Inhibition of 2-Oxoglutarate Dehydrogenase in Potato Tuber Suggests the Enzyme Is Limiting for Respiration and Confirms Its Importance in Nitrogen Assimilation[1][W][OA]

Wagner L. Araújo, Adriano Nunes-Nesi, Sandra Trenkamp, Victoria I. Bunik, and Alisdair R. Fernie*

Max-Planck-Institut für Molekulare Pflanzenphysiologie, 14476 Potsdam-Golm, Germany (W.L.A., A.N.-N., S.T., A.R.F.); and A.N. Belozersly Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russia (V.I.B.)

The 2-oxoglutarate dehydrogenase complex constitutes a mitochondrially localized tricarboxylic acid cycle multienzyme system responsible for the conversion of 2-oxoglutarate to succinyl-coenzyme A concomitant with NAD^+ reduction. Although regulatory mechanisms of plant enzyme complexes have been characterized in vitro, little is known concerning their role in plant metabolism in situ. This issue has recently been addressed at the cellular level in nonplant systems via the use of specific phosphonate inhibitors of the enzyme. Here, we describe the application of these inhibitors for the functional analysis of the potato (*Solanum tuberosum*) tuber 2-oxoglutarate dehydrogenase complex. In vitro experiments revealed that succinyl phosphonate (SP) and a carboxy ethyl ester of SP are slow-binding inhibitors of the 2-oxoglutarate dehydrogenase complex, displaying greater inhibitory effects than a diethyl ester of SP, a phosphono ethyl ester of SP, or a triethyl ester of SP. Incubation of potato tuber slices with the inhibitors revealed that they were adequately taken up by the tissue and produced the anticipated effects on the in situ enzyme activity. In order to assess the metabolic consequences of the 2-oxoglutarate dehydrogenase complex inhibition, we evaluated the levels of a broad range of primary metabolites using an established gas chromatography-mass spectrometry method. We additionally analyzed the rate of respiration in both tuber discs and isolated mitochondria. Finally, we evaluated the metabolic fate of radiolabeled acetate, 2-oxoglutarate or glucose, and ^{13}C-labeled pyruvate and glutamate following incubation of tuber discs in the presence or absence of either SP or the carboxy ethyl ester of SP. The data obtained are discussed in the context of the roles of the 2-oxoglutarate dehydrogenase complex in respiration and carbon-nitrogen interactions.

In plant heterotrophic tissues, energy metabolism is dominated by glycolysis, the tricarboxylic acid (TCA) cycle, and the mitochondrial electron transport chain (Tovar-Méndez et al., 2003; Plaxton and Podesta, 2006). However, despite the widespread adoption of reverse genetic approaches in the study of plant respiratory pathways (for review, see Fernie et al., 2004a; Sweetlove et al., 2007; Rasmusson et al., 2008), the metabolic impact of 2-oxoglutarate dehydrogenase in plants remains relatively poorly characterized. Studies in mammalian systems have firmly established that the 2-oxoglutarate dehydrogenase complex is dependent on Ca^{2+} (McCormack and Denton, 1979), is regulated by thioredoxins (Bunik, 2003), and is inactivated by oxidative species and toxins (for details, see Gibson et al., 2005; Bunik et al., 2006, 2007). Moreover, studies of the response of intact isolated nerve terminals to oxidative stress demonstrated that, although the aconitase enzyme was the most vulnerable to oxidative damage, it is the inhibition of the 2-oxoglutarate dehydrogenase complex that limits the amount of NADH available for the respiratory chain (Tretter and Adam-Vizi, 2000). Similar conclusions were also drawn following treatment of brain cells with monoamine oxidase (Kumar et al., 2003). These results suggest that in these systems the 2-oxoglutarate dehydrogenase complex exhibits a large proportion of the control resident in the TCA cycle. This hypothesis is in good accordance with the correlation of the 2-oxoglutarate dehydrogenase complex activity to flux through the TCA cycle in rat heart (Cooney et al., 1981).

While it has long been known that plant 2-oxoglutarate dehydrogenase requires thiamine pyrophosphate, NAD^+, and ADP (Bowman et al., 1976; Journet et al., 1982) and that the enzyme competes with pyruvate dehydrogenase

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* Corresponding author; e-mail fernie@mpimp-golm.mpg.de.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Alisdair R. Fernie (fernie@mpimp-golm.mpg.de).

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for intramitochondrial NAD^+ and CoA (Dry and Wiskich, 1985, 1987), there are very few studies concerning the broader metabolic function of this enzyme in plants. Among the few studies carried out, the in vitro characterization of partially purified cauliflower (*Brassica oleracea*) 2-oxoglutarate dehydrogenase revealed that it, unlike nonplant 2-oxoglutarate dehydrogenases, was activated by AMP (Wedding and Black, 1971; Craig and Wedding, 1980a, 1980b) and exhibited affinities for NAD^+ and NADH similar to other plant mitochondrial NAD^+-linked dehydrogenases (Pascal et al., 1990). The latter fact is particularly important given that 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase complex share a common subunit (E3).

In recent years, the adoption in plants of genomics, proteomics, and metabolomics approaches has provided a number of interesting observations suggesting the impact of the 2-oxoglutarate dehydrogenase complex on plant metabolism (Sweetlove et al., 2002; Balmer et al., 2004; Lemaire et al., 2004; Dutilleul et al., 2005; Fritz et al., 2006; Kolbe et al., 2006; Schneidereit et al., 2006). Several studies surveying metabolic changes of plants exposed to environmental or genetic perturbation have variously indicated important roles of 2-oxoglutarate in ammonium assimilation (Müller et al., 2001; Hodges, 2002; Hodges et al., 2003; Dutilleul et al., 2005; Fritz et al., 2006; Schneidereit et al., 2006; Noguchi and Yoshida, 2008), gibberellin biosynthesis (Hedden and Kamiya, 1997), and flavonoid and glucosinolate formation (Saito et al., 1999; Kliebenstein et al., 2001). However, as yet few studies have aimed at investigating the contribution of the 2-oxoglutarate dehydrogenase complex to the control of plant respiration. That said, recent studies have shown that the plant 2-oxoglutarate dehydrogenase complex is rapidly inhibited under oxidative stress (Sweetlove et al., 2002) and in particular by the cytotoxic lipid peroxidation product 4-hydroxy-2-nonenal (Millar and Leaver, 2000), and it is conceivable that this inhibition contributes to the reduced rates of respiration apparent under such conditions. Finally, treatment of leaf discs with dithiothreitol resulted in a decrease in the levels of organic acids involved in the first part of the TCA cycle (aconitate, isocitrate, and 2-oxoglutarate), while succinate and other intermediates of the second part of the TCA cycle increased (Kolbe et al., 2006). These changes imply an increased activity of the 2-oxoglutarate dehydrogenase complex in the presence of dithiothreitol, suggesting a redox-dependent protection of 2-oxoglutarate dehydrogenase complex in plants (Kolbe et al., 2006). This is in accordance both with earlier studies that proved that the mammalian 2-oxoglutarate dehydrogenase complex is subject to redox regulation (Bunik, 2000, 2003) and with the identification of the E2 subunit of the 2-oxoglutarate dehydrogenase complex in a proteomic survey that catalogued plant mitochondrial proteins capable of interacting with thioredoxins (Balmer et al., 2004; Lemaire et al., 2004).

In this article, we evaluate the in situ role of the 2-oxoglutarate dehydrogenase complex using phosphonate analogs of 2-oxoglutarate to specifically inhibit the reaction within intact cells (Bunik and Pavlova, 1997; Bunik et al., 2005; Santos et al., 2006). The consequences of this inhibition were monitored at the steady-state metabolite and flux levels, and the data collected are discussed in the context of current models of the metabolic regulation of respiration and amino acid metabolism in plant heterotrophic tissues.

