Biochem. J. (1986) 240, 57–61 (Printed in Great Britain)

Phosphorylation of purified bovine heart and rat liver 6-phosphofructo-2-kinase by protein kinase C and comparison of the fructose-2,6-bisphosphatase activity of the two enzymes

Mark H. RIDER and Louis HUE

Hormone and Metabolic Research Unit, University of Louvain Medical School, and International Institute of Cellular and Molecular Pathology, UCL-7529, Avenue Hippocrate 75, B-1200 Brussels, Belgium

Purified bovine heart 6-phosphofructo-2-kinase can be phosphorylated in the presence of protein kinase C and dephosphorylated by alkaline phosphatase; changes in phosphorylation state have no effect on enzyme activity. By contrast, the rat liver enzyme is a poor substrate for protein kinase C. Unlike the liver enzyme, which is bifunctional and is phosphorylated by fructose 2,6-[2-^{32}P]bisphosphate, the heart enzyme contains 10 times less fructose 2,6-bisphosphatase activity and is phosphorylated at a slower rate and to a lesser extent than the liver enzyme. Both rat liver and bovine heart enzymes catalyse a similar exchange reaction between [U-^{14}C]ADP and ATP.

INTRODUCTION

6-Phosphofructo-2-kinase (PFK-2) catalyses the formation of fructose 2,6-bisphosphate (Fru-2,6-P_2), a potent stimulator of 6-phosphofructo-1-kinase (PFK-1) [1]. In rat liver PFK-2 possesses fructose 2,6-bisphosphatase (FBPase-2) activity and is therefore a bifunctional enzyme [2]. Phosphorylation of the bifunctional enzyme with cyclic AMP-dependent protein kinase results in the activation of FBPase-2 and inactivation of PFK-2 [3,4]. Bovine heart PFK-2 differs from the rat liver enzyme in a number of respects. Compared with the liver enzyme, the heart enzyme has a smaller apparent M_r and different kinetic properties, is not inactivated by cyclic AMP-dependent protein kinase, and contains 10 times less FBPase-2 activity than does liver PFK-2 [5].

In the present paper, we report further differences between liver and heart PFK-2 regarding reaction mechanism and phosphorylation by cyclic AMP-dependent protein kinase and protein kinase C.

MATERIALS AND METHODS

Materials

Chemicals were obtained as cited previously [5]. Histone IIIS and ATP–agarose were from Sigma. [γ-^{32}P]ATP and [U-^{14}C]ADP were from Amersham International. Phenyl-Sepharose was from Pharmacia, and phosphatidylserine (PS) from Polylab (Antwerp, Belgium). Phosphocellulose paper P81 was from Whatman. Calf intestine alkaline phosphatase was from Boehringer.

Protein purification

Protein kinase C was purified by chromatography on DEAE-Trisacryl, Agarose A-0.5m and phenyl-Sepharose essentially as described previously [6]. The enzyme had an initial specific activity of 0.3 unit/mg of protein and lost 40% of its initial activity on storage for 2 months at −20 °C in the presence of 20% (v/v) glycerol and 0.1% (w/v) Triton X-100.

Rat liver and bovine heart PFK-2 were purified as described previously [5]. A further purification was achieved by chromatography on ATP–agarose. The enzyme was applied on a column (2.5 cm × 5 cm) of ATP-agarose equilibrated with 20 mM-Hepes/50 mM-KCl/2 mM-EDTA/5 mM-$MgCl_2$/20% glycerol/15 mM-2-mercaptoethanol/0.1 mM-phenylmethanesulphonyl fluoride/1 mM-potassium phosphate, pH 7.5 (buffer A). PFK-2 was eluted with a linear gradient of Fru-6-*P* (0–10 mM in buffer A). The active fractions were concentrated by ultrafiltration and dialysed against 500 vol. of buffer A to remove Fru-6-*P*. The enzymes were stored at −80 °C. The preparations were more than 90% homogeneous, as judged by peak integration of a 530 nm scan of a Coomassie Blue-stained SDS/polyacrylamide gel with a Joyce–Loebl Chromoscan gel scanner.

