Copolymers of glutamic acid and tyrosine are potent inhibitors of oocyte casein kinase II

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Polypeptides rich in glutamic acid are strong inhibitors purified from isolated nuclei of *Xenopus laevis* oocytes of casein kinase II. The presence of tyrosine in these peptides greatly enhances their inhibitory capacity. Using cascin as a substrate, copolyglu:tyr (4:1) has an I_{50} value of 20 nM, 250 fold lower than that of polyglutamic acid which is 5 μM. A similar large difference is observed when a synthetic peptide is used as substrate. The inhibition of copolyglu:tyr is competitive with casein and can be completely reversed by high ionic strength. The relative inhibitory capacity of the polypeptides tested, in descending order, is copolyglu:tyr (4:1) > copolyglu:tyr (1:1) > polyglu > copolyglu:phe (4:1) > copolyglu:ala (6:4) > copolyglu:leu (4:1). The high affinity for tyrosine-containing acid peptides is shared by rat liver and yeast casein kinase II so that it seems to be a general property of these enzymes.

Casein kinase II; *Xenopus laevis*; Oocyte; Nucleus; Polyglutamic acid; Protein kinase

1. INTRODUCTION

Casein kinase II (CK-II) is a messenger-independent protein kinase that is present in the cytoplasm and nuclear fractions of many cell types (for review see [1]). Although the mechanism for the regulation of this enzyme is not known, its activity can be increased significantly by treatment of the cells with hormones, growth factors or phorbol esters [2,3]. Additional interest in this enzyme arises from the fact that it has been shown to phosphorylate several key proteins involved in the replication and transcription of nucleic acids: RNA polymerases I and II [4,5], DNA topoisomerase II [6], transcription factor S-II [7]. It may also be relevant that several nuclear oncogenes (myc, fos, jun, SV-40 T antigen, and the adenovirus E1A protein) have amino acid sequences that are typical of those phosphorylated by CK-II [3,8].

The groups of Pinna and Krebs have analyzed the sequences phosphorylated by CK-II and have used synthetic peptides to define the specificity constraints of this enzyme [9,10]. These studies have indicated that CK-II phosphorylates either serines or threonines that are followed, towards the carboxyl end, by a cluster of acidic amino acids or by amino acids previously phosphorylated by other kinases.

The specificity of this enzyme for polyanionic peptide substrates explains its acute sensitivity to heparin inhibition which is competitive with the protein substrate [11]. Pinna and his collaborators have made an extensive study of the competitive inhibition of CK-II by synthetic acidic peptides, as well as by polyaspartic and polyglutamic acids [9,12]. However, recent observations that some specific nucleic acids are also potent inhibitors of this enzyme while others are not inhibitory indicate that the polyanionic character of a polymer is not the sole characteristic that determines its affinity for the enzyme [13].

CK-II is the most abundant protein phosphorylating activity present in the nuclei of the oocytes of *Xenopus laevis* [14], and it has been implicated in the process of the meiotic maturation of these cells [15]. In the present study we have used CK-II purified from the nuclei of *X. laevis* oocytes to determine the inhibitory effect of several synthetic mixed polypeptides containing high proportions of glutamic acid. A dramatic increase in the inhibitory capacity of these peptides is observed when tyrosine is included in their structure. When this work was being completed, a very recent publication by Meggio and Pinna [16] was brought to our attention. These researchers obtained similar results with rat liver CK-II. Our work, therefore, confirms and extends their observations and indicates that the high affinity for copolymers of glutamic acid and tyrosine is a general property of CK-II purified from different sources.

2. MATERIALS AND METHODS

Ovaries were obtained by surgery from adult female *X. laevis*. Stage 5 and 6 oocytes and nuclei were prepared as described by Burzio and Koide [17]. Highly purified oocyte CK-II was prepared from isolated *X. laevis* oocyte nuclei by chromatography on DEAE-Sephadex [18] and phosphocellulose [14] as described originally by Hathaway and Traugh [19]. Yeast CK-II was kindly donated by H.

