The Purine Nucleotide Cycle

CONTROL OF PHOSPHOFRUCTOKINASE AND GLYCOLYTIC OSCILLATIONS IN MUSCLE EXTRACTS*

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Linked oscillations of the glycolytic pathway and the purine nucleotide cycle were studied in particle-free extracts of rat skeletal muscle. Under the conditions used, an accumulation of about 1 μM fructose diphosphate can trigger a sudden increase in phosphofructokinase activity. The activation by fructose diphosphate depends on the presence of AMP. When the AMP concentration drops, phosphofructokinase becomes inhibited, even though the fructose disphosphate concentration remains high. It is concluded that the oscillatory behavior can be of advantage for maintaining a high average [ATP]/[ADP] ratio.

Oscillatory behavior of the purine nucleotide cycle (Reactions 1 to 3) has been demonstrated in extracts of muscle

Adenylate deaminase

\[ \text{AMP} + \text{H}_2\text{O} \rightarrow \text{IMP} + \text{NH}_3 \] (1)

Adenylosuccinate synthetase

\[ \text{IMP} + \text{GTP} + \text{aspartate} \rightarrow \text{adenylosuccinate} + \text{GDP} + \text{P}_i \] (2)

Adenylosuccinase

\[ \text{Adenylosuccinate} \rightarrow \text{AMP} + \text{fumarate} \] (3)

during glycolysis (1). Deamination of AMP is promoted by a low [ATP]/[ADP] ratio, whereas resynthesis of AMP is favored by a high [ATP]/[ADP] ratio (2). Oscillations of the purine nucleotide cycle are associated with oscillations in the [ATP]/[ADP] ratio, and these in turn occur in synchrony with oscillations of the intermediates of the glycolytic pathway (3). The relation between the changes in the concentrations of glycolytic and purine nucleotide cycle intermediates supported the proposal that phosphofructokinase is involved in controlling the oscillations. Phosphofructokinase from muscle and other tissues is inhibited by ATP and activated by AMP (4-17). It is also activated by fructose diphosphate (4, 11, 12, 15, 16). This implies that once a small amount of fructose diphosphate has accumulated, the activation of phosphofructokinase can become self-enhancing, so that the enzyme “switches on” suddenly. During the oscillations, as the ATP concentration decreased and the AMP concentration increased there was eventually a sudden burst of phosphofructokinase activity, presumably because of such activation by fructose diphosphate. This burst of activity produced a pool of 1,3-diphosphoglycerate, phosphoenolpyruvate, and their precursors, which served to convert AMP and ADP to ATP. When the pool of the high energy compounds was exhausted, ATP again dropped, ADP and AMP rose, and the process was repeated. An involvement of fructose diphosphate in producing the oscillations was supported by the phase shift that occurred on addition of fructose diphosphate (3). The purine nucleotide cycle oscillations were observed in the presence of 1 μM DPN, but were not apparent in the presence of 10 μM DPN (1). It was suggested that in the latter case the accumulation of sufficient fructose diphosphate to trigger phosphofructokinase might be prevented by the greater activity of glyceraldehyde-3-phosphate dehydrogenase.

The behavior of the system in the presence of 10 μM DPN has now been examined in greater detail, with emphasis on the factors that control the activity of phosphofructokinase. Two contrasting types of behavior were observed: (a) approach to an approximate steady state, which is characterized by little accumulation of the glycolytic intermediates from fructose diphosphate to phosphoenolpyruvate, by a linear accumulation of the end products lactate, pyruvate, and α-glycerophosphate, and by little change in the [ATP]/[ADP] ratio; (b) an oscillatory state, which is characterized by oscillations in the concentrations of all the glycolytic intermediates from glucose-6-P to phosphoenolpyruvate, by a corresponding stepwise accumulation of lactate, pyruvate, and α-glycerophosphate, and by oscillations in the concentrations of ATP, ADP, AMP, and IMP, and in the [ATP]/[ADP] ratio. Oscillations of the purine nucleotide cycle do occur under these conditions, but

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they are smaller in amplitude and require more sensitive spectrophotometric techniques than used previously. In the presence of 10 μM DPN it is possible to observe an initial rise in fructose diphosphate before the sudden activation of phospho-
fructokinase. Under our experimental conditions the accumu-
lation of as little as 1 μM fructose diphosphate can be sufficient to trigger the burst of phosphofructokinase activity. The activation by fructose diphosphate is apparently dependent on the presence of AMP, for phosphofructokinase became in-
hibited when the AMP level dropped, even though a high concentration of fructose diphosphate remained. It is con-
cluded that the fructose diphosphate activation of phospho-
fructokinase and the oscillatory behavior of the glycolytic pathway and the purine nucleotide cycle are of advantage for maintaining a high [ATP]/[ADP] ratio.

EXPERIMENTAL PROCEDURE

Methods—The preparation of the high speed supernatant of rat hind leg muscle was described previously (2). The reaction mixture was kept in a reservoir in a water bath, from which it was pumped at a rate of about 2 ml per min through a coarse Millipore prefilter into a flow-through cell, which was located in a Perkin-Elmer model 356 double beam spectrophotometer, and then back to the reservoir. The flow-through cell was water jacketed and kept at the same temperature as the water bath. The operation of the purine nucleotide cycle was monitored by one of the following methods. (a) A spectral scan was recorded from 310 to 290 nm at 2-min intervals. Changes in the concentrations (nm) of adenine nucleotide, adenylosuccinate, and IMP were calculated using the following equations for a 1-mm light path:

\[ \Delta \text{Adenylosuccinate} = \Delta A_{292}/1.0 \]  
\[ \Delta \text{Adenine nucleotide} = (A_{292} - 1.14A_{282})/0.88 \]  
\[ \Delta \text{IMP} = - (\Delta \text{adenylosuccinate} + \Delta \text{adenine nucleotide}) \]

Details of this method have been given previously (2). When the absorbance at 282 nm remains constant, the changes in adenine nucleotide are given by \[ \Delta A_{282}/0.88; \] this expression was used to calculate the scale for the small amplitude oscillations which were recorded at 292.5 nm in the inset to Fig. 1. (b) Changes in the concentration of adenylosuccinate were measured continuously by setting the double beam spectrophotometer to read changes in absorbance at 261 nm minus changes in absorbance at 282 nm. These wavelengths were chosen because adenine nucleotide and IMP absorb equally at 282 nm, and when IMP is converted to adenylosuccinate the change in absorbance is the same at 261 nm as at 282 nm. Therefore, the change in adenylosuccinate can be measured directly:

\[ \Delta \text{Adenine nucleotide} = \Delta (A_{261} - A_{282})/0.85 \]

The advantage of this method is that it can be used to record small amplitude oscillations of adenine nucleotide when the adenylosuccinate concentration may be changing. For this purpose the range of the spectrophotometer was set at 0.1 absorbance unit full scale. When changes in DPNH fluorescence were recorded, the reaction mixture was pumped from the reservoir through a second line also provided with a coarse Millipore prefilter to a flow-through cell located in a front surface fluorometer which was kept at the same temperature as the water bath.

