Oscillation in Fructose 2,6-Bisphosphate Levels and in the Phosphorylation States of Fructose 6-Phosphate,2-kinase:Fructose-2,6-bisphosphatase in Ischemic Rat Liver*

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In order to determine the role of fructose (Fruc) 2,6-P2 in stimulation of phosphofructokinase in ischemic liver, tissue contents of Fruc-2,6-P2, hexose-Ps, adenine nucleotides, and Fruc-6-P,2-kinase:Fru-2,6-bisphosphatase were investigated during the first few minutes of ischemia. The Fruc-2,6-P2 concentration in the liver changed in an oscillatory manner. Within 7 s after the initiation of ischemia, Fruc-2,6-P2 increased from 6 to 21 nmol/g liver and decreased to 5 nmol/g liver within 30 s. Subsequently, it reached the maximum value at 50, 80, and 100 s and decreased to the basal concentration at 60, 90, and 120 s. Oscillatory patterns were also observed with Glc-6-P and Fruc-6-P, but the ATP/ADP ratio decreased monotonically.

Determination of Fruc-6-P,2-kinase activity and the phosphorylation states of Fruc-6-P,2-kinase:Fru-2,6-bisphosphatase demonstrated that at 7 and 50 s, where Fruc-2,6-P2 was the highest, the enzyme was activated and mostly in a dephosphorylated form. On the other hand, at 0, 30, and 300 s, the enzyme was predominantly in the phosphorylated form. The concentration of cAMP in the liver also changed in an oscillatory manner between 0.5 to 1.3 nmol/g with varying frequency of 10 to 40 s. These results indicated that: (a) Fruc-2,6-P2 was important in rapid activation of phosphofructokinase in the first few seconds and up to 2–3 min, and (b) the oscillation of Fruc-2,6-P2 concentration was the result of activation and inhibition of Fruc-6-P,2-kinase:Fru-2,6-bisphosphatase, which was caused by changes in the phosphorylation state of the enzyme.

Fructose 2,6-P2 is the most potent activator of phosphofructokinase, which is one of the key regulatory enzymes of glycolysis (see Ref. 1, for review). The synthesis and degradation of fructose (Fruc)1-2,6-P2 are catalyzed by a bifunctional enzyme, Fruc-6-P,2-kinase:Fru-2,6-bisphosphatase (Fruc-6-P + ATP -> Fruc-2,6-P2 + ADP and Fruc-2,6-P2 -> Fruc-6-P + P) (2–7). Activity of the bifunctional enzyme is regulated by phosphorylation, which is catalyzed by cAMP-dependent protein kinase and results in inhibition of the kinase and activation of the phosphatase activities (8–10). The net result is a decrease in Fruc-2,6-P2 concentration and inhibition of phosphofructokinase and glycolysis, which explains the action of a hormone such as glucagon on the regulation of glucose metabolism in liver. However, whether Fruc-2,6-P2 plays any role in the regulation of carbohydrate metabolism in liver under other stimuli or interventions is still unclear. Hue (11) earlier investigated a possible role of Fruc-2,6-P2 in activating phosphofructokinase and glycolysis in liver under anaerobiosis. He found that the concentration of Fruc-2,6-P2 decreased in hepatocytes when measured 5 min after initiation of anoxia even though glycolysis was stimulated. Furthermore, Fruc-6-P,2-kinase activity was not affected by anoxia. Thus, he concluded that Fruc-2,6-P2 could not be responsible for stimulation of glycolysis in liver under anaerobiosis.

More recently, we investigated a possible role of Fruc-2,6-P2 in brain under anaerobic condition (12). A brain-blowing device (13) allowed the measuring of the changes in glycolysis within the first few seconds and up to a few minutes of ischemia. Results showed that Fruc-2,6-P2 does not change significantly in spite of the fact that phosphofructokinase and glycolysis are activated severalfold within the first 2–5 s (12). However, we found that a new activator of phosphofructokinase, Rib 1,5-P2, is formed within 2 s after the initiation of ischemia and reaches the maximum in 5 s (12). We concluded that Rib 1,5-P2 (also a potent activator of phosphofructokinase) rather than Fruc-2,6-P2, is important during the first few seconds of ischemia in brain.