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Sternbach (Max Planck Institute). CK-I was purified from oocyte nuclear extracts using DEAE-Sephadex chromatography [18]. cAMP dependent protein kinase was obtained from Sigma.

Amino acid polymers and copolymers are denoted by the component amino acid, their molar ratio in parentheses when pertinent, and the average relative molecular weight as determined by viscosity and reported by the manufacturer. Polymers purchased from Sigma were the sodium salts of polyglutamic acid, M_r 43,000; polyaspartic acid, 42,500; copolyglu:tyr (4:1), 46,000; copolyglu:phe (4:1), 16,000; copolyglu:leu (4:1), 10,000; copolyglu:ala (6:4), 30,000; copolyglu:lys:tyr (6:3:1), 23,000; copolyglu:ala:tyr (6:3:1), 25,000. Copolyglu:tyr (1:1), M_r 12,500; was prepared and kindly donated by Dr. M. Sela (Weizmann Institute). The synthetic oligopeptide used as substrate, Arg-Arg-Arg-Glu-Glu-Glu-Thr-Thr-Thr-Glu-Glu-Glu-Glu (RRREEETTTEEEE), was kindly donated by Dr. R. Bravo, then at EMBL. Polymers and synthetic peptides were dissolved in distilled water with the exception of the copolymers copolyglu:phe (4:1) and copolyglu:leu (4:1) which were dissolved in NH_4OH at pH 9 and the pH readjusted to 7.8 with the assay buffer.

[γ-^{32}P]ATP was prepared by the method of Walseth and Johnson [20] using [^{32}P] from Amersham. Dephosphorylated casein used as substrate in assays was prepared as described [19].

Casein kinase II assay: Reactions (50 μl) contained 50 mM Hepes, pH 7.8; 180 mM KCl, 7 mM $MgCl_2$, 0.5 mM dithiothreitol, and 100 μM [γ-^{32}P]ATP (500–1000 cpm/pmol). All assays contained 20–30 units/ml of CK-II [14] and either 5.0 mg/ml dephosphocasein or 3.5 mM synthetic peptide substrate, as indicated. All additions were made prior to the addition of the enzyme. Incubation times were routinely 15 min at 30°C and the reaction was linear for at least 30 min. The reaction was terminated by the removal of an aliquot to a 2×1 cm Whatman P81 phosphocellulose paper which was immersed in 75 mM phosphoric acid, washed 3 times in the same acid, dried and counted. Values reported have been corrected for controls run with heat-denatured enzyme. All assays were performed in duplicate and are representative of 2–4 experiments.

3. RESULTS

The results presented in Fig. 1 show the inhibition of oocyte CK-II by different polypeptides rich in glutamic acid. In Fig. 1A, the oocyte CK-II is assayed using casein as substrate. The enzyme is strongly inhibited by micromolar concentrations of all of these polymers. It is observed, however, that the introduction of tyrosine residues into the copolymer at a ratio of glu:tyr of 4:1 causes a dramatic enhancement of the inhibitory capacity. This copolymer is 250 times more efficient as an inhibitor when compared to polyglutamic acid of similar molecular weight. With a higher percentage of tyrosine, as in the copolyglu:tyr (1:1), the enhancement of inhibition is not as great, being only 4 fold more potent than polyglutamic. The presence of phenylalanine rather than tyrosine residues as in the polymer copolyglu:phe (4:1) results in an inhibitor which is less efficient than both polyglu:tyr (1:1) and polyglutamic acid. The introduction of alanine into a copolymer with a ratio of glutamic to alanine of 6:4 likewise decreases the inhibitory capacity of the polyanion (8 fold below that of polyglutamic).

