

Glycolytic Enzymes Associate Dynamically with Mitochondria in Response to Respiratory Demand and Support Substrate Channeling ^W

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In *Arabidopsis thaliana*, enzymes of glycolysis are present on the surface of mitochondria and free in the cytosol. The functional significance of this dual localization has now been established by demonstrating that the extent of mitochondrial association is dependent on respiration rate in both *Arabidopsis* cells and potato (*Solanum tuberosum*) tubers. Thus, inhibition of respiration with KCN led to a proportional decrease in the degree of association, whereas stimulation of respiration by uncoupling, tissue ageing, or overexpression of invertase led to increased mitochondrial association. In all treatments, the total activity of the glycolytic enzymes in the cell was unaltered, indicating that the existing pools of each enzyme repartitioned between the cytosol and the mitochondria. Isotope dilution experiments on isolated mitochondria, using ¹³C nuclear magnetic resonance spectroscopy to monitor the impact of unlabeled glycolytic intermediates on the production of downstream intermediates derived from ¹³C-labeled precursors, provided direct evidence for the occurrence of variable levels of substrate channeling. Pull-down experiments suggest that interaction with the outer mitochondrial membrane protein, VDAC, anchors glycolytic enzymes to the mitochondrial surface. It appears that glycolytic enzymes associate dynamically with mitochondria to support respiration and that substrate channeling restricts the use of intermediates by competing metabolic pathways.

INTRODUCTION

The classical view of metabolism is that enzymes are physically independent entities freely diffusing in the intracellular aqueous phase. Products of catalysis are released into the bulk aqueous phase and arrive at the next enzyme in the metabolic sequence by passive diffusion. However, it is increasingly apparent that many enzymes are not free in solution but rather interact with membrane structures (Martin et al., 2005) or with other proteins (Uhrig, 2006). Indeed, large-scale mapping of eukaryotic protein–protein interactions suggests that few proteins exist in isolation (Grigoriev, 2003). Interaction between sequential enzymes in metabolic pathways gives rise to the possibility of direct channeling of metabolites between enzymes, in which the metabolic intermediates are not released to the bulk solution phase of the cell (Srere, 1987). Such substrate channeling confers a number of benefits, from altered reaction kinetics, to savings in cellular solvation capacity (Ovadi and Srere, 2000) or sequestration of toxic intermediates (Moller and Conn, 1980). The highly dynamic nature of cellular protein–protein interactions (Han et al., 2004) means that metabolon formation could have implications for the

regulation of metabolic flux, either in terms of magnitude or direction of flux (Achnine et al., 2004). Such enzyme complexes (metabolons) have been identified in plants, and examples have been characterized from both primary and secondary metabolism (Winkel, 2004; Jorgensen et al., 2005).

Although a new view of plant metabolism as a structurally complex and dynamically regulated network is emerging (Sweetlove and Fernie, 2005), the molecular characterization of protein complexes and establishment of their functional significance is far from complete. For example, the respiratory pathways of glycolysis and the tricarboxylic acid (TCA) cycle that form the central backbone of heterotrophic carbon metabolism are arguably among the best studied of all plant metabolic pathways (Plaxton and Podesta, 2006). However, despite the fact that the respiratory pathways in mammals represent a substrate-channeling paradigm (Ovadi and Srere, 2000; Haggie and Verkman, 2002), little work has been done to investigate whether plant respiration is organized in a similar way. There is some evidence that points toward interaction between plant glycolytic enzymes, but direct evidence for substrate channeling is lacking. For example, the increase in respiratory rate that occurs when storage root slices are aged is accompanied by an association of glycolytic enzymes with a particulate fraction (Moorhead and Plaxton, 1988). In addition, coimmunoprecipitation experiments have revealed an interaction between aldolase and both ATP- and PP_i-dependent phosphofructokinases in carrot (*Daucus carota*) storage roots (Moorhead and Plaxton, 1992). Recently, a new dimension to the structural organization of glycolysis was

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^WOnline version contains Web-only data.

www.plantcell.org/cgi/doi/10.1105/tpc.107.053371

discovered (Giegé et al., 2003). In *Arabidopsis thaliana*, 5 to 10% of the cytosolic isoforms of each glycolytic enzyme is associated with the outer surface of mitochondria. This has been independently corroborated by green fluorescent protein tagging of certain isoforms of *Arabidopsis* phosphofructokinase (Mustroph et al., 2007). There is also good evidence from a range of plant species that hexokinase associates with the outer mitochondrial membrane (Dry et al., 1983; da-Silva et al., 2001; Damari-Weissler et al., 2006; Kim et al., 2006). While it has been demonstrated that the glycolytic enzymes associated with isolated *Arabidopsis* mitochondria are catalytically competent and constitute a functioning glycolytic pathway (Giegé et al., 2003), the functional significance of this microcompartmentation of glycolysis has not been investigated.

It could be argued that the localization of glycolytic enzymes to the mitochondrial surface provides a mechanism for generating pyruvate close to its site of uptake into the mitochondrion where it acts as a substrate for the TCA cycle. However, it is not clear why this demand for pyruvate cannot be satisfied by a cytosolic glycolytic pathway with simple diffusion of pyruvate through the bulk phase to the mitochondrial pyruvate transporter. A possible explanation is that the cytosolic glycolytic pathway is functionally distinct from the mitochondrially associated one. As well as providing pyruvate for respiration, glycolysis is a highly branched pathway and supplies intermediates for a number of biosynthetic pathways and the oxidative pentose phosphate pathway. The provision of intermediates for biosynthesis and pyruvate for respiration are effectively competing demands on the same pathway. To ensure that sufficient respiratory pyruvate is produced in the face of competing withdrawal of upstream intermediates, specific regulatory mechanisms would be required. Substrate channeling between glycolytic enzymes associated with the mitochondrial surface is one possible mechanism that would achieve this end. Moreover, if the glycolytic enzymes present in the cytosol catalyzed a conventional unchanneled metabolism, the demand for glycolytic intermediates upstream of pyruvate could be met. In this article, this hypothesis is investigated by establishing the extent to which the association of glycolytic enzymes with the mitochondrion relates to the respiratory demand for pyruvate and by examining the extent to which glycolytic intermediates are channeled between glycolytic enzymes when localized to the mitochondrion. We also provide direct evidence for protein–protein interactions between mitochondrially associated glycolytic enzymes.

RESULTS

The Degree of Association of Glycolytic Enzymes with *Arabidopsis* Mitochondria Correlates with Respiratory Rate

In our previous study (Giegé et al., 2003), we observed that a small proportion of each glycolytic enzyme in *Arabidopsis* is associated with the outer surface of the mitochondria. To investigate the functional significance of this localization, we examined whether different metabolic demands led to alterations in the subcellular partitioning of the glycolytic enzymes. The rate of respiration in a heterotrophic *Arabidopsis* cell suspension culture (May and Leaver, 1993; Giegé et al., 2003) was decreased by

addition of KCN, an inhibitor of complex IV of the mitochondrial respiratory chain (Villani and Attardi, 2007), or increased by the addition of carbonyl cyanide *m*-chlorophenylhydrazine (CCCP), a proton ionophore that uncouples mitochondrial electron transport from ATP synthesis (Felle and Bentrup, 1977). In each case, a titration of KCN or CCCP against respiration rate (measured as the rate of oxygen consumption of the cell culture) was performed to establish the concentrations of KCN or CCCP that gave an ~50% decrease or increase in respiration rate, respectively (Figures 1A and 1B). These concentrations were 5 mM KCN and 0.3 μ M CCCP. Neither of these treatments reduced cell viability during the experimental time frame (data not shown). Cells were treated with KCN or CCCP at these concentrations, and mitochondria were isolated from treated and control cells. The activities of glycolytic enzymes in the mitochondrial preparation were quantified and compared (having corrected for mitochondrial yield) with the total cellular activity of each enzyme (Figures 1C and 1D). Enolase was not measured because the substrate, 2-phosphoglycerate, was not commercially available.

Neither treatment altered the total activity of each glycolytic enzyme in the cell. However, there were significant changes in the association of glycolytic activities with isolated mitochondria. The degree of association of glycolytic enzymes with *Arabidopsis* mitochondria is in good agreement with our previously published estimates (Giegé et al., 2003), with at most ~12% of the total cellular activity associated with mitochondria (in the case of hexokinase and glyceraldehyde-3-phosphate dehydrogenase) and more usually 4 to 5%. Inhibition of respiration with cyanide resulted in a significant decrease in the association of glycolytic enzymes with mitochondria (Figure 1C). With the exception of hexokinase, the partitioning of all the glycolytic enzymes to mitochondria was decreased within 30 min of KCN treatment. In some cases, significant decreases in activity were observable after only 10 min of KCN treatment. Generally speaking, the association of most enzymes was decreased by about half, which correlates with the 50% decrease in respiration rate. Conversely, a stimulation of respiration with CCCP led to an increase in the association of glycolytic enzymes with mitochondria (Figure 1D). Again, the degree of change of association showed good correlation with the change in respiratory rate. However, in this case, only six of the measured nine enzymes were increased, with partitioning of hexokinase, phosphoglucose isomerase, and glyceraldehyde-3-phosphate dehydrogenase to mitochondria remaining unchanged.

