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PURIFICATION AND CHARACTERIZATION OF TWO CYCLIC AMP-INDEPENDENT CASEIN/GLYCOGEN SYNTHASE KINASES FROM RAT LIVER CYTOSOL

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Summary

Two cyclic AMP-independent protein kinases (ATP protein phosphotransferase, EC 2.7.1.37) (casein kinase 1 and 2) have been purified from rat liver cytosol by a method involving chromatography on phosphocellulose and casein-Sepharose 4B. Both kinases were essentially free of endogeneous protein substrates and capable of phosphorylating casein, phosvitin and I-form glycogen synthase, but were inactive on histone IIA, protamine and phosphorylase *b*. They were neither stimulated by cyclic AMP, Ca^{2+} and calmodulin, nor inhibited by the cyclic AMP-dependent protein kinase inhibitor protein. The casein and glycogen synthase kinase activities of each enzyme decreased at the same rate when incubated at 50°C.

Casein kinase 1 and casein kinase 2 showed differences in molecular weight, sensitivity to KCl, K_m for casein and phosvitin and K_a for Mg^{2+}, whereas their K_m values for ATP and I-form glycogen synthase were similar.

The phosphorylation of glycogen synthase by these kinases correlated with a decrease in the ∓ glucose 6-phosphate activity ratio (independence ratio). However, casein kinase 1 catalyzed the incorporation of about 3.6 mol of ^{32}P/85 000 dalton subunit, decreasing the independence ratio from 83 to about 15, whereas the phosphorylation achieved by casein kinase 2 was only about 1.9 mol of ^{32}P/85 000 dalton subunit, decreasing the independence ratio to about 23. The independence ratio decrease was prevented by the presence of casein but was unaffected by phosphorylase *b*.

Abbreviation EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid

These data indicate that casein/glycogen synthase kinases 1 and 2 are different from cyclic AMP-dependent protein kinase and phosphorylase kinase.

Introduction

The presence of multiple forms of cyclic AMP-independent glycogen synthase kinase has been observed in rabbit muscle [1,2]. One of these kinases catalyzes a total inactivation of glycogen synthase incorporating up to 4 mol phosphate/mol 85 000 dalton subunit [3—5]. Such a value is comparable to the alkaline labile phosphate content of purified D-form from rabbit muscle [6,7]. However, there is a discrepancy regarding the extent of phosphorylation and degree of inactivation described in other reports [8—11]. In addition, it has been recently demonstrated that glycogen synthase can be phosphorylated by muscle phosphorylase kinase in a reaction that is stimulated by Ca^{2+} and proceeds much faster at pH 8 2 than at pH 6.8 [12—15] Nevertheless, the stoichiometry of ^{32}P incorporation by phosphorylase kinase was about 1 mol/mol 85 000 dalton subunit or less, which promoted only a partial inactivation of glycogen synthase

Cyclic AMP-independent kinase activity has also been found to account for most of the glycogen synthase kinase activity present in other mammalian tissues [16] The presence of cyclic AMP-independent casein kinase(s) is known in a variety of mammalian tissues including rat liver [17—19], calf brain [20], human erythrocytes [21] and rabbit reticulocytes and erythrocytes [23]. Two casein kinases were detected in rat liver by means of gel filtration [18,24]. However, the physiological role of these kinases was unknown.

In a recent report [25] we have demonstrated the presence in rat liver cytosol of a cyclic AMP-independent casein kinase (casein kinase 1) able to incorporate up to 3.6 mol phosphate/mol 85 000 dalton subunit of glycogen synthase, which resulted in a great inactivation of the synthase.

In the present study we describe the presence in rat liver cytosol of another casein/glycogen synthase kinase (casein kinase 2) differing from casein kinase 1. We also report the purification of both kinases as well as some of their molecular and kinetic properties.

Experimental procedures

Materials

The sources of the materials used are those previously reported [25].

Rabbit muscle I-form glycogen synthase [26], phosphorylase *b* [27] and phosphorylase kinase [28] were purified to homogeneity by standard procedures. The cyclic AMP-dependent protein kinase inhibitor protein was purified up to the trichloroacetic acid precipitation step by the method of Walsh et al. [29]. Calmodulin was prepared from phosphorylase kinase according to Shenolikar et al. [30].