It has been previously demonstrated that CK-II can use synthetic model peptides as substrates [9,12]. Such a peptide, with the sequence Arg-Arg-Arg-Glu-Glu-Glu-Thr-Thr-Thr-Glu-Glu-Glu-Glu, has been tested with the oocyte CK-II and found to be a substrate with

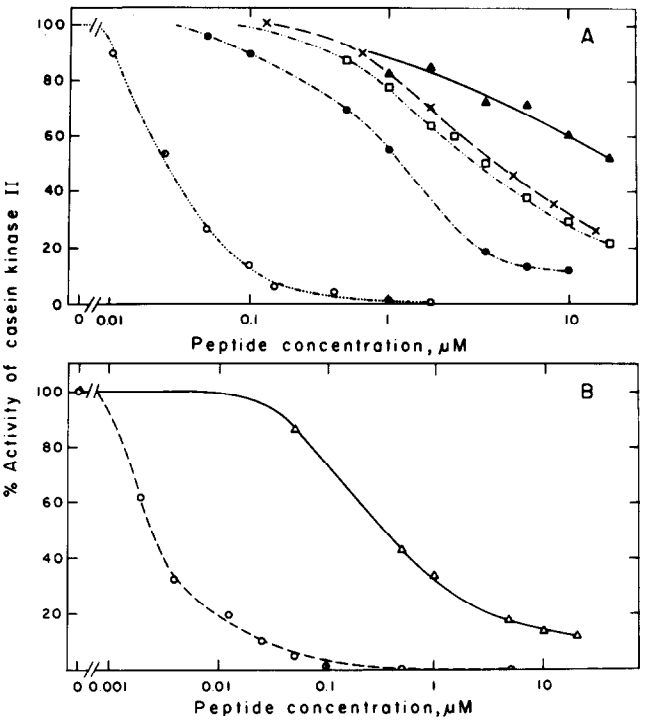

Fig. 1. Inhibition of oocyte nuclear CK-II activity by polymers rich in glutamic acid. (A) Assays were carried out using purified CK-II from oocyte nuclei as described in Materials and Methods with 5 mg/ml casein as substrate in the presence of the sodium salts of polymers of average M_r as indicated: polyglutamic acid, M_r 43,000 (□); copolyglu:ala (6:4), M_r 30,000 (▲); copolyglu:phe (4:1), M_r 16,000 (×); copolyglu:tyr (1:1), M_r 12,500 (●); copolyglu:tyr (4:1), M_r 46,000 (○). (B) Assays were carried out as in A except with the synthetic peptide substrate RRREEETTTEEEE at 3.5 mM and in the presence of the sodium salts of the polymers: polyglutamic acid, M_r 43,000 (△); copolyglu:tyr (4:1), M_r 46,000 (○).

a K_m of 0.33 mM (data not shown). In Fig. 1B, results are presented showing the effect of the copolyglu:tyr (4:1) and polyglutamic acid on the phosphorylation of this synthetic peptide. Again, the inhibitory effect of the mixed polymer is more than two orders of magnitude greater than that of the homopolymer, with I_{50} of 2 nM and 500 nM, respectively.

The nature of the inhibition of the copolyglu:tyr (4:1) was studied using casein as substrate. Fig. 2 demonstrates that the mixed polymer is competitive with the substrate casein and has a apparent K_i of 17 nM. We also observed competitive inhibition by polyglutamic acid with an apparent K_i of 3.8 μM (data not shown).

Table I presents the I_{50} of these polymers and of several others polypeptides analyzed. It can be seen that as has been observed with rat liver CK-II [9] the oocyte enzyme is more sensitive to polyaspartic acid than to polyglutamic acid. It is also evident that the presence of a positively charged side chain does not greatly reduce the affinity of the copolymer as seen by comparing the I_{50} values of copolyglu:lys:tyr (6:3:1) with that of copolyglu:ala:tyr (6:3:1).

