# Investigating Metabolite Channelling in Primary Plant Metabolism

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Thesis submitted for the degree of

Doctor of Philosophy, University of Oxford,

Department of Plant Sciences

Trinity Term 2012

# Abstract

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The tricarboxylic acid (TCA) cycle is one of the central pathways in respiration and also plays an important role in a variety of metabolic processes including the synthesis of secondary metabolites and the provision of carbon skeletons for ammonium assimilation and amino acid biosynthesis. Effective regulation of these multiple demands on the TCA cycle is likely to be very important for plant fitness. One way that this regulation could be achieved is through metabolite channelling. This occurs when metabolites are transferred between enzyme active sites without diffusing into the bulk aqueous phase of the cell, and is known to be important in regulating demands in metabolic pathways.

Although there is evidence that metabolite channelling exists in animals, there have been no attempts to investigate it in plant. The first aim of this thesis was therefore to investigate whether metabolite channelling exists in the plant TCA cycle. Isotope dilution experiments were developed to investigate metabolite channelling, and were able to show that metabolite channelling was present between certain enzymes of the TCA cycle in both *S. tuberosum* and *A. thaliana* mitochondria. The second aim of the thesis was investigate whether metabolite channelling is important in regulating the TCA cycle in plant mitochondria. The pattern of metabolite channelling did not change in mitochondria isolated from the light and the dark, or from mitochondria with increased or decreased TCA cycle rates, but it was not possible to say whether the metabolite channelling altered in a quantitative fashion. Overall the thesis provides the first direct evidence of channelling in the TCA cycle in plants, and further work should help to elucidate what role, if any, it plays.

# Acknowledgments

I would like to thank my supervisors, Dr Lee Sweetlove and Professor R. George Ratcliffe for all their guidance, help and patience over the course of my DPhil, and Dr Gwendoline Leach at Advanced Technologies Cambridge for useful discussions and advice.

Thanks also to Dr Tom Williams and Dr Shyam Masakapalli for invaluable discussions, advice and help with GC-MS and NMR, Pedro Bota for excellent technical support, Dr Chris Snowden for providing both *S. cerevisiae* and advice on working with them, and Dr Markus Schwarzländer and Dr Megan Morgan for advice and assistance with mitochondrial isolation. I also wish to thank everyone I have had the very great privilege of working with on the Biochemistry floor for the past four years and my friends and family for all their support and encouragement.

This work was funded by a BBSRC CASE studentship with Advanced Technologies Cambridge, UK.

## Abbreviations

| | |
|---|---|
| 2OG | 2-oxoglutarate |
| 2OGDH | 2-oxoglutarate dehydrogenase |
| αKGDH | α-ketoglutarate dehydrogenase |
| AAT | aspartate aminotransferase |
| ADP | adenosine-5'-diphosphate |
| ALA | 5-aminolevulinic acid |
| AOX | alternative oxidase |
| ATP | adenosine-5'-triphosphate |
| BCECF | 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein |
| BTC | 1,2,3-benzenetricarboxylate |
| BSA | bovine serum albumin |
| CCCP | carbonyl cyanide *m*-chlorophenylhydrazone |
| CHO | chinese hamster ovary |
| CK2 | casein kinase 2 |
| COX | cytochrome oxidase |
| CPS | carbamoyl phosphatate synthase |
| CS | citrate synthase |
| DHFR | dihydrofolate reductase |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| EDTA | ethylenediaminetetraacetic acid |
| $FADH_2$ | flavin adenine dinucleotide |
| FRET | förster (fluorescence) resonance energy transfer |
| GABA | γ-aminobutyric acid |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| GC-MS | gas chromatography mass spectrometry |
| GDC | glycine decarboxylase |
| GDH | glutamate dehydrogenase |
| GFP | green fluorescent protein |

| | |
|---|---|
| GOGAT | glutamate synthase |
| GS | glutamine synthetase |
| HPLC | high-performance liquid chromatography |
| HxK | hexokinase |
| ICDH | isocitrate dehydrogenase |
| MDH | malate dehydrogenase |
| MES | 2-[morpholino]ethanesulfonic acid |
| MFA | metabolic flux analysis |
| MSTFA | *N*-methyl-*N*-(trimethylsilyl)trifluroacetamide |
| NADH | nicotinamide adenine dinucleotide |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NIT4 | nitrilase 4 |
| NMR | nuclear magnetic resonance |
| OAA | oxaloacetic acid |
| OCT | orientation conserved transfer |
| PDC | pyruvate dehydrogenase complex |
| PDH | pyruvate dehydrogenase |
| PEG | polyethylene glycol |
| PEP | phosph*enol*pyruvate |
| PRK | phosphoribulokinase |
| PVP | polyvinylpyrrolidone |
| RFP | red fluorescent protein |
| SDH | succinate dehydrogenase |
| TCA | tricarboxylic acid cycle |
| TES | 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid |
| TPP | thiamine pyrophosphate |
| TS | thymidilate synthase |
| UDP | uridine diphosphate |
| YPD | yeast extract peptone dextrose |

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# Chapter 1: General Introduction

Plants produce a huge number of important compounds, including nutrients such as carbohydrates, oils and protein; vitamins and other health-beneficial micronutrients such as antioxidants; pharmaceuticals such as taxol and vincristine; and economically important products such as cotton. A goal of modern plant science is to engineer plants to our advantage through increasing yields, increasing the efficiency of resource utilisation and by changing the types or quantities of metabolites produced. Achieving this will involve altering the metabolic pathways of plants, either by genetic engineering or by targeted breeding approaches.

Many of the reactions that constitute plant metabolism have been known for some time, and recent progress in genomics mean that we understand far more about the genetic basis of many metabolic pathways. This has led to hopes that we would be able to manipulate metabolism more successfully. However, although there have been many attempts to engineer plant metabolism, there are actually very few examples of successful projects (Milo and Last 2012). Those that have been successful have involved expensive and lengthy research phases. For example, microbes have been successfully engineered to produce pre-cursors to the anti-malarial artemisinin (Ro *et al.* 2006). This was seen as a major achievement, as it has the potential to allow the drug to be produced more quickly and at lower cost. However, it was estimated that the project cost over $25 million and 150 'person-years' of research (Smolke and Silver 2011). Part of the reason for this was the difficulty involved in trying to express a large number of enzymes in the correct quantities to ensure high product yield and to avoid the build-up of toxic metabolic intermediates. It is becoming clear that there

are large gaps in our understanding about how metabolic pathways are organised and regulated *in vivo*. This makes it very difficult to successfully engineer metabolism, as it is harder to identify engineering targets, and to predict what effect engineering metabolism will have. Until we have a better understanding of the organisation and regulation of plant metabolic networks, it is likely that the full potential of plant science will not be reached.

## Primary metabolism is an important target for metabolic engineering

Primary metabolic pathways are those that are common to most plants and are essential for their survival, including respiration, photosynthesis and nitrogen assimilation. They are therefore targets themselves: increasing the efficiency of photosynthesis and nitrogen assimilation are major targets of engineering (Parry *et al.* 2011; Long *et al.* 2006; Foyer *et al.* 2010). Primary metabolic pathways are also important because they provide precursors for a wide range of secondary metabolites. These include phenolics, alkaloids and isoprenoids which are usually vital for plant fitness rather than directly for survival, for example by providing defence against infection and herbivory. These compounds are often the target of engineering due to their medicinal properties (Aharoni and Galili 2011). The availability of precursors from primary metabolism is important for the production of secondary metabolites. Evidence for this comes from that fact that transcription factors which regulate genes encoding enzymes involved in biosynthesis of certain glucosinolates also up-regulate enzymes in primary metabolism that are involved in the prediction of precursors for this pathway (Malitsky *et al.* 2008). Similarly, the ratio of amino acids to sugars in tobacco can affect the ratio of phenylpropanoids (which are rich in carbon) and

alkaloids (which are rich in nitrogen) (Matt *et al.* 2002). There is also evidence from unsuccessful attempts at engineering secondary metabolites, which have suggested that flux through the pathway may be limited by the supply of precursors (Stephanopoulos and Vallino 1991). The result of this is that understanding the regulation of primary metabolic pathways is particularly crucial if we want to successfully engineer plant metabolism.

## The TCA cycle plays an important role in primary plant metabolism

The TCA cycle was first discovered in plants in the mid twentieth century (see Beevers 1961 and references therein). It is one of the central pathways in respiration, where it converts pyruvate from glycolysis into a series of organic acids, producing carbon dioxide and reductant in the form of NADH and $FADH_2$. This reductant is used in the mitochondrial electron transport chain to produce ATP. Extra carbon can enter the cycle as malate or OAA via cytosolic phospho*enol*pyruvate (PEP) carboxylase and malate dehydrogenase. Although it is most well-known for its role in respiration, the TCA cycle also plays an important role in many different metabolic processes in the cell (Figure 1.1). These include the synthesis of secondary metabolites such as mevalonate and its derivatives, as well as in providing carbon skeletons for ammonium assimilation and amino acid biosynthesis.

In addition to these well-established roles, there have been a number of studies which highlight the role of the TCA cycle in a variety of other processes. Plants with reduced activity of TCA cycle enzymes exhibit changes in flowering (Landschutze *et al.* 1995), stomatal opening (Fernie and Martinoia 2009) and leaf pigmentation (Sienkiewicz-Porzucek *et al*. 2008), although the mechanisms which link the TCA cycle and these

processes are currently unknown. There is also evidence for TCA cycle metabolite involvement in processes such as fatty acid extension (Fatland *et al.* 2005), cell expansion (Fernie and Martinoia 2009) and maintaining pH (Hurth *et al.* 2005).

## Different flux modes through the TCA cycle exist in plants

The traditional view of the TCA cycle is one of a predominantly cyclic pathway, with carbon skeletons removed for biosynthesis being replaced by so-called anaplerotic reactions. However, the more modern view sees metabolism as a network, with connections to lots of other reactions. The development of more sophisticated techniques of metabolic flux analysis has allowed us to study the fluxes through the TCA cycle as part of this wider network (Allen *et al.* 2009; Kruger and Ratcliffe 2009). The results have shown that in several tissues fluxes through the TCA cycle differ greatly from the traditional view of the cycle (Sweetlove *et al.* 2010).

There is evidence that flux modes through the TCA cycle differ depending on the demand for ATP. For example, a partial TCA cycle was able to support the synthesis of all the components of dividing heterotrophic cells according to a flux-balance model by Poolman *et al.* (2009). In this model, ATP demand was relatively low (because cell maintenance costs were not included) and can be met through substrate level phosphorylation in glycolysis and low levels of oxidative phosphorylation. When the demand for ATP was increased, a cyclic flux mode was necessary.

Flux modes through the TCA cycle can differ greatly in different tissues. Developing embryos from several species including maize (Alonso *et al.* 2010), soybean (Allen *et al.* 2009) and sunflower (Alonso *et al.* 2007) show a greater flux between 2OG and malate than the rest of the cycle, reflecting the entry of glutamate into the TCA cycle.

Glutamate levels are high in embryos as a result of the metabolism of glutamine supplied from the mother plant as the major nitrogen source. In contrast, when oilseed rape seeds were grown on ammonium nitrate as a nitrogen source a cyclic flux mode was present in the TCA cycle. The largest fluxes occurred between malate and citrate, and between citrate and 2OG (Junker *et al.* 2007). However, when embryos were cultured with glutamine and alanine as the nitrogen source, the TCA cycle existed as a non-cyclic pathway. In this situation, there was a small flux to citrate from 2OG, and larger fluxes between 2OG and malate, and malate and citrate, the latter being exported to the cytosol to form acetyl CoA (Schwender *et al.* 2006). Similarly, *Arabidopsis thaliana* seedlings also have greater fluxes through citrate synthase to provide citrate for the cytosolic acetyl coA synthesis (Lonien and Schwender 2009). *Lotus japonicas* roots under anoxic conditions are also thought to exhibit non-cyclic carboxylic acid metabolism, with fluxes from OAA to malate, and 2OG to succinate but no significant cyclic flux (Rocha *et al.* 2010). There is also a considerable amount of evidence that the TCA cycle is non-cyclic in leaf tissue in the light (Hanning and Heldt 1993, Tcherkez *et al.* 2009). This is discussed in more detail in Chapter 5.

### Regulation of the TCA cycle is not well understood

Although we are starting to understand a lot more about the different flux modes through the TCA cycle under different conditions, we know very little about how these flux modes are regulated. It is clear that the TCA cycle needs to balance demand for oxidative cyclic flux for ATP production, and non-cyclic anabolic flux allowing the withdrawal of intermediate carboxylic acids for biosynthesis. However, currently it is not clear how this is achieved. Most of the studies looking at regulation of the TCA

cycle have focused on the regulation of ATP production. This is generally short term regulation of the TCA cycle in response to fluctuations in substrates or ATP demand (Hill 1998) or feedback regulation due to product inhibition. This feedback inhibition is important in coupling reductant production to the ATP demand to prevent the mitochondria becoming over-reduced. The link between reductant state and TCA cycle flux is demonstrated by analysis of transgenic plants over-expressing uncoupling protein, which weakens the coupling between ATP synthesis electron transport and in turn influences TCA cycle activity, increasing flux between pyruvate and citrate (Smith *et al.* 2004). Williams *et al.* (2008) showed that in cell cultures under higher $O_2$ concentrations there was a corresponding increase in net fluxes throughout the TCA cycle. However the relative fluxes remained the same, meaning that the balance between pathways providing carbon skeletons for biosynthesis and ATP production remained the same. This suggests that, at least in the short term, the TCA cycle is tightly regulated to ensure stability of fluxes through the network.

Changes in enzyme abundance could contribute to regulation of the TCA cycle. For example, when nitrogen starved plants were re-supplied with nitrogen the expression of genes encoding NAD-IDH, CS and aconitase all increased, as well as the genes for nitrate reductase and glutamine synthetase (Lancien *et al.* 1999). Similarly, Fukushima *et al.* (2009) found that *A. thaliana* plants with mutations in three circadian clock genes PRR 9,7,5, showed increases in TCA cycle intermediates, as well as down regulation of genes encoding fumarase and a putative 2OGDH. However some caution should be taken in interpreting these results, as increases in transcript abundance do not necessarily reflect an increase in enzyme abundance. There is also evidence that

the abundance of TCA cycle enzymes differs between root and shoot mitochondria (Lee *et al.* 2008). Similarly, increases in protein amount may be in caused by protein turnover rather than rate of synthesis, and even if enzyme levels do increase, this may not be reflected in increases in activity or flux.

In addition, we currently have a poor understanding of how the TCA cycle flux differs between tissues and cell types, as well as the distribution of TCA cycle enzymes and metabolites between different compartments. For example, with the exception of the reactions catalysed by SDH and citrate synthase, all the steps of the TCA cycle can also occur in the cytosol (Sweetlove *et al.* 2010). Many TCA cycle enzymes are known to exist in the cytosol as well as the mitochondria (Hodges 2002, Pracharoenwattana *et al*. 2010), but it is not clear to what extent this is universal, or species, tissue or cell type specific. It is known for example, that a cytosolic fumarase exists in the cytosol of *A. thaliana (*Pracharoenwattana *et al.* 2010), but not in tomato (Nunes-Nesi *et al.* 2007). There are also significant gaps in our understanding of the extent to which metabolites move between different compartments in the cell. This is illustrated by the work of Krueger *et al.* (2011) who used non-aqueous fractionation to analyse the sub-cellular distribution of different metabolites. A significant proportion of the metabolites identified were present in compartments which were unexpected and could not be explained by conventional understanding of metabolism.

### Metabolite channelling may exist between TCA cycle enzymes

One aspect of the TCA cycle that could be involved in its regulation is metabolite channelling between enzymes. There is increasing evidence that metabolite channelling is important in metabolic pathways. Metabolite channelling occurs when

metabolites are transferred between enzyme active sites without diffusing into the bulk aqueous phase of the cell, and is normally associated with enzymes which are physically co-localised in a complex. Srere (1985) introduced the term metabolon for these arrangements. It should be pointed out that not all enzyme complexes channel metabolites, although the two are sometimes confused in the literature.

Metabolons vary in their stability. At one end of the scale is a metabolon such as tryptophan synthase, which catalyses the final two steps in L-tryptophan synthesis. The two reactions are catalysed by separate alpha and beta subunits which combine to form a stable multi-enzyme complex (Hyde *et al.* 1998). During synthesis, indole (the intermediate) is transferred between the two active sites through a hydrophobic tunnel within the enzyme complex (Hyde *et al.* 1998). At this end of the scale, the distinction between a metabolon and what would traditionally be thought of as a multifunctional enzyme is quite blurred.

At the other end of the scale there are metabolons which are transient in nature. These may be held together by weak forces, or may require the binding of a substrate or ligand before they form (Spivey and Ovadi 1999). These metabolons, which are sometimes called dynamic metabolons, may be particularly useful when metabolite channelling is important in controlling flux through different branches of a pathway, or in regulating the pathway under different conditions in the cell (Srere 1987). For example, the enzymes of glycolysis are known to associate with the outside of the mitochondria in a metabolon (Giegé *et al.* 2003). The proportion of enzymes in the cell which are part of the metabolon is responsive to changes in the metabolic state of the

cell, and metabolite channelling through these enzymes could be observed (Graham *et al.* 2007).

There are numerous examples of metabolite channelling in plant metabolism. In secondary metabolism channelling occurs in the synthesis of flavonoids, alkaloids, isoprenoid, phenylpropanoids, cyanogenic glucosides (Winkel 2004, Jorgensen *et al.* 2005). There are also examples in primary metabolism, including glycolysis (Graham *et al.* 2007) and the Calvin-Benson cycle (Gontero *et al.* 1988, Süss *et al.* 1993).