Assays

Protein kinase activity was measured as described previously [3], except that

incubation was at 30°C In general, the reaction mixture (50 μl) contained 25 mM β-glycerol phosphate, pH 7 0/1 mM dithiothreitol/0.5 mM EDTA/0 3 mM EGTA/8 mM magnesium acetate/0.125 mM [γ-^{32}P]ATP (50—200 cpm/pmol)/4 mg/ml casein or the indicated amount of other protein substrate. At timed intervals, samples were removed and assayed for ^{32}P-labeled protein by the instant thin layer chromatography (ITLC) method [31] 1 unit of kinase activity is defined as the amount catalyzing the transfer of 1 nmol phosphate from [γ-^{32}P]ATP to casein per min at 30°C.

Glycogen synthase activity was determined in both the absence and the presence of 7.2 mM glucose 6-phosphate as described previously [32]. Glycogen synthase independence ratio is defined as the activity in the absence of glucose 6-phosphate divided by the activity in its presence, the result being multiplied by 100 as in Ref 3 Protein concentration was determined by the modified method [33]of Lowry et al. [34] in order to avoid the effects of interfering substances

Phosphorylation of glycogen synthase

Glycogen synthase phosphorylation was carried out under the standard assay conditions in 0.2 ml reaction mixture containing 0.1 mg/ml I-form glycogen synthase/0 02 mg/ml rabbit liver glycogen/0 7 units/ml kinase. At timed intervals, 20 μl aliquots were removed in order to measure ^{32}P incorporation as described earlier [31]. A similar assay, in which non-radioactive ATP was used, was carried out for the determination of glycogen synthase independence ratio change along the phosphorylation.

Purification casein kinases 1 and 2

(a) Initial steps The purification steps were performed at 4°C. Male Wistar rats (200—300 g) fed ad libitum were decapitated and, after bleeding, the livers (about 100 g) were removed, washed in 0.9% NaCl and homogenized in 200 ml 10 mM phosphate buffer, pH 7 5/1 mM EDTA using a homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 10 000 × *g* for 30 min and the supernatant was filtered through glass wool and then centrifuged at 100 000 × *g* for 90 min The supernatant was adjusted to 60% saturation with solid $(NH_4)_2SO_4$, and after stirring for 30 min, the precipitate was collected by centrifugation at 25 000 × *g* for 30 min, resuspended in 90 ml of 50 mM Tris-HCl buffer, pH 7 5/1 mM dithiothreitol/5% glycerol/0.1 mM phenylmethylsulphonyl fluoride (Buffer A), and extensively dialyzed against the same buffer.

(b) First chromatography on phosphocellulose The dialyzed material was applied to a 4 × 8 cm phosphocellulose column equilibrated with buffer A The column was washed with 150 ml of buffer A followed by 150 ml 0.2 M KCl, 225 ml 0.35 M KCl, a 300 ml linear gradient of 0.35—1.0 M KCl and finally 75 ml 1 0 M KCl, all in buffer A Fractions (5 ml) number 150—165 corresponded to casein kinase 1, and number 175—190 to casein kinase 2

(c) Further purification of casein kinase 1 and 2. (I) Pooled fractions corresponding to casein kinase 1 were dialyzed against buffer A and applied to a 4 × 8 cm casein-Sepharose 4B column equilibrated with the same buffer. The column was washed with 150 ml buffer A, and then with a 500 ml linear gradient of 0—1 M KCl in buffer A. Fractions (5 ml) number 110—135 were

pooled, diluted with 2 vol buffer A and applied to a 1.1 × 4 cm phosphocellulose column equilibrated with the same buffer. The column was washed with 10 ml 0.2 M KCl in buffer A, and the kinase eluted with 10 ml 1 M KCl in the same buffer 1-ml fractions were collected during the washing and elution steps, and those with casein kinase activity were pooled.

(II) The same procedure described in I was applied to the pooled fractions from phosphocellulose corresponding to casein kinase 2. Fractions 90—100 from casein-Sepharose 4B were pooled in this case.

