Short-term Changes in Hexose Phosphates and ATP in Intact Cells of *Acer pseudoplatanus* L. Subjected to Anoxia

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Abstract. Endogenous concentrations of hexosemonophosphates and ATP decline sharply and rapidly in intact cells of *Acer pseudoplatanus* subjected to anoxia, whereas fructose-1,6-diphosphate and pyruvate accumulate markedly. In view of gas exchange data indicating an apparent acceleration of glycolysis under anoxic conditions, the observed changes in glycolytic metabolite concentrations indicate regulation of glycolysis by phosphofructokinase.

Dennis and Coultate (6) have recently shown that phosphofructokinase derived from higher plants displays many of the same regulatory properties as phosphofructokinase derived from animals (17) and microorganisms (18). High levels of ATP reportedly inhibit the activity of the enzyme under conditions of an *in vitro* assay, as does citrate. Inorganic phosphate relieves ATP inhibition, but in the case of plant phosphofructokinase ADP and AMP do not do so (6). On the basis of their own evidence and that of others, Dennis and Coultate (6) have concluded that “glycolysis is controlled in plants by phosphofructokinase in a similar manner to yeast and animal tissues”. It appears, however, that although these results strongly suggest the possibility that phosphofructokinase is capable of regulating glycolytic flux in response to fluctuations in adenine nucleotide and ATP concentrations within the intact plant cell, it has not to date been made entirely clear whether such regulation is actually involved in controlling the rate of glycolysis *in vivo* (3). It therefore seemed desirable to attempt to ascertain whether the rate of glycolysis in intact *Acer pseudoplatanus* cells is controlled at the phosphofructokinase step.

Although the regulation of glycolysis under aerobic conditions is of intrinsic interest *per se*, it is of special importance in the metabolism of cultured *Acer pseudoplatanus* cells. Previous work employing inhibitors and exogenous substrates has demonstrated that the rate of glycolysis determines the endogenous respiration rate of these cells by controlling the rate at which pyruvate is supplied to the mitochondria (10,11). Since earlier studies with 2,4-dinitrophenol had pointed toward a regulation of glycolysis by intracellular levels of ATP or adenine nucleotides (10), additional investigation of the regulation of glycolysis in relation to adenine nucleotide concentrations appeared to be warranted. In the present work, the effect of anoxia has been investigated. Anoxia is believed to inhibit respiratory chain phosphorylation to approximately the same degree as an uncoupling compound, while also preventing the substrate level phosphorylation associated with the oxidation of ω-ketoglutarate (15).

Since Lynen’s classic studies (14) on yeast cells had indicated that uncoupler treatment and anoxia accelerated glycolysis to the same extent, the effect of anoxia on *Acer* cells has been investigated here in order to obviate certain difficulties associated with uncoupler studies. (These difficulties stem principally from the extremely pronounced concentration dependence of the response to dinitrophenol which leads to problems in standardizing experimental conditions so as to obtain satisfactorily reproducible data).

As discussed elsewhere (*e.g.*, 22), sites responsible for glycolytic control *in vivo* can be pinpointed by comparing intracellular concentrations of glycolytic intermediates under circumstances in which alterations in adenine nucleotide or ATP concentrations are brought about owing to anoxia. Facilitation of substrate flux through a rate-limiting step in response to altered adenine nucleotide concentrations will presumably result in a decrease in intracellular concentration of the substrate of this reaction accompanied by a relative increase in its product. Such a reaction is said to constitute a “crossover point” (22). By means of such metabolite analyses it has been possible to demonstrate that glycolytic flux in yeast (14), rat kidney (24), rat heart (22), ascites tumor cells (25), and other tissues is internally controlled at the phosphofructokinase step, with the control evidently being exerted by the prevailing levels of adenine nucleotides or ATP. The present results, indicating that this is likewise true of *Acer pseudoplatanus* cells, lend strong support to the proposal (6) that glycolysis in higher plants is

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regulated at the phosphofructokinase step under aerobic conditions. The present data do not, however, support a more recent assertion of Dennis and Coultert (7) that fluctuations in ATP are probably not involved with in vivo regulation of the phosphofructokinase reaction. Taken in conjunction with earlier studies (10), these results suggest that the phosphofructokinase is a critical step ultimately responsible for determining the rate of endogenous aerobic respiration in Acer cells.