Pure CK-II obtained from yeast was also tested with

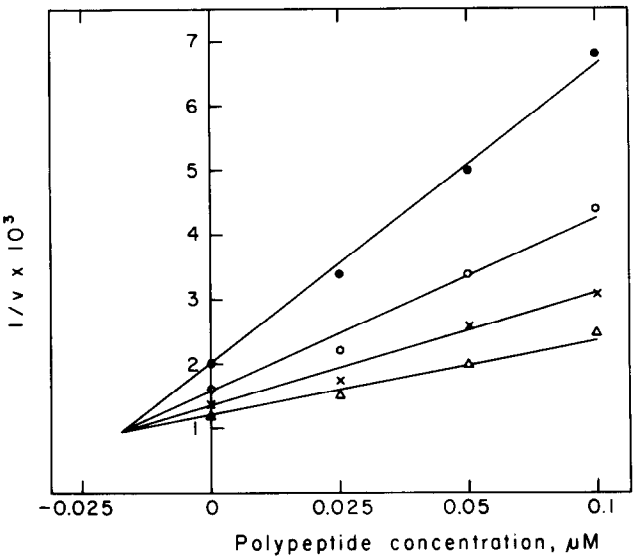

Fig. 2. Inhibition of oocyte CK-II phosphorylation of casein by copolyglu:tyr (4:1). Casein concentrations used were 2 (●), 4 (○), 6 (×) and 10 (△) mg/ml in the standard assay system, in the presence of varying concentrations of copolyglu:tyr (4:1), M_r 46,000. An apparent K_i of 0.017 μM was determined.

polyglutamic acid and copolyglu:tyr (4:1). The results were similar to those obtained with the amphibian enzyme in that the tyrosine-containing polymer was two orders of magnitude stronger as inhibitor than polyglutamic acid (results not shown). On the other hand two other protein kinases, CK-I from *X. laevis* oocyte nuclei [18] with casein as substrate, and commercial cAMP-dependent protein kinase from rabbit muscle with mixed histones as substrate, were tested with copolyglu:tyr (4:1). Concentrations of this copolymer that give complete inhibition of CK-II (3 μM), did not inhibit these other kinases (results not shown).

CK-II activity is highly dependent on ionic strength [19], reaching an optimum at 180 mM NaCl under our assay conditions. The effect of ionic strength on the capacity of both polyglutamic acid and copolyglu:tyr (4:1) to inhibit the amphibian CK-II was tested by increasing concentrations of NaCl. With both polymers inhibition is reverted by high salt. As seen in Fig. 3, levels of polymers which give 60% inhibition at or below optimal salt concentrations are not inhibitory over 250 mm NaCl.

Table I

Inhibition of CK-II by different polyanionic polymers

Polymer	M_r	I_{50}	
		μg/ml	μM
Polyaspartic acid	42,500	11	0.25
Polyglutamic acid	43,000	215	5
Copolyglu:tyr (4:1)	46,000	0.9	0.02
Copolyglu:phe (4:1)	16,000	102	6.4
Copolyglu:leu (4:1)	10,000	800	80
Copolyglu:tyr (1:1)	12,500	16	1.3
Copolyglu:ala (6:4)	30,000	1200	40
Copolyglu:ala:tyr (6:3:1)	25,000	250	10
Copolyglu:lys:tyr (6:3:1)	23,000	575	25

I_{50} is defined as the concentration required to give 50% inhibition of the CK-II activity, assayed under standard condition given in Materials and Methods. In these assays casein was used as substrate.

4. DISCUSSION

The results presented above demonstrate that the presence of tyrosine in glutamic acid-rich random copolymers greatly increases the inhibitory capacity of these polymers towards oocyte CK-II. The copolymer polyglu:tyr (4:1) has an I_{50} of 22 nM, and is approximately 250-fold more efficient than polyglutamic acid. The relative efficiency of these two polymers as inhibitors is the same for the phosphorylation of the protein substrate casein and a specific synthetic peptide substrate.

The finding that the inhibition caused by both polyglutamic and copolyglutyr is competitive with the substrate probably explains the finding that the concentration of either inhibitory copolymer required for 50% inhibition with the synthetic peptide substrate is one order of magnitude lower than that observed when casein is used as substrate. Synthetic peptides have been shown previously to have higher apparent K_m values as compared to protein substrates for CK-II [12].

