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ORIGINAL ARTICLE

On the role of the mitochondrial 2-oxoglutarate dehydrogenase complex in amino acid metabolism

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Abstract Mitochondria are tightly linked to cellular nutrient sensing, and provide not only energy, but also intermediates for the de novo synthesis of cellular compounds including amino acids. Mitochondrial metabolic enzymes as generators and/or targets of signals are therefore important players in the distribution of intermediates between catabolic and anabolic pathways. The highly regulated 2-oxoglutarate dehydrogenase complex (OGDHC) participates in glucose oxidation via the tricarboxylic acid cycle. It occupies an amphibolic branch point in the cycle, where the energy-producing reaction of the 2-oxoglutarate degradation competes with glutamate (Glu) synthesis via nitrogen incorporation into 2-oxoglutarate. To characterize the specific impact of the OGDHC inhibition on amino acid metabolism in both plant and animal mitochondria, a synthetic analog of 2-oxoglutarate, namely succinyl phosphonate (SP), was applied to living systems from different kingdoms, both in situ and in vivo. Using a high-throughput mass spectrometry-based approach, we showed that organisms possessing OGDHC respond to SP by significantly changing their amino acid pools. By contrast, cyanobacteria which lack OGDHC do not show perturbations in amino acids following SP treatment. Increases in Glu, 4-aminobutyrate and alanine represent the most universal change accompanying the 2-oxoglutarate accumulation upon OGDHC inhibition. Other amino acids were affected in a species-specific manner, suggesting specific metabolic rearrangements and substrate availability mediating secondary changes. Strong perturbation in the relative abundance of amino acids due to the OGDHC inhibition was accompanied by decreased protein content. Our results provide specific evidence of a considerable role of OGDHC in amino acid metabolism.

Keywords Amino acid metabolism · Mitochondria · 2-oxoglutarate dehydrogenase · Succinyl phosphonate · TCA cycle

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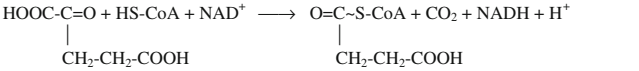
Introduction

The 2-oxoglutarate dehydrogenase reaction (Reaction 1) catalyzed by the highly regulated multi-enzyme 2-oxoglutarate dehydrogenase complex (OGDHC) belongs to an important branch point of central metabolism, present in a wide variety of species from different kingdoms, including bacteria, plants and animals.



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At this point, an intermediate of central metabolism 2-oxoglutarate may either be further degraded in the

tricarboxylic acid (TCA) cycle with energy production or provide the carbon skeleton for inorganic nitrogen assimilation via glutamate (Glu) biosynthesis. Hence, the flux through OGDHC can be assumed to be an important control point for a general flux distribution between the catabolic and anabolic pathways in the cell and whole organism. However, the level of the OGDHC control over the flux distribution has been only experimentally tested in bacteria, driven by a biotechnological aim to increase bacterial Glu production (Shiio and Ujigawa-Takeda 1980; Kataoka et al. 2006; Asakura et al. 2007; Bott 2007). Indeed, mutants lacking OGDHC exhibit increased Glu synthesis (Asakura et al. 2007). The first and second components of OGDHC are down-regulated under conditions of Glu production (Kataoka et al. 2006). Further support for the biological function and significance of the OGDHC-dependent switch came from a recent discovery of a natural system which regulates the Glu production in *Corynebacteria* through phosphorylation/dephosphorylation of the protein inhibitor of the 2-oxoglutarate dehydrogenase (Niebisch et al. 2006; Bott 2007; Schultz et al. 2007, 2009). Nevertheless, despite the universal nature of the 2-oxoglutarate relationship to Glu, the relative importance of the irreversible 2-oxoglutarate dehydrogenase reaction for the regulation of Glu homeostasis in eukaryotes has not yet received much attention. Accordingly, considerations of the eukaryotic processes controlling Glu metabolism are often limited to the reactions involving Glu and glutamine (Reissner and Kalivas 2010; Brauc et al. 2011; Karaca et al. 2011). In addition, generation of the Glu precursor 2-oxoglutarate by isocitrate dehydrogenase and transamination is often considered (Butow and Avadhani 2004; Rakhmanova and Popova 2006). Yet 2-oxoglutarate oxidation by OGDHC, providing irreversible Glu degradation via the TCA cycle, is rarely taken into account when regarding the control of Glu metabolism in eukaryotes. We have recently demonstrated, via use of specific in vivo inhibitors of OGDHC, namely the phosphonate analogs of 2-oxoglutarate, that in potato tubers OGDHC not only limits respiration, but also plays an important role in nitrogen assimilation (Araújo et al. 2008). Additionally, data obtained in animal systems also suggest that the OGDHC-dependent reaction is an important component of the equation defining the Glu/2-oxoglutarate ratio that in its turn is known to define retrograde signaling from mitochondria to nucleus (Butow and Avadhani 2004). For instance, the lethal outcome of OGDHC mutations in animals is associated with impaired development of the nervous system (reviewed in Bunik and Fernie 2009), known to be under significant control of Glu (Kwon and Sabatini 2011). Using the OGDHC inhibition by the phosphonate analogs of 2-oxoglutarate in animals and neuronal cultures, we established the developmental impact of OGDHC inhibition during organogenesis (Graf et al. 2009) and the role of OGDHC in Glu neurotoxicity (Kabysheva et al. 2009). We, therefore, hypothesize that it is the OGDHC-exerted control of the flux distribution between the oxidation of 2-oxoglutarate and biosynthesis of amino acids which significantly contributes to the OGDHC impact on the overall metabolic activity, causing in particular the maldevelopment of the nervous system and lethality due to the OGDHC mutations in vertebrates. In the present work, we use a specific in vivo inhibitor of OGDHC succinyl phosphonate (SP) in a comparative cross-kingdom metabolomics study in order to characterize the role of the 2-oxoglutarate dehydrogenase reaction on amino acid metabolism in eukaryotes. We also compare the changes induced by SP in eukaryotes and in organisms lacking OGDHC to provide evidence for the OGDHC dependence of the SP-induced changes in metabolite profiles. The data obtained are discussed in the context of the roles of OGDHC in carbon–nitrogen interactions in a cross-kingdom comparison.

Materials and methods

Materials

All biochemicals, substrates and co-factors were from Sigma-Aldrich (Taufkirchen, Germany) and of the highest quality available. Mass spectrometry (MS) grade chemicals were: acetonitrile from Riedel-de-Haen (Seelze, Germany); formic acid from Across Organics (Geel, Belgium); methanol, glacial acetic acid and methoxyamine hydrochloride from Sigma-Aldrich (Taufkirchen, Germany); pyridine from Merck (Darmstadt, Germany). Cell culture media were from Gibco (Carlsbad, CA, USA).

Plant and cell cultures and sampling

Arabidopsis thaliana Col-0 seedlings were grown in liquid Murashige and Skoog plant growth medium (Murashige and Skoog 1962) as described previously (Sweetlove et al. 2007) with the exception that agar was omitted from the medium. Seedlings were grown under continuous light. The 10-day-old seedlings were treated for 2 and 4 h with SP added directly to the growth medium at final concentrations of 0.05 or 0.1 mM. The treated seedlings were washed with water twice and dried using a combination of mild centrifugation and blotting on filter paper. The seedlings were rapidly frozen in liquid nitrogen and stored at −80 °C. The frozen samples were ground in a ball-mill precooled with liquid nitrogen. Metabolites were extracted following a modified method previously described (Lisec et al. 2006). For extraction, about 100 mg of plant material (fresh weight) was ground in 1.4 mL of methanol

supplemented with 0.05 mM ribitol added as an internal standard for the quantification of metabolite abundances. After incubation at 70 °C for 15 min, the extract was centrifuged at 11,000*g* for 10 min to remove tissue debris. The supernatant was carefully mixed with 0.75 ml of chloroform and 1.5 ml water to separate the polar and non-polar metabolites. The phases were separated by centrifugation for 15 min at 2,200*g*. For further analysis, aliquots of 0.15 ml of the upper (polar) phase were dried by vacuum centrifugation without heating using a Concentrator 5301 from Eppendorf (Hamburg, Germany). The tubes were flushed with argon and stored with a dessicant at −80 °C until derivatization and assay.

Cyanobacterial strains were obtained from the Pasteur Culture Collection (Pasteur Institute, Paris, France). Axenic cultures were grown photoautotrophically in 125-mL Erlenmeyer flasks shaken at 100 rpm with 25 µmol m^{-2} s^{-1} continuous soft white illumination in a growth chamber (GFL 3033, Progen Scientific, London, UK) at 30 °C. Three independent cultures were inoculated with 1 mL of a medium-dense pre-culture in 40 mL of BG11 and BG11o growth medium for *Synechocystis* sp. PCC 6803 and *Nostoc* sp. PCC 7120 sp., respectively (Krall et al. 2009). The cultures were exposed to SP in the exponential growth phase (OD_{730} ~ 0.8). Cell sampling, sample preparation, data acquisition by GC–TOF–MS, data processing, compound identification and data analyses were carried out exactly as described (Krall et al. 2009).