RESULTS

The Inhibitory Effects of Phosphonate Analogs of 2-Oxoglutarate on the in Vitro Activity of the 2-Oxoglutarate Dehydrogenase Complex

Phosphonate analogs of 2-oxoglutarate have previously been demonstrated to act as highly efficient specific inhibitors of the 2-oxoglutarate dehydrogenase complex in a range of in vitro and in situ systems, including purified enzyme from rat heart or pigeon breast muscle as well as in *Escherichia coli*, fibroblasts, and cerebellar neurons (Bunik et al., 1992, 2005; Biryukov et al., 1996; Santos et al., 2006). As a first experiment, we evaluated whether the previously described analogs had similar effects on the in vitro activity of the potato (*Solanum tuberosum*) tuber 2-oxoglutarate dehydrogenase complex. We extracted the enzyme from potato tuber tissue and assayed it following the protocol of Millar et al. (1999) in the presence of varying concentrations (0–100 μM) of the inhibitors succinylphosphonate (SP), a diethyl (carboxy and phosphono) ester of SP (DESP), a phosphono ethyl ester of SP (PESP), a triethyl ester of SP (TESP), and a carboxy ethyl ester of SP (CESP). These experiments revealed that DESP and PESP were poor inhibitors of the potato tuber 2-oxoglutarate dehydrogenase complex, TESP showed an intermediary inhibition efficiency, whereas SP and CESP inhibited the enzyme activity markedly (Fig. 1). On the basis of these experiments, therefore, we decided to concentrate our studies on these two analogs. Since earlier experiments with mammalian enzyme have revealed that the phosphonate analogs of 2-oxoglutarate are slow-binding inhibitors (Bunik et al., 1991; Biryukov et al., 1996), we next analyzed the time dependence of their interaction with the potato tuber enzyme. We assayed the activity after preincubation of the enzyme with inhibitor for periods of 30 to 240 s prior to starting the reaction by addition of substrate, revealing that the phosphonate analog maximal inhibition was concentration dependent within the range tested (10–100 μM; Fig. 2).

Having established the inhibitory effects of these phosphonate analogs of 2-oxoglutarate on the in vitro activity of the 2-oxoglutarate dehydrogenase complex, we next performed a broader screen of enzymes of the TCA cycle (and associated proteins) in order to evaluate if they affected other enzymes of this pathway. We chose to evaluate the effect of high concentrations (100

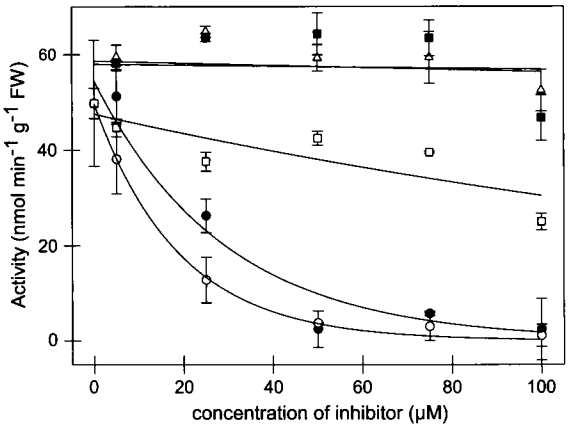

Figure 1. Effects of the concentration of SP (black circles), CESP (white circles), DESP (white triangles), PESP (black squares), and TESP (white squares) on the in vitro activity of 2-oxoglutarate dehydrogenase complex in the potato tuber extracts. The enzyme was assayed in the presence of the indicated concentrations of each inhibitor as described in "Materials and Methods." Each incubation was performed in four biological replicates, and data presented are means ± SE of these replicates. FW, Fresh weight.

μM) of the inhibitors on activities of the mitochondrial enzymes transforming organic acids that are structural analogs of 2-oxoglutarate: pyruvate dehydrogenase complex, citrate synthase, aconitase, isocitrate dehydrogenase, succinyl-CoA ligase, and malate dehydrogenase, as well as the important enzymes of nitrate metabolism Glu synthase (GOGAT) and Glu dehydrogenase (Table I). Neither SP nor CESP affected the activities of any of these enzymes. We additionally assayed all of the enzymes in the absence of their described substrates to verify that they were incapable of utilizing either of the phosphonate analogs as substrate. In no instance was a significant transformation of the phosphonate detected (data not shown).

The Inhibitory Effects of Phosphonate Analogs of 2-Oxoglutarate on the in Situ Activity of the 2-Oxoglutarate Dehydrogenase Complex

The fact that the above in vitro studies revealed that SP and CESP were potent inhibitors of the 2-oxoglutarate dehydrogenase complex activity suggests that they have the potential to be valuable tools for studying the metabolic impact of the function of this enzyme in situ. We next performed feeding experiments in which plant material was incubated in the presence or absence of 100 μM SP or CESP over a period of 4 h to evaluate whether we could effectively apply inhibitors to tuber tissue. For this purpose, we used discs isolated from growing potato tubers, since these are well documented to be highly homogenous and to provide a good model system for a highly metabolically active sink tissue (Geigenberger et al., 2000). Discs were sampled, rapidly washed, and snap frozen at time points during their incubation in phosphonate-supplemented medium, and the effect of the inhibitors was assessed by measuring the activity in extracts of these samples. These studies revealed that incubation of the discs with either SP or CESP effectively decreased the (subsequently extracted) 2-oxoglutarate dehydrogenase complex activity. After 1 h, the activity in the SP-fed discs was depleted to one-third of the control activity (Fig. 3A), while that in CESP-fed discs was reduced to approximately 40% of the control level (Fig. 3B), with levels after 4 h falling to an even greater extent.

Consequences of Inhibition of the 2-Oxoglutarate Dehydrogenase Complex on the Rate of Respiration

In order to gauge the effect of inhibition of the 2-oxoglutarate dehydrogenase complex on the rate of respiration, we performed an experiment wherein we incubated tuber discs in an oxygen electrode in the presence and absence of the inhibitors. The inhibitors decreased respiration rate over the entire observation period; however, the effect was more pronounced following 2 h of incubation, although after 3 h of incubation it was again less pronounced, suggesting an induction of compensatory mechanisms (Fig. 4). As has been shown previously, this is to meet the respiratory requirements imposed by the tuber wounding upon disc preparation (Kahl, 1974; Burton, 1989; Geigenberger et al., 2000). Remarkably, not only the respiration itself, but also this respiratory burst in response to wounding, was significantly slowed down by the phosphonates (Fig. 4).

In a complementary approach, we evaluated the rate of $^{14}CO_2$ evolution following incubation of potato tuber discs in [1,2-^{14}C]acetate or [1-^{14}C]2-oxoglutarate in the presence or absence of either SP or CESP (Fig. 5). We chose to use both label sources because 2-oxoglutarate is the direct substrate of the reaction but acetate is more often used and hence better characterized in studies of plant respiratory metabolism (Canvin and Beevers, 1961; Hooks et al., 2007). Both inhibitors resulted in

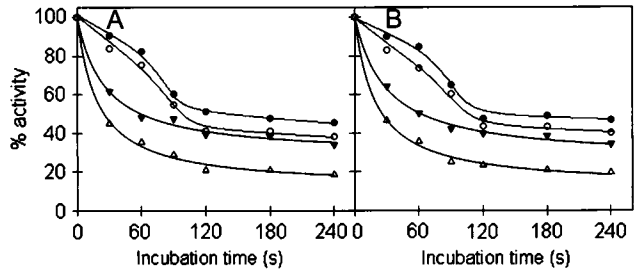

Figure 2. In vitro inhibition of 2-oxoglutarate dehydrogenase complex activity by SP (A) or CESP (B). 2-Oxoglutarate dehydrogenase complex activity was measured in the supernatant following centrifugation of the resultant extract, which was incubated with 10 μM (black circles), 25 μM (white circles), 50 μM (black triangles), or 100 μM (white triangles) SP or CESP for up to 240 s. At the times indicated, the enzymatic reaction was started by the addition of 1 mM sodium 2-oxoglutarate. Each value is the mean of four biological replicates.

Table I. *Enzyme activity determination of key enzymes in the absence (control) and presence of 100 μM SP or CESP*

Tuber tissue was harvested from 10-week-old nonsenescent plants. Values are presented as means ± SE of four biological replicates.