In contrast to brain, the preliminary studies have shown that Rib 1,5-P2 was not detectable in ischemic liver, ruling out its role in this tissue. Since the stimulation of glycolysis by ischemia in most tissues occurs within a few seconds, we investigated changes in Fruc-2,6-P2 and the activity of Fruc-6-P,2-kinase and phosphorylation states of the enzyme in liver from 5 s to 2 min after initiation of ischemia.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP (5000 Ci/mmol) and cAMP 32P-scintillation proximity assay system were purchased from Amersham Corp. The catalytic subunit of cAMP-dependent protein kinase, Fruc-2,6-P2, and benzamidine were purchased from Sigma. Fruc-2,6-[32P]P2 was prepared as described previously (14). Rabbit muscle phosphofructokinase was purified by the method of Uyeda et al. (15). Antiserum against rat liver Fruc-6-P,2-kinase:Fru-2,6-bisphosphatase were raised in a goat as described previously (16). Rabbit liver glycogen was purified by passing it through mixed-bed resin AG 501-X8 (17) (Bio-Rad), precipitating with ice-cold ethanol, and drying over P2O5. Rabbit anti-goat IgG was purchased from Miles-Yeda Ltd. (Elkhawk, IN). Other enzymes, substrates, and cofactors were purchased from Boehringer Mannheim, and all other chemicals were of analytical reagent grade and obtained from commercial sources.

Rats—Male Sprague-Dawley rats weighing 200–250 g were ob-
tained from Sasco Co. (Omaha, NE). Rats were fed ad libitum with the standard National Institutes of Health diet.

Preparation of Ischemic Liver—Rats were anesthetized by injection of nembutal (7.5 mg/100 g body weight). The abdominal cavity was opened and arteria hepatica and venae porta were clamped stopping the blood flow. At a given time interval, a piece of left lobe of liver weighing approximately 1-3 g was cut out and immediately freeze-clamped by tongs which were precooled in liquid nitrogen. The time between cutting and freezing was within 1 s. For zero time control samples, a piece of liver was quickly cut out and immediately freeze-clamped for 10 min, washed three times with PBS, once with 0.066 M Tris-HCl, pH 6.8, and dissolved in 40 μl of 0.066 M Tris-HCl, pH 6.8, containing 5% β-mercaptoethanol, 2% sodium dodecyl sulfate. A suitable aliquot of the dissolved enzyme solution was removed, and radioactivity was determined.

Oscillation of Fru-2,6-P₂—The concentration of Fru-2,6-P₂ in the liver underwent rapid pulsatory changes after the initiation of ischemia (Fig. 1). After a slight initial (3 s) decrease, it reached the maximum value of 21 nmol/g within 7 s and decreased to 5 nmol/g in 30 s. After the initial cycle the Fru-2,6-P₂ level increased to the maximum concentration of 13, 17, 16 nmol/g at 59, 80, and 100 s, respectively, and decreased to the basal values at 60, 90, and 120 s. Beyond 120 s the oscillatory change ceased and the Fru-2,6-P₂ concentration remained constant at 3 nmol/g up to 5 min. In contrast, the adenine nucleotides concentration changed rather monotonically (Fig. 2A). ATP decreased rapidly to 1.2 μmol/g from 2.6 μmol/g within the first 30 s and continued to decrease more slowly, while AMP increased rapidly for the first 30 s, followed with a slow increase. The concentrations of Glc-6-P and Fru-6-P changed in an oscillatory manner, reaching the maximum values at 7, 30, and 70 s, and the minimum at 10, 40, 80, and 120 s. It should be noted that the mean values of Glc-6-P and Fru-6-P in the peaks and valleys of oscillation are significantly less than the overall curves. Unlike Fru-2,6-P₂, however, the concentrations of these hexose phosphates did not change to the basal level at the minima (Fig. 2B). Glucose 1-phosphate and lactate continued to oscillate. However, it is difficult to decide whether Fru-1,6-P₂ underwent oscillation because of inaccuracy of the assay due to extremely low level in vivo (Fig. 2C). Comparison of the oscillations of Fru-2,6-P₂ and hexose-Ps revealed that their oscillatory pe-
phosphofructokinase activity. Preparations of liver samples and acid extracts of livers and assay methods for these metabolites were as described under "Experimental Procedures." The vertical lines are standard error and only show on one side of the curves for clarity.

Periods were similar (approximately 20–30 s), but the periods were out of phase except at 7 s of ischemia. Both hexose-Ps and Fru-2,6-P2 increased severalfold within 7 s, but Fru-1,6-P2 did not increase until 10 s. These changes in hexose-Ps suggest the activation and the inhibition, i.e. oscillation of phosphofructokinase activity.

Oscillation of Fru-6-P,2-kinase Activity—In order to determine if the oscillation of Fru-2,6-P2 is as a result of activation and inhibition of Fru-6-P,2-kinase by phosphorylation and dephosphorylation of the bifunctional enzyme rather than by changes in allosteric effectors, the activity of Fru-6-P,2-kinase at two different concentrations of Fru-6-P was determined in the ischemic livers. The kinase was assayed at 0.05 mM (v) and 1.0 mM (v) and inhibition of Fru-6-P,2-kinase by phosphorylation and dephosphorylation of the bifunctional enzyme rather than by changes in allosteric effectors, the activity of Fru-6-P,2-kinase was determined in the presence of [32P]ATP, precipitated with specific antibodies, and analyzed by polyacrylamide gel electrophoresis.