Samples for metabolite analysis were withdrawn from the reservoir and analyzed by enzymatic methods as described previously (3). The sum of [ADP] + [GDP] was measured directly because pyruvate kinase, which was used for assaying ADP, also rapidly phosphorylates GDP (18). Adenylosuccinate was assayed enzymatically by converting it to AMP plus fumarate with adenylosuccinase; for this analysis the Perkin-Elmer double beam spectrophotometer was set to measure \[ \Delta A_{340} - A_{340}. \] The [ATP]/[ADP] and [GTP]/[GDP] ratios were measured by comparing the areas of the respective peaks obtained by high pressure liquid chromatography using a Varian Aerograph model 1000 machine equipped with an anion exchange column (1 mm in internal diameter and 3 m in length) packed with Reeve Angel AS-Pelionex-SAX. Chromatography of a solution containing known amounts of ATP and ADP showed that the ratio of the peak areas was the same as the ratio of the concentrations. The conditions used for the liquid chromatography were as follows. The high concentration solution contained 0.2 M potassium acetate buffer, pH 4.1, 0.2 M potassium dihydrogen phosphate, and 0.3 M KCl. The mixing vessel held 40 ml of low concentration solution which was made by diluting the high concentrate 10-fold. A linear gradient was formed by pumping 30 ml per hour from the mixing vessel into the column, and 15 ml per hour from the high concentrate reservoir into the mixing vessel. The column temperature was 50°.

Protein was determined by the method of Lowry et al. (19).

Materials—Male rats of the Sprague-Dawley strain were obtained from Charles River Breeding Laboratory, GTP, DPN, ADP, and dithiothreitol were purchased from P-L Laboratories. L-Aspartic acid was obtained from Sigma; glucose from Fisher; and ATP and fructose diphosphate from Boehringer. The imidazole used in the first experiment and the last experiment was obtained from Eastman Organic Chemicals; fluorometric grade imidazole from Sigma was used in second and third experiments. The dimethylketal derivative of dihy-
droxyacetone P was obtained from Boehringer; after acid hydrolysis of the dimethylketal (pH 1.8 at 40° for 6 hours), the solution was neutralized and assayed enzymatically. A suspension of crystalline yeast, lactokinase in ammonium sulfate was purchased from Boehringer. The ammonium sulfate was removed by gel filtration as described previously (2). A preparation of the desalted enzyme lost activity gradually over a number of weeks or months; it was assayed within a day of each experiment. The adenylosuccinase used for assaying adenylosuccinate was prepared from yeast by Dr. M. N. Goodman according to the procedure of Miller et al. (20).

RESULTS

Approach to Steady State—Fig. 1 shows a comparison of the behavior of the purine nucleotide cycle in the presence of 1 and 10 μM DPN. The oscillations in metabolite concentrations

![Fig. 1. Effect of DPN on purine nucleotide cycle oscillations. The reaction mixture initially contained 0.3 mM ATP, 0.3 mM GTP, 4 mM aspartate, 10 mM glucose, 0.03 unit per ml of crystalline yeast hexokinase, 7.5 mM orthophosphate, 27 mM imidazole-HCl buffer, pH 6.8, 8.3 mM MgCl₂, rat muscle extract equivalent to 1.3 mg of protein per ml, and either 1 μM DPN (A, O) or 10 μM DPN (A, O). The protein extract contributed 47 mM KCl, 0.83 mM EDTA, 17 μM dithiothreitol, and 2.5 mM of the total orthophosphate to the reaction mixture. The reaction was started by adding the protein extract and then the glucose in rapid succession. The temperature was 30°. Changes in adenine nucleotide and adenylosuccinate concentrations were calculated from a series of spectral scans taken on a Perkin-Elmer model 356 recording spectrophotometer (see "Methods"). The spectral scans were started at the times indicated by the experimental points. The first scan, which was started 1 min after adding the glucose, was used as a baseline for the calculations. Changes in IMP are not shown, since they are simply opposite to the sum of the changes in adenine nucleotide and adenylosuccinate. The inset shows the changes in absorbance at 262.5 nm after 70 min in the reaction mixture with 10 μM DPN. Since the absorbance at 282 nm had been constant (as shown by the adenylosuccinate curve), the scale for the recording at 262.5 nm was calculated in terms of changes in adenine nucleotide concentration (see "Methods"). The first scan, which was started 1 min after adding the glucose, was used as a baseline for the calculations. Changes in IMP are not shown, since they are simply opposite to the sum of the changes in adenine nucleotide and adenylosuccinate. The inset shows the changes in absorbance at 262.5 nm after 70 min in the reaction mixture with 10 μM DPN. Since the absorbance at 282 nm had been constant (as shown by the adenylosuccinate curve), the scale for the recording at 262.5 nm was calculated in terms of changes in adenine nucleotide concentration (see "Methods"). The inset shows the changes in absorbance at 262.5 nm after 70 min in the reaction mixture with 10 μM DPN. Since the absorbance at 282 nm had been constant (as shown by the adenylosuccinate curve), the scale for the recording at 262.5 nm was calculated in terms of changes in adenine nucleotide concentration (see "Methods").
observed with 1 µM DPN are similar to those reported previously (1, 3). They involve transient bursts of phosphofructokinase activity, and each burst of activity ends when the hexose monophosphate is nearly exhausted. When the phosphofructokinase activity drops, there is a corresponding decrease in the rate of ATP consumption, at a time when ATP production from 1,3-diphosphoglycerate and phosphoenolpyruvate is high; this is reflected by the abrupt transitions from deamination to reamination (3). In contrast, in the experiment with 10 µM DPN the initial deamination shows a gradual slowing which is followed by a period of little change (an approximate steady state), and then a slow accumulation of adenine nucleotide. The pattern of the points obtained between 35 and 67 min suggested the occurrence of oscillations of short amplitude and period. The spectral scanning used for obtaining these points was stopped at 67 min, and the spectrophotometer was set to read continuously at 262.5 nm with a full scale deflection of 0.1 absorbance unit (Fig. 1, inset). The trace so obtained clearly showed oscillations with a period of 8 min.

