**Metabolic Activity Decreases as an Adaptive Response to Low Internal Oxygen in Growing Potato Tubers**

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Plants lack specialised organs and circulatory systems, and oxygen can fall to low concentrations in metabolically active, dense or bulky tissues. In animals that tolerate hypoxia or anoxia, low oxygen triggers an adaptive inhibition of respiration and metabolic activity. Growing potato tubers were used to investigate whether an analogous response exists in plants. Oxygen concentrations fall below 5% in the centre of growing potato tubers. This is accompanied by a decrease of the adenylate energy status, and alterations of metabolites that are indicative of a decreased rate of glycolysis. The response to low oxygen was investigated in more detail by incubating tissue discs from growing tubers for 2 hours at a range of oxygen concentrations. When oxygen was decreased in the range between 21% and 4% there was a partial inhibition of sucrose breakdown, glycolysis and respiration. The energy status of the adenine, guanine and uridine nucleotides decreased, but pyrophosphate levels remained high. The inhibition of sucrose breakdown and glycolysis was accompanied by a small increase of sucrose, fructose, glycerate-3-phosphate, phosphoenolpyruvate, and pyruvate, a decrease of the acetyl-CoenzymeA:CoenzymeA ratio, and a small increase of isocitrate and 2-oxoglutarate. These results indicate that carbon fluxes are inhibited at several sites, but the primary site of action of low oxygen is probably in mitochondrial electron transport. Decreasing the oxygen concentration from 21% to 4% also resulted in a partial inhibition of sucrose uptake, a strong inhibition of amino acid synthesis, a decrease of the levels of cofactors including the adenine, guanine and uridine nucleotides and CoenzymeA, and attenuated the wound-induced increase of respiration and invetase and phenylalanine lyase activity in tissue discs. Starch synthesis was maintained at high rates in low oxygen. Anoxia led to a diametrically opposed response, in which glycolysis rose 2-fold to support fermentation, starch synthesis was strongly inhibited, and the level of lactate and the lactate:pyruvate ratio and the triose-phosphate:glycerate-3-phosphate ratio increased dramatically. It is concluded that low oxygen triggers (i) a partial inhibition of respiration leading to a decrease of the cellular energy status and (ii) a parallel inhibition of a wide range of energy-consuming metabolic processes. These results have general implications for understanding the regulation of glycolysis, starch synthesis and other biosynthetic pathways in plants, and reveal a potential role for pyrophosphate in conserving energy and decreasing oxygen consumption.

**Keywords:** Adenylates / Glycolysis / Hypoxia / Oxygen / Potato tubers / Respiration.

**Introduction**

Animals have evolved specialised respiratory organs that absorb oxygen from the environment, and circulatory systems that pump fluids containing high concentrations of oxygen-binding proteins round the organism. These allow large amounts of oxygen to be delivered, and facilitate rapid changes in oxygen delivery in response to changes in metabolic activity. In contrast, plants lack specialised systems for oxygen delivery. Oxygen moves by diffusion from the surrounding air (21% oxygen, equivalent to 21 KPa at sea level) through regulated apertures in the epidermis and intercellular air spaces within the tissue. Although plants can adapt to low oxygen by producing larger intercellular air spaces (Laan et al., 1990; Drew et al., 1994, 1995), this response is slow and inefficient compared to the physiological adaptations in animals. The absence of specialised systems for oxygen delivery is not generally considered to be a problem for plant growth and metabolism because most plant organs have a relatively high surface-to-volume ratio, and their respiration rates per unit volume of tissue are usually lower than in animals because plant cells usually have a large vacuole. However, tissues with high rates of metabolism can become hypoxic (a severe decrease of the oxygen concentration) or anoxic (zero oxygen), especially when they lack large intercellular air spaces, contain cells that are not highly vacuolated, or are located in the centre of organs or at other sites remote from the sites at which oxygen enters the plant.

Low internal oxygen has been implicated in the development of seed dormancy in some species (Honek and Matinkova, 1992; Jauzein and Mansour, 1992; de Meillon et al., 1990; Drew et al., 1994, 1995) especially when external oxygen is low due to water logging or microbial activity. In vegetatively growing plants, low oxygen...
concentrations have been reported in meristems (Armstrong and Beckett, 1985; Gibbs et al., 1995) and vascular tissues (Kimmers and Stringer, 1988). Vascular tissues are especially prone to hypoxia in roots (Thomson and Greenway, 1991; Sorrel, 1994; Armstrong et al., 1994; Ober and Sharpe, 1996), where the external oxygen concentration is anyway often low and the phloem and xylem are located in the stele in the centre of the root. Low oxygen tensions in the stele interfere with sugar retention in the phloem and ion loading into the xylem (Drew, 1997; Gibbs et al., 1998). The potential importance of oxygen delivery for reproductive growth is revealed by the observation that seed production is totally inhibited at 2% external oxygen in rice (Akita and Tanaka, 1973), soybean, wheat, sorghum (Quebedeaux and Hardy, 1975, 1976), rape and Arabidopsis (Porterfield et al., 1999). When the external oxygen concentration is decreased in the range below 15% there is a linear reduction of Arabidopsis seed size (Porterfield et al., 1999). This implies that substantial concentration gradients are required to drive oxygen diffusion into these tissues. In agreement, when Arabidopsis and rape were grown in air the oxygen concentration in the airspace inside the silique in the dark was already decreased to 12% and 6%, respectively (Porterfield et al., 1999). Several indirect lines of evidence, including induction of enzymes involved in fermentation, accumulation of lactate or ethanol, and declining adenylate energy charge indicate that oxygen falls to low levels in developing seeds of Phaseolus (Boyle and Yueng, 1983), barley (Macnicol and J acobson, 1992), rape (Ching et al., 1974) and soybean (Quebedeaux, 1981; Shelp et al., 1995). Decreased internal oxygen concentrations have also been measured in bulky storage organs, including apples (Magness 1920), bananas (Banks, 1983), avocado (Ke et al., 1995), carrots (Lushuk and Salveit, 1991) and potato tubers (Magness 1920; Steward et al., 1932; Stiles, 1960; Burton, 1950, 1989). As these studies (see Stiles, 1960 for a review) focused on stored fruits and tubers at low temperatures, they may underestimate the severity of hypoxia in growing tubers and fruits at ambient growth temperatures.

Cytochrome oxidase catalyses the terminal reaction of the mitochondrial electron transport chain, which transfers electrons from NADH to oxygen. Cytochrome oxidase has a very high affinity for oxygen with a K_m(oxygen) of about 14 μM (equivalent to the oxygen concentration that is in equilibrium with a gas phase containing 0.013% oxygen at 20°C; see Drew, 1997). Unregulated operation of cytochrome oxidase would decrease internal oxygen to very low levels when the oxygen supply is restricted. Although this would promote oxygen delivery by diffusion, it carries the concomitant risk that sectors of the tissue will be driven into anoxia by small fluctuations of the external oxygen concentration, the resistance to oxygen entry, or oxygen consumption in other parts of the plant. Anoxia represents a stress because the efficiency of ATP formation is sharply reduced, cytosolic pH decreases, and lactate and ethanol accumulate. Furthermore, harmful oxygen radicals are formed when oxygen re-enters highly reduced anoxic tissues (Drew, 1997; Biemelt et al., 1998). Oscillation between anoxia and hypoxia would be particularly harmful, leading to fluctuations in the ATP supply and cellular pH, and repeated exposure to oxygen radicals (Drew, 1997). The importance of mechanisms to avoid, or at least delay, the depletion of oxygen to very low levels is also emphasised by the finding that a period of pre-adaptation in low oxygen is important for the development of tolerance to anoxia (Drew et al., 1997; Chang et al., 2000).

