Cyclic AMP-independent casein/glycogen synthase kinases from pig polymorphonuclear leucocytes

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1. Two cyclic AMP-independent casein/glycogen synthase kinases were purified from pig polymorphonuclear leucocytes by chromatography on phosphocellulose followed by affinity chromatography on casein–Sepharose 4B or gel filtration on Bio-Gel A-1.5 m. When the affinity step was used, the specific activities were 86 and 43 units/mg of protein for casein kinase 1 and 2, respectively, whereas these values were 94 and 90 units/mg of protein when the gel-filtration step was used. 2. These kinases differ as follows: (a) the molecular weight of casein kinase 1 (38 000) is very much lower than that of casein kinase 2 (185 000); (b) the K_m for casein (0.46 mg/ml) and K_a for Mg^{2+} (0.3 mM) of casein kinase 1 are lower than those of casein kinase 2 (0.90 mg/ml and 1.7 mM respectively); (c) KCl stimulates the phosphorylation of casein by casein kinase 1, whereas it inhibits phosvitin phosphorylation by this enzyme; on the contrary, the effect of KCl on casein kinase 2 is very similar with either casein or phosvitin as substrate; (d) although both kinases phosphorylate rabbit muscle glycogen synthase I, the ratio of glycogen synthase to casein phosphorylation by casein kinase 1 is about 4-fold greater than that by casein kinase 2. Furthermore, ^{32}P incorporation into glycogen synthase promoted by casein kinase 1 (3.6 mol of ^{32}P /mol of 85 000-dalton subunit) is twice that observed with casein kinase 2 (1.8 mol of ^{32}P /mol of 85 000-dalton subunit). Such a phosphorylation results in a decrease in the glucose 6-phosphate-independence ratio of glycogen synthase to 10–15 with casein kinase 1 and to 35–45 with casein kinase 2. 3. The activity of both kinases is neither stimulated by cyclic AMP, Ca^{2+} and calmodulin nor inhibited by cyclic AMP-dependent protein kinase inhibitor protein. 4. No phosphorylation kinase activity was observed with casein kinase 1 and 2 at either pH 6.8 or 8.2 in the presence of Ca^{2+} . 5. Activities of both kinases on casein and glycogen synthase decreased in parallel when incubated at 50°C.

Glycogen synthase (EC 2.4.1.11) has long been recognized to exist as forms with different phosphate contents, which determine their glucose 6-phosphate dependence (Villar-Palasi & Larner, 1961; Roach & Larner, 1976). The less phosphorylated of these forms (I-form) is independent of glucose 6-phosphate for activity, whereas the highly phosphorylated form (D-form) is totally dependent on the presence of this sugar phosphate.

The presence of these two forms was initially reported in human polymorphonuclear leucocytes, where they could be interconverted *in vitro* by phosphorylation/dephosphorylation reactions catalysed by endogenous protein kinases and phos-

phatases (Solling & Esmann, 1977). The presence of these two forms has also been demonstrated in pig polymorphonuclear leucocytes (Pegueroles & Cussó, 1979).

Three types of protein kinases have been shown to phosphorylate glycogen synthase in rabbit muscle: (i) cyclic AMP-dependent protein kinase or its catalytic subunit (EC 2.7.1.37) (Schlender *et al.*, 1969; Soderling *et al.*, 1970), (ii) cyclic AMP-independent casein/phosvitin kinases (EC 2.7.1.37) (Itarte *et al.*, 1977; Itarte & Huang, 1979; De Paoli-Roach *et al.*, 1979b) and (iii) phosphorylase kinase (EC 2.7.1.38) (De Paoli-Roach *et al.*, 1979a; Embi *et al.*, 1979). However, the extent of phos-

phate incorporation as well as the glucose 6-phosphate-independence-ratio change achieved by these kinases was different. Of these enzymes, only a cyclic AMP-independent casein kinase has been demonstrated to catalyse a total phosphorylation and conversion of glycogen synthase into its D-form (Itarte *et al.*, 1977; Itarte & Huang, 1979), at physiological pH, in the absence of any other protein kinase. The presence of such a kinase has also been reported in rat liver by our group (Itarte *et al.*, 1979).

