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Rabbit muscle fructose-1,6-bisphosphatase is phosphorylated *in vivo*★

Dariusz Rakus, Marek Zarzycki and Andrzej Dzugaj✉

Department of Animal Physiology, Zoological Institute, University of Wrocław, Z. Cybulskiego 30, 50-205 Wrocław, Poland

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Phosphorylated fructose-1,6-bisphosphatase (FBPase) was isolated from rabbit muscle in an SDS/PAGE homogeneous form. Its dephosphorylation with alkaline phosphatase revealed 2.8 moles of inorganic phosphate per mole of FBPase. The phosphorylated FBPase (P-FBPase) differs from the dephosphorylated enzyme in terms of its kinetic properties like K_m and k_{cat}, which are two times higher for the phosphorylated FBPase, and in the affinity for aldolase, which is three times lower for the dephosphorylated enzyme.

Dephosphorylated FBPase can be a substrate for protein kinase A and the amount of phosphate incorporated per FBPase monomer can reach 2–3 molecules.

Since interaction of muscle aldolase with muscle FBPase results in desensitisation of the latter toward AMP inhibition (Rakus & Dzugaj, 2000, *Biochem. Biophys. Res. Commun.* 275, 611–616), phosphorylation may be considered as a way of muscle FBPase activity regulation.

Fructose-1,6-bisphosphatase (FBPase) [EC 3.1.3.11] catalyses the hydrolysis of fructose-1,6-bisphosphate (Fru-1,6-P_2) to fructose-6-phosphate and inorganic phosphate. Liver and muscle isoenzymes have been found in mammalian tissues (Tejwani, 1983; Al-Robaiy & Eschrich,1999). Liver FBPase is recognised as a regulatory enzyme of gluconeogenesis, the muscle isoenzyme participates in glycogen synthesis from lactate (Ryan & Radziuk,1995; Gleeson, 1996). Both isoenzymes require divalent cations like mag-

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✉Corresponding author: Andrzej Dżugaj; phone: (48 71) 375 9213, fax: (48 71) 328 8246, e-mail: dzugajan@biol.uni.wroc.pl

Abbreviations: FBPase, fructose-1,6-bisphosphatase; Fru-1,6-P_2, fructose-1,6-bisphosphate; PKA, cAMP-dependent protein kinase.

nesium, manganese, cobalt or zinc to express their activity, are activated by monovalent cations, inhibited competitively by fructose-2,6-bisphosphate (Fru-2,6-P_2) and allosterically by AMP (Pilkis *et al.,* 1981; Van Schaftigen & Hers, 1981). A synergistic effect of both these inhibitors has also been reported by Van Schaftigen & Hers (1981) and Skalecki *et al.* (1995). The basic difference between the liver and muscle FBPase concerns their sensitivity to AMP inhibition. $I_{0.5}$ for AMP for the muscle isoenzyme is about 0.1 μM and this value is 50–100 times lower than that determined for the liver isoenzyme (Tejwani, 1983; Skalecki *et al.,* 1995; Rakus & Dzugaj, 2000). Taking into account that AMP level in muscle cells is within the range of 20 to 30 μM and Fru-2,6-P_2 is about 2.5 μM, the muscle FBPase should be almost completely inhibited, expressing no more than 0.1% of its activity (Skalecki *et al.*, 1995). This raises a question how the muscle FBPase may function *in vivo*. Searching for physiological activators of muscle FBPase we found that muscle aldolase desensitises muscle FBPase to AMP inhibition (Rakus & Dzugaj, 2000). In this paper we demonstrate that muscle FBPase is phosphorylated and the phosphorylated form has a higher affinity for aldolase. To the best of our knowledge this is the first report concerning muscle FBPase phosphorylation. The physiological meaning of this phenomenon is discussed.

MATERIALS AND METHODS

Phosphocellulose P-11 was purchased from Whatman (Maidstone, England), ammonium sulphate was from Fluka (Buchs, Switzerland). Malachite Green and ammonium molybdate were purchased from POCh (Gliwice, Poland). [γ^{32}P]ATP was from NEN (Boston, MA, U.S.A.). Other reagents were from Sigma (St. Louis, MO, U.S.A.). All the reagents were of the highest purity commercially available.

Enzyme purification and kinetic studies. Rabbit muscle FBPase was purified from fresh skeletal muscle under conditions protecting against dephosphorylation according to the method of Rakus & Dzugaj (2000).