Analyses of samples taken from the reaction mixture containing 10 µM DPN are shown in Fig. 2. The concentration of ATP measured just after the start of the reaction, namely 350 µM, is significantly lower than the concentration of ATP present initially, namely 500 µM. This was in part due to the rapid phosphorylation of the large impurity of GDP present in the GTP used in the experiment. The ATP concentration dropped rapidly from 350 µM to nearly 200 µM within the first 8 min; thereafter it declined slowly to about 170 µM at 28 min, and then rose gradually to about 200 µM. The AMP concentration rose to 30 µM within 6 min; it then declined slowly to about 10 µM at 20 min, and then remained fairly constant. The fructose-6-P concentration rose to 45 µM in 12 min; after a small drop at 16 min the accumulation continued but at about one-fourth of the former rate. The change in glucose-6-P concentration (not shown) was similar to that of fructose-6-P. The [fructose-6-P]/[glucose-6-P] ratio remained between 0.29 and 0.32 throughout the experiment. Dihydroxyacetone-P and fructose diphosphate began to appear at 8 to 10 min and accumulated slowly to about 1 µM fructose diphosphate and 4 µM dihydroxyacetone-P. There was an abrupt rise to 5 µM fructose diphosphate and 30 µM dihydroxyacetone-P at 16 min, but both dropped back to 1 µM fructose diphosphate and 5 µM dihydroxyacetone-P by 18 min. A second peak appeared at 22 min, when fructose diphosphate and dihydroxyacetone-P reached 2 and 12 µM, respectively. α-Glycerophosphate and pyruvate also began to appear at 8 min. Thereafter their concentrations rose steadily for more rapid rises between 14 to 16 min and 16 to 20 min, respectively. Lactate accumulated at a fairly constant rate after 10 min. The concentrations of phosphoenolpyruvate, and 2- and 3-phosphoglycerate were less than 1 µM throughout the experiment.

The results shown in Fig. 2 indicate that phosphofructokinase became activated gradually between 8 and 14 min. Between 14 and 16 min the enzyme activity increased suddenly, perhaps because a critical concentration of fructose diphosphate had been reached. In the presence of 10 µM DPN this activity increase did not result in a large consumption of the hexose monophosphate and a corresponding appearance of fructose diphosphate and triose phosphate, such as was found in the presence of 1 µM DPN (3). Since neither glyceraldehyde-3-P nor DPN are saturating glyceraldehyde-3-phosphate dehydrogenase (K_{app} for DPN is 20 to 100 µM (21–23)), it is to be expected that raising the DPN concentration from 1 to 10 µM would result in an increase in glyceraldehyde-3-phosphate dehydrogenase activity. This might be sufficient to balance the increased activity of phosphofructokinase. However, not only was the accumulation of fructose diphosphate and triose phosphate curtailed, but their concentrations dropped considerably after the sudden accumulation between 14 and 16 min. Since the levels of ATP, AMP, and fructose-6-P did not change in a manner that would inhibit phosphofructokinase, this suggests a delay in the rise of the activity of glyceraldehyde-3-phosphate dehydrogenase in relation to the rise in glyceraldehyde-3-P. (The latter is proportional to dihydroxyacetone-P; see below.) One possibility is that initially the rate of operation
of glyceraldehyde-3-phosphate dehydrogenase may have been limited by the rate of reoxidation of DPNH, which is itself an inhibitor of the enzyme ($K_i = 0.3$ to $1 \mu M$). Reoxidation of DPNH may in turn have been limited by the low concentrations of pyruvate and dihydroxyacetone-P. Once the dihydroxyacetone-P concentration rose, the oxidation of a certain amount of DPNH by $\alpha$-glycerophosphate dehydrogenase would allow the accumulation of an equivalent amount of pyruvate, and this in turn would lead to an increase in the capacity of lactate dehydrogenase to reoxidize DPNH. The drop of fructose diphosphate back to about $1 \mu M$, which may have resulted from such an increase in the activity of glyceraldehyde-3-phosphate dehydrogenase, could be responsible for the lowering of the rate of the phosphofructokinase reaction to a moderate level averaging about three-fourths the hexokinase rate, and thus for the establishment of an approximate steady state. This state is characterized by low concentrations of glycolytic intermediates from fructose diphosphate to phosphoenolpyruvate, and by an approximately linear accumulation of the end products pyruvate, lactate, and $\alpha$-glycerophosphate, in contrast to the stepwise accumulation observed during the oscillatory state (3). It is termed an approximate steady state because of the continued increase in fructose-6-P and glucose-6-P (which may be viewed as representing the excess hexokinase activity over the glycolytic rate necessary to balance ATP consumption in the steady state; see below), and the slow accumulation of adenine nucleotide (Fig. 1), and because of the recurring transient increases in dihydroxyacetone-P and fructose diphosphate which appear to indicate additional limited, transient increases in phosphofructokinase activity. It is likely that the increasing fructose-6-P concentration was in part responsible for the recurring increases in phosphofructokinase activity and for the accumulation in adenine nucleotide. Note that the steady state occurred in the presence of about $30 \mu M$ AMP, which is probably necessary for the continuing activity of phosphofructokinase. Accumulation of phosphoenolpyruvate and phosphoglycerate was prevented by the high concentration of ADP plus GDP of about $200 \mu M$ (not shown in Fig. 2).

