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The two forms of bovine heart 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase result from alternative splicing

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Purified bovine heart 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) showed two bands with subunit M_r of 58000 and 54000 when analysed by SDS/PAGE. Both the 58000- and 54000-M_r forms were phosphorylated by cyclic AMP-dependent protein kinase (PKA) and by protein kinase C (PKC) *in vitro*. Phosphorylation by PKA decreased the apparent K_m of PFK-2 for one of its substrates, fructose 6-phosphate, while phosphorylation by PKC did not correlate with any change in PFK-2 activity. The differences between the 58000- and 54000-M_r forms were studied by electroblotting, peptide mapping and microsequencing. Residues 451–510, which correspond to exon 15 in the rat and contain phosphorylation sites for PKA (Ser-466) and PKC (Thr-475), were absent from the 54000-M_r form. Peptide mapping after phosphorylation by [γ-^{32}P]MgATP and PKC showed a phosphorylated peptide containing Thr-475, which was present in the 58000-M_r form but not in the 54000-M_r form. The fact that the latter form was phosphorylated by PKC and PKA suggests that other phosphorylation sites for PKA and PKC are located outside the region encoded by exon 15. Finally, analysis of RNA from bovine heart showed that the tissue contains two PFK-2/FBPase-2 mRNAs, only one of which was recognized by a probe specific to the region coding for Ser-466 and Thr-475. Taken together, these findings demonstrate that the 58000- and 54000-M_r forms of bovine heart PFK-2/FBPase-2 result from alternative splicing of the same primary transcript.

INTRODUCTION

At least three isoenzymes of the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) are known to be present in mammalian tissues [1,2]. These have been termed the liver (L), muscle (M) and heart (H) type isoenzymes [1]. The three isoenzymes exist as homodimers and their amino acid sequences have been determined from their corresponding cDNA [3–6]. The isoenzymes differ in subunit M_r, kinetic properties and phosphorylation by protein kinases. The calculated subunit M_r values of the three forms are 54570 (L), 52011 (M) and 60679 (H). Furthermore, their PFK-2/FBPase-2 activity ratios are different: 1–2 for type L, 0.1–0.2 for type M and 80 for type H [1]. Types L and H are substrates of the cyclic AMP-dependent protein kinase (PKA) [7,8], while type M is not [9]. Phosphorylation of type L in the *N*-terminal domain at Ser-32 leads to PFK-2 inactivation and FBPase-2 activation [7]. Type M is not a substrate of PKA, because it possesses a different *N*-terminal sequence lacking the phosphorylation site [5]. Type H is phosphorylated in its *C*-terminal domain by PKA at Ser-466 [8]. Phosphorylation of this serine residue in the bovine heart enzyme has been reported to activate PFK-2 by decreasing its K_m for fructose 6-phosphate (Fru-6-*P*) [8]. In addition, results from this laboratory showed that bovine heart PFK-2/FBPase-2 is phosphorylated by protein kinase C (PKC), without an effect on PFK-2 and FBPase-2 activities [10], whereas the rat liver enzyme was a poor substrate [10]. By contrast, Uyeda and co-workers [8] reported that phosphorylation of bovine heart PFK-2/FBPase-2 by PKC resulted in an increase in the $V_{max.}$ of PFK-2, and they identified the site of phosphorylation by PKC as Thr-475. On the other hand, analysis of purified bovine heart PFK-2/FBPase-2 by SDS/PAGE revealed two bands with M_r values of 58000 and 54000 [11]. Phosphorylation has been reported to be restricted to the 58000-M_r form, thus implying that the 54000-M_r form is a proteolytic degradation product lacking the *C*-terminal end with the phosphorylation sites [8].

We have re-investigated the properties of bovine heart PFK-2/FBPase-2 and its phosphorylation by protein kinases. By using the techniques of electroblotting, microsequencing and PCR, we show here that the 54000- and 58000-M_r forms of bovine heart PFK-2/FBPase-2 actually result from alternative splicing of the same primary transcript. We also present evidence for new phosphorylation sites for PKA and PKC.

MATERIALS AND METHODS

Materials

The sources of enzymes and chemicals were as cited previously [10]. Sequencing-grade trypsin was from Boehringer, Sephacryl S-200 was from Pharmacia and radiochemicals were from Amersham. Immobilon poly(vinylidine difluoride) (PVDF) membranes were from Millipore. All other chemicals were from Boehringer or Sigma.

Enzyme purification

The catalytic subunits of PKA [12] and PKC [13] were purified as described. Bovine heart PFK-2/FBPase-2 was purified from freshly obtained tissue, as described in [10] with the following modifications. The buffers were supplemented with 0.5 mM-

Abbreviations used: diolein, *sn*-1,2-dioleoylglycerol; FBPase-2, fructose-2,6-bisphosphatase (EC 3.1.3.46); Fru-6-*P*, fructose 6-phosphate; Fru-2,6-P_2, fructose 2,6-bisphosphate; PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105); PKA, cyclic AMP-dependent protein kinase (EC 2.7.1.37); PKC, protein kinase C (EC 2.7.1.37); PS, phosphatidylserine; PVDF, poly(vinylidine difluoride).