Cyclic AMP-independent glycogen synthase kinase activity has also been recognized in a variety of other tissues, where it seems to account for a great percentage of the total glycogen synthase kinase activity (Schlender & Reimann, 1977).

In the present study the purification and characterization of cyclic AMP-independent casein/glycogen synthase kinases from pig polymorphonuclear leucocytes was undertaken.

Experimental

Materials

Blood was taken from pig jugular vein in the slaughterhouse; EDTA was used as anticoagulant. Pig polymorphonuclear leucocytes were isolated as described by Esmann (1964). The sources of all other materials used are those indicated by Itarte *et al.* (1979).

Enzyme assays

Protein kinase activity was measured at 30°C as described previously (Itarte *et al.*, 1977) in either the presence or absence of 20 μ M-cyclic AMP. When phosphorylase and glycogen synthase were the substrates their concentrations were 5 mg/ml and 0.2 mg/ml respectively. In all other cases protein substrate concentration was 4 mg/ml. One unit of casein kinase is defined as that amount catalysing the transfer of 1 nmol of phosphate from [γ -³²P]-ATP to casein/min at 30°C.

Glycogen synthase activity was determined at 30°C as described by Thomas *et al.* (1968). RI (independence ratio) is defined as the ratio of glycogen synthase activity in the absence of glucose 6-phosphate to that in the presence of 7.2 mM-glucose 6-phosphate, the result being multiplied by 100.

Glycogen phosphorylase activity was measured in either the absence or presence of AMP as indicated by Gilboe *et al.* (1972). One unit of glycogen phosphorylase *a* is that amount incorporating 1 μ mol of glucose 1-phosphate into glycogen/min at 30°C in the absence of AMP.

Phosphorylase kinase activity was determined as the rate of conversion of glycogen phosphorylase *b* into *a*; assays were carried out at either pH 6.8 or

8.2 in the presence of 0.5 mM-CaCl₂ with glycogen phosphorylase (5 mg/ml) as substrate. All other assay conditions were those described by Cohen (1973).

Purification of enzymes

Cyclic AMP-independent casein kinases were purified as follows, all procedures being performed at 4°C. About 1.2×10^{10} isolated pig polymorphonuclear leucocytes were homogenized in 60 ml of 50 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-EDTA, with a glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 27 000 *g* for 15 min. The resulting supernatant was centrifuged for 90 min at 50 000 *g*. The high-speed supernatant was adjusted to 60% saturation with 100%-satd. (NH₄)₂SO₄ and after gentle stirring for 20 min it was centrifuged at 25 000 *g* for 20 min. The precipitate was resuspended in 5 ml of 50 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-DL-dithiothreitol, 5% (v/v) glycerol and 0.1 mM-phenylmethanesulphonyl fluoride (buffer A) and extensively dialysed against the same buffer.

Dialysed material from the 60%-satd.-(NH₄)₂SO₄ precipitation was applied to a phosphocellulose column (2 cm $\times$ 4 cm) equilibrated with buffer A. The column was washed with 60 ml of buffer A, followed by 50 ml each of 0.1 M-KCl, 0.2 M-KCl and 0.3 M-KCl in buffer A, and finally a 100 ml linear gradient of 0.3–1.5 M-KCl in buffer A; 2 ml fractions were collected and samples were taken for protein kinase assay. Fractions corresponding to the casein kinases eluted at about 0.55 M-KCl and 0.70 M-KCl were pooled. Two alternative separation steps were used to achieve a better resolution and purification of both enzymes. In the first, pooled fractions from phosphocellulose were extensively dialysed against buffer A and then applied to a casein–Sephacrose 4B column (1.1 cm $\times$ 10 cm) equilibrated with the same buffer. The column was washed with 30 ml of buffer A, followed by a 30 ml linear gradient of 0–1 M-KCl in the same buffer. Fractions (1 ml) were collected and assayed for protein kinase activity. Fractions corresponding to the casein kinases eluted at about 0.25 M-KCl and 0.45 M-KCl were pooled separately, diluted with 2 vol. of buffer A and each was applied to a phosphocellulose column (1.1 cm $\times$ 2 cm) equilibrated with the same buffer. The column was washed with 5 ml of 0.4 M-KCl in buffer A and then the enzymes were eluted in each case with 5 ml of 1 M-KCl in the same buffer. Fractions (1 ml) were collected and those containing casein kinase activity were pooled.