## There is evidence that TCA cycle enzymes exist as a metabolon

The majority of evidence for metabolon formation in the TCA cycle comes from work done in mammalian tissue and yeast. Most of the experiments provide evidence that enzymes of the TCA cycle interact with each other and the inner mitochondrial membrane. Fumarase, malate dehydrogenase, citrate synthase and aspartate aminotransferase were found to associate together *in vitro* (Beeckmans and Kanareck 1981). Fumarase, MDH, Citrate Synthase, aconitase and ICDH were also found to be associated together in *Escherichia coli*. The complex was disrupted by ultrasonication (Barnes and Weitzman 1986). Malate dehydrogenase, citrate synthase, isocitrate dehydrogenase, fumarase and aconitase also interact with each other in *B. subtilis* (Meyer *et al.* 2011). Enzymes were found to be associated with the inner membrane after chemical crosslinking in isolated rat liver mitochondria (D'souza and Srere 1983). Malate dehydrogenase and citrate synthase have also been shown to bind to the inner membrane (Morgunov and Kamzolova 2009), as have aspartate aminotransferase and malate dehydrogenase (Teller *et al.* 1990). A proportion of the citrate synthase activity in mitochondria was found to be associated with the membrane fraction, and this

interaction was sensitive to ionic strength (Robinson and Srere 1985). Individual TCA cycle enzymes were also shown to associate with the membrane fraction of disrupted cells (Millar *et al.* 1999), although the authors didn't measure whether this was specific to the inner mitochondrial membrane. There is also evidence that cytochrome bc1 interacts with malate dehydrogenase in *Bos taurus* mitochondria *in vitro* (Wang *et al.* 2010). Kispal *et al.* (1989) showed that citrate synthase which was inactive restored the ability of yeast cells to grow on acetate compared to cells with no citrate synthase. They argue that this is because the enzyme restores a metabolon of TCA enzymes, which then allows acetate metabolism. There is some evidence that TCA cycle enzymes have restricted mobility in the mitochondrial matrix in CHO cells. Haggie and Verkman (2002) found that TCA cycle enzymes tagged with GFP had lower than expected recovery times after photo-bleaching, suggesting that diffusion through the matrix was restricted. This may be due to interactions with other TCA cycle enzymes, or with other matrix components or membranes.

Although these experiments suggest that TCA cycle enzymes are capable of interacting with each other and the membrane in mitochondria, this is not proof of metabolite channelling. This can only be demonstrated by directly studying fluxes through the TCA cycle.

### Very few attempts have been made to measure *metabolite channelling* in the TCA cycle in plants

There have been very few attempts to measure metabolite channelling directly in the plant TCA cycle. There is some evidence that 2OGDH associates with the mitochondrial membrane fraction in plants, but no evidence that it forms a metabolon (Millar *et*

*al.*1999). Wiskich *et al.* (1990) found evidence for separate pools of enzymes within the same matrix in mitochondria isolated from in pea leaves. Malate dehydrogenase was found to be able to operate in opposite directions simultaneously when oxidising malate and glycine. By using an inhibitor which blocks OAA transport across the inner mitochondrial membrane, they were able to show that the reactions occurred in the same mitochondrial matrix, rather than in separate mitochondria.

Currently, it is not clear whether metabolite channelling exists in the TCA cycle in plants, although the presence of metabolite channelling across a range of phyla suggests that it would not be a surprise if it did. Although there is some evidence for metabolite channelling in the TCA cycle, it is also unclear what role, if any, it plays.

## The role of metabolite channelling in metabolic pathways

Metabolite channelling appears to have evolved in many different pathways in a variety of organisms. There is considerable debate about the role of metabolite channelling, and it is likely that the role of metabolite channelling depends on the pathway and the metabolic context in which it occurs. It is therefore important not to generalise about the role of metabolite channelling in a pathway without testing it each time. However, there are some common themes which have been put forward to explain the existence of metabolite channelling across different pathways.

## Metabolite channelling may be a response to crowded cell environments

One hypothesis is that metabolite channelling is an inevitable outcome of the crowded environment of the cell (Ellis, 2001, Ovadi and Srere 1996): it has been suggested that more than 60% of the mitochondrial matrix volume might be made up of proteins (Goodsell 1991). There is some evidence that this environment encourages protein

interactions (Zimmerman 1993). The crowded mitochondrial matrix may also favour configurations which help preserve the limited solvent capacity of the matrix (Ovadi and Srere 1996).

However, if metabolite channelling was purely a response to solvation capacity, then we would not necessarily expect enzymes in the same pathway to be located near each other. The fact that they are suggests that such associations play a role in the function of the cell beyond preserving solvation capacity.

## Metabolite channelling may allow reactions to overcome diffusion limitations

Another hypothesis that has been forward to explain the existence of metabolite channelling is that it allows reactions to overcome diffusion limitations in cells (Ovadi and Saks 2004), and therefore increase the rate of reactions at lower effective substrate concentrations. Compartmentalising reactions would be one way to overcome barriers to diffusion (Verkman 2002), and metabolite channelling reactions would be one way to achieve this.

Some experiments have found that metabolite channelling increases the yield of product when enzymes are artificially co-localised on a protein scaffold. For example, Dueber *et al.* (2009) achieved 77-fold increases in the yield of mevalonate by attaching three enzymes of the metabolic pathway to a protein scaffold. This dramatic increase occurred even though expression levels were low. This suggests that, at least in theory, metabolite channelling is able to enhance flux through a pathway by raising the effective local concentrations of metabolites around the enzyme active site. This in turn increases the flux through the pathway compared to the same system of free

enzymes, where a higher concentration of metabolites is needed to provide the same effective concentration available to the enzymes.

This argument has been put forward to explain the existence of metabolite channelling in metabolic pathways. For example it has been argued that the expected rate of the citrate synthase reaction (based on measurements of the concentration of citrate synthase and free OAA) is an order of magnitude lower than the observed rate of the TCA cycle (Srere *et al.* 1997). The authors suggested that this result could be explained if OAA was passed directly between MDH and CS, therefore increasing the rate at lower measurable concentrations of free OAA. However, OAA is difficult to measure accurately inside the mitochondria, and as a result there is some question over the accuracy of these calculations.

This argument assumes that metabolic reactions are diffusion-limited in the cell. This is based on experiments which suggest that in crowded cell environments diffusion may be less efficient than is assumed in models (Ovadi and Srere 2000, Zimmerman 1993, Roosen-Runge 2011). Several experiments using fluorescence recovery after photo-bleaching have been used to investigate diffusion in the cytoplasm. BCECF, a fluorescent probe, diffused 3-4 times more slowly in cytoplasm than in water (Kao *et al.* 1993). After comparing diffusion in swollen cells with normal cells, the authors suggested that this effect was due to collisions between the probe and other components in the cell (Swaminathan *et al.* 1996). Similarly, GFP has been shown to diffuse more slowly in cytoplasm than in saline (Swaminathan *et al.* 1997). Experiments with GFP that was targeted to the mitochondrial matrix suggested that around 90% of the GFP was able to diffuse through the matrix (Partikian *et al.* 1998) In

contrast, when GFP was attached to enzymes of the TCA cycle (Haggie and Verkman 2002) there was a much slower recovery of fluorescence after photo-bleaching. It was suggested that this was because the diffusion of the enzymes was restricted in the matrix, possibly through attachment to a membrane. It was proposed that role of these complexes was to free up space in the matrix to facilitate more rapid diffusion of small molecules (Haggie and Verkman 2002).

However there are several criticisms of these approaches. Firstly, several experiments extrapolate from the diffusion of the large GFP molecule to the rate of diffusion of smaller metabolites, which are likely to diffuse differently through the cell. Recombinant GFP or other probes may also act very differently in cells to enzymes and proteins which are native to that environment. In addition, it is questionable whether the internal environment of the mitochondria is dynamic enough to allow observable recovery in the short time measured. Comparing enzyme-GFP complexes to free GFP is also problematic as GFP has very different properties to most enzymes, and is used precisely because it is able to diffuse through the cytosol with relative ease. Finally, even if the assumptions are correct, it is likely that GFP tags could cause disruption to any interactions which are occurring in the cell. As a result some of this experimental evidence may be of limited use. There is also some evidence that the reductions in diffusion measured in this way does not represent the physiological norm, as NMR experiments suggested that the diffusion coefficient of ATP and phosphocreatine in rat muscle is very similar to that *in vitro* (de Graaf *et al.* 2000).

A further criticism is that most of the evidence that metabolite channelling is able to increase rates by removing the limitations from diffusion come from experiments

where enzymes have been expressed as recombinant proteins *in vitro* or in yeast or *E. coli* and then artificially localised on scaffolds. The aim of these experiments is usually to maximise product titres, without increasing the levels of enzymes. In these situations, the enzyme expression levels are usually low, and the distance between enzymes is also likely to be large, meaning that they may be more likely to be limited by diffusion. Similarly, the enzymes are likely to be in a sub-optimal cellular environment compared to the one in which they have evolved. There is therefore greater scope to increase flux through pathways by localising them close together. In contrast, it is thought that very few enzymes are diffusion limited in the cell, and although there is some evidence that the diffusion rate of small molecules is slower in cells than in water (Kao *et al.* 1993) the rate is only 3 fold lower. The $K_{cat}$ of most enzymes of central metabolism is 100 $s^{-1}$ (Milo and Last 2012), suggesting that the rates of diffusion would need to be very slow to be limiting the reactions. For this reason, the argument that metabolite channelling evolved in response to limitations of diffusion is not a strong one.

## Metabolite channelling may prevent the diffusion of toxic or reactive intermediates

Another reason for the occurrence of metabolite channelling is protection from potentially toxic or reactive metabolic intermediates. For example, in sorghum, the cyanogenic glucoside dhurrin is made from tyrosine. One of the intermediates of this pathway is p-hydroxymandelonitrile, which can dissociate into hydrogen cyanide and p-hydroxybenzaldehyde, and is therefore toxic (Winkel 2004). The enzymes which catalyse the biosynthesis of dhurrin (two cytochrome P450 (called CYP79A1 and

CYP71E1), and a UDP-glucosyltransferase (called UGT85B1) are known to form a metabolon (Winkel 2004). This is thought to be important in preventing the toxic intermediate from diffusing into the rest of the cell. Evidence for this idea comes from the fact that an incomplete pathway consisting of just the two enzymes caused severe alterations in phenotype when it was heterologously expressed in *A. thaliana*. When the complete pathway was transferred this was not the case (Kristensen *et al.* 2005).

Similarily nitrilase 4 (NIT4) enzymes are important enzymes in many plants, as they are involved in cyanide detoxification. Metabolon formation is thought to be vital for their function, as protein extracts from *Poaceae* species exhibit detoxifying activity, but recombinant enzymes do not, and in sorghum three NIT4 enzymes are only able to hydrolyse β-cyanoalanine effectively if they are present in a complex. The presence of the metabolon is thought to be imporatnt in allowing plants to use cyanogenic glycosides as storage compounds, as it means they can be safely metabolised without releasing toxic hydrogen cyanide into the cell.

Another example is the carbamoyl phosphatate synthase complex, which catalyses four steps in the synthesis of carbamoyl phosphate (Anderson and Meister 1966). Here ammonia and carbamate, which are both reactive intermediates, are transferred directly between the three active sites of the complex through a hydrophobic tunnel (Thoden *et al.* 1997, Raushel *et al.* 1998). By preventing the diffusion of the reactive intermediates, the metabolon may be important in ensuring efficient synthesis of carbamoyl phosphate.

While metabolite channelling can clearly be important in preventing the escape of reactive or toxic intermediates in certain pathways, it is likely that these examples

constitute special cases in the evolution of metabolite channelling. Many pathways do not produce such toxic or labile intermediates, yet metabolite channelling is present across a wide range of pathways. This suggests that metabolite channelling plays another role in most metabolic pathways.

### Metabolite channelling is important in regulating flux through a pathway

Controlling flux through a pathway is another reason that metabolite channelling might exist. Studies on isolated chloroplasts suggest that enzymes of the Calvin cycle are organised into a multi-enzyme complex (Winkel 2004; Suss *et al.* 1993; Gontero *et al.* 1988). The complex is also associated with thylakoid membranes, near to where NADPH and ATP are synthesised. Assembly of the complex increases the activity of the enzymes, and also enhances the regulation of phosphoribulokinase (PRK) by reduced thioredoxin. In chlamydomonas PRK and GAPDH are regulated by NADPH when they are part of the complex, but by NADH when they are not (Graciet *et al.* 2002). Metabolon structure provides a mechanism for enhanced efficiency by modifying the activity and regulation of individual enzymes.

Metabolite channelling in glycolysis has also been shown to be important in ensuring demand for pyruvate is met, and in balancing the demand for glycolytic intermediates from other biosynthetic pathways. Glycolytic enzymes are known to interact on the surface of mitochondria in plants to form a metabolon which supports substrate metabolite channelling (Graham *et al.* 2007, Giegé *et al.* 2003). Graham *et al.* (2007) showed that the fraction of glycolytic enzymes associated with mitochondria increased with increasing respiration rate, and decreased with inhibition of respiration by KCN. This is thought to be to ensure glycolysis can fulfil the demand of the TCA cycle for

pyruvate. Metabolite channelling prevents the withdrawal of intermediates by competing pathways, such as the synthesis of amino acids or the oxidative pentose phosphate pathway. By changing the proportion of enzymes in the channelled pathway, fluxes into the TCA cycle and competing pathways can be regulated.

A third example in primary metabolism is seen in the work of An *et al.* (2008), who found that six enzymes of purine biosynthesis cluster together when HeLa cells were depleted of purine, but dissociated when the demand was low. Further work showed that formation of the complex was promoted by kinase inhibitors and phosphates, while dissociation was promoted by phosphorylation of the CK2 kinase (An *et al.* 2010).

This phenomenon is also seen widely in secondary metabolism. For example, calcone synthase is able to interact with both dihydroflavanol 4 reductase (which is involved in the anthocyanin biosynthetic pathway) and flavanol synthase 1 (which is part of the pathway that produces flavanols). These interactions have been shown to be competitive. This could be a mechanism to allow plants to divert flux into either pathway depending on the demand of the cell (Crosby *et al.* 2011). There is evidence that different interactions between phenylpropanoid biosynthesis enzymes on the ER facilitate the production of different classes of phenylpropanoids (Winkel 2004).

Similarly associations between enzymes of 5-Aminolevulinic acid (ALA) synthesis appear to be important in balancing heme and cholorophyll production in plants. GluTR is an important enzyme in controlling flux in the ALA synthesis. Czarnecki *et al.* (2011) found evidence that a GluTR binding protein was important in channelling flux into heme biosynthesis, by facilitating the binding of GluTR with the thylakoid

membrane. ALA produced via this enzyme appears to be used to synthesise heme, while ALA produced in the stroma produced chlorophyll.

Cyanogenic glucosides and glucosinolates are important compounds in plant defence, as they can be cleaved to produce hydorgen cyanide or isothiocyanates, which are important in insect defence. The production of cyanogenic glucoides is known to proceed via a metabolon (see above), which prevents reactive oximes from escaping into the cell. However, Møller (2011) has suggested that dissassembly of this metabolon may be important in producing oximes, which can be used as a defence against fungi, which are resistant to hydrogen cyanide or isothiocynanates. Regulation of metabolon assembly and disassembly may help to direct flux into oximes or cyanogenic glucosides and glucosinolates, depending on the type of predation.

### Metabolite channelling may be important in regulating the TCA cycle

The above examples illustrate the important role that metabolite channelling plays in regulating fluxes through different metabolic pathways in plants. It is becoming increasingly clear that this is the reason why metabolite channelling has evolved in many pathways across different organisms. It also provides the strongest case for the evolution of metabolite channelling in the TCA cycle.

There is evidence that metabolite channelling exists across several eukaryotic phyla, including yeast (Sumegi *et al.* 1990; Kispal *et al.* 1989), mammals (Sherry *et al.* 1994; Teller *et al.* 1990) and bacteria (Meyer *et al.* 2011, Barnes and Weitzman 1986). It is also present in a variety of cell types within the same phyla – for example, mammalian liver, heart and neurones. The widespread nature of metabolite channelling suggests that it may play a functional role in the TCA cycle.

As already discussed, the organisation of metabolons into functional groups suggests that channelling plays a role beyond that of maintaining solvation capacity. Similarily, the argument that metabolite channelling enables metabolic pathways to overcome diffusion limitations is not a strong one, and the role of metabolite channelling in mitigating the effects of toxic intermediates is unlikley to be important in the TCA cycle. However, there is a good case to be made that metabolite channelling is important in regulating fluxes through the TCA cycle.

TCA cycle intermediates are important for a variety of different metabolic processes in the cell, including the provision of carbon skeletons for ammonium assimilation and amino acid biosynthesis, and for the synthesis of secondary metabolites (see above). However, these same intermediates are also important in maintaining a cyclic flux through the TCA cycle. In order for the TCA cycle to work effectively, balancing the withdrawal of intermediates for biosynthesis with the maintenance of cyclic flux is crucial. If the correct balance is not achieved, it is likely to be detrimental to plant fitness.

Tight regulation of metabolic pathways is a feature of metabolism, but there are currently gaps in our understanding of how the TCA cycle is regulated. Metabolite channelling could provide an additional level of regulation beyond what is currently known: that is, the product inhibition that is common to all enzymes and some evidence that enzyme abundances change under different metabolic conditions. Metabolite channelling has been shown to be important in regulating other pathways that face multiple demands on their intermediates, including glycolysis.

Channelling could be important in regulating these different demands on TCA cycle intermediates. For example, 2-oxoglutarate is important in the production of many amino acids, but is also crucial in producing succinate for use in the electron transport chain and maintaining the cyclic flux through the TCA cycle for repsiration. Channelling could allow several pools of 2OG to exist in the mitochondria at the same time: one which provides carbon for amino acid synthesis, and another for respiration. The advantage of separating the two pools into different micro-compartments is that it would allow different demands on 2OG to be regulated independently. For example, an increased demand for amino acid synthesis could withdraw more 2OG from the TCA cycle, without compromising the production of ATP.