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EGTA, 0.1 mM-phenylmethanesulphonyl fluoride, 0.5 μg of leupeptin/ml and 2 mM-benzamidine hydrochloride to protect the enzyme from proteolysis. After elution from Blue Sepharose [9,10], the enzyme was applied to a column (90 cm × 1.5 cm) of Sephacryl S-200 equilibrated with 20 mM-Hepes, 50 mM-KCl, 2 mM-EDTA, 5 mM-$MgCl_2$, 0.5 mM-EGTA, 5 mM-potassium phosphate, 0.1 mM-phenylmethanesulphonyl fluoride, 20% (v/v) glycerol and 15 mM-2-mercaptoethanol, pH 7.5 (gel filtration buffer) at 4 °C. Fractions containing PFK-2 activity were pooled and concentrated to about 1 ml by ultrafiltration. The specific activity of PFK-2 in the enzyme preparations was about 40 munit/mg of protein.

Measurement of enzyme activities

PKA [14] and PKC [15] were assayed with 1.25 mg of histone IIA or histone IIIS/ml respectively. PFK-2 [16] and FBPase-2 [17] were assayed as described, with modifications given in the legends to the Figures. One unit of enzyme activity is the amount that catalyses the formation of 1 μmol of product/min under the stated conditions.

Protein phosphorylation

Bovine heart PFK-2/FBPase-2 (0.1 mg/ml) was incubated at 30 °C with protein kinases and [γ-^{32}P]MgATP in 50 mM-Tris/HCl, 5 mM-$MgCl_2$, 0.2 mM-EDTA and 2 mM-dithiothreitol, pH 7.5 (phosphorylation buffer), as described in the legends to the Figures and the Table. Incubations with PKC also contained 1.5 mM-$CaCl_2$, 0.5 mM-EGTA, 100 μg of phosphatidylserine (PS)/ml and 1 μg of *sn*-1,2-dioleoylglycerol (diolein)/ml. In some experiments the reaction was stopped after 30 min by adding 30 μl of 40 mM-Tris/HCl, 4 mM-EDTA, 4% (w/v) SDS, 20% (w/v) dithiothreitol, 40% (v/v) glycerol and 0.4% (w/v) Bromophenol Blue, pH 6.8 (SDS/PAGE sample buffer), to 50 μl of reaction mixture. Proteins were denatured by heating at 90 °C for 10 min prior to SDS/PAGE, followed by gel drying and autoradiography. In other experiments, heart PFK-2/FBPase-2 (0.1 mg/ml) was phosphorylated in the FBPase-2 domain (presumably on His-257) by incubation with 15 μM-[2-^{32}P]fructose 2,6-bisphosphate (Fru-2,6-P_2) (specific radioactivity 900 c.p.m./pmol) in 50 μl of phosphorylation buffer at 30 °C. The reaction was stopped after 30 s by adding 30 μl of SDS/PAGE sample buffer. The sample was denatured for SDS/PAGE as described above.

Electroblotting and microsequencing

The bovine heart PFK-2/FBPase-2 preparation (80 μg) was incubated at 30 °C in a final volume of 70 μl of phosphorylation buffer (see above) with either 0.65 munit of PKC or 0.35 munit of PKA. Incubations with PKC contained 1.5 mM-$CaCl_2$, 0.5 mM-EGTA, 100 μg of PS/ml and 1 μg of diolein/ml. Reactions were initiated with 0.1 mM-[γ-^{32}P]MgATP (specific radioactivity 500–1000 c.p.m./pmol) and terminated after 60 min by adding 30 μl of SDS/PAGE sample buffer (see above). Following SDS/PAGE in 10% acrylamide gels, proteins were electroblotted on to PVDF membranes [18]. The 58000- and 54000-M_r bands were excised and digested *in situ* with trypsin [18]. Peptides were separated by reverse-phase narrowbore h.p.l.c. on a Vydac C_{18} column (2.1 mm × 250 mm) with an acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid (solvent A). Elution was performed with the following gradient program: 5–100% B in 100 min (solvent B = 70% acetonitrile/0.1% (v/v) trifluoroacetic acid), at a flow rate of 80 μl/min generated by a model 140 A Applied Biosystems solvent delivery system. Peptides eluting from the column were monitored by their A_{214} (detector 759A; Applied Biosystems) and collected by hand in a volume of 30–50 μl. Aliquots (3 μl) were counted for radioactivity. Peptides were sequenced using a gas phase sequencer (Applied Biosystems model 470 A) equipped with a 120A phenylthiohydantoin amino acid analyser.