Animals that tolerate low oxygen tensions are able to decrease their oxygen consumption at low oxygen concentrations (Storey, 1996; Kwast and Hand, 1996; Hochachka et al., 1996; Hochachka, 1997). This adaptive response includes a partial inhibition of respiration, and a concomitant decrease of ATP consumption due to decreased protein synthesis and ion pumping activity. The inhibition of respiration is rapid, and occurs at oxygen concentrations well above the K_m(oxygen) of cytochrome oxidase. Characteristic changes in gene expression that are induced by hypoxia are not simulated when respiratory inhibitors are added to animal tissues (Land and Hochachka, 1995), indicating that an oxygen sensing system triggers a co-ordinated inhibition of ATP formation and consumption.

Biochemical adaptation to low oxygen might be especially important in plants because (see above) they lack sophisticated physiological mechanisms for oxygen transport. Although the response of plants to external hypoxia has been intensively studied (Crawford and Brandle, 1996; Drew et al., 1997), this research has been driven by questions related to adaptation to anoxia. Low external oxygen induces a characteristic set of anaerobic response (ANR) genes including lactate dehydrogenase (LDH), pyruvate decarboxylase, alcohol dehydrogenase (ADH) and several glycolytic enzymes (Andrews et al., 1993, 1994; Fennoy and Bailey-Serres, 1995; Dolferus et al., 1997; Germain et al., 1997b). On the other hand, it has also been reported that low oxygen inhibits respiration of tubers (Stiles, 1960; Burton, 1989), seeds (Raymond and Pradet, 1980; Al Ani et al., 1985) and roots (Saglio et al., 1983). In seeds, this is accompanied by a decrease of the adenylate energy charge (Raymond and Pradet, 1980; Saglio et al., 1985). In maize root tips, low oxygen inhibits protein synthesis (Chang et al., 2000). Low external oxygen has opposing effects on enzymes for sucrose breakdown, repressing invertase and inducing sucrose synthase (SuSy) in maize root tips (Germain et al., 1997a; Zeng et al., 1999). In ripening banana fruits low oxygen inhibits the synthesis and breakdown of starch, and activates cycles in sucrose metabolism and glycolysis (Hill and ap Rees, 1995). Some of the metabolic responses to low external oxygen, for example the induction of enzymes required for fermentation, represent a pre-adaptation to allow better survival under anoxia, (Drew, 1994; 1997; Chang et al., 2000). It is unclear whether the other changes represent an adaptation to decrease oxygen consumption and avoid anoxia. Although there are differences between the response to low and zero external oxygen (Andrews
et al., 1994; Dolferus et al., 1997; Germain et al., 1997b; Ying et al., 1998; Zeng et al., 1999), this may be because sudden anoxia interferes with adaptive responses (Chang et al., 2000). Interpretation is further complicated because some cells or tissues may become anaerobic when plants are exposed to low external oxygen concentrations (Armstrong and Beckett, 1987; Drew, 1997). Most previous studies have used rather low external oxygen concentrations, and did not investigate the effect on internal oxygen concentrations.

The following experiments ask whether the oxygen concentration is significantly decreased in growing potato tubers, and investigate the accompanying changes in energy status. In a complementary approach, tissue discs from growing tubers were exposed to a range of oxygen concentrations to explore the consequences for cellular energy status, metabolite levels, fluxes in sucrose, starch and respiratory metabolism, and the response to wounding.

Results

Oxygen Tension inside Growing Potato Tubers

Oxygen concentrations inside Solanum tuberosum cv. Desiree tubers were measured using an oxygen minielectrode. The oxygen concentration in the centre of tubers which had been harvested and stored for 4 weeks at 20 °C was 12 – 14% (Figure 1A), which is similar to values reported for tubers of other cultivars stored at this temperature (see e.g. Magness, 1920; Burton, 1950, 1989). The oxygen concentration in the centre of growing tubers at ambient growth temperature (20 °C) was below 5% (Figure 1A). The tubers were growing in well-aerated soil and the oxygen concentration surrounding the tubers in situ was > 18% (data not shown). Figure 1B shows a typical oxygen concentration profile through a growing tuber (fresh weight approx. 20 g). The oxygen concentration was 11 – 15% immediately under the periderm, falling to 5% in the centre. Figure 1C illustrates the interaction between tuber size and the internal oxygen concentration. As tuber size increased from 8 g to 32 g, the oxygen concentration immediately under the periderm decreased from 15% to 5% and the concentration in the centre decreased from 8% to 2%.

Adenine Nucleotide Levels along a Transect across Growing Tubers

To investigate gradients of metabolites, a cork borer was forced through the tuber, the tissue plug was immediately forced out and simultaneously sliced into discs that fell into successive containers filled with liquid nitrogen. In two tubers of ca. 20 g harvested from separate plants there was a decrease of ATP (Figure 2A), no clear change of ADP (Figure 2B), an increase of AMP (Figure 2C) and a decrease of the ATP/ADP ratio (Figure 2D) and the adenylate energy change (Figure 2E) in the centre compared to the peripheral zones. The total adenine nucleotide pool decreased slightly in the centre of the tubers (Figure 2F). Whereas the adenylate energy charge in the outer zones (0.75 – 0.85) was similar to that expected in aerobic tissues (see Raymond and Pradet, 1980), the energy charge in the centre (0.45 – 0.6) was much lower. This decrease occurred at internal oxygen concentrations (ca. 5%, see Figure 1B) two orders of magnitude above those needed to saturate cytochrome oxidase (ca. 0.04% oxygen, see Introduction).

In numerous experiments over a period of two years, we reproducibly found low ATP/ADP ratios (1.5 – 3) when discs from growing tubers were harvested immediately into liquid nitrogen, and much higher values (6 – 12) when the discs were allowed to equilibrate with air (data not shown). The tubers investigated in Figure 2 showed similar trends, even though there are differences in the exact magnitude from tuber to tuber. This may be due to differences in tuber size, position or (Geigenberger et al., 2000)
metabolic status. The internal oxygen gradient in one of the tubers was measured along a transect about 2 cm removed from that used to harvest discs for metabolite analysis (compare Figure 1B with the data set shown as solid squares in Figure 2). There was good agreement between the internal changes of oxygen and the changes in the adenylate energy status.

For comparison, a growing tuber was excavated and submerged for 24 h to severely restrict the external oxygen supply. During this period the tuber remained attached to the mother plant. Submergence led to a decrease of ATP, whereas ADP remained unaltered and AMP increased (Figure 2A–C). The ATP/ADP ratio (Figure 2D) and the adenylate energy charge (Figure 2E) were far lower and the total adenine nucleotide pool (Figure 2F) was slightly lower than in tubers in air. There was still a marked gradient, with a dramatic decrease of ATP and increase of AMP in the centre of the tuber, where the adenylate energy charge decreased to under 0.3. A similarly low adenylate status was found when tubers were pre-equilibrated in nitrogen gas (data not shown).