The protein purity and proteolysis level were checked by using 10% SDS/polyacrylamide gel electrophoresis according to Laemmli (1970).

All kinetic experiments were performed at pH 7.5 at 37°C using a glucose-6-phosphate isomerase–glucose-6-phosphate dehydrogenase coupled spectrophotometric assay (Skalecki *et al.*, 1995).

Determination of K_m and K_{is} for Fru-1,6-P_2 (inhibitory constant for the substrate) was carried out in 1 ml mixture containing: 50 mM bis-tris propane (BTP), 2 mM $MgCl_2$, 150 mM KCl, 1 mM EDTA, 0.2 mM NADP, 10 units glucose-6-phosphate dehydrogenase, 10 units glucose-6-phosphate isomerase, 1 μg of FBPase and variable concentration of Fru-1,6-P_2. The reaction was started by addition of the substrate and the reaction progress was recorded in intervals of 1 s. FBPase concentration was determined spectrophotometrically assuming that $A^{1\%}_{1cm}$ = 6.3 at 280 nm. V_{max}, k_{cat}, K_m and K_{is} were calculated on the basis of the recorded progress of the reaction with Excel and GraFit 3 (Leatherbarrow, 1992) accounting for the uncompetitive inhibition of the enzyme by substrate according to (Skalecki *et al.*, 1995).

One unit of enzyme activity is defined as the amount of the enzyme that catalyses the formation of 1 μmole of product per minute.

The determination of $I_{0.5}$ and Hill coefficient for AMP (n) as well as $A_{0.5}$ and n for Mg^{2+} was performed using the GraFit 3 programme.

AMP concentration was determined spectrophotometrically using 15 400 $M^{-1}cm^{-1}$ as the molar absorption coefficient at 259 nm.

FBPase dephosphorylation. To obtain the dephosphorylated form of FBPase, the enzyme was incubated for 3 h at 37°C with alkaline phosphatase from bovine intestinal mucosa. A 250 μl reaction mixture contained:

200 μg of FBPase, 10 U (5 μg) of alkaline phosphatase, 20 mM Tris, 5 mM $MgCl_2$, 100 mM KCl and 1 mM EDTA, pH 8.0. The reaction was terminated by heating at 56°C for 10 min, and denatured alkaline phosphatase was removed by centrifugation. There was no detectable alkaline phosphatase activity determined in the supernatant incubated for 30 min at 37°C in 50 mM Tris, 10 mM $ZnSO_4$, 1 mM EDTA, pH 8.0, with 200 μM Fru-1,6-P_2 and 10 μM AMP as substrates. FBPase concentration and activity in the supernatant were determined spectrophotometricaly as described above.

The control sample of non-dephosphorylated, native form of FBPase, was obtained by applying the above mentioned procedure, but omitting alkaline phosphatase in the reaction mixture.

Endogeneous phosphate contents. FBPase was initially extensively dialysed against 4 M urea in 20 mM Tris (pH 7.5), then against 20 mM Tris, 0.1 mM EDTA (pH 7.5, 4°C). This procedure was employed to remove traces of AMP and phosphosugars from the enzyme.

To determine the amount of phosphates in native FBPase, the enzyme was dephosphorylated for 3 h at 37°C with alkaline phosphatase. The dephosphorylating mixture (400 μl) contained: 2 U of alkaline phosphatase, 120 μg FBPase, 20 mM Tris, 0.1 mM EDTA, 5 mM $MgCl_2$, 100 mM KCl, pH 8.0. The reaction was stopped by heating at 100°C, proteins were discarded by centrifugation and the released phosphate was measured with Malachite Green.

Control experiments were performed incubating the dephosphorylating mixture without FBPase as well as incubating FBPase samples without alkaline phosphatase.

Binding experiment. Binding of the dephosphorylated and non-dephosphorylated rabbit muscle FBPase with rabbit muscle aldolase was performed in the presence of 10% polyethylene glycol (PEG 8000) as described by Rakus & Dzugaj (2000). Each sample (100 μl) contained 100 μg/ml (0.68 μM) FBPase and various concentrations of aldolase: 0.1–10 mg/ml (0.63–63 μM). Following incubation at room temperature for 120 min the samples were centrifuged at 35 000 r.p.m. in a Beckman TLA-100 rotor for 60 min at 25°C. The amount of FBPase in the supernatant and in the pellet was determined by measuring the enzyme activity.