Enzyme	Control	SP	CESP
	nmol min^{-1} g^{-1} fresh weight		
Aconitase	15.0 ± 1.3	14.5 ± 3.5	14.8 ± 2.6
Citrate synthase	40.5 ± 4.2	36.2 ± 4.6	37.5 ± 4.4
Glu dehydrogenase	412.6 ± 20.2	381.6 ± 28.3	477.4 ± 18.4
NAD-dependent isocitrate dehydrogenase	37.9 ± 1.6	34.8 ± 2.1	36.8 ± 1.9
Succinyl-CoA ligase	47.7 ± 3.4	43.0 ± 5.9	45.9 ± 2.8
GOGAT	21.0 ± 1.2	21.4 ± 1.9	22.9 ± 2.8
	μmol min^{-1} g^{-1} fresh weight		
Malate dehydrogenase	20.4 ± 3.2	24.3 ± 4.3	28.5 ± 4.0
Pyruvate dehydrogenase	18.2 ± 0.5	14.8 ± 2.2	13.9 ± 2.48

the reduction of $^{14}CO_2$ evolution following incubation in [1-^{14}C]2-oxoglutarate; however, at 2- and 3-h time points this inhibition was somewhat greater in the samples incubated in [1,2-^{14}C]acetate.

In a further approach, we isolated mitochondria following the protocol of Giegé et al. (2003) and measured their rate of respiration, on provision of citrate, 2-oxoglutarate, or succinate as substrate, in the presence or absence of the inhibitors (Table II). In both instances, the inhibitors decreased the rate of oxygen consumption when citrate or 2-oxoglutarate was supplied as respiratory substrate but had no effect when succinate was supplied. These results thus confirm the findings of the above experiments and provide further evidence for the specificity of the inhibition.

Consequences of Inhibition of the 2-Oxoglutarate Dehydrogenase Complex on Other Cellular Fluxes

In order to assess the general effects of inhibition of the 2-oxoglutarate dehydrogenase complex, we next analyzed the metabolic fate of [U-^{14}C]Glc supplied to isolated tuber discs. For this purpose, we preincubated tuber discs in 10 mM MES-KOH (pH 6.5) containing 2 mM Glc and 10, 25, 50, or 100 μM SP or CESP for 1 h and subsequently supplemented with [U-^{14}C]Glc (specific activity of 8.11 MBq mmol^{-1}) for a period of 1, 2, or 3 h. The presence of inhibitor had dramatic consequences at each time point, so for the sake of simplicity we only present the data obtained from the 2-h treatment (Fig. 6). Both SP and CESP treatments displayed a strong concentration-dependent decrease in the rate of Glc uptake (Fig. 6A). This was coupled to a dramatic decrease in $^{14}CO_2$ evolution (Fig. 6B) as well as decreases in the radiolabel incorporations in Suc (Fig. 6C), protein (Fig. 6D), and cell wall (Fig. 6F). Conversely, there was a minor increase in label incorporation in starch following incubations of low concentrations of the inhibitors (Fig. 6E); however, partitioning to other cellular components was unaltered (Supplemental Fig. S1).

Given that interpretation of the distribution of radiolabel can be complicated by differential mobilization of internal, unlabeled storage reserves (Geigenberger et al., 1997), we next measured the levels of phosphate esters in these samples. Following these measurements, we were able to determine that the specific activity of this pool, in the presence of SP and CESP, was significantly lower than that observed in the untreated control. However, estimation of the absolute fluxes revealed that there were no changes in the rates of Suc biosynthesis or glycolysis but minor yet significant decreases in the flux to starch coupled with a considerable reduction in the flux to cell wall (Supplemental Fig. S1).

Consequences of Inhibition of the 2-Oxoglutarate Dehydrogenase Complex on Primary Metabolism in Potato Tuber Tissue

We next utilized a gas chromatography-mass spectrometry (GC-MS)-based metabolic profiling method (Fernie et al., 2004b) to quantify the relative metabolite levels following incubation in buffer in the presence or absence of 100 μM SP or CESP for 1, 2, or 3 h. The data obtained are displayed in false color in the heat map of Figure 7 in order to provide an easy overview (the full data set is additionally available as Supplemental Table S1). From this display, it is notable that there were considerable changes in the levels of metabolites even in the absence of inhibitor. However, with the exception of the 3- and 4-fold increases in glycerol and guanidine and the 70% reduction in glucaric acid-1,4-lactone, these were relatively minor and very few of these changes were statistically significant. The changes in metabolite profiles were, by and large, qualitatively and quantitatively similar between the inhibitors, which is in good accordance with the similar level of enzyme inhibition displayed by the phosphonates (Fig. 3). Furthermore, inhibition of the 2-oxoglutarate dehydrogenase complex was evident from the fact that 2-oxoglutarate was significantly elevated at all time points following incubation with SP or CESP. Despite the large increases in Ala, glycerol, guanadine, and 2-oxoglutarate described above, there was a general trend toward a decrease in metabolite

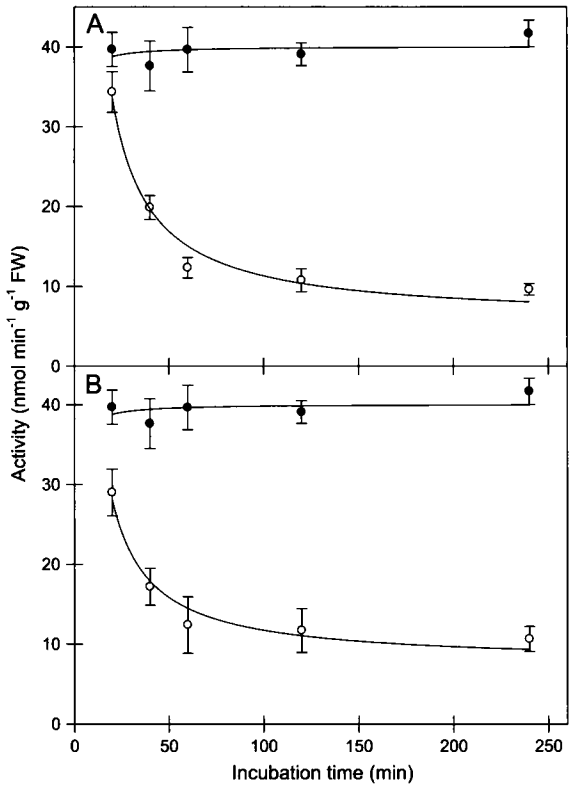

Figure 3. Effects of SP (A) or CESP (B) preincubation with the potato tuber discs on the extracted 2-oxoglutarate dehydrogenase complex activity. Potato tuber discs were incubated in 10 mM MES-KOH (pH 6.5) with 100 μM SP or CESP for up to 4 h. The control (black circles) was incubated in the absence of inhibitor. At the times indicated, the tuber discs were washed with 10 mM MES-KOH (pH 6.5) to remove excess inhibitors and then homogenized. 2-Oxoglutarate dehydrogenase complex activity of the extracts was measured in the standard assay medium without the inhibitors. Each value is the mean ± SE of four biological replicates. FW, Fresh weight.

levels. Within the TCA cycle, the intermediates citrate, isocitrate, and malate were generally, although not exclusively, decreased, whereas the level of fumarate remained constant. The levels of Gln were dramatically reduced following incubation (significantly so in all instances), declining to as low as 7% of those at the start of the incubation. Similarly, the levels of Asn fell sharply following incubation with either analog (although the difference in metabolite levels was only significant during the first hour of incubation, most probably due to a general decline in the level of this metabolite following incubation), and there was a parallel decrease in the levels of Lys in these samples. The levels of succinate were also elevated after inhibition of the 2-oxoglutarate dehydrogenase complex, albeit only following 2- or 3-h incubations. The most likely explanation for this observation is as a compensatory up-regulation of other pathways of succinate production, as was observed previously on the antisense inhibition of succinyl-CoA ligase (Studart-Guimarães et al., 2007). Intriguingly, the most prominent changes in the data set were observed for the levels of Ala, which increased more than 5-fold following incubation in SP and more than 7-fold following incubation in CESP, most likely reflecting an increased anaerobic fermentation in the treated samples. Looking at metabolites less intimately associated with mitochondria revealed a general decrease in Phe as well as an exacerbated reduction in the level of Glc. Furthermore, perhaps as a consequence of the latter, levels of galactinol were significantly elevated following both 2- and 3-h incubations in the presence of either inhibitor (Supplemental Table S1).

