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CHARACTERIZATION OF THE INHIBITOR OF CASEIN KINASES 1 AND 2 FROM RAT LIVER CYTOSOL

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The heat-labile inhibitor of casein kinases 1 and 2 from rat liver cytosol (J.F. Bertomeu et al., FEBS Lett., 124, 262-264) has been purified extensively and characterized. Analysis by gel filtration and SDS-polyacrylamide gel electrophoresis suggest that the inhibitor has an Mr of 30,000. It did not contain glycosaminoglycans, oligonucleotides or neutral sugars and was totally inactivated by digestion with trypsin. Besides casein kinases, the inhibitor also inhibited the catalytic subunit of cAMP-dependent protein kinase to the same extent. The data suggest that the inhibitor is a monomeric protein that could modulate intracellular protein phosphorylation by both casein kinases and cAMP-dependent protein kinase.

INTRODUCTION

Cyclic nucleotide-independent casein kinases from various sources, including rat liver cytosol (1-5), phosphorylate and inactivate I-form glycogen synthase, what suggests a possible role of these enzymes in the regulation of glycogen metabolism. Despite this fact, there is little information concerning the regulation of casein kinases by endogenous factors.

In a previous report (6) we have evidenced that rat liver cytosol contains an inhibitor of the two cytosolic casein kinases from the same tissue. The inhibitor is devoid of ATP-ase, phosphatase or protease activity and the enzyme-inhibitor complex is easily reversible. The presence of inhibitory factors has also been demonstrated in other mammalian tissues. Besides the inhibitor protein specific for cAMP-dependent protein kinase (7), rat skeletal muscle, pancreas, cerebellum and corpus luteum contain a 15,000-Mr thermostable protein which inhibits both cAMP- and cGMP-dependent or cyclic nucleotide-independent protein kinases (8). Bovine adrenal cortex contains a second type of inhibitor

Abbreviations ; CK-1 and CK-2, casein kinases 1 and 2; C, catalytic subunit of cAMP-dependent protein kinase.

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which is heat-stable (9), resistant to trypsin digestion and has been recently identified as an heterogeneous glycosaminoglycans structure (10). This inhibitor selectively inhibits a particular class of casein kinase, the G-type, which is similar to casein kinase 2 in many aspects (4,11). Two other inhibitors are present in rat liver nuclei, both of them specific only for nuclear enzymes and inert towards the cytosolic protein kinases (12). One of these inhibitors is a 150,000-Mr, heat-labile, acidic protein, whereas the other one has been characterized as a family of two oligonucleotides.

This paper describes the characterization of the inhibitor from rat liver cytosol, which is different from other inhibitors reported so far.

METHODS

Assays : Protein kinase and inhibitor assays were done at 30°C as described previously (6). Units of protein kinase are expressed as nmol of ^{32}P incorporated from [γ-^{32}P]ATP to protein substrate per min. One unit of inhibitor is the amount that promotes a 25% inhibition of CK-2 under standard assay conditions.

Preparation of protein kinases : CK-1 and CK-2 were isolated from rat liver cytosol as described in (4). C was prepared from rabbit muscle by chromatography on CM-Sephadex C-50 in the presence of cAMP as described previously (13).

Purification of the inhibitor : Partial purification of the inhibitor was accomplished in a way similar to that reported in (6). The inhibitor preparation corresponding to the flowthrough fraction from DEAE-cellulose (70 ml) was dialyzed against 20 mM potasium phosphate buffer (pH 6.5) containing 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and 5% glycerol (Buffer A) and applied to a CM-Sephadex C-50 column (1.5x10 cm) equilibrated with Buffer A. The column was washed with 50 ml of Buffer A and the inhibitor eluted with 50 ml of Buffer A at pH 7.5. Fractions with inhibitory activity (pH 6.9) were pooled and concentrated in an Amicon ultrafiltration chamber outfitted with a PM-10 membrane to a final volume of 1.6 ml. The inhibitor preparation was adjusted to 0.4 M KCl with a final volume of 2 ml and applied to a Bio-Gel A-0.5m column (1x90 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and 5% glycerol (Buffer B) plus 0.4 M KCl. The column was washed with the same Buffer B and fractions with maximal inhibitory activity were pooled and dialyzed against a buffer similar to Buffer B but at pH 8.5. The dialyzed inhibitor fraction was applied to a DEAE-Sepharose CL-6B column (1.2x2.5 cm) equilibrated with dialysis buffer. Then, the column was washed with 15 ml of the same buffer and the inhibitor eluted with 15 ml of Buffer B. Fractions with maximal inhibitory activity (pH 8.0) were pooled, concentrated to 1.5 ml as above and stored at -20°C. The purification achieved by this procedure was 15-fold with respect to the DEAE-cellulose step (6), with a recovery of 6%.

