The Role of Adaptive Enzymes in Carbohydrate Oxidation by Stressed and Senescing Plant Tissues

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Fruit ripening and leaf senescence are generally accompanied by the mobilization of stored carbohydrates and proteins. Exposure of plants to abiotic and biotic stresses also frequently leads to the mobilization of carbohydrates and proteins. Mobilization of stored carbohydrates may involve the hydrolysis of starch and other polysaccharides or simply the conversion of sucrose to reducing sugars. The accumulated reducing sugars, nevertheless, serve as respiratory substrates and enhance glycolysis. The respiratory rate of plant tissues, however, is controlled further by the respiratory capacity of the mitochondria and the demand for ATP (adenosine 5'-triphosphate). Thus, the overall rate of glucose oxidation in many plant tissues is in synchrony with the rate of ATP utilization. However, ATP still accumulates, concomitant with the increased respiratory rate in several organs, such as ripening fruit and senescing leaves, as well as in potato tubers (*Solanum tuberosum* L.) that have been exposed to ethylene or cyanide (Bennett et al., 1987; Chalmers and Rowan, 1971; Macnicol, 1973; Malik and Thimann, 1980; Solomos and Laties, 1975).

CONTROL OF GLYCOLYSIS

In normal respiratory metabolism, accumulated ATP slows the rate of glycolysis by inhibiting certain regulatory enzymes. How then is glycolysis enhanced in the presence of high levels of ATP in ripening, senescing, and stressed plant tissues? Glycolysis has two essentially irreversible reactions, the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate by phosphofructokinase and the conversion of phosphoenolpyruvate to pyruvate by pyruvate kinase (Fig. 1). A high concentration of ATP in cells inhibits phosphofructokinase and activates fructose 1,6-bisphosphatase, thereby diminishing glycolysis and promoting gluconeogenesis. Nevertheless, both fructose 1,6-bisphosphate and pyruvate accumulate in ripening fruit and senescing leaf tissues (Biale, 1975; Salminen and Young, 1975). Pyruvate also accumulates in chilling-sensitive cucumber (*Cucumis sativus* L.) and eggplant (*Solanum melongena* L.) fruits during cold storage, but not in chilling-insensitive carrot (*Daucus carota* L.) roots (Tsuchida et al., 1990). Apparently, the regu-

lation of glycolysis and the oxidation of pyruvate in ripening, senescing, and stressed plant tissues differ from that found in actively growing, unstressed plant tissues.

Two paths of glycolysis exist in plant cells, a maintenance pathway catalyzed by the classical ATP-dependent phosphofructokinase that is inhibited by high concentrations of ATP, and an adaptive path catalyzed by a pyrophosphate-dependent phosphofructokinase (Fig. 1) (Black et al., 1987). Activity of the constitutive maintenance path remains at a relatively constant level throughout the life of the tissue, whereas the adaptive path appears at certain stages of plant development and during environmental perturbations. Thus, the adaptive path enables plants to cope with both short-term and long-term environmental stresses.

The adaptive path is regulated by the intracellular level of fructose 2,6-bisphosphate (Fig. 1). This compound activates the pyrophosphate-dependent phosphofructokinase and inhibits the cytosolic fructose 1,6-bisphosphatase, thereby promoting glycolysis even in the presence of high levels of ATP. A constitutive regulator cycle is present in plant cells for the synthesis and hydrolysis of fructose 2,6-bisphosphate via a 2-kinase and a 2-phosphatase, respectively (Black et al., 1987). Thus, the fructose 2,6-bisphosphate levels change rapidly in tissues subjected to environmental perturbations or less rapidly during tissue ontogeny. Ethylene induces significant increases in the fructose 2,6-bisphosphate content of the nonphotosynthetic storage tissues, carrot roots and potato tubers (Stitt et al., 1986). Fructose 2,6-bisphosphate levels also increase during the climacteric ripening of avocado (*Persea americana* Mill.) (Bennett et al., 1987) and banana (*Musa* spp., AAA group) (Beaudry et al., 1989) and in carrot root shreds stored in low-O₂ atmospheres (Kato-Noguchi and Watada, 1996).