The amount of 32P incorporation was also determined by the filter assay for protein kinase according to the procedure of Corbin and Reimann (24). The Coomassie Blue-stained gel indicated that the protein band corresponding to the Fru-6-P,2-kinase:Fru-2,6-Pase was visible in all the samples (Fig. 3A). The autoradiogram of the same gel showed significant 32P incorporation into the enzymes after 7 s (lane 2) and 50 s (lane 4) of ischemia, indicating that these enzymes were mostly in dephospho forms in vivo. This was in contrast to the enzymes after 0, 30, and 300 s of ischemia, in which little 32P was incorporated, indicating that the enzymes were in phospho forms in these livers. Interestingly, the degree of incorporation decreased with time, indicating that the amount of phosphorylated enzyme increased with time of ischemia, and after 300 s of ischemia the enzyme was fully phosphorylated. The determination of the amount of [32P]phosphate incorporation demonstrated that at zero time 0.33 mol of phosphate incorporated/mol of enzyme subunit which indicated that 33% of the enzyme was in dephospho form in vivo, but within 7 s of ischemia 70% of the enzyme was in dephospho form. Similarly, 27, 50, and 0% of the enzymes were in dephospho forms at 30, 50, and 300 s, respectively (Table I).

Changes in cAMP in Ischemic Liver—Since the above results demonstrated that the phosphorylation of the bifunctional enzyme was involved when the Fru-2,6-P2 decreased, and thus far only cAMP-dependent protein kinase is known to phosphorylate the enzyme, it was important to determine
whether cAMP also undergoes oscillation. The results (Fig. 4) showed that oscillatory changes in cAMP concentration occurred in the ischemic livers. For comparison the changes in Fru-2,6-P₂ from Fig. 1 were also included in Fig. 4. The concentrations of cAMP reached the maximum level at 5, 10, 50, and 80 s and the minimum level at 7, 20, and 60 s under these conditions. One would expect that the oscillation of cAMP and Fru-2,6-P₂ is out of phase because of the opposite relationship between these compounds in liver. The expected opposite effect was observed only at 7 s at which time Fru-2,6-P₂ reached the maximum as cAMP decreased to the minimum value. At other time points, however, this opposite relationship was not maintained. For example, at 50 and 80 s both cAMP and Fru-2,6-P₂ reached the maxima and at 60 s both reached the minima.

Change in Glycogen Phosphorylase α in Ischemic Liver—Glycogen phosphorylase α in fresh ischemic liver was also determined. Results showed that activity increased from 6.5 units/g liver to 8.5 units/g within 7 s, but subsequent changes were relatively small (data not shown).

**DISCUSSION**

These results showed for the first time that sudden ischemic conditions in rat liver induced oscillatory changes in Fru-2,6-P₂ and glycolytic intermediates. The oscillatory cycle began within a few seconds after the initiation of ischemia and lasted up to 2 min. The oscillation period of Fru-2,6-P₂ was 20–30 s, which is extremely short compared to other known oscillations such as glycolysis in yeast (25–27), heart (28), and muscle (29, 30) extracts, insulin secretion from islets (31–33), and Ca²⁺ release in signal transduction (for a review, see Ref. 34). The oscillation periods in these systems range from 5 min to hours.

These results further demonstrated that Fru-2,6-P₂ appeared to play an important role in triggering the rapid activation of phosphofructokinase in liver following initiation of ischemic conditions. There was no significant amount of ribose 1,5-P₂ in any of the ischemic liver samples unlike ischemic rat brain (12). The observed changes in hexose phosphates were consistent with the activation of phosphofructokinase by the changes in the Fru-2,6-P₂ concentrations. Hue (11) concluded that Fru-2,6-P₂ is not responsible for the activation of glycolysis in ischemic liver because he failed to detect any change in Fru-2,6-P₂ in hepatocytes or in liver under ischemia. The likely explanation for the discrepancy is that he measured Fru-2,6-P₂ only at 5 and 10 min after initiation of ischemia. As shown in Fig. 1, the oscillation of Fru-2,6-P₂ and major changes in hexose-Ps and adenine nucleotides occurred within the first few seconds and persisted only for 2 min. As is the case in brain (12, 35), the present results with the ischemic liver emphasize the importance of determining the metabolic changes within the first 30 s in order to understand the mechanism of activation of phosphofructokinase and glycolysis. The Fru-2,6-P₂ oscillation ceased within 2 min, and beyond this time it remained at a basal level (Fig. 1). The question then is does the activation of phosphofructokinase beyond 2 min continue under these conditions? It is possible that a combination of increasing Fru-1,6-P₂, which activates the enzyme in an autocatalytic manner (especially in the presence of Fru-2,6-P₂ and increased AMP), and decreased ATP (an inhibitor), could account for the sustained activation of phosphofructokinase and glycolysis.