The Degree of Association of Glycolytic Enzymes with Potato Mitochondria Correlates with Respiratory Rate

Thus far, with the exception of hexokinase, the mitochondrial association of glycolytic enzymes has only been investigated in *Arabidopsis*. To check that the phenomenon is not restricted to *Arabidopsis*, the interaction of glycolytic enzymes with potato (*Solanum tuberosum*) tuber mitochondria was also investigated. Generally, a much lower proportion of the total activity of each enzyme was associated with potato mitochondria (0.1 to 2%) than in *Arabidopsis*. This may be because stored potato tubers are a dormant organ and the respiratory rate is much lower than in growing *Arabidopsis* cells (Figures 1A and 2A). The exception

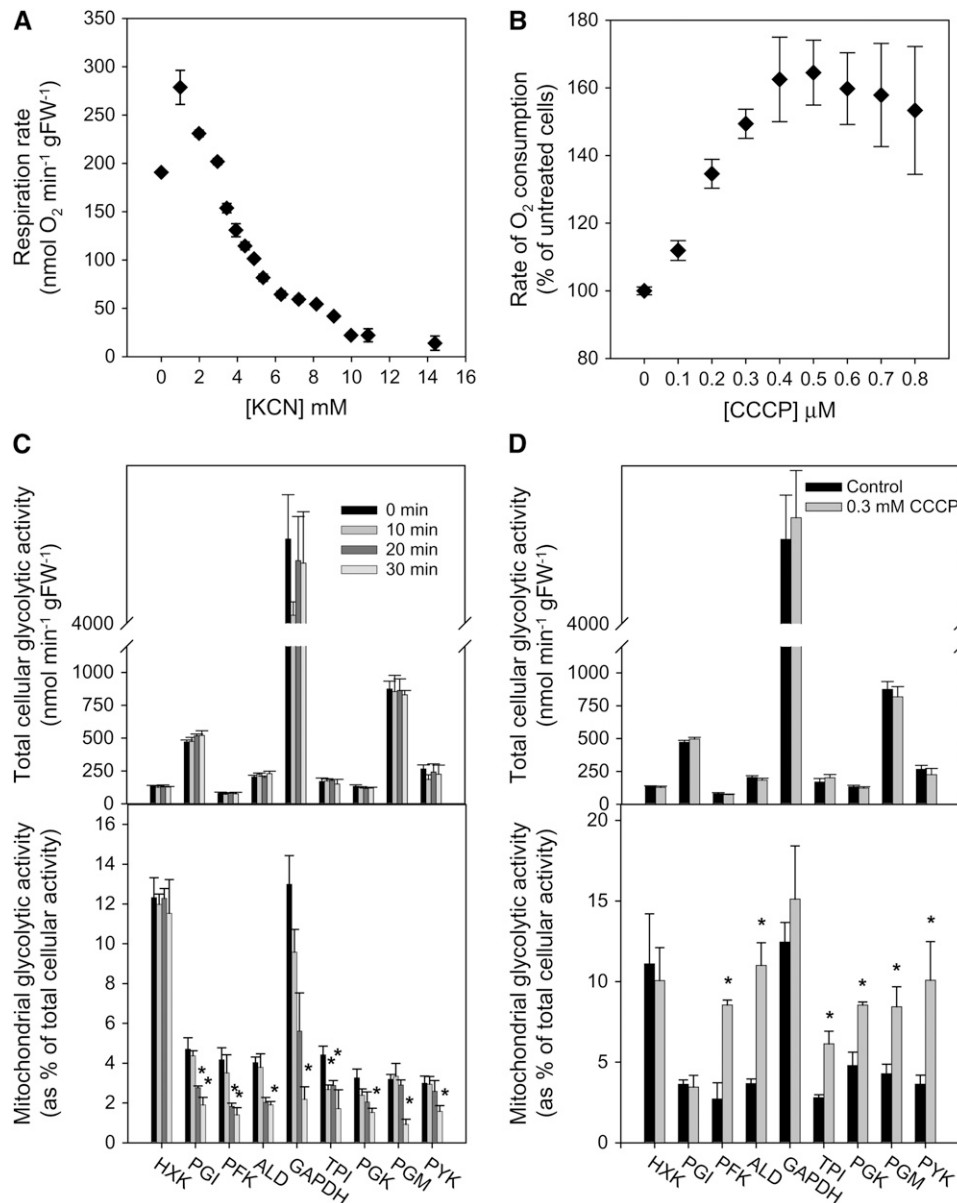


Figure 1. Effect of Manipulation of Respiration Rate in *Arabidopsis* Cell Suspensions on the Degree of Association of Glycolytic Enzymes with Mitochondria.

KCN (**A**) or CCCP (**B**) was added to heterotrophic *Arabidopsis* cell suspension cultures at the final concentrations indicated and the rate of oxygen consumption measured until a steady rate was obtained. *Arabidopsis* cell suspensions were treated with 5 mM KCN for the indicated time interval (**C**) or 0.3 μM CCCP for 30 min (**D**). Following these treatments, mitochondria were isolated, and the activities of glycolytic enzymes in the mitochondrial fraction relative to the total cellular activity of each enzyme were determined. HXK, hexokinase; PGI, phosphoglucose isomerase; PFK, phosphofructokinase; ALD, aldolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TPI, triose phosphate isomerase; PGK, phosphoglycerate kinase; PGM, phosphoglyceromutase; PYK, pyruvate kinase; FW, fresh weight. Values are mean of four independent samples ± SE. Asterisks indicate significant difference from untreated control (*t* test; *P* < 0.05).

was hexokinase, with nearly half the total cellular activity of this enzyme recovered in the mitochondrial preparations. The respiratory rate of potato tubers is known to increase substantially if the tissue is aged (Hackett et al., 1960). In our hands, ageing of potato tuber discs led to an ~2.5-fold increase in the rate of

respiration (Figure 2A). To see if the association of glycolytic enzymes with potato mitochondria increased with respiration rate, mitochondria were isolated from control and aged tuber discs and the recovery of glycolytic activities compared (Figure 2C). Ageing did not alter the total activity of each glycolytic

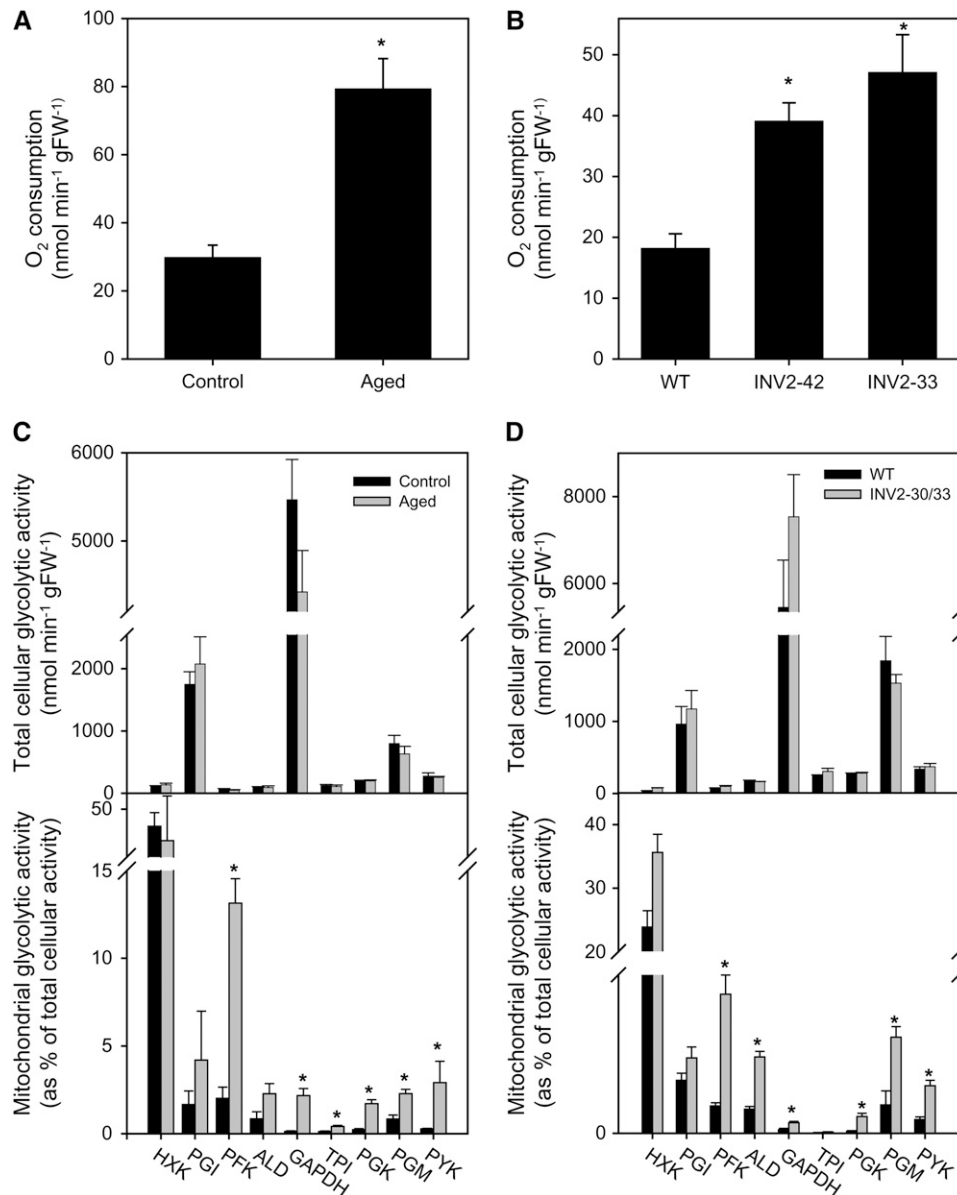


Figure 2. Effect of Manipulation of Respiration Rate in Potato Tubers on the Degree of Association of Glycolytic Enzymes with Mitochondria.