The pooled fractions of each kinase were extensively dialyzed against buffer A and stored at −40°C

Molecular weight determination

The characterization of the kinases was carried out with the highly purified preparations. Molecular weight determination by gel filtration was carried out in a Bio-Gel A 1.5 m column using yeast alcohol dehydrogenase (M_r 150 000), bovine serum albumin (M_r 68 000), ovalbumin (M_r 43 000) and chymotrypsinogen A (M_r 25 700) as standard proteins. Electrophoresis was carried out in slabs of 10% polyacrylamide gels containing 0.1% sodium dodecyl sulphate (SDS) as described [35]

Results

Purification. Protein kinase activity present in the rat liver preparations up to the 60% $(NH_4)_2SO_4$ precipitate was composed of about one-third casein kinase and two-thirds histone kinase

Several peaks of protein kinase activity were separated by chromatography on phosphocellulose (Fig. 1). The first one, which corresponded to the flow-through fraction, contained the cyclic AMP-stimulated histone kinase activity. The second one also contained a histone kinase but it was different from the first one in that it was not stimulated by cyclic AMP. A more detailed study of these kinases will be described elsewhere. The third and fourth peaks were two clearly separated cyclic AMP-independent casein kinases which had poor histone kinase activity. These peaks have been designated as casein kinase 1 and 2, respectively, according to their order of elution.

A greater purification of both casein kinase 1 and casein kinase 2 was achieved by chromatography on casein-Sepharose 4B (Fig 2). This step also served to eliminate cross-contamination between the two kinases since they showed different elution patterns We do not know whether the unbound component from casein kinase 2 is an altered form of the enzyme or a separate casein kinase. Only the fractions corresponding to the main bound component were pooled in each case. After this chromatography casein kinase 1 and casein kinase 2 were well separated from their endogenous protein substrates, but they were unstable.

In order to stabilize their activity, both kinases were concentrated by rechromatography on phosphocellulose. This step brought about a further purification of each enzyme, eluting both casein kinase 1 and casein kinase 2 as sharp peaks in a total volume of 2—3 ml.

A typical purification of casein kinase 1 and casein kinase 2 is summarized in

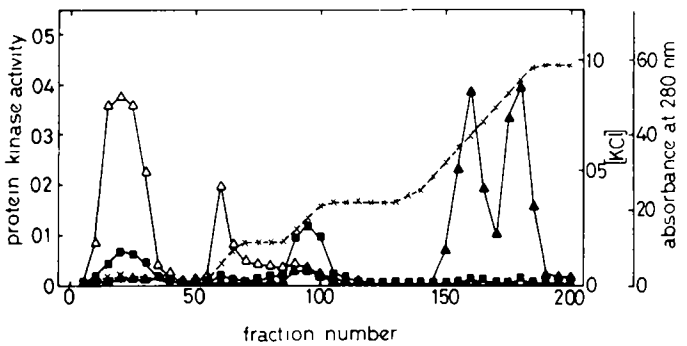

Fig 1 First chromatography on phosphocellulose Fractions were assayed for (■———■) histone kinase activity in the presence of 2 10^{-5} M cyclic AMP, (▲———▲) casein kinase activity in the absence of cyclic AMP, (△———△) protein, as absorbance at 280 nm, and (x———x) KCl concentration (M) Protein kinase activity is given as nmol of ^{32}P incorporated under standard assay conditions

Table I. It should be noted that the purification and recovery achieved for each kinase may be underestimated since all the data refer to the total casein kinase activity present in the crude extract, in which more than one kinase is present