It is important to understand whether metabolite channelling is involved in regulating the TCA cycle. The TCA cycle is an important target for metabolic engineering, through its role in nitrogen assimilation (Foyer *et al.* 2010), photosynthesis (Parry *et al.* 2011) and in providing precursors for valuable secondary metabolites (Aharoni and Galili 2011). The more we understand about how these processes are regulated, the more successful we are likely to be in manipulating them. If channelling is important in regulating the TCA cycle then it is vital to understand this process. For example, if increasing flux through a section of pathway relies on changes in metabolite channelling or metabolon formation, then it is important to understand this.

## Aim of this thesis

Currently there have been very few attempts to investigate whether metabolite channelling exists in the TCA cycle in plants. The first aim of this thesis is therefore to investigate whether metabolite channelling exists in the plant TCA cycle. If metabolite

channelling is found to exist in the plant TCA cycle, I will then attempt to investigate whether it plays any role in regulating the TCA cycle in plant mitochondria

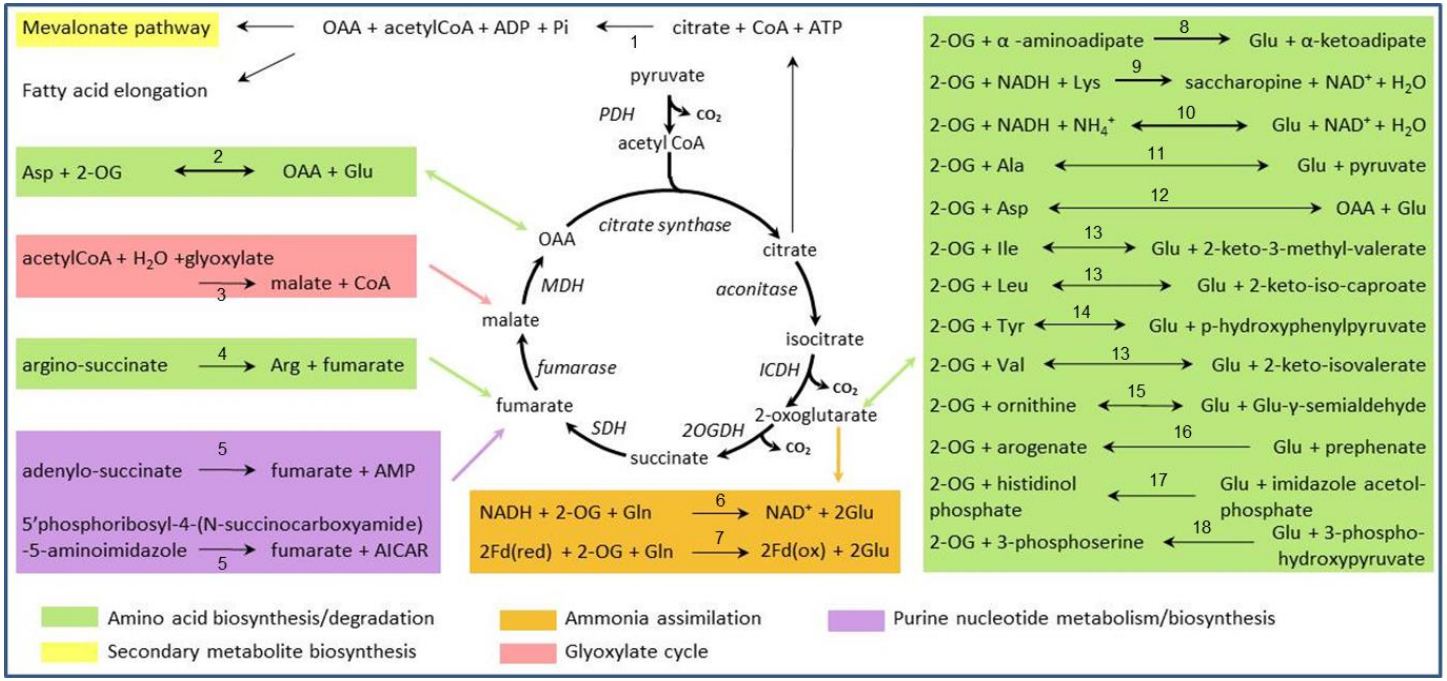


**Figure 1.1: Metabolic reactions that produce or consume TCA cycle intermediates** Abbreviations: ICDH, isocitrate dehydrogenase (NAD(P) dependent); MDH, malate dehydrogenase ($NAD^+$ dependent); 2OGDH, 2-oxoglutarate dehydrogenase; PDH, pyruvate dehydrogenase; SDH, succinate dehydrogenase. Metabolite abbreviations: AICAR, aminoimidazole carboxamide ribonucleotide; CoA, coenzyme A; Fd(ox), oxidized ferredoxin; Fd(red), reduced ferredoxin; 2-OG, 2-oxoglutarate; OAA, oxaloacetate; Pi, inorganic phosphate. Numbered reactions are catalysed by the following enzymes: 1, ATP citrate lyase; 2, Aspartate transaminase; 3, Malate synthase; 4, Arginosuccinate lyase; 5, Adenylosuccinate lyase; 6, Glutamate synthase (NADH); 7, Glutamate synthase (ferredoxin); 8, 2-aminoadipate transaminase; 9, Saccharopine dehydrogenase (NADH, L-lysine forming); 10, Glutamate dehydrogenase; 11, Alanine transaminase; 12, Aspartate transaminase; 13, Branched chain amino acid transaminase; 14, Aromatic amino acid transaminase; 15, Ornithine transaminase; 16, Glutamate-prephenate aminotransferase; 17, Histidinol-phosphate transaminase; 18, Phosphoserine aminotransferase.

# Chapter 2: Materials and Methods

## Reagents

General chemicals were purchased from Sigma-Aldrich, Gillingham, UK Fischer Scientific UK Ltd. Loughborough, UK or MERCK Chemicals Ltd, Nottingham, UK. unless otherwise stated. Enzymes were purchased from Roche Diagnostics, Burgess Hill, UK. [3-$^{13}$C]pyruvate (99 atom %) was purchased from Cambridge Isotopes, Anderstown, MA, USA and [3-$^{13}$C]glutamate (99 atom %) from Isotec (Sigma-Aldrich, Gillingham, UK)

## Biological material

### Growth of *A. thaliana* seedlings in liquid culture

Approximately 100 *A .thaliana* (ecotype Columbia) seeds were grown per 100ml Magenta vessels. Seeds were washed for 2 min in 70% (v/v) ethanol, then in 0.05 % (v/v) sodium hypochlorite solution with 0.1 % (v/v) TWEEN-20 for 15 minutes before being washed three times with sterile distilled $H_2O$. Seeds were transferred onto the surface of 0.44 % (v/v) Murashige and Skoog Medium (including vitamins) (Duchefa Biochimie, Haarlem, Netherlands) with 0.04 % (w/v) MES, 2 % (w/v) sucrose and 0.1 % (w/v) agar (pH 5.8). 100 ml media was used per vessel. Sown seeds were kept in the dark at 4 °C for 2 days, and then transferred onto a shaker at 80 rpm in a growth chamber, with a diurnal cycle of 16 h light at 18 °C and 8 h dark at 20 °C.

### Growth and harvesting of *S. cerevisiae*

Yeast (*S. cerevisiae*) was provided by Dr Chris Snowden, University of Oxford. Yeast strain BY47419 (MATα, his3Δ1, leu2Δ0, met 15Δ0, ura3Δ0) was plated on standard YPD plates (2 % (w/v) peptone, 1 % (w/v) yeast extract, 2 % (w/v) dextrose, 2 % (w/v)

agar ) and grown at 28 °C for 72 hours. Approximately 50 ml lactate medium (0.1 % (w/v) glucose, 1 g $KH_2PO_4$, 1 g/L $NH_4Cl$, 0.5 g/L $CaCl_2$ $2H_2O$, 0.5 g/L NaCl, 0.6 g $MgSO_4H_2O$, 0.3 ml 1 %(w/v) $FeCl_3$, 22 ml 90 % lactic acid pH 5.5) was inoculated with a few colonies from the YPD plates and incubated for approximately 6 hours at 30 °C with shaking at 150 rpm. When cells reached log phase ($OD_{600nm}$ of <1.0), 250 ml of lactate media was inoculated with the 50 ml starter culture and incubated at 30 °C with shaking. Yeast cells were harvested in log phase, centrifuged twice at 1500*g* in a Beckman J2-21 centrifuge with a JA-14 rotor (Beckman Coulter Ltd.) for 10 minutes, and washed with distilled $H_2O$ between each spin. Cells were weighed to determine the fresh weight.

## Isolation of mitochondria

*Solanum tuberosum* mitochondria were isolated as described in Considine *et al.* (2003). *A. thaliana* mitochondria were isolated as described by Day *et al*. (1985) and Sweetlove *et al.* (2007) with some modifications. All steps were carried out at 4 °C with detergent free equipment, and all equipment and solutions were pre-cooled to 4 °C before use. A Beckman J2-21 centrifuge with a JA-14 and a JA-20 rotor was used (Beckman Coulter Inc.).

## Isolation of mitochondria from *S. tuberosum* tubers

*S. tuberosum* tubers (cv. Desireé) were purchased from Marks and Spencer PLC, London, UK. Approximately 500 g fresh weight of potato tubers was homogenised in 500 ml extraction buffer (0.3 M mannitol, 50 mM TES, 0.5% (w/v) BSA, 0.5% (w/v) PVP-40, 2 mM EGTA, 20 mM cysteine, pH7.5) in a juicer. Homogenate was filtered through 1 layer of miracloth (MERCK Chemicals Ltd, Nottingham, UK) and 2 layers of

muslin and centrifuged at 1500*g* for 5 minutes. The pellet was discarded and the supernatant centrifuged at 18 000*g* for 10 minutes. The resulting pellet was gently re-suspended in 1 ml wash buffer (0.3 M mannitol, 10 mM TES, 0.1 % (w/v) BSA, pH7.5) using a soft brush. The suspension was loaded onto a stepped gradient of 5 ml 50%, 10 ml 28% and 15 ml 20% (v/v) Percoll (GE Healthcare, Amersham, UK) in wash buffer and centrifuged for 30 minutes at 40 000*g* with the rotor brake turned off. Mitochondria formed a pale band at the interface of the 50 % and 28 % Percoll layers. The upper layers were discarded and the mitochondrial band removed and transferred to a fresh centrifuge tube. 25 ml wash buffer was added and then centrifuge at 18 000*g* for 10 minutes. The supernatant was removed and the pellet re-suspended in 25 ml buffer. This was centrifuged again at 18 000*g* for 10 minutes and the supernatant removed. The pellet was suspended in 1 ml wash buffer. This was loaded onto 30 ml of sucrose buffer (0.3 M sucrose, 10 mM TES, 0.1% BSA pH 7.5 containing 28 % (v/v) Percoll and centrifuged for 40 000*g* for 30 minutes with the centrifuge brake turned off. Mitochondria formed an opaque a band at the top of the tube. This was transferred into a fresh centrifuge tube and suspended in 25 ml BSA-free wash buffer (0.3 M mannitol, 10 mM TES, pH 7.5) and centrifuged at 18 000*g* for 10 minutes. The supernatant was removed, the pellet re-suspended in wash buffer and centrifuged a second time at 18 000*g* for 10 minutes. The final pellet was re-suspended 1 ml BSA free wash buffer with 5 % (v/v) DMSO, frozen in liquid nitrogen and stored at -80°C.

### Isolation of mitochondria from *A. thaliana* seedlings

20-30 g fresh weight 14 day old *A. thaliana* seedlings were used in each preparation. For all experiments (unless stated), plants were removed 3 h into the light period. For

experiments where mitochondria were isolated from plant in the night, plants were removed 7 h into an 8 h night period. Plants were covered in foil while still in the dark, and foil was removed immediately prior to washing and grinding.

Seedlings were washed with water, weighed and ground using a pestle and mortar in 600 ml extraction medium (0.25 M sucrose, 15 mM MOPS, 0.4 % (w/v) BSA, 0.6 % (w/v) PVP-40, 1.5 mM EDTA, 100 mM ascorbate, and 10 mM dithiothreitol, pH 7.4). The suspension was filtered through 2 layers of muslin and 1 layer of Miracloth (MERCK Chemicals Ltd, Nottingham, UK). Filtered seedlings were ground again in extraction medium with sand and filtered as above.

The pooled suspension was centrifuged at 1100*g* for 5 minutes, the pellet discarded and the supernatant centrifuged at 18 000*g* for 20 minutes. The supernatant was discarded and the pellet re-suspended in around 50 ml of wash medium (0.3 M sucrose, 10 mM TES, 0.1% (w/v) BSA pH 7.5) using a soft brush and then centrifuged at 1100*g* for 5 minutes. The pellet was discarded and the supernatant centrifuged at 18 000*g* for 20 minutes. The pellet was re-suspended in a small amount of wash buffer using a soft brush. The suspension was loaded onto two 0-4.4 % (w/v) PVP-40 gradients. The gradients were centrifuged at 40 000*g* for 40 minutes with the rotor break turned off. The mitochondria were present as a very pale green band near the bottom of the tubes. The top layers of gradient were discarded and the mitochondrial band was removed. Mitochondria were suspended in approximately 30 ml of a second wash buffer (0.3 M sucrose, 10 mM TES, pH 7.5) and centrifuged for 15 min at 23 700*g.* The supernatant was removed re-suspended in buffer and centrifuged a second time at 23 700*g* for 15 min. The supernatant was removed and the mitochondrial

pellet suspended in approximately 1ml of the second wash buffer. Mitochondria were kept on ice at 4°C until use.

### Oxygen uptake measurements

Oxygen consumption in isolated mitochondria was measured using a Clarke type oxygen electrode (Hansatech Instruments, Pentney, Norfolk, UK). Electrodes were calibrated using air-saturated water and sodium hydrosulphite before each experiment. For all assays mitochondria were suspended in 1 ml buffer containing 5 mM $MgCl_2$, 0.2 M mannitol, 0.1M MOPS, 0.1% (w/v) BSA, 20 mM $KH_2PO_4$, (pH 7.2) in the electrode chamber at 22°C. The rate of oxygen consumption was monitored using Oxygraph software (Hansatech Instruments, Pentney, Norfolk, UK).

### Respiratory coupling assay

The rate of oxygen uptake was measured during and after the addition of 100 µM ADP in isolated mitochondria metabolising either: 10 mM pyruvate, 10 mM malate, 0.1 mM TPP and 0.3 mM $NAD^+$; or 10 mM succinate and 0.25 mM ATP; or 1 mM NADH. The respiratory control coefficient (RC) was calculated as the ratio of the rate before and after the addition of ADP.

### Outer membrane integrity assay

The outer membrane integrity of the mitochondria was measured by measuring the latency of cytochrome c oxidase. Oxygen uptake of mitochondria was measured after each addition of: 10 mM ascorbate, 0.05 mM cytochrome C, 0.05 % (v/v) Triton-X, and 0.05 mM KCN. The integrity was measured by expressing the rate after addition of Triton-X as a percentage of the rates before and after the addition of Triton-X.

## Isotope dilution experiments

For all experiments, mitochondria were incubated in buffer containing 5 mM $MgCl_2$, 0.2 M mannitol, 0.1 M MOPS, 0.1% (w/v) BSA, 20 mM $KH_2PO_4$, pH 7.2. Oxygen was bubbled slowly through the buffer to ensure complete oxygenation and mixing of the mitochondria, and a water bath was used to ensure the temperature was constant at 22°C. 100 µl samples were taken at each time point and snap-frozen in liquid nitrogen. For initial experiments, mitochondria were incubated with 10 mM [3-$^{13}$C]pyruvate, 60 mM malate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP, 0.15 U/ml hexokinase and 20 mM glucose.

For dilution experiments, mitochondria were incubated with either 10 mM [3-$^{13}$C]pyruvate, 10 mM malate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP, 0.15 U/ml hexokinase, 20 mM glucose and 10 mM malonate, or 10 mM [3-$^{13}$C]glutamate, 0.5 µM fluorocitrate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP, 0.15 U/ml hexokinase and 20 mM glucose. At 82 minutes, unlabelled intermediate (citrate, 2OG, succinate, fumarate or malate) was added to a final concentration of 0.1 mM.

## Measurement of the organic acid concentration in mitochondria

1ml of total suspension (including mitochondria) were incubated with 10 mM 1,2,3-benzenetricarboxylate (BTC) on ice for 5 minutes before being filtered on a membrane filter (0.22 µm pore size) (Millipore, Watford, UK) which had been pre-washed with buffer containing 10 mM BTC. Mitochondria were rapidly separated from the media by vacuum filtration, and then washed with 5ml buffer containing 10 mM BTC. Filters were removed; snap frozen and prepared for GC-MS analysis.

## Gas chromatography mass spectrometry (GC-MS)

### Sample preparation

Samples were prepared for GC-MS based on Lisec *et al.* (2006). Samples were lyophilised and dissolved in 1400 µl 100% methanol (HPLC grade) and vortexed. 60µl 0.2mg/ml Ribitol in water was added as an internal standard. The samples were heated for 10 min at 70°C at 950 rpm and centrifuged for 10 minutes at 1100*g*. 750 µl chloroform (HPLC grade) was added and mixed with 1500 µl dH2O before being centrifuged for 15 min at 2200*g*. 150 µl of the polar phase was dried in a speed-vac overnight for derivitisation. Dried samples were suspended in 40 µl of 20 $mg^{-1}ml^{-1}$ methoxyamine hydrochloride in pyridine (HPLC grade) and incubated for 2 hours at 37 $^{o}$C with shaking at 900rpm. 70 µl MSTFA (*N*-methyl-*N*-(trimethylsilyl)trifluroacetamide (HiChrom, Berkshire, UK))was added to each sample and incubated for 30 minutes at 37 $^{o}$C at 900 rpm. They were transferred to glass vials (Chromacol, Welwyn garden city, UK) with a septum cap for injection.

