Regulation of fructose-2,6-bisphosphate synthesis and breakdown in heart and skeletal muscle

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Introduction

Fructose-2,6-bisphosphate is a potent stimulator of 6-phosphofructo-1-kinase (PFK-1) in all mammalian tissues studied so far (Hers & Van Schaftingen, 1982). 6-Phosphofructo-1-kinase is generally regarded as an enzyme which participates in the control of glycolysis. In principle, thereafter, changes in the concentration of fructose-2,6-bisphosphate could cause parallel changes in glycolysis. Fructose-2,6-bisphosphate is also an inhibitor of fructose-1,6-bisphosphatase (FBPase-2) and, in general, changes in the concentration of hepatic fructose-2,6-bisphosphate correlate positively with glycolysis and inversely with gluconeogenesis (Hers & Hue, 1983). The purpose of this article is to review recent work on the enzymology of fructose-2,6-bisphosphate synthesis and degradation in heart and skeletal muscle and to summarize its role in these tissues. The liver system is briefly described for comparison.

The bifunctional liver PFK-2/FBPase-2

The steady-state concentration of fructose-2,6-bisphosphate depends on the activities of 6-phosphofructo-2-kinase (PFK-2), which catalyses its synthesis and fructose-2,6-bisphosphatase (FBPase-2) which catalyses its breakdown. PFK-2 and FBPase-2 are purified to homogeneity with a protein of M, 110000 composed of identical M, 55000 subunits (El-Maghrabi et al., 1982a). Limited proteolysis (El-Maghrabi et al., 1984a; Sakakibara et al., 1984) and treatment of the bifunctional enzyme with protein modifying reagents (El-Maghrabi et al., 1984a; b; Sakakibara et al., 1984a; Kitajima et al., 1985) indicates that the two reactions are catalysed at distinct sites on the same polypeptide chain.

Product inhibition studies suggest that the mechanism of the kinase reaction involves ternary complex formation (Kitajima et al., 1984; Kretschmer & Hofmann, 1984), whereas a phosphoryl enzyme (E-P) is formed in the bisphosphatase reaction (Pilkis et al., 1984). The rate of E-P formation from [2-3P]fructose-2,6-bisphosphate (100-times faster than the overall bisphosphatase reaction, consistent with its existence as a reaction intermediate (Stewart et al., 1985).

The activity of PFK-2 depends on the concentrations of its substrates, fructose-6-phosphate and MgATP, and the concentrations of both its positive effectors, AMP and P, and negative effectors, sn-glycerol-3-phosphate, citrate and phosphoenolpyruvate. Fructose-2,6-bisphosphate activity is stimulated by sn-glycerol-3-phosphate and P, and inhibited by the reaction product, fructose-6-phosphate (reviewed by Hers & Hue, 1983). In addition, PFK-2/FBPase-2 is an interconvertible enzyme regulated by phosphorylation/dephosphorylation. Treatment of the bifunctional enzyme with cyclic-AMP-dependent protein kinase results in phosphorylation of the enzyme (1 mol phosphate incorporated/mol enzyme subunit) and concomitant inactivation of PFK-2 and activation of FBPase-2 (Murray et al., 1984; Sakakibara et al., 1984a). The PFK-2 activity of phosphorylated enzyme has a decreased affinity for fructose-6-phosphate, a lower V, and is more susceptible to phosphoenolpyruvate and citrate inhibition (Van Schaftingen et al., 1981). The effects of phosphorylation on PFK-2 activity are less at pH 8 than at pH 7. The activity of FBPase-2 is increased by virtue of a change in V, and increased sensitivity to sn-glycerol-3-phosphate stimulation (Van Schaftingen et al., 1982). The bifunctional enzyme is dephosphorylated by phosphoprotein phosphatase(s) (Pelech et al., 1984; Mieskes et al., 1984). Reciprocal changes in PFK-2 and FBPase-2 described above are observed in hepatocytes treated with glucagon (Bartrons et al., 1983) and are probably the result of cyclic-AMP-dependent phosphorylation of the enzyme (El-Maghrabi et al., 1982a).