Molecular weight The molecular weights of casein kinase 2 were estimated

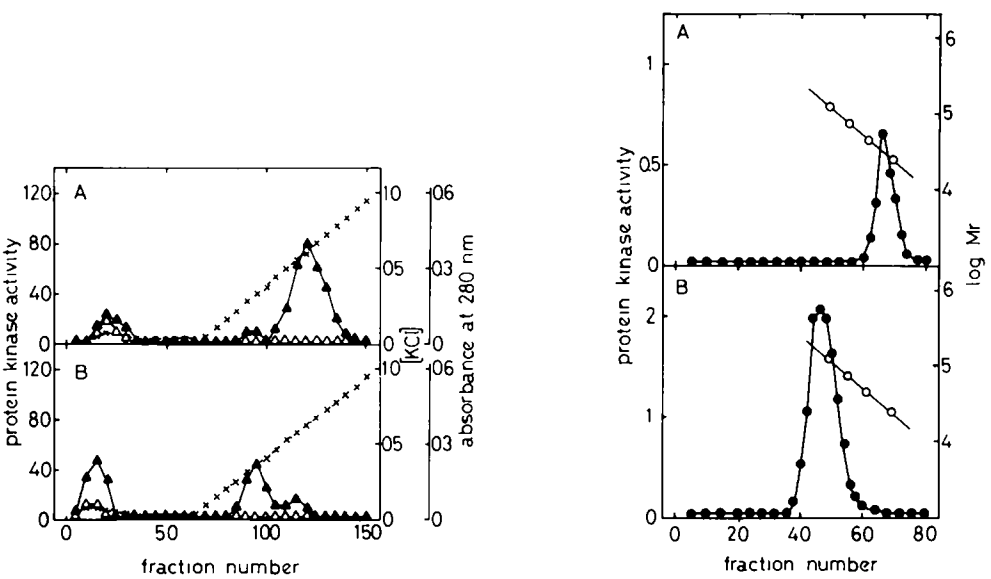

Fig. 2 Chromatography on casein-Sepharose 4B A, chromatography of casein kinase 1 B, chromatography of casein kinase 2 In both cases, fractions were assayed for (▲———▲) casein kinase activity, (△———△) protein, as absorbance at 280 nm and (x———x) KCl concentration (M) Protein kinase activity is given as pmol of ^{32}P incorporated into casein in the absence of cyclic AMP under standard assay conditions

Fig 3 Gel filtration of casein kinase 1 (A) and casein kinase 2 (B) in Bio-Gel A-1 5 m 1 ml of casein kinase 1 or casein kinase 2 from the second phosphocellulose step were chromatographed in a (1.5 X 90 cm) Bio-Gel A-1.5 m column equilibrated with buffer A containing 0,4 M KCl Fractions of 2 ml were collected and assayed for casein kinase activity (●———●) The elution volume of marker proteins is represented against the log of its M_r (○———○) Protein kinase activity is given as units of casein kinase per ml

TABLE I

SUMMARY OF PURIFICATION OF CASEIN KINASES FROM RAT LIVER CYTOSOL

Specific activity equals nmol ^{32}P incorporated into substrates/min per mg protein Casein kinase 1, CK-1, casein kinase 2, CK-2

Fraction	Total protein (mg)	Specific activity			
		Endogenous substrate	Casein	Purification **	Yield
Crude extract	15317	0 06 *	0 11 *	1	100
100 000 X *g* supernatant	9880	0 10 *	0 14 *	1 3	82 1
60% $(NH_4)_2SO_4$ ppt	5781	0 15 *	0 18 *	1 6	61 8
1st Phosphocellulose					
1st peak	2598	0 05 *	0 07 *	0 6	10 8
2nd peak	686	0 13	0 15	1 4	6 1
3rd peak (CK-1)	24	2 2	16 05	145 9	22 9
4th peak (CK-2)	13	2 9	22 37	203 4	17 3
Casein-Sepharose 4B					
CK-1	1 3	0 0	93 0	845 0	7 2
CK-2	1 6	0 0	36 2	329 0	3 5
2nd Phosphocellulose					
CK-1	0 12	0 0	198 7	1806 0	1 5
CK-2	0 60	0 5	58 4	531 0	2 1

* A 10-fold dilution of the samples was performed in homogenization buffer before assaying the activity

** Purification and yield were calculated relative to the total amount of casein kinase activity present in the crude extract

at 35 000 and 190 000, respectively, by gel filtration in Bio-Gel A 1.5 m as indicated under 'Experimental procedures' (Fig. 3). A single symmetrical peak of casein kinase activity was obtained in each case. When the peak fractions from each kinase were concentrated in an Amicon cell fitted with an UM-10 membrane, then dialyzed against buffer A and assayed for protein substrate specificity, the results obtained were similar to those reported in Table II.

Nevertheless, when casein kinase 1 and casein kinase 2 were analyzed by polyacrylamide gel electrophoresis in the presence of SDS, several bands were observed, especially with casein kinase 2. These results indicate that they are not homogeneous, although the possibility of their being composed of different subunits cannot be ruled out

Substrate preference, cyclic AMP-dependence and the effect of cyclic AMP-dependent protein kinase inhibitor protein. Purified casein kinase 1 and 2 preferred casein and phosvitin to histone and protamine as substrate acceptor proteins (Table II). They also phosphorylated I-form glycogen synthase and, when assayed under the same conditions, the latter was as good a subtrate as casein for both kinases. However, phosphorylase *b* was not a substrate for these kinases under our assay conditions (Table III). When the standard assay conditions described for phosphorylase kinase were used [28], the peak excluded from the first phosphocellulose promoted a conversion of phosphorylase *b* to *a*, increasing the −AMP/+AMP activity ratio from 10 to 33% in 30 min at either pH 6.8 or 8.2, whereas no conversion was observed with either casein kinase 1 or casein kinase 2.