Cultures of cerebellar granule neurons were prepared according to (Kabysheva et al. 2009) using neurobasal medium (NBM) with B-27 supplement and glutamax as the culture growth medium. 10–12 days old neurons grown in Petri dishes (100 × 15 mm) were used in the experiment. To ensure no stress effects on metabolite profile, renewal of culture medium was performed by exchanging half of the medium, with the last exchange 3 days before the experiment. SP was added directly into the culture medium and incubated for the time indicated. To prepare the neuronal extracts, the culture medium was removed, the neurons were washed twice with 16 mL of warm phosphate buffer saline (PBS), and 5 mL of cold methanol containing 0.05 mM ribitol was added per Petri dish. The dishes were slowly shaken in a cold room for an hour, the cells scraped and their methanol suspension transferred to a 15-mL Falcon tube. Protein was precipitated by a 20-min centrifugation at 6,000*g*, supernatants were transferred into a pure falcon tube, and both the extract and protein pellets were stored until assayed at −20 °C. Further treatment of the samples was performed as described for the seedling extracts.

Astrocytes were isolated from newborn rat pups as in (Bettendorff et al. 1991). The cells were suspended in Dulbecco's modified Eagle medium (DMEM) and seeded into 75-cm^2 cell culture flasks (Greiner Bio-One GmbH, Frickenhausen, Germany). After 6–10 days of growth at 37 °C in the presence of 10 % CO_2, the flasks were washed with warm PBS, and cells detached by 5 min of incubation with 0.1 % trypsin/EDTA (10 mL per flask). The cell suspension was diluted with DMEM to 200,000 cells per mL and seeded into six-well plates (5 mL of the cell suspension per well). The medium was exchanged on the third day. On the fourth day, astrocytes were washed with warm PBS, and incubated with or without 0.1 or 0.5 mM SP for 30 min and 2 h in Hank's Balanced Salt solution. After incubation, astrocytes were washed with PBS and lysed by addition of 0.2 mL per well of the modified radio-immunoprecipitation assay (RIPA) buffer without Triton X-100. The cell suspension was transferred to an Eppendorf tube, incubated for 15 min at 4 °C, and used for the OGDHC assays without centrifugation.

Animal experiments

All experiments were performed according to the guidelines of the Helsinki Declaration on the Guide for the Care and Use of Laboratory Animals, defining the conduct of ethical research on laboratory and other animals. Animals were housed at 21 ± 2 °C, fed a standard ration and subjected to a 12/12 h light/dark cycle. Wistar rats of about 250–300 g were used in the experiment. SP was administered to animals at 0.02 mmol/kg by intranasal application of a water solution of the trisodium salt, with the Ringer's solution substituting for SP in the control groups. Intranasal application is known to allow drugs to be delivered to the brain beyond the blood–brain barrier. The SP application to pregnant rats, which were supposed to be more reactive to the mitochondrial inhibitor SP due to the known increase in sensitivity to hypoxia, induced by pregnancy, was done on the 9–10th day of pregnancy, i.e., at the critical period of organogenesis. The control group in this case comprised pregnant rats treated with the Ringer's solution. At 24 h after the treatment, the animals were killed by decapitation; the cortexes were quickly excised on ice, frozen in liquid nitrogen and stored at −70 °C. For extraction, half of the brain cortex (approximately 470–500 mg of the tissue) was weighed in an ice-cold vial, 4.0 mL of 100 % methanol pre-cooled at −70 °C was added to the sample, and the tissue was homogenized using Ultra-Turrax T10 basic (IKA; Staufen, Germany) for 2 min at speed 4. While preparing different samples, the methanol homogenates were left on ice. Then, a 1-mL aliquot of each homogenate was transferred to a 15-mL Falcon vial, 1.5 mL of 0.2 % acetic acid (MS grade) was added, the mixture vortexed and left on ice with shaking for an additional 30 min. The samples were centrifuged for 30 min at 3,220*g* in an Eppendorf Centrifuge 5810 R (Hamburg, Germany). The

supernatants were transferred to clean vials and stored frozen at −70 °C until assay.

SP effect on the conversion of [^{14}C]glucose into amino acids and proteins

Potato tuber disks were washed three times with 10 mM MES-KOH (pH 6.5), following a 1-h pre-incubation in the presence or absence of 0.1 mM SP in 10 mM MES [2-(*N*-morpholino) ethane sulfonic acid]-KOH (pH 6.5) containing 2 mM glucose. After the pre-incubation, 1.00 μCi of [^{14}C] glucose (specific activity of 8.11 MBq mmol^{-1}) was added, and the disks were incubated for a further 3 h, with the reaction terminated by freezing in liquid nitrogen. The extraction and heavy label redistribution were studied as previously described (Fernie et al. 2001a). Briefly, frozen tuber tissue was extracted successively for 10 min in 5 ml of 80 % (v/v) ethanol, 2 ml of 50 % (v/v) ethanol, 2 ml of 20 % (v/v) ethanol, 2 ml H_2O and finally 2 ml of 80 % (v/v) ethanol. The supernatants were combined and dried under vacuum; the ethanol-soluble components were resuspended in 2 ml of H_2O and separated into neutral (soluble sugars), anionic (organic acids) and cationic (amino acids) fractions by ion-exchange chromatography (Fernie et al. 2001b). The ethanol-insoluble material was separated into neutral (starch), anionic (cell wall) and cationic (protein) components as described previously (Runquist and Kruger 1999). Quantitative recovery of radiolabel was acceptable in the present study (90–105 %) and the reliability of these fractionation techniques had been thoroughly documented previously (Runquist and Kruger 1999; Fernie et al. 2001b; Araújo et al. 2008).

Protein concentration

Protein content was calculated per gram FW or as total protein per neuronal culture dish. Bovine serum albumin was used as a standard with either BioRad protein assay kit (Bio-Rad Laboratories GmbH, Munich, Germany) for the plant and neuronal samples or Micro BCA^{TM} protein assay kit (ThermoFischer Scientific, Rockford, IL, USA) for brain samples.

OGDHC activity assay

Extraction and assay of the potato and brain OGDHC activity was done according to the established tissue-specific protocols as described in (Araújo et al. 2008); (Graf et al. 2009), respectively. The assay was performed after disruption of mitochondria by detergents or sonication. In animal experiments, the OGDHC coenzyme thiamine diphosphate (ThDP) was omitted from the brain extract assays to ensure determination of the enzymatic activity under physiological levels of ThDP, which were shown to decrease as a result of SP treatment (Mkrtchyan et al. 2011). Tight ThDP binding to mammalian OGDHC enables assaying endogenous activity levels of brain OGDHC in the absence of the coenzyme in the medium (Bunik and Strumilo 2009). Astrocytes were extracted as described above, and the OGDHC activity was assayed in the reaction medium described earlier (Graf et al. 2009).

Amino acid assay in brain extracts

Amino acids in the brain extracts were quantified using 3200 Q TRAP LC–MS/MS system of Applied Biosystems/MDS SCIEX (Foster City, CA, USA). Chromatographic separation of the extracts was done using a LUNA-C18(2) column from Phenomenex (Torrance, CA, USA) and a 50 % aqueous solution of acetonitrile supplemented with 0.1 % acetic acid as the mobile phase. A mixture of amino acid standards dissolved in the mobile phase was used for calibration. A 0.02-mL aliquot of the brain extract was applied to the column. The eluted amino acids were identified using electrospray ionization (ESI) in the positive mass spectrometry (MS) mode by the mass to charge ratio for the primary and secondary ions as given in Table 1. Quantification of the amino acids was done per gram of fresh tissue weight (FW) using the calibration curve obtained in the same experiment. The groups to be compared were analyzed within the same run. Except for Glu, amino acids showed a satisfactory linearity between their concentration and the MS-determined peak area within the concentration interval 0.1–5 μM. Due to the non-linear dependence and high concentration of Glu in the brain extracts even after a tenfold dilution, the changes in the Glu levels could not be correctly determined by the MS approach described above. Glu levels were therefore assayed enzymatically using commercial preparation of bovine glutamate dehydrogenase (Sigma-Aldrich, Taufkirchen, Germany). Glutamate dehydrogenase (0.1 mg/mL, final concentration) was added to 100 mM Tris–HCl buffer, pH 8.0, containing 1.5 mM NAD^+ and a 120-fold diluted brain extract. The quantity of Glu was calculated from the maximal increase in optical density at 340 nm after completion of the reaction, using the molar extinction coefficient of NADH 6220 M^{-1} cm^{-1}. The calculation was based on equimolar production of NADH from Glu in the glutamate dehydrogenase reaction. Using the Glu standard solution, we showed that under our conditions, the Glu oxidation in the glutamate dehydrogenase reaction occurred to completion up to 0.02 mM Glu in the assay medium, i.e., at ≥75-fold excess of NAD^+ to Glu. The brain extract dilution used resulted in Glu concentrations in the assay medium which did not exceed this upper limit. The Glu underestimation due to the shift in the equilibrium of the

Table 1 Mass to charge ratio of the primary and secondary ions used for ESI-MS identification of amino acids in the eluate after HPLC separation of the samples

Amino acid	Mass to charge ratio of the primary/secondary ions
Ala	90.2/44.0
Arg	175.4/70.0
Asn	133.3/74.2
Asp	134.1/42.8
Cys	122.0/58.9
GABA	104.3/45.1
Gln	147.3/84.0
Glu	148.4/56.0
Gly	76.2/30.2
His	156.3/81.2
Ile	132.1/86.0
Leu	132.1/41.0
Lys	147.4/56.2
Met	150.3/61.1
Phe	166.5/102.9
Pro	116.3/70.0
Ser	106.3/42.0
Thr	120.3/56.1
Trp	205.2/115.1
Tyr	182.4/90.9
Val	118.3/57.1

glutamate dehydrogenase reaction in the backward direction started at about 0.04 mM Glu in the medium.