The close agreement of the results, irrespective of the phosphonate inhibitor applied, alongside the fact that the inhibitors do not affect other enzymes of the TCA cycle afford some confidence in their potential for the evaluation of the 2-oxoglutarate dehydrogenase complex in respiration. That said, as is always the case with experiments using pharmacological inhibitors, we cannot rule out the possibility that they could have potential secondary effects. For this reason, we carried out a broad correlation analysis in an attempt to determine which changes were most closely associated with the change in 2-oxoglutarate activity. Given that the mechanism of the inhibitors renders it highly difficult to infer the exact extent to which the enzyme is inhibited, we instead performed this analysis by correlating the relative levels of each metabolite to the relative levels of 2-oxoglutarate in all experimental samples. When evaluating the strength of these correlations and their significances, it becomes apparent that only 10 of the metabolic changes (those in Glc, inositol, γ-aminobutyrate [GABA], glutarate, Met, Ser, Glu, Gln, malate, and succinate) are closely associated

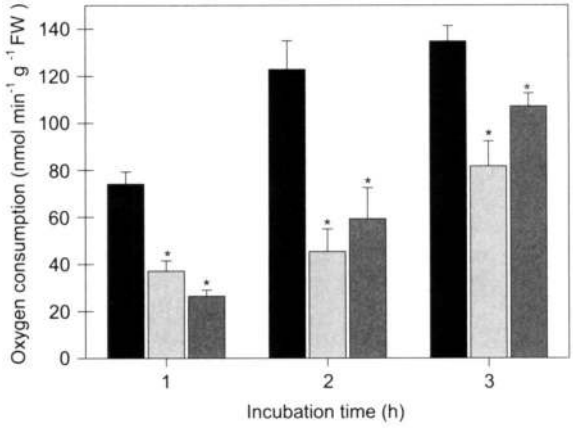

Figure 4. Respiration of tuber discs incubated in the absence (black bars) or presence of 100 μM SP (gray bars) or CESP (dark gray bars). Freshly prepared potato tuber slices were transferred into the temperature-controlled measuring chamber of an oxygen electrode containing 1 mL of 10 mM MES-KOH, pH 6.5. Each value is the mean ± SE of four biological replicates. The asterisks demarcate values that were judged to be significantly different from the control ($P < 0.05$) following the performance of Student's *t* tests. FW, Fresh weight.

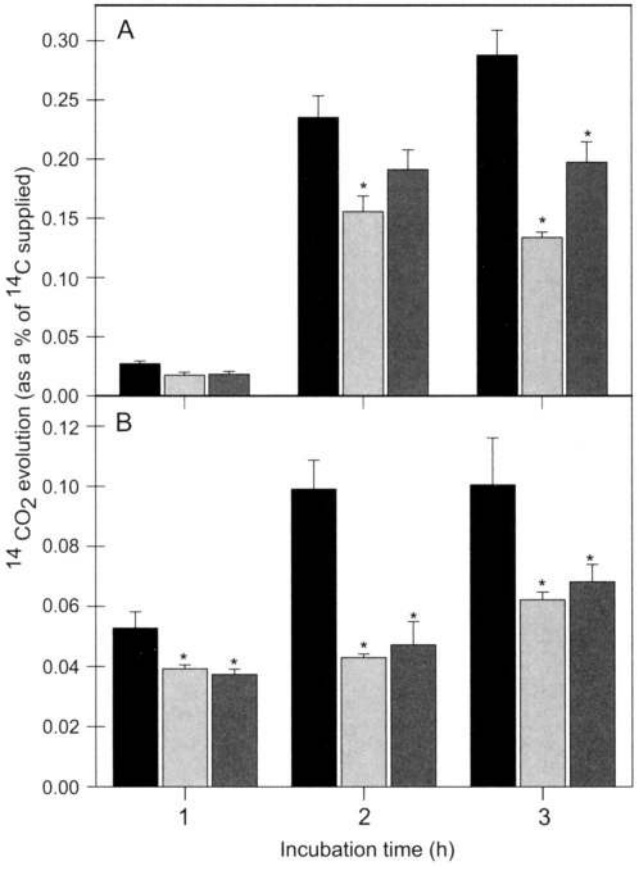

Figure 5. $^{14}CO_2$ evolution following incubation of potato tuber discs in [1-^{14}C]2-oxoglutarate (A) or [1,2-^{14}C]acetate (B) in the absence (black bars) or presence of 100 μM SP (gray bars) or CESP (dark gray bars). Each value is the mean ± SE of four biological replicates. The asterisks demarcate values that were judged to be significantly different from the control ($P < 0.05$) following the performance of Student's *t* tests.

to the change in 2-oxoglutarate concentration (Table III).

In order to provide corroborative evidence for some of the changes highlighted above, we performed three further experiments. Namely, in order to gain further evidence for a restriction in nitrate assimilation, we directly determined the level of this ion following incubation in the phosphonate analogs, while we also assessed the fermentative and GABA shunt fluxes by following the metabolic fate of ^{13}C-labeled substrates. As can be seen in Figure 8, following incubation with both inhibitors, the total steady-state cellular nitrate content was higher than that of the control.

Give that the levels of nitrate increased during the treatment, it seems likely that nitrate is taken up from the incubation medium, since MES buffer is a rich source of nitrate. In addition, we incubated tuber discs in the presence or absence of the analogs in either [^{13}C]pyruvate (in order to address the flux through fermentation) or [^{13}C]Glu (in order to address the flux through the GABA shunt). In both instances, the rate of label transfer between representative metabolite pairs (pyruvate to Ala and Glu to succinate) revealed that these fluxes were indeed increased in the presence of the inhibitors, confirming the importance of the 2-oxoglutarate dehydrogenase complex in these metabolic pathways (Fig. 9).

DISCUSSION

Inhibition of the Plant 2-Oxoglutarate Dehydrogenase Complex with Phosphonates

As part of an ongoing project, we have in recent years assessed the metabolic role of the component enzymes of the mitochondrial TCA cycle, paying particular attention to their influence both on the rate of respiration and on primary metabolism in general. The aim of this work was to characterize the importance of the 2-oxoglutarate dehydrogenase complex in plant heterotrophic metabolism by following the consequences of its in situ inhibition in potato tubers. These phosphonate analogs have previously been studied in animal and *E. coli* cells, cellular homogenates, and using the purified complex itself (Bunik et al., 1992, 2005; Biryukov et al., 1996). They inhibit the 2-oxoglutarate dehydrogenase complex in a mechanism-based manner by targeting the catalytic center (i.e. the C2 atom of the thiamine diphosphate coenzyme, which is responsible for the high specificity of the inhibition;

Table II. *Analysis of mitochondrial respiration rates of isolated potato mitochondria utilizing different substrates in the absence (control) or presence of 100 μM SP or CESP*

Oxygen consumption was determined in mitochondria isolated from tubers of 10-week-old nonsenescent plants. Values are presented as means ± SE of three independent mitochondrial isolations. The values that are significantly different from the control ($P < 0.05$), following the performance of Student's *t* tests, are shown in boldface type.

Substrate	O_2 Consumption		
	Control	SP	CESP
	nmol O_2 min^{-1} mg^{-1} protein		
Succinate	220.17 ± 6.61	191.74 ± 8.18	198.55 ± 6.85
Citrate	154.72 ± 1.76	**92.14 ± 3.27**	**92.35 ± 2.97**
2-Oxoglutarate	208.41 ± 9.71	**133.15 ± 16.93**	**108.77 ± 2.04**

