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Islet Fructose 6-Phosphate, 2-Kinase:fructose 2,6-Bisphosphatase: Isozymic Form, Expression, and Characterization

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Polymerase chain reaction analysis of the mRNA isolated from rat islets demonstrated that the major isozyme of Fructose 6-P,2-kinase:Fructose 2,6-bisphosphatase was the heart type enzyme, and that the liver type enzyme was not detectable. The islet enzyme was expressed in *Escherichia coli* and purified to homogeneity. The islet enzyme showed the highest Fructose 6-P,2-kinase activity (478 milliunits/mg) compared to the other isozymes and Fructose 2,6-Pase activity (39 milliunits/mg). Fructose 6-P,2-kinase showed $K_m^{F6P} = 17\ \mu M$, which is within the range of *in vivo* Fru 6-P concentrations in islets. 6-P-Gluconate was a potent inhibitor of Fructose 2,6-Pase. The data suggest that Fructose 6-P,2-kinase activity of the bifunctional enzyme was high and Fructose 2,6-Pase activity was inhibited under physiological variations of blood glucose concentration.

Liver and islets of Langerhans are two tissues which must rapidly respond to acute changes in blood glucose concentrations in the 5–10 mM range, and these two tissues share many of the same isozymic forms of the glycolytic enzymes. An important control point of glycolysis is PFK. While much is known about its regulation in liver, little is known about its regulation in β-cells.

Especially important in the regulation of glycolysis in liver is Fru 2,6-P_2, the most potent known activator of PFK (1). The synthesis and degradation of Fru 2,6-P_2 is controlled by a bifunctional enzyme with both Fru 6-P,2-kinase and Fru 2,6-Pase activities. There are several known isozymes of this enzyme expressed in different tissues. The liver bifunctional enzyme is phosphorylated by cAMP-dependent protein kinase resulting in inhibition of Fru 6-P,2-kinase activity and activation of Fru 2,6-Pase activity. This decreases Fru 2,6-P_2 levels causing inhibition of PFK and glycolysis in liver. Glucose administration increases Fru 2,6-P_2 and activates PFK and glycolysis. This was previously thought to be due to increased levels of the substrate, Fru 6-P (2), but Fru 6-P formation is immediate while Fru 2,6-P_2 formation has a lag period. Recently we have shown that glucose stimulates dephosphorylation of the liver bifunctional enzyme through Xu 5-P activation of a specific protein phosphatase 2A and that the time required for Xu 5-P formation after glucose administration accounts for the lag period in Fru 2,6-P_2 formation (3). These data suggest an important regulatory role for pentose shunt intermediates in liver glycolysis.

Glucose-stimulated insulin secretion by β-cells of islets requires glucose metabolism *via* glycolysis (4), and many of the glycolytic characteristics of these cells are similar to liver. As seen in liver, Fru 2,6-P_2 levels increase with glucose administration, but unlike liver, there is no lag period of formation (5). Also unlike liver, glucagon fails to decrease Fru 2,6-P_2 levels in islets (5). This suggests that the bifunctional enzyme of islets differs from liver, and we have sought to characterize this enzyme in islets to deduce its regulation and its role in islet glycolysis.

EXPERIMENTAL PROCEDURES

cDNA synthesis kit was purchased from Amersham Corp. (Arlington Heights, IL); SuperScript RNase H reverse transcriptase from Gibco-BRL (Gaithersburg, MD); Taq DNA polymerase from Perkin-Elmer Cetus Instruments (Norwalk,

Abbreviations used: PCR, polymerase chain reaction; *E. coli, Escherichia coli:* PFK, phosphofructokinase; Fru 2,6-P_2, fructose 2,6-bisphosphate; Fru 6-P,2-kinase, Fructose 6-P,2-kinase; Fru 2,6-Pase, Fructose 2,6-phosphatase; Xu 5-P, xylulose 5-P; PEP, phosphoenolpyruvate.