Alternatively, pooled fractions from the first phosphocellulose column were applied to a Bio-Gel A-1.5 m column (1.5 cm $\times$ 90 cm) equilibrated with buffer A containing 0.4 M-KCl. The column was then washed with the same buffer and 2 ml fractions were

collected. Fractions corresponding to each of the two casein kinase peaks were pooled separately, diluted with 1 vol. of buffer A and chromatographed on a second phosphocellulose column as indicated above. In both cases, the final enzyme preparations were extensively dialysed against buffer A and stored at -40°C .

Glycogen synthase I form was purified from fresh rabbit skeletal muscle as previously described (Takeda *et al.*, 1975). The specific activity of the enzyme was $25\text{--}30\ \mu\text{mol}/\text{min}$ per mg of protein at 30°C , with an independence ratio (RI) of 82.

Glycogen phosphorylase was purified from rabbit muscle as previously described (Fischer & Krebs, 1958). The enzyme preparation had a specific activity of $132\ \mu\text{mol}/\text{min}$ per mg of protein and a $-\text{AMP}/+\text{AMP}$ activity ratio of 0.02.

A crude preparation of pig polymorphonuclear leucocytes, corresponding to the $27\,000\text{g}$ supernatant, obtained as indicated above, was used as a source of phosphorylase kinase activity.

Calmodulin was prepared from purified rabbit muscle phosphorylase kinase as described by Shenolikar *et al.* (1979).

Miscellaneous

Protein was measured by the modification (Bensaoud & Weinstein, 1976) of the method of Lowry *et al.* (1951).

Molecular weights of casein kinase 1 and 2 were determined by gel filtration in a $1.5\text{cm} \times 90\text{cm}$ Bio-Gel A-1.5m column equilibrated with buffer A containing 0.4M -KCl. Aldolase (mol.wt. 150000),

bovine serum albumin (mol.wt. 68000), ovalbumin (mol.wt. 43000) and chymotrypsinogen A (mol.wt. 25700) were used as marker proteins.

Results

Purification

Pig leucocytes contained different types of histone kinases and casein kinases, which could be partially resolved by chromatography on phosphocellulose (Fig. 1). The first three peaks of protein kinase activity, corresponding to the flow-through fraction and those eluted at 0.12M -KCl and 0.3M -KCl, contained both histone kinase and casein kinase to varying degrees.

Two other protein kinases, which phosphorylated casein but not histone, were eluted at 0.55M -KCl and 0.70M -KCl. According to their order of elution we will refer to these kinases as casein kinase 1 and 2 respectively.

The resolution of these casein kinases was not complete on phosphocellulose. However, a clear separation as well as a greater purification of both enzymes could be achieved when the phosphocellulose peak containing both kinases was either chromatographed on casein-Sepharose 4B (Fig. 2a) or gel-filtered in Bio-Gel A-1.5m (Fig. 2b). Two types of casein kinase, eluted from casein-Sepharose 4B at 0.2M -KCl and 0.5M -KCl, were resolved in the first case. A third minor peak of casein kinase, which was not adsorbed to the column, was also observed. When peak fractions from phosphocellulose corresponding to casein kinase 1 alone were

Fig. 1. Separation of cytoplasmic protein kinases on phosphocellulose

The resuspended $(\text{NH}_4)_2\text{SO}_4$ precipitate was applied to a phosphocellulose column ($2\text{cm} \times 4\text{cm}$). The column was washed with buffer A, followed by 0.1M -, 0.2M - and 0.3M -KCl and a linear gradient of $0.3\text{--}1.5\text{M}$ -KCl in buffer A. Fractions (2ml) were assayed for ($\square$) histone kinase activity in the presence of $20\ \mu\text{M}$ -cyclic AMP, ($\triangle$) casein kinase activity in the absence of cyclic AMP and (---) KCl concentration.