In addition to cyclic-AMP-dependent phosphorylation and regulation of liver PFK-2/FBPase-2, phosphorylase kinase has been reported to phosphorylate and inactivate PFK-2 (Uyeda et al., 1982). However, this effect is probably of little physiological significance since in hepatocytes incubated with A23187, vasopressin or angiotensin II, all of which stimulate phosphorylase kinase, no change in PFK-2/FBPase-2 phosphorylation is observed (Garrison et al., 1984).

Heart PFK-2: an isoenzyme distinct from liver PFK-2

Evidence for distinct isoenzymes of PFK-2 was first suggested when purified bovine heart PFK-2 was compared with the rat liver enzyme (Rider et al., 1985). The two enzymes were originally reported to differ in molecular mass (Rider et al., 1985), but this was not confirmed (Rider & Hue, 1986) and in addition El-Maghrabi et al. (1986) report a similar Stokes radius for PFK-2 from several rat tissues including heart and liver. However, compared with rat liver PFK-2, the bovine heart enzyme has a lower K for fructose-6-phosphate, is barely inhibited by sn-glycerol-3-phosphate and is more sensitive to citrate inhibition (Rider et al., 1985). Unlike rat liver PFK-2, bovine heart PFK-2 is not inactivated on incubation with cyclic-AMP-dependent protein kinase (Rider et al., 1985) and is phosphorylated to a lesser extent (0.29 mol phosphate incorporated/mol enzyme subunit) than the liver enzyme (0.83 mol phosphate incorporated/mol enzyme subunit) (Rider & Hue, 1986). On the contrary, when the two enzymes are incubated with the calcium- and phospholipid-dependent protein kinase, protein kinase C, heart PFK-2 is phosphorylated to the same extent as liver PFK-2 (1 mol phosphate incorporated/mol enzyme subunit), whereas liver PFK-2 is a relatively poor substrate (0.19 mol phosphate incorporated/mol enzyme subunit) (Rider & Hue, 1986). The latter finding complements the work of Garrison et al. (1984), who showed that PFK-2 is not phosphorylated in hepatocytes stimulated with phorbol ester. Incubation with protein kinase C has no effect on the activities of either heart or liver PFK-2 (Rider & Hue, 1986).

A striking difference between the two enzymes is their FBPase-2 content. Heart PFK-2 has a PFK-2/FBPase-2 activity ratio which is 20-times that of the liver enzyme. Measurement of E-P formation from [2-3P]fructose-2,6-bisphosphate supports the low FBPase-2 activity of heart PFK-2 (Rider & Hue, 1986). Liver FBPase-2 is phosphorylated to the extent of 0.25 mol/mol holoenzyme within 10s, whereas for heart enzyme the rate of labelling is at least 10-times slower and the extent of labelling is 6-fold less. Is heart PFK-2 a bifunctional enzyme? The low FBPase-2 activity of bovine heart PFK-2 has been consistently observed (Rider et al., 1985; Rider & Hue, 1986); however, one could argue that the FBPase-2 activity is the result of contamination of the heart PFK-2 preparation by the liver enzyme. Alternatively, the FBPase-2 activity of bovine heart PFK-2 could be a genuine characteristic of the enzyme. In this case, heart PFK-2 would represent a bifunctional
enzyme, albeit with a low bisphosphatase activity. Table 1 shows that the latter possibility is probably true. Incubation of rat liver PFK-2/FBPase-2 with cyclic-AMP-dependent protein kinase resulted in a 4-fold activation of FBPase-2, whereas incubation of bovine heart enzyme under identical conditions caused no change in bisphosphatase activity. These findings contradict those of El-Maghrabi et al. (1986) who were unable to detect any bisphosphatase activity in heart PFK-2 either by activity measurement or E-P formation.