### GC-MS analysis

GC-MS analysis was carried out on an Agilent 79890 GC coupled to an Agilent 5975 quadrupole MS detector (70 eV). Either an Agilent HP 5-ms column (30 m, 0.25 mm inner diameter) or a Varian VF5-ms column (30 m, 10 m guard column, 0.25 mm inner diameter) was used. The Agilent autotune method was used to calibrate the mass spectrometer before each run, and 1µl sample was injected each time. During the run, the oven was heated to 70°C for 5 min, then increased at a rate of 5°C/min until a temperature of 280°C, held at this temperature for 3 min before being decreased to 70°C at 120°C/min with a helium gas flow of 0.6 ml $min^{-1}$ and a solvent delay of 10

minutes. Mass spectra were acquired using a scan and/or single ion monitoring (SIM) method (with a dwell time of 10 to 30 ms for each ion group).

Elution times and mass fragments of organic acids were determined by comparison with organic acid standards, and by reference to the Golm Metabolome Database (Kopka *et al*. 2005). The GC peaks and ion abundances of each metabolite were obtained using Chemstation software (Agilent Technologies, Wokingham, UK) and expressed relative to the internal ribitol standard. Mass ions were corrected for naturally occurring heavy isotopes using MSCorr software (Wahl *et al.* 2004). MSCorr software was also used to deduce the proportional abundance of the mass isotopomers. Concentrations of organic acids were calculated by reference to standard curves of pure organic acids.

### $^{13}$C NMR

#### Orientation conserved transfer experiments

Experiments were carried out as described in Smith *et al.* 2004 and Wirtz *et al.* 2012 with some modifications. For all experiments, mitochondria were incubated in 5 mM $MgCl_2$, 0.2 M mannitol, 0.1M MOPS, 0.1% (w/v) BSA, 20 mM $KH_2PO_4$, in 10% $D_2O$, (pH 7.2). An airlift system was used to ensure continuous oxygenation and mixing of the sample in a 10 mm diameter NMR tube. For preliminary experiments the incubation medium also contained 2.1 mM citrate, 1.3 mM succinate, 0.6 mM malate, 0.02 mM fumarate and 0.02 mM isocitrate (Farré *et al.* 2001, Smith et al. 2004). For orientation conserved transfer experiments, mitochondria were incubated with 10 mM [3-$^{13}$C]glutamate, 0.5 µM fluorocitrate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP, 0.15 U/ml hexokinase and 20 mM glucose. Proton-decoupled $^{13}$C NMR spectra were

recorded at 150.9 MHz using a broadband probe on a Varian Unity Inova 600 spectrometer. A 90° pulse, 1.016-s acquisition time, and a 6-s relaxation delay was used. Low power frequency-modulated decoupling was applied during the relaxation delay but was switched to higher power Waltz decoupling during the acquisition time. Spectra were recorded in 15-min blocks over a period of 5 h.

### Other experiments

In experiments investigating the effect of EDTA on the line broadening of malate, freeze-dried samples from the orientation conserved transfer experiments were re-suspended in 10% $D_2O$.A single 2 hour spectra was run using the same conditions as above. EDTA was added in 5mM increments and the line broadening was checked until it was approximately 3 Hz, and a second 2 hour spectra was recorded. The range of EDTA concentrations was 10-15 mM depending on the sample. In experiments to test whether changing the acquisition delay altered the ratio of label in malate carbons, 100 mM malate was dissolved in standard buffer with 10% $D_2O$. The acquisition parameters were as above, but the relaxation delay was altered between 2-12 seconds (in 2 second increments).

### Orientation conserved transfer experiments in *S. cerevisiae*

The experiment was carried out as described in Sumegi *et al.* 1993, although [3-$^{13}$C]glutamate was used as a substrate, instead of [4-$^{13}$C]glutamate. 1 g of packed cells was re-suspended in 6 ml minimal media (0.67 % (w/v) YNB without amino acids pH 6.2) containing [3-$^{13}$C]glutamate and fluorocitrate. Cells were incubated for 30 minutes at 30 °C with vigorous shaking, after which the reaction was stopped by adding 4% perchloric acid. This was neutralised with 2 M KOH, and the precipitate removed by

centrifuging the sample for 15 min at 2200*g*. The supernatant was freeze-dried and stored at -80°C for NMR experiments. The freeze dried samples were dissolved in 3.3 mL of 10 mM $KH_2PO_4$ buffer with 10 mM EDTA, 25 mM 1,4-dioxane, and 10% $2H_2O$ (pH 7.5). 1H-decoupled 13C NMR spectra were recorded at 150.9 MHz using a 10-mm-diameter broadband probe. A 90° pulse, an acquisition time of 1.016 s and a relaxation delay of 6 s was used. Spectra were recorded for a total of 20h, and chemical shifts were given relative to the 1,4-dioxane signal at 67.30 ppm.

## Spectral processing

NMR spectra were analysed using NUTS PRO software (Acorn NMR, Professional version 20070315). Spectra were summed, zero-filled and baseline corrected, and a line-broadening of 1 Hz was applied before being fourier transformed, and corrected both automatically and manually. A linear regression was also applied, to ensure the baseline was flat. Chemical shifts were assigned relative to the internal mannitol signal at 63.90 ppm, and the signal intensities of peaks were measured relative to the intensity of this peak.

# Chapter 3: Developing a method to investigate metabolite channelling in the TCA cycle

## Introduction

There have been no previous attempts to investigate metabolite channelling in the TCA cycle in plants. The first aim of this thesis is therefore to develop a method to investigate whether channelling occurs in the plant TCA cycle.

### Methods of measuring metabolite channelling

Several different methods have been used to investigate whether metabolite channelling exists between enzymes. One way is to study physical interactions between the enzymes under investigation through the use of various techniques including pull-down experiments with selected enzymes or cell extracts (Meyer *et al.* 2011); attempts to isolate a complex of enzymes directly from the cell or organelle (Robinson and Srere 1985), FRET experiments (Winkel 2004), and investigating the co-localisation of enzymes tagged with GFP or through immuno-gold labelling (Haggie and Verkman 2002, Suss *et al.* 1993). These methods have resulted in the discovery of many putative metabolons. However, channelling is only one reason why enzymes may interact *in vivo*, and these experiments do not prove that channelling is occurring: this can only be achieved through studying the way that metabolites behave *in vivo*.

There are several methods that can be used to investigate whether channelling is occurring (Ovadi and Spivey 1999). The effectiveness of each method depends upon the nature of the metabolic pathway under investigation, the stability of the proposed metabolon, and how much information is available about the kinetics of the enzymes involved. Some of the different approaches are discussed below.

### *Estimation of metabolite channelling in vivo through flux analysis*

Williams *et al.* (2011) have recently demonstrated that it is possible to investigate metabolite channelling using steady state metabolic flux analysis (MFA). In this approach existing models of metabolic networks were modified to include channelling. Models were created for two types of channel. One type where the rotation of symmetrical intermediates was restricted resulting in unequal labelling of symmetrical carbons. The other set of channels were ones that bypass a branch point in the network. If the channel was active, this led to an altered pattern of redistribution of label in the network. This approach was extended to pre-existing models of metabolism in *A. thaliana* cell cultures. In one example, a metabolite channel was included in glycolysis which bypassed triose phosphate and was found to significantly improved the fit of the model. Similarly, a channel between cytosolic hexose phosphate and OAA carried a significant flux, suggesting that, metabolite channelling between these metabolites could exist in the cell cultures. This method has also been used to assess the likelihood of channelling in the TCA cycle. Channelling between different pairs of metabolites was included in the model. The flux model showed that a channel between fumarate and citrate and malate and citrate carried a significant flux, while channels between 2OG and citrate and succinate and citrate did not.

This method provides a useful way of investigate channelling, both in investigating the feasibility of proposed channels, and in complementing experimental methods. However, in order to carry out metabolic flux analysis, the cultures need to be at isotopic steady state, which limits the extent to which the method can be used to measure channelling under transient metabolic conditions (such as changes between

the light and the dark). It also limits the choice of material to that which can be labelled to isotopic steady state, such as cell cultures.

### *Kinetic analyses*

There have also been attempts to study channelling through comparing the kinetics of channelled enzymes with predicted or measured kinetics in unchannelled pathways. For example, Srere *et al.* (1973) measured the rate of citrate production when MDH and CS were immobilised next to each other on gels or columns, and found that the activity of the immobilised enzymes was 100% greater than that of the free enzymes. If pyruvate and lactate dehydrogenase were included to re-oxidise NADH, the rate increased by 400%. These results have been used as evidence that metabolite channelling will increase the rate of reactions compared to an unchannelled pathway, and that this could be used to detect channelling in metabolons. However, artificially constructing metabolons in this way also tells us very little about channelling *in vivo* because the conditions are so different to those experienced inside the cell. It is also not clear whether the changes in rate are due to channelling or to changes in the kinetics of the individual enzymes after they have been immobilised.

### *Fusion proteins*

There have been some attempts to investigate metabolite channelling *in vivo* using artificial fusion proteins of TCA cycle enzymes. Lindbaldh *et al*. (1994) expressed a DNA fusion of CS1 and MDH1 in *E. coli*. They found that the fusion proteins had a higher Km for acetyl CoA and OAA than the equivalent non fusion proteins, and that AAT was a less effective competitor for OAA in the fusion protein. The same fusion protein was expressed in an *S. cerevisiae* strain in which the original proteins had been deleted.

The ratio of label in carbons 2 and 3 of alanine was also measured. An unequal ratio of label in C2 and C3 of alanine is thought to be an indication of channelling, as it results from the restricted rotation of the symmetrical intermediate succinate and fumarate (see chapter 6 for a more detailed explanation). In this case, the labelling was unequal in both cases, suggesting that a degree of metabolite channelling was present between the enzymes. The drawback to using fusion proteins is that they do not test whether channelling is occurring in the endogenous pathways cycle *in vivo*, as fusion proteins are an artificial construct. This means that although fusion proteins are able to provide a model for how channelled pathways might operate *in vivo*, they are of limited use beyond this.

### *Competing enzyme experiments*

Another way that metabolite channelling can be investigated is through the introduction of an enzyme or chemical which reacts with the intermediate in the pathway under investigation. If channelling is occurring, we would expect the rate of the competing reaction to be lower than when compared with the same system of 'free' enzymes. In order for this to work properly, the experiment requires an excess of the competing enzyme so that the rate is only limited by the provision of the intermediate as a substrate.

This approach has been used to investigate the way that TCA cycle enzymes behave when they are localised closely together. Datta *et al.* (1985) used a competing enzyme experiment to investigate channelling between MDH and CS after polyethylene glycol (PEG) had been added to generate a solid state enzyme complex *in vitro* to simulate channelling. The rate of citrate synthesis was measured in the presence of an excess of

aspartate aminotransferase and glutamate (which compete with citrate synthase for OAA). The results were compared to the free enzyme system where no PEG was added. They found that concentrations of AAT and glutamate which reduced the rate of citrate synthesis by 90% in the unchannelled system had no effect in the channelled one. Morgunov and Srere (1998) used a similar experiment to investigate channelling between CS and mitochondrial MDH, and mitochondrial CS and cytosolic MDH. Aspartate aminotransferase and oxaloacetate decarboxylase were used as the competing enzymes. The enzymes were found to be less effective at competing with MDH and CS for OAA when the mitochondrial forms were precipitated in PEG at low ionic strength, when compared with CS and cytosolic MDH or with free enzymes.

One advantage of this method is that it directly tests whether intermediates are able to diffuse into the bulk aqueous phase between active sites, which is one of the definitions of channelling. A further advantage is that it does not require any information about the kinetics of the enzymes involved in the pathway. This means it can be used across a range of pathways and metabolons, provided there is a suitable competing reaction, and the metabolon can either be isolated, or the competing reaction successfully introduced into the cell in which the reaction occurs. As a result, these competing enzyme methods work well *in vitro*, where competing enzymes can easily be introduced into the same vessel as the enzymes under investigation. However, they are harder to use *in vivo* or in isolated organelles or cells, as they require competing enzymes to be introduced in excess into the cell or organelle. This can pose a challenge to carrying out this method *in vivo*, and is a barrier to carrying out competing enzyme methods in mitochondria where the already high matrix

protein concentration limits the amount of heterologous protein that can be introduced.

### *Isotope dilution*

Another way to investigate channelling is to use an isotope dilution approach. This approach works by measuring the dilution of labelled product by an unlabelled intermediate. If a pathway is completely channelled, then the labelled intermediate will not have access to the bulk phase, and will therefore not be diluted by the unlabelled intermediate. This means that the product will be fully labelled. In contrast, if the pathway is not channelled then the unlabelled intermediate will have access to the enzymes in the channel. This means that the unlabelled intermediate will also be converted to product, and the labelling in the product will be diluted. The result of this is that by measuring the labelling in product after the addition of unlabelled intermediate it is possible to assess whether channelling occurs in the pathway.

Isotope dilution has several advantages. Firstly, it can be carried out *in vivo* or in isolated cells or mitochondria, meaning that it can be used to measure metabolite channelling in metabolons that require specific cellular conditions or are disrupted when they are isolated from the cell. Secondly it does not require detailed knowledge of the kinetics of the system. The greatest advantage is that it directly measures whether pathway intermediates have access to the bulk phase, which is the most widely used definition of channelling.

As well as being able to identify whether channelling is present, isotope dilution methods can also be used to estimate the proportion of enzymes which are channelling metabolites. This is because a dilution of the labelled product could be the

result of either a completely un-channelled pathway, or pathway which is partially channelled (i.e. the channel is 'leaky'). If the concentration of the labelled and unlabelled intermediates and products, and the rate that they are being consumed are known, then it is possible to calculate a theoretical dilution. If the measured dilution is less than this theoretical one, then it is thought that the pathway is partially channelled.

Graham *et al.* (2007) used isotope dilution to calculate the percentage channelling in the glycolytic metabolon attached to the outside of isolated mitochondria. Channelling was measured between glucose and 3-PGA after mitochondria were fed with [1-$^{13}$C ]fructose 1,6-bisphosphate or [1-$^{13}$C ]glucose. NMR was used to measure the change in rate of accumulation of label in downstream products before and after the addition of unlabelled intermediates. This was used to calculate the percentage channelling between the enzyme pairs. Provided that the enzymes are saturated (which was demonstrated in the Graham *et al.* (2007) study), the change in rate of labelled product production is directly related to the percentage dilution of the intermediate (i.e. a 50% dilution of label in an intermediate metabolite would cause a 50% decrease in the rate of production of labelled product). By measuring the rate of label accumulation and the concentration of unlabelled intermediate in the system they were able to calculate the effective concentration of labelled intermediate (both channelled and un-channelled). This was used to calculate % channelling using the formula:

$$\% \text{ channelling} = 100 \{1 - ([I^*]ext/[I^*]eff)\}$$

Where ([I*]ext is the amount of labelled intermediate I* present in the suspending medium, and [I*]eff is the sum of the I* that is directly channelled to the active site and ([I*]ext. [I*]eff can be calculated from the ratio of the rate of label accumulation in the product in the presence and absence of the unlabelled intermediate.

## Aim of this chapter

Isotope dilution is an effective way of measuring channelling, and the advantages of the method meant that it was chosen to measure channelling experimentally in the plant TCA cycle. The aim of this chapter was to develop an isotope dilution method that is applicable to the TCA cycle in isolated plant mitochondria.

# Results

## Integrity and coupling of isolated mitochondria

Mitochondria were isolated from tubers of *S. tuberosum* as described in materials and methods. I tested the integrity and respiratory coupling (RC) of fresh mitochondria and mitochondria that had been frozen overnight at -80°C. RC of mitochondria incubated with malate and pyruvate was 8.92 ±2.37 for fresh mitochondria and 5.02 ±2.89 for frozen. RC of mitochondria incubated with succinate was 3.57 ±1.11 for fresh and 2.06 ± 0.29 for frozen, while RC of mitochondria incubated with NADH was 9.04 ±0.25 for fresh and 6.8 ±1.22 for frozen. The outer membrane integrity of the mitochondria was also tested, and was found to be 97.9± 1.15% for fresh mitochondria, and 95.7 ±2.93% for frozen. The values of RC and outer membrane integrity were comparable to published data for potato mitochondria. The fact that there was only a slight decrease in these values upon freezing and that the frozen mitochondria were still well coupled upon thawing allowed me to utilise frozen mitochondria for the experiments in this thesis. The integrity and coupling of fresh mitochondria isolated from 14 day old *A. thaliana* seedlings were also tested. RC for malate/pyruvate was 2.34± 0.05, and outer membrane integrity was 99.2± 0.6%. In addition, the integrity and coupling of the mitochondria was tested prior to each individual experiment to check that the preparations were suitable for use. Fresh mitochondria were used in the experiment because the integrity and coupling of *A. thaliana* mitochondria is known to deteriorate after freezing.

## Developing an isotope dilution method

The system was adapted from published NMR experiments with isolated potato mitochondria in which the metabolism of labelled pyruvate into labelled TCA cycle

organic acids was demonstrated (Smith *et al.* 2004). GC-MS rather than NMR was used to measure the organic acids. This allowed me to measure organic acids at lower concentrations than with NMR, and to measure the total pool of organic acids (unlike NMR which is only sensitive to the organic acids that are exported from the mitochondria). It also enabled me to measure both the labelled and unlabelled pools of organic acids.

### *Mitochondria can respire over several hours in vitro with oxygen and pyruvate*

*S. tuberosum* mitochondria are known to respire for up to 5 hours in at 25 $^{o}$C supplied with appropriate substrates co-enzymes and oxygen (Smith *et al.* 2004). It was necessary to show that mitochondria in my experimental set up retained metabolic activity over the proposed time course of the experiment. Mitochondria from *S. tuberosum* were incubated in buffer with 10 mM [3-$^{13}$C]pyruvate, 0.6 mM malate, 0.3 mM $NAD^{+}$, 0.2 mM ADP, 0.1 mM TPP 0.15 U/ml hexokinase and 20 mM glucose. Oxygen was bubbled slowly through the buffer to ensure complete oxygenation and mixing of the mitochondria, and a water bath was used to ensure the temperature was constant. 100 μl samples were taken every 20 min over 5 h, snap-frozen in liquid nitrogen and analysed by GC-MS as described in materials and methods. Figure 3.1 shows the that both labelled malate and citrate increased over the time course, showing that the TCA cycle was active and converting labelled pyruvate into organic acids. As the production of labelled citrate levelled off after 2h, all subsequent experiments were terminated at 2h or earlier.