0006-291X/96 $12.00

CT); restriction enzyme and bacteriophage T4 DNA ligase from Boehringer Mannheim (Indianapolis, IN). The pT7-7 RNA polymerase/promoter plasmid (6) was a gift of Dr. Stan Tabor (Harvard Medical School).

Methods

Isolation of rat pancreatic islets. Pancreatic islets were isolated from male Sprague-Dawley rats (200–250 g) by the collagenase digestion method of Naber *et al.* (7).

Assay method for Fru 6-P,2-kinase. The reaction mixture in a final volume of 50 μl contained 100 mM Tris/HCl (pH 7.5), 0.1 mM EDTA, 10 mM $MgCl_2$, 2 mM ATP, and 2 mM Fru 6-P. The mixture was incubated at 30°C for 10 min. At the end of the reaction 0.1 N NaOH (50 μl) was added, and the mixture was heated for 90 s at 80°C. Suitable aliquots were assayed for Fru 2,6-P_2. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of Fru 2,6-P_2/min under these conditions.

Assay method for Fru 2,6-Pase. The reaction mixture (in a final volume of 1.0 ml) contained 100 mM Tris/HCl (pH 7.5), 0.2 mM EDTA, 100 μM NADP, 0.4 unit of Glu 6-P dehydrogenase, 1 unit of phosphoglucose isomerase, and varying amounts of Fru 2,6-P_2. The reaction was initiated with the addition of enzyme and was followed at 25°C by measuring the NADPH formation at 452 nm emission and excitation at 350 nm using an Aminco-Bowman Series 2 luminescence spectrometer.

Synthesis and PCR amplification of cDNA. The oligo(dT)-primed cDNA was synthesized from poly(A)$^+$-RNA ($\sim$5 μg), using 200 units SuperScript RNase H reverse transcriptase in 20 μl of reaction mixture according to the manufacturer's instructions. An aliquot (5 μl) of the reaction mixture was amplified in 100 μl of 10 mM Tris-HCl buffer (pH 8.3) containing 50 mM KCl, 1.5 mM $MgCl_2$, 0.001 (w/v)% gelatin, 0.2 mM of each deoxyribonucleotide, and 2.5 units Taq DNA polymerase. Synthetic oligonucleotide primers (1 μM) are as follows.

For rat heart Fru 6-P,2-kinase:Fru 2,6-Pase cDNA: 5′- CTCCACATCATCTTCAA-3′ and 5′-TGACAGCGTCAACA-GGC-3′ corresponding to #1447–1463 and complimentary to #2512–#2528, respectively of the heart enzyme cDNA (8); for the liver enzyme cDNA (full length): 5′-TGGAGATAATCTGTGAGAGGCTCCG-3′ and 5′- TGGCTAGGAATT-GAGACCTGTTTGG-3′ (M8) corresponding to #14–#38 and complimentary to #1727–#1751, respectively for the rat liver enzyme (1.7 kb, full length); for the liver enzyme (1.0 kb), the P_2 primer and 5′-GCTAGTGCATAGGCATACTGCTTGC-3′ which is complimentary to #1012–#1036 (10); for the skeletal muscle enzyme, 5′-GGCACACGCTACATGGTAAA-TCG-3′ corresponding to #843–#865 and M8 (11). The PCR was performed as previously described (8). Amplified DNA fragments were separated and analyzed by agarose gel electrophoresis.

Construction and expression of rat islet Fru 6-P,2-kinase:Fru 2,6-Pase. Unless otherwise noted, molecular biology methods were from Sambrook *et al.* (12). To construct an expression vector carrying the rat brain Fru 6-P,2-kinase:Fru 2,6-Pase gene, the full length cDNA cloned into pUC18 (RB10/pUC18) was used. To create NdeI site, the oligonucleotide primer 5′-GTGCCTCCTGAAGAACTCATATGTCT-3′, encoding an NdeI site at Met191 and the complimentary oligo-nucleotide primer 5′-CCACGCCTTACTGTTGGTAGCC-3′, corresponding to #1723–#1744 of the rat heart enzyme cDNA (9) containing an HindIII site were used for PCR. After digestion with NdeI and HindIII, the DNA fragment was ligated into NdeI/HindIII double-digested pT7-7 DNA vector, and *E. coli* was transformed with the ligation mixture, selecting ampicillin-resistant colonies.