The small amplitude oscillations in total adenine nucleotide shown in the inset of Fig. 1 involved changes in rate from $-11 \mu M$ per min to $-5 \mu M$ per min. Such a range requires fairly large changes in the relative rates of the purine nucleotide cycle enzymes. Unless adenylate deaminase exhibits pronounced sigmoidal kinetics with respect to AMP, this would be incompatible with the limited change in the AMP and ATP concentrations observed up to 50 or 60 min (Fig. 2). It is possible that the phosphofructokinase activity might have been increased to such an extent by the doubling of the ACC phosphate dehydrogenase reactions. Hence, a lower rate of phosphofructokinase production necessitates that there be a greater conversion of pyruvate to lactate, and thus a higher [lactate]/[pyruvate] ratio.

The establishment of an approximate steady state must depend critically on the relative activities of phosphofructokinase and glyceraldehyde-3-phosphate dehydrogenase, which determine whether and to what degree fructose diphosphate accumulates and triggers a sudden activation of phosphofructokinase. The experiment shown in Fig. 2 is especially interesting in that it represents a borderline case: sudden activations of phosphofructokinase did occur, as shown by the transient increases in dihydroxyacetone-P and fructose diphosphate, but were quickly aborted either by a compensatory rise in the glyceraldehyde-3-phosphate dehydrogenase activity or by shifts in the ATP and AMP concentrations which were too small to detect clearly. Maintenance of the steady state also requires that other enzyme activities be suitably balanced. For example, since large changes in ATP and AMP would be likely to cause the phosphofructokinase activity to change greatly, it is necessary that ATP production by pyruvate...
kinase and phosphoglycerate kinase, which in the experiment shown in Fig. 2 was very nearly twice the rate of the glyceraldehyde-3-phosphate dehydrogenase reaction, be equal to ATP consumption by hexokinase, phosphofructokinase, adenylosuccinate synthetase (via GTP), and ATPase. Furthermore, the rates of adenylate deaminase and adenylosuccinase must be approximately equal. Such balances depend in part on the experimental conditions used, but also on the activities of the enzymes present in the muscle extract. As shown below, experimental conditions which yield steady state behavior with one extract (Fig. 2) may give oscillatory behavior with another, most likely because of some difference from preparation to preparation in the amount of phosphofructokinase or glyceraldehyde-3-phosphate dehydrogenase. The amount of fructose diphosphatase may also be a variable factor.

Oscillatory State—The experiment shown in Figs. 3 and 4 had the same starting conditions as the experiment with 10 μM DPN shown in Figs. 1 and 2, except that another muscle preparation was used. Changes in adenine nucleotide were measured continuously by recording (ΔA_{254} - ΔA_{252}) on the double beam spectrophotometer (see “Methods”). Oscillations of small amplitude were observed (Fig. 3). The shape of the oscillations was more complex than that observed in the experiment shown in the inset of Fig. 1. Each deamination was followed first by a rapid increase in adenine nucleotide that lasted about 1 min, and then by a longer interval of slow amination, which ended in a gradual transition to deamination. A tiny peak was usually visible at the end of the fast amination. It was first thought that the transition from fast to slow accumulation of adenine nucleotide might be due to the exhaustion of adenylosuccinate, after which the adenylosuccinate synthetase reaction would be rate-limiting because of the low concentration of IMP produced in this experiment. (It follows from Equation 6 that the IMP concentration is less than or equal to the loss of adenine nucleotide, which Fig. 3 shows to have been less than 60 μM.) However, the adenylosuccinate concentration was found to be too small to measure (<2 μM) even in samples taken during or just before the fast amination (see below). It follows that the abrupt change in the rate of accumulation of adenine nucleotide shown in Fig. 3 was probably due to a sudden change in the rate of operation of adenylosuccinate synthetase; the cause of this change in activity is not readily apparent.

The upper part of Fig. 3 shows a recording of the DPNH fluorescence in the same experiment. The sharp drop in DPNH that occurs in each oscillation corresponds exactly with the transition from fast to slow amination in the adenine nucleotide curve (dashed lines). For the first three oscillations the sudden rise in DPNH corresponds with the transition from deamination to reamination (dotted lines), but, as the successive DPNH peaks broaden, the rise in DPNH starts progressively earlier in the deamination phase.

Changes in metabolite concentrations were examined in detail for one complete oscillation. Samples were taken for analysis between 34½ and 47 min (Fig. 3, interval A), and the results are shown in Fig. 4. The interval was chosen so as to begin just after a downsweep in DPNH and a transition from fast to slow amination (Fig. 3). In the first part of this interval, ATP fell and fructose-6-P and AMP rose. These changes can be expected to lead to an activation of phosphofructokinase, and this occurred quite suddenly between 42 and 43 min; at which point there was a sharp drop in fructose-6-P and a sudden appearance of dihydroxyacetone-P and fructose diphosphate.

An upswing in DPNH occurred at 42 min, presumably because substrate for glyceraldehyde-3-phosphate dehydrogenase suddenly became available. The rise in DPNH occurred slightly before the rise in fructose diphosphate and dihydroxyacetone-P (and glyceraldehyde-3-P; see below), which indicates that at this point the phosphofructokinase activity was so low that the glyceraldehyde-3-phosphate dehydrogenase activity was sufficient to prevent accumulation of much triose phosphate. Fructose diphosphate and triose phosphate would be expected to rise only as the phosphofructokinase activity increased further and exceeded the capacity of glyceraldehyde-3-phosphate dehydrogenase.