PYRUVATE OXIDATION

Pyruvate kinase, which catalyzes the second essentially irreversible reaction in glycolysis, is activated by the accumulation of glycolytic intermediates, particularly fructose 1,6-bisphosphate. Pyruvate, the end product of glycolysis, can be metabolized by plant tissues in at least three different ways (Fig. 2). Under normal aerobic conditions, pyruvate is oxidized through the TCA cycle (tricarboxylic acid cycle) to CO₂ with the conservation of energy through the electron transport chain. Electron transport through the cytochromes to O₂ develops a proton gradient across the mitochondrial inner membrane that is dissipated with the synthesis of ATP. Unless there is an increased demand for ATP by the cell during

the enhanced glycolytic activity, however, electron flow through the mitochondrial electron transport chain will be limited by the availability of ADP and/or inorganic phosphate. Although anabolic reactions are involved in the response to stress and during ripening and senescence, overall metabolism is catabolic. Potato tubers utilize the enhanced ATP levels following exposure to ethylene, cyanide, or low temperature in the biosynthesis and accumulation of sucrose or "sweetening" of the potato (Amir et al., 1977; Solomos and Laties, 1975).

Pyruvate can also be transaminated to alanine (Fig. 2). Alanine accumulates in the tissues of sensitive plants, but not in the tissues of resistant plants, when they are exposed to chilling temperatures (Kozukue et al., 1984; Patterson et al., 1981). Transamination of pyruvate, however, does not utilize ATP or reductant.

Pyruvate can also undergo decarboxylation in the cytosol to produce acetaldehyde, which is reduced subsequently to ethanol (Fig. 2). Ethanol and acetaldehyde are produced by chilling-sensitive citrus fruits in proportion to the storage temperature and length of exposure to low temperatures (Eaks, 1980). Ethanol accumulates in the leaves of transgenic tobacco (*Nicotiana tabacum* L.) that lack the alternative oxidase when the cytochrome path is blocked by antimycin A (Vanlerberghe et al., 1995). The leaves develop necrotic lesions, but whether the accumulated ethanol is directly responsible for stress-induced tissue injury is not clear. The leaves of transgenic plants with elevated levels of the alternative oxidase appear normal and do not accumulate ethanol when their cytochrome electron transport chain is blocked by antimycin A. Restriction of cytochrome electron transport by inhibitors or by an insufficient level of ADP can lead to aerobic fermentation in plant tissues.

During glycolysis, NAD⁺ is reduced to NADH (nicotinamide adenine dinucleotide), which has to be reoxidized for glycolysis to continue. Reduction of acetaldehyde to ethanol provides a mechanism for the cycling of cytosolic NADH-NAD⁺ and the continuation of glycolysis. Plant mitochondria are unique, however, in that they possess an NADH dehydrogenase on the outside of the inner mitochondrial membrane and are able to oxidize cytosolic NADH (Palmer and Ward, 1985). Electrons from cytosolic NADH are transferred directly to ubiquinone, bypassing complex I and the first phosphorylation site. Since the electrons pass through complex III and the other two phosphorylation sites, however, the oxidation of cytosolic NADH by the mitochondria still is restricted by the availability of ADP.

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ALTERNATIVE PATH OF ELECTRON TRANSPORT

How then can the accumulated pyruvate and NADH be oxidized without producing potentially harmful levels of acetaldehyde and ethanol? Another adaptive path, the alternative path of electron transport, is induced during exposure of plants to various environmental and biotic stresses as well as during fruit ripening (Purvis and Shewfelt, 1993). Furthermore, greater steady-state levels of the alternative path are found in those cultivars and tissues that appear to be more resistant to the stress. The alternative oxidase is induced in plant tissues by several stress-related molecules, including ethanol (Janes et al., 1981; van der Plas and Wagner, 1980), superoxide anions (Minagawa et al., 1992), and hydrogen peroxide (Vanlerberghe and McIntosh, 1996; Wagner and Krab, 1995), as well as by antimycin A (Minagawa and Yoshimoto, 1987; Vanlerberghe and McIntosh, 1992), sulfhydryl reductants, and TCA cycle intermediates (Vanlerberghe and McIntosh, 1996). Whether transduction of the alternative oxidase by these molecules is direct or indirect is not known.

Because electron transport through the alternative path does not develop a proton gradient across the membrane, it is nonphosphorylating. Thus, electron transport through the alternative path is not restricted by the avail-

ability of ADP and can occur even at high levels of ATP.