Dissociation constant (K_d) for the aldolase–FBPase complex was calculated using the GraFit 3 programme.

The effect of aldolase on the inhibition of non-dephosphorylated and dephosphorylated FBPase by AMP. The effect of aldolase on FBPase activity was measured as the change in the rate of NADP reduction coupled to 6-phosphogluconate formation from dihydroxyacetone phosphate as described previously by Rakus & Dzugaj (2000). Constant FBPase and aldolase concentrations, 1 μg/ml and 1 mg/ml respectively, were used in the assay system.

Phosphorylation of FBPase. Various amounts (6.8 pmole to 27.3 pmole) of rabbit muscle FBPase dephosphorylated with alkaline phosphatase were phosphorylated with 5 units of cAMP-dependent protein kinase (PKA) for 60 min at 30°C, pH 7.0. The reaction took place in the presence of 0.2 mM ATP, 5 μM cAMP, 0.035 μCi [γ^{32}P]ATP, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mg/ml BSA, 50 μM EDTA, 50 μM EGTA, 25 mM Hepes in 25 μl. The reaction was stopped by precipitation with 15% trichloroacetic acid in the presence of 1 mg/ ml BSA. The precipitate was dissolved in 50 μl of 0.5 M NaOH and reprecipitated three times.

The amount of phosphate incorporated into FBPase depends on time. The progress of FBPase (1 μg, 27.5 pmole) phosphorylation by PKA (5 U) was monitored between 0 and 90 min. The conditions of the reaction were as above.

Labeled phosphate incorporation into the enzyme was estimated by the method of Corbin & Reimann (1974).

Rabbit muscle FBPase modelling and phosphorylation sites prediction. The T-state structure of rabbit muscle FBPase was constructed on the basis of 1RDY.pdb using the SPDBV software (Peitsch, 1995; Guex & Peitsch, 1997; Guex *et al.,* 1999).

The prediction for serine, threonine and tyrosine phosphorylation sites in rabbit muscle FBPase was performed with the NetPhos 2 WWW server (Blom *et al.,* 1999).

RESULTS AND DISCUSSION

Phosphorylated FBPase was isolated from rabbit muscle in an SDS/PAGE homogeneous form. Its dephosphorylation with alkaline phosphatase revealed 2.8 moles of inorganic phosphate per mole of FBPase, which means 0.7 moles of phosphate per FBPase monomer. The effect of substrate concentration on the activity of phosphorylated and dephosphorylated FBPase is presented in Fig. 1a and 1b. Dephosphorylation slightly increases the FBPase affinity for the substrate and decreases k_{cat} (Table 1). Mammalian liver and muscle FBPases are inhibited by excess of the substrate (Skalecki *et al.,* 1995; Mizunuma & Tashima, 1978; Rakus *et al.,* 2000) but the dephosphorylated rabbit muscle FBPase is insensitive to inhibition by Fru-1,6-P_2 (Table 1). Dephosphorylation does not change the FBPase affinity for AMP. $I_{0.5}$ of the phosphorylated and dephosphorylated enzyme as well as Hill coefficients are nearly the same (Fig. 2, Table 1). On the other hand, the phosphorylated enzyme has a significantly higher affinity for aldolase (Fig. 3), which results in higher desensitisation to AMP inhibition in the presence of aldolase (Fig. 2). The determined K_d values were 19 μM and 53 μM for the phosphorylated and dephosphorylated FBPase, respectively.

Phosphorylation of mammalian liver FBPases has been previously investigated (Ekdahl, 1987; 1988). It has been postulated that phosphorylation plays a role in regulating the enzyme activity *in vivo*. It turned out that only rat liver FBPase, which extends 24–26 residues beyond the C-terminal amino acids of other mammalian liver FBPases, is a substrate for protein kinases (Vidal *et al.,* 1986). The phosphorylation sites are located in this extension. Phosphorylation slightly decreased the rat liver FBPase affinity for the substrate but the remaining kinetic parameters of the phosphorylated and unphosphorylated enzyme were practically the same.