Levels of Glycolytic Metabolites and Organic Acids along a Transect across Growing Tubers

Sugars and glycolytic metabolites were investigated along the transects through the aerated tubers (Figure 3, solid symbols). Both tubers contained slightly elevated sucrose levels (Figure 3A) in the central zone. Fructose was 3-fold higher in the central zone of one tuber, but only slightly increased in the other (Figure 3B). The central region contained unaltered or slightly increased glucose-1-phosphate (Glc1P, data not shown), glucose-6-phosphate (Glc6P, Figure 3C) and fructose-6-phosphate (Fru6P, data not shown). Fructose-1,6-bisphosphate (Fru1,6P₂) (Figure 3D) and triose phosphates (Figure 3E) were below the limit of detection (ca. 1 nmol/gFW). The centre contained higher glyceraldehyde-3-P (3PGA, Figure 3F) and phosphoenolpyruvate (PEP, Figure 3G) than the periphery, with a large increase in one and a small increase in the other tuber. Increased 3PGA and PEP are typically seen when glycolysis is inhibited in plants (Dennis and Greyson, 1987; Hatzfeld and Stitt, 1991; Dennis et al., 1997). Pyruvate was unaltered or increased slightly in the centre (Figure 3H). Lactate (Figure 3I) remained very low in the centre of one tuber and rose slightly in the centre of the other. The lactate:pyruvate (Figure 3J) ratio remained very low. Although similar trends were found in both tubers, the extent of the changes varied, with one containing slightly more lactate and showing a much more marked increase of fructose, 3PGA and PEP in the centre. This may reflect a slightly lower internal oxygen concentration.

Submergence of tubers for 24 h led to a slight decrease of Glc1P (data not shown), Glc6P (Figure 3C) and Fru6P (data not shown), a dramatic increase of Fru1,6P₂ (Figure 3D) and triose phosphates (Figure 3E), no change or a slight decrease of 3PGA and PEP (Figure 3F - G), a decrease of pyruvate (Figure 3H), and a 30-fold increase of lactate (Figure 3I). The lactate:pyruvate ratio (Figure 3J) and the triose phosphate:3PGA ratio (compare Figures 3E and 3F) rose dramatically. Inspection of the transect across the submerged tuber reveals a marked increase of triose phosphate (Figure 3E), no unaltered or inconsistent changes of 3PGA and PEP (Figure 3F – G), and a marked decrease of pyruvate (Figure 3H) and dramatic increase of lactate (Figure 3I) in the central zone.

The low lactate content of aerated tubers was not due to absence of lactate dehydrogenase (LDH). Aerated and...
submerged tubers contained similar activities of LDH (Figure 4A) and alcohol dehydrogenase (ADH, Figure 4B). Phosphofructokinase (PFK) activity was identical in aerated and submerged tubers (Figure 4C). As the reactions catalysed by lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase are close to equilibrium in vivo, the low lactate:pyruvate ratio (Figure 3J) and very low triose phosphate:3PGA ratio (compare Figures 3E and 3F) in the centre of tubers in air shows that the NAD⁺ system is highly oxidised. Due to uncertainties about the subcellular location of metabolites in tubers, the precise cytosolic \((\text{NADH})/\text{(NAD⁺NADH)}) ratio could not be calculated.

**Influence of the Oxygen Concentration on Adenine Nucleotide Levels in Discs**

A complementary approach was taken to allow a more precise analysis of the effect of different oxygen concentrations on metabolism. Tissue discs (1 mm thick, 8 mm diameter) were prepared from small growing tubers (ca. 15 g FW) and incubated for 2 h with 20 mM sucrose in the presence of 0, 4%, 8%, 12%, 21% or 40% oxygen. For comparison, metabolites were investigated in discs that

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**Fig. 3** Metabolite Levels in Transects of Growing Potato Tubers.
In the same samples as in Figure 2, metabolite levels were analysed. (A) sucrose, (B) fructose, (C) Glc6P, (D) Fru-1,6-P₂, (E) Triose-P, (F) 3-PGA, (G) PEP, (H) pyruvate, (I) lactate, (J) lactate/pyruvate ratio. For details, see legend of Figure 2.

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**Fig. 4** Enzyme Activities in Transects of Growing Potato Tubers.
In samples taken in parallel to those in Figure 2, enzyme activities were analysed. (A) lactate dehydrogenase, (B) alcohol dehydrogenase, and (C) PFK. For details, see legend of Figure 2.
were quenched immediately after removing them from the centre of the tubers used for these experiments (shown on the right hand side of the following Figures).

Decreasing oxygen concentrations led to a progressive decrease of ATP (Figure 5A), an increase of ADP (Figure 5B) and AMP (Figure 5C), and a marked decrease of the ATP/ADP ratio (Figure 5D) and the adenylate energy charge (Figure 5E). Marked changes of the adenine nucleotide levels occurred when oxygen was decreased from 40% to 12%, and dramatic changes occurred when oxygen was decreased below 12%. The overall adenylate content declined (Figure 5F), even though the incubation lasted only 2 h. GTP, GDP and the overall guanine nucleotide content were 90% lower than the corresponding adenine nucleotide pool, and showed a similar response to oxygen (data not shown).

Adenine nucleotide levels respond in a similar manner to a low oxygen concentration in discs, and low internal oxygen in intact tubers. The absolute levels of ATP, ADP and AMP and the values of the ATP/ADP ratio and adenylate energy charge in tissue harvested immediately from the centre of tubers (right hand data point, Figure 5A – F) resemble those found in discs incubated with 4 – 8% oxygen (Figure 5A – F). Based on our previous measurements (see Figure 1), the oxygen concentration in the centre of these tubers would have been in this range. The value of the ATP/ADP ratio and the adenylate energy charge also resemble those found in the centre of the two tubers shown in Figure 2, although the absolute levels of adenylates varied by a factor of two. This may reflect differences between separate batches of tubers or plants.

**Influence of the Oxygen Concentration on Metabolites Involved in Sucrose Breakdown**

Sucrose breakdown in growing potato tubers occurs via sucrose synthase (SuSy), fructokinase (FK) and UDP-glucose pyrophosphorylase (UGPase). SuSy catalyses the reversible conversion of sucrose and UDP to UDP-glucose (UDP-Glc) and fructose, FK phosphorylates fructose to Fru6P, and UGPase catalyses the reversible conversion of pyrophosphate (PPi) and UDPGlc to Glc1P and UTP. Plants contain a significant pool of PPi, that, at least in leaves, is located in the cytosol (Weiner et al., 1987).

Sucrose was not measured because it was supplied in the medium, and repeated washing to remove extracellular sucrose would have introduced the risk of changing the oxygen concentration. Fructose (Figure 6A) increased by about 50% when the oxygen concentration was decreased to 8% and by 2- to 3-fold when oxygen was decreased to 4 and 1%, and decreased again by 50% in zero oxygen. For comparison, fructose increased slightly (Figure 5A, Figure 3B) or markedly (Figure 3B) in the centre of intact tubers.

The overall uridine nucleotide pool declined in parallel with the total adenine nucleotide pool (Figure 5K). UDP-glucose (UDP-Glc) accounted for most of the uridine nu-

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**Fig. 5** Levels of Adenine and Uridine Nucleotides in Tuber Slices Incubated at Different Oxygen Tensions. Freshly cut slices of growing potato tubers were either immediately quenched in liquid nitrogen ($t_0$), or incubated in buffer including 20 mM sucrose under continuous aeration using premixed gases containing 0, 4, 8, 12, 21, or 40% oxygen. After 2 h incubation, slices were harvested by immediately quenching in liquid nitrogen, and then extracted to measure nucleotide levels (nmol gFW$^{-1}$). (A) ATP, (B) ADP, (C) AMP, (D) ATP/ADP ratio, (E) adenylate energy charge, (F) total adenylates, (G) UTP, (H) UDP, (I) UTP/UDP ratio, (J) UDP-Glc, and (K) total uridylnylates. (L) ADPGlc. Data are mean ± SE ($n = 3$).
discs even when they are incubated in air (see also Loef et al., 1999), and UTP and UDP levels are sensitive to the sucrose concentration supplied to the discs. When discs were supplied with zero, 25 and 100 mM sucrose for 20 min at 21% oxygen, the UDP level decreased from 3.25 ± 0.024 to 3.08 ± 0.5 and 2.37 ± 0.01 nmol/g FW, UTP increased from 29.7 ± 0.2 to 29.9 ± 1.7 and 31.3 ± 0.3 nmol/g FW, and the UTP/UDP ratio increased from 8.6 ± 0.7 to 11.0 ± 1.7 and 13.3 ± 0.1, respectively (mean ± SE, data not shown).