In most plant tissues, the alternative path appears not to be as accessible to NADH oxidation as it is to succinate and malate oxidation (Lance et al., 1985). In addition, high threshold levels of reduced ubiquinone are necessary for alternative oxidase activity (Day et al., 1991). Such activity, however, is tightly regulated by the redox status of the protein as well as by pyruvate and other α -ketoacids (Millar et al., 1993; Siedow and Umbach, 1995; Umbach et al., 1994). When activated, the enzyme competes with cytochrome oxidase for electrons (Hoefnagel et al., 1995). Furthermore, electrons from cytosolic NADH can be transferred to O_2 via the alternative oxidase following its activation by pyruvate, thus bypassing all three phosphorylation sites in the mitochondrial electron transport chain. Therefore, the alternative path offers a mechanism for cycling cytosolic NADH-NAD⁺ during times of enhanced glycolytic activity, even when the energy charge of the cell is high. The feed-forward activation of the alternative oxidase by pyruvate ensures a balance between glycolysis and the capacity of the electron transport chain in the presence of high levels of ATP. The alternative oxidase also provides for the operation of the TCA cycle during ripening, senescence, and exposure to stress.

MITOCHONDRIAL PRODUCTION OF ACTIVE OXYGEN SPECIES

How does the alternative oxidase reduce stress-induced injury? Another consequence of restricted electron transport through the cytochromes is that some components of the electron transport chain will be mostly reduced and susceptible to autooxidation and the generation of superoxide in the presence of molecular oxygen. Mitochondria are major producers of superoxide in plant cells (Boveris and Cadenas, 1982; Puntarulo et al., 1991; Purvis et al., 1995; Rich and Bonner, 1978). Superoxide is produced by cyanide-sensitive mitochondria when electron transfer through the cytochromes is inhibited with antimycin A (Minagawa et al., 1992). Superoxide is dismutated to hydrogen peroxide by Mn-superoxide dismutase in the mitochondrial matrix. Because the mitochondrial membranes are permeable to the more stable hydrogen peroxide, mitochondria are a major source of cytosolic hydrogen peroxide (Puntarulo et al., 1991). In the presence of iron, superoxide and hydrogen peroxide also produce the highly reactive hydroxyl radical. Reactive oxygen species have been implicated in several stress-induced injuries, as well as in ripening and senescence processes (Scandalios, 1993). Controlling oxygen-free radical activity, therefore, is crucial not only in reducing stress-

Fig. 1. Regulation of glycolysis in plant tissues by phosphofructokinase and pyruvate kinase and the regulation of phosphofructokinase by fructose 2,6-bisphosphate. Glu = glucose; PFK = phosphofructokinase; FBptase = fructose 1,6-bisphosphatase; F-1,6-P₂ = fructose 1,6-bisphosphate; F-2,6-P₂ = fructose 2,6-bisphosphate; PPi = pyrophosphate; PEP = phosphoenolpyruvate; PK = pyruvate kinase; F-6-P = fructose-6-phosphate; ATP = adenosine 5'-triphosphate.

Fig. 2. Metabolic disposition of pyruvate accumulating during enhanced glycolysis in plant tissues. PDC=pyruvate decarboxylase; PDH=pyruvate dehydrogenase; ADH = alcohol dehydrogenase; AAT = alanine aminotransferase; TCA = tricarboxylic acid cycle; EC = Enzyme Commission.

induced injuries but also in slowing down the rate of ripening and senescence. Hence, cells contain several antioxidants and active oxygen-scavenging enzymes that control the oxygen-free radicals generated in normal metabolic processes (Winston, 1990). During exposure to stress the level of antioxidants sometimes increases. The most logical defense system, however, also includes mechanisms to reduce the production of active oxygen species.

We postulated that the alternative path of electron transport in plant mitochondria reduces the potential production of reactive oxygen species in plant tissues (Purvis and Shewfelt, 1993). During normal metabolism, pyruvate is oxidized by the mitochondria to CO₂ and H₂O, with the energy released conserved as ATP. Although some active oxygen species are produced, the cell has active oxygen-scavenging enzymes and antioxidants, e.g., vitamins E and C, sufficient to prevent extensive and detrimental peroxidation of membrane lipids and proteins. When stored carbohydrates are mobilized in stressed plant tissues and during ripening and senescence, glycolysis is stimulated and more NADH and pyruvate are available to the mitochondria. Stress-induced physical changes in the membrane components, such as those occurring at chilling temperatures or when ADP and/or inorganic phosphate are limiting, may occur during ripening and senescence, thereby restricting electron transport through the cytochrome path. Reduced electron transport components can autoxidize in the presence of molecular oxygen and generate excessive active oxygen species. If the defense systems of cells are overwhelmed, membrane lipids and

enzymic proteins are peroxidized and become nonfunctional. The feed-forward activation of the nonphosphorylating alternative path of respiratory electron transport by pyruvate, however, allows the cell to oxidize cytosolic NADH directly. Furthermore, the increased level of pyruvate can be oxidized aerobically without producing a harmful excess of active oxygen species or acetaldehyde and ethanol.