Metabolite profiling

The relative levels of metabolites were determined after derivatization with methoxyamine hydrochloride (20 mg ml^{-1} stock solution in pyridine) using an established procedure followed by gas chromatography time-of-flight mass spectrometry (GC–TOF–MS) as described by Lisec et al. (2006). As with mass spectrometry-based methods in general, this method is also best suited to detect differences between metabolite profiles rather than absolute concentrations of metabolites (Lisec et al. 2006). Therefore the results of comparative analysis are presented as the fold changes between the control and SP-treated samples after their incubation under otherwise identical conditions. Chromatograms and mass spectra were evaluated by Chroma TOF 1.6 (Leco, St Joseph, MI) and TagFinder 4.0. (Luedemann et al. 2008). Metabolites were identified by comparison with mass spectral tags represented in the query database, including recent additions to our mass spectral libraries which cover data from mammals, yeast, corynebacterium, model plants and related wild species, as well as required non-sample controls (Kopka et al. 2005; Schauer et al. 2005; Erban et al. 2007). The libraries currently feature more than 5,000 evaluated mass spectra from two technology platforms which represent 1,089 non-redundant and 360 identified mass spectral tags (Kopka et al. 2005). Before subjection to further data analysis, the processed data were checked manually for possible peak shifts, with the peak finding method corrected as routinely done on optimization for novel tissues (Lytovchenko et al. 2009). Due to technical reasons, such as detection limit and/or different matrix interaction, the exact set of metabolites detected in different systems could vary. Owing to this, certain metabolites could be absent from one system, while present in others, as seen from the results presented. It should, however, be noted that when a metabolite is not detected by the MS analysis of an extract, it does not necessarily mean that the metabolite is below its detection limit, but may be due to specific composition of the extract affecting its interaction with the matrix (Lisec et al. 2006). The relative metabolite abundance was calculated by normalization of the metabolite signal intensity to that of the internal ribitol standard and to the FW of the material.

Statistical analysis

Data are presented as mean ± SEM. Student's test was used to estimate statistical significance of the differences, with $P \leq 0.05$ considered statistically significant.

Results

Accumulation of 2-oxoglutarate and Ala in situ and in vivo are markers of OGDHC inhibition and perturbed TCA cycle function upon SP application

Figure 1a shows that treatment of the heterotrophic plant tissue (potato tubers), autotrophic photosynthetic organisms (*Arabidopsis* seedlings) and animal cell culture (neurons) with SP universally leads to 2-oxoglutarate accumulation. The data obtained with seedling and neurons demonstrate the dependence of the 2-oxoglutarate accumulation on the SP concentration and incubation time (Fig. 1a). Because the increased steady-state level of 2-oxoglutarate is the direct consequence of the SP-induced inhibition of OGDHC-catalyzed 2-oxoglutarate degradation, it can be taken as a measure of the OGDHC inhibition by SP in complex biological systems in vivo (seedlings) *and* in situ (tissue slices and cell culture). The resulting inhibition by SP of the flux through the TCA cycle where OGDHC is often rate limiting (Bunik and Fernie 2009) is evident from the Ala accumulation (Table 2). Usually, Ala

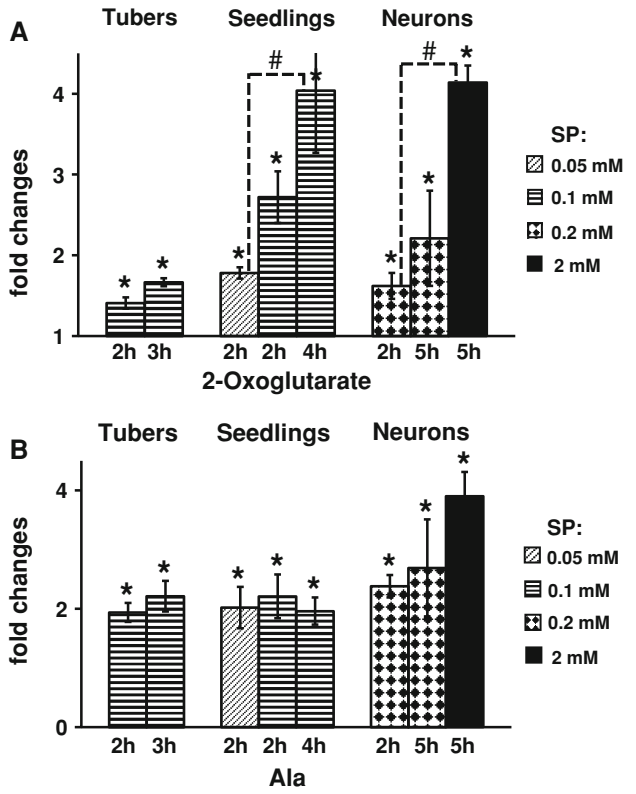

Fig. 1 Metabolic indicators of the SP action in plant and animal systems: 2-oxoglutarate (**a**) and Ala (**b**). Dependence of the changes in the relative metabolite content on the concentration and/or incubation time with SP is given. Media to which SP was added are given in Table 2

increases upon inefficient mitochondrial oxidation of the glycolytic product pyruvate, leading to increased transamination of pyruvate to Ala (Rocha et al. 2010). The widespread accumulation of Ala in the SP-treated systems (Fig. 1b) is thus another indicator of the SP-induced inhibition of OGDHC, leading to impaired flux through the TCA cycle and hence decreased pyruvate oxidation in the cycle. However, in contrast to the 2-oxoglutarate accumulation as an immediate consequence of the OGDHC inhibition, the increase in Ala represents a distal change dependent not only on the OGDHC inhibition, but also on other reactions of the metabolic network involved. Due to their buffering effect, this metabolic indicator does not show the same concentration/time dependence on SP as the immediate marker 2-oxoglutarate (Fig. 1).

Comparing the SP concentration dependence of 2-oxoglutarate accumulation in different species shows that the plant systems are more sensitive to SP than are animal systems. For instance, a similar increase in 2-oxoglutarate after 2 h of incubation with SP in rich culture medium is observed at a fourfold lower SP concentration applied to the *Arabidopsis* seedlings as compared to neuronal cultures (0.05 vs. 0.2 mM, Fig. 1). Increasing the SP incubation time to 4–5 h reveals an even greater difference, with a 20-fold lower SP in seedlings compared to neurons (0.1 vs. 2 mM SP, Fig. 1) resulting in a fourfold increase in 2-oxoglutarate. Also, the potato tubers incubated in the minimal medium show a higher sensitivity to SP treatment compared to neurons in rich medium. A twofold lower SP concentration in the tubers versus neurons (0.1 vs. 0.2 mM SP, Fig. 1) is required for a similar 1.6- to 1.7-fold increase in 2-oxoglutarate.

In contrast to plants, animal OGDHC exhibits up-regulation upon SP treatment

Although SP is a tight-binding inhibitor, its reaction with OGDHC is not irreversible (Bunik et al. 1992, 2005). This may cause a partial or complete dissociation of the enzyme–inhibitor complex upon extraction and activity assay, preventing a direct correlation between the level of extracted activity and degree of inhibition in vivo. Nevertheless, we screened different plant and animal systems with detectable OGDHC activity in extracts (Fig. 2) to determine whether interaction of OGDHC with SP in vivo affects the enzyme activity in extracts and whether the influence of the SP treatment on the extracted activity could be correlated with the metabolic indicators of the treatment. The OGDHC activity assays in plants (Fig. 2) showed that in spite of the dilution of enzyme–inhibitory complex upon extraction and further dilution in the assay medium, OGDHC activity decreases even at a relatively short incubation time (40 min) and a low concentration of SP (0.1 mM). In animal systems, the decrease is not so pronounced. Although we could observe a decrease in the OGDHC activity at increasing SP concentrations and/or incubation time (to 0.5 mM and/or 2 h in astrocytes, Fig. 2), animal systems repeatedly exhibited an increase in the OGDHC activity extracted after application of low SP doses, a result not observed in plant systems. This higher OGDHC activity detected in the standard assay at saturating substrate concentrations corresponds to an increase in the catalyst quantity or catalytic constant k_{cat}, which we shall further call the OGDHC up-regulation. Figure 2 shows that the OGDHC up-regulation was measured in astrocytes after 0.5 h of incubation with 0.1 mM SP or in brain cortex of pregnant rats 24 h after introduction of SP at 0.02 mmol kg^{-1}. Increasing the SP dose decreased the OGDHC up-regulation in animal experiments (Trofimova et al. 2010) and made apparent a decrease in the OGDHC activity in the astrocyte extracts (Fig. 2), suggesting that increased inhibition may mask the up-regulation and vice versa. At the same low SP dose, the increase in the brain OGDHC activity in response to SP depended on the initial OGDHC activity level. That is, the non-pregnant rats possess a higher ($P \leq 0.05$) OGDHC activity in brain

Table 2 Comparative analysis of changes in the levels of 2-oxoglutarate and amino acids due to incubation with SP in biological systems from different kingdoms