(A) Respiration rate of potato tuber discs after ageing for 16 h.

(B) Respiration rate of potato tuber discs from transgenic tubers expressing a yeast invertase in the cytosol. Data shown are pooled from two tubers each of lines INV2-30 and INV2-33 (Roessner et al., 2001).

(C) Activities of glycolytic enzymes associated with mitochondria isolated from aged tubers relative to the total cellular activity of each enzyme.

(D) Activities of glycolytic enzymes associated with mitochondria isolated from transgenic tubers expressing yeast invertase, relative to the total cellular activity of each enzyme.

Abbreviations are as given in Figure 1. Data are the mean of four independent samples \pm SE. Asterisks indicate significant difference from control value prior to treatment (**A**) and (**C**) or from the wild type (**B**) and (**D**) (*t* test, $P < 0.05$).

enzyme in the tissue. However, for the majority of the enzymes, there was a significant increase in the percentage association of enzyme activity with potato mitochondria (the exceptions being hexokinase, phosphoglucose isomerase, and aldolase). In some cases, the increase in association was dramatic, being several-

fold greater than the increase in respiration. For example, the degree of association of pyruvate kinase with mitochondria increased ~ 10 -fold. This may reflect the fact that tissue ageing is associated with a number of other physiological and biochemical changes in addition to respiratory increase. To manipulate

respiration in a more specific manner, we exploited existing transgenic potato lines expressing a yeast invertase in the cytosol (Sonnewald et al., 1997; Roessner et al., 2001). The lines carry a severalfold increase in invertase activity, which causes an increase of entry of carbon into glycolysis and is associated with an increased glycolytic capacity and increased glycolytic flux (Junker et al., 2004). Tubers from these lines respired more than twice as fast as wild-type tubers (Figure 2B). Correlating with this increased respiratory rate, there was a greater than twofold increase in the association of the majority of glycolytic enzymes with mitochondria from the transgenic lines (Figure 2D). The exceptions were hexokinase, phosphoglucose isomerase, and triosephosphate isomerase.

The Degree of Association of Glycolytic Enzymes with *Arabidopsis* Mitochondria Is Affected by Altered Flux through a the Oxidative Pentose Phosphate Pathway

To investigate whether the degree of association of glycolytic enzymes with mitochondria is affected by flux through nonrespiratory pathways, the oxidative pentose phosphate (OPP) pathway was stimulated by incubating *Arabidopsis* cells with nitrite. This is a well-established response (Averill et al., 1998; Garlick et al., 2002) in which an increased demand for NADPH for nitrite assimilation leads to an increased flux through the OPP pathway via the response of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase to the NADPH/NADP ratio. Titration of KNO_2 concentration against OPP pathway flux in an *Arabidopsis* cell suspension culture revealed that 5 mM nitrite is sufficient to lead to a significant increase in flux within 3 h (Figure 3A). This treatment did not have a significant effect on cell viability or on the total glycolytic activity within the cells (Figure 3B). When mitochondria were isolated after this treatment, it was apparent that there was a significant increase in the association of the majority of glycolytic activities with the isolated mitochondria (Figure 3B). As before, hexokinase was an exception. However, in contrast with other treatments, in which the activity of mitochondrially associated phosphoglucose isomerase was unchanged, there was a pronounced increase in the association of this enzyme with mitochondria following nitrite feeding.

Mitochondrially Associated Glycolytic Enzymes Form a Functioning Glycolytic Pathway That Can Support Mitochondrial Respiration

In our previous work, we provided limited evidence through isotopic labeling experiments that mitochondrially associated glycolytic enzymes constitute a functioning glycolytic pathway (Giegé et al., 2003). We wished to investigate the functionality of mitochondrial glycolysis further. In particular, we wanted to address two key questions. (1) Can the mitochondrially associated glycolytic pathway support mitochondrial TCA cycle and electron transport activity? (2) Does substrate channeling occur between mitochondrially associated glycolytic enzymes? In our previous work, mitochondria were incubated at high density with $[\text{U}-^{13}\text{C}]$ glucose and labeled pathway products detected with gas chromatography–mass spectrometry after a 16 h incubation (Giegé et al., 2003). Although these experiments showed that

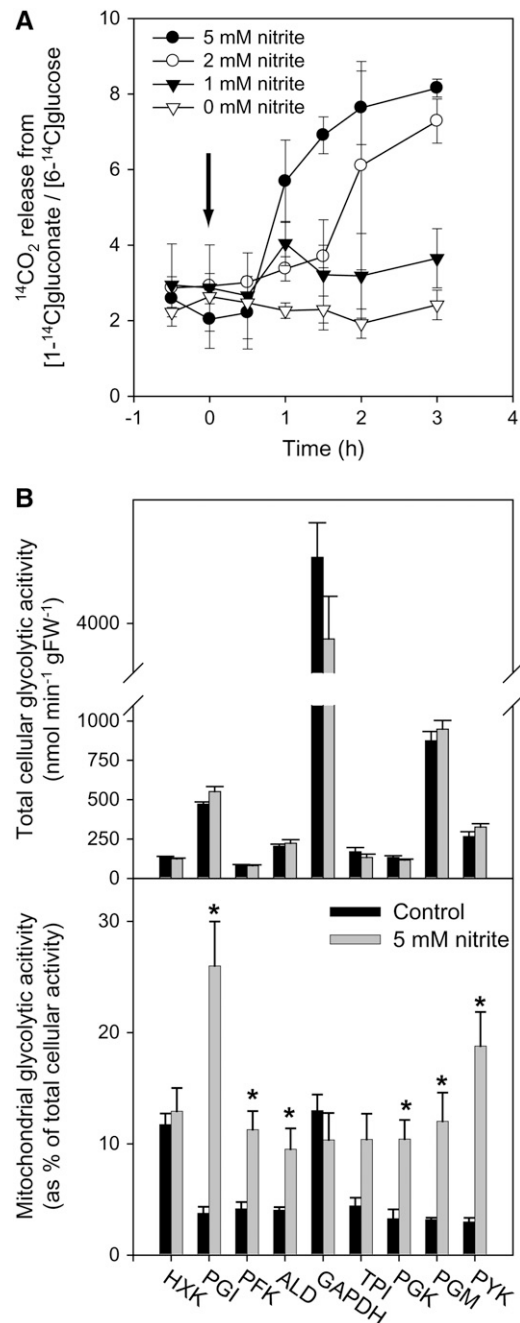


Figure 3. Effect of Stimulation of the OPP Pathway in *Arabidopsis* Cell Suspensions on the Degree of Association of Glycolytic Enzymes with Mitochondria.

(A) Different concentrations of KNO_2 were added to heterotrophic *Arabidopsis* cell suspensions and the flux through the OPP pathway measured (as the ratio of release of $^{14}\text{CO}_2$ from cells incubated with $[1\text{-}^{14}\text{C}]$ gluconate or $[6\text{-}^{14}\text{C}]$ glucose) over a 3-h time period. The arrow indicates the point of addition of KNO_2 .

(B) *Arabidopsis* cell suspensions were incubated with 5 mM KNO_2 for 3 h and the activity of glycolytic enzymes associated with mitochondria isolated from these cells measured. Abbreviations are as given in Figure 1. All data are the mean of three independent samples \pm SE. Asterisks indicate significant difference from control value prior to treatment (t test, $P < 0.05$).

isolated mitochondria supported glycolytic flux, it was not possible to quantify the flux accurately. For this, a labeling time series is required to demonstrate that the accumulation of labeled glycolytic pathway products is linear. We therefore decided to use a nuclear magnetic resonance (NMR)-based approach (Smith et al., 2004) that allowed the accumulation of labeled products to be monitored directly when isolated mitochondria were incubated with labeled substrate in an NMR spectrometer. In optimizing the experimental conditions, the following aspects were considered: provision of a labeled glycolytic precursor ($[1-^{13}\text{C}]$ glucose or $[1-^{13}\text{C}]$ fructose 1,6-bisphosphate), provision of cofactors necessary for glycolysis (ATP, ADP, and NAD^+), provision of a TCA cycle organic acid (to spark the TCA cycle), and addition of uncoupler (CCCP) to ensure that mitochondrial electron transport rate did not limit pyruvate consumption. The most critical factors were (1) the use of a relatively low concentration of TCA cycle organic acid (citrate at 0.5 mM) to avoid restriction of glycolytic flux via allosteric inhibition of pyruvate kinase (Podesta and Plaxton, 1992) and (2) the provision of sufficient ATP in the labeled glucose experiments for the two phosphorylation steps catalyzed by hexokinase and phosphofructokinase.