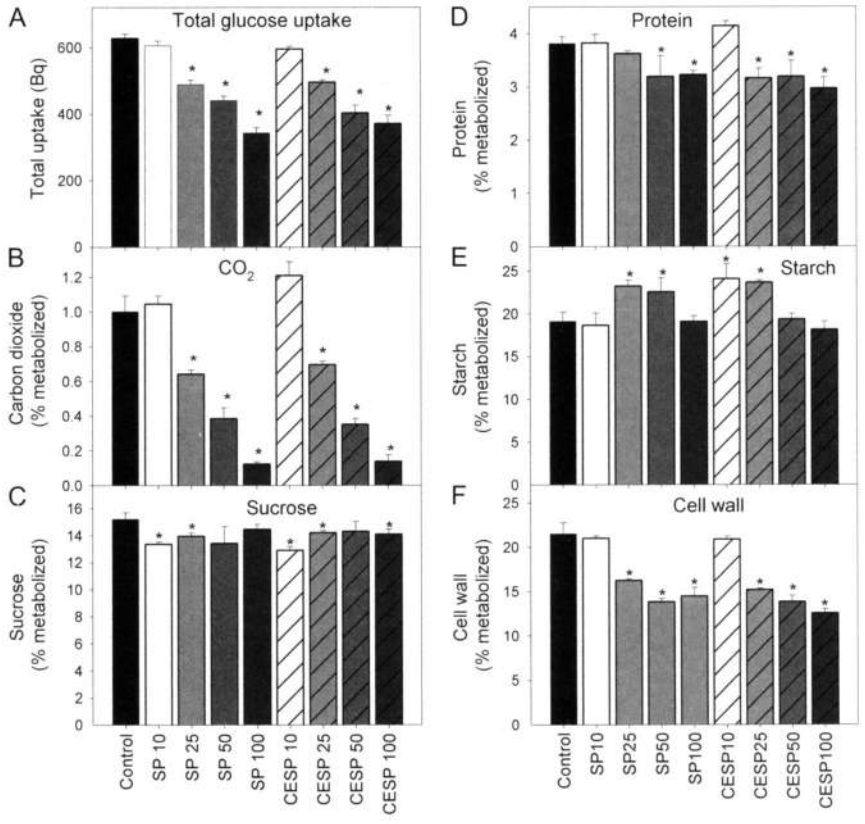

Figure 6. Effects of SP or CESP on metabolism of [U-^{14}C]Glc by potato tuber discs. Tuber discs were preincubated in 10 mM MES-KOH (pH 6.5) containing 2 mM Glc in the absence (control) or presence of varying concentrations of SP or CESP for 1 h, and then [U-^{14}C]Glc (specific activity of 8.11 MBq mmol^{-1}) was added. Each sample was extracted with boiling ethanol, and the amount of radioactivity in each metabolic fraction was determined as described in "Materials and Methods." Values are expressed as percentages of the total radiolabel metabolized and are means ± SE of three biological replicates. The asterisks demarcate values that were judged to be significantly different from the control ($P < 0.05$) following the performance of Student's *t* tests.

Bunik et al., 2005). It is worth noting that in neither instance did the time dependence of inhibition change linearly with increasing phosphonate concentration. While at high concentrations (50–100 μM) the activity decays according to the exponential law (as observed previously for the phosphonate analog of the pyruvate dehydrogenase substrate; Schönbrunn-Hanebeck et al., 1990), deviations from the exponential decay are obvious at low concentrations (10–25 μM; Fig. 2). This lag period, however, is undetectable at high inhibitor concentrations (50–100 μM) and as such is reminiscent of the mechanism of inhibition previously described for the 2-oxo analogs of 2-oxoglutarate in nonplant systems (Bunik et al., 2000). When taken together, these observations provide a likely mechanism to explain the nonlinear nature of the time-dependent inhibition on the inhibitor concentration (Fig. 2). Further kinetic studies of the interaction, however, were beyond the goal of this work, the focus of which was rather to characterize the effects of inhibiting the plant 2-oxoglutarate dehydrogenase complex in situ. Such studies using the phosphonate analogs have greatly enhanced the understanding of the role of the 2-oxoglutarate dehydrogenase complex in the central metabolism of animal cells. A structurally distinct fluorinated derivative of 2-oxoglutarate, 2,2-difluoropentanedioic acid, has additionally been used in the study of cyanobacterial function (Laurent et al., 2005). However, this previous study was largely concerned with a signaling function for 2-oxoglutarate, using the analog to investigate the function of the metabolite itself rather than the enzymes that utilize it. In order to specifically address the metabolic role of the 2-oxoglutarate dehydrogenase complex, we first surveyed the effectiveness of a range of previously used inhibitors in the plant system, since the formation and stability of the phosphonate adducts are highly conditional (Bunik et al., 2005). Indeed, the selection of SP and CESP for further studies of the potato tuber enzyme provides further support for this hypothesis, since it indicates that the potato tuber enzyme demonstrates different structural requirements than those described previously (Bunik et al., 1991, 2000, 2005; Biryukov et al., 1996). To corroborate the specific action of the phosphonate analogs of 2-oxoglutarate, we demonstrated that neither inhibitor had any effect on Glu dehydrogenase, GOGAT, or any enzymes of the TCA cycle that we tested, nor were they transformed by these enzymes (Table I). Furthermore, we showed that the inhibitors were readily taken up by the potato tuber discs (Fig. 3). Since current technologies do not allow us to comprehensively characterize the effect of the phosphonates on the activity of every enzyme of the cell, we cannot formally exclude the possibility that they could also produce side effects elsewhere in metabolism. For this reason, the interpretation of the results of such experiments needs to be made with caution. That said, when taken together, the results from both in vitro and in situ experiments indicate that both SP and CESP are appropriate for the study of a

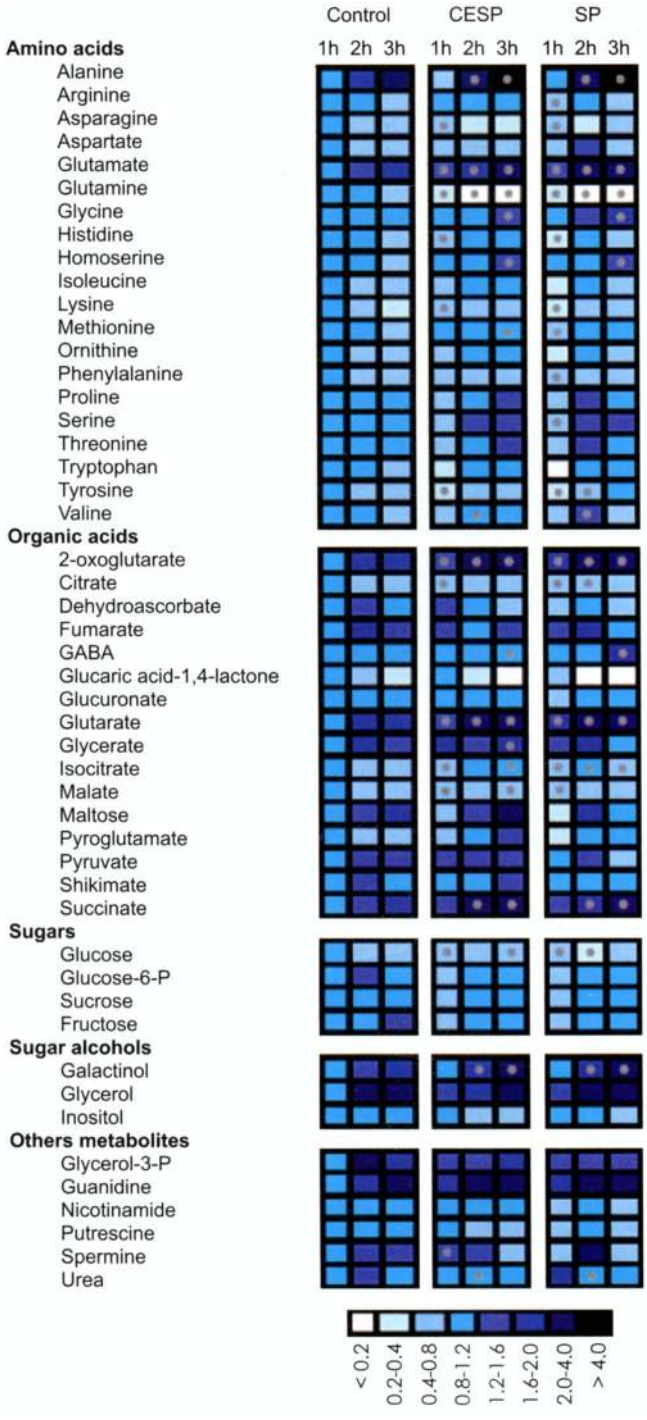

Figure 7. Heat map representing the changes in relative metabolite contents of treated and control tuber discs. Tuber discs were cut directly from growing tubers washed three times with 10 mM MES-KOH (pH 6.5) and then incubated for up to 3 h in 10 mM MES-KOH buffer (pH 6.5) containing 2.0 mM Glc and 100 μM SP or CESP. Metabolites were determined as described in "Materials and Methods." Data are normalized with respect to the mean response calculated for the control at 1 h; values presented are means of four biological replicates. The asterisks demarcate values that were judged to be significantly different from the control ($P < 0.05$) at the same time point following the performance of Student's t tests.

short-term specific inhibition of the 2-oxoglutarate dehydrogenase complex in the potato tuber.