PPi was very low in discs incubated in 40% oxygen, increased markedly in 21% oxygen, rose further to a maximum at 4 – 8% oxygen, and fell to low levels in zero oxygen (Figure 6B). The PPi level in discs incubated with 4 – 8% oxygen resembled those in the centre of intact tubers (Figure 6B). PPi levels in plant tissues usually do not change greatly (Stitt, 1998). It is striking that PPi remains high or

cleotide pool (see also Loef et al., 1999). UDPGlc declined slightly as the oxygen concentration decreased (Figure 5J). UTP decreased progressively as the oxygen concentration decreased (Figure 5G). UDP rose slightly as oxygen was decreased from 40% to 21%, remained unaltered as oxygen was decreased from 12% to 4%, and rose markedly in zero oxygen (Figure 5H). The UTP/UDP ratio fell progressively as the oxygen concentration was decreased (Figure 5I). Uridine nucleotide levels in discs incubated with 4 – 8% oxygen differed from those found in the centre of intact tubers (Figure 5G – K). The overall pool was smaller, UDPGlc was lower, UDP was higher and the UTP/UDP ratio was lower. This may be due to secondary changes triggered by disc preparation, and because the sucrose supplied to the discs does not fully replace the inflow of sucrose from the phloem in intact tubers. There is a rapid decrease of the overall uridine nucleotide pool in discs even when they are incubated in air (see also Loef et al., 1999), and UTP and UDP levels are sensitive to the sucrose concentration supplied to the discs. When discs were supplied with zero, 25 and 100 mM sucrose for 20 min at 21% oxygen, the UDP level decreased from 3.25 ± 0.024 to 3.08 ± 0.5 and 2.37 ± 0.01 nmol/g FW, UTP increased from 29.7 ± 0.2 to 29.9 ± 1.7 and 31.3 ± 0.3 nmol/g FW, and the UTP/UDP ratio increased from 8.6 ± 0.7 to 11.0 ± 1.7 and 13.3 ± 0.1, respectively (mean ± SE, data not shown).

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even increases slightly at low oxygen concentrations even though the energy status of the adenine, guanine and uridine nucleotides (see Figure 5) decreases. Independent changes of PPi and adenine nucleotides have also been seen in response to uncouplers and anoxia in pea roots and Arum maculatum spadices (Dancer and ap Rees, 1989), where PPi remained high even under anoxia.

**Influence of the Oxygen Concentration on the Levels of Glycolytic Metabolites and Organic Acids in Discs**

Glc1P (Figure 6C), Glc6P (Figure 6D) and Fru6P (Figure 6E) showed a slight decrease at 8% and 4% oxygen, and rose again slightly in 1% and zero oxygen. Fru1,6P2 (Figure 6F) and triose phosphates (Figure 6G) remained low until 1% oxygen, and rose 2-fold in zero oxygen. 3PGA (Figure 6H) and PEP (Figure 6I) increased progressively as oxygen was decreased, and decreased in zero oxygen. The triose phosphate:3PGA ratio was very low, except in zero oxygen (compare Figures 6G and 6H). Pyruvate decreased slightly between 21% and 8% oxygen, increased at 4% and 1% oxygen, and decreased in zero oxygen (Figure 6J). Lactate was low except in zero oxygen, where it rose dramatically (Figure 6I). The lactate:pyruvate ratio (Figure 6J) was very low in high oxygen, rose very slightly as oxygen was decreased below 12%, rose slightly in 1% oxygen, and increased dramatically in zero oxygen.

Potato tubers contain large pools of malate (Figure 6M) and citrate (Figure 6P), and much smaller pools of isocitrate (Figure 6N) and 2-oxoglutarate (Figure 6O). Most of the citrate and malate are in the vacuole (Martinoia and Rentsch, 1994). Decreasing concentrations of oxygen led to a progressive decrease of malate, whereas citrate remained unaltered. Isocitrate and especially 2-oxoglutarate increased slightly in 8% oxygen, and decreased in lower oxygen and zero oxygen. Acetyl-CoA decreased slightly as oxygen was reduced from 40% to 12%, decreased 50% between 12% and 4% oxygen, and remained low in 1% and zero oxygen (Figure 6Q). This was accompanied by a slight increase of CoA-SH (Figure 6R). As a result, there was a 75% decrease of the acetyl-CoA/CoA-SH ratio as oxygen decreased (compare Figures 6Q and 6R). The total CoA pool (the sum of acetyl-CoA plus CoA-SH) decreased by 40% at low oxygen (values calculated from Figures 6Q and R). The transient increase of isocitrate and 2-oxoglutarate in 8% oxygen (Figures 6N – O) occurred when acetyl-CoA was still relatively high and pyruvate (Figure 6J) was relatively low. When oxygen was decreased further there was a larger decrease of acetyl-CoA, 2-oxoglutarate and isocitrate decreased, and pyruvate rose (compare Figures 6N – O with Figure 6J).

Most glycolytic metabolites and organic acids respond in a similar manner to low oxygen in discs (Figure 6) and to depletions of oxygen in the centre of intact tubers (Figures 3, 6). In particular, 3PGA and PEP increase, lactate remains low, and the lactate:pyruvate and triose phosphate:3PGA ratios remain very low in both experimental systems. This contrasts with the response of discs to zero oxygen (Figure 6) and the changes in the centre of submerged tubers (see Figure 3), when 3PGA and PEP remain unaltered or fall, lactate increases, and the lactate:pyruvate and triose phosphate:3PGA ratios rise dramatically. Although a precise comparison is complicated by differences between tubers and by the dynamic changes in discs in the range between 12 and 4% oxygen, most metabolites including the hexose phosphates, 3PGA, pyruvate, lactate, malate and isocitrate were present at similar concentrations in discs incubated at 4 – 8% oxygen (Figure 6) and in the midst of intact aerated tubers (Figures 3 and 6). The major exceptions were Fru1,6P2 and triose phosphates which were 2- and 3-fold higher, and acetyl-CoA and 2-oxoglutarate which were slightly lower in discs than in the centre of tubers. Elevated levels of Fru1,6P2 and triose phosphates are typically seen in discs (data not shown), possibly due to a wounding-related stimulation of respiration.

**Influence of the Oxygen Concentration on the Level of ADPGlc in Discs**

The first committed reaction in starch biosynthesis is the conversion of ATP and Glc1P to ADPGlc and PPI, which is catalysed by ADP glucose pyrophosphorylase (AGPase). ADPGlc decreased slightly at 12% oxygen, fell further at 4 – 8% oxygen, and decreased markedly at zero oxygen (Figure 5L).

**Influence of the Oxygen Concentration on the Metabolism of [U-14C]Sucrose by Discs**

To investigate the effect of the oxygen concentration on fluxes, discs were incubated with 20 mM [U-14C] sucrose for 2 h at 40%, 21%, 12%, 8%, 4%, 1% or zero oxygen (Figure 7). Sucrose uptake was partially inhibited when oxygen was decreased from 12 to 8% (Figure 7A), but did not decrease further at 4%, 1% or zero oxygen. High oxygen (40%) also inhibited sucrose uptake. Figure 7B shows the proportion of the absorbed [U-14C] sucrose that was metabolised to other compounds. Only a small proportion of the absorbed sucrose is metabolised during the 2 h incubation (Figure 7B, see also Geigenberger et al., 1997; Geiger et al., 1998). The proportion that was metabolised showed a slight decrease as oxygen was decreased from 21% down to 4%, and decreased markedly at 1% and zero oxygen.