Analysis of bovine heart PFK-2/FBPase-2 mRNA

A bovine heart was obtained from a local slaughterhouse less than 15 min after the animal was killed. Samples (about 10 g) of ventricular muscle were immediately frozen in liquid N_2 and stored at −80 °C before preparing total RNA [19]. Reverse transcription and PCR were carried out using a kit from Perkin–Elmer Cetus. A 1 μg sample of RNA was reverse-transcribed with an oligonucleotide (oligo A) complementary to nucleotides 1606–1622 of the bovine heart cDNA described by Sakata & Uyeda [6]. The reaction product was submitted to 30 cycles of PCR using two primers deduced from nucleotides 1264–1281 (oligo B) and 1582–1600 (oligo C) of the bovine heart cDNA [6]. PCR was conducted in an automatic DNA thermal cycler (Hybaid) with the following step cycle program: denature at 94 °C for 2 min, anneal at 60 °C for 2 min, and extend at 72 °C for 2 min. The PCR reaction product was electrophoresed on a 1.5% agarose gel and the DNA was transferred to a nylon filter (Amersham) [20]. The filter was washed with 0.3 M-NaCl/30 mM-sodium citrate, pH 7.0 (double-strength SSC medium), followed by 2 mg of Ficoll/ml, 2 mg of polyvinylpyrrolidone 40/ml and 2 mg of BSA/ml. The DNA was hybridized with a ^{32}P-labelled oligonucleotide rat probe (oligo D) corresponding to nucleotides 1416–1459 of bovine heart cDNA [6] which encode residues 462–477, including the phosphorylation sites for PKA (Ser-466) and PKC (Thr-475). The filter was washed in double-strength SSC medium containing 0.1% (w/v) SDS at 60 °C prior to drying and autoradiography.

Other methods

Protein was determined [21] using γ-globulin as a standard. The statistical significance of results was determined by Student's *t* test. Curves describing the saturation of PFK-2 activity by Fru-6-*P* and MgATP were analysed by the method of non-linear least-squares fitting to a hyperbola using a computer program [22].

RESULTS AND DISCUSSION

Relative M_r and phosphorylation of heart PFK-2/FBPase-2

In our original studies [10,23] we reported M_r values of 57000 and 52000 for the bovine heart PFK-2/FBPase-2 subunits. On the other hand, Uyeda and co-workers identified two forms with subunit M_rs of 58000 and 54000 [8,11]. We have now investigated the relationship between the smaller and the larger form and their phosphorylation by protein kinases.

Bovine heart PFK-2/FBPase-2 was purified from fresh tissue in the presence of protease inhibitors. All the preparations contained two forms of subunits whose average M_r values were 58600 ± 800 and 54300 ± 500 (n = 5) as measured by SDS/PAGE and staining, or after labelling the intact protein by incubation with [2-^{32}P]Fru-2,6-P_2, which phosphorylates the protein in the FBPase-2 domain. Fig. 1 shows a Coomassie Blue-stained gel of a PFK-2/FBPase-2 preparation of bovine heart compared with one from rat liver (subunit M_r 55000). The latter gave a major band migrating between the two bands of the bovine heart enzyme, and a band of about 35000 M_r that was not seen consistently. The 54000-M_r form was about twice as abundant as the 58000-M_r form. The same proportion was observed after labelling from [2-^{32}P]Fru-2,6-P_2 (Fig. 2, lane 1).

Kitamura & Uyeda [11] reported that the 58000-M_r but not the 54000-M_r form was phosphorylated by PKA, suggesting that

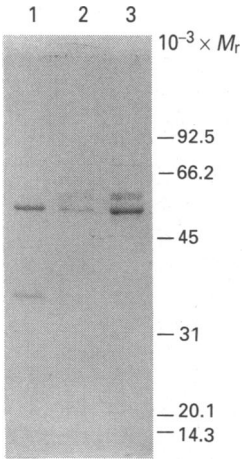

Fig. 1. SDS/PAGE of PFK-2/FBPase-2

Purified bovine heart and rat liver PFK-2/FBPase-2 (5 μg per lane) were subjected to SDS/PAGE followed by Coomassie Blue staining. Lane 1, rat liver PFK-2/FBPase-2; lane 2, bovine heart PFK-2/FBPase-2 before gel filtration; lane 3, bovine heart PFK-2/FBPase-2 after gel filtration.