Fig. 2. Resolution of casein kinases on casein-Sepharose 4B (a) and Bio-Gel A-1.5m (b)

The pooled fractions from the phosphocellulose column were applied to (a) a casein-Sepharose 4B column (1.1 cm × 10 cm) or (b) a Bio-Gel A-1.5m column (1.5 cm × 90 cm). In (a) the column was washed with buffer A and a linear gradient of 0–1 M-KCl in the same buffer. In (b) the column was washed with 0.4 M-KCl in buffer A. In both cases fractions were assayed for (○) casein kinase activity in the absence of cyclic AMP. The broken line in (a) indicates KCl concentration. In (b), the elution volumes of marker proteins are plotted against log of the molecular weight (●).

applied to casein-Sepharose 4B, a major peak of casein kinase eluted at 0.5 M-KCl was obtained (results not shown). In contrast, when those phosphocellulose fractions corresponding to casein kinase 2 alone were applied to the column, the major activity peak was eluted at 0.2 M-KCl (results not shown). Thus the casein kinase eluted at 0.5 M-KCl was considered as casein kinase 1, and that eluted at 0.2 M-KCl corresponded to casein kinase 2.

Gel filtration chromatography on Bio-Gel A-1.5m of the phosphocellulose peak containing both casein kinases 1 and 2 also resolved two clearly differentiated peaks of kinase activity (Fig. 2b). By separate chromatography of phosphocellulose peak fractions corresponding to casein kinases 1 and 2 it was observed that the first peak obtained by gel filtration corresponded to casein kinase 2, whereas casein kinase 1 was eluted as the second peak (results not shown).

Data on the purification of casein kinases 1 and 2 are given in Table 1. The specific activity of the casein kinase 1 preparation obtained by either affinity chromatography or gel filtration was similar, whereas that of casein kinase 2 was higher when the gel-filtration step was used.

Molecular weight

Molecular weights of casein kinase 1 and 2 were estimated as 38 000 and 185 000 respectively by gel filtration in Bio-Gel A-1.5m (Fig. 2b). Identical results were obtained for each kinase independently of the method used for their purification.

Catalytic properties

Casein kinase 1 and 2 catalysed a time-dependent phosphorylation of casein and phosvitin (Figs. 3a and 3b). Both kinases were free of endogenous protein substrates and showed little activity (less than 5% of that on casein) on histone II A.

I-form glycogen synthase was also a substrate for both kinases (Figs. 3c and 3d); however, the glycogen synthase/casein kinase activity ratio of casein kinase 1 was at least 2–3-fold higher than that of casein kinase 2. When assayed at 0.2 mg of casein/ml, the activities of casein kinase 1 and 2 were 1/2.7 and 1/4.5 of those observed at 4.0 mg/ml. Thus, at this low protein substrate concentration the ratio of glycogen synthase/casein kinase activity was 0.9 for casein kinase 1 and 0.4 for casein kinase 2. None of these kinases phosphorylate b-form glycogen phosphorylase to any significant extent under the standard assay conditions.

The activity of casein kinases 1 and 2 on any of the substrates assayed was not stimulated by the presence of cyclic AMP. Cyclic AMP-dependent protein kinase inhibitor protein, purified as described by Walsh *et al.* (1971), did not inhibit either casein kinase 1 or 2 activity. Ca²⁺, calmodulin or a combination of both did not stimulate phosphorylation of casein and glycogen synthase by either casein kinase 1 or 2 (Table 2). On the other hand, EGTA plus EDTA, at concentrations of 0.3 mM and 0.5 mM respectively, did not inhibit such phosphorylations.

The K_m for casein of casein kinase 1 was determined as 0.46 mg/ml and that of casein kinase 2 was 0.90 mg/ml. The K_m values for ATP of casein kinases 1 and 2 were determined as 14 μ M and 15 μ M

Table 1. *Summary of purification of casein kinases from pig polymorphonuclear leucocytes*

Specific activity is given as nmol of ^{32}P incorporated into casein/min per mg of protein. In (a) the casein-Sepharose 4B step was used after the first phosphocellulose column, whereas in (b) the Bio-Gel A 1.5 m step was used instead. The results in (a) and (b) refer to the activity of the enzymes after concentration by phosphocellulose and dialysis against buffer A.