Using this system with mitochondria isolated from *Arabidopsis* cell suspensions, it was possible to detect rapid metabolism of the $[1-^{13}\text{C}]$ glucose and the appearance of label in the downstream metabolites glucose 6-phosphate (G6P), fructose 6-phosphate (F6P), fructose 1,6-bisphosphate (F16BP), and dihydroxyacetone phosphate (DHAP) (Figure 4A). This confirms that the glycolytic enzymes when localized to mitochondria constitute a functional metabolic pathway (Giegé et al., 2003). Moreover, it was possible to detect linear accumulation of label in these glycolytic products (Figure 4C), meaning that it was possible to measure glycolytic flux using this experimental setup. To improve the chances of detecting label in glycolytic intermediates downstream of DHAP, $[1-^{13}\text{C}]$ fructose 1,6-bisphosphate was supplied as the precursor molecule rather than $[1-^{13}\text{C}]$ glucose. Because this bypasses the ADP-generating reactions catalyzed by hexokinase and phosphofructokinase, 2-deoxyglucose was also added (this is phosphorylated by hexokinase, thus regenerating ADP for the ATP-synthesizing glycolytic reactions but is not further metabolized by subsequent glycolytic enzymes). Using this experimental setup, it was possible to detect label not only in DHAP but also in 3-phosphoglyceric acid (3PGA) (Figure 4B). These labeled intermediates accumulated in a linear manner and to a much higher concentration than when $[1-^{13}\text{C}]$ glucose was the precursor (Figure 4D), making quantitation more reliable. Labeling of glycolytic intermediates was linear for at least 10 h (Figures 4C and 4D), and the mitochondria maintained the ability to respire over this time period, albeit at a reduced rate (see Supplemental Figure 1 online). Despite many optimization experiments and testing of different parameters and strategies, we were unable to find any experimental conditions that allowed us to detect intermediates downstream of 3PGA (data not shown). It was concluded that using either labeled glucose or labeled F16BP, it was possible to quantify glycolytic flux as far as 3PGA and therefore to investigate substrate channeling of intermediates between glucose and 3PGA. This covers the majority of the pathway and only the final two intermediates,

2-phosphoglyceric acid and phosphoenolpyruvate, remain out of reach of this experimental system.

To establish whether these glycolytic reactions are able to provide substrate for the TCA cycle, we looked for evidence of labeling of TCA cycle organic acids. The NMR approach used here is only able to detect labeled TCA cycle acids that are exported from the mitochondria into the medium (Smith et al., 2004), and the amounts of detected TCA cycle acids were relatively low. However, when spectra were summed across the 10-h time course, we were able to detect labeled succinate when $[1-^{13}\text{C}]$ glucose was supplied and labeled citrate, succinate, and malate when $[1-^{13}\text{C}]$ fructose 1,6-bisphosphate was supplied (Figures 5A and 5B). Furthermore, it was apparent that under the same conditions, provision of fructose 1,6-bisphosphate led to an increased rate of oxygen consumption by *Arabidopsis* mitochondria (Figure 5D), indicating that mitochondrial glycolytic enzymes were able to support both TCA cycle flux and electron transport chain activity. However, no significant oxygen consumption was observed in the presence of glucose as a substrate (Figure 5C). The observation that isolated mitochondria can use glycolytic substrates as respiratory substrates is novel and provides strong support for the hypothesis that mitochondrially associated glycolytic enzymes function to provide pyruvate for respiration.

Substrate Channeling between Mitochondrially Associated Glycolytic Enzymes

An isotope dilution approach (Czichi and Kindl, 1977; Clegg and Jackson, 1990; Shearer et al., 2005) was used to determine whether glycolytic intermediates are channeled between glycolytic enzymes localized to *Arabidopsis* mitochondria. The principle of the experiment is as follows: the flux through a pathway is followed by measuring the rate of conversion of an isotopically labeled precursor to a labeled product. The effect of adding an unlabeled pathway intermediate on the rate of accumulation of labeled product is then determined. In an unchanneled system, labeled pathway intermediates will enter the bulk phase and the label will be diluted by the additional unlabeled intermediate. Assuming that the enzyme is saturated, this will lead to a decrease in the rate of labeled product accumulation. By contrast, in a completely channeled system, with no exchange between intermediates and the bulk phase, there will be no isotope dilution.

In the experiments, unlabeled glycolytic intermediates were added to the mitochondrial suspension and the effect on the rate of labeled product formation was measured by NMR (Figure 6). In the absence of channeling, a 50% dilution of the label in the suspending medium would cause a 50% dilution in the rate of labeled product formation, reflecting competition for the binding site of the enzyme that converts the intermediate to the product; whereas in a fully channeled system, unlabeled intermediate would have no effect. This method assumes that each enzyme is saturated with substrate. If this is not the case, the effect of the unlabeled intermediate would be negated because the overall rate of catalysis would be able to increase. The assumption of saturation is reasonable in this case because substrate (glucose or F16BP) was supplied *in vitro* at a high concentration (10 mM). This is more than sufficient to saturate the respective enzymes

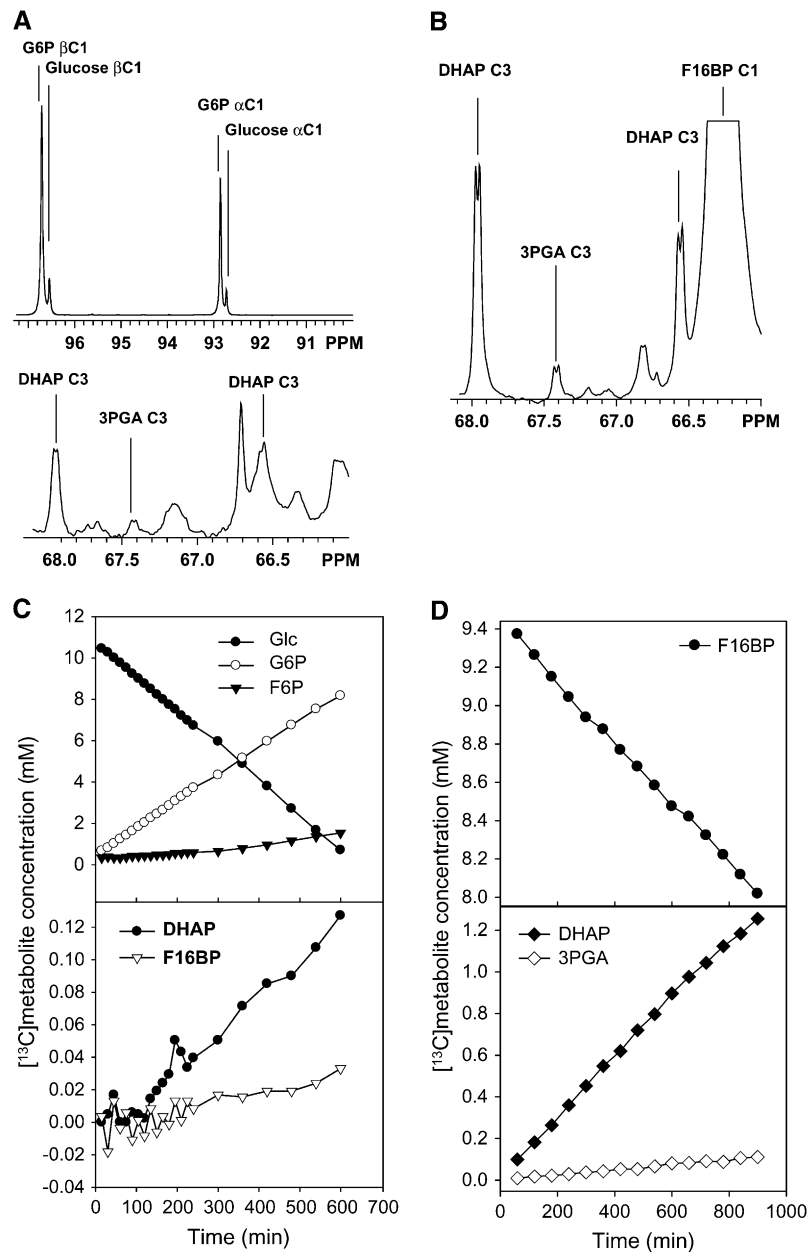


Figure 4. Measurement of Glycolytic Flux in Isolated *Arabidopsis* Mitochondria Using NMR.

Mitochondria isolated from *Arabidopsis* cells were incubated in situ in the NMR spectrometer in an oxygenated medium containing cofactors necessary for glycolysis and TCA cycle activity plus either [1- ^{13}C]glucose or [1- ^{13}C]fructose 1,6-bisphosphate.

(A) ^{13}C -NMR spectra showing ^{13}C -labeled metabolites after 600 min of incubation with [1- ^{13}C]glucose.

(B) ^{13}C -NMR spectrum showing ^{13}C -labeled metabolites after 600 min incubation with [1- ^{13}C]fructose 1,6-bisphosphate. Labeled peaks are annotated. Other peaks are present due to natural abundance of ^{13}C .

(C) Time-dependent labeling of glycolytic intermediates following incubation with [1- ^{13}C]glucose.

(D) Time-dependent labeling of glycolytic intermediates following incubation with [1- ^{13}C]fructose 1,6-bisphosphate.

that use these substrates and makes it likely that downstream enzymes will also operate close to saturation.