Media	17 amino acids, alanyl-glutamine, glucose		Gly, sucrose				Glucose		Citrate			
System Treatment conditions	Neurons 0.2 mM SP 2 h ($n = 4$)/($n = 5$)		*Arabidopsis* seedlings 0.1 mM SP 2 h ($n = 4$)/($n = 4$)		*Arabidopsis* seedlings 0.1 mM SP 4 h ($n = 4$)/($n = 4$)		Potato tuber discs[a] 0.1 mM SP 3 h ($n = 6$)/($n = 4$)		*Nostoc* 0.2 mM SP 6 h ($n = 6$)/($n = 6$)		*Synechocystis* 0.2 mM SP 6 h ($n = 6$)/($n = 6$)	
Metabolite	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
2-Oxoglutarate	1.62*	0.08	2.72*	0.32	4.04*	0.77	1.67*	0.06	ND		ND	
Ala	2.18*	0.10	2.21*	0.37	1.96*	0.23	2.21*	0.30	1.05	0.12	0.86	0.14
Arg	1.57*	0.20	2.25*	0.21	1.32#	0.01	1.14	0.09	1.19	0.15	1.16	0.18
Asn	1.50*	0.20	1.98*	0.33	1.58	0.25	1.06	0.06	0.84	0.17	1.21	0.23
Asp	ND		1.03	0.32	0.84	0.33	1.04	0.03	1.11	0.14	0.86	0.14
Cys	1.14	0.10	1.78*	0.11	1.78*	0.09	ND		ND		ND	
GABA	2.67*	0.11	2.10*	0.07	1.73*,#	0.06	2.08*	0.02	ND		ND	
Gln	1.01	0.08	1.02	0.08	0.55*,#	0.03	0.21*	0.03	0.86	0.13	1.28	0.19
Glu	1.48*	0.03	1.96*	0.07	1.57*,#	0.12	1.77*	0.10	0.95	0.14	0.89	0.12
Gly	1.64*	0.15	0.86	0.19	0.45*	0.01	2.14*	0.06	1.14	0.17	0.88	0.11
His	1.19*	0.06	0.91	0.14	0.63*	0.04	1.15	0.08	ND		ND	
Ile	1.16	0.04	2.82*	0.19	1.13#	0.03	1.13	0.20	0.92	0.23	0.84	0.20
Leu	1.09	0.08	ND		ND		ND		1.09	0.25	0.88	0.22
Lys	1.22	0.23	1.42	0.11	1.12	0.11	1.37	0.17	ND		ND	
Met	1.68*	0.13	2.66*	0.26	2.11*	0.14	1.38*	0.05	ND		ND	
Phe	1.29*	0.14	1.03	0.11	0.69*,#	0.06	1.43*	0.18	0.85	0.21	1.08	0.13
Pro	1.38*	0.11	1.15	0.17	1.72*	0.20	0.87	0.03	1.07	0.14	0.91	0.16
Ser	1.20*	0.03	2.60*	0.52	2.49*	0.18	1.35	0.14	1.26	0.18	0.87	0.16
Thr	1.83*	0.17	2.26*	0.20	1.96*	0.10	0.82	0.02	1.13	0.17	0.85	0.18
Trp	1.27*	0.09	1.23	0.15	0.92	0.19	1.40	0.25	ND		ND	
Tyr	1.14	0.10	1.02	0.08	0.37*,#	0.04	1.49	0.21	0.98	0.19	0.94	0.21
Val	0.93	0.02	1.15	0.12	0.92	0.07	1.01	0.05	1.01	0.02	0.83	0.18

The values represent fold differences to the corresponding controls. Metabolized substrates of the experimental media employed with different systems are indicated

ND not detected

Numbers of experiments (n) in the treated/control groups are given in parenthesis

Statistical significance: * $p \leq 0.05$ versus control. # $p \leq 0.05$ between the two SP treatment conditions

[a] Values for potato tubers are calculated using the data from (Araújo et al. 2008)

cortex (1.59 ± 0.16 μmol min^{-1} g FW^{-1}), compared to pregnant rats (1.19 ± 0.14 μmol min^{-1} g FW^{-1}), and SP treatment up-regulated the OGDHC activity in pregnant rats only (Fig. 2). As a result, the post-treatment level of the OGDHC activity in the cortex of pregnant rats (1.54 ± 0.14 μmol min^{-1} g FW^{-1}) increased to that inherent in the control non-pregnant rats (1.59 ± 0.16 μmol min^{-1} g FW^{-1}). This finding suggests that the OGDHC up-regulation takes place only when inhibition of the flux through OGDHC approaches a certain critical level. Thus, detection of the OGDHC up-regulation can be considered as an additional marker of the SP-induced perturbation in the OGDHC reaction in vivo. Besides, in addition to the reversibility of the enzyme–inhibitor complex formation, the OGDHC up-regulation observed in animal systems may mask the decrease in the extracted OGDHC activity due to SP treatment.

As a result, metabolic indicators of the SP effects in situ and in vivo (Fig. 1) show a certain correspondence to the changes in the extracted OGDHC activity (Fig. 2). However, the activity assays cannot be taken as a direct measure of the SP inhibition. At least in animal systems, not only the potential dissociation of the enzyme–inhibitory complex during extraction and assay, but also the OGDHC up-

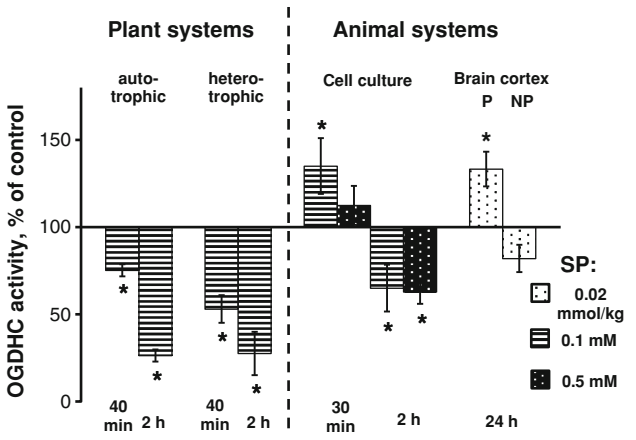

Fig. 2 Changes in the OGDHC activity (relative to the control values), as assayed in the extracts from the *Arabidopsis* leaves (autotrophic plant tissue), potato tuber disks (heterotrophic plant tissue), cultured astrocytes (animal cell culture) and brain cortex of pregnant (P) and non-pregnant (NP) rats, treated with the indicated SP concentration/dose for the time shown

regulation may interfere with using the in vitro assays of extracted OGDHC as a measure of enzyme inhibition by SP.

Targeting of OGDHC by SP in vivo and in situ perturbs the amino acid pool

Table 2 shows that along with the 2-oxoglutarate accumulation due to the OGDHC inhibition by SP, both animal and plant systems possessing the SP target, OGDHC, show pronounced changes in their amino acid pools. Although specific changes are condition dependent, a rather general increase in the amino acid pools is observed at moderate SP doses (up to 3 h at 0.1–0.2 mM). In contrast to the animal and plant systems, cyanobacterial strains lacking OGDHC (Laurent et al. 2005; Zhang and Bryant 2011) did not exhibit the amino acid changes, when treated with 0.1 or 0.2 mM SP for 1, 2, 3 or 6 h. Accordingly, even the maximal SP dose applied to cyanobacteria did not change the amino acid pool (Table 2). Unfortunately, we could not unambiguously quantify the SP peak in the metabolome of the treated cyanobacteria to estimate the inhibitor intracellular level, because the peak showed interference with other compound(s). However, the candidate SP peak was observed in all the SP-treated samples, in accordance with the inhibitor having entered the cell.

The systems which were directly exposed to $\approx 10^{-4}$ M SP concentrations for several hours were not the only ones to show changes in the amino acid pool. The changes could also be detected in intact animals exposed to a single low SP dose (0.02 mmol $\times$ kg^{-1}), with brain tissue taken for analysis 24 h after the exposure. Changes in the amino acid pool after SP treatment were detected in brain cortexes of pregnant rats (Table 3), confirming metabolic perturbation suggested by the OGDHC up-regulation due to the SP treatment of these rats (Fig. 2). Significant similarity in the changes in the amino acid pools in the brain of pregnant rats (Table 3) and neuronal culture (Table 2) is impressive if one takes into account that neurons were permanently exposed to 0.2 mM SP for 2 h, while animals were administered a single low SP dose that should be mostly cleared in 24 h. Nevertheless, in both cases the SP application led to statistically significant increases in Glu, GABA, Gly, Phe and Trp (Tables 2, 3). In addition, statistically significant increases in neuronal Ala, His, Met, Pro and Thr (Table 2) were matched by similar trends ($P < 0.1$) in the increases in these amino acids in the extracts of brain cortex. Unlike the case with pregnant rats, up-regulation of the brain OGDHC in vitro did not occur in non-pregnant rats (Fig. 2). As discussed in the previous section, this would be expected if the change in the in vivo flux through OGDHC was within the spare threshold capacity, i.e., did not induce gross metabolic perturbations. Indeed, only the proximal metabolic derivative of 2-oxoglutarate Glu was affected by the SP treatment of non-pregnant rats, while other amino acids of the brain cortex showed no trend to change (Table 3). Thus, the observed SP effect on the brain amino acid pools (Table 3) correlated with the response to SP of the brain OGDHC, detected by the activity assay in vitro (Fig. 2): the up-regulation of OGDHC was associated with more pronounced metabolic perturbation.