The difference between bovine heart and rat liver PFK-2 could be due to a species rather than a tissue difference. However, like bovine heart PFK-2, the rat heart enzyme is not inactivated by cyclic-AMP-dependent protein kinase (Rider et al., 1985; El-Maghrabi et al., 1986), contains little FBPase-2 activity and is more sensitive to citrate inhibition than rat liver PFK-2 (Rider & Hue, 1987). The PFK-2 isoenzymes present in heart and liver have been designated type 'H' and type 'L', respectively (Rider & Hue, 1986).

The significance of heart PFK-2 phosphorylation by protein kinase C described above is unclear since no changes in the activities of PFK-2 (Rider et al., 1986) or FBPase-2 (M. H. Rider & L. Hue, unpublished work) have been observed. It has yet to be shown that conditions which increase phosphorytidylinositol turnover in intact heart, such as α-adrenergic, muscarinic or electrical stimulation (Poggioli et al., 1986) or treatment with phorbol ester, lead to phosphorylation of heart PFK-2. In skeletal muscle, phosphorylation of PFK-1 by cyclic-AMP-dependent protein kinase increases the affinity of the enzyme for F-actin, thereby activating the enzyme (Luther & Lee, 1986). Moreover, in pig heart, PFK-2 also binds to myofibrils (M. H. Rider & L. Hue, unpublished work) and by analogy, phosphorylation by protein kinase C might regulate binding of PFK-2 to these structures, resulting in a change in enzyme activity. Alternatively, the phosphorylation of heart PFK-2 by protein kinase C might affect its activity in the long term by altering its susceptibility to proteolysis.

Fructose-2,6-bisphosphate hydrolysis in heart

In perfused rat hearts, the addition of ketone bodies to the perfusate decreased fructose-2,6-bisphosphate concentration from 1.9 to 0.5 mmol/g wet wt. in 20 min, indicating that an active bisphosphatase is present in the tissue (L. Hue & M. H. Rider, unpublished). The activity of PFK-2-1 in bovine heart is about 1 mmol fructose-2,6-bisphosphate produced/min per g wet wt. of tissue (M. H. Rider & L. Hue, unpublished work) and if the FBPase-2 activity of PFK-2 represents 1/80th of the kinase activity in the intact tissue (about 0.01 mmol produced/min per g wet wt.) this would clearly be insufficient to account for the hydrolysis of fructose-2,6-bisphosphate observed after perfusion with ketone bodies. However, the low FBPase-2 activity of heart PFK-2 might be regulated. It is possible that metabolite(s) or covalent modification could unmask the activity. Citrate, an inhibitor of PFK-2 which might be expected to increase in hearts perfused with ketone bodies, has no effect on FBPase-2 activity (M. H. Rider & L. Hue, unpublished work).

In pig heart, most of the FBPase-2 activity is associated with myofibrils (M. H. Rider & L. Hue, unpublished work). However, the activity is not saturable, even with 0.3 mm-fructose-2,6-bisphosphate and is 50% inhibited by 1 mm-sn-glycerol-3-phosphate and other phosphomonoesters. Although this activity (0.1 nmol P, produced/min per g wet wt. of tissue) would be sufficient to account for fructose-2,6-bisphosphate hydrolysis (M. H. Rider & L. Hue, unpublished work), it is likely to represent non-specific phosphatase activity and therefore be unimportant physiologically since the tissue concentration of other phosphohostes is 100-1000 times that of fructose-2,6-bisphosphate.

In addition to phosphomonoesterase activity, other means of fructose-2,6-bisphosphate removal could be envisaged. For example, analogues with the glucose-1,6-bisphosphate-dependent family of enzymes can be made (Rose, 1986). Like glucose-1,6-bisphosphate, fructose-2,6-bisphosphate could act as phosphoryl donor. The phosphate could be transferred to a sugar-1, 6 or -5 phosphate or other acceptor. Alternatively, osyl transfer could occur in which the furanose ring is transferred to an acceptor. The analogy can be made with ADP-ribosylation, whereby the ADP moiety of NAD+ is attached to an acceptor protein via the ribose ring (Ueda & Hayashi, 1985). Such a mechanism might be probed experimentally by incubating tissue fractions with [U-14C]fructose-2,6-bisphosphate. Whether such mechanisms for fructose-2,6-bisphosphate disposal exist in heart or other tissues remains to be determined.