RESULTS AND DISCUSSION

Fru 6-P,2-kinase:Fru 2,6-Pase mRNA in Rat Islets

Reverse transcribed strands of cDNA were synthesized from the poly (A)$^+$-RNAs and PCR amplified using the synthetic oligonucleotide primers for liver, skeletal muscle, and heart bifunctional enzyme. Figure 1 shows that a 1.1 kb band was obtained using the heart primers (lane 2) while no DNA was detected when the liver (lanes 3 and 4) or skeletal muscle (lane 1) primers were used. Increasing the amount of reverse transcribed DNA and changing the PCR conditions gave the same result; while the use of comparable amounts of mRNA from liver always showed detectable liver isozyme DNA (data now shown). The conclusion from Figure 1 is that the major form of Fru 6-P,2-kinase:Fru 2,6-Pase found in islets is the isozyme found in heart (9). This isozyme has also been found in most tissues, including brain, testis, liver, and skeletal muscle (9). The 1.1 kb size indicates that it is the form of the protein missing the protein kinase A and C phosphorylation sites which explains why glucagon fails to decrease Fru 2,6-P_2 levels in islets (5). The absence of the liver form of the enzyme in islets suggests that the mechanism for regulating glucose-stimulated Fru 2,6-P_2 formation differs from liver and does not involve Xu 5-P activation of protein phosphatase 2A (3).

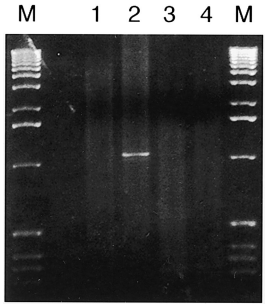

FIG. 1. Analysis of islet mRNA by PCR. The synthesis and PCR amplification were performed as described in Experimental Procedures. The PCR products were: (1) skeletal muscle; (2) islet; (3) liver (1.0 kb); and (4) liver (1.7 kb). M, molecular size standard.

Expression and Purification of Islet Fru 6-P,2-kinase:Fru 2,6-Pase

The bifunctional enzyme from islet cDNA was expressed in *E. coli* BL21 cells, and the enzyme was purified to homogeneity according to the procedure described previously (13). Table 1 compares the kinetic parameters of the Fru 6-P,2-kinase activity and the Fru 2,6-Pase activity of bifunctional enzyme from islets with isozymes from other tissues. Islet Fru 6-P,2-kinase activity was found to be the highest among these isozymes and, surprisingly, exceeded the bovine heart enzymes by at least 10-fold. Although not as dramatic, the Fru 2,6-Pase activity is 2-fold higher than the bovine heart enzyme (Table 1).

Assuming that *in vitro* kinetic constants reflect *in vivo* activity, Fru 6-P,2-kinase activity will vary with changes in Fru 6-P concentration since its K_m^{F6P} was found to be 17 μM, and Fru 6-P concentrations are reported to be 11–30 μM in islets under both low and high glucose conditions (45). Similarly, it appears that Fru 2,6-Pase will be active since its K_m^{F26P2} was found to be 0.3 μM, and Fru 2,6-P_2 concentrations in islets have been reported to be 0.6–1.3 μM in islets exposed to low and high glucose (15). However, since most Fru 2,6-P_2 is bound to PFK, the effective intracellular concentration of Fru 2,6-P_2 will be much lower than 0.6 μM. Taken together with the

TABLE 1
Comparison of the Kinetic Constants of Expressed Rat Islet, Testis, Liver, Muscle, and Bovine Heart Fru 6-P,2-kinase:Fru 2,6-Pase