Figures 7C – F depict the fate of the metabolised sucrose. The label recovered in each fraction is shown as a percentage of the metabolised [U-14C] sucrose. Part of the label is retained as phosphorylated intermediates (Figure 7D). This pool is dominated by the hexose phosphates (see Figure 5). As seen previously (Geigenberger and Stitt, 1993; Geigenberger et al., 1997; Geiger et al., 1998), starch (Figure 7C) represents the single largest product. A considerable proportion of the metabolised label is also converted to organic acids (Figure 7E) and amino acids (Figure 7F), representing the flux through glycolysis. Very
the proportion of the label that was retained in phosphorylated intermediates (Figure 7D). The proportion recovered in organic acids (Figure 7E) decreased slightly as oxygen was decreased down to 1%, and rose dramatically in zero oxygen. The marked increase of label in organic acids in discs at zero oxygen coincided with the accumulation of lactate (Figure 5K). The proportion of the metabolised label recovered in amino acids (Figure 7F) decreased continuously as the oxygen concentration was decreased.

Absorbed [U-14C]sucrose will mix with internal unlabelled pools, so movement of the label will not necessarily reflect fluxes into the various pools (Geigenberger and Stitt, 1993; Geigenberger et al., 1997). Interpretation of labelling experiments is especially complicated when little [14C]CO2 was released during the 2 h incubation (data not shown) because most of the label is initially trapped in the large pools of organic acids. Less than 2% of the label is converted to protein or cell wall components (data not shown, see Geigenberger et al., 1997).

Label allocation to starch increased slightly as the oxygen concentration was decreased from 40% to 12%, and decreased as the oxygen concentration was decreased to 8, 4 and 1% (Figure 7C). Labelling of starch was nevertheless high, even in 1% oxygen. Zero oxygen led to a strong inhibition of label incorporation into starch (Figure 7C). The residual level of label incorporation into starch in zero oxygen (<10%) is similar to that seen in stored tubers (see e.g. Hajirezaei et al., 1994). Low oxygen slightly increased the proportion of the label that was retained in phosphorylated intermediates (Figure 7D). The proportion recovered in organic acids (Figure 7E) decreased slightly as oxygen was decreased down to 1%, and rose dramatically in zero oxygen. The marked increase of label in organic acids in discs at zero oxygen coincided with the accumulation of lactate (Figure 5K). The proportion of the metabolised label recovered in amino acids (Figure 7F) decreased continuously as the oxygen concentration was decreased.

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Metabolic Adaptation to Low O2 in Potato Tubers 731

**Fig. 7** Metabolism of [14C]Sucrose in Tuber Slices Incubated at Different Oxygen Tensions. Freshly cut slices of growing potato tubers were incubated in a medium containing 20 mM sucrose under continuous aeration using pre-mixed gases containing 0, 1, 4, 8, 12, 21, or 40% oxygen as in Figure 6. After 20 min, [U-14C]sucrose was added and the incubation was continued for 2 h before slices were washed and extracted to determine label distribution. (A) [%14C]sucrose absorbed by the tissue, (B) percentage of the absorbed label that is metabolised to other compounds. Incorporation of [14C] into (C) starch, (D) phosphate ester, (E) organic acids and (F) amino acids is expressed as a percentage of the label metabolised. The specific activity of the hexose phosphate pool (G) was estimated by dividing the label retained in the phosphate ester pool by the summed carbon of the hexose phosphates (see Figure 6C – E). The values were divided by 2 to give the mean specific activity during the course of the 2 h. The specific activity of the hexose phosphate pool and label incorporation into the relevant fractions was used to calculate (H) the absolute rate of starch synthesis and (I) glycolytic flux (the sum of the flux to the organic acids and amino acids). Panel (J) shows the relative rates of starch synthesis and glycolysis and (K) the summed fluxes to starch and glycolysis. The results are given as mean ± SE (n = 4).
treatments are compared that modify label uptake or the turnover of internal pools, as is the case in the experiment of Figure 7. The label in the phosphorylated intermediate fraction (see Figure 7E) was divided by the total carbon found in Glc1P, Glc6P and Fru6P (Figures 6C – E) to calculate the specific activity of the hexose phosphate pool (Figure 7G) (see Geigenberger et al., 1997, for a discussion of the assumptions involved in these calculations). To estimate the absolute rate of starch synthesis (Figure 7H), the label in starch was divided by the specific activity of the phosphorylated intermediate pool. Starch synthesis increases slightly as the oxygen concentration is decreased from 40% to 12%, decreases slightly as the oxygen concentration decreases from 12% down to 8%, 4% and 1%, and falls sharply in zero oxygen. To estimate glycolytic flux (Figure 7I), label in organic acids and amino acids was summed and divided by the specific activity of the phosphorylated intermediate pool. The estimated rate of glycolysis fell almost linearly as the oxygen concentration was decreased from 40% to 1%, and then increased 2-fold in zero oxygen. In addition to lactate, some ethanol is produced during fermentation in potato tubers. It was not possible to accurately measure ethanol formation in our experimental system because it was volatilised by the gas passed through the solution. Our results may therefore underestimate the stimulation of glycolysis during fermentation. The ratio between the rate of starch synthesis and the rate of glycolysis is shown in Figure 7I. Starch synthesis rose relative to glycolysis as the oxygen concentration was decreased from 40% to 12%, remained high as the oxygen concentration was decreased from 12% down to 1%, and fell dramatically in zero oxygen.

The estimated fluxes to starch and through glycolysis are summed in Figure 7K. As these are the two major fluxes in potato tubers, their sum provides an estimate of the net rate of sucrose breakdown. The combined flux is inhibited when oxygen is decreased below 12%. This shows that the inhibition of [U-14C] sucrose breakdown observed when oxygen falls below 12% (see Figures 7A – B) is not an artefact due to increased equilibration of the incoming labelled sucrose with unlabelled endogenous sucrose pool. Indeed, comparison of Figure 7G with Figures 7A – B reveals that the specific activity of the phosphorylated intermediate pool decreases less than the rate of [U-14C] sucrose metabolism, showing that low oxygen also restricts mobilisation of endogenous carbohydrates. Zero oxygen did not lead to a further inhibition of sucrose breakdown (Figure 7K). Carbon is available for the 2-fold stimulation of glycolysis in zero oxygen (Figure 7I) because starch synthesis (Figure 7H) is strongly inhibited.

**Influence of the Oxygen Concentration on the Rate of Respiration of Tuber Discs**

To measure respiration, discs (1 mm thick, 8 mm diameter) were prepared from growing tubers, placed in an oxygen electrode at pre-adjusted oxygen concentrations, and oxygen uptake was measured during the next 20 min (Figure 8). Compared to 21% oxygen, respiration was inhibited by 22 and 53% when the oxygen concentration was decreased to 8 and 4% oxygen, respectively. In a separate experiment, 20 mM sucrose was included in the suspending medium. Decreasing the oxygen concentration from 21% to 8% resulted in a 40% inhibition of respiration (data not shown). The inhibition of respiration in low oxygen is of a similar order to the inhibition of glycolytic flux estimated from the labelling experiment (Figure 7H). This inhibition occurs within minutes. When oxygen uptake was monitored over a longer period in the oxygen electrode, respiration decreased as oxygen was depleted and the inhibition was rapidly reversed when oxygen was re-introduced (data not shown).