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)
Crude extract	463	92.6	0.20	1
50000g supernatant	340	88.4	0.26	1.3
60%-satd.-(NH_4) ₂ SO ₄ ppt.	142	82.4	0.58	2.9
First phosphocellulose				
Casein kinase 1	3.6	30.2	8.5	42.5
Casein kinase 2	3.1	22.0	7.1	35.5
(a) Casein-Sepharose 4B				
Casein kinase 1	0.21	18.1	86	430
Casein kinase 2	0.10	4.3	43	215
(b) Bio-Gel A 1.5 m				
Casein kinase 1	0.072	6.8	94	470
Casein kinase 2	0.082	7.4	90	450

Table 2. *Effect of Ca^{2+} , calmodulin and cyclic AMP-dependent protein kinase inhibitor on activity of casein kinases 1 and 2*

Activity of casein kinases 1 and 2 was assayed under standard assay conditions, except for the absence of EDTA and EGTA when indicated. When added, EDTA and EGTA concentrations were 0.5 mM and 0.3 mM respectively. Ca^{2+} was 0.5 mM, calmodulin was 10 $\mu\text{g}/\text{ml}$ and cyclic AMP-dependent protein kinase inhibitor was 0.5 mg/ml when indicated. Activity is given as pmol of ^{32}P incorporated/min per ml of assay mixture.

Additions	Casein kinase 1		Casein kinase 2	
	Casein	Glycogen synthase I	Casein	Glycogen synthase I
None	195.3	51.3	475.4	38.9
Ca^{2+}	151.8	49.9	438.9	35.2
Calmodulin	152.4	48.1	506.2	36.4
Ca^{2+} + calmodulin	175.7	46.7	456.3	34.8
EDTA + EGTA	182.7	48.4	464.2	34.8
Cyclic AMP-dependent protein kinase inhibitor	193.2	51.7	476.8	38.4

respectively. A great difference was observed in the K_a for Mg^{2+} of casein kinases 1 and 2 (0.3 mM and 1.7 mM respectively). Furthermore, casein phosphorylation by casein kinase 2 was always stimulated by the increasing Mg^{2+} concentrations assayed, whereas the activity of casein kinase 1 was maximal in the range 2–8 mM- Mg^{2+} , higher concentrations being inhibitory.

Heat-inactivation

Activity of casein kinases 1 and 2 on casein and glycogen synthase decreased drastically on preincubation of the kinases at 50°C. In both cases, there was a similar decrease in casein kinase and glycogen synthase kinase activity. However, casein kinase 2 required longer preincubation times than casein kinase 1 to become inactivated.

Effect of KCl

The activities of casein kinases 1 and 2 were differently affected by KCl, and this effect depended on the protein substrate used (Figs. 4a and 4b). Although the phosphorylation of casein by both casein kinase 1 and 2 was stimulated by KCl, the salt effect on casein kinase 1 was maximum at KCl concentrations lower than those required for casein kinase 2. With phosvitin as substrate, KCl always inhibited casein kinase 1 activity. In contrast, phosvitin phosphorylation by casein kinase 2 was stimulated by KCl concentrations below 0.25 M, higher concentrations being inhibitory.

When glycogen synthase was used as substrate, KCl inhibited its phosphorylation by either casein kinase 1 or 2. In both cases a 50% inhibition of

Fig. 3. *Substrate specificity and effect of cyclic AMP on the activities of casein kinases 1 (a, c) and 2 (b, d)* Casein kinase concentrations were 0.4 unit/ml in (a) and (b), and 1.0 unit/ml in (c) and (d). Exogenous protein substrate concentration was 4 mg/ml, except for glycogen synthase I and glycogen phosphorylase *b*, where concentrations of 0.2 mg/ml and 5 mg/ml were used respectively. The open and solid symbols represent the absence and presence of 20 μ M-cyclic AMP respectively. The protein substrates in (a) and (b) were: (O, ●) casein, (Δ , $\blacktriangle$) histone, (∇ , $\blacktriangledown$) phosvitin and ($\square$, $\blacksquare$) endogenous protein. In (c) and (d) protein substrates were: (O, ●) I-form glycogen synthase and (Δ , $\blacktriangle$) *b*-form glycogen phosphorylase.

glycogen synthase phosphorylation was achieved at about 0.15 M-KCl.