Between 43 and 43½ min ATP rose sharply and AMP dropped to less than 1 μM. These changes were probably responsible for the inhibition of phosphofructokinase after 43½ min, which is indicated by the drop in fructose diphosphate.
and the steady accumulation of fructose-6-P and glucose-6-P (glucose-6-P was proportional to fructose-6-P; see below). Note that fructose diphosphate fell by a factor of two between 43 and 43½ min, while dihydroxyacetone-P rose slightly; this indicates that the burst of phosphofructokinase activity was so great as to exceed the capacity of aldolase to maintain equilibrium. (The triose phosphate isomerase reaction remained at equilibrium; see below.) The rapid accumulation of ATP and the drop in nucleoside triphosphate (ADP + GDP) stopped between 43½ and 44 min. The fast amination lasted from 43 to 44 min, and the DPNH downsweep extended from 43½ till nearly 44 min (Fig. 3). (The DPNH trace from Fig. 3 is repeated in Fig. 4 for reference.) The slowing of the energy demand as phosphofructokinase became inhibited and the nucleoside triphosphate concentration dropped, together with the observed accumulation of phosphoenolpyruvate and 3-phosphoglycerate, suggests a condition in which 1,3-diphosphoglycerate might accumulate sufficiently to inhibit glyceraldehyde-3-phosphate dehydrogenase. This would require less than 1 μM 1,3-diphosphoglycerate (21, 22). Such an inhibition could easily account for the rapid drop in DPNH, since the flux through the dehydrogenase, as indicated by the rate of lactate accumulation before the drop in DPNH, was about 300 times the rate of the DPNH change. The close agreement between the drop in DPNH and the transition from fast to slow accumulation of adenine nucleotide (Fig. 3) may in part be due to the fact that the reaction sequence IMP → adenylosuccinate → AMP → ATP consumes 3 eq of 1,3-diphosphoglycerate or phosphoenolpyruvate. Therefore a sudden decrease in the amination rate should favor the accumulation of 1,3-diphosphoglycerate.

Note that α-glycerophosphate accumulated only between 42 and 43½ min; prior to that time there was no dihydroxyacetone-P available, and after that the DPNH concentration had dropped (Fig. 4). DPNH produced by glyceraldehyde-3-phosphate dehydrogenase after 43½ min was reoxidized by lactate dehydrogenase; this is indicated by the observation that the rise in 3-phosphoglycerate and phosphoenolpyruvate after that time was accompanied by an equivalent drop in the pyruvate concentration. Later, when 3-phosphoglycerate and phosphoenolpyruvate fell, there was a corresponding rise in pyruvate.

The concentrations of glucose-6-P, glyceraldehyde-3-P, and 2-phosphoglycerate were also measured but are not shown in Figs. 4 or 5, because they were generally in equilibrium with fructose-6-P, dihydroxyacetone-P, and 3-phosphoglycerate, respectively, as reported earlier (3). The [fructose-6-P]/[glucose-6-P] ratio was 0.28 to 0.30, except during the rapid drop in fructose-6-P when it fell to 0.25 to 0.27. When detectable, glyceraldehyde-3-P was about ½ the concentration of dihydroxyacetone-P, and 2-phosphoglycerate was ½ the concentration of 3-phosphoglycerate.

Activation of Phosphofructokinase by Accumulating Fructose Diphosphate—Prior to the sudden increase in phosphofructokinase activity in the experiment shown in Fig. 4, the concentrations of AMP, ATP, fructose-6-P, and fructose diphosphate were all changing in the direction which could be expected to lead to the activation of the enzyme. In this instance no single effector can be said to be responsible for causing the activation of the enzyme. An experiment in which fructose diphosphate could be unambiguously identified as the effector responsible for triggering the sudden activation will now be described. The starting conditions were the same as for the experiment shown in Figs. 3 and 4, except that the pH was 0.2 unit lower. Both experiments were performed on the same day, using the same muscle extract. Oscillations in the adenine nucleotide level were observed, as in Fig. 3. A detailed analysis of one oscillation is shown in Fig. 5. The time scale has been expanded between 89 and 92 min in order to show changes in metabolite concentrations more clearly. Fructose-6-P rose to reach a constant level at 86 min. The drop in ATP and rise in AMP stopped by 88 min. (The ATP concentration was lower than in the experiment shown in Fig. 4 because a more prolonged deamination occurred at the start of the experiment as a consequence of the lower pH (1).) However, between 87 and 89½ min there was a slow accumulation of fructose diphosphate and dihydroxyacetone-P; this is shown in expanded scale on the right of Fig. 5. A sudden increase in phosphofructokinase activity occurred at 90 min, as can be seen from the drop in fructose-6-P and the sharp rise in fructose diphosphate and dihydroxyacetone-P; it was apparently triggered by the accumulation of about 1 μM fructose diphosphate. This conclusion is unambiguous since during the burst of phosphofructokinase activity the ATP concentration remained constant, and AMP actually dropped by about 10%, leaving fructose diphosphate as the only modifier whose concentration...
The steadiness of the ATP and AMP concentrations indicates that energy production and consumption remained nearly balanced during the burst of phosphofructokinase activity, which was tortuous. In other experiments such as that shown in Fig. 4, the ATP concentration dropped, and AMP often rose, because the sudden increase in ATP utilization by phosphofructokinase was not immediately balanced by an adequate flux through the lower glycolytic pathway.

Between 91½ and 92 min phosphofructokinase became inhibited again, as indicated by the linear rise in fructose-6-P after that point. The fructose diphosphate level remained high, although it was declining slowly. ATP had risen by 11% at 91½ min. However, AMP had dropped by 40% at 91½ min, and by 90% at 92 min. Hence, it is probably the drop in AMP that is largely responsible for ending the burst of phosphofructokinase activity under these conditions. This interpretation implies that the effect of fructose diphosphate in activating phosphofructokinase is strongly dependent on the presence of AMP.

The suddenness of the decrease in the rate of operation of phosphofructokinase may be partially due to the following self-enhancing process. A reduction in the rate of ATP utilization by the enzyme would shift the balance between ATP regeneration and consumption toward regeneration at the expense of ADP and AMP, thus further reducing the rate of the phosphofructokinase reaction. This shift is probably triggered when the fructose-6-P concentration decreases to a critical value. The enzyme exhibits positive cooperativity with respect to fructose-6-P; as a consequence, a decrease in the fructose-6-P concentration can lead to a greater decrease in activity than would otherwise be the case (4, 7, 12, 13, 17).

In the experiments with 10 μM DPN shown in Figs. 4 and 5, a drop in the fructose-6-P concentration to about 60 μM was apparently sufficient to trigger the inhibition of phosphofructokinase. In contrast, in the presence of only 1 μM DPN the burst of phosphofructokinase activity continued until most of the hexose monophosphates were exhausted, to give a fructose-6-P concentration of 4 μM or less (3). In the latter case the rate of ATP production was probably severely limited by the rate at which glyceraldehyde-3-phosphate dehydrogenase could operate. Therefore a much more drastic drop in the fructose-6-P concentration was necessary to reduce the phosphofructokinase activity to the point where ATP would start to rise and AMP would fall.