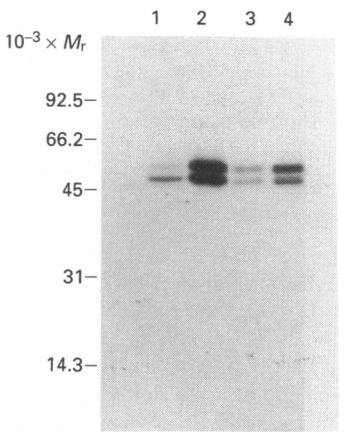

Fig. 2. SDS/PAGE and autoradiography of bovine heart PFK-2/FBPase-2 labelled by [γ-^{32}P]MgATP and PKA or PKC, or by [2-^{32}P]Fru-2,6-P_2

Bovine heart PFK-2/FBPase-2 (5 μg) was incubated for 30 min at 30 °C in a final volume of 50 μl with PKA (35 μunit) or PKC (70 μunit) and 0.1 mM-[γ-^{32}P]MgATP (200 c.p.m./pmol) as described in the Materials and methods section. The enzyme was also phosphorylated from [2-^{32}P]Fru-2,6-P_2 to form the phosphoenzyme (EP). Lane 1, EP; lanes 2 and 3, phosphorylation by PKC in the presence and absence respectively of PS/diolein; lane 4, phosphorylation by PKA.

the 54000-M_r form might lack the phosphorylation site and thus be a proteolytic fragment. We have not been able to confirm this difference, and found that both the 58000-M_r and 54000-M_r forms were always phosphorylated, not only by PKA but also by PKC (Fig. 2). Fig. 2 shows that the two bands phosphorylated by protein kinases corresponded to the bands which were specifically labelled by [2-^{32}P]Fru-2,6-P_2.

The stoichiometry of phosphorylation of the 54000- and 58000-M_r forms by PKA and PKC was estimated by scanning the Coomasie Blue-stained gel to determine the relative amounts of the two forms and by counting the radioactivity incorporated into the 54000- and 58000-M_r bands after complete phosphorylation from [γ-^{32}P]MgATP and each protein kinase. The 54000- and 58000-M_r forms were phosphorylated to the extent of 0.08 ± 0.01 and 0.36 ± 0.02 mol/mol of subunit by PKA, and 0.25 ± 0.02 and 0.85 ± 0.07 mol/mol of subunit by PKC (means $\pm$ S.E.M. for three experiments).

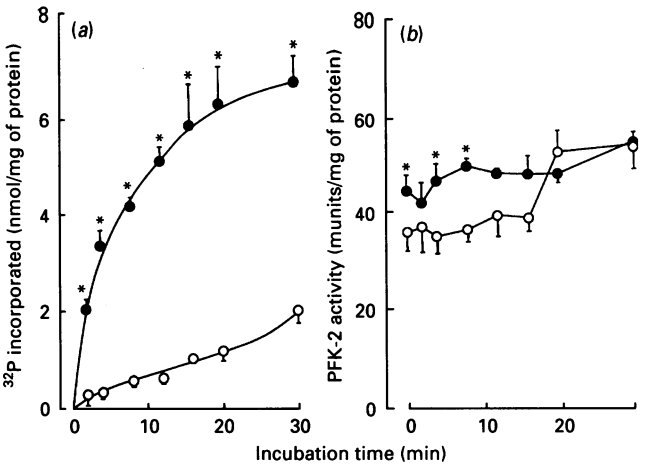

Fig. 3. Effect of phosphorylation of bovine heart PFK-2/FBPase-2 by PKC on PFK-2 activity

Bovine heart PFK-2/FBPase-2 (10 μg) was incubated in a final volume of 0.1 ml containing 0.1 mM-[γ-^{32}P]MgATP (specific radioactivity 300 c.p.m./pmol) (*a*) or 1 mM non-radioactive MgATP (*b*) and 0.13 munit of PKC in the presence (●) or absence (○) of PS/diolein at 30 °C. At the indicated times, aliquots of reaction mixture (10 μl) were removed for the determination of ^{32}P incorporation into protein [14] (*a*) or PFK-2 activity (*b*). PFK-2 was assayed at pH 7.1 under optimal conditions with 2 mM-Fru-6-*P* and 5 mM-MgATP in the presence of 5 mM-potassium phosphate. * indicates a significant effect ($P < 0.05$) of phosphorylation by PKC versus controls in the absence of PS/diolein. The results are the means $\pm$ S.E.M. for three separate experiments.

Effect of phosphorylation by PKC on PFK-2 activity

We [10] and others [24] reported that phosphorylation of bovine heart PFK-2/FBPase-2 by PKC was without effect on the activities of PFK-2 and FBPase-2. Later on, Uyeda and co-workers reported that this phosphorylation by PKC activated PFK-2 [8]. They interpreted our failure to observe changes in kinetic properties of bovine heart PFK-2 upon phosphorylation as being due to partial proteolysis of the enzyme [8]. This interpretation cannot hold, since the two forms of PFK-2/FBPase-2 are clearly phosphorylated (Fig. 2). Fig. 3 shows the phosphorylation of bovine heart PFK-2/FBPase-2 by PKC. Phosphorylation occurred in a time-dependent manner and depended upon the presence of PS/diolein. Comparison of panels (*a*) and (*b*) of Fig. 3 shows that there was no correlation between the $V_{max.}$ of PFK-2 and the extent of phosphorylation. However, a small increase in PFK-2 activity was already observed with PS/diolein alone at zero time. A similar effect was also obtained with albumin (results not shown). Therefore we suggest that the increase in the $V_{max.}$ of PFK-2, which Uyeda and co-workers observed, might have been due to an effect of PS/diolein and not to phosphorylation by PKC. When PFK-2 activity was measured with suboptimal concentrations of Fru-6-*P*, no PS/diolein-dependent change was observed in the presence of PKC, suggesting that the K_m was not affected either.