Glycogen synthase and phosphorylase kinase activity of casein kinases 1 and 2

Neither casein kinase 1 nor casein kinase 2 promoted any significant change in glycogen phos-

phorylase activity either at pH 6.8 or 8.2. Under similar conditions a clear increase in *a*-form glycogen phosphorylase was observed with a crude phosphorylase kinase preparation from pig leucocytes. The $-AMP/+AMP$ activity ratio increased to 29 at pH 6.8 and to 22 at pH 8.2 after incubation for 3 h with the crude extract, whereas it did not change

Fig. 4. Effect of KCl on casein kinases 1 (a) and 2 (b)

The activity of each kinase was determined under standard assay conditions, with casein (O), phosvitin (Δ) or I-form glycogen synthase ($\square$) as substrate in the presence of increasing concentrations of KCl. Data are represented as percentage activities relative to activities obtained with each substrate in the absence of KCl.

Table 3. Phosphorylation and change in RI of glycogen synthase by casein kinases 1 and 2

Reaction mixtures containing 0.2 mg of I-form glycogen synthase/ml were incubated under standard protein kinase assay conditions either alone or in the presence of 1 unit of casein kinase 1 or 2/ml. At indicated times samples were removed and assayed for ^{32}P incorporation. A parallel experiment was performed with non-radioactive ATP. Samples from this experiment were diluted with 9 vol. of 50 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-EDTA, 5 mM-EGTA and 0.1% rabbit liver glycogen and assayed for glycogen synthase independence ratio (RI) as indicated in the Experimental section.

Time (h)	Control		+Casein kinase 1		+Casein kinase 2	
	^{32}P incorporated (mol/mol of 85 000-dalton subunit)	RI	^{32}P incorporated (mol/mol of 85 000-dalton subunit)	RI	^{32}P incorporated (mol/mol of 85 000-dalton subunit)	RI
0	0.04	76	0.05	75	0.03	76
1	0.02	74	2.64	25	0.89	51
2	0.08	73	3.40	15	1.66	44
3	0.04	77	3.61	14	1.77	40
4	0.06	75	3.55	14	1.81	38

when the enzyme was incubated with either casein kinase 1 or 2.

In contrast, casein kinases 1 and 2 promoted a decrease in glycogen synthase RI (Table 3), although the maximum decrease achieved by casein kinase 1 (to about 10–15) was always greater than that obtained with casein kinase 2 (to about 35–45). No significant decrease in RI was observed with glycogen synthase in the absence of added casein kinases. In all of the cases total glycogen synthase activity remained constant throughout the preincubation time.

The decrease in glycogen synthase RI promoted by casein kinases 1 and 2 correlated with a phosphorylation of the enzyme, reaching a maximum value of 3.6 mol of ^{32}P incorporated/mol of 85 000-dalton subunit with casein kinase 1, whereas with casein kinase 2 the value was only 1.8.

Discussion

In the present study we demonstrate that pig polymorphonuclear leucocytes contain two types of casein kinase (1 and 2), both of them able to phosphorylate I-form glycogen synthase.

These enzymes can be initially distinguished by their behaviour on phosphocellulose chromatography. However, there was always some extent of overlapping between their elution peaks in this step. A better resolution of both enzymes was achieved by either gel filtration on Bio-Gel A-1.5 m or affinity chromatography on casein–Sephacrose 4B.

Clear differences were observed in the molecular and kinetic properties of casein kinases 1 and 2. (i) The molecular weight of casein kinase 1 (38 000) is about one-fifth of that of casein kinase 2 (185 000). (ii) The K_m for casein as well as the K_a for Mg^{2+} of