**Advantage of Oscillatory State for Maintaining High [ATP]/[ADP] Ratio**—The experiments presented in Figs. 1 to 5 illustrate two types of behavior: the approach to approximate steady state and the oscillatory state. A metabolic parameter which provides an important comparison of the two states is the [ATP]/[ADP] ratio. In the experiment shown in Fig. 2 (approach to steady state), the [ATP]/[ADP] ratio dropped to 2 in the first several minutes of the experiment and remained between 1.5 and 2 for the duration of the experiment (Fig. 6). In the experiment shown in Fig. 5 (oscillatory state), the [ATP]/[ADP] ratio also fell to 1.5 in the course of an oscillation, but the ratio rose to 6 after the activation of phosphofructokinase (Fig. 6). The time-averaged [ATP]/[ADP] ratio was about 4 (the oscillation is repeated by dashed lines in Fig. 6 in order to show this more clearly). Thus, although the [ATP]/[ADP] ratio dropped to about the same level before the activation of phosphofructokinase in both experiments, the oscillatory state produced an average [ATP]/[ADP] ratio twice as high as that observed for the steady state behavior.

Fig. 7 gives the changes in the [ATP]/[ADP] ratio for the oscillatory experiment shown in Fig. 4. Three values of interest are indicated. The trigger level is the value to which the ratio falls before phosphofructokinase becomes activated, about 4 in this case. The time-averaged ratio in this experiment was 14, that is about 3 times higher than the trigger level. (Note: The average [ATP]/[ADP] ratio is not the same as the ratio of average [ATP] to average [ADP], which was about 9 in this experiment.) The energy maximum designates the highest values of the [ATP]/[ADP] ratio. In this experiment a ratio of

![Fig. 6. Comparison of changes in the [ATP]/[ADP] ratio during oscillatory and steady state behavior. The steady state experiment was the one described in Fig. 2; the time scale is at the bottom of the figure. The oscillatory state experiment was the one described in Fig. 5; the time scale is at the top of the figure.](http://www.jbc.org/)
about 25 (6 times the trigger level) was maintained for about one-third of the oscillation period.

The \([\text{GTP}]/[\text{GDP}]\) ratio (not shown in Figs. 6 and 7) generally agreed with the \([\text{ATP}]/[\text{ADP}]\) ratio during the oscillations, as was shown previously (3). However, in the steady state experiment of Fig. 2 the \([\text{GTP}]/[\text{GDP}]\) ratio (not shown) was generally 10 to 20% less than the \([\text{ATP}]/[\text{ADP}]\) ratio. This may reflect the preference of pyruvate kinase for ADP over GDP (18).

Effect of Adding Fructose Diphosphate or Dihydroxyacetone-P on Time Course of Oscillations—It was shown previously that addition of 14 or 70 \(\mu\)M fructose diphosphate can cause an activation of phosphofructokinase several minutes in advance of the time at which the enzyme would become activated in the absence of added fructose diphosphate (3). However, the accumulation of only 1 \(\mu\)M fructose diphosphate was sufficient to trigger the activation of phosphofructokinase in the experiment shown in Fig. 5. The fructose diphosphate addition experiment was therefore repeated in order to determine the critical concentration of fructose diphosphate necessary to activate the enzyme. It was found that addition of 10 \(\mu\)M fructose diphosphate caused the early activation of phosphofructokinase, but addition of 1 or 3 \(\mu\)M fructose diphosphate had no effect. However, assays of samples taken from the reaction mixtures showed that 80% of the added fructose diphosphate was converted to dihydroxyacetone-P within 25 s after adding 1 or 3 \(\mu\)M fructose diphosphate. Aldolase and triose phosphate isomerase are very active in skeletal muscle extracts, and the combined equilibrium of these reactions is heavily in favor of dihydroxyacetone-P formation at low substrate concentrations (3). This raised the question as to whether phosphofructokinase was activated by the high concentration of fructose diphosphate present for a short period of time, or by the much lower concentration of fructose diphosphate present after the aldolase and triose phosphate isomerase reactions had reached equilibrium. This question was answered by approaching the equilibrium by addition of dihydroxyacetone-P instead of fructose diphosphate.

The results of such an experiment are shown in Fig. 8. The operation of the purine nucleotide cycle was followed by taking spectral scans at 2-min intervals, beginning 1 min after the start of the reaction (see "Methods"). A sample for metabolite analysis was taken at 6 min. Dihydroxyacetone-P was added at 7½ min, and further samples for metabolite analysis were taken at 8½, 10, and 12 min. The behavior of the purine nucleotide cycle observed on addition of 20 \(\mu\)M dihydroxyacetone-P was very similar to that observed with no addition (Fig. 8). Glucose-6-P and fructose-6-P accumulated steadily; this indicates that phosphofructokinase was not activated by the addition of 20 \(\mu\)M dihydroxyacetone-P. Only half of the added dihydroxyacetone-P remained ½ min after adding 20 \(\mu\)M dihydroxyacetone-P, and most of this was lost in the following 2 min. Only 0.2 \(\mu\)M fructose diphosphate was present ½ min after adding 20 \(\mu\)M dihydroxyacetone-P.

On the other hand, addition of 40 \(\mu\)M dihydroxyacetone-P caused AMP deamidation to stop earlier, and reamination to start earlier, than was observed in the control. Metabolite assays confirmed that addition of 40 \(\mu\)M dihydroxyacetone-P caused a rapid activation of phosphofructokinase. Within ½ min after adding dihydroxyacetone-P (that is, at 8½ min) the fructose diphosphate plus triose phosphate pool contained 50% more carbon than was added as dihydroxyacetone-P. In the time interval between 8½ and 10 min, glucose-6-P and fructose-6-P dropped by at least 85%, and fructose diphosphate and dihydroxyacetone-P accumulated rapidly. The glyceraldehyde-3-P concentration (not shown) was about ½ of the dihydroxyacetone-P concentration. Between 10 and 12 min, fructose diphosphate and triose phosphate dropped, while glucose-6-P and fructose-6-P accumulated at the same rate as was observed when 20 \(\mu\)M dihydroxyacetone-P was added. This suggests that phosphofructokinase was inhibited between 10 and 12 min, which corresponded to the change from deamination to reamination.