Effect of phosphorylation by PKA on PFK-2 activity

Fig. 4(*a*) shows that, at pH 7.1, PKA treatment activated PFK-2 by decreasing ($P < 0.05$) its K_m for Fru-6-*P* from 71 ± 8 μM (control) to 42 ± 3 μM (mean $\pm$ S.E.M. for five experiments). In these experiments, the individual curves describing the saturation

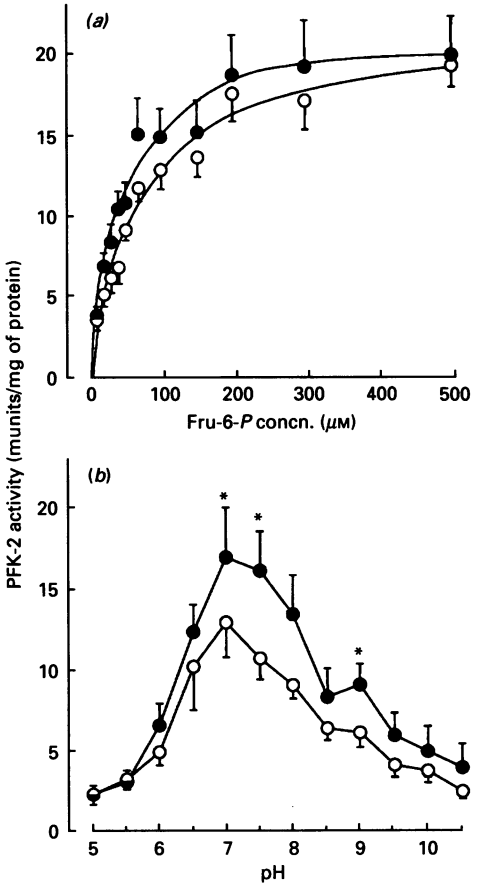

Fig. 4. Effect of treatment of bovine heart PFK-2/FBPase-2 with PKA on PFK-2 activity

Bovine heart PFK-2/FBPase-2 (5 μg) was incubated in a final volume of 50 μl containing 1 mM-MgATP in the presence (●) or absence (○) of 70 μunits of PKA at 30 °C. After 30 min, the reaction mixture was diluted 6-fold in gel filtration buffer (see the Materials and methods section) and placed on ice. Aliquots of reaction mixture (20 μl) were assayed for PFK-2. In (*a*), PFK-2 activity was measured at pH 7.1 in buffer containing 25 mM-Mes, 25 mM-glycylglycine, 25 mM-Hepes, 25 mM-borate, 50 mM-KCl, 20 mM-KF, 1 mM-dithiothreitol, 5 mM-potassium phosphate, 5 mM-MgATP and the indicated concentrations of Fru-6-*P*. Five individual curves describing the saturation of PFK-2 activity by Fru-6-*P* were each fitted by computer to give estimates of K_m and $V_{max.}$, which were tested for significant differences with respect to PKA treatment (see text). The values in (*a*) are the means ± S.E.M. for the five separate experiments. In (*b*), PFK-2 activities were measured with the buffer mixture described above, adjusted to the indicated pH values. These suboptimal assays contained 50 μM-Fru-6-*P*. The results are the means ± S.E.M. for four separate experiments. * indicates a significant effect ($P < 0.05$) of PKA treatment.

of PFK-2 activity by Fru-6-*P* were analysed separately to give estimates of $K_m^{app.}$, from which the means ± S.E.M. and the statistical significance of the differences were calculated. This effect of PKA confirms an earlier report [8]. PKA treatment had no effect on the $V_{max.}$ of PFK-2 (Fig. 4*a*), its K_m for MgATP, or its sensitivity towards inhibition by citrate (results not shown). Fig. 4(*b*) shows a pH profile of PFK-2 activity from control- and PKA-treated enzyme measured under suboptimal assay conditions. PKA treatment caused a small (30–50%) but statistically significant increase in PFK-2 activity between pH 7.0 and 7.5. This confirms our previous observation that treatment of a preparation of purified heart PFK-2/FBPase-2 with PKA resulted in a small (20–30%) increase in activity when measured under suboptimal conditions [23].