TABLE II

SUBSTRATE SPECIFICITY AND EFFECTS OF CYCLIC AMP AND CYCLIC AMP-DEPENDENT PROTEIN KINASE INHIBITOR PROTEIN ON CASEIN KINASE 1 AND CASEIN KINASE 2

Activity is pmol ^{32}P incorporated into substrates/min per ml reaction mixture

Protein substrate	Concentration in assay mg/ml	Activity					
		Casein kinase 1			Casein kinase 2		
		—cAMP	+cAMP *	+cAMP * +inhibitor **	—cAMP	+cAMP *	+cAMP * +inhibitor **
Casein	4 0	601	476	516	528	564	488
Phosvitin	4 0	376	336	408	572	580	532
Histone II A	4 0	12	8		16	8	
Protamine	4 0	0 4	1 2		0	0	
Bovine serum albumin	4 0	0	2 8		2	1 6	
Casein	0 1	72			66		
Glycogen synthase I	0 1	63	39	67	54	42	62

* When added, cyclic AMP concentration was 2 10^{-5} M

** Cyclic AMP-dependent protein kinase inhibitor protein concentration was 0 5 mg/ml

TABLE III

EFFECT OF Ca^{2+}, CALMODULIN, EGTA AND EDTA ON CASEIN KINASE 1 AND CASEIN KINASE 2

Protein kinase activity was assayed using EDTA (0 5 mM) and EGTA (0 3 mM) in the assay mixture only where indicated, Ca^{2+} and calmodulin concentrations were 0 5 mM and 10 mg/ml, respectively, when added When casein (4 0 mg/ml) was the substrate, the reaction was run for 10 min, whereas when glycogen synthase (0 1 mg/ml) and phosphorylase *b* (4 0 mg/ml) were used, the reaction was run for 30 min

Kinase	Protein substrate	Additions				
		None	+EDTA +EGTA	Ca^{2+} pmol/min per ml assay	Calmodulin	Calmodulin $+Ca^{2+}$
CK-1	Casein	242 8	285 7	230 0	210 0	190 6
	Glycogen synthase I	21 4	29 6	19 6	20 6	19 9
	Phosphorylase *b*	0	0 4	0	0 5	0 2
CK-2	Casein	300 5	372 6	251 4	305 4	303 1
	Glycogen synthase I	65 8	65 6	64 3	65 0	66 3
	Phosphorylase *b*	1 5	1 3	0	1 5	0

None of the activities of casein kinase 1 and casein kinase 2 on any of the substrates assayed (Table II) were stimulated by cyclic AMP at concentrations ($2 \cdot 10^{-5}$ M) known to produce a 3-fold stimulation of a crude preparation of rat liver cyclic AMP-dependent protein kinase [25]. Cyclic AMP-dependent protein kinase inhibitor protein did not produce any inhibition of either casein kinase 1 or casein kinase 2 when assayed with either casein, phosvitin or I-form glycogen synthase as a substrate. The same inhibitor preparation caused over 90% inhibition of the activity of a crude preparation of rat liver cyclic AMP-dependent protein kinase [25].

Effect of Ca^{2+} and calmodulin The activities of casein kinase 1 and casein kinase 2 were not stimulated by Ca^{2+} in either the absence or the presence of calmodulin when casein or glycogen synthase were used as substrates (Table III). No phosphorylase *b* kinase activity was observed even under those conditions. The lack of Ca^{2+} stimulation was not due to the presence of saturating endogenous Ca^{2+} levels since the activities of casein kinase 2 did not decrease when EGTA and EDTA were present in the assay mixture.

Heat stability of casein kinase 1 and 2. Incubation at 50° C has been used as a criterion for the characterization of the activity of protein kinases against different substrates [4,8,11]. When casein kinase 1 and casein kinase 2 were incubated at 50° C in buffer A, the activities on casein and glycogen synthase I of each enzyme decreased at the same rate to about 10% with casein kinase 1 and 20% with casein kinase 2 after 20 min.