casein kinase 1 were lower than those of casein kinase 2. (iii) KCl affected differently the activity of both enzymes on phosvitin and casein, increasing 3.6-fold the ratio of casein/phosvitin phosphorylation by casein kinase 1 in the presence of 0.2 M-KCl, but this ratio scarcely changed for casein kinase 2 (1.1–1.3-fold) under the same conditions. This fact suggests that the salt effect on the kinase's activity could be due to differences in the interaction of the protein substrates with KCl, rendering the proteins with a higher or a lower number of specific phosphorylatable sites exposed to kinases. (iv) Casein kinases 1 and 2 also phosphorylated I-form glycogen synthase from rabbit muscle. However, the ratio of glycogen synthase/casein (or phosvitin) phosphorylation rate for casein kinase 1 is about 3-fold higher than that for casein kinase 2. Furthermore, the total ^{32}P incorporation as well as the decrease in the glucose 6-phosphate-independence ratio of the synthase observed with casein kinase 1 was much higher than that with casein kinase 2. Thus casein kinase 1 should be considered as a better glycogen synthase kinase than casein kinase 2.

In general, the characteristics of casein kinases 1 and 2 are similar to those reported by Juhl (1979) for the so-called peak-A and peak-B enzymes from human leucocytes. However, several differences were observed between casein kinase 2 and peak-B enzyme, such as molecular weight, casein/phosvitin activity ratio and glycogen synthase kinase activity. This could be due to the fact that the characterization of the enzymes from human leucocytes was carried out with preparations from the first phosphocellulose step, where cross-contamination between both kinases could not be ruled out. The specific activities of the final preparations of pig leucocyte casein kinases 1 and 2 reported in the present paper are severalfold higher than those indicated for the peak-A and peak-B enzymes (with casein as substrate) from human leucocytes (Juhl, 1979). On the other hand, they are comparable with those of casein/glycogen synthase kinase from rabbit muscle (Itarte *et al.*, 1977).

Several reports have given evidence that phosphorylase kinase may catalyse the phosphorylation of glycogen synthase (De Paoli-Roach *et al.*, 1979a; Embi *et al.*, 1979). It has also been demonstrated that a cyclic AMP-independent glycogen synthase kinase present as a trace contaminant in certain glycogen synthase preparations (Nimmo & Cohen, 1974) is in fact a modified form of phosphorylase kinase (Embi *et al.*, 1979). Concomitantly with the phosphorylation by phosphorylase kinase, the independence ratio for glycogen synthase decreases to a minimum around 35 (Embi *et al.*, 1979), at pH 8.2. This value is similar to that observed with pig leucocyte casein kinase 2, at pH 7.0. However, neither casein kinase 1 nor casein kinase 2 is

contaminated with phosphorylase kinase, for the following reasons.

(a) They do not phosphorylate phosphorylase under conditions under which they act on glycogen synthase, casein and phosvitin.

(b) They do not promote any significant change in phosphorylase activity under standard phosphorylase kinase assay conditions, at either pH 6.8 or 8.2 in the presence of Ca^{2+} . Under these conditions a marked increase in phosphorylase *a* activity was observed with a crude preparation from pig leucocytes as a source of phosphorylase kinase activity. This increase was more pronounced at pH 6.8 than at pH 8.2, as indicated in human leucocytes (Sorensen, 1979).

(c) Their activity on casein and glycogen synthase is not stimulated by 0.5 mM- Ca^{2+} , calmodulin (10 $\mu\text{g}/\text{ml}$) or both, nor is it inhibited by 0.5 mM-EDTA plus 0.3 mM-EGTA.

It has been reported that casein kinase and phosvitin kinase activity, but not glycogen synthase kinase activity, of peak-B enzyme from human leucocytes (Juhl, 1979) was relatively insensitive to inactivation at 50°C. On the other hand, it has been shown that the glycogen synthase kinase activity of the so-called PC_{0.7} casein kinase from rabbit muscle (De Paoli-Roach *et al.*, 1979b) was more stable at 50°C than its casein kinase activity. These results could indicate that the casein kinase and glycogen synthase kinase activities of these enzyme preparations may correspond to the presence of different kinases. In contrast, our results on heat-stability suggest that casein kinase and glycogen synthase kinase activities of both casein kinases 1 and 2 could be due to a single kinase, different in each case.

Thus it is concluded that pig leucocyte casein kinases 1 and 2 should be considered as two separate glycogen synthase kinases, different from phosphorylase kinase and cyclic AMP-dependent protein kinase.

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