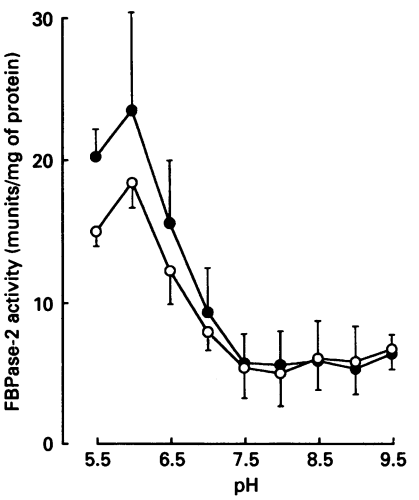

Fig. 5. Effect of PKA treatment on bovine heart FBPase-2 activity

Bovine heart PFK-2/FBPase-2 was incubated at 30 °C with (●) or without (○) PKA as described in the legend to Fig. 4. Aliquots of reaction mixture (10 μl) containing 0.9 μg of protein were assayed for FBPase-2 in a final volume of 0.5 ml of 20 mM-Mes, 20 mM-Hepes, 20 mM-Tris/HCl, 100 mM-KCl, 5 mM-$MgCl_2$, 2 mM-EDTA and 1 mM-dithiothreitol, at the pH indicated. To remove endogenous Fru-6-*P*, FBPase-2 assays contained 5 mM-potassium phosphate, 5 mM-$NADP^+$, 2 units of glucose-6-phosphate dehydrogenase and 4 units of phosphoglucoisomerase, and were started with 20 μM-[2-^{32}P]Fru-2,6-P_2 (specific radioactivity 30 c.p.m./pmol). After 15 min, the reactions were stopped and [^{32}P]P_i was separated from [2-^{32}P]Fru-2,6-P_2 [17]. The results are the means ± S.E.M. for three separate experiments.

FBPase-2 activity

We have previously shown that bovine heart contains about 80 times less FBPase-2 activity than PFK-2 activity, whereas the L-type isoenzyme has a PFK-2/FBPase-2 activity ratio of 1–2 [10,23]. These ratios were obtained when the FBPase-2 activity was measured at pH 7.5 and at a physiological concentration of Fru-2,6-P_2, i.e. in the micromolar range, which is at least 10-fold below the apparent estimated K_m (40 μM) for the heart FBPase-2 [11]. By contrast, the K_m of L-type FBPase-2 for Fru-2,6-P_2 is below micromolar [25]. When the FBPase-2 activity is measured under $V_{max.}$ conditions, the PFK-2/FBPase-2 activity ratio of the bovine heart enzyme decreases considerably. This explains why a PFK-2/FBPase-2 activity ratio close to 1 was reported for the heart enzyme, since the estimated $V_{max.}$ value for FBPase-2 was taken into consideration [11].

Bovine heart FBPase-2 activity was maximal at pH 6.0 (Fig. 5). Treatment with PKA caused a small but insignificant increase in FBPase-2 activity measured at pH 5.5 and 6 (Fig. 5). When the pH profile of the PKC-treated enzyme was studied, no PS/diolein-dependent change in FBPase-2 activity was observed (results not shown). The K_m for Fru-2,6-P_2 could not be estimated because the specific radioactivity of the [2-^{32}P]Fru-2,6-P_2 was not high enough to measure FBPase-2 activity with accuracy at concentrations of Fru-2,6-P_2 greater than 30 μM. Therefore we were unable to determine whether PKA or PKC has any effect on the K_m and/or $V_{max.}$ of bovine heart FBPase-2 activity.

Structure and origin of the 58000- and 54000-M_r forms

We have used the technique of electroblotting from SDS/polyacrylamide gels on to PVDF membranes, followed by trypsin digestion and microsequencing [18], to study the difference in structure between the 58000- and 54000-M_r forms of bovine heart PFK-2/FBPase-2.

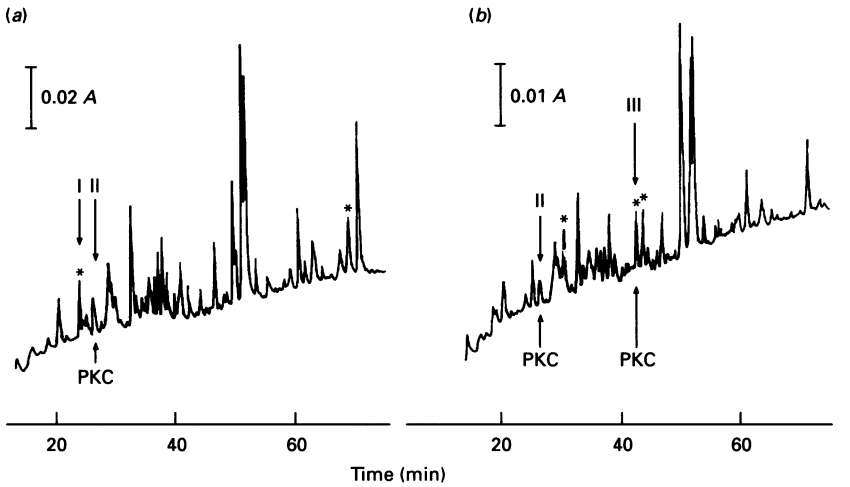

Fig. 6. Peptide mapping of the 54000- and 58000-M_r forms of bovine heart PFK-2/FBPase-2 after electroblotting and trypsin digestion

