

is increased from 0.30 μg/ml for the wild type to 4 μg/ml for the mutant (Fig. 2A).

The effect of other inhibitors was also tested. Fig. 2B shows the effects of poly U and Fig. 3A of the copolymer glutamic acid:tyrosine (ratio 4:1) on the activity of the wild type and mutant α subunits. It is evident that the mutations also affect very significantly the capacity of these other polyanions to inhibit the enzyme. Reduction in the I_{50} values of Poly U from 8 μg/ml to 125 μg/ml and from 0.025 μM to 0.2 μM for copolyglu:tyr were observed.

Inhibition by 2,3-diphosphoglycerate was also tested and again the mutant α subunit was an order of magnitude less susceptible to inhibition by this compound (not shown).

Fig. 3B shows the inhibition of the activity of the wild type and mutant α subunits by quercitin, a polycyclic flavone that is not polyanionic in nature. In this case, there is no difference in the inhibition of the two α subunits. Similar results were obtained with the nucleotide analog 5,6 dichloro-1-α-D-ribofuranosyl benzimidazole (not shown).

4. Discussion

The generation of site-directed mutations in the recombinant subunits of casein kinase II permits the study of some structure-function relationships in this enzyme. Comparison of the amino acid sequences that are not part of the conserved catalytic domains of different

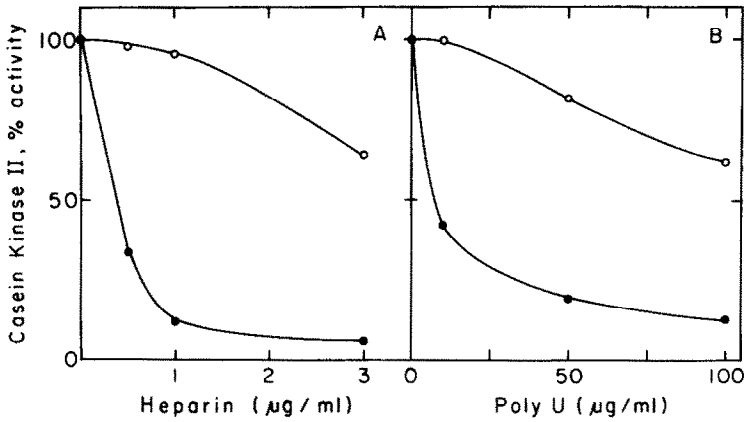

Fig. 2. The effect of heparin and poly U on the activity of CKII α_{WT} and CKII $\alpha E^{75}E^{76}$. The activity of CKII α_{WT} (●) and of CKII $\alpha E^{75}E^{76}$ (○) were measured in the presence of different concentrations of heparin (A) or polyuridylic acid (B). The activity assay was as described in section 2. In A, 100% activity was 16.5 and 15.7 pmol of ^{32}P incorporated for α_{WT} and $\alpha E^{75}E^{76}$, respectively. In B the 100% values were 8.8 and 7.7 of ^{32}P incorporated for the fractions in the same order.

kinases has led to the postulation that the highly basic region of CKII α that stretches from amino acid 71 to amino acid 78 may be involved in the interaction with the acidic regions that are present in CKII substrate proteins (1 or 2). Presumably this same region would also be responsible for interaction with polyanionic inhibitors and with the acidic clusters present in the β subunit.

The results presented confirm and extend those reported by Hu and Rubin [5] who had prepared a similar mutant of the α subunit of CKII of *C. elegans* in which Lys^{74} and Lys^{75} were substituted with glutamic acid. These authors only tested this mutant with regards to its app. K_m for casein and for its I_{50} by the potent inhibitor, heparin. Our results agree with the fact that the mutant shows a small increase in the apparent K_m for casein and for a model peptide substrate while there is a large reduction in the inhibitory potency of heparin. It is clearly demonstrated, in addition, that the mutation affects the interaction of α with other polyanionic inhibitors such as poly U, copolyglu:tyr (4:1) and 2,3 phosphoglycerate, while the activity of non polyanionic inhibitors is not affected by this mutation.

The fact that the app. K_m for the casein is not significantly affected by the mutation is unexpected because it was thought that the same region of the enzyme was responsible for its interaction with the acidic sequences of the substrate as with the polianionic inhibitors. The results obtained would argue that the enzyme-substrate interaction is influenced by structural features that go beyond the ionic interactions of this charged region of the molecule. Similarly, the interaction with the β subunit is not affected significantly by the mutation indicating that the interaction with the regulatory subunit also involves other aspects of its structure. Previous studies from this laboratory [15,16] have indicated that in addition to their acidic character, the presence of aromatic structures such as those found in copolyglu:tyr peptides, in nucleic acids and in polyglutamic derivatives of folic acid considerably influence their affinity for CKII.

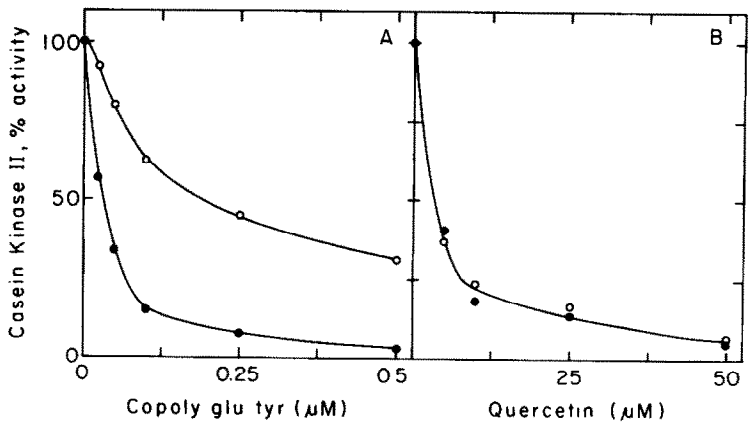

Fig. 3. The effect of copolyglutamic acid:tyrosine (4:1) and of quercetin on the activity of α_{WT} and CKII $\alpha E^{75}E^{76}$. The activity of CKII α_{WT} (●) and CKII $\alpha E^{75}E^{76}$ (○) was assayed in the presence of increasing concentrations of the random copolyglutamic acid:tyrosine in a ratio of 4:1 (A) and of quercetin (B). The 100% activities in (A) were 7.1 and 7.0 pmol, and in (B) 8.8 and 7.8 pmol of ^{32}P incorporated into casein for α_{WT} and $\alpha E^{75}E^{76}$, respectively.

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