The Plant 2-Oxoglutarate Dehydrogenase Complex Has a Large Role in the Regulation of Respiration

We demonstrate here that the inhibition of the 2-oxoglutarate dehydrogenase complex had a far greater consequence on the rate of respiration than the transgenic inhibition of the mitochondrial isoforms of malate dehydrogenase (Nunes-Nesi et al., 2005), fumarase (Nunes-Nesi et al., 2007), citrate synthase (Sienkiewicz-Porzucek et al., 2008), and succinyl-CoA ligase (Studart-Guimarães et al., 2007) or the reduced activities of the NAD-dependent isocitrate dehydrogenase (Lemaitre et al., 2007) and aconitase (Carrari et al., 2003) in mutants and transgenics of Arabidopsis (*Arabidopsis thaliana*) and tomato (*Solanum lycopersicum*), respectively. Given that we demonstrated that the phosphonates had no impact on other enzymes of the TCA cycle, this allows us to draw important conclusions concerning the prime importance of the reactions catalyzed by this complex in comparison with other enzymes of the cycle. This hypothesis is further supported by the reduced $^{14}CO_2$ evolution from potato tuber discs incubated in [^{14}C]acetate or [^{14}C]2-oxoglutarate and [^{14}C]Glc following inhibition of the 2-oxoglutarate dehydrogenase complex. Quantitative differences, however, were observed in the level of inhibition obtained depending on the substrate fed. There are two possible explanations for this observation. First, it may merely reflect the differences in the labeling of the substrate. Second, it may also indicate a reduced flux through the reaction catalyzed by isocitrate dehydrogenase. However, it is important to note that if the latter is true, this is due to an effect on the 2-oxoglutarate dehydrogenase complex per se and is not merely a consequence of a direct inhibition of isocitrate dehydrogenase. Indeed, Table I shows no inhibition of NAD-dependent isocitrate dehydrogenase by the phosphonates. On the other hand, the inhibited flux through isocitrate dehydrogenase, which produces 2-oxoglutarate, seems a logical consequence under conditions under which 2-oxoglutarate accumulates due to the inhibition of the 2-oxoglutarate dehydrogenase complex. These data were supported by respiration measurements carried out on isolated mitochondria, which revealed that inhibition of the complex resulted in reduced oxygen consumption when citrate or 2-oxoglutarate was provided as respiratory substrate but not when succinate was supplied (Table II). The rate of respiration was not the only metabolic flux affected, with considerable decreases being seen in cell wall biosynthesis and minor decreases observed in both starch and protein biosynthesis (Fig. 6). These changes are most likely a result of the massive decrease in the rate of the respiration. The fact that cell wall biosynthesis is decreased to a greater extent than the other processes is intriguing and suggests that the cell prioritizes protein and starch over

Table III. *Pairwise correlation coefficients, with their corresponding P values, calculated between the relative metabolite contents of 2-oxoglutarate and all other metabolites*

Correlations were determined using a combined data set including data obtained from treated and control tuber discs. ns, Not significant; *, significant at $P < 0.05$; **, significant at $P < 0.01$.

Metabolite	r	P	Significance
Amino acids			
Ala	−0.1845	0.9660	ns
Arg	0.1055	0.4567	ns
Asn	0.0425	0.7647	ns
Asp	−0.0477	0.7372	ns
Glu	**0.8594**	**3.50E-16**	**
Gln	**0.2870**	**0.0391**	*
Gly	0.2163	0.1234	ns
His	−0.0088	0.9509	ns
Homo-Ser	0.0965	0.4960	ns
Ile	0.1137	0.4223	ns
Lys	−0.1549	0.2730	ns
Met	**0.3003**	**0.0306**	*
Orn	0.0815	0.5656	ns
Phe	−0.0600	0.6726	ns
Pro	0.2315	0.0987	ns
Ser	**−0.2896**	**0.0373**	*
Thr	0.2692	0.0536	ns
Trp	0.1544	0.2745	ns
Tyr	0.1684	0.2328	ns
Val	0.1629	0.2487	ns
Organic acids			
Citrate	−0.2240	0.3510	ns
Dehydroascorbate	−0.2889	0.0378	ns
Fumarate	0.0227	0.8733	ns
GABA	**−0.6704**	**1.86E-07**	**
Glucaric acid-1,4-lactone	−0.1008	0.4772	ns
Glucuronate	−0.0385	0.7867	ns
Glutarate	**0.9803**	**1.4E-16**	**
Glycerate	0.1326	0.3487	ns
Isocitrate	0.0364	0.8125	ns
Malate	**−0.3998**	**6.52E-03**	**
Maltose	0.1014	0.5074	ns
Pyro-Glu	0.3412	0.0133	ns
Pyruvate	0.1261	0.4092	ns
Shikimate	0.1341	0.3799	ns
Succinate	**0.9379**	**1.85E-04**	**
Sugars			
Glc	**−0.5971**	**2.38E-05**	**
Glc-6-P	−0.1144	0.4192	ns
Suc	−0.0657	0.6435	ns
Fru	−0.1324	0.3493	ns
Sugar alcohols			
Galactinol	−0.2378	0.0896	ns
Glycerol	−0.0438	0.7578	ns
Inositol	**−0.3708**	**6.81E-03**	**
Other metabolites			
Glycerol-3-phosphate	−0.0569	0.6888	ns
Guanidine	−0.0323	0.8200	ns
Nicotinamide	−0.1621	0.2508	ns
Putrescine	−0.1763	0.2113	ns
Spermine	−0.1790	0.2042	ns
Urea	0.0118	0.9336	ns

growth under these circumstances. However, the data presented here do not allow us to speculate further on this. In addition to the changes in steady-state fluxes, tissue incubated with inhibitor displayed a clearly reduced increase in respiratory activity over time coupled to the decreased levels of Phe at latter stages of the experiment, suggesting a regulatory role for the 2-oxoglutarate dehydrogenase complex within the ox-

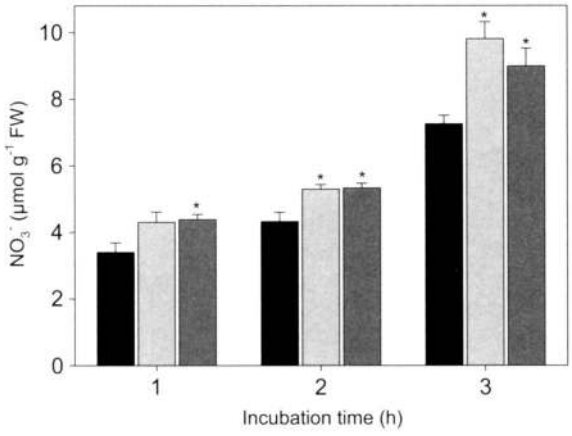

Figure 8. Effects of SP or CESP on nitrate content following incubation of potato tuber discs in the absence (black bars) or presence of 100 μM SP (gray bars) or CESP (dark gray bars). Each value is the mean ± SE of four biological replicates. The asterisks demarcate values that were judged to be significantly different from the control ($P < 0.05$) following the performance of Student's *t* tests. FW, Fresh weight.

idative burst that has been well defined to be associated with the wounding response of potato tuber tissue (Kahl, 1974).