The 54000- (*a*) and 58000- (*b*) M_r forms of bovine heart PFK-2/FBPase-2 were separated by SDS/PAGE and electroblotted on to PVDF membranes. The two bands were excised and digested for 2 h at 37 °C with 1 μg of sequencing-grade trypsin in 0.2 ml of 0.1 M-Tris/HCl, pH 8.5. Narrow-bore h.p.l.c. of the 54000-M_r (*a*) and 58000-M_r (*b*) protein digests was performed as described in the Materials and methods section. For experiments in which the preparation was treated with [γ-^{32}P]MgATP and either PKA or PKC prior to SDS/PAGE and electroblotting, the positions of the labelled peptides are indicated. Peptides which were taken for microsequencing (Table 1) are shown by roman numerals. * indicates peptides which were different in the two forms. PKC refers to PKC-labelled peptides

Fig. 6 shows a narrowbore h.p.l.c. elution profile of electroblotted 58000- and 54000-M_r forms digested with trypsin. The peptide maps are similar except for a number of peptides indicated in the Figure. The fact that several different peptides were found in the 54000-M_r form rules out the possibility that the latter is a proteolytic degradation product of the 58000-M_r form. Peak I, which is present in the 54000-M_r form but not in the 58000-M_r form, was sequenced. It contained one peptide whose sequence was DKPTAETSR. Cleavage by trypsin did not occur after the lysine residue because it is followed by proline. This sequence was compared with that derived from bovine heart cDNA [6]. This comparison identified the position of the DKPT half of the peptide at residues 447–450 and the AETSR half at residues 511–515, demonstrating that residues 451–510, containing the PKA and PKC phosphorylation sites, were absent from the 54000-M_r form. This deletion of a 6788-M_r peptide would be consistent with the difference in M_r between the two forms. In the rat PFK-2/FBPase-2 gene, the nucleotide sequence coding for the amino acid sequence DKPT is present at the end of exon 14, while the sequence coding for PGPAR, which replaces the bovine sequence AETSR, is located at the beginning of exon 16 [26]. This suggests that the 58000- and 54000-M_r forms arise from alternative splicing of the same transcript, such that the 54000-M_r form lacks exon 15 containing the PKA and PKC phosphorylation sites.

If the 54000-M_r form arises from splicing between exon 14 and exon 16 with loss of the PKC-phosphorylated Thr-475, then a PKC-labelled peptide present in the 58000-M_r form should be missing from the 54000-M_r form. Such a peptide (peptide III) was identified (Fig. 6*b*) and sequenced (Table 1). Its sequence spanned residues 464–476, as expected. Moreover, Thr-475 was not detected as the phenylthiohydantoin residue, in agreement with the fact that it was indeed phosphorylated.

If our interpretation holds true, then the mRNA for the 54000-M_r form should lack a portion that is present in the mRNA for the 58000-M_r form and corresponds to exon 15. To investigate this hypothesis, total bovine heart RNA was first reverse-transcribed from a primer (oligo A in Fig. 7) situated beyond exon 15 in the *C*-terminus. The cDNA product was then subjected to 30 rounds of PCR using two oligonucleotides on either side of exon 15, i.e. oligo B (exon 13) and oligo C (exon 16) of the bovine heart PFK-2/FBPase-2 cDNA sequence [6]. Fig. 8(*a*) shows that two cDNA species were observed, as expected. The lengths of these fragments, 370 and 180 bp respectively, are close to the values predicted from the bovine heart PFK-2/FBPase-2 cDNA sequence, namely 354 bp (containing exon 15) and 174 bp (exon 15 absent). It is remarkable that the smaller PCR product was more abundant than the longer product, in keeping with the observation (see above) that the PFK-2/FBPase-2 preparations always contained more 54000-M_r than 58000-M_r form. To confirm that the longer, but not the smaller, cDNA contained exon 15, a Southern blot of the PCR products was hybridized with a probe (oligo D) specific for exon 15 [26]. As expected (Fig. 8*b*), only the upper band was labelled. These results, together with the microsequencing data, demonstrate that the 58000- and 54000-M_r forms result from alternative splicing of the same transcript.

New phosphorylation sites

The fact that the 54000-M_r form was phosphorylated by PKA and PKC (Fig. 2) despite the loss of Ser-466 and Thr-475 prompted us to search for other phosphorylation sites on the protein. The bovine heart enzyme preparation was incubated with [γ-^{32}P]MgATP and either PKA or PKC, prior to electroblotting and microsequencing. Peak II was labelled by PKC in both the 58000- and 54000-M_r forms and should therefore contain phosphorylation site(s) other than Thr-475. This peak contained three peptides, whose sequences were identified (Table 1) by comparison with the published amino acid sequence [6], and which are potential phosphorylation sites for PKC [27].