For analysis of channeling of G6P, F6P, and F16BP, labeled glucose was supplied and the rate of accumulation of labeled F6P or DHAP monitored (Figures 6A to 6C). For the other

intermediates, labeled F16BP was supplied and the rate of accumulation of labeled 3PGA monitored (Figures 6D and 6E). For each intermediate, two replicate experiments were performed on independent mitochondrial preparations, and the results of both replicates are shown. It can be seen that the

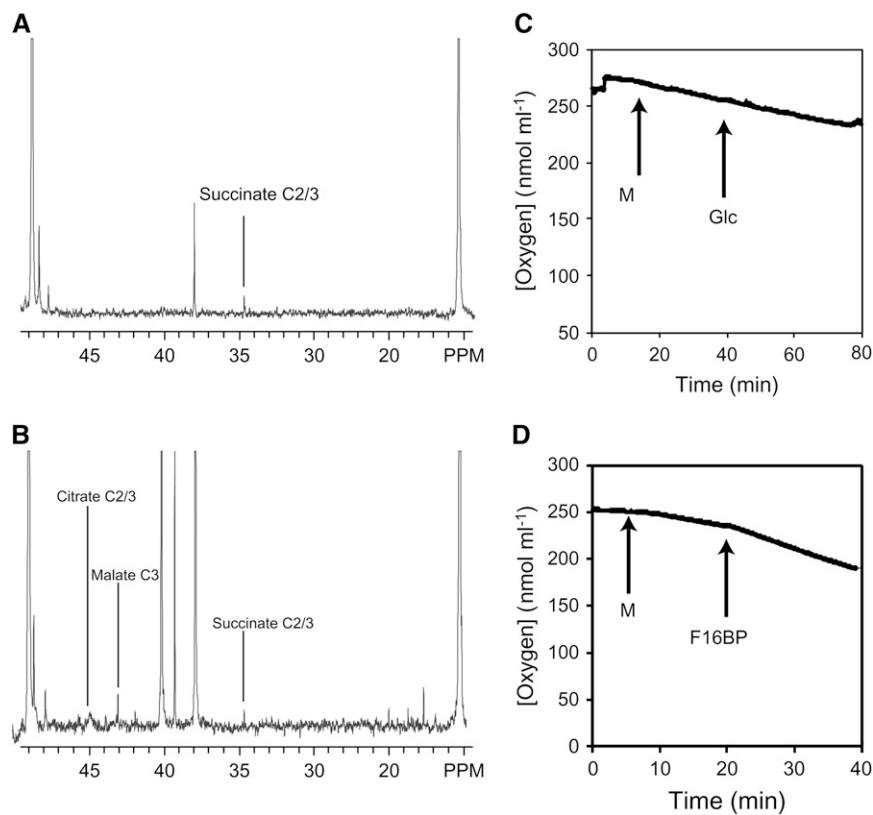


Figure 5. Glycolysis-Supported Respiration in Isolated *Arabidopsis* Mitochondria.

Mitochondria isolated from *Arabidopsis* cells were incubated in an oxygenated medium containing cofactors necessary for glycolysis and TCA cycle activity and either glucose (**A**) and **C**) or fructose 1,6-bisphosphate as a substrate. For analysis of TCA cycle activity supported by glycolysis, mitochondria were incubated in situ in the NMR spectrometer with $[1-^{13}\text{C}]$ glucose (**A**) or $[1-^{13}\text{C}]$ fructose 1,6-bisphosphate (**B**). ^{13}C spectra were acquired at 15-min intervals for the first 2 h and then at 60-min intervals for the next 8 h. **A**) and **B**) show a region between 50 and 10 ppm of the spectra, summed over the 10-h period. Labeled TCA cycle acids are indicated. For analysis of electron transport activity supported by glycolysis, mitochondria were incubated in an oxygen electrode and the rate of oxygen consumption measured when either glucose (**C**) or fructose 1,6-bisphosphate (**D**) (both at 10 mM final concentration) was supplied. The arrows indicate the addition of mitochondria (M), glucose, or fructose 1,6-bisphosphate (F16BP). All experiments were replicated three times, and representative data are shown.

addition of different unlabeled intermediates had different effects. In some cases (e.g., F16BP) glycolytic flux was not reduced after the addition of the unlabeled intermediate, which suggests that efficient substrate channeling is occurring. However, in other cases, there was a decrease in the rate of accumulation of label in downstream intermediates. The degree of substrate channeling in each case was calculated from the data in Figure 6. Derived data and the calculated percentage of substrate channeling are shown in Table 1. The data provide strong evidence that substrate channeling occurs between adjacent glycolytic enzymes when associated with isolated mitochondria. The intermediates between phosphofructokinase, aldolase, triosephosphate isomerase, and glyceraldehyde-3-phosphate dehydrogenase (F16BP, DHAP, and GAP) were channeled with high efficiency, with 92 to 100% of the intermediates being channeled. By contrast, substrate channeling was less efficient between the initial enzymes of the pathway, with only 38% of G6P being channeled between hexokinase and phosphoglucose isomerase and 63% of F6P being chan-

neled between phosphoglucose isomerase and phosphofructokinase.

Protein-Protein Interactions between Glycolytic Enzymes Associated with Mitochondria

The existence of substrate channeling between glycolytic enzymes demonstrates that there must be physical interactions between the proteins. To further investigate the nature of this glycolytic complex and how it might be anchored to the outer mitochondrial membrane, an antibody against castor bean (*Ricinus communis*) aldolase (Hodgson and Plaxton, 1998) was used to affinity purify aldolase and its interacting partners from a mitochondrial protein extract (Figure 7A, Table 2). Purified proteins were fractionated by two-dimensional gel electrophoresis, and several protein spots were observed. Some of these spots were also present in a control purification using anti-FLAG, indicating nonspecific interactions with either IgG or the agarose support (data not shown). Six spots specific to the aldolase

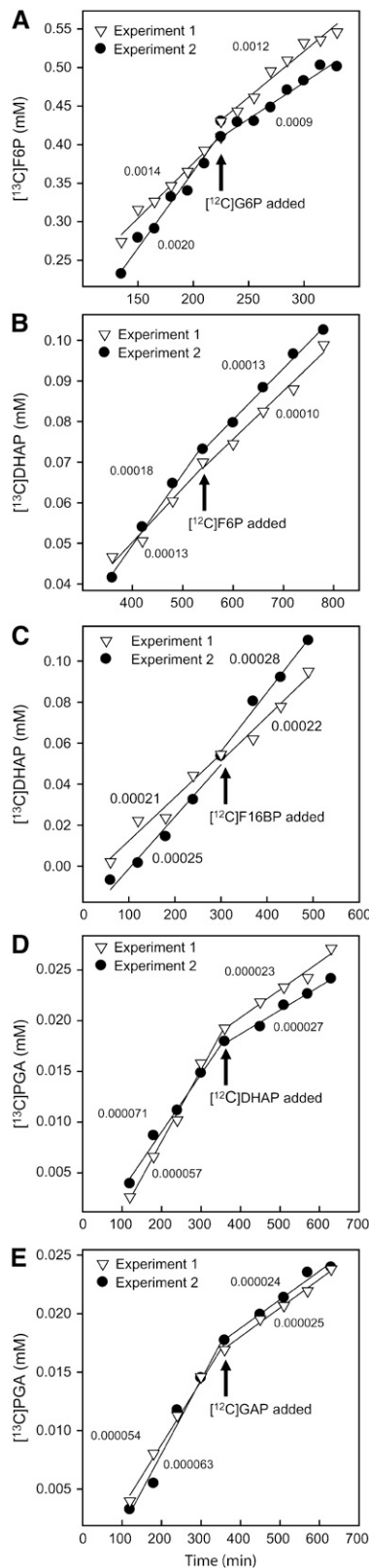


Figure 6. Substrate Channeling of Glycolytic Intermediates in Isolated *Arabidopsis* Mitochondria.

affinity purification are indicated (Figure 7A). To demonstrate the specificity of the aldolase antibody, a duplicate gel was analyzed by protein gel blotting (Figure 7A). It can be seen that the anti-aldolase antibody only reacted with a single protein that corresponds to the position of the aldolase spot (Table 2). This means that the other circled protein spots are interaction partners of aldolase rather than being copurified by nonspecific interaction with the aldolase antibody. As well as aldolase, four isoforms of GAPDH, the adjacent enzyme in the glycolytic pathway, were identified in addition to an isoform of the mitochondrial outer membrane protein, VDAC (for voltage-dependent anion channel) (Table 2).

The glycolytic complex was also investigated by blue-native PAGE. Mitochondrial proteins were extracted and fractionated by blue-native PAGE in the first dimension and SDS-PAGE in the second (Figure 7B). Protein gel blotting of replicate gels with anti-aldolase and anti-enolase antibodies revealed that these two proteins were present at a similar native molecular mass of ~ 350 kD (using the mitochondrial respiratory complexes as molecular mass markers). This is greater than the molecular mass of the individual enzymes (154 and 96 kD, respectively), indicating their interaction with other proteins. To determine whether other glycolytic proteins are also present at the same native molecular mass, a track from the appropriate portion of the gel was cut into 2-mm slices and proteins in each slice identified by tryptic digestion and matrix-assisted laser-desorption ionization time of flight (MALDI-TOF) mass spectrometry (Table 3; see Supplemental Table 1 online). In total, seven of the 10 glycolytic enzymes were identified at this same native molecular mass.

DISCUSSION

In this article, we investigated the functional significance of the partitioning of the glycolytic enzymes between the cytosol and the surface of mitochondria. Given that mitochondria require pyruvate to support respiration, the partitioning of glycolytic enzymes between the mitochondrial surface and cytosol could be a means of generating pyruvate at the point of demand while preventing withdrawal of upstream glycolytic intermediates by competing reactions. If this explanation is correct, then one might expect two predictions to hold true. First, the amount of each glycolytic enzyme associated with mitochondria would change in response to the respiratory demand for pyruvate. Second, substrate channeling would occur between mitochondrially localized glycolytic enzymes protecting the channeled metabolites from use by competing cytosolic pathways. Such a

Isolated mitochondria were incubated in the NMR spectrometer in a buffered oxygenated medium containing glycolytic cofactors (ATP, ADP, and NAD^+), citrate, CCCP, and either $[1\text{-}^{13}\text{C}]$ glucose (**A**) to **C**) or $[1\text{-}^{13}\text{C}]$ fructose 1,6-bisphosphate (**D**) and **E**). The accumulation of label in the indicated downstream glycolytic metabolite was quantified, and unlabeled intermediate was added as indicated. Each experiment was replicated with independent mitochondrial preparations, and both replicates are shown. The rate of labeled metabolite accumulation before and after addition of unlabeled intermediate was analyzed by linear regression, and the slopes of the fitted lines are shown.