**Influence of the Oxygen Concentration on the Wounding Response**

Slicing of tubers leads to a wound response in which respiration is stimulated, the activities of glycolytic enzymes increase, and secondary metabolism is induced (Kahl, 1974; Burton, 1989). Anaerobiosis inhibits these wounding-induced changes in gene expression (Butler et al., 1990), including the induction of PAL (Rumeau et al., 1990). We investigated whether the wounding response is attenuated when discs are incubated at oxygen concentrations similar to those found inside growing tubers (Figure 9).

When control discs were incubated for 8 and 24 h at 21% oxygen, there was a marked increase of respiration (Figure 9A), invertase activity (Figure 9B) and phenylalanine lyase (PAL) activity (Figure 9C). As expected (see above), these changes were completely suppressed in zero oxygen. They were also attenuated at 4% and 8% oxygen. Incubation at low oxygen attenuated the rise of respiration during the first 8 h, and led to a decline of respiration between 8 and 24 h. When tuber discs that had been incubated for 24 h at 4% or 8% oxygen were transferred...
sequences of internal oxygen gradients in plants. The oxygen concentration is significantly decreased in the outer zones of growing tubers, and there is a further decline between the outer and inner zones where the oxygen concentration drops below 5%. These low oxygen concentrations are accompanied by a decrease of the adenylate energy status and changes of metabolites, that are indicative of a partial inhibition of respiration. The magnitude of the changes varied between tubers, presumably depending on their size and metabolic activity. To allow more precise analysis of the effect of decreased oxygen on metabolism, freshly cut tuber slices were incubated for a short time at a range of oxygen concentrations. This model system provided three lines of evidence that low oxygen restricts respiration. First, respiration was inhibited when the oxygen concentration was reduced from 21% to 4–8%. Second, labelling experiments revealed a progressive inhibition of glycolysis across a wide range from 40% down to 1% oxygen. Third, decreasing oxygen led to a progressive and marked decrease of the ATP/ADP ratio and the adenylate energy charge.

The inhibition of respiration in low oxygen is not due to direct oxygen limitation of cytochrome oxidase. The inhibition of respiration and the decrease of the adenylate status is already evident in discs incubated with 8% oxygen, which is almost three orders of magnitude above the $K_m$ (oxygen) of cytochrome oxidase. Further, it occurs in the absence of lactate accumulation, and in the presence of low lactate:pyruvate and triose-P:3PGA ratios which are indicative of a low (NADH)/(NAD + NADH) ratio. This rules out the possibility that oxygen falls to very low levels inside the tissue discs. Our results provide indirect evidence that respiration is also inhibited by falling oxygen concentrations inside intact tubers, where there is a similar decrease of the adenylate energy status even though the oxygen concentration is still in the range of 5%, and low lactate levels and low lactate:pyruvate and triose-P:3-PGA ratios show that the tissue has not become anaerobic. Earlier studies, which found that low external oxygen reduces the rate of respiration of intact potato tubers but assumed this occurred because zones inside the tuber were becoming anoxic (Stiles, 1960; Burton, 1989), may require reinterpretation in the light of our results.

The responses to low oxygen and anoxia are actually diametrically opposed. Low oxygen leads a progressive inhibition of glycolysis and an increase of the terminal glycolytic intermediates (3PGA, PEP) as is typically seen when glycolysis is inhibited in plants (Dennis and Greyson, 1987; Hatzfeld and Stitt, 1991; Dennis et al., 1997). In contrast, zero oxygen leads to a dramatic increase of the lactate:pyruvate and triose-phosphate:3-PGA ratios, a further decrease of ATP, a stimulation of glycolysis, a decrease of 3PGA and PEP, and accumulation of lactate. Glycolysis is presumably stimulated in zero oxygen because ATP formation is less efficient during fermentation. There is an interesting parallel to the differing effect of low oxygen and anoxia on the levels of the transcripts for two glycolytic enzymes (enolase, aldolase), LDH and ADH in maize roots.
(Andrews et al., 1994). Whereas anoxia led to an increase of all four transcripts, low oxygen led to a marked increase of the transcripts for LDH and ADH as a pre-adaptation to anoxia (see also Chang et al., 2000), whereas transcript for enolase increased only slightly and transcript for aldolase decreased.

Low oxygen also inhibits a range of metabolic processes that consume ATP in potato tuber discs. When oxygen was decreased below 12% there was a partial inhibition of sucrose uptake (Figure 7A). Amino acid synthesis was progressively inhibited as oxygen was decreased below 21% (Figure 7F). The overall adenine, uridine and guanine nucleotide pools, and the CoA pool decreased within 2 h (Figures 5 - 6), revealing that synthesis or turnover of these cofactors responds rapidly to low oxygen. A similar decrease of CoA was observed during anaerobic incubation of bacteria cells (Chohnan et al., 1997). Biosynthesis of nucleotides and CoA requires energy (Brown, 1959; Stryer, 1990), and decreased levels of these cofactors may also represent a mechanism to decrease fluxes through the biosynthetic pathways in which they participate (Loef et al., 1999, 2000). Further experiments are needed to investigate the impact of low oxygen on cell wall synthesis, RNA synthesis and the overall rate of protein synthesis.

Chang et al. (2000) recently showed that hypoxia leads to an approximately 50% inhibition of protein synthesis in maize roots, due to inhibition of translation of a wide range of proteins. We investigated the effect of low oxygen on the wounding induced stimulation of respiration and activation of phenylpropanoid metabolism (Kahl et al., 1974; Burton et al., 1989), an example for a cellular response that requires a large increase of metabolic activity. Low oxygen decreased the wounding-induced increase of respiration and invertase activity by 50%, and strongly inhibited the wounding-induced increase of PAL activity.

Mechanisms Contributing to the Inhibition of Respiration and Metabolism at Low Oxygen

Our results indicate that low oxygen concentrations are sensed in plants, leading to an inhibition of respiration and a lowering of the adenylate energy status. Although accumulation of carbon dioxide, ethylene or other gases might play a role in triggering morphological adaptation to low oxygen (Drew et al., 1994, 1997), this cannot be responsible for the changes of metabolism in tuber discs because the oxygen concentration was decreased without decreasing the rate at which other gases exit the tissue. Studies with protoplasts and cell suspension cultures (Paul and Ferl, 1991; Bailey-Serres and Dawe, 1996) indicate that LDH expression is regulated in response to low oxygen, because low oxygen cannot be mimicked by respiratory inhibitors. LDH and ADH activity were already high in tubers in air, indicating that they are induced at the oxygen concentrations found inside growing tubers. Further studies are needed to characterise the oxygen sensing mechanism in plants. E. coli senses oxygen via a two component sensor-response system (Guest, 1992), heme-containing proteins are implicated in the oxygen-regulation of gene expression in Rhizobium melliloti (Monsen et al., 1992; Lois et al., 1993) and yeast (Zlotomer and Lowry, 1992; Sabova et al., 1993) and in the depression of metabolic activity in reptiles (Hand and Hochachka, 1995), and oxygen-sensitive ion channels have been implicated in oxygen sensing in mammals (Lopez-Bameo et al., 1994).

