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A new heparin-inhibited and polyamine-activated protein kinase from bovine kidney

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Two casein kinases, casein kinase-1 (CK-1) and casein kinase-2 (CK-2), have been characterized from many sources. In this study we describe the properties of a third casein kinase, designated casein kinase-3 (CK-3). CK-3 (M_r 32 000) is readily separated from CK-2 by gel filtration and from CK-1 by hydroxyapatite chromatography. CK-3 phosphorylates several proteins, including phosphorylase kinase. Phosphorylation of phosphorylase kinase by CK-3 results in a 10-fold enzyme activation. CK-3 is activated by spermine and inhibited by heparin, ADP, and divalent metal ions (Mn^{2+}, Zn^{2+}). Heparin inhibition of the kinase is reversed by spermine. The physical and regulatory properties of CK-3 are very similar to CK-1, suggesting that these kinases may be closely related.

Casein kinase-1; Heparin inhibition; Spermine activation; Casein kinase-2; Protein kinase; Casein kinase-3

1. INTRODUCTION

Two casein kinases, casein kinase-1 (CK-1) and casein kinase-2 (CK-2), have been characterized from different sources [1,2]. These kinases bind to phosphocellulose from which CK-1 and CK-2 elute at approx. 0.5 and 0.7 M KCl, respectively. CK-1 from different sources is apparently monomeric with M_r in the range 30000–40000. By comparison, CK-2 is an oligomeric protein (M_r 135000) containing two types of subunits termed α (M_r 42000) and β (M_r 27000). Both CK-1 and CK-2 have wide substrate specificities [1–3].

The mechanisms by which CK-1 and CK-2 are regulated are still unclear. Recent studies, however, have shown that when responsive cells are treated with insulin the activities of both CK-1 [4] and CK-2 [5] are stimulated. The mode of activation of these kinases by insulin remains unknown. In vitro both CK-1 and CK-2 are activated by polyamines [3,6] and inhibited by heparin [7,8]. In the special case when mixed casein is the substrate CK-1 is resistant to inhibition by heparin whereas CK-2 is readily inhibited [8]. Glycogen [9], divalent metal ions [10] and different phosphorylated metabolites [11] have also been shown to inhibit CK-1.

Here, we describe the properties of a third casein kinase from bovine kidney. This kinase, designated casein kinase-3 (CK-3), shares with CK-1 and CK-2 the twin characteristics of heparin inhibition and polyamine activation.

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2. MATERIALS AND METHODS

Nonactivated phosphorylase kinase was prepared from rabbit skeletal muscle [12] and its activity [13] assayed as described. Calcineurin was prepared from bovine brain [14]. Spermine, heparin, ADP, ATP, GTP, and all other protein substrates were purchased from Sigma. CK-1 and CK-2 were prepared from bovine kidneys (Singh, T.J. submitted). Briefly, an extract from renal cortex was prepared and batch adsorbed to phosphocellulose. Total casein kinase activity was eluted from the column with 1.2 M KCl. After concentration by ultrafiltration CK-1 and CK-2 were separated on a Bio-Gel A-1.5 m column. The CK-1 activity peak was applied to a hydroxyapatite column (1.5 × 5 cm) which separated CK-1 from CK-3 (fig.1). These kinases were separately pooled and further purified on a casein-agarose column. The purified kinases were concentrated and stored at −70°C in buffer containing 0.2 M KCl. CK-1 activity was assayed in a reaction mixture containing mixed casein (4 mg/ml), 25 mM Tris-HCl (pH 7.5), 10 mM 2-mercapto-

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ethanol, 5 mM $MgCl_2$, 5 mM NaF, 10 mM KCl, 0.1 mM [γ-^{32}P]ATP and kinase. When calcineurin was used as a substrate it was present at 0.25 mg/ml. Reactions were initiated at 30°C by the addition of [γ-^{32}P]ATP and aliquots removed at different times for the determination of ^{32}P incorporation into protein [15]. One unit of CK-1, CK-2, or CK-3 is defined as the amount of enzyme catalyzing the incorporation of 1 nmol ^{32}P from [γ-^{32}P]ATP into casein (4 mg/ml) per min at 30°C. Protein was determined by the method of Bradford [16].

