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Folylpolyglutamate analogs can inhibit casein kinase II from *Xenopus laevis*

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Polyglutamate analogs of folate and related compounds were tested as inhibitors of casein kinase II (CK II) obtained from *Xenopus laevis*. The inhibitory capacity of the pteroyl, 4-amino-10-methyl pteroyl (the methotrexate aromatic moiety), and *p*-aminobenzoil derivatives increased as the number of γ-glutamates attached went from 2 to 7. The nature of the aromatic head group was also important since hexa-γ-glutamatic acid had no inhibitory activity while the folylhexaglutamate derivatives were strong inhibitors with relative potency of methotrexate > pteroyl > *p*-aminobenzoic acid. The inhibition of CK II by methotrexate γ-pentaglutamate was competitive with casein and showed an apparent K_i of 90 μM.

Folylpolyglutamate; Methotrexate; Casein kinase II; *Xenopus laevis*; Pteroylpolyglutamate

1. INTRODUCTION

Casein kinase II (CK II) is a ubiquitous eukaryotic protein kinase that phosphorylates serine or threonine in the acidic regions of proteins. This enzyme has been shown to phosphorylate many key enzymes involved in cell division such as DNA and RNA polymerases, topoisomerases, and the products of nuclear oncogenes such as myc, myb, jun and fos (for reviews see [1,2]). In addition, CK II appears to be regulated during the cell division cycle and by factors that induce proliferation [3,4].

The work of the laboratories of Pinna [5] and Krebs [6] has demonstrated that CK II phosphorylates protein sequences in which the acceptor serine or threonine is followed towards the carboxyl end by several acidic residues such as aspartic or glutamic acids or previously phosphorylated amino acids. The affinity of CK II for these polyanionic sequences explains the very potent inhibitory effect of heparin [7], nucleic acids [8,9], and polyglutamic and polyaspartic peptides [10] on this enzyme.

However, the polyanionic nature of the compounds is not the only factor that influences the potency of CK II inhibitors. Recent evidence has demonstrated that the presence of the phenolic side chain of tyrosine in copolymers of glutamic acid and tyrosine greatly enhances the capacity of these compounds to inhibit CK II [11,12].

For a number of years it has been known that the important coenzyme tetrahydrofolate is present in the cell as a mixture of polyglutamated derivatives that contain an average of 4 to 7 residues of glutamic acid attached to the tetrahydrofolate moiety by amide bonds involving their γ-carboxyl group [13].

The demonstration that these polyglutamate derivatives constitute the true physiological substrates of the enzymes involved in one-carbon metabolism [14] has added importance to the study of these compounds. In addition, the finding that methotrexate, a well-known cancer chemotherapy drug used to block folate functions, is also polyglutamated in the cell [14], has also increased the interest in determining the effects that these derivatives might have on cellular metabolism.

In this communication we report that folate and methotrexate γ-polyglutamates can strongly inhibit CK II purified from *Xenopus laevis*. The inhibitory capacity of these compounds is influenced both by the length of the γ-glutamate substitution as well as by the nature of the folate analog moiety that is chemically attached to these polyanionic derivatives.

2. MATERIALS AND METHODS

2.1. *Preparation of casein kinase II*

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 [15]. Highly purified oocyte CK II was isolated from *X. laevis* oocyte nuclei by chromatography on DEAE-Sephadex and phosphocellulose columns [16].

2.2. *Assay of the activity of casein kinase II*

Reactions were carried out in a final volume of 50 μl containing 50 μM 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). In addition, the assays included purified CK II (20–30 U/ml [16]), 5.0 mg/ml of des-

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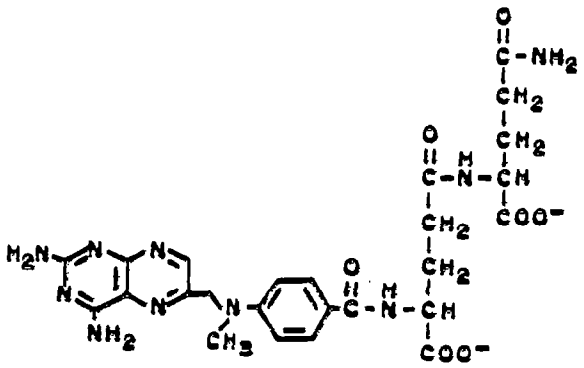

Fig. 1. The structure of 4-amino-10-methyl-pteroyl-di-γ-L-glutamatic acid (metnotrexate mono-γ-glutamate).

phosphorylated casein and the specified amount of the folate analog inhibitor. The reaction was started by addition of the enzyme and incubations were carried out at 30°C for 10 min. The reaction was terminated by 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.