Methods and Materials

The experimental material employed in the present work consisted of the strain of Acer pseudoplatanus cells originally isolated by Lamport (13) cultured as a liquid suspension and prepared for experiments according to methods described previously (10). Aliquots of cell suspension buffered at pH 4.1 in citrate-phosphate buffer were pipetted into Warburg flasks and used for measurements of gas exchange or for extraction to obtain estimates of intracellular levels of various glycolytic intermediates under aerobic and anoxic conditions. (The respiration rate of these cells is unaffected by the citrate present in the buffer). Anoxic conditions were achieved by flushing flasks with high purity N₂ for 15 minutes while they were being shaken at 25°. Anaerobic CO₂ evolution was calculated directly on the basis of observed pressure changes; CO₂ evolution under aerobic conditions was estimated by the "2-flask" method, in which KOH is omitted from the center well in 1 set of flasks (21). Gas exchange rates were determined over a 2 hour period following equilibration (and gassing with N₂ where applicable) with manometer readings taken every 20 minutes. Gas exchange rates were essentially linear over this period.

For metabolite analyses, cells subjected to anoxia were deproteinized and extracted with perchloric acid, after 15 minutes gassing, according to the techniques previously described (10,11). Control (aerobic) cells were treated and extracted in the same manner as cells subjected to anoxia, except that they were not gassed with N₂ prior to deproteinization.

Concentrations of glycolytic intermediates were determined enzymatically in neutralized extracts by enzymatic procedures described in the compendium edited by Bergmeyer (5). Glucose-6-P and fructose-6-P were estimated by following the reduction of TPN⁺ upon addition of glucose-6-P dehydrogenase, followed by phosphohexoisomerase; ATP was determined by addition of hexokinase in the presence of glucose, after the glucose-6-P initially present in the extract had been oxidized in the presence of glucose-6-P dehydrogenase. Dihydroxyacetone-P, glyceraldehyde-P, fructose-1, 6-diP, and pyruvate were determined by addition of α-glycerol-P dehydrogenase, triose-P isomerase, aldolase, and lactate dehydrogenase in that order, with the oxidation of DPNH being observed after the addition of each enzyme. Pyridine nucleotide oxidation or reduction was monitored by the dual monochromator spectrophotometric technique described by Klingenberg and Slenczka (12).

Results

The initial rate of CO₂ evolution under anoxic conditions (N₂) was approximately 45 to 50% of the rate of CO₂ evolution in air (Table I). Since the anaerobic degradation of a mole of hexose yields 2 moles of CO₂ whereas under aerobic conditions the same mole of hexose would be fully oxidized to yield 6 moles of CO₂, the fact that anaerobic CO₂ evolution exceeds one-third of aerobic CO₂ evolution is often interpreted to mean that more total carbohydrate is degraded under anoxia than under aerobic conditions. However, this cannot be taken as a certainty on the basis of gas exchange data alone (4). But leaving aside the question of the total amount of carbohydrate degraded, the increase in substrate flux through the Embden-Meyerhof-Parnas pathway in Acer cells subjected to anoxia is probably much greater than would be estimated on the basis of a simple comparison of rates of CO₂ evolution in air and under nitrogen. Previous work with fluoride has indicated that 40 to 50% of the aerobic respiration of these cells is independent of glycolysis (11). Hence there must be a fairly marked increase in the flow of substrate specifically through the Embden-Meyerhof-Parnas pathway in order to account for the rate of CO₂ evolution observed when cells are subjected to anoxia. This general question has been discussed by Effer and Ranson (8, 9), who point out that the suppression of the oxidative hexosemonophosphate pathway under anaerobic con-