### *Label in organic acids can be detected by GC-MS and tracked through the TCA cycle*

Labelling in malate and citrate was followed over 80 minutes. Mitochondria from *S. tuberosum* were incubated in buffer with 10 mM [3-$^{13}$C]pyruvate in the conditions described above. The masses of malate and citrate were measured using GC-MS, and corrected for fractional enrichment to show the effect that labelled pyruvate had on the labelling of malate and citrate. Figure 3.2 shows the proportion of label present in malate and citrate over the time course. Malate M+0, which corresponds to unlabelled malate molecules, decreased over the time course as the unlabelled malate was consumed by the mitochondria. At the same time, malate M+1 increased as the labelled pyruvate was consumed and converted through the TCA cycle into labelled malate. After 45 minutes, the proportion of M+1 decreased, and malate M+2 started to increase, as single labelled malate continued through the TCA cycle and was labelled again by [3-$^{13}$C]pyruvate. Citrate M+0 was not detected in the samples, as the system only contained labelled pyruvate. The proportion of M+1 citrate decreased after 10 minutes, as citrate was recycled through the TCA cycle and converted into citrate M+2 and M+3, which both increased over the time course

### *Malonate can be used to inhibit the TCA cycle*

In order to simplify the isotope dilution experiment, it was decided to use malonate (a competitive inhibitor of succinate dehydrogenase) to prevent multiple turns of the cycle. The experiment was carried out as before, but 10 mM malonate was also included in the buffer. In contrast to experiments with no malonate the proportion of M+0 malate remained high, and the proportion of M+1 was very low. No M+2 could be measured. Malate was consumed over the time course (Figure 3.3). The concentration

of succinate increased when malonate was present in the system, and the fractional enrichment increased until it was constant after 40 minutes.

One problem with this experiment is that the concentration of malate is very low after 40 minutes. This is likely to limit the further production or organic acids, as no new malate is produced when the TCA cycle is blocked. Figure 3.4 shows what happened when the experiment was repeated with 10 mM malate (i.e. equal to the concentration of labelled pyruvate) instead of 0.6 mM malate. The concentration of succinate increased over the time course, and malate decreased. The fractional enrichment of succinate and citrate was constant (data not shown). As a result, 10 mM malate was included in subsequent experiments.

### *Measuring metabolite channelling using [3-$^{13}$C]glutamate*

In order to fully investigate metabolite channelling in the TCA cycle, it was necessary to additionally investigate the reactions between succinate and citrate (which is not possible in the presence of malonate). To do this [3-$^{13}$C]glutamate was used to supply the label, and fluorocitrate was used to inhibit the TCA cycle at aconitase. Fluorocitrate binds irreversibly to aconitase and inhibits its activity (Elliott and Kalnitsky 1950). Fluorocitrate was added to a concentration of 5µM, which has been shown to fully inhibit aconitase in isolated mitochondria (Smith *et al.* 2004). It is already known that glutamate can support the TCA cycle in isolated mitochondria (Aubert *et al.* 2001).

Figure 3.5 shows the results of a preliminary experiment. Mitochondria were incubated in buffer with 10 mM [3-$^{13}$C]glutamate, 5 µM fluorocitrate , 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP 0.15 U/ml hexokinase and 20 mM glucose. Oxygen was bubbled slowly through the buffer to ensure complete oxygenation and mixing of the

mitochondria, and a water bath was used to ensure the temperature was constant. The concentration of malate and citrate increased over the time course, showing that citrate and malate were produced, and that the fluorocitrate did not prevent the metabolism of glutamate. The proportion of M+1 of malate and citrate remained constant over the time course, showing that label from glutamate was entering the TCA cycle. However, the lack of M+2 showed that labelled citrate and malate were not recycled through the TCA cycle: i.e. fluorocitrate was effective in blocking the TCA cycle. Figure 3.6 shows that the concentrations of malate and citrate increased over the time course and reached levels that could be robustly quantified by GC-MS. There was some variation between the replicates: this is likely to be due to small differences in the activity of the individual mitochondrial preparations.

### *Assessing the appropriate concentration of unlabelled citrate to use in the isotope dilution experiment*

The next step was to try and measure the concentration of organic acids that would be appropriate for an isotope dilution experiment. The aim was to increase the internal concentration of the relevant intermediate in the mitochondria by approximately double, in order to ensure that the unlabelled intermediate could dilute the labelled pool sufficiently.

The first step was to try and measure the internal concentration of organic acids using by rapidly filtering the mitochondria from the reaction buffer using a vacuum filtration system. The amount of organic acids left on the filter can then be measured. Citrate was measured in a preliminary experiment. Mitochondria were incubated with inhibitors of mitochondrial carboxylic acid transporters and then washed with buffer

also containing the inhibitor to prevent internal citrate leaking out. 1,2,3-benzenetricarboxylate (BTC) is a specific inhibitor of the mitochondrial di-tricarboxylic acid transporter (DTC) (Sandor *et al.* 1994, Vincent *et al.* 2000) and is known to block citrate export in isolated mitochondria at a concentration of 10 mM (Smith *et al.* 2004). Isolated S. *tuberosum* mitochondria were incubated with 10 mM [3-$^{13}$C]pyruvate, 10 mM malate, 10 mM malonate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP 0.15 U/ml hexokinase and 20 mM glucose. After 80 minutes the experiment was stopped. 1ml samples of the mitochondria suspension were removed. One sample was added to 100 µl of 100 mM BTC and incubated on ice for 5 minutes, before being filtered on a membrane which had been pre-washed with buffer containing 10 mM BTC. Mitochondria were rapidly separated from the media by vacuum filtration, and then washed with 5ml buffer containing 10 mM BTC. Filters were removed; snap frozen and prepared for GC-MS analysis as described. The internal concentration was estimated by quantifying the amount of citrate present, and correcting this for the internal volume of mitochondria per mg of protein. This was estimated to be 3.5ul/mg mitochondrial protein (Sweetlove LJ, unpublished). These were compared to mitochondria which were not incubated with BTC. Figure 3.7 showed that BTC increased the amount of citrate present on the filter, suggesting that the use of carboxylic transport inhibitors is necessary to prevent loss of carboxylic acids during filtration. The mitochondria were also washed with buffer containing 10 mM BTC to try and remove any experimental buffer surrounding the mitochondria that might artefactually increase the concentration that was measured. Samples which had been incubated with and without BTC and washed with buffer containing BTC were compared with samples which had not been washed. The results in Figure 3.7 show

that washing the mitochondria decreased the concentration when compared to experiments where the mitochondria were not washed with buffer.

A preliminary experiment was carried out to assess the increase of citrate in the mitochondria after different concentrations of unlabelled citrate was added. Mitochondria were incubated as described previously. After 80 minutes, different concentration of unlabelled citrate were added to the reaction buffer. Immediately before and after the addition of unlabelled citrate, 1ml of the medium was removed and internal citrate was measured as described. An external concentration of 0.1 mM citrate was found to approximately double the internal concentration of citrate in the mitochondria (Figure 3.8). However, the results showed that the concentration measured in this experiment was much lower than the concentrations measured in the trial experiments shown in Figure 3.7. This suggests that this method of measuring the internal concentration of citrate may not be very accurate.

Subsequently, the experiment was repeated with unlabelled 2OG, succinate and fumarate. However these could not be measured reliably using this approach (data not shown). It was therefore decided to use 0.1 mM as the unlabelled concentration for all added unlabelled intermediates in subsequent isotope dilution experiments.

### Preliminary isotope dilution experiment

A preliminary isotope dilution experiment was carried out using 0.1 mM citrate added at 82 minutes to potato mitochondria respiring labelled pyruvate. 80 minutes was chosen as by this point fractional enrichment of the organic acids were constant and the concentrations could be measured. This was compared with a control where an equal volume of buffer was added (Figure 3.9). In the first preliminary experiment,

there was no change in the fractional enrichment of succinate. In contrast, the fractional enrichment of 2OG fell when 0.1 mM citrate was added, but there was no change in the fractional enrichment in the control.

### Measuring metabolite channelling

The next step was to try and develop a way to get quantitative information about metabolite channelling from the isotope dilution experiment. To do this, we developed a simple model to calculate the expected fall in fractional enrichment if no metabolite channelling was present. The results of the model could then be compared with the fall in fractional enrichment that was measured from the preliminary experiment.

Assume:

1) succinate is 100% labelled
2) there is no metabolite channelling
3) there is free exchange of citrate between the mitochondria and the external media

Let:

R = rate

[citrate]mit = concentration of citrate in the mitochondria

Vsucc = the term which relates the production of succinate from citrate

Km = Michaelis-Menten constant for the enzyme metabolising the unlabelled intermediate

Then the rate of succinate production before unlabelled citrate is added is:

$$Rsucc = \frac{Vsucc \cdot [citrate]mit}{km + [citrate]mit}$$

Rsucc and [citrate]mit can be measured from the experiment. Vsucc can be calculated by re-arranging the equation to give:

$$Vsucc = \frac{Rsucc\ (\ km + [citrate]mit)}{[citrate]mit}$$

At time point t* 0.1 mM citrate is added to the mitochondria. This means:

- [citrate]mit will change to a new value: [citrate*]mit;
- the fractional enrichment of succinate will fall;
- the rate of succinate production may change

We can calculate the new rate of succinate production (called R*succ) based on:

$$R * succ = \frac{Vsucc \cdot [citrate *]mit}{km + [citrate *]mit}$$

These values allow us to estimate the fractional enrichments after unlabelled citrate is added to the system. If:

t* = time at which unlabelled citrate is added,

t= any time point after t*

[citrate]ext = external citrate concentration before t*

[citrate*]ext = external citrate concentration after time t*

At time t:

$$total\ succinate\ = \{Rsucc\ \cdot t *\} + \{R * succ \cdot (t - t *)\}$$

And

$$labelled\ succinate = Rsucc \cdot t* + \frac{[citrate]ext}{[citrate*]ext} \cdot R * succ\ (t - t*)$$

And

$$Fractional\ enrichment = \frac{labelled\ succinate}{total\ succinate}$$

By plotting the fractional enrichments using different values of t, it is possible to estimate the decrease in fractional enrichment over the time course of the experiment if there is no channelling.

Using the model we were able to predict the fall in fractional enrichment of downstream products if there was no metabolite channelling. I used the data from the preliminary experiment to estimate the expected dilution in succinate and 2OG in the case of zero channelling. The rate of succinate production before and after the addition of the unlabelled intermediate and the internal concentration of succinate was measured and used to calculate the expected fall in fractional enrichment. The Km's used were from the BRENDA database: no published valued were available for *S. tuberosum*, so the values from the closest species were used: in this case, *Acer pseudoplatanus.* Figure 3.10 shows the comparison between the predicted and actual fall in dilution. The dilution from the control experiment where no citrate was added was also included as a comparison. The model showed that we would expect a small fall in the fractional enrichment of succinate, whereas no fall was measured. The model also predicted a greater fall in the fractional enrichment of 2OG than the one we measured.

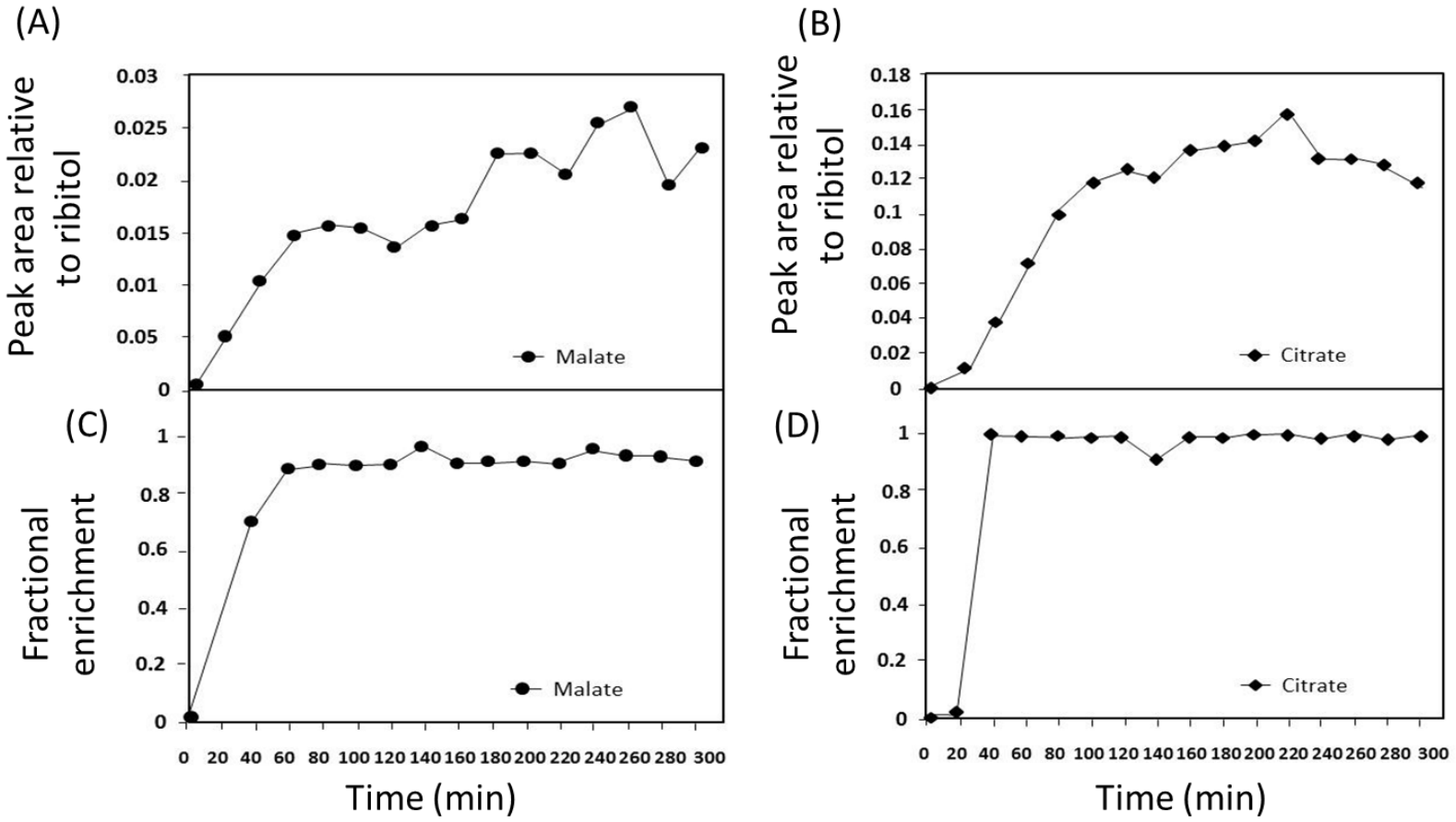


**Figure 3.1**: **Amount and enrichment of malate and citrate over time in isolated *S. tuberosum* mitochondria fed with 10 mM [3-$^{13}$C]pyruvate.** Peak area relative to internal ribitol peak of (A) malate and (B) citrate, and fractional enrichment of (C) malate and (D) citrate. Mitochondria were incubated in buffer with 10 mM [3-$^{13}$C]pyruvate, 0.6 mM malate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP 0.15 U/ml hexokinase and 20 mM glucose.

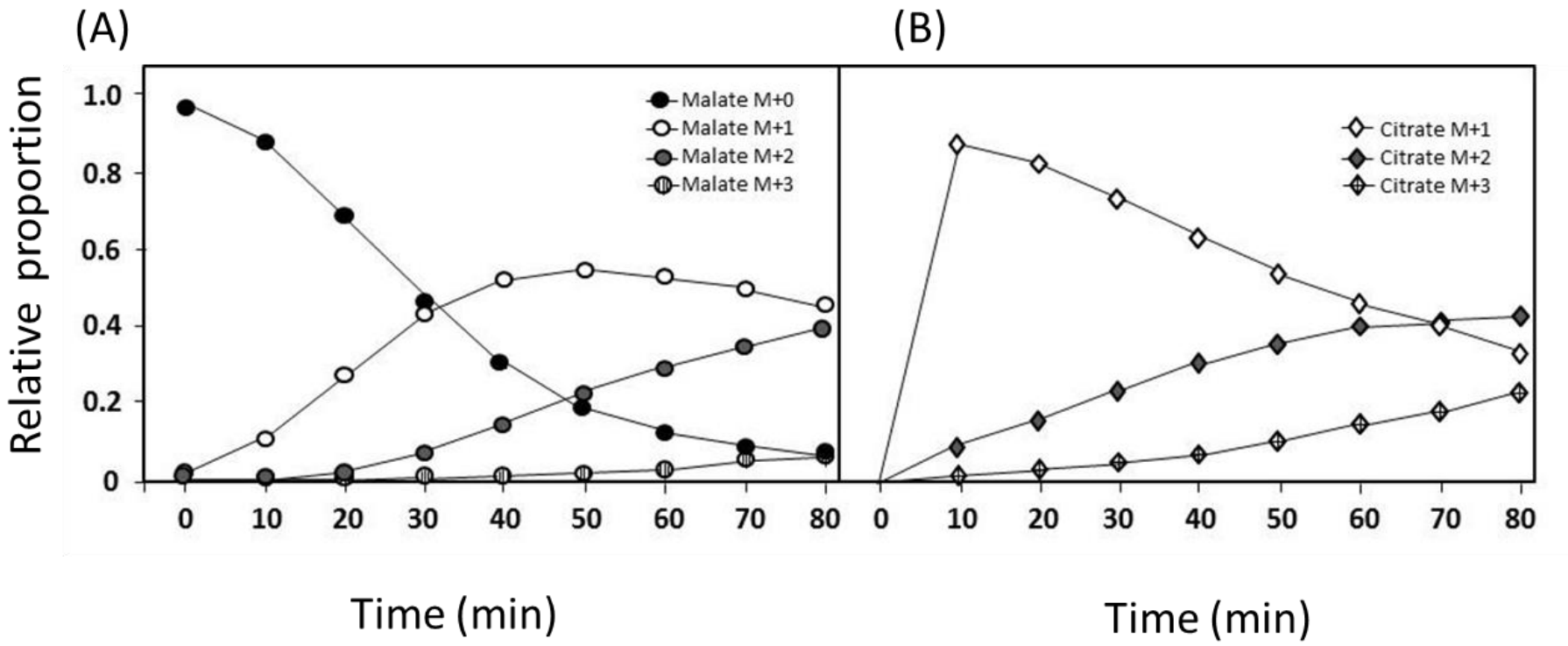


**Figure 3.2**: **Relative proportion of labelled molecules of malate and citrate in isolated *S. tuberosum* mitochondria fed with 10 mM [3-$^{13}$C]pyruvate.** Abundance of mass isotopomers of (A) malate and (B) citrate at each time point were measured and corrected for natural abundance. Mitochondria were incubated in buffer with 10 mM [3-$^{13}$C]pyruvate, 0.6 mM malate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP 0.15 U/ml hexokinase and 20 mM glucose.