The catalytic subunit of cyclic AMP-dependent protein kinase was purified as described previously [7].

Assay of enzyme activities

The activities of the cyclic AMP-dependent protein kinase catalytic subunit [8], protein kinase C [9], PFK-2 [10] and FBPase-2 [11] were measured as described previously. $NADP^+$ (0.125 mM), Glc-6-*P* dehydrogenase (0.2 unit) and phosphoglucoisomerase (0.4 unit) were added to the FBPase-2 assay in order to remove Fru-6-*P*, a potent inhibitor of the reaction.

One unit of enzyme is the amount that catalyses the transformation of 1 μmol of substrate/min under the assay conditions.

Protein phosphorylation

PFK-2 (10 μg) was incubated at 30 °C in a final volume of 0.2 ml containing 50 mM-Hepes, 4 mM-$MgCl_2$,

Abbreviations used: FBPase-2, fructose-2,6-bisphosphatase (EC 3.1.3.46); Fru-2,6-P_2, fructose 2,6-bisphosphate; Fru-6-*P*, fructose 6-phosphate; Glc-6-*P*, glucose 6-phosphate; PFK-1, 6-phosphofructo-1-kinase (EC 2.7.1.11); PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105); PS, phosphatidylserine.

0.1 mM-EDTA and 1 mM-dithioerythritol, pH 7.4, and the indicated amount of protein kinase. Incubations with protein kinase C included 0.25 mg of PS/ml, 1.5 mM-$CaCl_2$ and 0.5 mM-EGTA, except when otherwise stated. Reactions were initiated with 0.25 mM-[γ-^{32}P]ATP (200 c.p.m./pmol), and samples (20 μl) were removed at the indicated times for measurement of ^{32}P incorporation [8]. After 30 min, the remaining reaction mixture was denatured with 1 vol. of 0.02% (w/v) Bromophenol Blue/4% (w/v) SDS/20% glycerol/1% (v/v) 2-mercaptoethanol/0.15 M-Tris/HCl, pH 6.8, and heated at 80 °C for 30 min for SDS/polyacrylamide (10%)-gel electrophoresis [12]. Proteins were detected with Coomassie Blue before gel drying and autoradiography.

Labelling from [2-^{32}P]Fru-2,6-P_2

PFK-2 (1 μg) was incubated in 0.1 ml containing 20 mM-Tes, pH 7.5, 100 mM-KCl, 5 mM-$MgCl_2$, 1 mM-dithioerythritol, 1 mM-*sn*-glycerol 2-phosphate, 1 mM-GTP, 5 mM-potassium phosphate, 0.125 mM-$NADP^+$, 0.1 unit of Glc-6-*P* dehydrogenase, 0.2 unit of phosphoglucoisomerase and 7 μM-[2-^{32}P]Fru-2,6-P_2 (4000 c.p.m./pmol) at 30 °C. Samples (10 μl) of the reaction mixture were removed at the indicated times for determination of ^{32}P-labelled protein [8]. Radioactive Fru-2,6-P_2 was prepared as described elsewhere [11].

Dephosphorylation by alkaline phosphatase

PFK-2 which had been phosphorylated in the presence of protein kinase C was dialysed against 100 vol. of 50 mM-Tris/HCl (pH 8.5)/5 mM-$MgCl_2$/0.1 mM-$ZnCl_2$ at 4 °C. Samples of the dialysed mixture corresponding to 10 μg of phosphorylated PFK-2 were incubated at 30 °C with 1 unit of alkaline phosphatase, and samples were removed for measurement of ^{32}P-labelled protein [8].

