Polyglutamyl peptides: a new class of inhibitors of type-2 casein kinases

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Casein kinase-TS (Ck-TS), a type-2 casein kinase purified from rat liver cytosol which phosphorylates seryl and threonyl residues N-terminal to acidic clusters, is specifically inhibited by polyglutamyl peptides which are ineffective both on type-1 casein kinase and on cAMP-dependent protein kinase. The inhibition is competitive toward the protein substrate and non-competitive toward ATP. Among the polyglutamates tested $(Glu)_{70}$ is the most effective (K_i 0.11 μM). $(Glu)_{10}$ and $(Glu)_5$ are also inhibitors, though less powerful than $(Glu)_{70}$, while $(Glu)_3$, $(Glu)_2$ and free glutamic acid up to 5 mM are ineffective. These results disclose the possibility that naturally occurring polypeptides containing long stretches of acidic residues may act as physiological inhibitors of type-2 casein kinases.

Casein kinase-2 *Protein kinase* *Protein phosphorylation* *Polyglutamate* *Phosphorylation site* *Inhibitors of protein kinases*

1. INTRODUCTION

Casein kinases are multifunctional and widespread protein kinases, preferring casein and phosvitin over histones as model substrates, and not sensitive either to the cyclic nucleotides or to any other known effector of protein kinases (review [1]). Consequently the precise physiological role(s) and the regulatory mechanisms of such enzymes remain to be elucidated.

According to their different structure and specificity casein kinases can be grouped into two different classes: type-1 casein kinases, termed also S or A, are monomeric enzymes affecting only seryl residues of casein and using only ATP as phosphate donor; whereas type-2 casein kinases, termed also TS or G, are oligomeric enzymes affecting both Thr and Ser residues of casein and capable of utilizing also GTP [1–3]. An additional feature of type-2 casein kinases is their remarkable inhibitability by heparin which is almost ineffective on type-1 casein kinases [4–6]. The physiological relevance of such an inhibition, however, is questionable since heparin and heparin like compounds are absent in tissues free of mast cells [7] and their translocation across the plasma membranes has not yet been proved.

On the other hand, type-2 casein kinases have been shown to require two or more acidic residues C terminal to the target residues in model substrates [8,9]. Subsequently 3, 5 and 6 acidic residues have been found close to the C-terminal side of the seryl residues affected by type-2 casein kinases in troponin T [10] glycogen synthase [11] and the regulatory subunit of type-2 cAMP-dependent protein kinase [12,13], respectively, which are among the physiological targets of this enzyme. Moreover, clusters of more than 10 acidic residues characterize the phosphorylation sites of some acidic nucleolar proteins [14] affected by a protein kinase(s) possibly related to casein kinases. These findings prompted us to check whether

Abbreviations: Ck-TS, casein kinase TS; R_{II}, regulatory subunit of cAMP-dependent protein kinase, type II; SDS, sodium dodecyl sulfate

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acidic peptides may act as competitive inhibitors of type II casein kinases. The present paper reports on the inhibition of rat liver casein kinase TS (Ck-TS), a well characterized type-2 casein kinase [2], by polymers of glutamic acid. The results show that while free glutamic acid as well as the di- and tri-peptides $(Glu)_2$ and $(Glu)_3$ are ineffective, the larger peptides $(Glu)_5$, $(Glu)_{10}$ and $(Glu)_{70}$ act as specific competitive inhibitors whose efficiency increases with the length of the polypeptide chain. Such a finding discloses the possibility that very acidic polypeptides including clusters of Glu residues may represent physiological inhibitors of type-2 casein kinases.