Gel electrophoresis : Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate was carried out by the method of Laemmli (14) with 12.5% acrylamide in the separating gel.

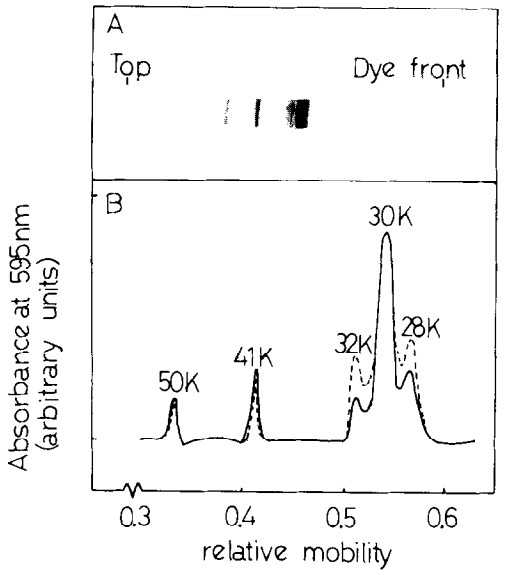

Fig.1 - Gel electrophoresis of the inhibitor. SDS-gel electrophoresis was carried out as indicated in Methods and protein bands were detected by staining with Coomassie Brilliant blue. In A 100 µg of purified inhibitor were applied to the gel. In B the scanning at 595 nm of two preparations of inhibitor with specific activities of 350 (- - -) and 700 units/mg of protein (——) are shown. The numbers on top of the peaks indicate Mr in kilodaltons.

Bovine serum albumin (Mr 68,000), ovalbumin (Mr 45,000), carbonic anhidrase (Mr 30,100) and trypsin inhibitor (Mr 21,500) were used as protein markers for molecular weight determination. Quantification of stained protein bands in the gels was performed by scanning at 595 nm in an Spay Unicam SP-1700 spectrophotometer.

Trypsinization of the inhibitor : Digestion was carried out at a trypsin:inhibitor ratio of 1/50 (w/w) with an aliquot of the inhibitor preparation which contained 0.2 mg/ml of protein. At indicated times aliquots were removed and the reaction was stopped by mixing with 10 µl of 50 µg/ml trypsin inhibitor. Controls were carried out in the absence of trypsin. The different aliquots were assayed immediately for inhibitory activity.

Other methods : Protein was determined according to Bradford (15) or by absorbance at 280 nm, using an $E^{1\%}_{280nm} = 10.5$, as indicated in the text. Total glycosaminoglycans were estimated by the o-toluidine blue method (16). Neutral sugars were determined by the orcinol method (17).

RESULTS

Molecular properties of the inhibitor : The Mr of the inhibitor was estimated as 31,000 from calibration of the Bio-Gel A-0.5m column used for the purification (not shown). Analysis of the purified inhibitor by SDS-polyacrylamide gel electrophoresis showed three major components, corresponding to Mr of 28,000-32,000, 41,000 and 50,000 (Fig. 1). The smaller molecular weight component

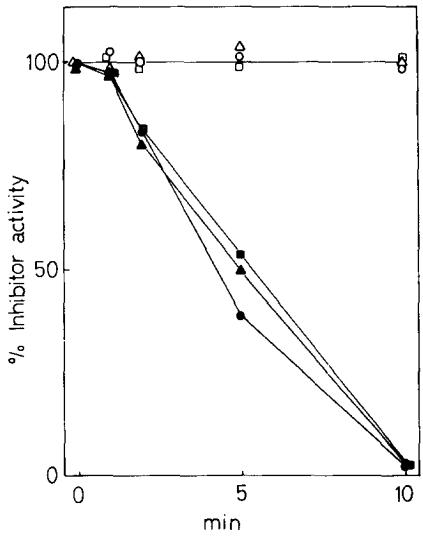

Fig.2 - Digestion of the inhibitor with trypsin.
A sample containing 0.2 mg/ml of inhibitor was incubated either alone (open symbols) or in the presence of 4 µg/ml of trypsin (solid symbols) and at indicated times aliquots were removed and assayed for inhibitory activity on CK-1 (○,●), CK-2 (△,▲) or C (□,■).

was always obtained as a triplet band and accounted for more than 75% of the total protein in the gel on the basis of Coomassie blue staining. A correlation between the inhibitory activity and the 30,000-Mr band was envisaged by comparison of the specific inhibitor activity of different preparations with the amount of this band relative to the 28,000-Mr and 32,000-Mr bands in the gels.

Digestion of the inhibitor with trypsin caused a time-dependent inactivation of the inhibitor. Such inactivation was similar when either CK-1, CK-2 or C were used in the assay (Fig. 2). Heating the inhibitor at 95°C for 2 min also promoted a total loss of its activity on these three kinases.