The concentration of fructose diphosphate produced by adding 40 \(\mu\)M dihydroxyacetone-P was measured by assaying samples from a reaction mixture identical with that used in the experiment shown in Fig. 8, except that it lacked ATP, GTP, DPN, glucose, and hexokinase. The first sample, taken 15 s after adding dihydroxyacetone-P, gave a concentration of 0.5 \(\mu\)M fructose diphosphate. However, at this time nearly half the added dihydroxyacetone-P had already disappeared. The value of [dihydroxyacetone-P]/[fructose diphosphate] was 0.7 mM, which is close to the value of 0.8 mM calculated for the last
If the original 40 μM dihydroxyacetone-P had reached equilibrium with fructose diphosphate and glyceraldehyde-3-P before any carbon was lost by side reactions, the highest concentration of fructose diphosphate would have been 4 times larger than the concentration of 0.5 μM measured at 15 s, that is to say 2 μM fructose diphosphate. Two minutes after adding 40 μM dihydroxyacetone-P, nearly half of the added carbon was present as glucose-6-P and fructose-6-P, indicating the existence of a relatively high activity of fructose diphosphatase. It is concluded that the fructose diphosphatase concentration generated by the addition of 40 μM dihydroxyacetone-P was in the range of 0.5 to 2 μM. This is in good agreement with the value of about 1 μM fructose diphosphate indicated by the experiment shown in Fig. 5. The critical concentration at which fructose diphosphatase triggers the burst of phosphofructokinase activity is no doubt a function of the concentrations of the other activators and inhibitors present.

The experiments with cell-free muscle extracts described under “Results” show that a rise in the concentration of fructose diphosphate to around 1 μM can trigger a sudden burst of phosphofructokinase activity. Activation of phosphofructokinase by fructose diphosphatase has been reported for the enzymes from muscle (4), heart (15), brain (11, 12), and liver (16), and the effect is often seen at micromolar concentrations of fructose diphosphate. Such strong product activation must be highly significant to the control of phosphofructokinase, and hence to the control of glycolysis and energy metabolism. A possible metabolic advantage of activation of phosphofructokinase by fructose diphosphate is suggested by a comparison of the experiments shown in Figs. 2, 4, and 5.

In the experiment shown in Fig. 2, the expression of the fructose diphosphatase activation was greatly curtailed. A fairly steady, intermediate activity of phosphofructokinase was maintained after 18 min by balancing the ATP inhibition with AMP activation. This required a high concentration of AMP, and the [ATP]/[ADP] ratio was 1.5 to 2 (Fig. 6). In contrast, in the experiments shown in Figs. 4 and 5, phosphofructokinase operated in strong bursts triggered by accumulation of fructose diphosphate. The resulting overshoots provided a pool of high energy precursors that kept AMP low and the [ATP]/[ADP] ratio was about 4, twice the value for the steady state experiment and in an oscillatory experiment just before the activation of phosphofructokinase by accumulating fructose diphosphate, then whichever has the higher [ATP]/[ADP] ratio should have the lower phosphofructokinase activity. (It is assumed that the concentration of the enzyme is the same.) A steady state condition is approached if the rate of the phosphofructokinase reaction (minus loss of carbon through the α-glycerophosphate dehydrogenase reaction) approaches one-fourth the rate of total ATP consumption. The situation is similar to simple feedback inhibition of a pathway, or to a servo mechanism: by increasing the rate of the phosphofructokinase reaction, a decrease in the [ATP]/[ADP] ratio tends to inhibit further decrease in the ratio, until the stable balance point is reached; further increase in the phosphofructokinase activity would then be countered by a resulting rise in the [ATP]/[ADP] ratio. The phosphofructokinase activity at the time in an oscillation just before fructose diphosphate activates the enzyme can have risen no higher than the steady state value, and therefore the trigger level of the [ATP]/[ADP] ratio must be higher than or equal to the [ATP]/[ADP] ratio in the corresponding steady state. The average [ATP]/[ADP] ratio in an oscillatory experiment is higher than the trigger level under the conditions used here (Figs. 6 and 7), and hence is higher than the steady state ratio. A more detailed theoretical treatment will be presented elsewhere (K. Tornheim, manuscript to be submitted for publication).

In many tissues increases in the rate of glycolysis are associated with large increases in fructose diphosphate. This applies, for example, to the transition from aerobic to anaerobic conditions, and to the transition from rest to exercise in muscle. However, even the lowest values reported for the fructose diphosphate content of various tissues correspond to concentrations that are much higher than the trigger point concentration of 1 μM determined in our in vitro experiments. For example, the content of fructose diphosphate (in nanomoles per g wet weight) has been reported to be 30 in resting skeletal muscle (25); 40 to 60 in perfused skeletal muscle at rest (26); 50 to 100 in diaphragm (27), perfused heart (28, 29), and brain (30, 31); and 10 to 20 in liver and kidney (32, 33). These values do not rule out a role for fructose diphosphate in the control of glycolysis in vivo in view of the following considerations. First, the in vitro measurements depend in part on the rapidity of the method of processing the tissue; for example, use of a “freeze-blowing” apparatus, designed to remove and freeze brain tissue in less than 1 s, results in a much lower level of fructose diphosphate, namely 9 nanomoles per g wet weight (34, 35), than values reported earlier. Second, in our in vitro experiments the concentration of ATP was only 1/5 that found in vivo. A report on muscle phosphofructokinase noted that the enzyme could be activated 3-fold by 1 μM fructose diphosphate in the presence of 1 mM ATP, but even 100 μM fructose diphosphate was ineffective in the presence of 4 mM ATP (no AMP was present) (4). Citrate is another inhibitor which counteracts the fructose diphosphatase activation of phosphofructokinase. It does so in part by enhancing the ATP inhibition of the enzyme (11, 16, 17, 36). This means that the fructose depending on the concentrations of the various modifiers of phosphofructokinase activity, such as hydrogen ion, fructose-6-P, orthophosphate, citrate, ammonium ion, and potassium ion, as well as AMP, ADP, and ATP. However, the oscillatory state can produce a higher average [ATP]/[ADP] ratio than the steady state behavior under corresponding conditions. For, if the concentrations of total adenine nucleotide and the other modifiers are similar in a steady state experiment and in an oscillatory experiment just before the activation of phosphofructokinase by accumulating fructose diphosphate, then whichever has the higher [ATP]/[ADP] ratio should have the lower phosphofructokinase activity. (It is assumed that the concentration of the enzyme is the same.) A steady state condition is approached if the rate of the phosphofructokinase reaction (minus loss of carbon through the α-glycerophosphate dehydrogenase reaction) approaches one-fourth the rate of total ATP consumption. The situation is similar to simple feedback inhibition of a pathway, or to a servo mechanism: by increasing the rate of the phosphofructokinase reaction, a decrease in the [ATP]/[ADP] ratio tends to inhibit further decrease in the ratio, until the stable balance point is reached; further increase in the phosphofructokinase activity would then be countered by a resulting rise in the [ATP]/[ADP] ratio. The phosphofructokinase activity at the time in an oscillation just before fructose diphosphate activates the enzyme can have risen no higher than the steady state value, and therefore the trigger level of the [ATP]/[ADP] ratio must be higher than or equal to the [ATP]/[ADP] ratio in the corresponding steady state. The average [ATP]/[ADP] ratio in an oscillatory experiment is higher than the trigger level under the conditions used here (Figs. 6 and 7), and hence is higher than the steady state ratio. A more detailed theoretical treatment will be presented elsewhere (K. Tornheim, manuscript to be submitted for publication).