Kinetic properties. K_m values of casein kinase 1 and casein kinase 2 for casein, phosvitin, glycogen synthase I and ATP, as well as their K_a for Mg^{2+} are indicated in Table IV. For the calculation of K_m for the protein substrates molecular weights of 23 000, 42 000 and 340 000 were assumed for casein, phosvitin and glycogen synthase (tetrameric form), respectively. It can be observed that casein kinase 1 had a somewhat higher affinity for casein and

TABLE IV

KINETIC CONSTANTS OF CASEIN KINASE 1 AND CASEIN KINASE 2

Substrate(s) concentration required for half-maximal activity (K_m) and concentration of activator necessary for half-maximal activation (K_a) were obtained from the double-reciprocal plots of duplicated measurements using 0 5 units/ml of casein kinase 1 or casein kinase 2 The appropriate concentration of the substrate or effector was varied The protein concentrations were varied between 0 1 and 4 0 mg/ml for casein and phosvitin, whereas for glycogen synthase they were between 0 01 and 0 18 mg/ml In these cases ATP and Mg^{2+} concentrations were kept at 0 125 mM and 8 0 mM, respectively When Mg^{2+} was varied between 0 1 and 8 0 mM, casein and ATP concentrations were 4 0 mg/ml and 0 125 mM, respectively

Parameter	Casein kinase			
	Casein kinase 1		Casein kinase 2	
	mg/ml	μM	mg/ml	μM
K_m casein	0 3	15	0 6	28
K_m phosvitin	0 5	12	0 2	5
K_m glycogen synthase	0 15	0 44	0 13	0 40
K_m ATP		16		13
K_a Mg^{2+}		800		2000

Mg^{2+} than casein kinase 2, while the affinity of casein kinase 2 for phosvitin was higher than that of casein kinase 1. The K_m for ATP and glycogen synthase of both kinases were very similar

Effect of salt concentration. The activities of both casein kinase 1 and casein kinase 2 were greatly affected by the presence of KCl in the assay (Fig. 4). The effect of salt on both enzymes depended upon the protein substrate used. Whereas the phosphorylation of casein by casein kinase 1 was stimulated by salt concentrations that inhibited its phosphorylation by casein kinase 2, the activity of casein kinase 1 on phosvitin was more sensitive to salt inhibition than that of casein kinase 2.

The phosphorylation of glycogen synthase by both kinases was always inhibited by the presence of salt. Nevertheless, the activity of casein kinase 1 on this substrate was more sensitive to the presence of salt than that of casein kinase 2.

Independence ratio change of glycogen synthase by phosphorylation The phosphorylation of glycogen synthase I by casein kinase 1 and casein kinase 2 correlated with a decrease in the glycogen synthase independence ratio (Fig 5) However, the amount of ^{32}P incorporated/mol 85 000 dalton subunit of glycogen synthase averaged 3.6 ± 0.3 mol when casein kinase 1 was used (see also Ref. 25), whereas it was only 1.9 ± 0.2 mol with casein kinase 2. Data are mean ± S.D. of six experiments using two different preparations of glycogen synthase and kinase.

When compared in terms of the maximum independence ratio change of the synthase promoted by the kinases, the differences were less marked. Whereas the independence ratio of the synthase decreased to 15 ± 3 when phosphorylated by casein kinase 1, it decreased to 23 ± 3 when casein kinase 2 was used.