Mitochondrial 2-Oxoglutarate Is Utilized during the Process of Nitrate Assimilation

While it was relatively easy to ascribe the changes in respiration to be a direct consequence of the inhibition of the 2-oxoglutarate dehydrogenase complex, this is not so straightforward in the case of the metabolite and flux profiling data. Since we cannot rule out the notion that the inhibitors have side effects in some of the other pathways we are looking at, we took a correlation analysis approach to look for metabolites that change in a linear fashion with changes in 2-oxoglutarate levels. This approach was taken since the fact that the 2-oxoglutarate dehydrogenase complex displays complex inhibitory kinetics renders it far from easy to determine the exact degree of inhibition of the enzyme itself; thus, we used the levels of its substrate as a proxy for the degree of inhibition. While this method is likely to reveal several changes that are directly related to the inhibition, it is unlikely to reveal them all, since nonlinear relationships are commonplace in metabolism. Taking a cautious approach, we will only discuss those metabolites that respond in a linear fashion; however, it is likely that a future reverse genetic approach may provide evidence that some of the other changes are also direct. Evaluation of the metabolites that correspond with the cellular levels of 2-oxoglutarate in the mitochondria revealed three sets of compounds: not surprisingly, those intimately involved in respiration (malate, succinate, GABA, and glutarate), a handful of amino acids (Glu, Gln, Met, and Ser), and two sugars/sugar derivatives (Glc and inositol phosphate). When taken together, several important conclusions can be made from these data. First, they clearly indicate that, at least in this tissue, the 2-oxoglutarate dehydrogenase complex activity plays an important role in modulating the rate of flux from 2-oxoglutarate into amino acid metabolism. This was evident from the responses of the levels of both Glu and Gln but also in Ser and Met, supporting the developing view that amino acid metabolism is a tightly controlled network (Coruzzi and Last, 2000; Foyer et al., 2003; Sweetlove and Fernie, 2005; Less and Galili, 2008). The clear impact that reducing tissue 2-oxoglutarate dehydrogenase complex activity has on carbon-nitrogen interactions contrasts somewhat with the long-held view that NAD-dependent isocitrate dehydrogenase is the major regulatory step at the juncture of the TCA cycle and nitrogen assimilation (Dry and Wiskich, 1985; Nichols et al., 1994; Cornu et al., 1996; Gálvez et al., 1999; Igamberdiev and Gardeström, 2003), since it clearly implies the additional importance of the 2-oxoglutarate dehydrogenase complex in both pro-

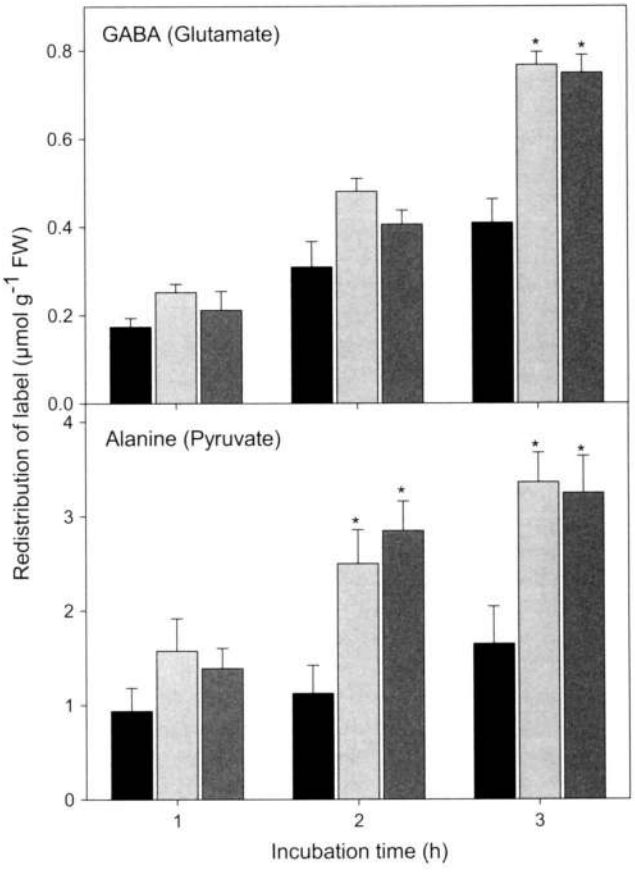

Figure 9. Redistribution of label following incubation of potato tuber discs in 10 mM [^{13}C]Glu or [^{13}C]pyruvate in the absence (black bars) or presence of 100 μM SP (gray bars) or CESP (dark gray bars). The substrate is indicated in parentheses following the name of the product. Each value is the mean ± SE of four biological replicates. The asterisks demarcate values that were judged to be significantly different from the control ($P < 0.05$) following the performance of Student's *t* tests. FW, Fresh weight.

cesses. Measurement of nitrate levels in tuber discs following inhibition of the 2-oxoglutarate dehydrogenase complex further supported this conclusion, since the ion increases considerably following inhibition (Fig. 8). A recent study of Arabidopsis knockouts of various subunits of NAD-dependent isocitrate dehydrogenase revealed that this isoform was quantitatively more important in plant metabolism under heterotrophic conditions (Lemaitre et al., 2007). When taken together, these studies suggest that the activities of both enzymes are crucial in determining the relative partitioning between these metabolic pathways. These findings thus reveal the importance of mitochondrial reactions involving 2-oxoglutarate and by implication of the mitochondrial membrane protein, which mediates its transport into the cytosol, such as that characterized by Picault et al. (2002).

Looking specifically to the role of the 2-oxoglutarate dehydrogenase complex in the TCA cycle, we found that inhibition of this step apparently evokes an upregulation of the GABA shunt that presumably also explains the increased levels of succinate. Remarkably, a similar conclusion was made following the inhibition of the 2-oxoglutarate dehydrogenase complex in neurons (Santos et al., 2006). We have additionally documented such a relationship previously in transgenic plants exhibiting antisense repression of succinyl-CoA ligase (Studart-Guimarães et al., 2007) and have furthermore recently speculated in detail on the general importance of the GABA shunt for the respiratory process (Fait et al., 2008). The findings of this study reveal that inhibition of succinate production can be compensated even within the short term with an elevated flux from Glu to succinate apparent in our ^{13}C feeding experiments. These data suggest that this compensation is likely to occur at the posttranslational level, most probably at the level of allostery or transaminase reactions. Conversely, as would perhaps be anticipated, the level of malate decreased on inhibition of the 2-oxoglutarate dehydrogenase. Keeping with the theme of energy metabolism, the coregulation of 2-oxoglutarate with glutarate, which is a breakdown product of Lys degradation, is intriguing, since it suggests the mobilization of Lys as an alternative respiratory substrate at very short time points. Further evidence for a modification in energy metabolism was provided by flux measurements, which revealed that inhibition resulted in an increase in the fermentative flux between pyruvate and Ala, although it is important to note that this was not supported in our correlation analysis. The other conserved metabolite changes observed following inhibition are currently less easy to explain. While we can speculate that the increased level of Met may be due to an increased degradation of protein to compensate for the block in the TCA cycle, the lack of concerted changes in the levels of the other amino acids suggests that the metabolite profiles are not diagnostic of such a switch in metabolism (for details, see Ishizaki et al., 2005, 2006). Similarly, the decreased levels of Ser may be indicative of its breakdown. However, further experimentation is clearly required in order to completely understand the full complexity and diversity of plant respiratory substrates (Rasmusson et al., 2008) before we can assess the quantitative importance of such changes. Similarly, the reason for the coregulation of 2-oxoglutarate levels with those of Glc and inositol are unclear at the moment, despite the fact that these changes probably explain the observed reduction in the rate of cell wall biosynthesis.

In conclusion, we have shown here that the phosphonate analogs of 2-oxoglutarate, SP and CESP, are efficient tools for probing the metabolic impact of the 2-oxoglutarate dehydrogenase complex function in plants. Inhibition of the enzyme using these analogs resulted in a dramatic reduction of the rate of respiration, coupled to alterations in levels of intermediates of the TCA cycle and amino acids crucial to nitrate assimilation. When compared with the results of previous studies, these findings indicate that 2-oxoglutarate plays a critical regulatory role in the rate of respiration. These findings may be of particular pertinence given the reasonable indirect evidence suggesting that the plant enzyme is subject to complex metabolic regulation at both allosteric and posttranslational levels (Craig and Wedding, 1980a; Millar and Leaver, 2000; Balmer et al., 2004) in a manner analogous to that characterized in animal systems (Bunik et al., 2000). Given that the plant 2-oxoglutarate dehydrogenase complex had additionally been reported to be a target of oxidative stress in plants (Sweetlove et al., 2002), a fuller understanding of the consequences of its inhibition is also of likely agronomic importance. While the observations of this study clearly have important implications for the understanding of primary metabolism in plant heterotrophic tissues, questions concerning its role in photosynthetically active tissues or in plant development in general remain to be addressed in future studies.