ADP/ATP exchange

PFK-2 (5 μg) was preincubated with 0.125 mM-$NADP^+$, 0.2 unit of Glc-6-*P* dehydrogenase and 0.4 unit of phosphoglucoisomerase for 5 min at 30 °C in 0.2 ml of 20 mM-Tes (pH 7.5)/100 mM-KCl/5 mM-$MgCl_2$/1 mM-dithioerythritol/1 mM-potassium phosphate/0.2 mM-ATP. The reactions were started by the addition of 20 μM-[U-^{14}C]ADP (100 c.p.m./pmol), stopped at various times up to 90 min with 1 vol. of 50 mM-NaOH and heated at 80 °C for 10 min. The samples were diluted 5-fold in 20 mM-Tris/HCl, pH 8.2, and applied to a column (1 cm × 5 cm) of DEAE-Sephacel. The column was developed with a linear gradient of NaCl (0–0.4 M in 20 mM-Tris/HCl, pH 8.2), which resolved ADP (eluted at 0.14 M-NaCl) from ATP (eluted at 0.175 M-NaCl).

Protein determination

Protein was measured [13] with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Phosphorylation by protein kinases

Previous studies have shown that rat liver PFK-2 can be phsophorylated by cyclic AMP-dependent protein kinase to the extent of about 1 mol/mol of enzyme subunit [3,4]. In agreement with these studies, it was found that treatment of purified rat liver PFK-2 with 0.6 munit of cyclic AMP-dependent protein kinase for

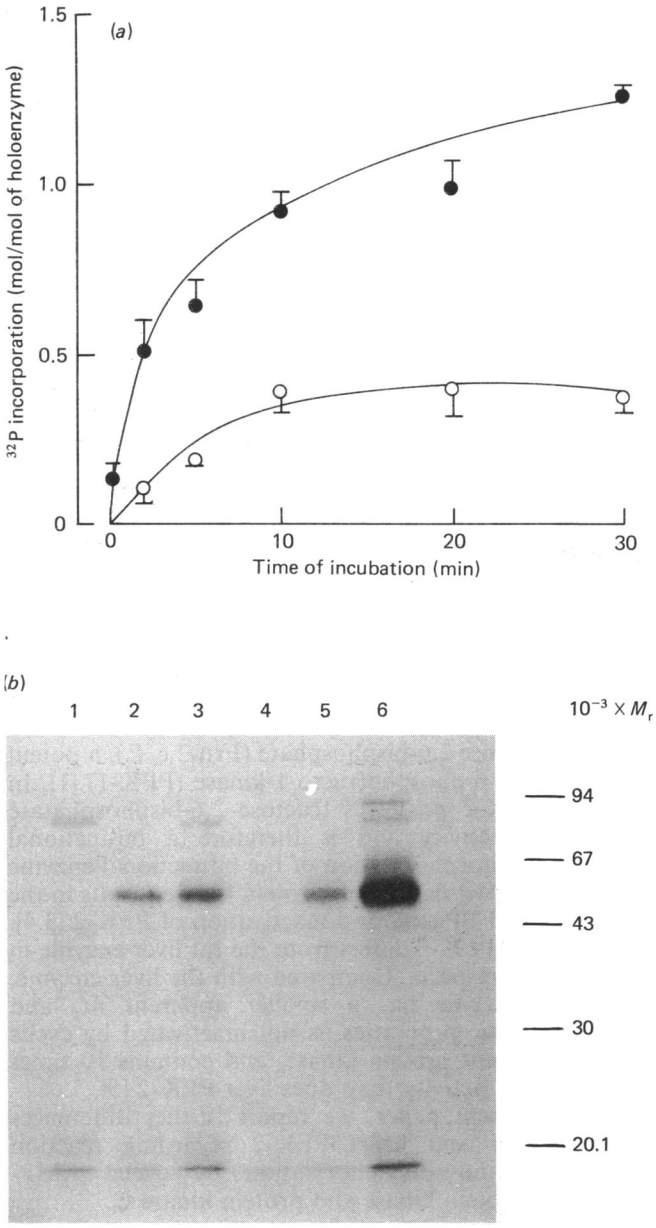

Fig. 1. (*a*) Time course of phosphorylation of purified bovine heart PFK-2 by protein kinase C and (*b*) identification of ^{32}P-labelled protein by autoradiography of SDS/polyacrylamide gel