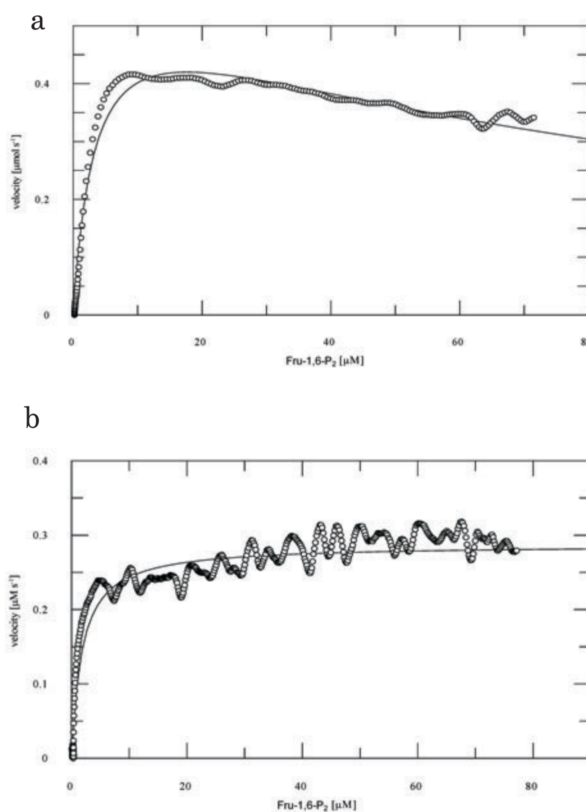

Figure 1. Dependence of velocity of reaction catalysed by phosphorylated (a) and dephosphorylated (b) rabbit muscle FBPase on substrate concentration.

Experimental points of the spectrophotometrically registered progress of the reaction were used to determine substrate concentration and the velocity of catalysed reaction. Sets of five experimental points were used for substrate concentration calculation. For each of those points the velocity of the reaction was calculated taking additionally the five preceding and five subsequent points. The calculated values are presented as open circles. The parameters of the presented curve were calculated as described in Materials and Methods.

Table 1. Kinetic properties of phosphorylated and dephosphorylated rabbit muscle FBPase.

Dephosphorylated FBPase has lower K_m and k_{cat} and is not inhibited by substrate.

FBPase	k_{cat} (s^{-1})	K_m (μM) Fru-1,6-P_2	K_{is} (mM) Fru-1,6-P_2	$I_{0.5}$ (nM) AMP	Hill constant (n)	$A_{0.5}$ (mM) Mg^{2+}	Hill constant (n)
Phosphorylated	21	3	95	69	2.24	0.25	2.32
Dephosphorylated	12.5	1.5	not inhibited	83	2.16	0.29	2.30

Therefore, most authors have concluded that mammalian liver FBPase activity is regulated only indirectly by phosphorylation of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2/FBPase2), which affects Fru-2,6-P_2 concentration.

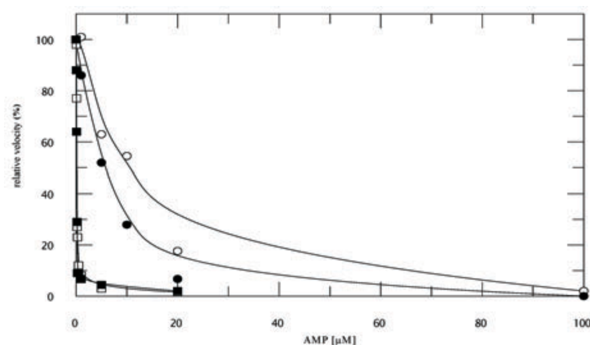

Figure 2. Inhibition of phosphorylated and dephosphorylated rabbit muscle FBPase by AMP in the absence of aldolase (respectively: empty and full squares) and in the presence of 6 μM rabbit muscle aldolase (respectively: empty and full circles).

Curves were plotted employing nonlinear regression analysis using the GraFit 3 programme.

Regulation of muscle FBPase activity is still an open question. Because of its high sensitivity to AMP inhibition and the synergistic effect of AMP and Fru-2,6-P_2, the muscle enzyme should be almost completely inhibited under physiological conditions (Skalecki *et al.*, 1995). On the other hand, in muscle cells glycogen synthesis from noncarbohydrate precursors is observed (Ryan & Radziuk, 1995; Gleeson, 1996) and thus muscle FBPase must be active *in vivo*. Recently we have described an interaction of muscle aldolase with muscle FBPase that results in a partial desensitisation of the latter enzyme to AMP inhibition (Rakus & Dzugaj, 2000). In this paper we present evidence that phosphorylated rabbit muscle FBPase has an increased affinity for