MATERIALS AND METHODS

Materials

Potato plants (*Solanum tuberosum* 'Desirée'; Saatzucht Fritz Lange) were grown in well-aerated soil (3-L pots) supplemented with Hakaphos grün slow-release fertilizer (100 g per 230 L of soil; BASF) in a greenhouse during the summer (16 h of light/8 h of dark, 20°C/18°C day/night, 60% relative humidity) with supplementary light as described by Bologa et al. (2003). Growing tubers from 10-week-old plants watered daily with high activities of Suc synthase, which is taken as an indicator for rapidly growing tubers (Merlo et al., 1993), were used for the experiments. All biochemicals, substrates, cofactors, and ion-exchange resins were from Sigma-Aldrich, with the exception of lipoamide dehydrogenase from porcine heart, which was from Calzyme Laboratories. Radiochemicals were from Amersham International (http://www.amersham.com/), with the exception of [1-^{14}C]2-oxoglutarate from Perkin-Elmer. The phosphonate inhibitors were synthesized according to Bunik et al. (2005), with their identity and purity proven by NMR spectra. Mitochondria were isolated exactly as described by Giegé et al. (2003).

Enzyme Assays

Enzymes were extracted from potato tubers exactly as described by Jenner et al. (2001). The assay media used were to determine maximal catalytic

activities of the enzymes. 2-Oxoglutarate dehydrogenase activity was measured essentially following the protocol of Millar et al. (1998). In brief, 2-oxoglutarate dehydrogenase activity was measured by determining NADH formation at 340 nm in a reaction medium containing 75 mM TES-KOH (pH 7.5), 0.05% (w/v) Triton X-100, 0.5 mM $MgCl_2$, 2 mM NAD^+, 0.12 mM lithium-CoA, 0.2 mM thiamine pyrophosphate, 2.5 mM Cys-HCl, 1 mM AMP, 1 mM sodium-2-oxoglutarate, and 3 units of lipoamide dehydrogenase (as described in Millar et al., 1999). If not specified otherwise, the reaction was started by 2-oxoglutarate after the enzyme was preincubated in the reaction medium with or without inhibitors for 15 min. The initial linear part of the product accumulation curves (within 10 min of the reaction) was used to calculate the reaction rates. Pyruvate dehydrogenase was assayed as described by Randall and Miernyk (1990), Glu dehydrogenase as described by Purnell et al. (2005), aconitase as described by Carrari et al. (2003), citrate synthase as described by Gibon et al. (2004), NAD-dependent isocitrate dehydrogenase as described by Jenner et al. (2001), NADH-GOGAT as described by Chen and Cullimore (1988), and malate dehydrogenase as described by Nunes-Nesi et al. (2005).

Metabolism of [1,2-^{14}C]Acetate and [1-^{14}C]2-Oxoglutarate by Potato Tuber Discs

Tuber discs (diameter of 10 mm, thickness of 2 mm) were cut directly from growing tubers attached to the fully photosynthesizing mother plant, washed three times with 10 mM MES-KOH (pH 6.5) following a 1-h preincubation in the presence or absence of 100 μM SP or CESP, and then incubated (eight discs) in 2 mL of 10 mM MES-KOH buffer (pH 6.5) containing 1 mM acetate or 2-oxoglutarate in a 100-mL Erlenmeyer flask shaken at 90 rpm containing 0.25 μCi of [1,2-^{14}C]acetate or [1-^{14}C]2-oxoglutarate (specific activity of 2.79 or 1.53 MBq $mmol^{-1}$, respectively). The $^{14}CO_2$ liberated was captured (in hourly intervals) in a KOH trap, and the amount of radiolabel was subsequently quantified by liquid scintillation counting.

Metabolism of [^{14}C]Glc by Potato Tuber Discs

Tuber discs were cut and treated as described above with 2 mM Glc substituting for acetate/2-oxoglutarate. A 100-mL Erlenmeyer flask containing 1.00 μCi of [^{14}C]Glc (specific activity of 8.11 MBq $mmol^{-1}$) was shaken at 90 rpm. The $^{14}CO_2$ liberated was captured (in hourly intervals) in a KOH trap, and the amount of radiolabel was subsequently quantified by liquid scintillation counting. After this step, the discs were harvested, washed three times in buffer (eight discs per 100 mL), and frozen in liquid nitrogen to enable further analysis.

Extraction and Fractionation of Radiolabeled Material

Tissue was fractionated exactly as described by Fernie et al. (2001b), with the exception that hexoses were fractionated enzymatically rather than utilizing thin-layer chromatography. Labeled Suc levels were determined after 4 h of incubation of 200 μL of total neutral fraction with 4 units mL^{-1} hexokinase in 50 mM Tris-HCl, pH 8.0, containing 13.3 mM $MgCl_2$ and 3.0 mM ATP at 25°C. For labeled Glc and Fru levels, 200 μL of neutral fraction was incubated with 1 unit mL^{-1} Glc oxidase and 32 units mL^{-1} peroxidase in 0.1 M potassium phosphate buffer, pH 6, for a period of 6 h at 25°C. After the incubation time, all reactions were stopped by heating at 95°C for 5 min. The label was separated by ion-exchange chromatography as described by Fernie et al. (2001c). The reliability of these fractionation techniques has been thoroughly documented (Runquist and Kruger, 1999; Fernie et al., 2001a) previously, with the exception of the hexose fractionation. Recovery experiments performed in this study determined that the quantitative recovery of radiolabel following this novel method of hexose fractionation was acceptable (90%–105%). Fluxes were calculated as described by Fernie et al. (2001a), following the assumptions detailed by Geigenberger et al. (1997, 2000).

Metabolic Profiling

Metabolite extraction was carried out exactly as described previously (Roessner et al., 2001; Schauer et al., 2006). As described above, tuber discs were cut directly from growing tubers attached to the fully photosynthesizing mother plant, washed three times with 10 mM MES-KOH (pH 6.5), and then incubated (eight discs) in 2 mL of 10 mM MES-KOH buffer (pH 6.5) in a 100-mL Erlenmeyer flask shaken at 90 rpm containing 2.0 mM Glc in the absence or presence of 100 μM SP or CESP. These tuber discs were incubated for 1, 2, and 3 h and then frozen in liquid nitrogen until further analysis. Approximately 100 mg of tuber discs was homogenized using a ball mill precooled with liquid nitrogen. Derivatization and GC-MS analysis were carried out as described previously (Lisec et al., 2006). The GC-MS system was composed of a CTC CombiPAL autosampler, an Agilent 6890N gas chromatograph, and a LECO Pegasus III time-of-flight mass spectrometer running in EI+ mode. Metabolites were identified in comparison with database entries of authentic standards (Kopka et al., 2005; Schauer et al., 2005). Nitrate was measured as described by Sienkiewicz-Porzucek et al. (2008).

Measurement of Redistribution of Stable Isotope

The fate of ^{13}C-labeled pyruvate or acetate was traced following incubation of tuber discs in 10 mM labeled substrate in 10 mM MES-KOH (pH 6.5) for 1, 2, and 3 h. Fractional enrichment of metabolite pools was determined and label redistribution was expressed exactly as described previously (Roessner-Tunali et al., 2004; Tieman et al., 2006).

Respiration Measurements

Potato tuber respiration and respiration in the isolated mitochondria were measured in an oxygen electrode following the protocol detailed by Geigenberger et al. (2000).

Statistical Analysis

Standard procedures were carried out using functions of the Microsoft Excel program. Where two observations are described as different, this means that they were determined to be statistically different ($P < 0.05$) by the performance of Student's t tests. Heat maps were generated in Excel using the appropriate preavailable algorithm. Correlation analyze (Pearson parametric method) was calculated using the relative metabolite content of 2-oxoglutarate and all other metabolites from the combined data set, including data obtained from treated and control tuber discs.

Supplemental Data

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

Supplemental Figure S1. Effect of SP or CESP on metabolism of [U-^{14}C]Glc by potato tuber slices.

Supplemental Table S1. Relative metabolite contents of treated and control tuber discs.

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