3. RESULTS AND DISCUSSION

In an earlier study [2] we have shown that kidney is an especially rich source for CK-1. Hence, our current efforts have been directed at obtaining homogeneous preparations of CK-1 from this tissue. One of the steps used in our purification scheme (Singh, T.J., submitted) is the fractionation of CK-1 (separated from CK-2 by gel filtration on Bio-Gel A-1.5 m) on hydroxyapatite (fig.1). When the column fractions were assayed using mixed casein as a substrate two peaks of kinase activity were observed. A prominent peak eluting at 132 mM potassium phosphate was followed by a second smaller peak eluting at 180 mM potassium phosphate (fig.1A). When the column fractions were reassayed using calcineurin as a substrate [18] two equally prominent kinase peaks are observed. The first calcineurin kinase coeluted with the main peak of casein kinase activity. This peak has been called CK-1 and characterized in earlier studies [6,8,10]. The second calcineurin kinase coelutes with the second peak of casein kinase (fig.1). This kinase has not been previously described. It will be designated here as casein kinase-3 (CK-3). Both CK-1 and CK-3 were separately pooled, purified further on a casein-agarose column and used for the studies described below.

We have previously shown that CK-1 can phosphorylate several proteins [2,18,19]. It was therefore of interest to compare the substrate specificities of CK-1 and CK-3. Mixed caseins, phosvitin, β-casein, α-casein and calcineurin are relatively good substrates for both kinases (table 1). In addition, we found that phosphorylase kinase (see below), microtubule-associated protein-2, and smooth muscle myosin light chains are also phosphorylated by both kinases (not shown).

We have shown that phosphorylase kinase can

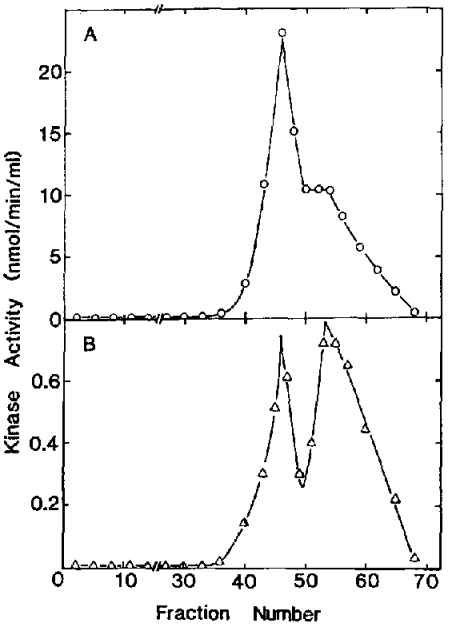


Fig.1. Separation of CK-1 and CK-3 by chromatography on hydroxyapatite. Peak fractions of CK-1 from a Bio-Gel A-1.5 m column were pooled and applied directly to a hydroxyapatite column (1.5 × 5 cm) equilibrated with buffer A (20 mM potassium phosphate, pH 7.2, 10 mM 2-mercaptoethanol, 0.5 mM EDTA, 0.2 mM PMSF, 250 mM sucrose). The column was washed with three bed volumes of buffer A, then eluted with an 8-bed volume linear gradient of 20–500 mM potassium phosphate (pH 7.2) containing 10 mM 2-mercaptoethanol, 0.5 mM EDTA, and 250 mM sucrose. Fractions of 2 ml were collected and assayed for casein kinase (A) or calcineurin kinase (B).

Table 1

Substrate specificities of CK-1 and CK-3

Substrate	Kinase activity (%)	
	CK-1	CK-3
Mixed casein	100	100
Phosvitin	92	111
β-Casein	87	89
α-Casein	49	47
Calcineurin	37	43
Troponin	2.3	4.8
Histone IIAS	0.9	2.5
Histone IIIS	0.9	2.1
Protamine	0.7	0.9

The different substrates (1 mg/ml) were phosphorylated by CK-1 (10 mU) and CK-3 (10 mU) under standard conditions. The rates of phosphorylation of the different substrates are compared with that observed with mixed casein which is taken as 100%

be phosphorylated and activated by CK-1 [11]. In these studies CK-1 was purified by a scheme that did not use hydroxyapatite chromatography [20]. Hence these earlier preparations of CK-1 may also have contained CK-3. It was therefore of interest to determine whether CK-1 and CK-3 can separately phosphorylate and activate phosphorylase kinase. As shown in fig.2, both kinases catalyze the incorporation of several moles of phosphate into phosphorylase kinase. Further, CK-1-catalyzed phosphorylation results in an 8-fold activation of phosphorylase kinase (fig.2A) compared with the 10-fold activation achieved by CK-3 (fig.2B). ^{32}P incorporation catalyzed by either kinase continues to increase even after maximal activation of phosphorylase kinase is observed (fig.2).

CK-2 is a well-known heparin-inhibited and polyamine-activated kinase [3,7]. Recently, we have shown that CK-1 is similarly regulated [6,8]. It was therefore of interest in the present study to ascertain whether CK-3 is also regulated by polyamines and heparin. As shown in table 2 a 3-fold activation of CK-3 by 1 mM spermine is observed when mixed casein is used as the substrate. This is comparable to the 3- and 4-fold activation observed for CK-1 and CK-2, respectively, by the polyamine. With mixed casein as the substrate CK-1 and CK-3 are insensitive to inhibition by heparin whereas CK-2 is inhibited by 92%. Inhibition of CK-1 and CK-3 by heparin is observed when calcineurin is used as the substrate. Further, this inhibition can be reversed by 1 mM spermine (table 2). A similar reversal of heparin inhibition of CK-2 by spermine has been documented [21]. These results indicate that CK-1, CK-2, and CK-3 are all heparin-inhibited and polyamine-activated kinases.