2.3. *Materials*

All folate-polyglutamate derivatives and the hexa-γ-glutamate were purchased from B. Schircks Laboratories, Switzerland. All the other materials were obtained from Sigma Chemical Co.

3. RESULTS AND DISCUSSION

Fig. 1 shows the structure of 4-amino *N*-10-methylpteroyl di-γ-L-glutamate as an example of the structure of the compounds used in these studies.

The results presented in Fig. 2 show the rise in the potency in the inhibition of *X. laevis* CK II by methotrexate derivatives that contain increasing numbers of γ-glutamic acid residues. It is clear from these results that the elongation of the γ-glutamate polymer significantly raises their inhibitory capacity. The I_{50} value of the heptaglutamate derivative is approximately 5-fold lower than that of the diglutamate compound (0.36 mM vs. 2 mM).

Similar experiments were performed with pteroyl and *p*-aminobenzoyl derivatives containing different lengths of γ-polyglutamate chains (not shown). The results obtained were qualitatively similar in the sense that an increasing number of glutamates resulted in stronger inhibitory compounds.

However, there are some interesting differences in the inhibitory capacity depending on the nature of the folate analog head group. Fig. 3 shows a comparison of the effect on the activity of CK II of three different derivatives all containing hexa-γ-glutamate chains. The figure also includes the effect of hexa-γ-glutamate itself. Analysis of these results demonstrates that hexa-γ-glutamate has no detectable inhibitory capacity, a finding

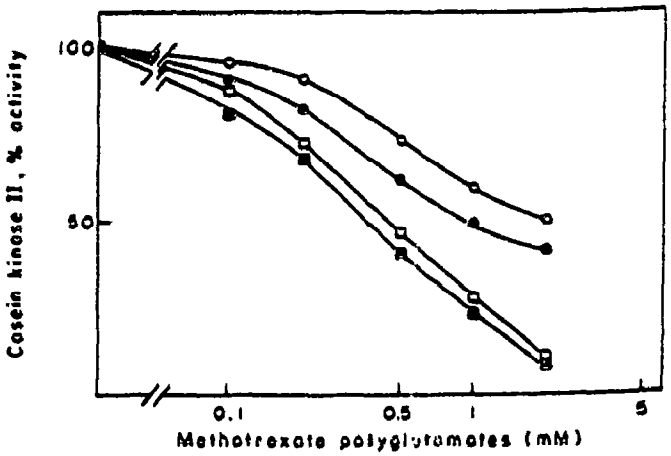

Fig. 2. The inhibitory effect on CK II of various concentrations of methotrexate polyglutamates with different numbers of γ-glutamate residues. The activity of CK II was measured as described in section 2. The value for 100% activity in the absence of inhibitor was approximately 32,500 cpm of ^{32}P incorporated into casein in 10 min which corresponds to 4.06 pmol/min incorporated. The symbols correspond to derivatives of methotrexate containing: (○) di-γ-glutamate; (●) tri-γ-glutamate; (□) hexa-γ-glutamate and (■) hepta-γ-glutamate.

which is in agreement with the report by Pinna's laboratory on the very low inhibitory capacity of penta-α-glutamates [10]. The aromatic head group therefore plays a key role in determining the inhibitory capacity of the hexaglutamate with the relative potency of the compounds being: 4-amino *N*-10 pteroyl > pteroyl > *p*-aminobenzoyl.

The mechanism of inhibition of the methotrexate with 5 additional γ-glutamates was assayed by analyzing its effect on the kinetic parameters of the enzyme by