(*a*) Purified bovine heart PFK-2 was incubated as described in the text with protein kinase C (0.3 munit) in the presence (●) or absence (○) of 0.25 mg of PS/ml. Values are means ± S.E.M. for three separate experiments. Values of phosphate incorporation in the presence of PS were different ($P < 0.05$) from those in its absence at all times of incubation except at 20 min. (*b*) After incubation of purified bovine heart and rat liver PFK-2 with protein kinase C, the reaction mixture was denatured as described in the text. A sample containing approx. 1 μg of PFK-2 was subjected to SDS/polyacrylamide (10%)-gel electrophoresis. The gel was dried and autoradiographed. Lanes: 1, protein kinase C + PS + Ca^{2+}; 2, liver PFK-2 + protein kinase C + Ca^{2+}, no PS; 3, liver PFK-2 + protein kinase C + PS + Ca^{2+}; 4, heart PFK-2 + PS + Ca^{2+}; 5, heart PFK-2 + protein kinase C + Ca^{2+}, no PS; 6, heart PFK-2 + protein kinase C + PS + Ca^{2+}. Positions of M_r standards are shown on the right.

30 min resulted in the incorporation of 0.83 mol of ^{32}P/mol of enzyme subunit. By contrast, heart PFK-2 incubated under the same conditions was phosphorylated to only 0.29 mol of ^{32}P/mol of subunit (result not shown). In agreement with out previous ovservation [5], it was confirmed that treatment with cyclic AMP-dependent protein kinase did not affect the activity of heart PFK-2, whereas it did inactivate liver PFK-2.

The opposite situation was observed when liver and heart PFK-2 were treated with protein kinase C. Incubation of bovine rat heart PFK-2 in the presence of [γ-^{32}P]ATP and protein kinase C resulted in a time-, PS- and Ca^{2+}- (not shown) dependent incorporation of ^{32}P into the protein, which reached 1.3 mol of phosphate/mol of holoenzyme (Fig. 1*a*). With 0.5 munit of protein kinase C, 1.9 mol/mol of holoenzyme was incorporated after 30 min of incubation, suggesting that bovine heart PFK-2 can be phosphorylated stoichiometrically to the extent of 1 mol of phosphate/mol of enzyme subunit. When the phosphorylated enzyme was dialysed to remove ATP and then incubated with alkaline phosphatase, protein-bound phosphate decreased in a time-dependent manner (0.74 mol/mol remaining after 10 min), indicating that phosphorylation involved the formation of phosphomonoester bond. No dephosphorylation was observed in the presence of 10 mM-EDTA.

When phosphorylated enzyme was analysed by SDS/polyacrylamide-gel electrophoresis followed by autoradiography, a major band was observed whose phosphorylation was dependent on the presence of PS (Fig. 1*b*) and Ca^{2+} (not shown), and which migrated with an apparent M_r of 57000, corresponding to the value for the enzyme subunit. By contrast, when purified rat liver PFK-2 was treated with 0.3 munit of protein kinase C for 30 min, phosphorylation of the enzyme reached 0.39 ± 0.09 mol/mol of holoenzyme in the presence of PS, compared with 0.13 ± 0.03 mol/mol in its absence. These results complement previous studies [14] which showed that treatment of isolated rat hepatocytes with phorbol esters, known stimulators of protein kinase C, increased the phosphorylation of several proteins, but not of PFK-2.

These differences in phosphorylation of liver and heart PFK-2 by the two protein kinases can be added to the previously established criteria [5] indicating that the two tissues contain distinct isoenzymes (Table 1). Fig. 1(*b*) shows that there was no difference in M_r of the phosphorylated subunit of liver and heart PFK-2. This was also the case in Coomassie Blue-stained polyacrylamide gels of two different enzyme preparations. Therefore we have been unable to confirm a difference in M_r between the two enzymes [5]. The previous difference could have resulted from partial proteolysis of the heart enzyme.