CK-1 and CK-3 also show similar responses to other effector molecules. We have previously shown that Mn^{2+}, Zn^{2+}, and other divalent metal ions inhibit CK-1 [10]. CK-3 was found to be inhibited to the same extent as CK-1 by 0.2 mm Mn^{2+} (table 2) or 0.2 mM Zn^{2+} (not shown). The effects of two nucleotides, ADP and GTP, on the kinase activities were also investigated. CK-1 and CK-3 were inhibited to the same extent by 0.1 mM ADP but were unaffected by 0.2 mM GTP. By contrast, CK-2 was inhibited 54% by GTP (table 2). Since GTP, like ATP, is known to be a substrate for CK-2 but not CK-1 [1], our data suggest that CK-3 may not use GTP as a nucleotide substrate.

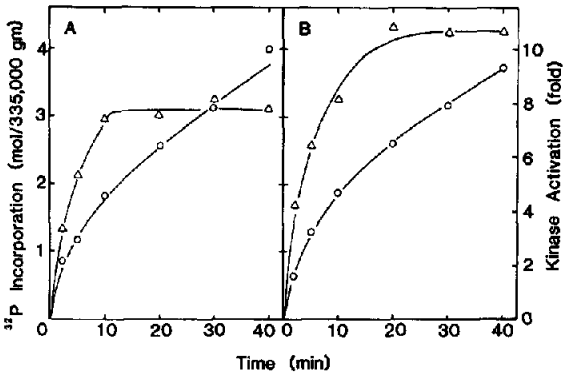


Fig.2. Phosphorylation and activation of phosphorylase kinase by CK-1 and CK-3. Phosphorylase kinase (0.3 mg/ml) was phosphorylated either by 40 mU CK-1 (A) or 40 mU CK-3 (B). Reactions were carried out under standard conditions with the exception that 0.5 mM EGTA was included in the assays to suppress autophosphorylation of phosphorylase kinase. The level of phosphorylation (○) and resultant activation (△) of phosphorylase kinase is shown.

The results of this study clearly show that a third casein kinase, CK-3, exists in bovine kidney and perhaps other mammalian tissues. CK-3 shares the properties of heparin inhibition and polyamine ac-

Table 2

Effect of different ligands on CK-1, CK-2, and CK-3

Additions	Substrate	Kinase activity (%)		
		CK-1	CK-2	CK-3
None	casein	100	100	100
Heparin	casein	94	8	98
Spermine	casein	325	403	284
GTP	casein	97	46	100
ADP	casein	45	ND	46
Mn^{2+}	casein	12	ND	15
None	calcineurin	100	ND[a]	100
Spermine	calcineurin	138	ND	138
Heparin	calcineurin	17	ND	14
Heparin + spermine	calcineurin	99	ND	98

[a] Calcineurin has been shown [17] to be a very poor substrate of CK-2

ND, not done. Mixed casein (4 mg/ml) and calcineurin (0.25 mg/ml) were phosphorylated by CK-1 (10 mU), CK-2 (10 mU), and CK-3 (10 mU) in either the absence or presence of heparin (2 μg/ml), spermine (1 mM), GTP (0.2 mM), ADP (0.1 mM), and Mn^{2+} (0.2 mM)

tivation with CK-1 and CK-2. CK-3 can be distinguished from CK-2, however, by using mixed casein as a substrate. With this substrate CK-2 is inhibited by heparin and GTP whereas CK-3 is insensitive to these effectors. Further, CK-3 has an M_r of approx. 32000 as determined by gel filtration on Bio-Gel A-1.5m (not shown) compared with an M_r of 135000 for CK-2 [1]. CK-3 can also be differentiated from CK-2 by the finding that calcineurin is a good substrate for CK-3 but is only poorly phosphorylated by CK-2 [17].

By contrast with CK-2, the physical and regulatory properties of CK-1 and CK-3 are very similar. Both kinases have M_r values of approx. 32000 as determined by gel filtration. These kinases have very similar substrate specificities (table 1) and show the same responses to various effectors (table 2). However, they can readily be resolved from one another by chromatography on hydroxyapatite (fig.1). The similarities between CK-1 and CK-3 in physical and regulatory properties suggest that these kinases may be closely related. One possibility is that CK-1 and CK-3 may be isozymes. Further characterizations using homogeneous enzyme preparations should illuminate this problem.

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