Cross-kingdom comparison of the SP effects on the amino acid pool

Given that the use of SP in animal experiments is associated with uncertainties regarding the drug concentration, distribution and clearance, simplified model systems presented in Table 2 were chosen for the cross-kingdom comparison of the SP action. Taking into account the different physiology of the biological systems compared in this study, the SP application to seedlings and neurons was done under the system-specific conditions known to be optimal for growth in culture (Table 2). The nutrient-rich growth media of these systems enable more adaptations of metabolism to the SP-induced perturbation than the minimal medium where the experimentation is possible with the storage tissue (potato tubers). Owing to this, one may expect less secondary adaptations of metabolic network to the SP treatment in tubers compared to neurons and seedlings. Indeed, tuber slices showed minimal number of changed amino acids, with the largest differences observed for Ala and proximal metabolites of 2-oxoglutarate, such as Glu, GABA and Gln (Table 2; Fig. 3). Mostly, these amino acids were affected in a similar manner in all the OGDHC-

Table 3 Comparative analysis of changes in the levels of amino acids in brain cortex of non-pregnant and pregnant rats 24 h post-treatment with 0.02 mmol kg^{-1} SP

Animal group	Pregnant ($n = 4$)/($n = 6$)		Non-pregnant ($n = 4$)/($n = 9$)	
Amino acid	Mean	SEM	Mean	SEM
Ala	1.43	0.24	0.94	0.04
Arg	0.95	0.09	0.92	0.06
Asn	1.48	0.34	1.02	0.06
Asp	1.53	0.42	1.06	0.09
Cys	1.66	0.39	0.89	0.07
GABA	1.34*	0.16	0.87	0.05
Gln	1.40	0.28	1.05	0.08
Glu	1.23*	0.09	0.69*	0.04
Gly	1.60*	0.21	0.99	0.06
His	1.18	0.14	0.95	0.02
Ile	1.37	0.27	1.02	0.06
Leu	1.39	0.28	1.03	0.06
Lys	1.23	0.21	1.03	0.06
Met	1.46	0.26	0.96	0.09
Phe	1.33*	0.11	0.93	0.06
Pro	1.37	0.21	0.93	0.05
Ser	1.42	0.26	1.05	0.07
Thr	1.52	0.32	1.06	0.06
Trp	1.45*	0.17	0.97	0.02
Tyr	1.19	0.16	0.86	0.04
Val	1.33	0.27	1.01	0.07

The values represent fold differences compared to the corresponding controls, i.e., non-pregnant or pregnant rats which were not treated with SP

Numbers of experiments (n) in the treated/control groups are given in parenthesis

* $p < 0.05$ versus control

possessing systems presented in Table 2 and in animal experiments (Table 3, pregnant rats). That is, independent of the medium conditions or metabolic differences between heterotrophic (neurons, potato tubers) and autotrophic (seedlings) or animal (neurons) and plant (seedlings, potato tubers) systems, the proximal metabolites of 2-oxoglutarate, i.e., Glu and its decarboxylation product GABA, are universally increased (Table 2). General to all systems is also the Ala increase, an indicator of the perturbed function of the TCA cycle as discussed above. In the potato tubers incubated in the absence of a nitrogen source, a concomitant decrease in Gln is detected (Table 2). Excessive Gln consumption at the increased steady-state level of 2-oxoglutarate is also observed in other systems, either as an obvious decrease (seedlings, 4 h at 0.1 mM SP, Table 2; Fig. 3d) or as decreased ratios of Gln/2-oxoglutarate (Fig. 3e–h). These data indicate that the SP-induced nitrogen flow to the accumulated 2-oxoglutarate, resulting in the amino acid increases, is first of all supported by Gln.

In contrast to the common effects of the SP treatment on Ala and proximal 2-oxoglutarate metabolites, i.e., Glu, GABA and Gln, changes in other amino acids are more species specific. One of the most pronounced differences between the compared systems involves Gly. It increases in heterotrophic systems, such as potato tubers, neurons and the brain cortex of pregnant rats (Tables 2, 3). However, in the photosynthetic seedlings, Gly is decreased with increasing SP exposure (Table 2, seedlings). Because Gly is added to the medium of the seedlings' incubation with SP, the depletion suggests an increased consumption of Gly to compensate for the OGDHC inhibition by SP. NADH, methylenetetrahydrofolate and ammonia resulting from oxidative decarboxylation of Gly are obviously used for the amino acid biosynthesis, as seedlings show a very different pattern of the amino acid increases due to the SP action compared to the heterotrophic systems. Indeed, under conditions where Gly is not limiting, Ile, Met and Ser increased in seedlings much more than Ala, Glu and GABA (Fig. 3, seedlings 2 h). Another conspicuous difference between neurons and seedlings is the SP influence on the Cys level. Cys remains constant in neurons, but greatly increases in seedlings.

Nitrogen accumulation within amino acids due to SP action coincides with decreased protein content

It is important to note that the metabolic shift, induced by the 2-oxoglutarate accumulation, significantly changes the relative abundance of amino acids, as their general increase is not equally distributed among the different amino acids (Fig. 3; Table 2). We suggest that this may interfere with protein synthesis, which is known to be inhibited under

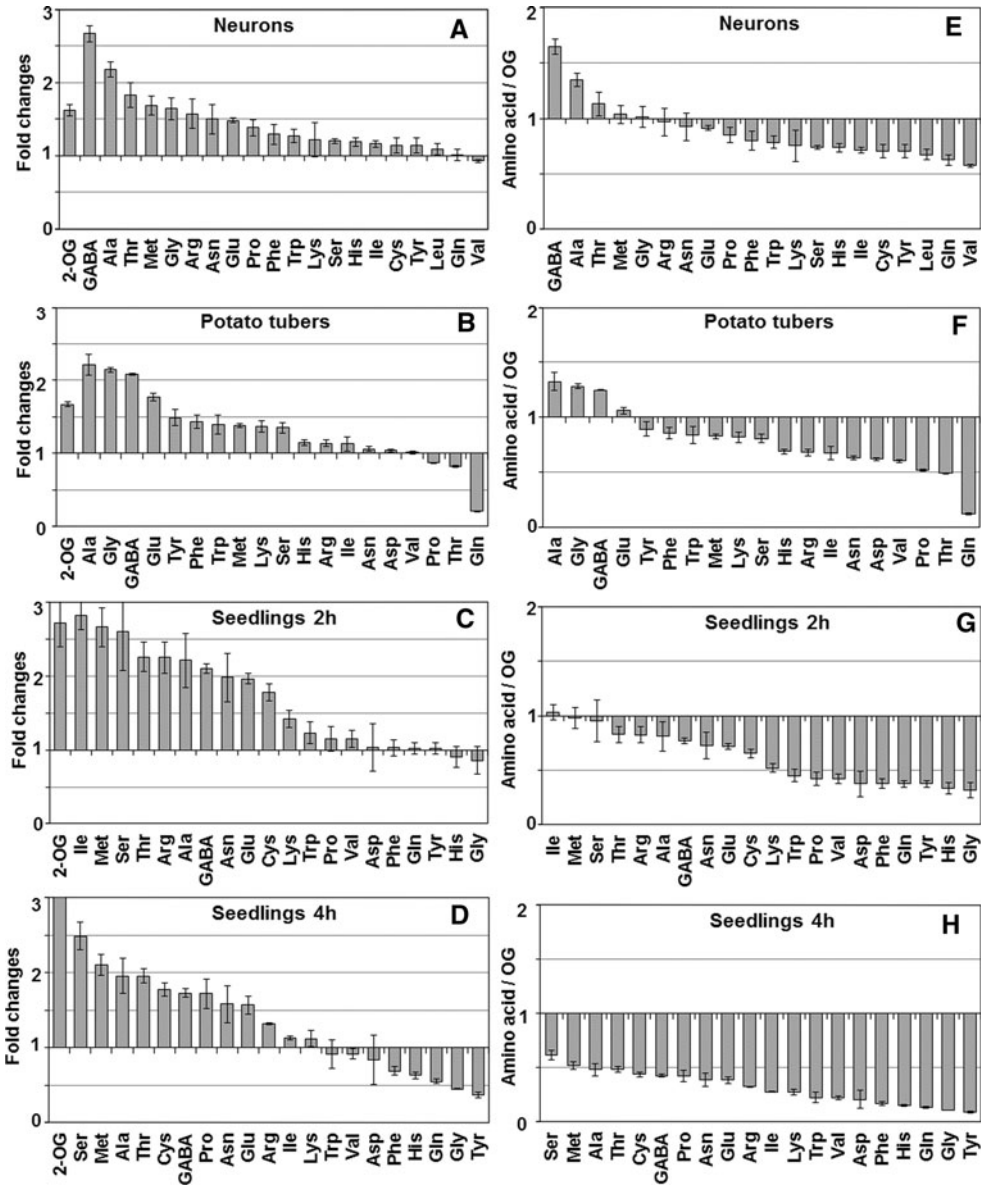

Fig. 3 Depiction of the SP-induced changes in the amino acid levels (**a**–**d**) and their ratios relative to 2-oxoglutarate (**e**–**h**) according to the magnitudes of the effect. Media to which SP was added are given in Table 2

conditions where certain amino acids are depleted more than others (Iskakova et al. 2006; Swartz 2006). Figure 4 shows that the SP-treated systems with significant deviations in the amino acid pool, i.e., neuronal culture, tuber slices and seedlings, showed decreased protein content, while intact animals exposed to a low SP dose resulting in less pronounced changes within the amino acid pool (Table 3) did not (Fig. 4). The largest protein decrease was observed in seedlings (Fig. 4), which roughly correlated with the highest 2-oxoglutarate accumulation in this system (2.7-fold at 2 h in seedlings vs. 1.6- to 1.7-fold in neurons and tubers, Table 2). More precisely, the protein decrease (Fig. 4) correlated with the decreased ratios of amino acids to 2-oxoglutarate (Fig. 3e, f, g). That is, 18, 35 and 60 % decreases in neuronal, tuber and seedlings protein (Fig. 4)