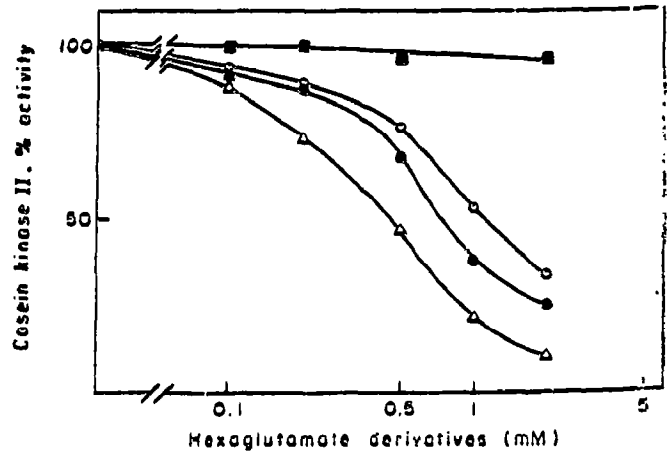

Fig. 3. The inhibitory effect on CK II of different concentrations of hexa-γ-glutamates. The assay of CK II was carried out as described in section 2. The value of 100% activity in the absence of inhibitor was approximately 30,500 cpm of ^{32}P incorporated into casein in the 10 min reaction time. This corresponds to 3.8 pmol/min incorporated. The inhibitors tested were: (■) hexa-γ-glutamate; (○) *p*-nitrobenzoyl-hexa-γ-glutamate; (●) pteroyl-hexa-γ-glutamate; and (△) 4-amino-10-methyl-hexa-γ-glutamate.

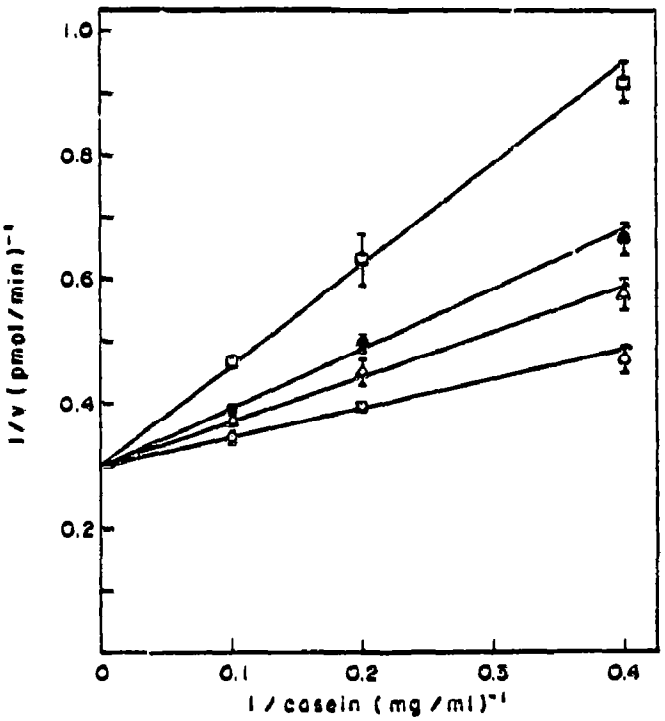

Fig. 4. The effect of methotrexate penta-γ-glutamate on the velocity of the CK II reaction measured with different casein concentrations. The assays were carried out as described in section 2 except for the specified casein concentrations. The amount of the methotrexate penta-γ-glutamate (4-amino-10-methyl-pteroyl-hexa-γ-glutamate) used in each curve was as follows: (○) none; (△) 50 μM; (●) 100 μM; (□) 200 μM. The standard deviation of the values is indicated.

using different concentrations of casein as a substrate. The results obtained are shown in Fig. 4. It is clear that this methotrexate derivative is a competitive inhibitor of casein, a protein substrate of casein kinase II. The apparent K_i calculated from these studies is of 90 μM, while similar studies carried out with *p*-aminobenzoyl-tri-γ-glutamate (not shown) produced an apparent K_i of 1.7 mM. These inhibitory activities can be compared with those of synthetic ordered peptides such as tyr · tyr · $(glu)_{n=9}$ which has an apparent K_i of 0.15 mM (Tellez et al., in preparation).

The results presented above have demonstrated that naturally occurring and pharmacologically active folate polyglutamate derivates have a significant capacity to inhibit CK II. This capacity is influenced both by the nature of the aromatic head group as well as by the length of the γ-polyglutamate chain. The influence of the substituted benzene ring and polycyclic structures of these derivatives is noteworthy in relation to the previous findings that the presence of tyrosine greatly increases the inhibitory capacity of polyglutamic acid peptides [11,12].

The physiological relevance of this inhibitory capacity cannot be established by the present study. The concentrations of the polyglutamates required for significant inhibition in the in-vitro assays used here is an order of magnitude higher than the concentrations of the folate derivatives known to exist in mammalian cells which are of the order of 15–35 μM [17]. These latter values agree well with those found for methotrexate taken up by cells in culture [18]. However, since these assays were carried out with high concentrations of casein and the inhibition is competitive, it is possible that physiological protein substrates of CK II might be at low enough concentrations to achieve significant inhibition by the folylpolyglutamates present in cells. It is also known that an important fraction of these folate derivatives is bound to dehydrofolate reductase under in-vivo conditions [19]. It is possible that the protein-bound polyglutamate derivatives may have considerably greater inhibitory power for CK II than the free compounds that we have studied.

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