<table>
<thead>
<tr>
<th>Total CO₂ evolved or O₂ consumed per flask</th>
<th>CO₂ (Air)</th>
<th>O₂ (Air)</th>
<th>CO₂ (N₂)</th>
<th>R. Q. (Air)</th>
<th>CO₂ (N₂)/CO₂ (Air)</th>
</tr>
</thead>
<tbody>
<tr>
<td>µl</td>
<td>µl</td>
<td>µl</td>
<td>Ratio</td>
<td>Ratio</td>
<td></td>
</tr>
<tr>
<td>Expt.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>208 ± 3</td>
<td>180 ± 3</td>
<td>101 ± 4</td>
<td>1.15</td>
<td>0.49</td>
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<tr>
<td>II</td>
<td>386</td>
<td>355 ± 2</td>
<td>188 ± 10</td>
<td>1.09</td>
<td>0.48</td>
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</table>
Fig. 1. Concentrations of glucose-6-P (G-6-P), fructose-6-P (F-6-P), dihydroxyacetone phosphate (DHAP), pyruvate (pyruv), and ATP in extracts of anoxic cells, expressed as percent of aerobic control. Mean values for 6 experiments are given. Vertical lines indicate 2 × standard deviation. (ATP and F-6-P were determined in 5 experiments only. In 2 experiments, DHAP and glyceraldehyde-3-P were assayed together).

Conditions would tend to mask an increase in flux through the conventional glycolytic pathway insofar as CO₂ evolution is concerned. Although the exact increase in total glycolytic flux cannot be accurately determined from the gas exchange data, it seems reasonable to estimate that roughly a doubling of the glycolytic flux occurs under anaerobic conditions. The objective of the present study, however, has been to examine alterations in the concentrations of specific metabolites rather than to establish the absolute magnitude of the change in overall glycolytic flux under anoxia.

Figure 1 shows the combined results of 6 typical experiments in which the levels of certain glycolytic intermediates and ATP were determined in cell suspensions gassed with nitrogen for 15 minutes. The concentrations of these compounds are plotted as percent of control (cell suspension maintained under aerobic conditions). In comparison to aerobic cells, cells deprived of O₂ showed marked decreases in levels of glucose-6-P and fructose-6-P, whereas there were sizable increases in fructose-1,6-diP and pyruvate. Triose-P (dihydroxyacetone-P plus glyceraldehyde-P) concentrations showed less marked changes, though a relatively small increase in dihydroxyacetone-P could often be discerned. The glyceraldehyde-P concentration was very low under both aerobic and anaerobic conditions. Since measurements of the latter intermediate were fairly close to the noise level of the measuring instrument, the glyceraldehyde-P data are not included in figure 1, owing to the questionable reliability of the assay. The ATP content of cells subjected to anoxia fell markedly to approximately one-third of the aerobic control level (fig 1). In 1 experiment the same levels of these intermediates were found after 10, 15, and 20 minutes gassing with N₂, suggesting that they represent anaerobic steady-state values rather than fast transients.

Table II summarises data from a single experiment and shows the relative molar concentrations of the intermediates present in extracts of aerobic and anaerobic cells. By far the largest change in reactant-product ratio brought about by anoxia was in the ratio of fructose-6-P to fructose-1,6-diP. This ratio fell from approximately 7.5:1 under aerobic conditions to about 1.4:1 under anoxia, a difference of about 5-fold. Since the equilibrium position of plant phosphofructokinase is believed to be heavily in favor of the diphosphate ester (e.g. 20), the present data suggest that under aerobic conditions the reaction is greatly displaced from equilibrium and therefore greatly inhibited. The decline in ATP significantly relieves the inhibition of the enzyme so that the fructose-6-P:fructose-diP ratio drops markedly. Williamson (22, 23) has discussed the apparent deviations of glycolytic reactions from equilibrium in rat heart and has drawn special attention to the phosphofructokinase reaction. The sharp drop in intracellular concentration of glucose-6-P and fructose-6-P and the marked rise in fructose-1,6-diP indicate a crossover point in the linear sequence of glycolytic metabolites at the step catalyzed by phosphofructokinase (cf. 22). This observation suggests that in Acer cells, as in various microorganisms, glycolysis is controlled at the phosphofructokinase step. In Acer cells this control is presumably exerted at least in part by the inhibitory action of ATP upon the enzyme, since ATP is present at a relatively high concentration under