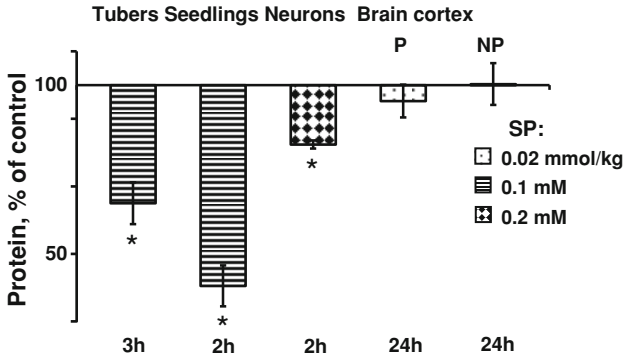

Fig. 4 Changes in the protein content of selected plant and animal systems due to SP treatment. Media to which SP was added are given in Table 2. Details of the SP application to animals are described in "Materials and methods"

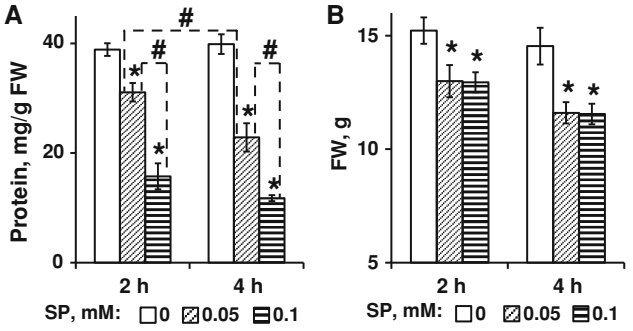

Fig. 5 Dependence of the protein content (**a**) and fresh weight (**b**) of *Arabidopsis* seedlings on the SP concentration and incubation time

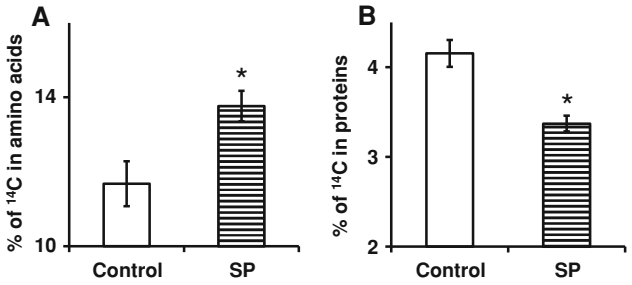

Fig. 6 Incorporation of ^{14}C label from uniformly labeled glucose into the fractions of amino acids (**a**) and protein (**b**) of the potato tubers treated with 0.1 mM SP for 3 h. The percentage of the label in the fraction to that of the metabolized glucose is given

correlated correspondingly to two amino acids (Fig. 3e), six amino acids (Fig. 3f) and 10 amino acids (Fig. 3g) having their ratios to 2-oxoglutarate ≤ 0.6. This result indicates that the decreased protein content may depend on reactions between 2-oxoglutarate and amino acids, presumably involving transamination.

Using the in vivo system of the intact plant organism (seedlings), we studied how the decreased protein content affected the overall biomass. Figure 5 demonstrates that in the SP-treated seedlings, a statistically significant dose-dependent decrease in the protein content calculated per gram FW (Fig. 5a) was accompanied by a decrease in the fresh weight of the seedlings (Fig. 5b). With increasing SP dose, both effects showed saturation, although not through identical mechanisms. While the protein content was dependent on both the SP concentration and incubation time (Fig. 5a), the decrease in the fresh weight was maximal at the lowest SP dose (2 h at 0.05 mM), showing no further dependence on the SP concentration and incubation time in the interval studied (Fig. 5b). This finding indicates that after a certain threshold in the weight decrease concomitant with the decreased protein, the contribution of decreased protein synthesis to the seedling biomass is compensated by an alternative weight gain, probably including osmotic effects due to the increased intracellular amino acid concentration.

Thus, biochemical reactivity of amino acids may define their differential involvement in the reactions with accumulated 2-oxoglutarate, contributing to perturbed relative abundance of the amino acids in the SP-treated systems (Fig. 3). The perturbation in the amino acid pool is associated with decreased protein content (Figs. 4, 5a), affecting the organism's weight (Fig. 5b).

In general, the observed decreases in the total protein (Fig. 4) reflect the changed balance between protein synthesis and degradation, which may involve either decreased synthesis or increased degradation of intracellular proteins, or both. To understand whether the decrease in the protein synthesis does occur under SP treatment, we used the simplest system of the SP action in the minimal medium, i.e., potato tubers. We compared the amino acid and protein data obtained in this system (Table 2; Fig. 4) with the distribution of ^{14}C atoms from uniformly labeled glucose to amino acids and proteins under the same conditions (Fig. 6). This experiment showed that the detected increases in amino acids due to the SP treatment (Table 2; Fig. 3) are also observed as an increase in the total fraction of the ^{14}C-labeled amino acids from the metabolized ^{14}C-labeled glucose (Fig. 6a). The result confirms the increased synthesis of amino acids from glucose upon accumulation of 2-oxoglutarate. However, in spite of the increased synthesis of amino acids (Fig. 6a), the label incorporation into the protein was reduced (Fig. 6b), indicating that the accumulated amino acids are less efficiently incorporated into protein in the SP-treated tubers. Thus, decreased protein synthesis (Fig. 6b) contributes to the reduction in total protein (Fig. 4) observed upon accumulation of 2-oxoglutarate and amino acids (Figs. 1, 6a). Increased protein degradation to compensate for the perturbed glucose oxidation due to impaired TCA cycle may contribute to the decreased protein content as well.

Discussion

General implications of the OGDHC inhibition for amino acid metabolism

2-Oxoglutarate is a known precursor for the biosynthesis of many amino acids. This metabolic function of 2-oxoglutarate may also be linked to the effect of its dietary supplementation on mTOR (mammalian Target Of Rapamycin) signaling (Hou et al. 2010, 2011), which controls growth and proliferation in mammals depending on the nutrient availability. However, the role of the highly regulated mitochondrial system for the irreversible degradation of 2-oxoglutarate, namely OGDHC, in the general and species-specific interactions between the catabolic and anabolic

pathways of the 2-oxoglutarate metabolism, has not received much attention in eukaryotes. In the present work, we used comparative metabolomics to address this problem. We applied an established pharmacological approach to specifically inhibit OGDHC by a synthetic substrate analog SP (Bunik and Fernie 2009). The consequences of such inhibition for a specific subset of pathways, i.e., those of the amino acid metabolism, were characterized using another established approach, namely the metabolic profiling (Lisec et al. 2006). Comparative metabolomics was done on a set of autotrophic and heterotrophic systems from the plant and animal kingdoms. Besides, we evaluated the SP action on cyanobacteria known to lack OGDHC (Laurent et al. 2005), to obtain metabolic evidence on the in vivo specificity of SP action, which was previously shown in studies of enzymes, 2-oxoglutarate transporters and regulatory protein binding to 2-oxoglutarate (Bunik et al. 2005; Araújo et al. 2008, 2012; Bunik and Fernie 2009). Although one should always be aware that a drug could affect targets beyond those studied, to date, biological activities of none of the tested proteins binding or transforming 2-oxoglutarate or its structural analogs were affected by SP. This high selectivity is due to the known mechanism of the SP inhibition, involving formation of the 2-oxoglutarate dehydrogenase transition state analog (Bunik et al. 1992; Bunik and Fernie 2009). Because this type of inhibition is based on catalytic events occurring specifically at the active site of the 2-oxoglutarate dehydrogenase, even the close catalytic relative of 2-oxoglutarate dehydrogenase, a ThDP-dependent 2-oxoglutarate decarboxylase, has orders of magnitude lower affinity to SP (Fang et al. 2010). Comparison of the metabolic profiles characterized in the present work further supports the high specificity of the SP action in vivo. Indeed, the SP treatment of cyanobacteria, which do not have OGDHC, does not induce metabolic perturbation, as it does in the plant and animal systems that possess OGDHC (Table 2). It is worth noting that at the concentrations employed ($\geq 10^{-4}$ M), the phosphonate derivatives of 2-oxoglutarate inhibit the growth of *Mycobacterium tuberculosis* (Fang et al. 2010). The SP penetration through the bacterial cell wall even in the case of a microorganism known to be highly resistant to permeation (Fang et al. 2010) does not support the assumption that the absence of the SP effects on the cyanobacterial amino acid pool (Table 2) is due to a permeability problem. The SP penetration into cyanobacteria is also consistent with our observation of the SP candidate peak in the metabolome of the SP-treated cyanobacteria, although due to technical reasons we could not unambiguously quantify the peak. The absence of metabolic changes in the SP-treated cyanobacteria (Table 2) is in good accordance with the specificity of the SP action shown earlier (Bunik et al. 2005; Araújo et al. 2008, 2012; Bunik and Fernie 2009) and the fact that the phosphonate analogs of 2-oxo acids are much more powerful inhibitors of the 2-oxo acid dehydrogenases, compared to the non-oxidative 2-oxo acid decarboxylases transforming the same substrate (O'Brien et al. 1980). Indeed, in the medium with 1–2 mM 2-oxoglutarate, SP significantly inhibits the eukaryotic 2-oxoglutarate dehydrogenases at concentrations as low as 10^{-6} to 10^{-5} M (Bunik et al. 2005, 2009; Araújo et al. 2008), while even at a 100-fold lower 2-oxoglutarate concentration (0.02 mM), the inhibition of a ThDP-dependent 2-oxoglutarate decarboxylase from *Mycobacterium tuberculosum* requires SP at 10^{-4} to 10^{-3} M (Fang et al. 2010). This difference in the efficiency of the SP action, which is due to its mimicking catalysis-specific transition state of OGDHC but not of the bacterial 2-oxoglutarate decarboxylase, is in accordance with the data of Table 2, showing the SP effect only upon the metabolite profile of the systems possessing the multienzyme complex for the 2-oxoglutarate oxidative decarboxylation, but not on the metabolite profile of cyanobacteria possessing the non-oxidative 2-oxoglutarate decarboxylase (Zhang and Bryant 2011).