Skeletal muscle PFK-2: a third isoenzyme?

Rat skeletal muscle PFK-2 resembles both type H and type L PFK-2. It is not inactivated by cyclic-AMP-dependent protein kinase (Rider et al., 1985) and its sensitivity to citrate inhibition is similar to that of type H PFK-2 (Rider & Hue, 1987). On the other hand, significant PFK-2 shares many of the properties of muscle PFK-2 with cyclic-AMP-dependent protein kinase (Van Schaftingen & Hers, 1986). Such a mechanism might be probed experimentally by incubating tissue fractions with [U-14C]fructose-2,6-bisphosphate. Whether such mechanisms for fructose-2,6-bisphosphate disposal exist in heart or other tissues remains to be determined.

Table 1. Effect of cyclic-AMP-dependent protein kinase on the FBPase-2 activity of heart and liver PFK-2

<table>
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<tr>
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<th>Heart</th>
<th>Liver</th>
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<tr>
<td>FBHase-2 activity (nmol P, produced/min per mg protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>− Catalytic subunits</td>
<td>1.4</td>
<td>8.2</td>
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<tr>
<td>+ Catalytic subunits</td>
<td>1.3</td>
<td>35.5</td>
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HTC cells, a liver-to-muscle-type PFK-2 transition may be involved.

**Role of fructose-2,6-bisphosphate in heart and muscle**

The basal tissue concentrations of fructose-2,6-bisphosphate in heart and skeletal muscle are about 1 nmol/g (Hue et al., 1982; Rider & Hue, 1984; Narabayashi et al., 1985) and 0.5 nmol/g (Hue et al., 1982; Minatogawa & Hue, 1984; Bosca et al., 1985) representing concentrations 1/10 and 1/20, respectively of its concentration found in the livers of fed rats. However, these values are within the range of concentrations which stimulate PFK-1 in heart (Clark & Patten, 1984; Narabayashi et al., 1985) and muscle (Uyeda et al., 1981). Changes in the concentration of fructose-2,6-bisphosphate can be brought about by changes in the concentrations of substrates and effectors of PFK-2/FBPase-2 or through covalent modification or any combination of these.

Adrenaline increases the concentration of fructose-2,6-bisphosphate in skeletal muscle by 2-4-fold (Hue et al., 1982; Bosca et al., 1985), while in heart, the hormone has been reported to double (Narabayashi et al., 1985) or cause no change (Hue et al., 1985) in fructose-2,6-bisphosphate content. These increases cannot be the result of cyclic-AMP-dependent protein kinase activation by adrenaline since treatment of muscle and heart PFK-2/FBPase-2 with the protein kinase causes no change in the enzyme activities (see above). The rise in fructose-2,6-bisphosphate elicited by adrenaline in heart and muscle is probably secondary to the increased rate of glycogen breakdown which leads to a 2-6-fold rise in the hexosemonophosphate pool (Hue et al., 1982; Bosca et al., 1985; Narabayashi et al., 1985).

In both muscle (Hue et al., 1982; Bosca et al., 1985) and heart (Rider & Hue, 1984; Rider et al., 1986), insulin doubles the tissue content of fructose-2,6-bisphosphate. The doubling is unlikely to be the consequence of the 30-40% increase in hexosemonophosphates (Rider & Hue, 1984; Bosca et al., 1985), which probably occurs as a result of an increase in glucose transport. Therefore other mechanism(s) must be involved. Insulin doubles the V_{max} of PFK-2 in rat heart, possibly resulting from covalent modification of the enzyme (Rider & Hue, 1984). Whether a similar effect of insulin on skeletal muscle PFK-2 is responsible for the rise in fructose-2,6-bisphosphate has yet to be tested. In electrically stimulated muscles, the fructose-2,6-bisphosphate content is not changed by frequencies which cause tetanic contractions, but at low frequencies resulting in a small stimulation of glycolysis, it increases transiently (Minatogawa & Hue, 1984). The high FBPase-2:PFK2 activity ratio of muscle PFK-2 and decreased sensitivity of FBPase-2 to fructose-6-phosphate inhibition (Rider & Hue, 1984), may explain why the fructose-2,6-bisphosphate does not always rise in muscle when fructose-6-phosphate is increased.