Table 1. Parameter Values Used to Estimate Percentage of Channeling of Glycolytic Intermediates

Enzyme Pair	Intermediate (I)	[I] _{ext}		R		[I*] _{ext}		Channeling (%)	
		Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2
HXK:PGI	G6P	2	2	0.64	0.60	2.02	1.98	43.2	33.0
PGI:PFK	F6P	1	1	0.71	0.76	0.99	1.04	59.6	67.2
PFK:ALD	F16BP	1	1	1.12*	1.05*	0.01	0.01	100.0	100.0
ALD:TPI	DHAP	1	1	0.44	0.39	0.05	0.05	93.6	92.2
TPI:GAPDH	GAP	1	1	0.40	0.38	ND	ND	98.5**	98.4**

Unlabeled intermediates for each enzyme pair were added to give [I]_{ext}. The values for R and [I*]_{ext} were determined from the analysis of the NMR spectra (Figure 6), and the percentage of channeling was calculated as described in Methods. A single asterisk indicates that R was set to a value of 1 because R cannot be >1, and percentage of channeling was set to 100%. A double asterisks indicates that [I*]_{ext} was set to the detection threshold, 0.01 mM, because the signal was undetectable (ND). Abbreviations for glycolytic enzymes and intermediates are stated in the legends for Figures 1 and 4.

channeling mechanism would further imply the existence of specific interactions between the mitochondrially associated glycolytic enzymes and components of the mitochondrial membrane.

The Proportion of Cellular Glycolytic Activity Associated with the Mitochondrion Correlates with the Rate of Respiration

The rate of respiration in *Arabidopsis* cells or potato tubers was manipulated using chemical inhibition, uncoupling, wounding, or genetic manipulation and the relationship between the rate of respiration and the recovery of glycolytic activities with isolated mitochondria investigated. A good correlation was found between the two parameters. Generally speaking, when the respiration rate was reduced, there was a reduction in the activity of enzymes associated with mitochondria, and when respiration was increased, the association of glycolytic activities with mitochondria also increased. Importantly, in all treatments, the total activity of each glycolytic enzyme in the cell was unaltered. This suggests that the change in activity associated with mitochondria is not due to a change in either the total amount of each glycolytic enzyme (due to protein synthesis or turnover) or the activation state of the enzyme, but rather that there is repartitioning of enzyme pools such that more or less of each enzyme is associated with mitochondria. With the exception of potato tuber ageing, the change in association of glycolytic enzymes with mitochondria was quantitatively proportional to the change in respiration. These data strongly suggest that the reason glycolytic enzymes are localized to the mitochondrial surface is related to the supply of pyruvate for respiration.

Within the general pattern of correlation, there were some exceptions and variable behavior of individual enzymes. Most notable among the exceptions was hexokinase. Specific isoforms of hexokinase are known to associate with mitochondria (Claeyssen and Rivoal, 2007; Damari-Weissler et al., 2007), but the amount of hexokinase associated with mitochondria was unaffected by any of the treatments or transgenic manipulations. This may imply that the association of hexokinase with mitochondria is unconnected with respiration. Indeed, if sucrose is considered to be the starting point of glycolysis and is metabo-

lized by sucrose synthase to generate UDP glucose and fructose, hexokinase is not required as part of the glycolytic pathway (Plaxton and Podesta, 2006). Hexokinase is well known to have an alternative function as a glucose sensor (Rolland et al., 2006), but this appears to be connected to a nuclear-localized form of the enzyme (Cho et al., 2006). However, a role for mitochondrial-localized hexokinase in the regulation of programmed cell death has recently been uncovered (Kim et al., 2006), and this may be the main function of the hexokinase protein in this location. Hexokinase has also been proposed to play a specific role in generating ADP to support oxidative phosphorylation, thereby avoiding an ATP synthesis-related limitation of respiration and subsequent reactive oxygen species production (da-Silva et al., 2004).

The degree of association of glycolytic enzymes with mitochondria is also responsive to changes in nonrespiratory metabolism with a significant increase in response to stimulation of the OPP pathway. Given the tight linkage between glycolysis and the OPP pathway and the sharing of several intermediates, this is perhaps not surprising. The increased partitioning of glycolytic enzymes to mitochondria may reflect the increased competition from the OPP pathway for shared intermediates. It is therefore significant that there is a substantially greater increase in the association of phosphoglucose isomerase to mitochondria than that of the other glycolytic enzymes (phosphoglucose isomerase catalyzes the conversion of G6P to F6P, and the former is an intermediate shared with the OPP pathway). As well as increased demand for shared intermediates, the addition of nitrite to stimulate the OPP pathway will result in an increased demand for TCA cycle intermediates for nitrogen assimilation, and the increased partitioning of glycolytic enzymes to mitochondria could also reflect this demand.

Substrate Channeling in Mitochondrially Associated Glycolysis: Implications for Regulation of Glycolytic Flux

While a tight colocalization of glycolysis with mitochondria makes logical sense in terms of providing pyruvate at the point of demand, this alone is not a sufficient reason for having a dynamic interaction that is sensitive to the respiratory demand. There is no a priori reason why the mitochondrial demand for pyruvate cannot be met by glycolysis in the cytosol. However, if

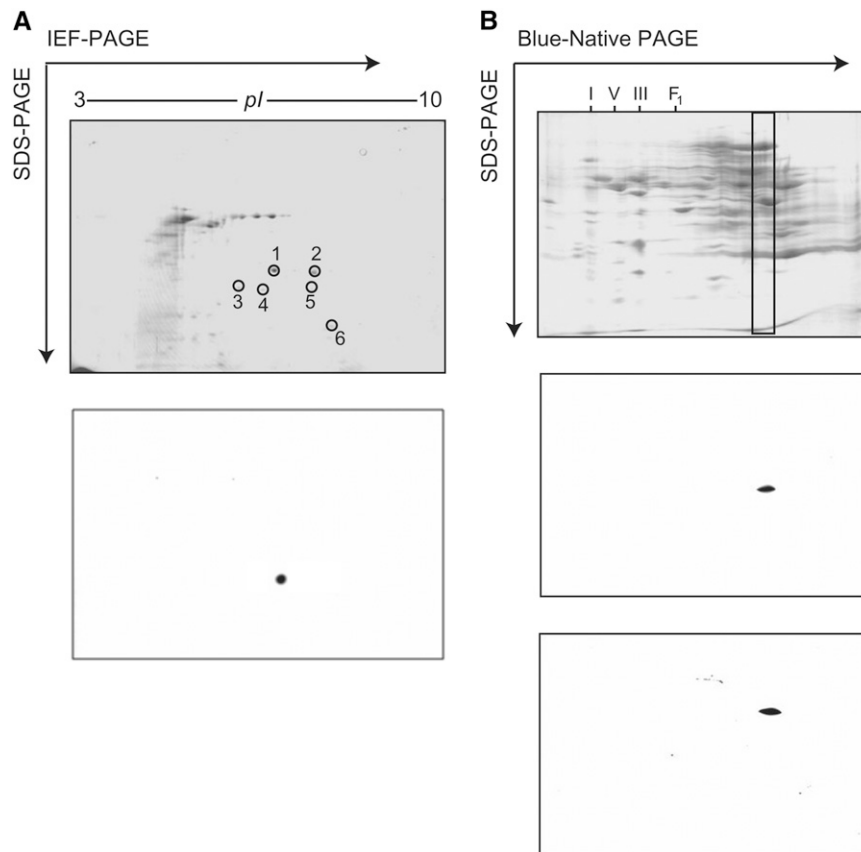


Figure 7. Protein-Protein Interactions between Glycolytic Enzymes Associated with Isolated Mitochondria.

(A) Affinity purification of mitochondrial aldolase and interacting proteins. Top panel: mitochondrial proteins eluted from an anti-aldolase-agarose column, fractionated by two-dimensional electrophoresis (isoelectric focusing [IEF] in the first dimension and SDS-PAGE in the second), and stained with colloidal Coomassie blue. Bottom panel: a replicate gel was transferred to a nitrocellulose membrane and probed with anti-aldolase antibody.

(B) Fractionation of mitochondrial proteins by blue-native PAGE (top panel). The position of mitochondrial respiratory complexes I, III, and V and the F_1 subunit of complex V are indicated. The box indicates a region of the gel that was excised and cut into 2-mm slices and proteins identified by MALDI-TOF mass spectrometry (Table 2; see Supplemental Table 1 online). Replicate blue-native gels were transferred to nitrocellulose membranes and probed with anti-aldolase (middle panel) and anti-enolase (bottom panel) antibodies.

the association of glycolytic enzymes with the outer mitochondrial membrane enhances protein-protein interactions between the enzymes, then the purpose of this localization of glycolytic enzymes could be to form a glycolytic complex that supports substrate channeling. This may be necessary because there are

multiple demands for intermediates of glycolysis for use as carbon skeletons in various biosynthetic pathways. In situations in which biosynthetic fluxes are high, there needs to be a regulatory mechanism to ensure that sufficient carbon flows right through the pathway to generate respiratory pyruvate in the

Table 2. Identity of Proteins Copurifying with Mitochondrial Aldolase

Protein Spot (see Figure 7A)	<i>Arabidopsis</i> Gene	Protein Name	Number of Matching Peptides	Coverage of Protein (%)	MOWSE Score
1	At3g52930	Fructose-bisphosphate aldolase	11	48	145
2	At1g13440	Glyceraldehyde-3-phosphate dehydrogenase	13	53	156
3	At3g52930	Glyceraldehyde-3-phosphate dehydrogenase	10	44	128
4	At3g52930	Glyceraldehyde-3-phosphate dehydrogenase	9	39	113
5	At3g52930	Glyceraldehyde-3-phosphate dehydrogenase	9	39	113
6	At5g15090	VDAC	6	29	127

Mitochondrial proteins eluted from an anti-aldolase-agarose column were fractionated by two-dimensional gel electrophoresis (Figure 7A), and the indicated proteins excised, digested with trypsin, and identified from their peptide mass fingerprint obtained using MALDI-TOF mass spectrometry.