Two additional copolymers of glu:X (4:1) ratio were tested and it was seen that the substitution of phenylalanine or leucine for the tyrosine moiety greatly reduced the inhibitory capacity of the polyanion. In order to obtain a closer approximation of the relative effectiveness of these three compounds as inhibitors, independent of the degree of polymerization of the polypeptides which is known to affect the affinity for the enzyme [12], the I_{50} values were calculated on the basis of the monomer unit, $glu_4:X_1$. (The difference in the

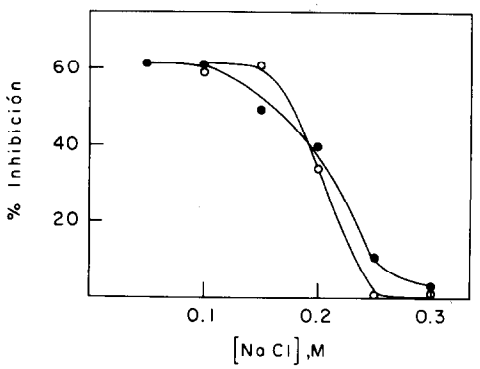

Fig. 3. Reversal of inhibition of oocyte CK-II activity by polyglutamic acid and copolyglu:tyr (4:1) at increasing salt concentrations. Polymer concentrations used were those required to give 60% inhibition of CK-II in the standard assay system (at 180 mM NaCl) and were 35 nM for copolyglu:tyr (4:1) (●) and 8.5 μM for polyglutamic acid (○). Enzyme activity was then determined in the presence and absence of the polymers at each salt concentration.

monomer molecular weight due to the contribution of the varied amino acid is minimal.) On this basis, the midpoints of inhibition for the three copolymers glu:tyr, glu:phe, and glu:leu are 1.3, 153 and 1263 μM, respectively, clearly demonstrating the notable effect of the tyrosine residue. The relative amount as well as the nature of the nonpolar amino acid in the polypeptides appears to be important in determining their inhibitory efficiency. Copolyglu:tyr (1:1) is less effective than the 4:1 copolymer of the same composition.

The fact that copolyglu:tyr (4:1) is also a stronger inhibitor of yeast CK-II than polyglutamic acid and a previous similar observation with the rat liver CK-II [16] indicate that the effect of tyrosine on the affinity is a general property of CK-II. However, the finding that this same polymer under similar conditions does not inhibit oocyte CK-I or rabbit cAMP-dependent protein kinase demonstrates that this phenomenon does not apply to all other protein kinases.

The observation that the introduction of tyrosine or other nonpolar amino acids into polyglutamic acid copolymers greatly enhances the inhibitory capacity of these polyanionic molecules was made by Sela and his collaborators more than 25 years ago, when a similar phenomenon was described for the inhibition of ribonuclease, trypsin and lysozyme by polyglutamic acid and copolymers rich in this amino acid [21,22]. These authors interpreted their results by postulating that the large aromatic or aliphatic hydrophobic side chains of tyrosine, phenylalanine or leucine interspersed among the negative charges of the glutamic acid residues could greatly favor the interaction of these polymers with many basic enzymes, and that both electrostatic and hydrophobic forces are important in the inhibitory process. In the present case, there seems to exist an additional factor related to the specificity of the active site of CK-II since tyrosine residues are clearly different from phenylalanine and leucine in their potentiation of the inhibition of this enzyme by polyanionic peptides. However, electrostatic properties of these polyanions indeed must play a role in their interaction with the enzyme since high ionic strength can completely reverse their effect.

The fact that copolymers of glutamic acid and tyrosine are very potent inhibitors of CK-II is very suggestive in view of the fact that these same copolymers are substrates of tyrosine kinaseas [23]. In fact, of the synthetic tyrosine polymers tested as substrates for insulin receptor kinase the most efficient was found to be copolyglu:tyr (4:1) [23]. The best characterized tyrosine kinases are the membrane-bound enzymes that are involved in transducing external mitogenic signals. Many of the membrane-located proteins that are products of oncogenes have tyrosine kinase activity, but the identity of the substrates that they phosphorylate and that are responsible for relaying the proliferative signal to the nucleus has remained elusive. It seems possible, therefore, that proteins containing tyrosines imbedded in highly acid sequences may provide a link between the membrane-bound tyrosine kinases and nuclear protein kinases which, like CK-II, may be involved in phosphorylating nuclear oncogenes. It would be of considerable interest to determine whether phosphorylation of the tyrosine residues present in copolyglu:tyr peptides or in natural proteins with similar sequences influences their affinity for CK-II.

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