Table II. Concentrations of Hexose Phosphates, Dihydroxyacetone Phosphate, Pyruvate, and ATP in Extracts of Aerobic and Anoxic Acer Cells

The data are mean values with range of duplicate assays. Abbreviations as in figure 1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>G-6-P</th>
<th>F-6-P</th>
<th>FDP</th>
<th>DHAP</th>
<th>Pyruvate</th>
<th>ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic</td>
<td>50 ± 1</td>
<td>18 ± 0</td>
<td>2.4 ± 0.2</td>
<td>3.6 ± 0.4</td>
<td>13.5 ± 0.5</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>Anoxic</td>
<td>21 ± 1</td>
<td>7.5 ± 0.0</td>
<td>5.2 ± 0.1</td>
<td>4.2 ± 0.0</td>
<td>55 ± 4</td>
<td>2.5 ± 0.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reactant/Product Ratios</th>
<th>G-6-P/F-6-P</th>
<th>F-6-P/FDP</th>
<th>FDP/DHAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic</td>
<td>2.8:1</td>
<td>7.5:1</td>
<td>0.67:1</td>
</tr>
<tr>
<td>Anoxic</td>
<td>2.8:1</td>
<td>1.4:1</td>
<td>1.2:1</td>
</tr>
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</table>
aerobic conditions. The depletion of ATP under anaerobic conditions permits an acceleration of the phosphofructokinase reaction, thereby allowing an increase in the overall rate of glycolysis.

Discussion

Insofar as the present experiments can be compared to the investigations of Lynen and his collaborators on yeast metabolism (14, 15), the degree of correspondence between the responses of *Acer* and yeast cells to anoxia is surprisingly close. In both *Acer* and yeast cells, suppression of respiration leads at once to increases in fructose-1,6-diP and pyruvate, and to a decrease in hexosemonophosphates, with the change in triosephosphates being relatively minor. In yeast the declines in hexosemonophosphates and ATP are less pronounced after steady-state conditions have been reached following the transition to anaerobic metabolism. This difference probably reflects the inherently high capacity of yeast cells for fermentative metabolism as compared to most higher plant cells. It should be pointed out that the observation of a decline in ATP under anoxic conditions necessitates reconsideration of a recent assertion by Dennis and Coultate (7) that ATP is maintained at a constant level by glycolytic regulation and can therefore not be considered to play a role in controlling the activity of phosphofructokinase *in vivo*. This is probably true of yeast cells (14), but the evidence presented here indicates that the situation in higher plant cells differs in this regard.

Most studies on the effect of anoxia on glycolytic metabolite profiles in higher plants have consisted of long-term experiments in which the effects of anoxia have been observed over periods of many hours or even days. Although the early work of Rowan and co-workers (19) indicated a decrease in fructose-1,6-diP under anaerobic conditions and an increase in the hexosemonophosphates, a number of more recent reports have shown that increases in fructose-1,6-diP occur when various higher plant tissues are subjected to anoxia. Barker and his collaborators (1) found this to be true of peas, though glucose-6-P and fructose-6-P remained constant or even increased slightly so that there was no short-term crossover point as such between fructose-6-P and fructose-1,6-diP. These investigators did not explain the observed increase in the diphosphate ester as being the result of a decrease in ATP-inhibition of phosphofructokinase, but rather thought it necessary to invoke the existence of a "glycolytic granule". This largely hypothetical organelle was assumed to prevent glycolytic kinases from having free access to cytoplasmic ATP, so that only the ATP produced by the glycolytic reactions themselves was available for the initial phosphorylation of hexoses and hexosemonophosphates (1).