Some of the changes in low oxygen will be secondary responses that are triggered by the decreased adenylate energy status or other consequences of a decreased rate of respiration. For example, changes in adenylate status explain the inhibition of sucrose breakdown. SuSy and UGPase catalyse reversible reactions that are close to equilibrium in vivo (Geigenberger and Stitt, 1993; Geigenberger et al., 1994), so sucrose breakdown will depend strongly on the removal of fructose by FK. ADP inhibits potato tuber FK, acting competitively to ATP (Renz et al., 1993). The physiological significance of these properties was previously unclear, because inhibition is negligible until the ATP/ADP ratio falls to low values. It is now evident that they facilitate inhibition of FK in hypoxic conditions. The observed increase of fructose in discs incubated with less than 12% oxygen provides strong evidence that FK is inhibited in vivo at low oxygen concentrations. Fructose is, in turn, a potent feedback inhibitor of SuSy (Doehlert 1987, Dancer et al., 1990). Comparison of the $K_{\text{fructose}}$ (1 - 5 mM) with the $K_{m}(\text{sucrose})$ (50 - 100 mM; Avigad, 1982) of SuSy and the overall level of sucrose in tubers (ca. 40 mM, Figure 2) indicates that the increase of fructose from 0.8 mM in high oxygen to over 2 mM under hypoxic conditions will lead to an approx. 50% inhibition of SuSy activity. This is in good agreement with the inhibition measured in the labelling experiment of Figure 7. The changes of sucrose and fructose (Figures 3A - B and 6A) in the centre of tubers indicate that this mechanism is switched on with varying intensity in intact growing tubers.

Low oxygen led to a series of small changes in many intermediates of glycolysis and the Krebs cycle, revealing that these pathways are being coordinately regulated at multiple sites and indicating that the primary site is likely to be in the mitochondrial electron chain. UDP Glucose and hexose phosphates showed only slight changes, showing that the rate of carbon consumption for glycolysis and starch synthesis is regulated in parallel with the rate of sucrose breakdown. There was a 2-fold increase of 3PGA and PEP, showing that pyruvate kinase and/or PEP carboxylase activity has been decreased. The maintenance of high pyruvate and the decrease of the acetyl-CoA:CoA ratio pinpoint pyruvate dehydrogenase as a further important site for regulation. The precise reasons for the inhibition of pyruvate kinase, PEP carboxylase and pyruvate dehydrogenase are not revealed by our results. Nevertheless, organic acids that participate in the tricarboxylic acid cycle including 2-oxoglutarate and isocitrate remain high or even increase slightly, especially in 8% oxygen when 3PGA and PEP have only increased slightly and acetyl-CoA has decreased only slightly. This may be-
because glycolysis is inhibited less than respiration, or because consumption of organic acids for biosynthetic processes including amino acid synthesis is decreased (see Figure 6F), or because malate is re-mobilised (see Figure 6M) to compensate for the short fall of carbon from glycolysis. Increased malate mobilisation via NAD-malic enzyme has been reported in response to extreme hypoxia (Edwards et al., 1996). The slight differences in the responses of different metabolite pools as the oxygen concentration is decreased indicates that the inhibition of the various enzymes in carbon metabolism is not totally synchronous. This may contribute to the variable responses of individual metabolites in intact tubers.

Starch synthesis is well adapted to the hypoxic conditions inside growing potato tubers. The highest rates of starch synthesis, both in absolute terms and relative to other fluxes, were found at 12% oxygen. Although a further decrease of the oxygen concentration down to 1% slightly inhibited the absolute rate, starch synthesis remained high relative to other fluxes. The changes in the rate of starch synthesis are in agreement with the changes of the ADPGlc level. Although ADPGlc decreases slightly under hypoxic conditions, the in vivo level remains above 1.5 nmol/gFW, which is sufficient to support near-maximal rates of starch synthesis in vivo (Geigenberger et al., 1997). Two mutually compensating changes explain why starch synthesis operates effectively down to very low oxygen concentrations. Although low ATP will restrict AGPase activity (see Loef et al., 2000), this is compensated because low oxygen leads to a restriction of glycolysis and an increase of 3-PGA, which is a potent allosteric activator of AGPase (Preiss, 1988). Starch synthesis is not severely inhibited until tuber tissue becomes anoxic, when ATP decreases even further and there is also a decrease of 3-PGA because glycolysis is stimulated to support fermentation. Our results differ from those obtained by Hill and ap Rees (1995) with ripening banana fruit; however, they did not monitor the internal oxygen concentrations, and there may be differences between tissues which are accumulating starch and tissues which are ripening and re-mobilising starch.

**General Implications for the Regulation of Plant Metabolism**

Many important plant tissues become hypoxic in the presence of a high external oxygen concentration (see Introduction). The finding that growing potato tubers respond by decreasing their rate of respiration and adenylate status has general implications for our understanding of plant metabolism.

First, it provides a rationale for the rather unusual way in which glycolysis is regulated in plants. PFK is subject to exquisite regulation by the ATP/AMP ratio in most microbes and animals (Stryer, 1990), allowing sensitive activation of glycolysis and respiration in response to falling ATP. In plants, however, cytosolic and plastid PFK are only weakly regulated by adenylates (Dennis and Greyson, 1987), and our results demonstrate in vivo that a declining adenylate energy status does not override the partial inhibition of glycolysis in low oxygen. When the oxygen supply is restricted, activation of PFK by falling ATP and rising ADP and AMP would be counterproductive because the resulting stimulation of glycolysis would either increase oxygen consumption or lead to accumulation of NADH and require fermentation. Low sensitivity of PFK and other glycolytic enzymes to regulation by adenylates may be an important component of the regulatory networks that allow respiration to be decreased in response to low oxygen.

Second, plants will require regulatory systems that inhibit energy-consuming processes (see above for examples) when oxygen is low. The plant SNF1 homolog resembles the mammalian counterpart in being activated by AMP (Sudgen et al., 2000). SNF1-related kinases have been shown in vitro to phosphorylate and inactivate key enzymes in the pathways of sucrose synthesis, nitrate assimilation and isoprenoid synthesis in plants (Sugden et al., 1999). Based on the role of the AMP kinase in animals (Hardie et al., 1998), it will be important to investigate whether plant SNF1 kinase homologs facilitate a global regulation of biosynthetic processes to allow ATP consumption to be reduced when respiration falls and AMP rises.

Thirdly, there will be a premium on biochemical adaptations that conserve energy and allow oxygen consumption to be decreased. This may explain the unusual use of PPi, as an alternative energy donor in plants (Stitt, 1998). PPi is a side product of numerous biosynthetic reactions and, in most organisms, is hydrolysed to Pi (Stryer, 1990). The cytosol of plant cells contains significant levels of PPi, which is utilised as an energy donor for sucrose mobilisation via SuSy and UGPase (see above), for glycolysis via pyrophosphate:fructose-6-phosphate phosphotransferase (PFPP), and for tonoplast energisation via a PPi-dependent proton pump (Stitt, 1998). Each of these PPi-dependent reactions duplicates an ATP-consuming reaction (Stitt, 1998). Substitution of part of the ATP consumption by PPi will recycle ‘waste’ energy to support important central metabolic and cellular functions, and allow ATP and oxygen consumption to be decreased. PPi is maintained at high levels in hypoxic tissues (Figure 6B), in contrast with the progressive decrease of the energy status of the adenine, uridine and guanine nucleotide systems (Figure 5). The stimulation of substrate cycles in sucrose metabolism and glycolysis in hypoxic banana fruit (Hill and ap Rees, 1995) is also consistent with our proposal. The cycle between sucrose and hexose phosphates is mainly due to label equilibration via the PPi-dependent sequence catalysed by the reversible SuSy and UGPase reactions (Geigenberger and Stitt, 1993), and the substrate cycle between hexose phosphates and triose phosphates is due to the reversible PPi-dependent reaction catalysed by PFPP (Hajirazaei et al., 1994).