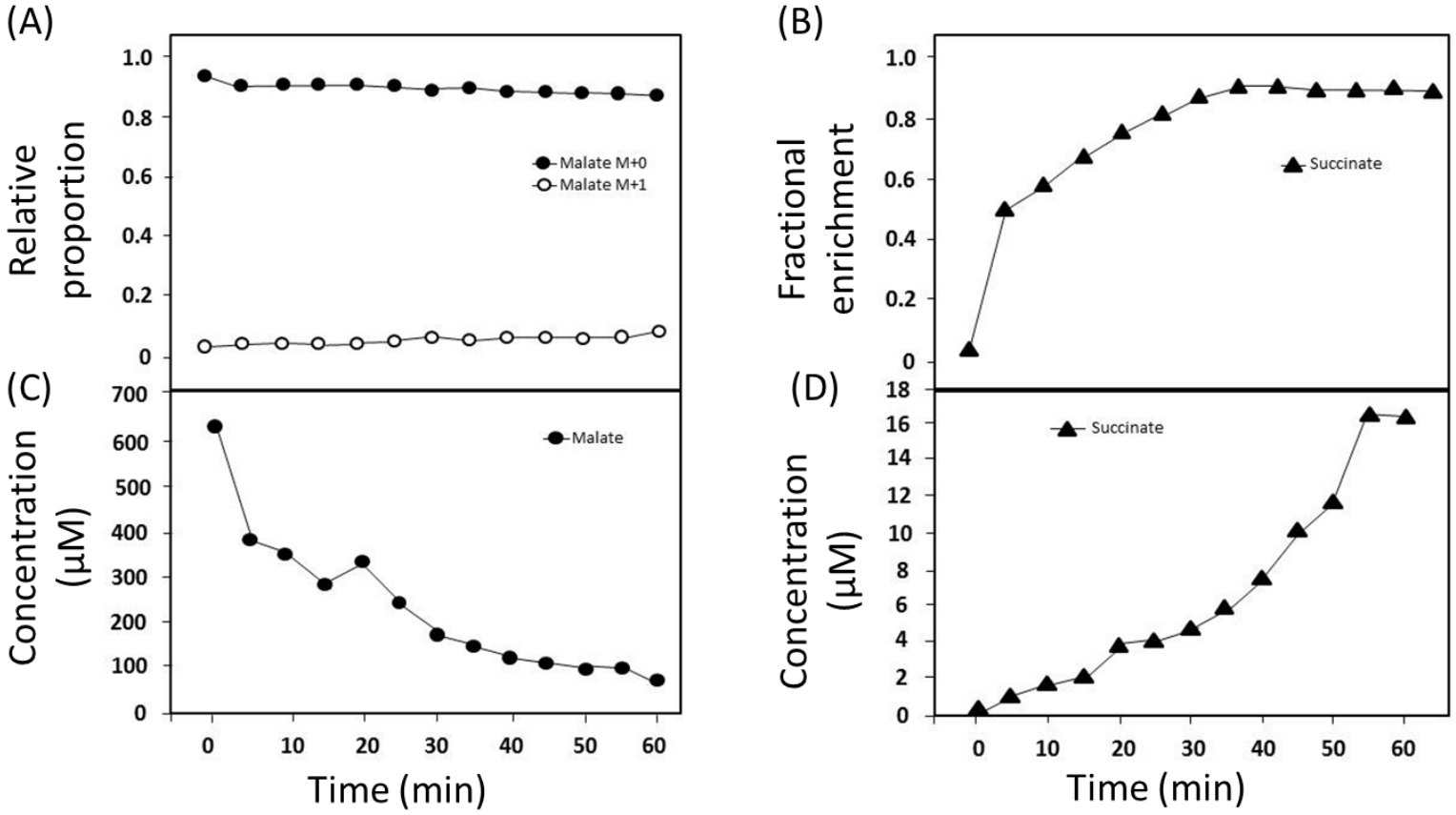


**Figure 3.3**: **Effect of 10 mM malonate on concentration and enrichment of malate and succinate in isolated *S. tuberosum* mitochondria incubated with 10 mM [3-$^{13}$C]pyruvate and 10 mM malate.** (A) Relative proportion of malate M+0 and M+1 corrected for natural abundance; (B) fractional enrichment of succinate corrected for natural abundance; concentration of (C) malate and (D) succinate. Mitochondria were incubated in buffer with 10 mM [3-$^{13}$C]pyruvate, 10 mM malate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP 0.15 U/ml hexokinase, 20 mM glucose and 10 mM malonate.

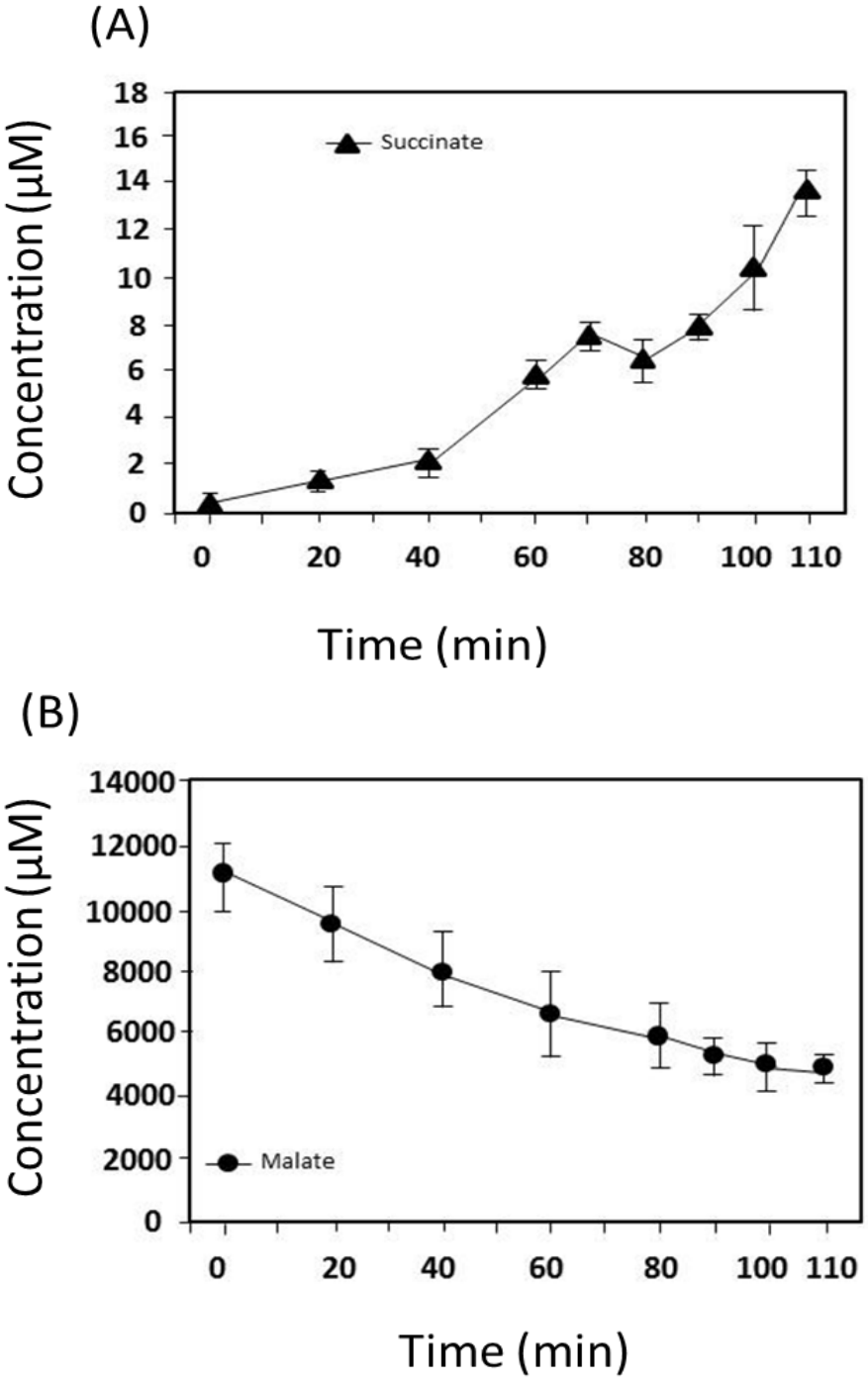


**Figure 3.4**: **Concentration of organic acids in isolated *S. tuberosum* mitochondria incubated with 10 mM malonate, 10 mM [3-$^{13}$C]pyruvate and 10 mM malate.** Concentration of (A) malate and (B) citrate produced. In both cases *n*=2. Mitochondria were incubated in buffer with 10 mM [3-$^{13}$C]pyruvate, 10 mM malate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP 0.15 U/ml hexokinase, 20 mM glucose and 10 mM malonate.

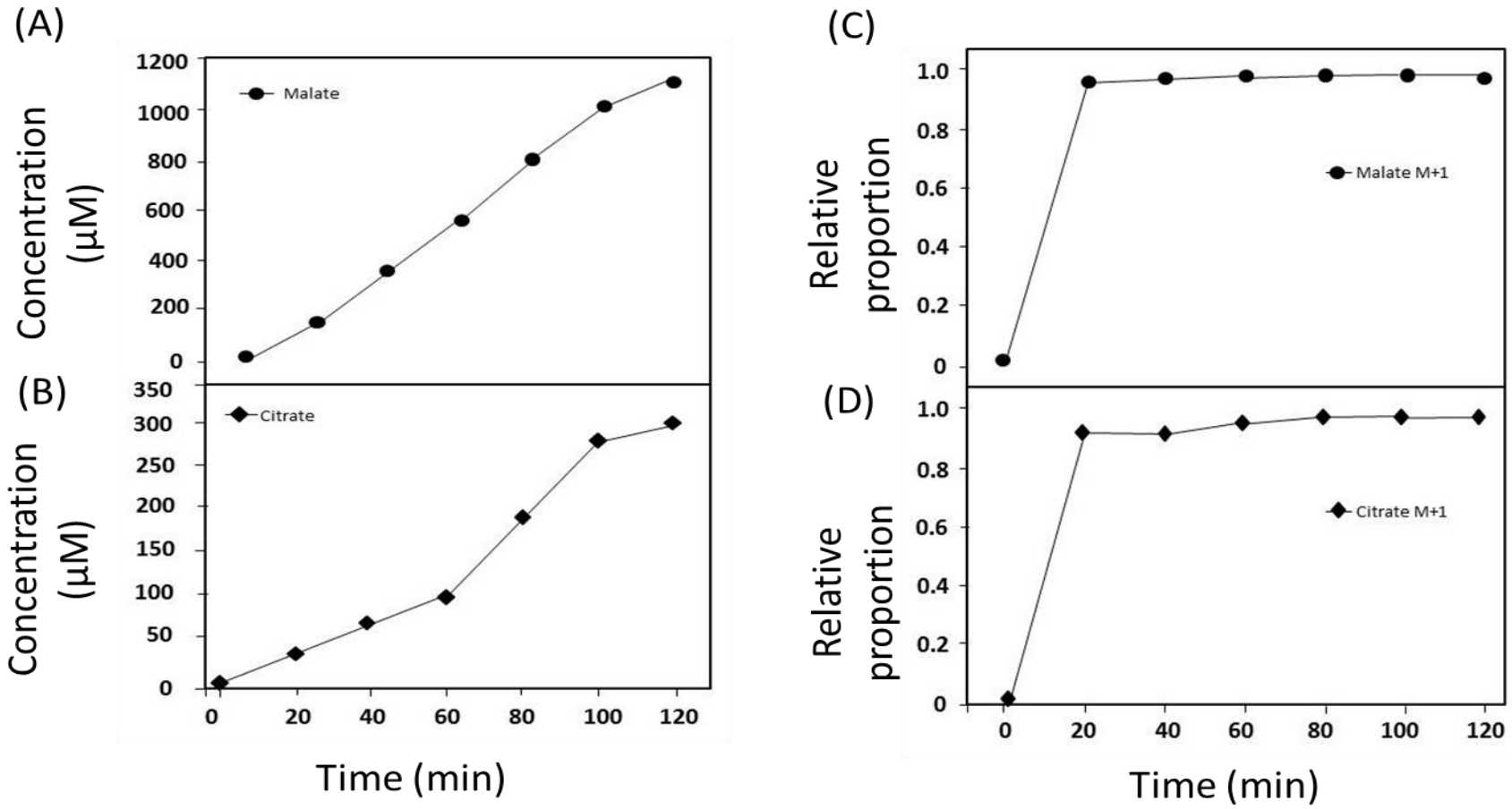


**Figure 3.5: Preliminary experiment of *S. tuberosum* mitochondria incubated with [3-$^{13}$C]glutamate and 5 µM fluorocitrate.** Concentration of (A) malate and (B) citrate; and the relative proportion of M+1 isotopomer of (C) malate and (D) citrate corrected for natural abundance.

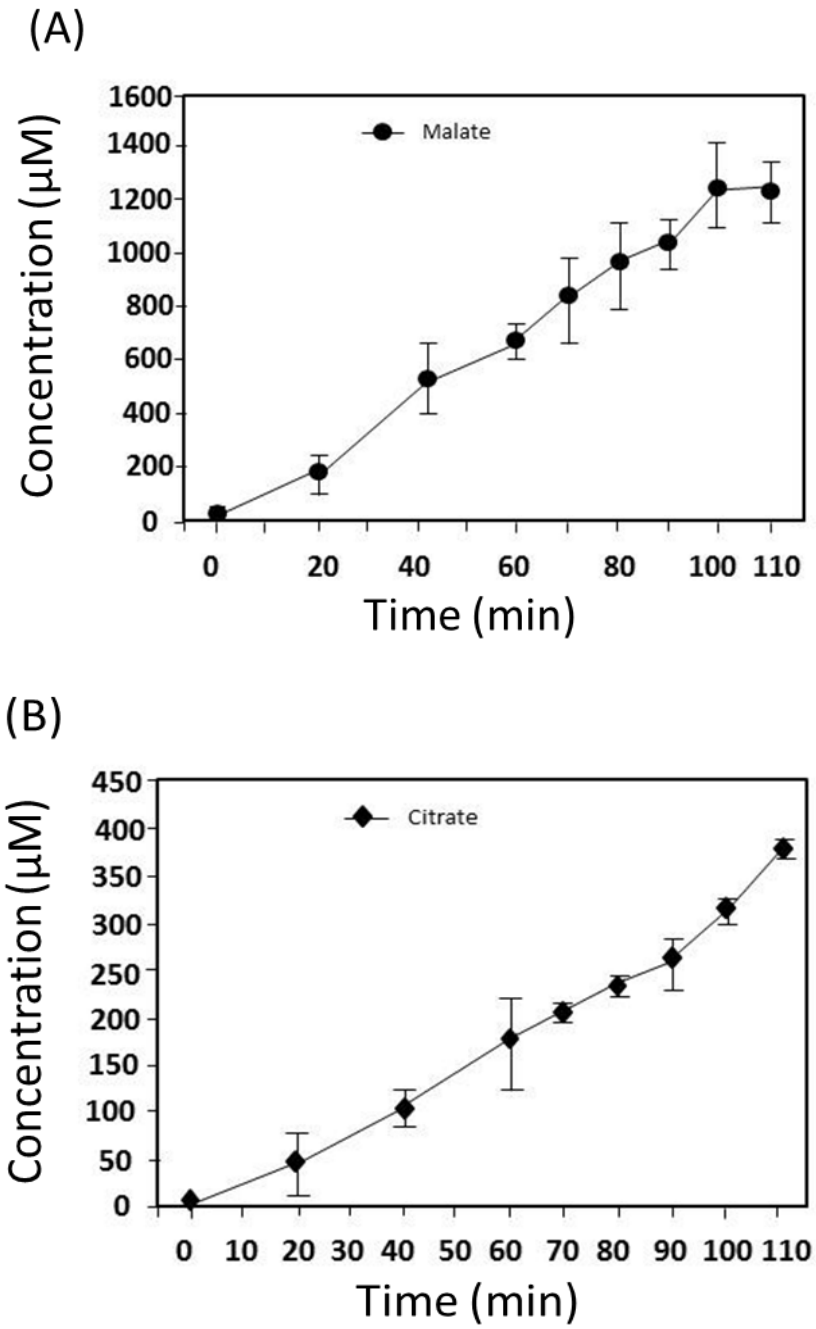


**Figure 3.6**: **Concentration of organic acids in isolated *S. tuberosum* mitochondria incubated with 10 mM [3-$^{13}$C]glutamate and 5 µM fluorocitrate.** Concentration of (A) malate and (B) citrate produced. In both cases *n*=2. Mitochondria were incubated in buffer with 10 mM [3-$^{13}$C]glutamate, 5 µM fluorocitrate , 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP 0.15 U/ml hexokinase and 20 mM glucose.