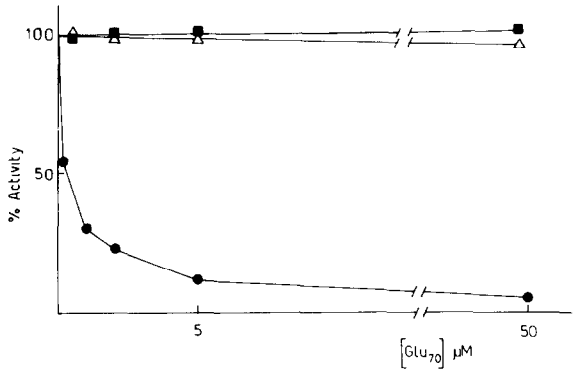

Fig.1. Specific inhibition of Ck-TS by polyglutamate. The activities of casein kinases-TS (●—●) and S (△—△) and of cAMP-dependent protein kinase (■—■) were tested as in section 2.

2. MATERIALS AND METHODS

The isolation and purification of rat liver casein kinases TS (Ck-TS) and S (Ck-S), as well as the assay of casein kinase activity and 10% polyacrylamide gel electrophoresis in SDS of radiolabeled substrates were either described or quoted in [2].

cAMP-dependent protein kinase was isolated from rat liver according to [15] and tested on histones as in [16]. Glycogen synthase was purified from rat liver as in [17]. A sample of the regulatory subunit of type II cAMP-dependent protein kinase (R_{II}), prepared from bovine cardiac muscle to >95% homogeneity was kindly provided by Professor F. Hofmann (Heidelberg).

Di- and triglutamic acids were from Serva. Pentaglutamic acid and decaglutamic acid, over 95% pure, were prepared by a procedure to be described (unpublished). Polyglutamic acid was kindly provided by Professor E. Peggion (Padova); its av. M_r was 10000, corresponding to sequences of 60–70 residues; it will be also referred to as $(Glu)_{70}$.

3. RESULTS AND DISCUSSION

Fig.1 shows that a mixture of large polymers of glutamic acid, with an av. M_r of ~10000, drastically inhibits Ck-TS but neither Ck-TS, a type-1 casein kinase, nor the cAMP-dependent protein kinase. Inhibition of Ck-TS occurs with either casein or phosvitin as phosphorylatable substrates. The concentration of polyglutamate inducing a 50% inhibition is below 1 μM; i.e., of the same order of the I_{50} values reported for heparin [6], which is an extremely powerful inhibitor of type-2 casein kinases.

The influence of the length of the polyglutamate chain on the inhibitory efficiency is shown in fig.2. $(Glu)_{10}$ and $(Glu)_5$ are still inhibitory agents, though increasingly less powerful than $(Glu)_{70}$. No inhibition could be evidenced with either free glutamate, $(Glu)_2$ or $(Glu)_3$, even increasing the concentrations up to 5 mM.

Fig.3 also shows that the phosphorylation of physiological substrates of Ck-TS, like glycogen synthase and R_{II}, is efficiently prevented by polyglutamate. The autophosphorylation of Ck-TS at its β-subunit is inhibited as well (not shown).

The type of inhibition was investigated by kinetic studies, employing as inhibitors either

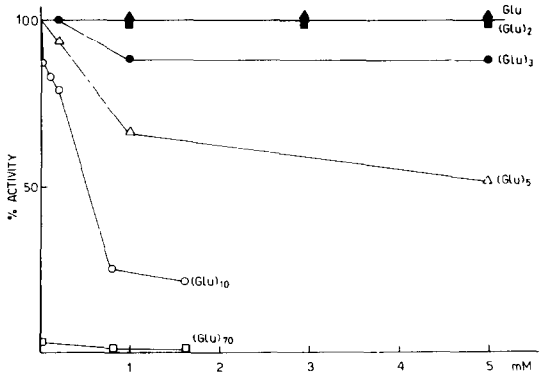

Fig.2. Inhibition of Ck-TS by glutamyl peptides of increasing length.