Another significant result concerned the mechanism of Fru-2,6-P₂ oscillation in the liver. It was shown (Fig. 3, Table 1) that cAMP or Fru-2,6-P₂ were a result of activity and inhibition of Fru-6-P₂-kinase:Fru-2,6-bisphosphatase. This rapid activation and inhibition of the bifunctional enzyme caused mainly by the phosphorylation and dephosphorylation of the enzyme other than changes in concentration of Fru-6-P or other metabolites. The following lines of evidence support this conclusion: (a) phosphorylation of the bifunctional enzyme, catalyzed by cAMP-dependent protein kinase, causes inhibition of Fru-6-P₂-kinase and activation of Fru-2,6-Pase activities and results in a decrease in Fru-2,6-P₂ concentration. Dephosphorylation of the bifunctional enzyme by a protein phosphatase causes the opposite effect resulting in increased Fru-2,6-P₂ levels. This study demonstrated that when the Fru-2,6-P₂ level was low at 0, 30, and 300 s post-ischemia, the bifunctional enzyme was in the phosphorylated state, but when the level was high at 7 and 50 s, the enzyme was in a dephosphorylated state (Fig. 3). (b) The concentration of Fru-6-P, the substrate for the bifunctional enzyme, did not oscillate in conjunction with Fru-2,6-P₂ levels. For example, Fru-2,6-P₂ reached a maximum concentration of 21 μM at 7 s, while Fru-6-P increased from 70 to 100 nmol/g, and then continued to increase reaching 200 nmol/g at 70 s. (c) The Fru-6-P level was at least four to five times higher than the estimated Kₘ value of 16 μM as determined with purified enzyme in vitro (24). (d) Rapid decrease in Fru-2,6-P₂, which is catalyzed by Fru-2,6-Pase, was observed in the oscillation in spite of the presence of a high concentration of Fru-6-P, a potent inhibitor of Fru-2,6-bisphosphatase with Kᵢ = 1.2 μM (36). All these results argue against the possibility that Fru-6-P concentration regulates the Fru-6-P₂-kinase activity in liver under these conditions.

Oscillation in the phosphorylation state of the bifunctional enzyme indicates that either the activities of both protein kinase A and protein phosphatase are coordinately regulated in a reciprocal manner or one of these enzymes undergoes oscillation while the other remains at a constant activity. To our knowledge such a coordinated regulation of protein kinase A and protein phosphatase has not been reported. Closer examination of the oscillation of cAMP and Fru-2,6-P₂ (Fig. 4) showed a reciprocal relationship between these two compounds at 7 s. At 7 s of ischemia cAMP dropped rapidly, presumably inhibiting protein kinase A, which resulted in activation of Fru-6-P₂-kinase and inhibition of Fru-2,6-bisphosphatase activities and increased levels of Fru-2,6-P₂. Whether the protein phosphatase was activated or remained the same during this period remains to be determined. Beyond 7 s of ischemia, the reciprocal relationship between cAMP and Fru-2,6-P₂ did not occur, but instead they changed in synchrony. The changes in the protein kinase and the protein phosphatase during this period also need to be elucidated.

The physiological significance of the oscillation of Fru-2,6-P₂ in liver under ischemia is not clear at present. However,

![Fig. 4. Effect of ischemia on cAMP and Fru-2,6-P₂ levels in liver. The cAMP (A) in the freeze-clamped liver was determined as described under "Experimental Procedures." Fru-2,6-P₂ (O) was the same as in Fig. 1.](image-url)
oscillatory behavior usually serves to trigger rapid and efficient stimulation of physiological and metabolic changes. It is possible that the Fru-2,6-P₂ oscillation serves to trigger the activation of phosphofructokinase and thus glycolysis in liver during the initial phase of aerobic to anaerobic shift. The oscillation of glycolysis caused by a series of bursts of phosphofructokinase activity in crude muscle extract has been shown to respond to ATP/ADP, and thus the tissues can maintain high ATP/ADP (37). This is not the case in the ischemic liver since the ratio and the ATP concentration continued to decrease in the tissue. In the liver it appears that Fru-2,6-P₂ is the major triggering factor for the rapid activation of phosphofructokinase. As Berridge (34) and Rapp (38) pointed out that the advantage of oscillation in signal transmission is higher signal to background ratio. Relatively infrequent large bursts of Fru-2,6-P₂ as in the case of the oscillation may be more effective than steady smaller increases in activation of phosphofructokinase in ischemic liver. The important question remains to be answered, i.e. the mechanism of regulation of protein kinase A and the protein phosphatase, whose activities control the relative activity of Fru-6-P₂-kinase and Fru-2,6-P₂ase and ultimately the level of Fru-2,6-P₂.

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