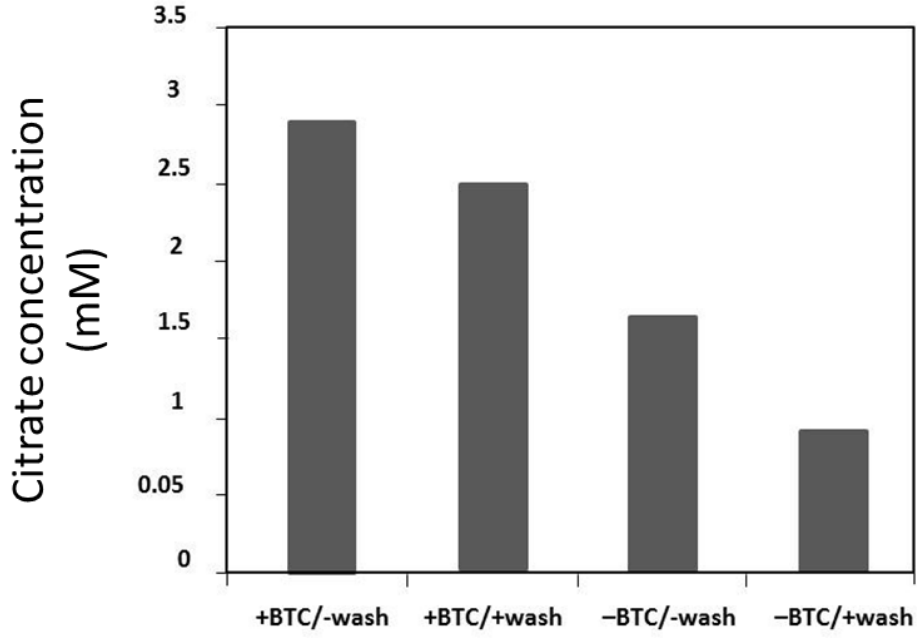


**Figure 3.7**: **Effect of BTC and washing on the measured internal concentration of citrate in isolated *S. tuberosum* mitochondria incubated with 10 mM [3-$^{13}$C]pyruvate, 10 mM malate and 10 mM malonate.**

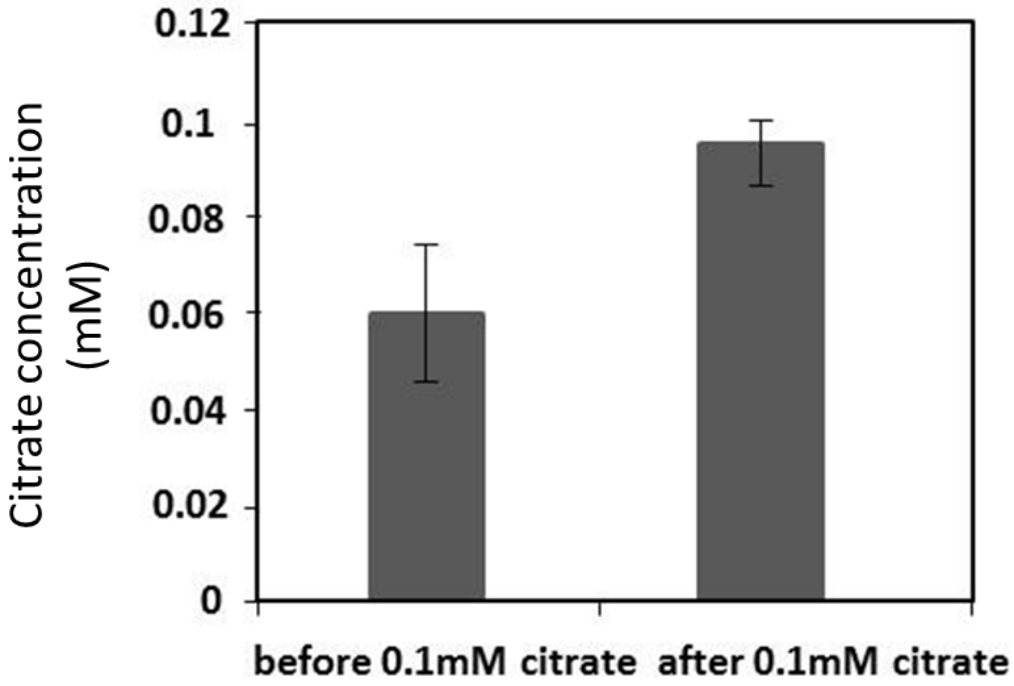


**Figure 3.8**: **Internal concentration of citrate before and after the addition of 0.1 mM unlabelled exogenous citrate to isolated *S. tuberosum* mitochondria incubated with 10 mM [3-$^{13}$C]pyruvate, 10 mM malate and 10 mM malonate.** Mitochondria incubated with 10 mM BTC on ice for five minutes before being rapidly separated from the media**.** The concentration of citrate associated with the mitochondria was measured by GC-MS. *n*=2

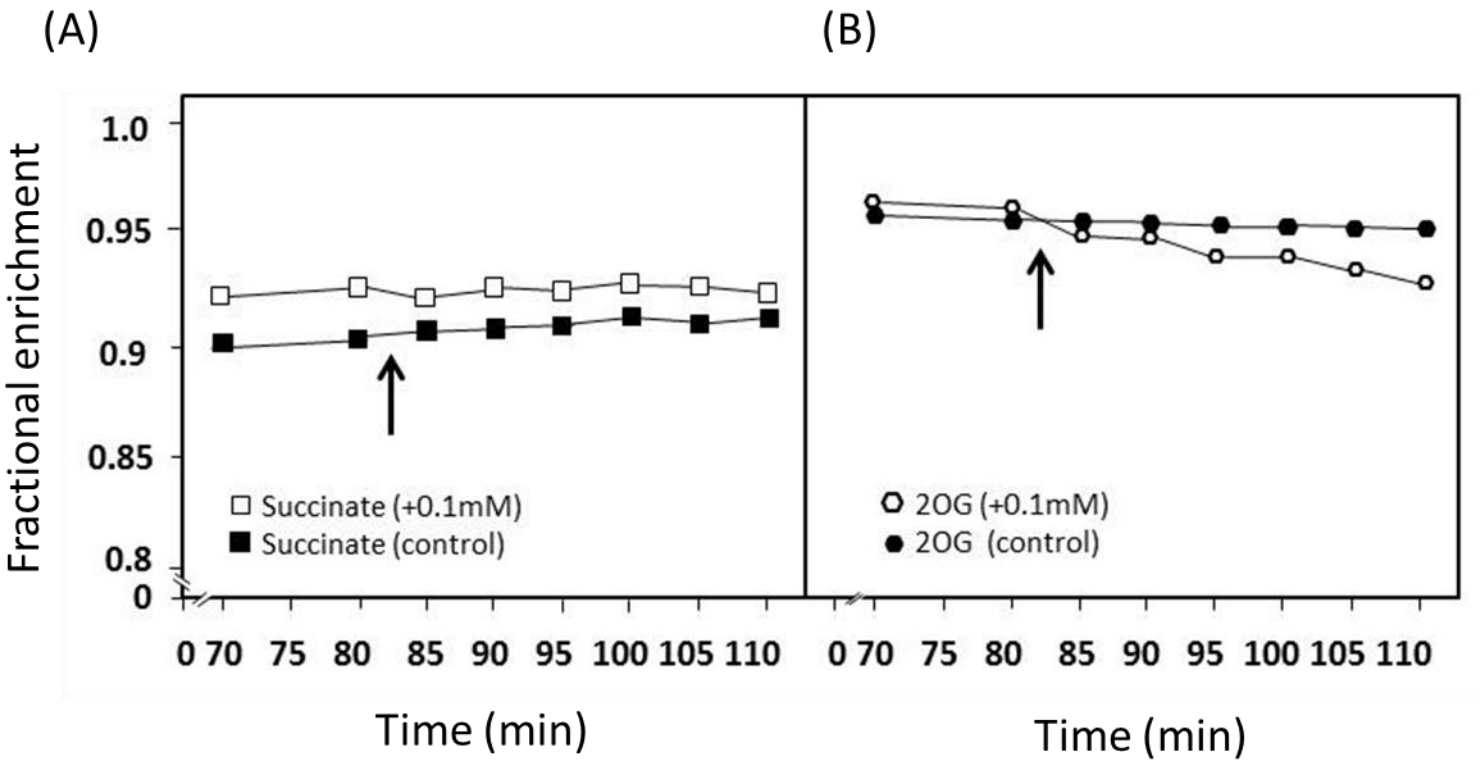


**Figure 3.9: Effect of addition of unlabelled 0.1 mM citrate on the fractional enrichment of succinate and 2OG in *S. tuberosum* mitochondria fed with 10 mM [3-$^{13}$C]pyruvate and 10 mM malonate compared with a control.** Fractional enrichment of (A) succinate (B) 2OG. Arrow denotes addition of 0.1 mM unlabelled citrate. Mitochondria were incubated in buffer with 10 mM [3-$^{13}$C]pyruvate, 10 mM malate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP 0.15 U/ml hexokinase, 20 mM glucose and 10 mM malonate.

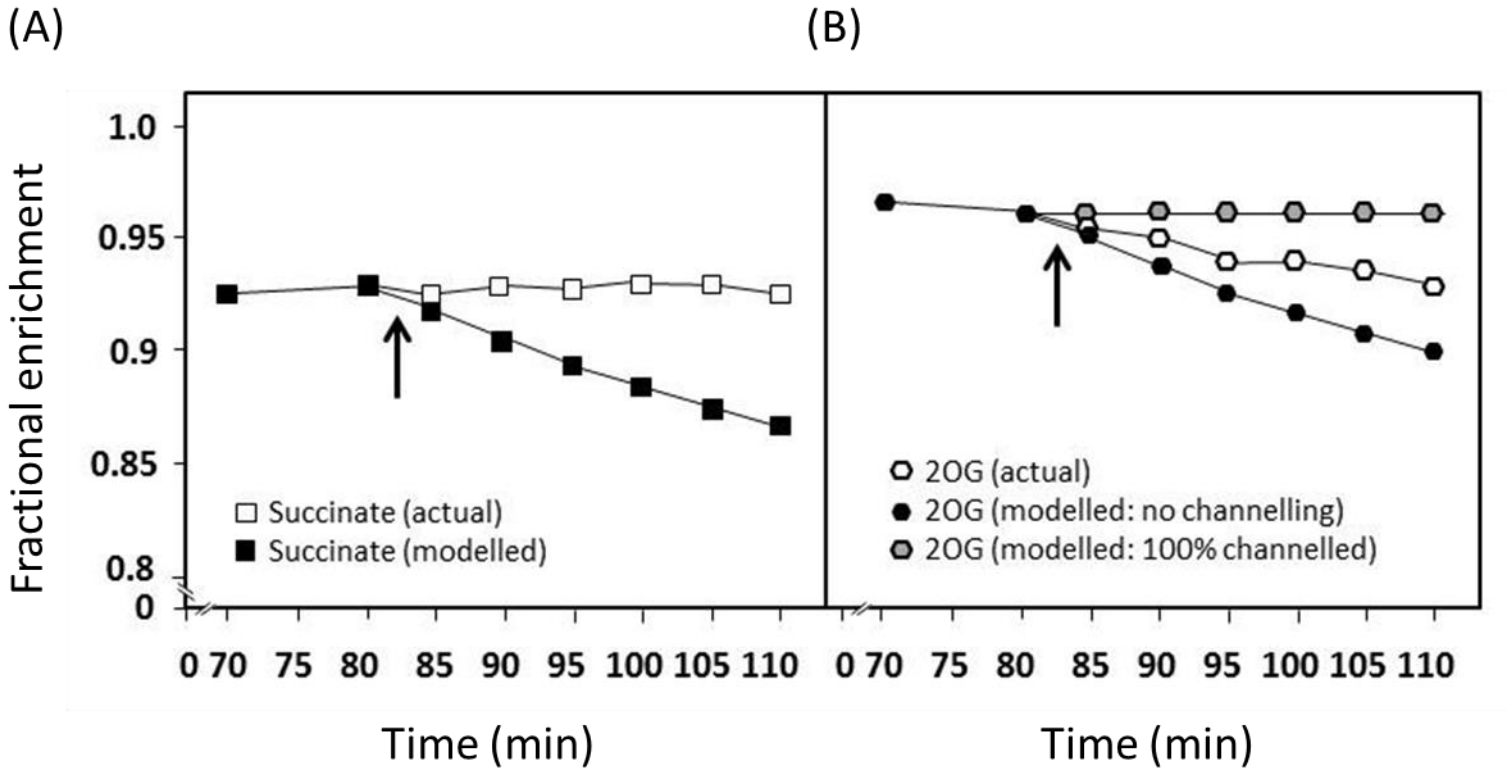


**Figure 3.10: Effect of addition of unlabelled 0.1 mM citrate on the fractional enrichment of succinate and 2OG in *S. tuberosum* mitochondria fed with 10mM [3-$^{13}$C]pyruvate and 10 mM malonate compared with fractional enrichments predicted from a simple model where no channelling was present.** Fractional enrichment of (A) succinate (B) 2OG. Arrow denotes addition of 0.1 mM unlabelled citrate. Mitochondria were incubated in buffer with 10 mM [3-$^{13}$C]pyruvate, 10 mM malate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP 0.15 U/ml hexokinase, 20 mM glucose and 10 mM malonate.

## Discussion

### An isotope dilution approach can be used to assess whether channelling is present in the TCA cycle.

The results show that isolated potato mitochondria can produce labelled organic acids, and that the effect of unlabelled exogenous organic acids on the internal mitochondrial pools can be measured. The TCA cycle could be successfully blocked by malonate or fluorocitrate to simplify the labelling pattern (by preventing multiple turns of the cycle) and so measure the isotopic dilution of the product. The experiments showed that the unlabelled citrate was able to dilute the 2OG pool but not the succinate pool, demonstrating that the approach is able to discriminate whether channelling is present in the TCA cycle, and to make a qualitative assessment of channelling.

### It was harder to make quantitative assessments of channelling using this method.

I developed a simple model in an attempt to gain quantitative information about metabolite channelling. The model showed that a fall in the fractional enrichment of succinate would be expected if channelling was not present. The fact that no dilution was measured suggests that channelling is present in this section of the TCA cycle.

However there are several difficulties in using this model to estimate the percentage metabolite channelling in the TCA cycle. The first challenge is that it cannot be assumed that the concentration present in the medium was equivalent to the concentration experienced by the enzymes. This is because the unlabelled

intermediate has to cross the mitochondrial membrane through a transporter protein. Although the internal concentration of citrate in the mitochondria could be measured, it was not possible to quantify the internal concentrations of succinate, fumarate or 2OG, as the concentrations were too low to reliably detect by GC-MS. As these measurements are critical for the prediction of isotope dilution in the model this was a significant barrier to measuring percentage metabolite channelling. A further difficulty is that the model is relatively simplistic. For example, the model relies on information about the concentration of unlabelled intermediate that is present in the mitochondria immediately after it has been added. This is then used to calculate the fall in dilution over the rest of the time course. However, in reality the concentration of unlabelled intermediate in the system decreases quite quickly, meaning that the fall in enrichment is on over-estimate. The model can still be used to give a maximum dilution in the system, but may not be useful in allowing me to calculate % channelling. In addition, the model relies on accurate values for the $K_m$s of the enzymes. As $K_m$s are not the same for the same enzyme across species, it is important to use $K_m$s for *S. tuberosum* and *A. thaliana*. However, there are very few published $K_m$s for the correct enzymes of the TCA cycle, and this limits the accuracy of the model.

## Future developments

Other methods were considered for measuring channelling in the TCA cycle. One option would have been to try and model the TCA cycle more fully, for example by using programmes such as Gepasi (Mendes 1993). This may also have allowed me to measure channelling without the use of inhibitors. However both the lack of time and reliable information about the enzyme Km's meant that this method was not used.

The approach of Williams *et al.* (2011) would also have been a useful way to measure metabolite channelling in the TCA cycle. However it was developed too late in my work to allow me to take advantage of it in the time I had left.

## Summary

The approach developed in this chapter is able to assess whether channelling is present in the TCA cycle. The preliminary experiment showed that the unlabelled intermediate diluted the 2OG pool but not the succinate pool (the experiment is repeated and discussed in chapter 4). This result shows that it is still possible to gain information about channelling from the TCA cycle without measuring % channelling. As a result it was decided to use isotope dilution to investigate whether channelling was present or absent in the TCA cycle, rather than attempting to measure it quantitatively using isotope dilution.

# Chapter 4: Measuring metabolite channelling in the plant TCA cycle

## Introduction

In the previous chapter I showed that it was possible to use an isotope dilution method to measure metabolite channelling in a qualitative manner in isolated mitochondria, although measuring metabolite channelling quantitatively proved much harder. The aim of this chapter is to use the method described in chapter 3 to investigate metabolite channelling in the TCA cycle in plants.

Preliminary experiments (chapter 3) focused on measuring metabolite channelling through the addition of unlabelled citrate, in order to test whether metabolite channelling was present between citrate synthase and aconitase. However in order to investigate metabolite channelling in the TCA cycle fully we need to investigate whether metabolite channelling is present between other pairs of TCA cycle enzymes. As outlined in the general introduction different parts of the TCA cycle are subject to different metabolic demands. As a result, metabolite channelling may be necessary or beneficial in some parts of the pathway but not others. This could result in differences in metabolite channelling between enzymes. This has been shown to be the case in the glycolytic metabolon, where percentage channelling between glycolytic enzymes in *A. thaliana* varied between 33 and 100 between different enzyme pairs (Graham *et al.* 2007).

Many of the TCA cycle enzymes have been found to interact with each other in eukaryotes (Srere *et al.* 1999) However experiments in different species have

identified a number of different combinations of TCA cycle enzymes that appear to be able to interact *in vitro* (see general introduction). Interaction experiments are known to be potentially unreliable and produce a high level of false positives. There is also no information about which TCA cycle enzymes, if any, form a metabolon in plants, and no attempts to investigate metabolite channelling directly, though methods such as isotope dilution. It is therefore necessary to investigate metabolite channelling between as many enzymes as possible.