A CASE STUDY

Grapefruit (*Citrus ×paradisi* MacFady), like the fruit of many other tropical and subtropical species, is injured when exposed to low, but above freezing, temperatures. Although chilling injury is primarily a storage disorder that occurs during storage of the fruit at low temperatures, it can also occur on fruit still attached to the tree (Purvis and Yelenosky, 1993). Low temperature is generally believed to have a direct, physical effect on mitochondrial membrane lipids and certain enzymic proteins of chilling-sensitive plants (Raison and Orr, 1990). Consequently, mitochondrial respiration, i.e., electron transfer and oxygen uptake, is impaired at chilling temperatures whereas glycolysis continues at a reduced rate. The accumulated intermediary metabolites, including pyruvate, then are respired rapidly when the fruit is removed from chilling temperatures (Lyons and Breidenbach, 1990). Visible manifestation of chilling injury, appearing randomly as pits on the peel, occurs along with the increased respiratory activity. In contrast, chilling-resistant plant tissues generally do not exhibit such an anomalous respiratory activity when exposed to low temperatures.

Exposure of grapefruit to temperatures of 10 °C or lower, either on the tree (Purvis et al., 1979; Purvis and Grierson, 1982; Purvis and Yelenosky, 1982) or during postharvest storage (Purvis, 1989; Purvis and Rice, 1983), results in the hydrolysis of sucrose in the peel tissues. The accumulated reducing sugars stimulate respiration (Purvis, 1985).

Grapefruit harvested from the interior canopy of the tree are more resistant to chilling injury than those harvested from the exterior (Purvis, 1980). The level of reducing sugars accumulating during low-temperature storage, however, is similar in the peel tissues of both interior and exterior fruit (Purvis, 1989). The alternative path of respiratory electron transport also is present at about the same level in the peel tissues of both interior and exterior fruit at harvest, but after storing the fruit at low temperature for only 1 week, the capacity of the alternative path is significantly greater in chilling-resistant interior than in chilling-sensitive exterior fruit (Purvis, 1985). Thus, the ability to synthesize the alternative oxidase enzyme during exposure to low temperature appears to be more important in inducing chilling tolerance than the inherent level of the enzyme at the time of exposure.

In summary, two adaptive alternative paths of carbohydrate oxidation serve as homeostatic mechanisms in plants exposed to biotic and abiotic stresses as well as during ripening and senescence. The two paths can operate in the presence of high levels of ATP when maintenance pathways are usually restricted. The adaptive pyrophosphate-dependent phosphofructokinase favors glycolysis when respiratory substrates are abundant. The accumulated fructose 1,6-bisphosphate activates pyru-

vate kinase resulting in the production and accumulation of pyruvate. Pyruvate activates the alternative path of electron transport in the mitochondria and allows for the oxidation of pyruvate through the TCA cycle and the turnover of cytosolic NADH without the production of excessive potentially harmful active oxygen species and anaerobic metabolites. Furthermore, the alternative path is induced by metabolites, such as ethanol, superoxide, hydrogen peroxide and citrate, which are produced when electron transport through the cytochrome pathway is restricted. Thus, plant tissues are able to metabolize reducing sugars when the demand for respiratory energy is diminished during ripening, senescence, and exposure to stress. The critical question is whether transgenic plants with enhanced alternative oxidase activity better cope with stress and ripen and senesce at slower rates than those with low levels of alternative oxidase. With the alternative oxidase gene having been cloned from several species, including *Sauromatum guttatum*, *Hansenula anomala*, *Arabidopsis thaliana*, soybean [*Glycine max* (L.)], potato, tobacco, and rice (*Oryza sativa* L.) (McIntosh, 1994), the answer to that question appears to be within reach.

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