The physiological significance of the phosphorylation of heart PFK-2 is unclear, since the enzyme treated with protein kinase C showed no change in kinetic properties measured with either saturating or sub-saturating concentrations of substrates, or in the presence of 0.1 mM-citrate, an inhibitor of the enzyme. There was, however, a time-dependent loss of activity when PFK-2 was incubated with protein kinase C, and PS protected the enzyme against this inactivation, giving the impression of a protein kinase C, and PS protected the enzyme against this inactivation, giving the impression of a protein kinase C-dependent activation of PFK-2. When the incubation was conducted in the presence of 1 mg of albumin/ml, which also protected PFK-2 activity, no effect of PS was observed.

Treatment of rat liver PFK-2 with protein kinase C did not change enzyme activity.

FBPase-2 activity

Rat liver PFK-2 is a bifunctional enzyme catalysing the synthesis and hydrolysis of Fru-2,6-P_2, whereas the bovine heart enzyme contains 10 times less FBPase-2 activity than does the liver enzyme [5]. Fig. 2(*a*) shows the saturation curves of the two enzymes for Fru-2,6-P_2, and Table 1 summarizes the differences in $V_{max.}$ and K_m and several other differences between the two isoenzymes. The PFK-2/FBPase-2 activity ratio is 80:1 and 4:1 for the heart and liver preparations respectively. The high ratio in cardiac muscle is, however, not typical of muscle tissue, since the ratio is 0.2 in purified rat hindlimb muscle PFK-2 (M. H. Rider & L. Hue, unpublished work).

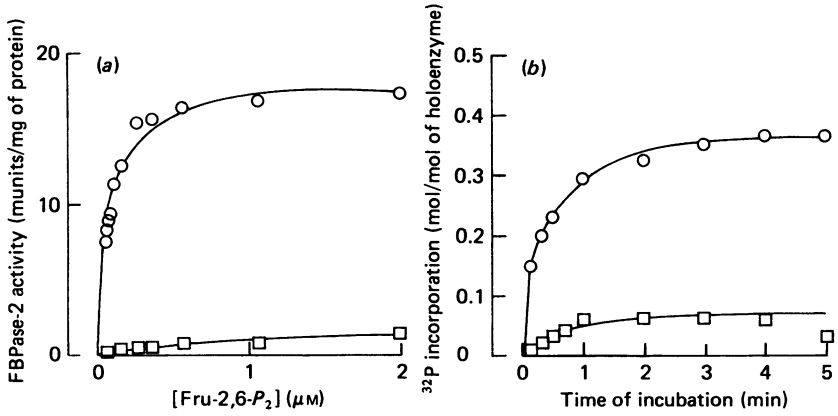

Fig. 2. (*a*) Fru-2,6-P_2 saturation curves of bovine heart and rat liver FBPase-2 and (*b*) ^{32}P-labelling of the two enzymes by [2-^{32}P]Fru-2,6-P_2

(*a*) Bovine heart (□) and rat liver (○) PFK-2 were assayed for FBPase-2 activity as described in the Materials and methods section. Curves were fitted to hyperbolae [22]. Kinetic parameters obtained are summarized in Table 1. (*b*) Bovine heart (□) and rat liver (○) PFK-2 were incubated with [2-^{32}P]Fru-2,6-P_2, and ^{32}P incorporation into protein was measured as described in the Materials and methods section.

Table 1. Evidence for different PFK-2 isoenzymes in heart and liver

Data are taken from this paper and from [5].