	Fru 6-P, 2-kinase:Fru 2,6-Pase				
	Rat				Bovine
	Islet	Testis	Liver	Muscle	Heart
Fru 6-P,2-kinase					
K_m^{F6P} (μM)	17 ± 0.7	40	16	56	23
K_m^{ATP} (μM)	328 ± 12	100	310	48	83
V_{max} (mU/mg)	478 ± 14	100	57	66	42
Fru 2,6-Pase					
K_m^{F26P2} (μM)	0.3 ± 0.01	0.03	<1	0.4	0.09
V_{max} (mU/mg)	39 ± 2.3	17	45	154	17
Kinase/Phosphatase	12	5.9	1.3	0.4	2.5

Fru 6-P,2-kinase and Fru 2,6-Pase were assayed as described in Experimental Procedures except for the determination of K_m^{F6P} in that 2 mM ATP was used and for K_m^{ATP} determination 0.2 mM Fru 6-P was used. These values were the average ± S.D. of at least three determinations. The values for the testis, the liver, the muscle, and the heart enzymes were from Tsujikawa *et al.* (17), Kitamura *et al.* (18), and Abe *et al.* (13), respectively.

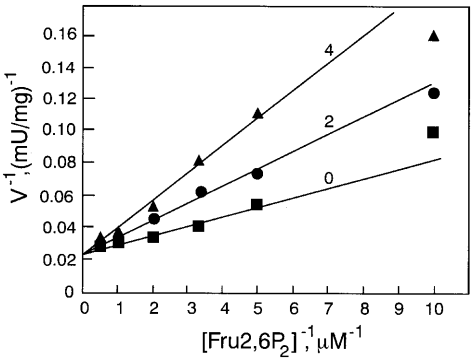

FIG. 2. Inhibition of Fru 2,6-Pase by 6-P-gluconate. Fru 2,6-Pase activity was assayed as described in *Methods* except Fru 2,6-P_2 concentration was varied in the presence of constant concentrations of 6-P-gluconate. (■), 0; (♦), 2 μM; and (▲), 4 μM 6-P-gluconate.

fact that Fru 6-P,2-kinase activity is 12-fold higher than Fru 2,6-Pase activity, conditions within islet cells appear to favor Fru 2,6-P_2 synthesis.

Because the islet bifunctional enzyme lacks protein kinase phosphorylation sites, regulation of the enzyme must involve other mechanisms. PEP and citrate are known to inhibit liver Fru 6-P,2-kinase and activate Fru 2,6-Pase *in vitro* (16). These effectors also inhibit islet enzyme with a $K_i^{PEP} = 80$ μM and $K_i^{CITRATE} = 50$ μM. In testing intermediates of the pentose shunt pathway for effects on the enzyme, we found that 6-P-gluconate was a competitive inhibitor of Fru 2,6-Pase with respect to Fru 2,6-P_2, had a K_i of 2.3 μM (Fig. 2), and had no effect on Fru 6-P,2-kinase activity. Since 6-P-gluconate levels in islets have been shown to increase from 8 μM to 180 μM when glucose concentrations are raised from 4–40 mM, it is possible that 6-P-gluconate completely inhibits Fru 2,6-Pase *in vivo* when blood glucose concentrations rise.

In summary, our findings indicate that the islet bifunctional enzyme is the heart type isozyme lacking protein kinase A and C phosphorylation sites. The kinetic constants derived from purified enzyme indicate that Fru 6-P,2-kinase activity will be greatly favored, and studies of effector molecules indicate that Fru 2,6-Pase activity may be inhibited under *in vivo* conditions. These studies explain why there is no lag in Fru 2,6-P_2 formation after glucose administration in islets and why Fru 2,6-P_2 levels are not decreased by glucagon (5). These studies provide a basis for regulation of the enzyme through intracellular metabolite levels. This is attractive teleologically since the function of the islet is to rapidly respond to acute changes in glucose availability.

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