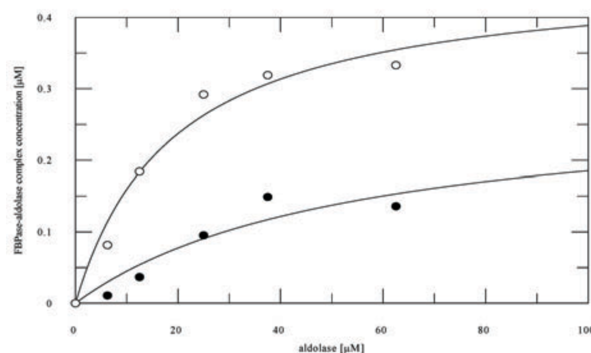

Figure 3. Saturation of phosphorylated (empty circles) and dephosphorylated (full circles) muscle FBPases (both at 0.68 μM) with muscle aldolase.

The experiment was performed in 10% polyethylen glycol.

aldolase. Assuming that phosphorylation of muscle FBPase is reversible, its hormonal control might be expected. In the liver signals from β-adrenergic receptors are transferred to phosphorylation of PFK2/FBPase2 which results in decreasing Fru-2,6-P_2 concentration. Muscle PFK2/ FBPase2 does not contain the phosphorylation domain (Rousseau & Hue, 1993) and therefore it cannot be regulated by hormone dependent phosphorylation. On the other hand, muscle FBPase seems to be a good substrate for kinases. Employing the NetPhos 2.0 (Blom *et al.,* 1999)

programme we found that Ser 148, Ser 270 and Thr 105 might be a target for protein kinase C. Thr 142 and Thr 143 might be phosphorylated by cAMP-dependent protein kinase (Fig. 4).

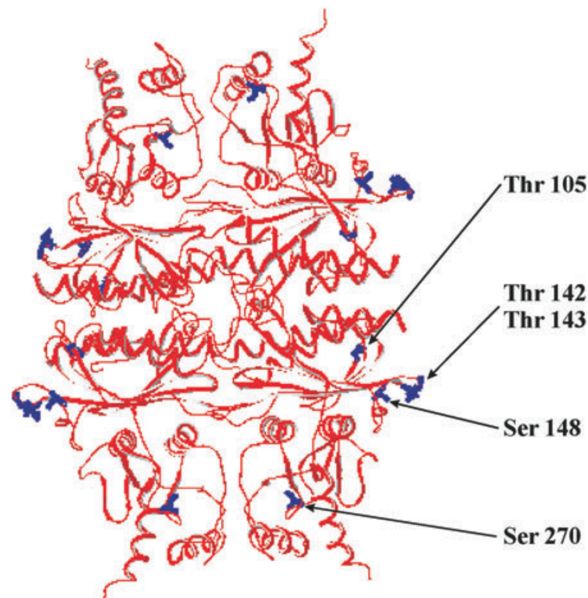

Figure 4. A model of rabbit muscle FBPase, with hypothetical phosphorylation sides.

Thr 143 (or 142) could be phosphorylated by cAMP-dependent protein kinase. Thr 105, Ser 148, Ser 270 may be the substrates for protein kinase C.

The time course of phosphorylation of rabbit muscle FBPase with PKA revealed that up to 3.2 phosphate molecules can be incorporated into FBPase monomer (Table 2). (The observed decrease of the phosphorylation level after 60 min of incubation was supposedly caused by instability of PKA and traces of alkaline phosphatase in the sample). Although four amino-acid residues on the surface of FBPase monomer can serve as substrates for phosphorylation, the results of the phosphorylation of various amounts of FBPase (Table 3) suggest that only two phosphate molecules are incorporated specifically. The FBPase in living muscle cells is probably composed of unphosphorylated and phosphorylated forms. Since phosphorylated FBPase has a higher affinity for aldolase and FBPase in a complex with aldolase has a decreased affinity for AMP, the reversible phosphorylation might be a way of regulation of FBPase activity in the muscle cell. Further studies are necessary to answer the question if FBPase phosphorylation and dephosphorylation are hormonally controlled.

Table 2. Rabbit muscle FBPase phosphorylation with PKA

Time of phosphorylation (min)	The amount of incorporated phosphate residues per monomer of FBPase
0	0.0
10	0.84
20	1.08
40	1.68
60	3.16
90	2.38

Table 3. PKA catalysed phosphate (^{32}P) incorporation into FBPase

FBPase monomer concentration [μM]	The amount of incorporated phosphate residues per monomer FBPase
6.81	1.82
13.6	1.93
20.4	1.64
27.3	1.85

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