Table 3. Identity of Mitochondrially Associated Glycolytic Proteins at a Native Molecular Mass of ~350 kD

<i>Arabidopsis</i> Gene	Protein Name	Number of Matching Peptides	Coverage of Protein (%)	MOWSE Score
At4g29130	Hexokinase	10	44	72
At3g52930	Fructose bisphosphate aldolase	8	31	80
At3g55440	Triosephosphate isomerase	7	26	69
At1g13440	Glyceraldehyde-3-phosphate dehydrogenase	9	29	75
At1g79550	Phosphoglycerate kinase	10	37	79
At2g36530	Enolase	14	37	71
At2g36580	Pyruvate kinase	6	28	62
At5g63680	Pyruvate kinase	8	30	67

Mitochondrial proteins were fractionated by blue-native PAGE and a track excised from the gel (see Figure 7B). The track was cut into 2-mm slices and proteins in each slice digested with trypsin and identified from their peptide mass fingerprint obtained by MALDI-TOF mass spectrometry. Peptides were matched against the National Center for Biotechnology Information (NCBI) protein database using an in-house MASCOT server. All proteins shown have MOWSE scores that indicated a significant identification ($P < 0.05$). Identified glycolytic proteins are shown. For a full list of identified proteins, see Supplemental Table 1 online.

face of this competing demand. A possible way in which this could be achieved is to organize glycolysis differently for the two roles. Localization of a proportion of glycolytic enzymes to the mitochondrial surface to promote substrate channeling would ensure that sufficient carbon is channeled directly from glucose or hexose phosphates to pyruvate and that glycolytic intermediates are protected from competing enzymes in biosynthetic pathways.

Using a ^{13}C -NMR approach, we have provided direct evidence for substrate channeling between glycolytic enzymes associated with isolated mitochondria. Using the principle of isotope dilution, the extent to which intermediates are passed directly between sequential enzymes without release into the bulk phase can be quantified. We were able to test the extent of channeling of five glycolytic intermediates from G6P to GAP. In every case, there was appreciable channeling of these intermediates. Several intermediates were channeled with high efficiency, while more leaky channeling was observed for G6P and F6P. Our estimates of substrate channeling assume that the enzymes of glycolysis are saturated with substrate at the point at which unlabeled intermediate is added to the system. For G6P, F6P, and F16BP, there is direct evidence to support this assumption: the measured concentration of labeled G6P, F6P, and F16BP in the bulk phase ($[I]_{\text{ext}}$, Table 1) was sufficient to saturate the enzymes phosphoglucose isomerase, phosphofructokinase, and aldolase, respectively, based on published K_m values (Noltmann, 1972; Schnarrenberger and Kruger, 1986; Knowles et al., 1990). However, it was not possible to confirm that triose phosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase were saturated: the concentration of labeled DHAP in the bulk phase (Table 1) was less than the K_m of triose phosphate isomerase for DHAP (0.88 mM) (Tomlinson and Turner, 1979), and we were unable to detect GAP. This is not to say that these enzymes were not saturated. If there is considerable substrate channeling at these enzymes then the effective concentration of labeled intermediate available to the enzyme would be higher than the concentration in the bulk phase. Moreover, the decrease in the rate of labeled 3PGA production after the addition of unlabeled DHAP or GAP suggests that these enzymes must reach saturation at this point

(otherwise the unlabeled intermediate would have no impact on the rate of production of labeled product due to the extra catalytic capacity available). However, without direct proof of saturation, we regard the data as providing qualitative evidence in support of substrate channeling of DHAP and GAP, and it is possible that the quantitative values of channeling for these two intermediates (Table 1) are overestimates.

The fact that only F16BP and not glucose was able to act as an efficient respiratory substrate for isolated mitochondria in vitro (Figure 5) provides additional support for leaky channeling of G6P and F6P and tighter channeling of intermediates from F16BP onwards. Without tight substrate channeling, intermediates will be considerably diluted by equilibration with the incubation medium. Thus, the rate of production of glycolytic intermediates will become progressively slower further down the pathway due to the lag generated as the preceding metabolite is diluted into the medium. Thus, the in vitro rate of the reactions between glucose, G6P, and F6P where substrate channeling is leaky will occur progressively more slowly. This explains the failure to detect substantial production of TCA cycle acids or oxygen consumption when glucose is supplied as a substrate. By contrast, the problem of intermediate dilution will be less prevalent in the reactions downstream of F16BP where much tighter substrate channeling occurs and, thus, F16BP is more effective as a respiratory substrate in vitro.

The channeling of intermediates between glycolytic enzymes associated with mitochondria provides strong support for the hypothesis that the mitochondrial localization of glycolytic enzymes is to provide a dedicated route of pyruvate production for respiration that is protected from competition from competing biosynthetic pathways. The corollary of this hypothesis is that the majority of the glycolytic pathway that remains free in the cytosol should be unchanneled or at least much less efficiently channeled to allow provision of intermediates for other pathways. This is difficult to test directly because the isotope dilution approach would require an experimental mechanism to allow intact cells or tissues to take up glycolytic intermediates. Most of these phosphorylated intermediates are not naturally transported across the plasma membrane, and so artificial ways of allowing their uptake

would be required. Possible approaches include permeabilization of the plasma membrane using pore-forming proteins (Matic et al., 2005) or expression of heterologous transgenes encoding appropriate transporter proteins (Shearer et al., 2005). This is outside of the scope of this study. However, it is worth noting that substrate channeling in mammalian glycolysis is considerably more leaky than we have measured here (Clegg and Jackson, 1990). If we assume that glycolytic enzymes are localized to mitochondria in mammalian cells (Taylor et al., 2003) to a similar extent as we have observed for *Arabidopsis* (i.e., ~5 to 10% of the total cellular pool of glycolytic enzymes are mitochondrially associated), then the whole-cell isotope dilution experiments of Clegg and Jackson (1990) will report mainly on the free cytosolic pathway. Thus, it seems likely that the cytosolic glycolytic pathway in mammals is not tightly channeled. By contrast, whole-cell isotope dilution experiments in *Escherichia coli* suggest that cytosolic glycolysis is tightly channeled (Shearer et al., 2005). However, because prokaryotes do not have mitochondria, the physical arrangement of the glycolytic pathway is likely to be different (Norris et al., 1999).

Regulation of Mitochondrial Localization and Protein-Protein Interactions of Glycolytic Enzymes

The observation of tight substrate channeling between many of the mitochondrially associated glycolytic enzymes implies the existence of direct physical interactions between glycolytic enzymes (since enzyme-enzyme interactions are a prerequisite for substrate channeling). However, we were unable to isolate an intact glycolytic complex from mitochondria using affinity purification or blue-native PAGE approaches. This is most likely because the interactions between the enzymes are relatively labile. In particular, because the glycolytic enzymes are associated with a membrane, it may be that protein-lipid interactions are required to stabilize the complex and both of the techniques use detergents to solubilize proteins from lipids. However, the affinity purification experiments did reveal a strong interaction between aldolase and glyceraldehyde-3-phosphate dehydrogenase, the subsequent enzyme in the glycolytic pathway. Moreover, there was also evidence of an interaction between aldolase and/or glyceraldehyde-3-phosphate dehydrogenase and the outer mitochondrial membrane protein, VDAC. This interaction has also been observed in yeast two-hybrid experiments (Holtgrawe et al., 2005) and is significant because it may represent a mechanism by which glycolytic enzymes are anchored to the outer mitochondrial membrane. Indeed, it is known that mammalian hexokinase is anchored to the outer mitochondrial membrane via an interaction with VDAC and that this interaction is modulated by G6P concentration (Wilson, 2003). It is possible that glycolytic enzymes are organized in a modular fashion, with pairs or small sets of enzymes interacting strongly with each other and with VDAC for anchoring to the outer mitochondrial membrane. Weaker (possibly lipid-dependent) interactions could then bring these modules together. This arrangement is supported by the copurification experiments and also by the blue-native gel experiments in which many of the glycolytic enzymes appeared at the same native molecular mass that was larger than the native mass of the individual holo-

zymes but not large enough to indicate a complete glycolytic complex (which would be in excess of 1200 kD).