In addition to its stimulatory effect on PFK-1, fructose-2,6-bisphosphate has been reported to stimulate or inhibit muscle FBPase-1, depending on the assay conditions (Bosca et al., 1985). It increases FBPase-1 activity at high fructose-1,6-bisphosphate concentrations. Whether a rise in fructose-2,6-bisphosphate could increase the rate of cycling at the level of fructose-6-phosphate/fructose-1,6-bisphosphate in the presence of elevated concentrations of the latter remains to be determined. Since the cycling rate is low compared to the glycolytic flux in vertebrate skeletal muscle it is probably not important. However, in bumble bee flight muscle, the substrate cycle may be important for thermogenesis which helps to maintain the thorax temperature at 30°C required for flight (Clark et al., 1973). Substrate cycling is inversely related to the ambient temperature and abolished during flight (Clark et al., 1973). Insect flight muscles display some of the highest rates of glycolysis when active. In cockroach flight muscles which power flight by glucose oxidation, the fructose-2,6-bisphosphate content increases 2-fold during flight (Storey, 1983). Locust flight muscles, on the other hand, progressively oxidize lipid substrates as the carbohydrate reserves are depleted. In these muscles, fructose-2,6-bisphosphate decreases steadily over 15 min of flight and may signal the change from carbohydrate to lipid oxidation (Wegener et al., 1986). Results from this laboratory (L. Hue & M. H. Rider, unpublished work) indicate that fructose-2,6-bisphosphate decreases in hepatocytes incubated with fatty acid and in hearts perfused with ketone bodies. Therefore, in addition to its role as a signal for glucose availability, it may be involved in the glucose-sparing effect of fatty acids.

**Conclusions**

Fructose-2,6-bisphosphate has a unique role in the liver in the fine control of the relative activities of PFK-1 and FBPase-1. It is not surprising, therefore, that the properties of the bifunctional liver PFK-2/FBPase-2 are the exception rather than the rule. In heart and muscle, the characteristics of PFK-2/FBPase-2 are quite different. PFK-2s from liver, heart and muscle (El-Maghrabi et al., 1986; Rider & Hue, 1986; Rider, Hue, 1986, Van Schaftingen & Hers, 1986) all have similar molecular mass, but differ dramatically in their regulatory properties and FBPase-2 content. Anti-rat liver PFK-2 polyclonal antibody does not precipitate rat heart PFK-2 (El-Maghrabi et al., 1986) and clearly a detailed immunological investigation is called for to determine whether muscle PFK-2 represents a third isoenzyme.

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Mapping motion in large proteins by single tryptophan probes inserted by site-directed mutagenesis: lactate dehydrogenase

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It is possible to make a formal distinction between two classes of enzyme: in one class, typified by a tyrosyl tRNA synthetase (Fersht, 1987), the protein provides a relatively rigid active site which has overlapping binding areas which match the substrate, transition state and product, and it is the shape change of the substrate which populates these in order; in a second type, typified by the simple ω-ketoacid dehydrogenases, there is only one binding site for the substrate, but a movement of a mobile and non-rigid protein domain distorts the site so that in time it successively matches the shape of the substrate, the transition state and the product. Characterization of this class of enzyme catalysts requires that the rate of change of protein structure be known, and that it is related to the velocity of defined steps in the enzyme mechanism. We describe a general method to make a map of the rate of defined movements in protein domains. It requires the following. (i) Site-directed

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