This is not to imply that the average [ATP]/[ADP] ratio in every oscillatory experiment will be higher than the ratio maintained in every steady state experiment; in either case the ratio can vary widely depending on the concentrations of the various modifiers of phosphofructokinase activity, such as hydrogen ion, fructose-6-P, orthophosphate, citrate, ammonium ion, and potassium ion, as well as AMP, ADP, and ATP. However, the oscillatory state can produce a higher average [ATP]/[ADP] ratio than the steady state behavior under corresponding conditions. For, if the concentrations of total adenine nucleotide and the other modifiers are similar in a steady state experiment and in an oscillatory experiment just before the activation of phosphofructokinase by accumulating fructose diphosphate, then whichever has the higher [ATP]/[ADP] ratio should have the lower phosphofructokinase activity. (It is assumed that the concentration of the enzyme is the same.) A steady state condition is approached if the rate of the phosphofructokinase reaction (minus loss of carbon through the α-glycerophosphate dehydrogenase reaction) approaches one-fourth the rate of total ATP consumption. The situation is similar to simple feedback inhibition of a pathway, or to a servo mechanism: by increasing the rate of the phosphofructokinase reaction, a decrease in the [ATP]/[ADP] ratio tends to inhibit further decrease in the ratio, until the stable balance point is reached; further increase in the phosphofructokinase activity would then be countered by a resulting rise in the [ATP]/[ADP] ratio. The phosphofructokinase activity at the time in an oscillation just before fructose diphosphate activates the enzyme can have risen no higher than the steady state value, and therefore the trigger level of the [ATP]/[ADP] ratio must be higher than or equal to the [ATP]/[ADP] ratio in the corresponding steady state. The average [ATP]/[ADP] ratio in an oscillatory experiment is higher than the trigger level under the conditions used here (Figs. 6 and 7), and hence is higher than the steady state ratio. A more detailed theoretical treatment will be presented elsewhere (K. Tornheim, manuscript to be submitted for publication).

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diphosphate concentration needed for the activation of the enzyme in vivo, where citrate is present, can be expected to be greater than it was in our experiments, where citrate was absent. Third, in vivo only a portion of the total fructose diphosphate may be accessible, a conclusion drawn by Lowry and co-workers from the observation that the fructose diphosphate content of brain, liver, and kidney was 100 times that expected from the dihydroxyacetone-P content, irrespective of the direction of carbon flow through the aldolase reaction (30, 32). Fourth, in vivo measurements are always an average. If the glycolytic oscillations occur in vivo, and are not in synchrony from cell to cell, the high values of fructose diphosphate in some cells at a given moment would dominate and obscure the picture. For example, in Fig. 5 the time-averaged fructose diphosphate concentration (which is the same as the average fructose diphosphate concentration for an asynchronous population of oscillating cells) is 30 μM, though the activation of phosphofructokinase clearly occurs at around 1 μM fructose diphosphate.

Glycolytic oscillations have also been observed in heart and yeast extracts, and in yeast cells. The metabolite patterns reported for the oscillations in heart extracts (37) are similar to those reported previously for the muscle extracts (3), and thus appear to be consistent with the activation of phosphofructokinase by fructose diphosphate. However, fructose diphosphate is probably not involved in generating the oscillations in yeast extracts, where the fructose diphosphate concentration is very high (3 to 8 mM) and changes only slightly (38). In intact yeast cells the fructose diphosphate concentration is lower and oscillates with a greater relative amplitude than in the extracts. Nevertheless, phosphofructokinase was not considered to be the generator of the oscillations, because of the observation that changes in DPNH and pyruvate preceded changes in glyceraldehyde-3-P. Inhibition of glyceraldehyde-3-phosphate dehydrogenase by both its product DPNH and its substrate glyceraldehyde-3-P was proposed to account for the results (39). However, a similar relationship of changes in DPNH and pyruvate preceding changes in glyceraldehyde-3-P is found in the muscle extracts (Figs 4 and 5; note that glyceraldehyde-3-P is proportional to dihydroxyacetone-P); yet, as is shown under "Results," this is consistent with the model in which phosphofructokinase is the generator of the oscillations.

It was pointed out previously that the purine nucleotide cycle is regulated in a manner that helps to maintain a high [ATP]/[ADP] ratio (1-3, 40). Conversion of AMP to IMP by adenylate deaminase leads to the formation of ATP from ADP by a readjustment of the myokinase equilibrium. During a period of high energy demand the purine nucleotide cycle may thus exert a regulating influence on the relative concentrations of AMP, ADP, and ATP, but also by the production of ammonium ions.

REFERENCES


*M. N. Goodman and J. M. Lowenstein, unpublished observations.
The purine nucleotide cycle. Control of phosphofructokinase and glycolytic oscillations in muscle extracts.
K Tornheim and J M Lowenstein


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