We also searched for a second phosphorylation site for PKA in the 58000- and 54000-M_r forms. In the 54000-M_r form no phosphorylated peptide was detected in blots similar to that shown in Fig. 6(*a*). However, Fig. 2 clearly shows that the 54000-M_r form was phosphorylated by PKA. Therefore we suggest that this phosphorylated peptide was hydrophobic and was not released from the PVDF membrane. Apart from Ser-466 encoded by exon 15, Ser-29 is in a favourable consensus sequence for

Table 1. Sequences of bovine heart PFK-2/FBPase-2 peptides after trypsin digestion of the 58000- and 54000-M_r forms

The peaks taken for amino acid sequencing are shown in Fig. 6. X could not be identified, consistent with the fact that this residue (Thr-475) was phosphorylated. S refers to serine and to dehydroalanine.

Peak	Amino acid sequence	Position
I	DKPTAETSR	447–450 and 511–515
II	ASLR	19–22
	KLTR	57–60
	QAVKSYK	80–86
III	RNSFTPLSSSNXI	464–476

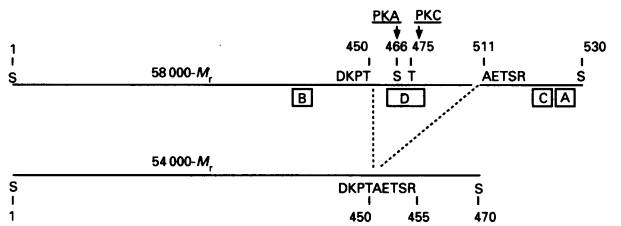

Fig. 7. Schematic representation of the two forms of bovine heart PFK-2/FBPase-2

The amino acids are indicated by the single-letter code. The squares show the location, with respect to the amino acid sequence, of the oligonucleotides described in the Materials and methods section.

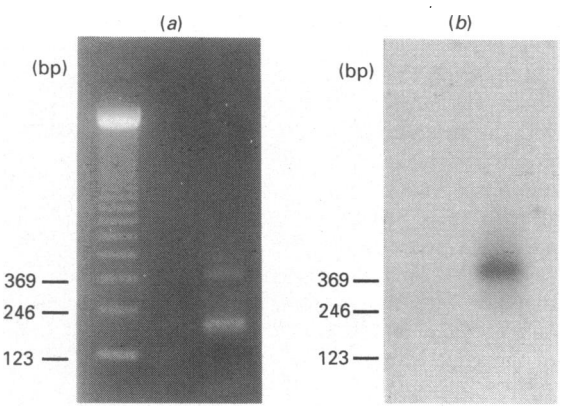

Fig. 8. Identification by PCR of two forms of bovine heart PFK-2/FBPase-2 mRNA

(*a*) Ethidium bromide staining of a 1.5% agarose gel electrophoresis of the PCR product (see the Materials and methods section) alongside a 180 bp DNA ladder (BRL). The right lane shows a major band of about 220 bp and a minor band of about 370 bp. (*b*) Southern blot of the same gel after hybridization with oligo D (see Fig. 7).

PKA [27]. This serine residue corresponds to Ser-32 in rat liver PFK-2/FBPase-2, which is phosphorylated by PKA. Ser-29 is located in a long hydrophobic tryptic fragment (residues 28–49), which could easily be retained by the PVDF membrane. Therefore we suggest that Ser-29 could represent another phosphorylation site for PKA, in addition to Ser-466 in the *C*-terminal domain.

Conclusions

The evidence that the 58000- and 54000-M_r forms of bovine heart PFK-2/FBPase-2 result from alternative splicing can be summarized as follows. First, the 54000-M_r form contains a peptide which is not present in the 58000-M_r form. Inspection of the rat gene and of the bovine cDNA coding for the heart isoenzyme shows that the nucleotides coding for the first half of this peptide occur at the end of exon 14, while those coding for the other half occur at the beginning of exon 16. This suggests that in the 54000-M_r form the region encoded by exon 15 is absent. Second, analysis of the heart RNA suggests the presence of two PFK-2/FBPase-2 mRNAs, only one of which hybridizes with a nucleotide probe corresponding to a sequence in exon 15. Third, the 58000-M_r form contains a peptide labelled by PKC which is not present in the 54000-M_r form and which is coded by exon 15.

The fact that the 54000-M_r form was phosphorylated by PKA and PKC indicated that additional phosphorylation sites for both protein kinases should be located outside the missing peptide. As to the effects of phosphorylation on activity, they should be interpreted with caution. First, the stoichiometry of phosphorylation by PKA and PKC might be underestimated because the two forms could already be partially phosphorylated. Second, there is multisite phosphorylation of the two forms. Third, the relative proportion of the two forms might be regulated. Fourth, the existence of heterodimers of the two forms cannot be excluded at present. One way of approaching these problems would be to study the properties of the expressed recombinant proteins.

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