A significant shift in the amino acid pools due to specific inhibition of OGDHC by SP, common for the animal (rat brain, neurons) and plant (*Arabidopsis* seedlings, potato tubers) systems, is established in the present work. Several other studies focusing on particular amino acids or their small sets are in good agreement with our metabolic profiling of the amino acid pool (Tables 2, 3). For instance, an independent in vivo study showed that introduction of 2-oxoglutarate solution directly to the crop of turkey increased plasma concentrations of Pro and Leu, decreasing those of taurine and Gln (Tatara et al. 2005). Taurine was also decreased in the cortex of mice with a reduced activity of OGDHC due to disrupted expression of the core component of the complex, dihydrolipoyl succinyl transferase, although the brain levels of GABA in these animals were not different from controls (Nilsen et al. 2011). It should be stressed that the same defect in the OGDHC expression in cultured cells increased GABA and Ala (Shi et al. 2009; Nilsen et al. 2011) similar to our results (Tables 2, 3). Moreover, the OGDHC inhibition-affected metabolites could either increase or decrease depending on the surrounding medium. For instance, the levels of GABA and Ala upon OGDHC inhibition in the cerebellar granule neurons decreased in the minimal experimental medium (Sá Santos et al. 2006), but increased in the rich amino acid-supplemented growth medium (Table 2). Furthermore, the profile of the changed metabolites could vary depending on the system where the OGDHC inhibition occurred. As already mentioned, upon the changed expression of OGDHC, different metabolites were perturbed in cell culture (Shi et al. 2009; Nilsen et al. 2011) and in animals (Nilsen et al. 2011). Besides, the same SP exposure of the animals in different physiological states

also resulted in different responses of the brain amino acids to the treatment (Table 3). The observed variations in the metabolic responses to the OGDHC inhibition indicate that the buffering capacity of the metabolic network to compensate for metabolic perturbations greatly depends on conditions, including both the external supply of substrates (e.g., rich vs. minimal medium) and internal properties of the metabolic network (e.g., different patterns of the enzyme expression in the pregnant vs. non-pregnant rats). The condition-dependent consequences of the OGDHC inhibition are supported by modeling of the mitochondrial function under the OGDHC flux restrictions, which showed, e.g., that the 2-oxoglutarate efflux from the system occurs only when the flux through the GABA shunt is limited (Smith and Robinson 2011).

Compensatory pathways activated as a result of the OGDHC inhibition in plant and animal systems

Several ^{13}C-label distribution studies of plant and animal systems pointed to activation of the GABA shunt upon the OGDHC inhibition (Araújo et al. 2008, 2012; Sá Santos et al. 2006), which is supported by the perturbations in the Glu and GABA levels observed in different experiments on the OGDHC inhibition discussed above. Modeling the consequences of the OGDHC inhibition for the heart mitochondrial function also revealed the compensatory activation of the GABA shunt (Smith and Robinson 2011). Thus, the succinate-producing GABA shunt appears to be activated universally in response to the OGDHC inhibition. Because the decrease in mitochondrial respiration, which is observed upon the OGDHC inhibition in plant and animal tissues, is substrate-dependent (Araújo et al. 2008; Cheshchevik et al. 2010), with the succinate-supported respiration not affected, the compensatory activation of the GABA shunt producing succinate indicates that the OGDHC inhibition does not necessarily cause a decrease in cellular ATP. Moreover, the modeling showed that even the complete block of the OGDHC flux had only a minor effect on ATP production (Smith and Robinson 2011). This agrees with the high buffering capacity of the metabolic network regarding the ATP synthesis (Brown 1992; Rolfe and Brown 1997). Interestingly, in some cases, when the respiration was decreased due to a short-term (5 min) hypoxia (Zeiger et al. 2010) or when OGDHC in animals was inhibited by pyrithiamine (McCandless 1982), increases in ATP were even observed in neuronal culture and brain, respectively. Activation of the energy-consuming biosynthetic processes, suggested by our results and discussed below, also indicates that alternative respiratory substrates are provided and pathways activated to compensate for the energy deficit upon OGDHC inhibition.

The species- and/or condition-specific pathways compensating for the OGDHC inhibition are important to note in view of the potential applications of the OGDHC regulation to metabolic engineering and for treating metabolic disorders. In a photosynthetic system with highly active biosynthetic machinery (seedlings), we observed Gly depletion, which did not occur in the heterotrophic systems (Table 2; Fig. 3). This finding is consistent with the high abundance of the system of oxidative Gly decarboxylation, generating NADH, ammonium and one carbon synthetic unit transferred to tetrahydrofolate, specifically in the plant photosynthetic cell (Douce et al. 2001; Bauwe et al. 2010). As a result, NADH derived from oxidative decarboxylation of Gly may contribute to the compensation for insufficient energy production resulting from the OGDHC reaction. At the same time, other products of Gly degradation reaction, methylenetetrahydrofolate and ammonium, may allow the seedlings to synthesize amino acids beyond the proximal 2-oxoglutarate metabolites Glu and GABA. Indeed, the large relative increases in Ile, Met and Ser occur under sufficient Gly supply (within 2 h of the SP treatment), pointing to the SP-elevated biosynthesis of these amino acids in seedlings at the expense of the Gly decarboxylation. Ser may be directly formed from Gly according to the serine hydroxymethyltransferase-catalyzed Reaction 2, which uses the product of Gly degradation bound to THF (i.e., methylenetetrahydrofolate; MTHF).

$$\mathrm{Gly} + \mathrm{MTHF} \leftrightarrows \mathrm{Ser} + \mathrm{THF} \quad (2)$$

Other products of Gly degradation, i.e., ammonium and NADH, may be used in Reactions 3–5, which utilize also the SP-accumulated 2-oxoglutarate for the Glu synthesis in the plant-specific Reaction 5.

$$\text{2-Oxoglutarate} + \mathrm{NH_3} + \mathrm{NADH} + \mathrm{H^+} \leftrightarrows \mathrm{Glu} + \mathrm{NAD^+} + \mathrm{H_2O}, \quad (3)$$

$$\mathrm{Glu} + \mathrm{NH_3} + \mathrm{ATP} \rightarrow \mathrm{Gln} + \mathrm{H_2O} + \mathrm{ADP} + \mathrm{P_i}, \quad (4)$$

$$\text{2-Oxoglutarate} + \mathrm{Gln} + \mathrm{NADPH} + \mathrm{H^+} \leftrightarrows 2\ \mathrm{Glu}, \quad (5)$$

$$2\ \text{2-Oxoglutarate} + 2\mathrm{NH_3} + \mathrm{ATP} + 2\mathrm{NAD(P)H} + 2\mathrm{H^+} \rightarrow 2\mathrm{Glu} + 2\mathrm{H_2O} + \mathrm{ADP} + \mathrm{P_i} + 2\mathrm{NAD(P)^+} \quad (6)$$

Thus, the Gly-derived ammonium may stimulate the plant-specific Glu synthesis from 2-oxoglutarate (Reaction 6), alternative to the Asp-involving 2-oxoglutarate transamination. As a result, Asp may be used for biosynthesis of Ile and Met, which indeed increase in response to the SP treatment of a plant system (Fig. 3c). Interestingly, branched chain amino acids are known to accumulate in plants under stresses including decreased respiration (Araújo et al. 2011), and a rapid threefold increase in the Ile level of

seedlings (Table 2) treated with the respiratory inhibitor SP (Araújo et al. 2008) may thus be considered as a component of the stress response. With increasing the SP incubation time, Gly becomes depleted (Table 2; Fig. 3). This correlates with the decreases in the levels of many amino acids and in their ratios to 2-oxoglutarate, observed between 2 and 4 h of incubation of seedlings with SP (Table 2; Fig. 3). In particular, the Ile level at 4 h of incubation of seedlings with SP returns to the control (Fig. 3; Table 2). The finding indicates that with Gly depletion, the system begins to lack energy and nitrogen to be incorporated into the carbon skeleton of accumulated 2-oxoglutarate. Remarkably, after 2 h of the SP action, the Gln level in seedlings is kept constant, while its ratio to 2-oxoglutarate is reduced (Fig. 3), pointing to an increased usage of Gln as a nitrogen source under conditions of 2-oxoglutarate accumulation. When Gly is depleted, not only the Gln/2-oxoglutarate ratio, but also the Gln level is reduced in seedlings (Fig. 3; Table 2). Thus, whereas NADH from Gly degradation can compensate for the inhibited NADH production by OGDHC (Reaction 1), the other two products are used by seedlings for the amino acid biosynthesis in response to accumulation of an amphibolic intermediate, namely 2-oxoglutarate.