	Heart (type H)	Liver (type L)
PFK-2 activity		
K_m for Fru-6-*P* (μM)	50	140
Inactivation by 1 mM-*sn*-glycerol 3-phosphate (%)	15	75
Inactivation by cyclic AMP-dependent protein kinase	No	Yes
Protein phosphorylation (mol/mol of enzyme subunit)		
Cyclic AMP-dependent protein kinase	0.29	0.83
Protein kinase C	0.95	0.19
FBPase-2 activity		
K_m for Fru-2,6-P_2 (μM)	1.4	0.1
$V_{max.}$ (munits/mg of protein)	2	19
Labelling from [2-^{32}P]Fru-2,6-P_2	10-fold slower and 6-fold less than liver	0.15 mol/mol of subunit
PFK-2/FBPase-2 activity ratio	80	4

Labelling of liver PFK-2 by [2-^{32}P]Fru-2,6-P_2 [15,16] suggested that the bisphosphatase reaction proceeds through the formation of a phosphoenzyme intermediate (substituted-enzyme mechanism). The rate of phosphorylation of the enzyme is about 100 times faster than the overall bisphosphatase reaction rate [15], consistent with the existence of the intermediate in the reaction mechanism and suggesting that its formation is not rate-limiting for the reaction. Fig. 2(*b*) shows that liver PFK-2 can be phosphorylated from [2-^{32}P]Fru-2,6-P_2 and that 50% of the total incorporation was achieved within 10 s. By contrast, the rate of phosphorylation of heart PFK-2 was at least 10 times slower than for the liver enzyme, and the extent of phosphorylation was 6-fold less. The difference in labelling rate by [2-^{32}P]Fru-2,6-P_2 represents further evidence for the low FBPase-2 content of heart PFK-2. The existence of a relatively small FBPase-2 activity in heart PFK-2, as well as the low extent of labelling by [2-^{32}P]Fru-2,6-P_2, may be due to the kinetic characteristics of the heart enzyme itself or may indicate the presence of a small amount of liver-type isoenzyme in the heart PFK-2 preparation. Our results do not allow us to distinguish between these two possibilities. On the other hand, it cannot be excluded that some labelling could have occurred through the reversal of the kinse reaction, since a phosphoryl-enzyme has also been suggested for the kinase reaction on the basis of an exchange between [^{32}P]Fru-6-*P* and Fru-2,6-P_2 [15].

The exchange reaction between ADP and ATP has been taken as another argument in favour of the existence of a phosphoryl intermediate in the kinase reaction [15,17]. However, the involvement of the ADP/ATP exchange in the kinase reaction mechanism is questionable. Firstly, the rate of the exchange is less than the overall reaction rate [15,17]. Furthermore, thermolysin, enzyme oxidation and 5′-*p*-fluorosulphonylbenzoyladenosine, which abolish the kinase activity, do not affect the exchange [18,19].

In our hands incubation of rat liver PFK-2 with [U-^{14}C]ADP resulted in the formation of labelled ATP at a rate of 6.5 nmol/min per mg of protein, representing 9% of the kinase rate. Similarly, heart PFK-2 catalysed the ADP/ATP exchange at a rate of 8.7 nmol/min per mg of protein, i.e. 5% of the overall reaction rate. For the reasons listed above, it is unlikely that the ADP/ATP exchange is involved in the kinase reaction mechanism. It may indeed represent exchange not related to the kinase activity or may result from a contaminating activity. These findings, together with our measurements of initial rates (results not shown), support the previous conclusion [17] that a substituted-enzyme mechanism is unlikely.

General conclusions

The results confirm and further support our previous conclusion, namely that liver and heart contain distinct PFK-2 isoenzymes. They display different kinetic properties (Table 1), and phosphorylation by protein kinases is different: liver, but not heart, PFK-2 is a good substrate for cyclic AMP-dependent protein kinase, whereas the opposite is true for protein kinase C. Moreover, the PFK-2/FBPase-2 activity ratio of the heart enzyme is 20 times that of the liver enzyme preparation (Table 1), suggesting that 'bifunctionality' might be restricted to liver (type L) PFK-2. In addition, the rate of phosphorylation of the two enzymes by [2-^{32}P]Fru-2,6-P_2 is also different. These differences could be explained by the hypothesis that the two enzyme preparations are contaminated by a small amount of the other isoenzyme. In this case, heart (type H) PFK-2 would be devoid of any FBPase-2 activity. Alternatively, the characteristics of the two isoenzymes could be such that type L PFK-2 is a poor substrate of protein kinase C and type H PFK-2 contains some FBPase-2 activity.