Subsequently, Barker *et al.* (2) further considered the evidence for the existence of a distinct glycolytic organelle and also considered the view that altered hexosephosphate concentrations induced by anoxia are the result of a reduction in ATP-inhibition of phosphofructokinase. The latter view was rejected by these workers, who preferred to ascribe the rise in fructose-1,6-diP to inhibition of the triosephosphate dehydrogenase reaction owing to accumulation of DPNH under anaerobic conditions. This interpretation would not be compatible with the data of Lynen (14) who found that in yeast cells the diphosphate ester accumulated when aerobic glycolysis was accelerated in the presence of di-nitrophenol, which did not interfere with DPNH oxidation via the respiratory chain. In the present experiments the increase in fructose-1,6-diP under anoxia was much more pronounced than the increase in dihydroxyacetone phosphate, suggesting that the increase in fructose-1,6-diP could not be ascribed solely to inhibition of triosephosphate utilization.

Effer and Ranson (9) demonstrated that glucose-6-P and fructose-6-P levels declined in anoxic buckwheat seedlings, while in some cases there was an increase in the level of fructose-1,6-diP. This was evidently indicative of a crossover point at the phosphofructokinase step, though these authors did not use this terminology in reference to their findings. ATP was presumed to decline in the absence of O2.

The problem of glycolytic regulation in higher plants has been discussed recently by Marre *et al.* (16) and by Barker *et al.* (3). Marre and collaborators raised the possibility of phosphofructokinase regulation upon finding that there was an apparent deviation of the phosphofructokinase reaction from equilibrium in various aerobic tissues, while the fructose-diP concentration rose under anoxic conditions. Barker *et al.* (3) acknowledged the possibility that a rise in fructose-diP under anaerobic conditions might result from a diminution in ATP-inhibition of phosphofructokinase but felt that in their pea material the decline in glucose-6-P and fructose-6-P was far too slow to indicate activation of phosphofructokinase as being involved in the initial acceleration of glycolysis under anaerobic conditions. Barker *et al.* (3) still appear, on balance, to favor their earlier explanation of glycolytic control based upon the postulation of a glycolytic organelle.

The present data on the response of intact cells of *Acer pseudoplatanus* to anoxia indicate that phosphofructokinase of higher plants is subject to metabolic control *in vivo*. The short-term changes in hexosemonophosphate and diphosphate levels occurring in response to anoxia are readily explained on the basis of a relief of ATP-inhibition of phosphofructokinase. The decline in ATP content of anaerobic cells presumably permits accelerated flux through the phosphofructokinase step, just as is true of the isolated carrot enzyme (6). The rapidity of the decline in hexosemonophosphates, concurrent with a decline in ATP and an increase in fructose-diP, stands in contrast to the slower response shown in the work on peas cited above (3). Hence one of
the chief objections to the phosphofructokinase activation theory (3) evidently does not apply to the experimental material employed in the present study. There is adequate evidence, therefore, to sustain the proposal (6) that aerobic glycolysis is subject to control at the phosphofructokinase step in higher plants just as in other taxonomic groups of organisms. It does not seem necessary to postulate the existence of a distinct glycolytic organelle, although this possibility, as well as the possibility of additional glycolytic control mechanisms is by no means precluded. It may be well to note that there is no a priori reason to suppose that aerobic glycolysis in all higher plant material will prove to be controlled in precisely the same fashion. Wu (24), for example, has reported that the factors regulating glycolysis in rat liver and rat kidney are not at all the same. Very possibly the rate-limiting factors in other higher plant material will not invariably turn out to be identical to those characterizing the *Acer* cells studied in the present work.

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Literature Cited