Ultraviolet spectra of the inhibitor showed a peak with a maximum at 280.5 nm and an $E^{1\%}_{280nm}$ of 10.5. Its absorbance ratio at 280 nm/260 nm was 1.87, what indicates the absence of oligonucleotides. No detectable glycosaminoglycans were present in the purified inhibitor preparations when assayed by the o-toluidin method using heparin as standard. The inhibitor gave a negative orcinol reaction when assayed for neutral sugars.

Characteristics of the inhibition : The inhibition of CK-1, CK-2 and C was proportional to the amount of inhibitor present in the assay and the degree of inhibition promoted by a given amount of inhibitor was similar for the three kinases (Fig. 3). By extra-

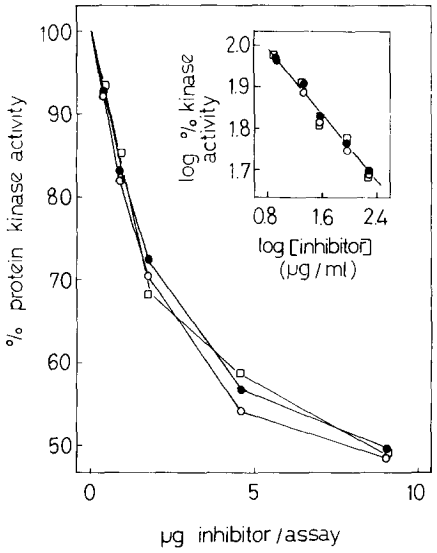

Fig.3 - Inhibition of casein kinases 1 and 2 as a function of inhibitor protein concentration. The activity of purified CK-1 (○), CK-2 (●) or C (□) (0.6 units/ml in each case) was measured in the presence of various amounts of purified inhibitor protein under standard conditions, using casein (○,●) or histone II A (□) as substrate. Inset, the same data repploted on a log-logit scale.

polation from the log-logit plot a Ki apparent of 180 µg/ml was estimated for all these enzymes.

The degree of inhibition of CK-1 and CK-2 by a fixed amount of inhibitor was independent on the substrate (either casein, phos vitin or glycogen synthase I) used (Table I).

TABLE I

1 and 2 by the inhibitor protein

Protein kinase	Substrate[a]	pmols ^{32}P/min x ml of assay		
		control	+inhibitor[b]	% inhibition
CK-1	casein	312	144	46
	phosvitin	112	61	50
	glycogen synthase	29	12	41
CK-2	casein	376	165	44
	phosvitin	230	83	36
	glycogen synthase	27	11	41

[a]The concentration of substrates was 4 mg/ml except for glycogen synthase I that was 0.08 mg/ml.

[b]The concentration of the inhibitor was 0.14 mg/ml.

DISCUSSION

The casein kinase inhibitor from rat liver cytosol is heat-labile, does not contain glycosaminoglycans or oligonucleotide structures, shows an absorbance spectra typical of proteins and is very sensitive to trypsinization. All these data lead us to conclude that the inhibitor is a protein. The similarity between the molecular weight estimated by gel filtration (31,000) and that of the major component observed in SDS-polyacrylamide gel electrophoresis (30,000) suggests a monomeric structure for the inhibitor protein.

The inhibitor protein inhibits the two casein kinases as well as the catalytic subunit of cAMP-dependent protein kinase. The parallel decrease of inhibitory activity on these three kinases by trypsinization or heat-treatment of the inhibitor, together with their Ki apparent agrees with the presence in the inhibitor preparation of a single inhibitor protein equally effective on these three enzymes.

The characteristics of the inhibitor are different from those of other inhibitors reported so far. Its proteic nature makes it different from the inhibitor present in bovine adrenal cortex, which is a mixture of glycosaminoglycans (10), and from the small molecular weight inhibitor present in rat liver nuclei, which is a family of two oligonucleotides (12). On the other hand, it is different from the inhibitor protein present in several rat tissues (8) in its molecular weight and heat lability, and from the inhibitor protein from rat liver nuclei (12) in its molecular weight and specificity for the source of casein kinase.

It is worthwhile to note that the extent of inhibition of rat liver casein kinases by the inhibitor protein was similar when either casein, phosvitin or glycogen synthase I were used as substrate, what corroborates that the activity of each casein kinase on these three substrates could reside, in each case, in a single catalytic entity, as we have suggested previously (4).

The inhibitor reported herein is the first protein inhibitor observed in rat liver cytosol which could affect the activity of both cAMP-dependent and independent protein kinases, and in consequence the extent of phosphorylation and degree of activity of phosphorylatable enzymes, such as glycogen synthase.

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