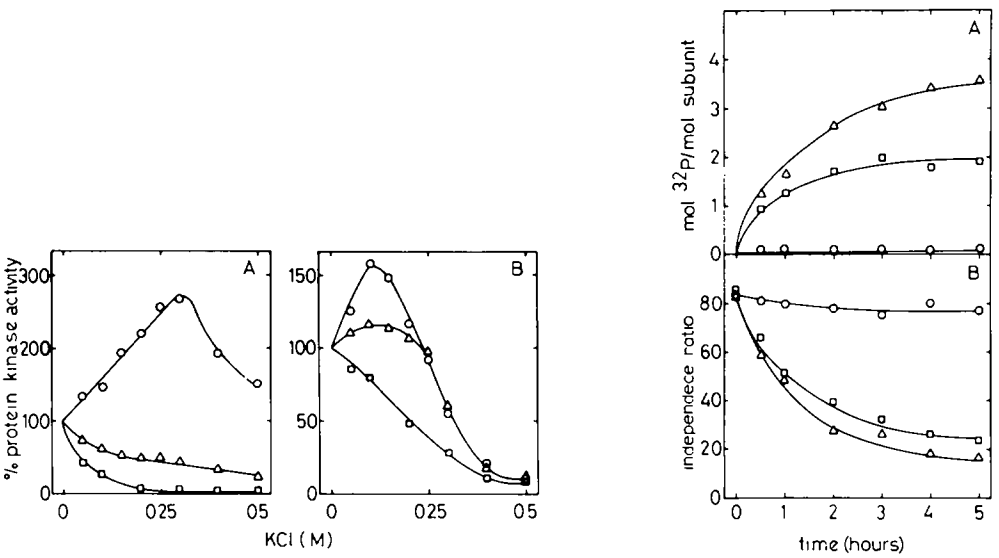

Fig. 4 Effect of KCl on casein kinase 1 (A) and casein kinase 2 (B). The activity of casein kinase 1 and casein kinase 2 was measured under the standard assay conditions using casein (○———○), phosvitin (△———△) or I-form glycogen synthase (□———□) as a substrate in the presence of increasing concentrations of KCl Percentages are expressed relative to activities obtained with each substrate in the absence of KCl

Fig 5 Phosphorylation (A) and change in glucose 6-phosphate dependence, (B) of glycogen synthase by casein kinase 1 and casein kinase 2. The reactions were carried out using casein kinase 1 (△———△) or casein kinase 2 (□———□) The control experiment was carried out using glycogen synthase alone (○———○). ^{32}P incorporation per 85 000 subunit of glycogen synthase and independence ratio were determined at indicated times

Effect of casein and glycogen phosphorylase b on glycogen synthase inactivation by casein kinase 1 and 2. Since it has been reported that glycogen synthase phosphorylation by rabbit muscle phosphorylase kinase was prevented

TABLE V

EFFECT OF CASEIN AND PHOSPHORYLASE ON GLYCOGEN SYNTHASE I TO D CONVERSION

Glycogen synthase I (0 1 mg/ml) was incubated in the presence and absence of 0 8 units/ml of either casein kinase 1 or casein kinase 2 supplemented with 4 0 mg/ml casein or 4 0 mg/ml phosphorylase *b* as indicated Data are means of two separate experiments with two different preparations of each protein kinase

Protein kinase	Additions	Independence ratio		
		20 min	40 min	60 min
none	none	77	75	80
	casein	74	78	75
	phosphorylase *b*	76	78	76
Casein kinase 1	none	43	35	22
	casein	74	73	70
	phosphorylase *b*	45	36	24
Casein kinase 2	none	46	35	29
	casein	61	47	43
	phosphorylase *b*	48	36	31

by the addition of phosphorylase *b* to the reaction mixture [16], the ability of glycogen phosphorylase and casein to inhibit the inactivation of glycogen synthase by casein kinase 1 and casein kinase 2 was tested (Table V). The addition of 4 0 mg/ml of phosphorylase *b* did not modify the rate of glycogen synthase inactivation promoted by either casein kinase 1 or casein kinase 2. On the contrary, casein at 4.0 mg/ml prevented the inactivation almost completely in the case of casein kinase 1 and partially in the case of casein kinase 2 Because of the large amount of ^{32}P incorporated into casein, the phosphorylation of glycogen synthase in the presence of 4.0 mg/ml of casein was not measured. Thus, casein, but not phosphorylase *b*, could interfere with the ability of glycogen synthase to be modified by either casein kinase 1 or casein kinase 2. This would be expected if, in both cases, casein and glycogen synthase kinase activities resided in a single kinase, other than phosphorylase *b* kinase.

Discussion

The aim of the work presented here was to characterize the two cyclic AMP-independent casein/glycogen synthase kinases (casein kinase 1 and casein 2) purified from rat liver and to compare their properties with those of cyclic AMP-dependent protein kinase, phosphorylase kinase and casein kinases whose presence in mammalian tissues has been previously described

Several criteria used to distinguish between casein kinase 1 and casein kinase 2 showed that they were clearly different in molecular weight, response to KCl and kinetic parameters for casein, phosvitin and Mg^{2+} Both kinases were able to phosphorylate glycogen synthase, however, the total number of phosphates incorporated by casein kinase 2 was about one-half of those incorporated by casein kinase 1.

The results obtained in substrate specificity and effect of cyclic AMP-dependent protein kinase inhibitor protein, summarized in Table III, make it difficult to conceive that casein kinase 1 or casein kinase 2 could correspond to, or derive from, the cyclic AMP-dependent protein kinase.