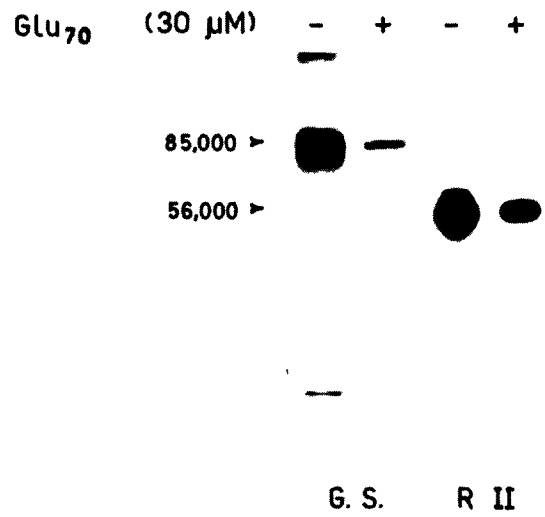

Fig.3. Phosphorylation of glycogen synthase (left) and the regulatory subunit of type II cAMP-dependent protein kinase (right) by Ck-TS: inhibition by polyglutamate. Phosphorylation was performed by replacing casein with either glycogen synthase (0.4 mg/ml) or R_{II} (0.5 mg/ml). The autoradiographies of the 10% polyacrylamide gel electrophoresis in SDS of the phosphorylated proteins, are presented. (⟶) M_r of the phosphorylated proteins estimated by calibration of the SDS gels with proteins of known M_r-value [2].

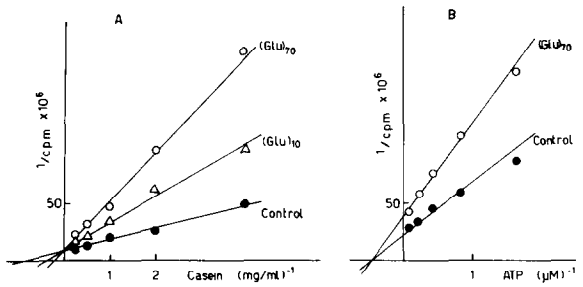

Fig.4. Double reciprocal plots for the inhibition of Ck-TS by polyglutamates: (A) ATP was 10 μM; (B) casein was 2 mg/ml; $(Glu)_{70}$ and $(Glu)_{10}$ were 1 μM and 0.42 mM, respectively.

$(Glu)_{70}$ or $(Glu)_{10}$. In both cases the inhibition was competitive with respect to casein and non-competitive with respect to ATP (fig.4). Quite similar results were obtained using phosvitin instead of casein as phosphorylatable substrate (not shown). A competition between the glutamyl peptides and the protein substrate was expected considering that all the phosphorylation sites of type-2 casein kinases are characterized by clusters of acidic residues which are supposed to be critical for the binding to the enzyme [8–13]. Rather, the negligible inhibitory power of $(Glu)_2$ and $(Glu)_3$ might suggest that the fast phosphorylation of sites with only two or three acidic residues [9] could depend on their inclusion within larger polypeptide chains: intact proteins were reported to be preferred over their proteolytic derivatives by Ck-TS [18]. Thus it will be interesting to see whether short polyglutamyl peptides, like $(Glu)_3$ and $(Glu)_5$ can improve their inhibitory efficiency once included into larger non-acidic sequences.

Several proteins possessing continuous sequences of >40 acidic amino acids and/or an extremely high content (up to 50%) of glutamyl residues, have been detected in many tissues [19,20]. It is conceivable therefore that some of them might act as physiological inhibitors of type-2 casein kinases. Although the physiological concentrations of such putative inhibitors of Ck-TS are not known, the concentrations of other inhibitor polypeptides, like the heat-stable inhibitor of cAMP-dependent protein kinase and protein phosphatase inhibitors 1 and 2, have been calculated to be very similar, ranging from 0.5–1.5 μM [21–23]. On the other hand, the K_i of $(Glu)_{70}$ for Ck-TS, calculated from the experiment of fig.4A, is 0.11 μM, a value quite consistent with the hypothesis that structurally related compounds might act as inhibitors of type-2 casein kinases in vivo.

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