The differences between the two isoenzymes confirm the previous observation that treatment of perfused rat hearts with adrenaline did not decrease Fru-2,6-P_2 concentration [20]. In addition, it is known that, in rat hepatocytes, insulin has little effect on Fru-2,6-P_2 concentration and PFK-2/FBPase-2 activities, but antagonizes the effect of glucagon on these parameters [21]. By contrast, when administered to overnight-starved rats, insulin doubles Fru-2,6-P_2 concentration and PFK-2 activity in the hearts of these animals [10].

M.H.R. thanks the International Institute of Cellular and Molecular Pathology for financial support. L.H. is Maître de Recherches of the Belgian Fonds National de la Recherche Scientifique. This work was supported in part by the Belgian Fonds de la Recherche Scientifique Médicale.

REFERENCES

1. Van Schaftingen, E. & Hers, H. G. (1981) Biochem. Biophys. Res. Commun. **101**, 1078–1084
2. El-Maghrabi, M. R., Claus, T. H., Pilkis, J., Fox, E. & Pilkis, S. J. (1982) J. Biol. Chem. **257**, 7603–7607
3. Murray, K. J., El-Maghrabi, M. R., Kountz, P. D., Lukas, T. J., Soderling, T. R. & Pilkis, S. J. (1984) J. Biol. Chem. **259**, 7673–7681

4. Sakakibara, R., Kitajima, S. & Uyeda, K. (1984) J. Biol. Chem. **259**, 41–46
5. Rider, M. H., Foret, D. & Hue, L. (1985) Biochem. J. **231**, 193–196
6. Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S. & Nishizuka, Y. (1982) J. Biol. Chem. **257**, 13341–13348
7. Reimann, E. M. & Behman, R. A. (1983) Methods Enzymol. **99**, 51–55
8. Roskoski, R., Jr. (1983) Methods Enzymol. **99**, 3–6
9. Parker, P. J., Stabel, S. & Waterfield, M. D. (1984) EMBO J. **3**, 953–959
10. Rider, M. H. & Hue, L. (1984) FEBS Lett. **176**, 484–488
11. Van Schaftingen, E., Davies, D. R. & Hers, H. G. (1982) Eur. J. Biochem. **124**, 143–149
12. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
13. Bradford, M. (1976) Anal. Biochem. **72**, 248–254
14. Garrison, J. C., Johnsen, D. E. & Campanile, C. P. (1984) J. Biol. Chem. **259**, 3283–3292
15. Pilkis, S. J., Regen, D. M., Stewart, H. B., Pilkis, J., Pate, T. M. & El-Maghrabi, M. R. (1984) J. Biol. Chem. **259**, 949–958
16. Stewart, H. B., El-Maghrabi, M. R. & Pilkis, S. J. (1985) J. Biol. Chem. **260**, 12935–12941
17. Kitajima, S., Sakakibara, R. & Uyeda, K. (1984) J. Biol. Chem. **259**, 6896–6903
18. El-Maghrabi, M. R., Pate, T. M., Murray, K. J. & Pilkis, S. J. (1984) J. Biol. Chem. **259**, 13096–13103
19. El-Maghrabi, M. R., Pate, T. M. & Pilkis, S. J. (1984) Biochem. Biophys. Res. Commun. **123**, 749–756
20. Hue, L., Blackmore, P. F., Shikama. H., Robinson-Steiner, A. & Exton, J. H. (1982) J. Biol. Chem. **257**, 4303–4313
21. Pilkis, S. J., Chrisman, T. D., El-Maghrabi, M. R., Colosia, A., Fox, E., Pilkis, J. & Claus, T. H. (1983) J. Biol. Chem. **258**, 1495–1503
22. Barlow, R. B. (1983) Biodata Handling with Microcomputers, pp. 116–121, Elsevier, Cambridge

Received 7 July 1986/11 August 1986; accepted 19 August 1986