Independent evidence that PPi may play a role in conserving oxygen was recently provided by Zeng et al. (1999), who showed that hypoxia and anoxia repress invertase and induce a specific SuSy gene in maize roots.
Whereas breakdown of a molecule of sucrose via invertase requires 2 molecules of ATP, breakdown via SuSy and UGPase requires only one molecule of PPi (Stitt, 1998). The development- and cell-specific expression patterns of these two enzymes also takes on a new significance when they are related to oxygen delivery. In seeds and tubers, invertase is typically expressed during the early stages and SuSy during the later stages of development (Appeldoorn et al., 1997; Weber et al., 1997). This switch correlates with a transition from cell division to cell expansion and storage, and to date has been interpreted in terms of the regulation of seed development by sugar-related signals. It will be interesting to learn whether the switch from invertase to SuSy is also related to problems that develop with respect to oxygen delivery as seeds (Ching et al., 1974; Quebedeaux, 1981; Macnicol and Jacobson, 1992; Shelp et al., 1995; Porterfield et al., 1999) and tubers (see Figure 1) grow. Intriguingly, ectopic overexpression of invertase in tubers failed to improve starch synthase, which is taken as an indicator for rapidly growing tubers (Merlo et al., 1993) were used for the experiments.

Analysis of Oxygen Tensions in Potato Tubers

Intact tubers growing near the surface of the soil (where the oxygen concentration of the soil was above 18%, data not shown) were excavated. The internal oxygen tension was measured 1 - 2 min later by introducing an O2 microelectrode (diameter of the tip <1 mm; Toepfer Lab Systems, Germany) into the tuber tissue. When gradients through the tuber were investigated, the electrode was pushed gradually through the tuber, pausing for ca. 3 - 4 minutes at each position to obtain a measurement.

Sampling of Tissues from Intact Tubers

To investigate adenine nucleotide levels inside the tuber, a tuber growing at the surface of the pot and that was still attached via the stolon to the plant was excavated and exposed to air for ca. 2 min. A cork borer (diameter 8 mm) was forced through the middle, removed, and the tissue plug rapidly forced out and simultaneously sliced into ca. 1 mm thick discs which fell directly into liquid nitrogen. For each sample, three subsequent discs were pooled and stored in liquid nitrogen until extraction.

Labelling Experiments with Tuber Slices

Tuber discs (diameter 8 mm, thickness 1 mm) were cut directly from a core removed in a cork borer from the centre of growing tubers (ca. 15 g FW) attached to the fully photosynthesising mother plant, washed quickly with 10 mM 2-(N-morpholino) ethanesulfonic acid (MES) (pH 6.5; KOH), pre-incubated for 20 min in buffer containing 20 mM sucrose using 50 ml Falcon tubes in a water bath at 25 °C (ca. 8 discs in 20 ml), and U-14C]sucrose (final specific activity 1.4 KBq/μmol) (Amersham-Buchler, Freiburg, Germany) was added and incubation continued for another 2 h. During the whole incubation and pre-incubation time, discs were aerated by a continuous stream of pre-mixed gases containing 0, 1, 4, 8, 12, 21 or 40% oxygen. The oxygen concentration in the solution was routinely checked using an oxygen electrode. After 2 h, discs were immediately washed 3 times with buffer to remove external radioactivity, and frozen in liquid nitrogen to analyse label distribution.

Tissue slices were prepared and incubated in a similar manner to measure metabolite levels in dependence on oxygen, except that no radioactivity was added and a subset of discs from the centre of the tubers were allowed to fall directly after cutting into liquid nitrogen. Tissue slices (30 discs in ca. 80 ml medium) were incubated using glass vessels allowing continuous aeration with premixed gases (see above). The oxygen concentration was checked using an oxygen electrode. Slices were harvested by pouring the medium immediately through a strainer and throwing the slices into liquid nitrogen in 1 second. The slices were stored in liquid nitrogen until extraction.
Fractionation of 14C-Labelled Tissue Extracts

Discs were extracted with 80% (v/v) ethanol at 80°C (1 ml per 2 discs), re-extracted in two subsequent steps with 50% (v/v) ethanol (1 ml per 2 discs for each step), the combined supernatants dried under an air stream at 40°C, taken up in 1 ml H2O (‘soluble fraction’), and separated into neutral, anionic, and basic fractions by ion-exchange chromatography; the neutral fraction (3.5 ml) was freeze dried, taken up in 100 μl water, and further analysed by thin-layer chromatography (Geigenberger et al., 1997). To measure phosphate esters, esters (150 μl) of the soluble fraction were incubated in 50 μl buffer (10 mM Mes-KOH, pH 6.0) with or without 1 unit potato acid phosphatase (Grade II, Boehringer Mannheim) for 3 h at 37°C, boiled for 2 min, and analysed by ion-exchange chromatography (Geigenberger et al., 1997). The insoluble material left after ethanol extraction was homogenised, taken up in 1 ml water and counted for starch. In discs from growing tubers, starch accounts to over 90% of the label in the insoluble fraction (Geigenberger et al., 1994).

Metabolite and Nucleotide Analysis

Tissue slices were extracted with trichloroacetic acid, and metabolites and nucleotides measured as given in Geigenberger et al. (1998). The recovery of small, representative amounts of each metabolite through the extraction, storage, and assay procedures has been documented previously (see et al. et al., 1992; Merlo et al., 1994; Hajeirezaei et al., 1994; Geigenberger et al., 1994; Faire et al., 2000). Acetyl-CoA and CoA-SH were measured according to Bergmeyer (1987), using the same extracts.

Enzyme Analysis

Acid invertase and PFK activities were analysed in tuber extracts as in Geigenberger et al. (1998). PAL activity was analysed according to Lamb et al. (1979), lactate dehydrogenase and alcohol dehydrogenase according to Bergmeyer (1987).

Respiration Measurements

Two freshly prepared potato tuber slices (1 mm thick, 8 mm diameter) were transferred into the temperature controlled measuring chamber of an oxygen electrode (Hansatech, Kings Lynn, Norfolk, UK) containing 1 ml buffer solution (10 mM MES-KOH, pH 6.5 and different sugars) preequilibrated to 4, 8 and 21% oxygen by using premixed gases, to measure O2 consumption of the discs. Each measurement took ca. 5 min, during which time oxygen depletion in the buffer solution was less than 20% of the initial value.

Incubation for Inducing the Wounding Response

Discs from the middle of intact tubers (1 mm thick, 8 mm diameter) were sliced into 80 ml incubation medium (10 mm MES-KOH, pH 6.5). The whole procedure took place inside a plastic bag, and both the atmosphere inside the bag and the incubation medium was equilibrated to 21, 8, 4 or zero oxygen by using premixed gases passing through the bag and the medium. The temperature was 20°C. Modified glass vials were used allowing complete aeration of the medium via a continuous air stream. After ca. 20 min (t0), one set of discs was harvested and frozen immediately in liquid nitrogen to analyse enzyme activities, whereas a second set of discs was directly transferred into the measuring chambers of oxygen electrodes (pre- equilibrated at the different oxygen concentrations, respectively) to measure O2 consumption at 21, 8 and 4% oxygen, respectively. The remaining discs were incubated for another 8 or 24 h, before they were sampled in the same way as above.

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References


Lof, I., Stitt, M., and Geigenberger, P. (2000). Adenine leads to an increase in adenine nucleotide levels and modifies the interaction between respiration and starch synthesis in growing tubers. Planta, in press.


and Erwinia carotovora infection. Plant Physiol. 93, 1134 - 1139.