A compensatory response to the OGDHC inhibition, detected specifically in animal systems, comprises activation of OGDHC (Fig. 2). Comparison of the changes in the physiologically different animals (pregnant vs. non-pregnant rats) indicates that the up-regulation occurs after the OGDHC-dependent flux decreases to a critical level causing significant perturbation in the metabolic network (Fig. 2; Table 3). Taking into account the fact that this response is activated over a short time period (Fig. 2, astrocytes after 30 min of incubation with 0.1 mM SP) and under conditions inhibiting protein synthesis (Figs. 4, 5), a post-translational modification of OGDHC seems likely.

It is worth noting that the changes in the in vitro OGDHC activity were not in accordance with the straightforward expectation that the higher the activity, the lower is the Glu level. Indeed, when the extracted activity of OGDHC increased in response to SP, Glu increased as well (pregnant rats), whereas no up-regulation of OGDHC in response to SP was associated with the SP-induced decrease in the Glu content (non-pregnant rats; Fig. 2; Table 3). This could indicate that in pregnant rats with initially lower OGDHC activity, the in vivo flux through OGDHC was indeed inhibited by SP, causing the detected increase in Glu. However, in the non-pregnant rats with the initially higher OGDHC activity, the metabolic perturbation due to SP inhibition could have been compensated by the changed substrate saturation of other members of the metabolic network, such as the Glu/2-oxoglutarate aminotransferases and GABA shunt enzymes, resulting in a decrease in the Glu level without changes in other amino acids (Table 3). Thus, comparison of the SP effects in physiologically different animals suggests an interplay between the brain OGDHC activity and Glu levels in vivo, which may involve also other member(s) of the metabolic network transforming 2-oxoglutarate and its closest metabolites Glu and GABA.

2-Oxoglutarate is known to stimulate initiation of catabolism of the branched chain amino acids via the branched chain amino acid transaminase (Hou et al. 2011). Indeed, in spite of the general increase in the amino acid levels and the possibility of their delivery from the neuronal culture medium, the branched chain amino acids Ile, Leu, Val are maintained at a constant level in heterotrophic systems or decrease in seedlings (notably Ile) after the biosynthetic resources are depleted (Table 2). This finding points to efficient transamination of the branched chain amino acids with the SP-accumulated 2-oxoglutarate, followed by the irreversible degradation of the corresponding 2-oxo acids through the branched chain 2-oxo acid dehydrogenase complex. The compensatory importance of the universal activation of this pathway in plant and animal systems is apparently due to the fact that it not only generates NADH that is lost upon blocking the 2-oxoglutarate oxidation, but also ultimately leads to succinyl-CoA when Ile and Val are degraded. This product can enter the TCA cycle at the level of succinyl-CoA ligase following OGDHC. As a result, Ile and Val degradation may restore the TCA cycle at the level of succinyl-CoA ligase, providing for the generation of mitochondrial A(G)TP at the substrate level in Reaction 7.

$$\text{Succinyl} - \text{CoA} + \text{A(G)DP} = \text{CoA} + \text{A(G)TP} \quad (7)$$

Thus, by compensating for the OGDHC inhibition through the restoration of both the energy production and TCA cycle in plants and animals, transamination of Ile and Val with 2-oxoglutarate, followed by oxidation of the corresponding 2-oxo acids is likely an efficient way to overcome the SP-induced block of the TCA cycle at the OGDHC level.

An important difference between the animal (neurons) and photosynthetic (seedlings) systems concerns the SP-induced changes in Cys. The constant level of Cys in neurons contrasts with its twofold accumulation in seedlings (Table 2), suggesting increased Cys consumption upon the neuronal incubation with SP. Indeed, the glutathione level was affected by the OGDHC inhibition, showing a decrease upon cellular incubation with SP in a minimal medium (Sá Santos et al. 2006), but increasing when cells with compromised OGDHC were in the rich growth medium (Shi et al. 2009). Because the OGDHC inhibition activates compensatory pathways involving the amino acid degradation, the increased intracellular

glutathione of cells cultivated in the rich medium may suggest the glutathione carrier function to be employed for the amino acid uptake through the γ-glutamyl cycle (Orlowski and Meister 1970). Indeed, the SP-treated neurons, where Cys does not increase (Table 2), strongly depend on the amino acid supply from the medium which therefore contains 17 amino acids and Ala-Gln dipeptide. In contrast, the experimental medium of the SP-treated seedlings, where a twofold increase in the Cys is observed (Table 2), contains Gly only. The active amino acid uptake through the γ-glutamyl cycle may thus explain increased consumption of neuronal Cys for the glutathione synthesis as compared to that of seedlings (Table 2; Fig. 3). Although the physiological role of the γ-glutamyl cycle in mammals has been questioned (Hanigan and Ricketts 1993), our observations rather support the view that the process may have a role in the amino acid uptake, at least under some conditions (Viña et al. 1989). The proposed function of the cycle to use extracellular glutathione as a source to increase intracellular glutathione (Hanigan and Ricketts 1993) cannot be employed under our conditions, as glutathione is absent in the neuronal incubation medium.

OGDHC inhibition has an important impact on the biosynthetic ability of eukaryotic systems

Bacterial synthesis of Glu and arginine is known to be induced by OGDHC inhibition (Kataoka et al. 2006; Asakura et al. 2007; Bott 2007; Schultz et al. 2007). As discussed above, increased Gly degradation in the SP-treated *Arabidopsis* seedlings (Table 2) provides for the biosynthesis of Ile, Met and Ser (Table 2; Fig. 3). Thus, OGDHC inhibition may be used to increase the plant biosynthesis of essential amino acids at the expense of Gly, which is likely to be of biotechnological importance. Additionally, the OGDHC-dependent effects on the amino acid biosynthesis in animal systems may have biomedical implications. In particular, the SP-induced changes in amino acids of neurons and brain, which either are neurotransmitters themselves, such as GABA, Glu and Gly, or neurotransmitter precursors, such as Phe or Trp (Tables 2, 3), may be responsible for the behavioral and physiological effects of SP, observed previously in rats (Bunik et al. 2009; Graf et al. 2009; Trofimova et al. 2010). For instance, the SP-induced up-regulation of brain OGDHC was accompanied by increased exploratory activity and decreased anxiety of the experimental animals (Trofimova et al. 2010), with the SP pre-treatment protecting from behavioral stress due to hypoxia or alcohol intoxication (Bunik et al. 2009).

While the total synthesis of amino acids from glucose is increased due to OGDHC inhibition, protein synthesis is decreased under these conditions (Fig. 6). Indeed, apart from the consumption of Gln as the nitrogen source, the amino acids undergoing transamination with 2-oxoglutarate, such as Asp and branched chain amino acids Leu and Val, universally show large decreases in their ratios to 2-oxoglutarate (Fig. 3e–h). Phe, Tyr, His, Met and Cys are also known to be transaminated with 2-oxoglutarate by aspartate transaminase (Cooper 2004). The efficiency of these reactions in vivo is affected by the relative concentrations of these amino acids and their salvage pathway through the action of glutamine transaminase K (Cooper 2004; Kuhara et al. 2011). The latter enzyme catalyzes the Gln-dependent transamination of the 2-oxo analogs of amino acids, with those of Phe, Tyr, His, Met and Cys, all being good substrates. Species-specific differences in the steady-state levels of the amino acids determining the aspartate aminotransferase saturation with different amino acids, and efficiency of the glutamine transaminase K salvage of the amino acids from their 2-oxo analogs may thus explain different positioning of these amino acids as shown in Fig. 3. Apart from transamination, 2-oxoglutarate may condense with Lys producing Glu and 2-aminoadipate 6-semialdehyde in the saccharopine dehydrogenase-catalyzed reaction. This reaction is in line with the finding of no significant increases in Lys upon the 2-oxoglutarate accumulation (Table 2; Fig. 3a–d) and a decrease in the Lys/2-oxoglutarate ratio, similar to that inherent in the amino acids transaminating with 2-oxoglutarate (Fig. 3e–h).

The observed relationship between the decrease in total protein of the SP-treated systems (Fig. 4) and the degree of perturbation in the amino acid pool (Tables 2, 3; Fig. 3) suggests that the protein decrease is most likely due to the changed relative abundance of intracellular amino acids, induced by the 2-oxoglutarate accumulation. Although regulation of the metabolic network by the signaling role of amino acids and 2-oxoglutarate (Wu 2009; Hou et al. 2010, 2011; Araújo et al. 2011) may provide additional mechanism(s) for the observed protein decrease, competition for the branched chain amino acids between transamination and protein synthesis pathways could be a straightforward mechanism accounting for the observed changes in the protein content of the SP-treated systems. Indeed, decreased protein synthesis is known to occur in situations where some amino acids are depleted more than others (Iskakova et al. 2006; Swartz 2006). The significance of the issue is further highlighted by the above-mentioned fact that biological systems possess glutamine transaminases for the salvage of a number of essential amino acids undergoing non-specific transamination with the abundant metabolites 2-oxoglutarate and pyruvate (Cooper 2004; Kuhara et al. 2011). So far, the OGDHC inhibition-induced shift in the transaminase reaction provides a simple mechanism to switch between different ratios of protein synthesis to degradation.

Independent of specific regulatory mechanisms existing in different metabolic networks, our data show that distribution of 2-oxoglutarate between its transformations to either succinyl-CoA in Reaction 1 or Glu is essential for interaction between the carbohydrate and amino acid metabolic pathways in both plants and animals. The regulatory consequences of this interaction extend to controlling such general features of biological systems as the respiratory substrate choice and overall biosynthetic ability.

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