Summary

This work demonstrates that interaction of glycolytic enzymes with the surface of the mitochondrion is highly dynamic and therefore regulated. The regulatory mechanism is based on repartitioning of existing pools of glycolytic enzyme and occurs on a timescale of minutes. The short timescale over which repartitioning of enzymes occurs suggests that posttranslational modification of the proteins or metabolite-induced conformational changes may be responsible for regulating their localization. Aldolase interacts strongly with the outer mitochondrial membrane protein, VDAC, and this interaction presumably serves to anchor this enzyme onto the outer mitochondrial surface. The fact that the enzymes support substrate channeling means that there must be direct physical interaction between adjacent enzymes in the pathway, although the failure to recover a complete glycolytic complex by affinity purification or blue-native PAGE suggests these interactions may be labile or require protein-lipid interactions for stabilization. The coordinated nature of the repartitioning of glycolytic enzymes argues against individual enzymes playing secondary moonlighting roles when localized to the mitochondrial surface (Jeffery, 2003). In a general context, this work provides additional evidence that association of enzymes with membrane structures promotes protein-protein interactions and substrate channeling (Winkel, 2004; Jorgensen et al., 2005). The work also suggests that metabolon formation may be an important mechanism of regulation of metabolic flux and one that will have to be considered when attempting to construct a systems view of the behavior of plant metabolic networks.

METHODS

Plant Material

Heterotrophic *Arabidopsis thaliana* cells were cultured in conical flasks under aseptic conditions containing 100 mL of Murashige and Skoog basal media supplemented with 3% (w/v) sucrose, 0.5 mg/L of naphthaleneacetic acid, and 0.05 mg/L of kinetin (May and Leaver, 1993). The cell cultures were maintained in the dark at 22°C with shaking at 150 rpm. After 7 d of growth, each flask (120 mL) contained 8 to 10 g FW cells, and growth was approximately in the middle of the log phase. Subculture of 20 mL of culture to 100 mL of fresh media began the cycle again. Potato tubers (*Solanum tuberosum* cv Desirée) were either purchased from the local supermarket or wild-type and transgenic plants expressing yeast invertase (Sonnewald et al., 1997; Roessner et al., 2001) were grown in a glasshouse at 25°C with a 16-h photoperiod of natural daylight supplemented to give a minimum irradiance of 150 $\mu\text{E m}^{-2} \text{s}^{-1}$. Developing tubers were harvested after 10 weeks. Aged tissue was obtained by cutting discs from potato tuber cores and incubating overnight in oxygenated sterile water.

Isolation of Mitochondria and Assay of Glycolytic Enzymes

Mitochondria were isolated from *Arabidopsis* cells using density gradient centrifugation exactly as described by Giegé et al. (2003) and from potato tubers as described by Considine et al. (2003). Glycolytic enzyme activities were measured by spectrophotometric assay as described by

Giegé et al. (2003). The percentage association of these activities with mitochondria was calculated by comparison of the activity recovered with isolated mitochondria (having corrected for mitochondria yield) with the total cellular activity present in the crude homogenate.

Measurement of Respiration

Respiration was measured as the rate of oxygen consumption using a Clark oxygen electrode (Hansatech). For *Arabidopsis* cells, 1 mL of cell suspension in growth medium was added to the oxygen electrode, and for potato tubers, five discs of tuber tissue (1 mm thick, 1 cm diameter, ~0.1 g FW) threaded on cotton were suspended in 900 μ L of sterile deionized water.

Measurement of OPP Pathway Flux

Flux through the OPP pathway was estimated based on the method of Garlick et al. (2002) in which the labeling of CO₂ when cells are incubated with either [1-¹⁴C]gluconate or [6-¹⁴C]glucose is monitored.

Measurement of Glycolytic Flux in *Arabidopsis* Mitochondria

Isolated mitochondria from heterotrophic *Arabidopsis* cells (7 mg of mitochondrial protein in 1 mL of 300 mM mannitol and 10 mM TES buffer, pH 7.5) were added to 4 mL of an NMR buffer containing a ¹³C-labeled substrate. The final composition of the suspending medium was 0.2 M mannitol, 0.1 M MOPS, 20 mM KH₂PO₄, 5 mM MgCl₂, 0.5 mM citrate, 0.3 mM NAD⁺, 0.1 mM TPP, 4 μ M CCCP, 0.1% (w/v) BSA, 10% (v/v) D₂O, pH 7.20, plus either 10 mM [1-¹³C]glucose (99 atom%; Omicron Biochemicals), 20 mM ATP, and 20 mM ADP or 10 mM [1-¹³C]fructose 1,6-bisphosphate (99 atom%; Omicron Biochemicals), 40 mM 2-deoxyglucose, and 0.8 mM ADP. The mitochondrial suspension was oxygenated in a 10-mm-diameter NMR tube using an air-lift system (Fox et al., 1989; Smith et al., 2004), and the metabolism of the ¹³C-labeled substrate was followed by recording ¹H-decoupled ¹³C-NMR spectra at 20°C and 150.9 MHz on a Varian Unity Inova 600 spectrometer equipped with a dual frequency ¹³C/³¹P switching probe. Spectra were recorded in 15-min blocks over a period of up to 10 h using a 90° pulse angle, a 1.016-s acquisition time and a 6-s relaxation delay. Low-power frequency-modulated decoupling was applied during the relaxation delay to maintain the nuclear Overhauser effect before switching to higher-power Waltz decoupling during the acquisition time to remove the proton couplings. Spectra were processed with a line broadening of 2.5 Hz using NUTS software (Acorn NMR). Chemical shifts are quoted relative to the mannitol CH₂OH signal at 63.90 ppm, and peaks were assigned on the basis of literature values and authentic standards.

Measurement of Mitochondrial Respiration

The rate of oxygen consumption by isolated mitochondria when supplied with glycolytic substrates or succinate was measured using a Clark oxygen electrode (Hansatech) at 20°C. Mitochondria were suspended in 1 mL of a buffered medium of the same composition as for the NMR experiments. Glucose or fructose 1,6-bisphosphate (10 mM) was supplied as substrate in the presence of appropriate glycolytic cofactors (see above) or succinate (10 mM) and ATP (0.5 mM).

Estimation of Substrate Channeling

Substrate channeling was analyzed by the isotope dilution method (Clegg and Jackson, 1990; Shearer et al., 2005). The rate of labeled product formation before and after the addition of an unlabeled pathway intermediate (I) was measured from the NMR time courses. Defining R as the ratio of the rate in the presence and absence of I and assuming saturation of the enzyme that catalyzes the formation of the product, then $R = [I^*]_{\text{eff}} /$

$([I^*]_{\text{eff}} + [I]_{\text{ext}})$, where $[I^*]_{\text{eff}}$ is the effective concentration of the labeled intermediate, and $[I]_{\text{ext}}$ is the concentration of I in the suspending medium. $[I^*]_{\text{eff}}$ is the sum of the I* that is directly channeled to the active site and the I* present in the suspending medium ($[I^*]_{\text{ext}}$). Defining channeling as the fraction of I* that does not have its immediate origin in the bulk phase: % channeling = $100\{1 - ([I^*]_{\text{ext}}/[I^*]_{\text{eff}})\}$.

Immunoaffinity Purification of Aldolase

Immunoaffinity purification was performed using the Seize Coimmunoprecipitation kit (Pierce Biotechnology) according to the manufacturer's instructions. Two hundred micrograms of anti-aldolase antibody (Hodgson and Plaxton, 1998) was coupled to 100 μ L of antibody-coupling agarose and stored at 4°C until used. Triton X-100 (0.05% [v/v] final) was added to isolated mitochondria (10 mg of mitochondrial protein mL⁻¹). Samples were then vortexed, sterile-filtered, and diluted 1:3 with phosphate-buffered saline. One milligram of mitochondrial protein was batch loaded onto 100 μ L of anti-aldolase-agarose. The anti-aldolase-agarose was washed and bound proteins eluted according to the manufacturer's instructions. Several parallel purifications were pooled to provide sufficient material for subsequent analysis by gel electrophoresis.

Gel Electrophoresis and Protein Gel Blotting

Isoelectric focusing/SDS-PAGE was done as described by Millar et al. (2001). Blue-native PAGE of mitochondrial total protein was done as described by Heazlewood et al. (2003). Protein gel blotting was done using nitrocellulose membranes and visualization using enhanced chemiluminescence using standard protocols. Anti-castor bean (*Ricinus communis*) aldolase antibody was a gift from W.C. Plaxton, (Departments of Biology and Biochemistry, Queen's University, Ontario, Canada). Anti-human enolase antibody was from Santa Cruz Biotechnology.

Protein Identification by Mass Spectrometry

Protein spots or bands were excised from polyacrylamide gels, in-gel digested with trypsin, and identified from their peptide mass fingerprint as described by Millar et al. (2001). Peptides were analyzed using an Axima CFR MALDI-TOF mass spectrometer (Shimadzu Biotech) and monoisotopic peak lists searched against the NCBI nonredundant protein database using an in-house MASCOT server.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Capacity of Isolated Mitochondria to Respire Succinate Over Time.

Supplemental Table 1. Identification of *Arabidopsis* Mitochondrial Proteins at a Native Molecular Weight of ~350 kD.

ACKNOWLEDGMENTS

We thank Uwe Sonnewald (Universität Erlangen-Nürnberg, Germany) and Lothar Willmitzer (Max-Planck Institute for Molecular Plant Physiology, Golm, Germany) for access to the transgenic potato lines overexpressing yeast invertase. We acknowledge the gift of the anti-aldolase antibody from Bill Plaxton (Queens University, Kingston, Canada). J.W.A.G., R.G.R., L.J.S., and T.C.R.W. acknowledge the financial support of the Biotechnology and Biological Sciences Research Council, UK.

Received June 5, 2007; revised September 21, 2007; accepted October 15, 2007; published November 2, 2007.

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