Rabbit muscle phosphorylase kinase may also phosphorylate glycogen synthase in a reaction that is stimulated by the presence of Ca^{2+} and calmodulin and inhibited by EGTA [12—15,36]. Much less is known about phosphorylase kinase from liver. However, it has recently been demonstrated that its activity on phosphorylase *b* was inhibited by 0 05 mM EGTA and stimulated by 10^{-6} M concentrations of Ca^{2+} [37]. Our results clearly rule out the possibility of glycogen synthase phosphorylation by casein kinase 1 and casein kinase 2 being due to the presence of contaminating phosphorylase kinase in the casein kinase 1 and casein kinase 2 preparations, since neither 1 nor 2 phosphorylated phosphorylase *b* or promoted any increase in phosphorylase —AMP/+AMP activity ratio under any of the conditions assayed Furthermore, synthase phosphorylation by casein kinase 1 and casein kinase 2 was not stimulated by 0.5 mM Ca^{2+}/10 μg/ml calmodulin, or a combination of the two, nor was it inhibited by 0.3 mM EGTA, showing that such a phosphorylation was not due to contamination of glycogen synthase by phosphorylase kinase Similar results were obtained with casein as a substrate, indicating that neither

casein kinase 1 nor casein kinase 2 activity corresponds to calmodulin-dependent protein kinase.

Soderling et al. [9] reported the presence in rabbit muscle of a cyclic AMP-independent synthase kinase distinguishable from casein kinase by its instability at 50°C, among other criteria However, two lines of evidence suggest that casein and glycogen synthase kinase activity of casein kinase 1 and casein kinase 2 could reside, in each case, in a single protein kinase.

(1) There is a parallel decrease in activity on both substrates when the kinases are incubated at 50°C

(2) The addition of casein prevents the inactivation of glycogen synthase by casein kinase 1 and casein kinase 2, presumably acting as an alternate substrate

The comparison of casein kinase 1 and casein kinase 2 with the phosvitin/casein kinases previously reported as present in mammalian tissues is of obvious interest. There has been a great diversity in the particular properties studied by the various groups and, thus, a general criterion cannot be established. Nevertheless, the enzymes from different sources [22,38,39] can be classified into two types on the basis of their molecular weights and subunit composition. Type I kinases have a molecular weight of 37 000—42 000 and contain a single type of subunit, whereas type II kinases have a much larger molecular weight (123 000—168 000 or even 250 000) containing two or three different types of subunits The presence of multiple forms of casein kinases in rabbit muscle has been reported [3,4]. One of these enzymes, having a molecular weight of 34 000, has been shown to phosphorylate glycogen synthase incorporating up to 4 mol of ^{32}P/mol 85 000 dalton subunit. Such a phosphorylation leads to a decrease in the independence ratio to 5% or less, affecting the kinetic properties of the enzyme [5]. The multiplicity of rabbit muscle casein/glycogen synthase kinases has also been reported by De Paoli-Roach et al. [11], who indicate that these enzymes are different from phosphorylase kinase and are not affected by Ca^{2+}/calmodulin. However, no molecular or kinetic data are given by the authors for comparison.

Rat liver cytosol has been shown to contain two types of casein kinases which differ in their catalytic properties [24]. On the other hand, a phosvitin/casein kinase with a molecular weight of 150 000 has also been described as present in this tissue [40]. However, this last enzyme is different from casein kinase 2 and other Type I and II casein kinases [22,38,39] in that it has a much higher K_m for ATP

When considering all the data previously reported along with those we obtained with casein kinase 1 and casein kinase 2, it may be conjectured that these enzymes correspond to the type I and II casein kinases present in other mammalian tissues

The fact that both casein kinase 1 and casein kinase 2 are able to phosphorylate and inactivate glycogen synthase in a cyclic AMP-independent manner, suggests that these enzymes could play a part in the control of hepatic glycogen metabolism by α-adrenergic agents which do not produce changes in the levels of cyclic AMP or in the activity of cyclic AMP-dependent protein kinase [42,43].

Since casein kinase 1 and casein kinase 2 activities on glycogen synthase are affected by KCl concentrations in the physiological range, the possibility of K^+

acting as a modulator of their activities 'in vivo' cannot be disregarded. Recent reports [44,45] indicating that changes in the fluxes of K^+ may play a role in the α-adrenergic stimulation of hepatic glycogenolysis [44] underline this possibility.

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