Another aim of this chapter is to investigate metabolite channelling in both *S. tuberosum* and *A. thaliana* mitochondria. One hypothesis of this thesis is that metabolite channelling has a functional role in plants. If this hypothesis is correct then metabolite channelling might be different in mitochondria which have been isolated from tissues which experience different metabolic conditions (this is discussed in more detail in the general introduction). One way to investigate this is to compare mitochondria from different plant species and tissues which will be under different selection pressures and face different metabolic demands. By comparing metabolite channelling in more than one plant tissue it may be possible to uncover more about the role of metabolite channelling in plants.

There are also more pragmatic reasons for choosing to measure metabolite channelling in *S. tuberosum* and *A. thaliana. S. tuberosum* is a very convenient organism to use for mitochondrial experiments, because the tubers are an excellent source of stable, metabolically active mitochondria which can be isolated reliably in large quantities. However there is also less information available about the fluxes through the TCA cycle in *S. tuberosum* compared with *A. thaliana*. In contrast *A.*

*thaliana* mitochondria are less stable and harder to isolate in large quantities. However as a model organism more is known about demands on, and fluxes through, the TCA cycle. It is also easier to test whether changes in metabolism affect metabolite channelling in *A. thaliana* mitochondria as the plants can be grown easily in the lab, and it is easier to apply treatments to smaller plants grown in sterile conditions. Using the method developed in chapter 3, I will be able to compare whether metabolite channelling is present in both species, and if so, whether the type and pattern of metabolite channelling is the same.

# Results

## Investigating metabolite channelling in *S. tuberosum* mitochondria

Mitochondria were isolated from *S. tuberosum* (v.*Desireé*) tubers as described in materials and methods. Isolated mitochondria were incubated with either 10 mM [3-$^{13}$C]pyruvate, 10 mM malate and 10 mM malonate (which inhibits succinate dehydrogenase and so blocks the TCA cycle) or 10mM [3-$^{13}$C]glutamate and 5µM fluorocitrate (which inhibits aconitase). In all cases the incubation medium contained 0.3 mM $NAD^+$, 0.2mM ADP, 0.1 mM TPP, 20 mM glucose and 0.15U/ml hexokinase. After 80 minutes the unlabelled intermediate (citrate, 2OG, succinate, fumarate or malate) was added to a concentration of 0.1 mM. After extraction and derivitisation, the concentration and fractional enrichment of organic acids in the combined suspension and mitochondria was measured using GC-MS.

### *Investigating channelling using unlabelled citrate and fumarate*

When unlabelled citrate or unlabelled fumarate was added to the mitochondria, a dilution effect was apparent for some organic acids but not others (Figure 4.1a, Figure 4.2a). In both cases no dilution was seen in the final organic acid produced in the linearized pathway. When unlabelled citrate was added to the mitochondria there was fall in the fractional enrichment of 2OG but not succinate in both replicates. The fall in fractional enrichment in 2OG was very similar in the two replicates (Figure 4.1a). In contrast, the fractional enrichment of succinate remained constant after the addition of unlabelled citrate (Figure 4.1a). Succinate continued to be produced after

unlabelled citrate was added, showing that the constant fractional enrichment was not because the production of succinate had stopped (Figure 4.1b).

Similarly the fractional enrichment of malate fell in both replicates after the addition of fumarate (Figure 4.2a). The fall in enrichment was fastest immediately after the addition of citrate and then slowed as the unlabelled intermediate was used up. The fractional enrichment of the two replicates was very similar before the addition of fumarate, and then the rates diverge slightly. The difference between the two replicates could be due to differences in the pool sizes of malate or the activity of the individual mitochondria preps. The fractional enrichment of citrate remained fairly constant after the addition of fumarate. There were some very small changes in the fractional enrichments but no clear downward trend (Figure 4.2a). Again the concentration of citrate increased after fumarate was added, and so the constant enrichment was not due to a lack of citrate production (Figure 4.2b). There was also a fall in the concentration of citrate in the final time point, possibly due to a fall in mitochondrial activity towards the end of the experiment.

The best explanation for the dilution pattern is that more than one pool of TCA cycle enzymes exist, either in the same mitochondria, or within different mitochondria in the population. Externally added metabolites such as fumarate and citrate which enter the mitochondria via transporters have access to some of these enzymes, but the fact that they do not dilute citrate or succinate respectively suggests that they do not have access to all of the TCA cycle enzymes in the mitochondria. This suggests that at least some of the TCA cycle enzymes channel metabolites through the TCA cycle.

### *Investigating channelling using unlabelled succinate*

When unlabelled succinate was added to the mitochondria there was a fall in the fractional enrichment of malate (Figure 4.3a). The fall in enrichment was similar to the experiment when fumarate was added to the mitochondria. The standard deviation was larger than when fumarate was added, but again this could be due to difference in the pool size of malate and the activity of the separate mitochondrial preparations. However, unlike in previous experiments there was also a fall in the fractional enrichment of citrate (Figure 4.3a). The greatest fall in dilution was in the penultimate time point in both experiments. However the fall in fractional enrichment varied considerably between the two replicates. This suggests that unlike in the previous experiments unlabelled succinate has access to enzymes which produce citrate. However the large variation between the two experiments makes it harder to draw any firm conclusions. In both experiments citrate continued to be produced after the unlabelled intermediate was added, although the rate of increase was lower than when fumarate was added as an intermediate (Figure 4.3b).

### *Investigating channelling using unlabelled 2-oxoglutarate and malate*

When unlabelled 2-OG was added to the mitochondria, there was a small fall in the fractional enrichment of succinate (Figure 4.4a). When unlabelled malate is added to the mitochondria there is also a fall in the fractional enrichment of citrate (Figure 4.4b). However the fall in enrichment is very small and the final three time points show an increase in the fractional enrichment. This could be the result of a very small fall in fractional enrichment in the citrate pool; however it is also small enough for it to

be the result of noise. It is therefore difficult to say with any confidence that it is a real effect.

In both these experiments it is difficult to draw any conclusion as to whether metabolite channelling is occurring or not. In isotope dilution experiments a fall in the fractional enrichments of organic acids is thought to show that the enzymes are not fully channelled. This could occur if there is one set of enzymes which is fully or partially un-channelled. However it could also occur if there are two separate sets of enzymes: one which is fully or partially un-channelled, and one set which is channelled. In both cases a fall in fractional enrichment would occur. When citrate or fumarate was used as an unlabelled intermediate, some pools of organic acids were diluted while others were not. This suggests that two sets of enzymes are present. However, when 2OG or malate were used as an unlabelled intermediate, it was only possible to measure the fractional enrichment of one pool of organic acids (because the TCA cycle was blocked). This means that it is not possible to tell whether metabolite channelling is present between 2-OGDH and SDH or MDH and CS using this method.

### Investigating metabolite channelling in *A. thaliana* mitochondria

Some of the experiments were repeated in *A. thaliana* mitochondria. Trials showed that the amount of labelled succinate produced by mitochondria incubated with [3-$^{13}$C]pyruvate was very low, and therefore it was difficult to accurately measure the fractional enrichment. I therefore repeated the isotope dilution experiments where unlabelled succinate and fumarate were added, and the fractional enrichment of malate and citrate measured. I did not repeat the experiment with 2-OG or malate, as

the experiment in *S. tuberosum* showed that it wasn't possible to deduce anything about metabolite channelling from these experiments.

Mitochondria were isolated from 14 day old *A. thaliana* seedlings grown as described in materials and methods, and experiments were carried out as described for *S. tuberosum*. Organic acid production was similar to that seen in the preliminary experiments, with lower organic acids production in *A. thaliana* compared to *S. tuberosum*.

### *Investigating channelling using unlabelled fumarate*

After unlabelled fumarate was added to *A. thaliana* mitochondria metabolising [3-$^{13}$C]glutamate the fractional enrichment of malate fell and then remained constant. (Figure 4.5a). The fractional enrichment of citrate remained constant after the addition of fumarate. As in potato, the concentration of citrate increased after the addition of unlabelled intermediate (not shown). This result shows that the pattern of dilution after unlabelled fumarate is added is similar for both *A. thaliana* and *S. tuberosum* mitochondria

### *Investigating channelling using unlabelled succinate*

After succinate was added to isolated *A. thaliana* mitochondria metabolising [3-$^{13}$C]glutamate there was a significant fall in the fractional enrichment of malate (Figure 4.6a). There was also a fall in the fractional enrichment of citrate, although the fractional enrichment rose slightly immediately after succinate was added (Figure 4.6b). The reason for this is not clear. However, unlike in potato mitochondria there was a substantial fall in the dilution of citrate after succinate was added.

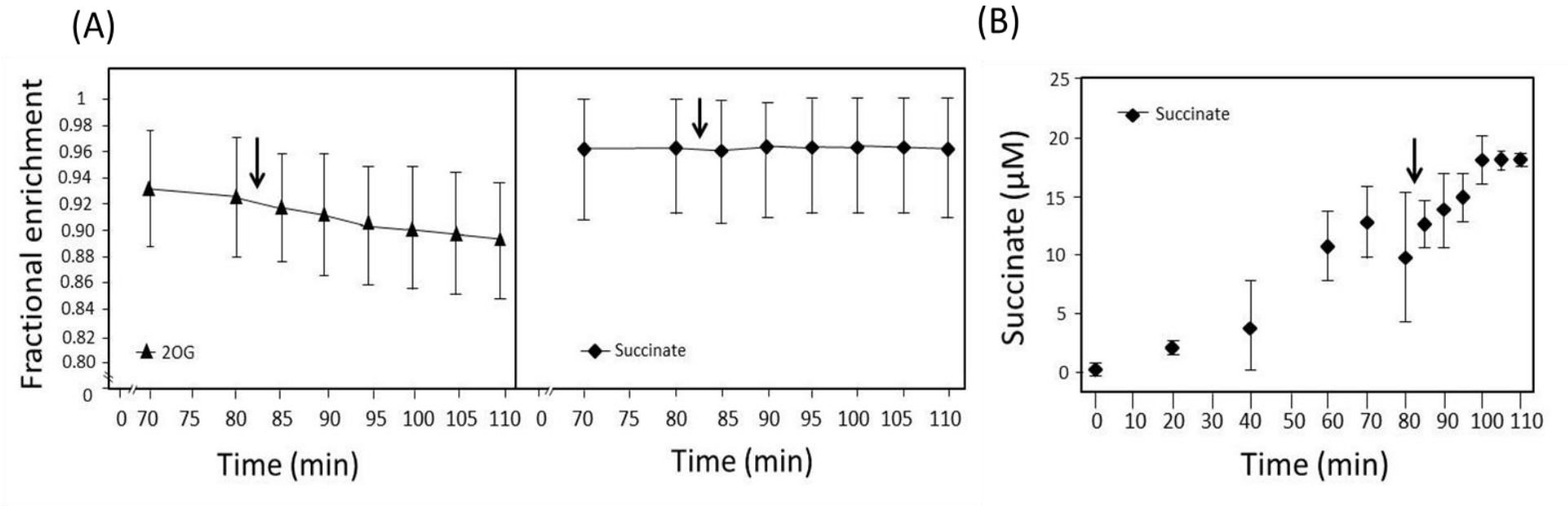


**Figure 4.1 Isotope dilution after addition of unlabelled citrate to isolated *S. tuberosum* mitochondria metabolising [3-$^{13}$C ]pyruvate.** (A) Average fractional enrichment of succinate and 2OG before and after the addition of 0.1mM unlabelled citrate; (B) average concentration of succinate over the course of the experiment. In both cases *n*=2, error bars denote standard deviations. The arrow denotes the addition of unlabelled citrate Isolated mitochondria were incubated with 10 mM [3-$^{13}$C ]pyruvate, 0.3 mM $NAD^{+,}$ 0.2 mM ADP, 0.1 mM TPP, 0.15 U/ml hexokinase and 10 mM malonate to block the TCA cycle at SDH.

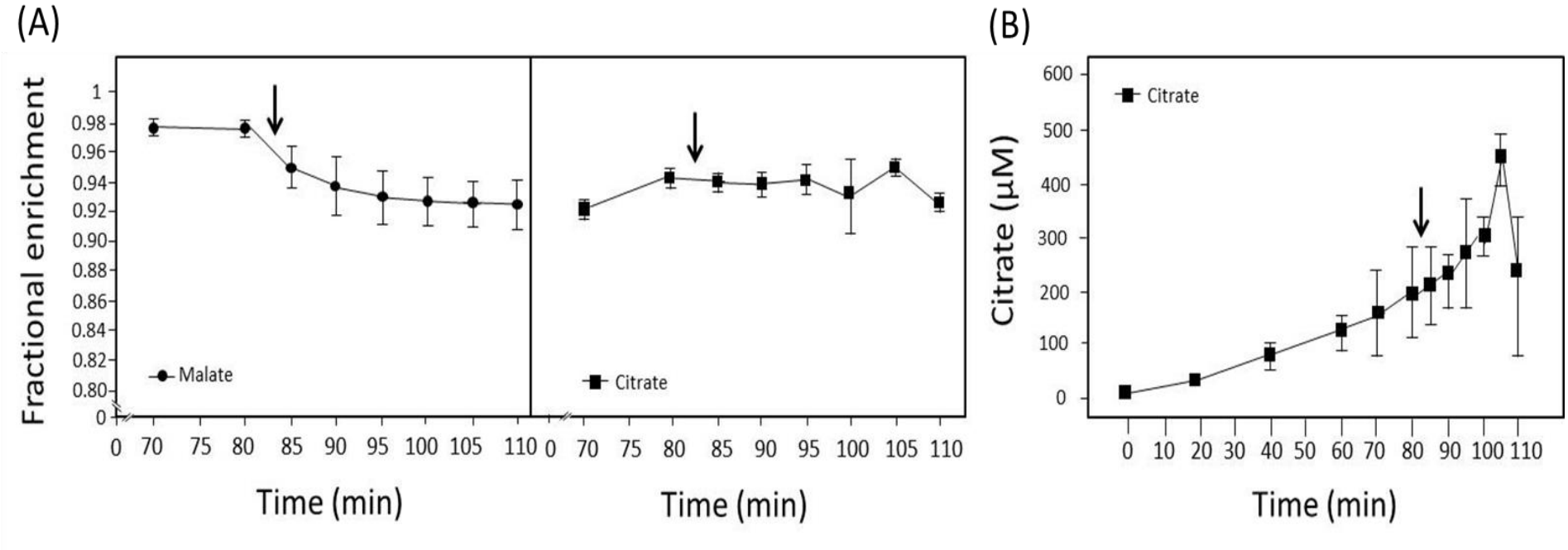


**Figure 4.2 Isotope dilution after addition of unlabelled fumarate in isolated *S. tuberosum* mitochondria metabolising [3-$^{13}$C]glutamate.** (A) Average fractional enrichment of malate and citrate before and after the addition of 0.1 mM unlabelled fumarate; (B) Average concentration of citrate over the course of the experiment. In both cases *n*=2, error bars denote standard deviations The arrow denotes the addition of unlabelled fumarate. Isolated mitochondria were incubated with 10 mM [3-$^{13}$C]glutamate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP, 20 mM glucose , 0.15 U/ml hexokinase and 5 µM fluorocitrate to block the TCA cycle at aconitase.

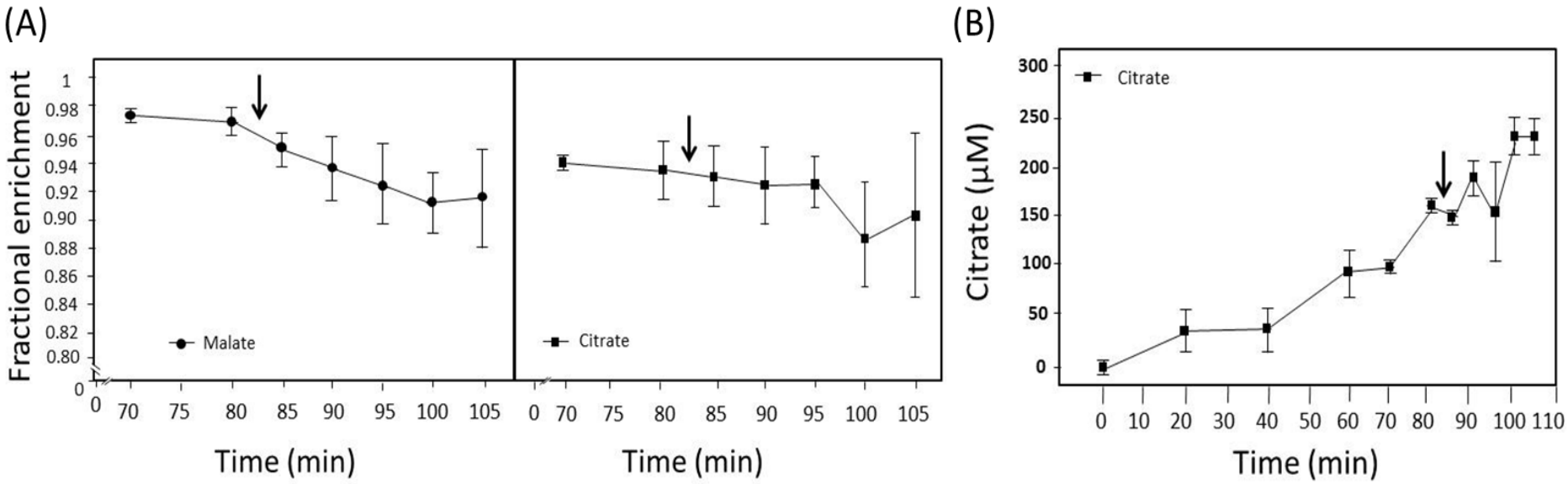


**Figure 4.3 Measuring isotope dilution after addition of unlabelled succinate in isolated *S. tuberosum* mitochondria metabolising [3-$^{13}$C]glutamate.** (A) Average fractional enrichment of malate and citrate before and after the addition of 0.1 mM unlabelled succinate; (B) Average concentration of citrate over the course of the experiment. In both cases *n*=2, error bars denote standard deviations. The arrow denotes the addition of unlabelled succinate. Isolated mitochondria were incubated with 10 mM [3-$^{13}$C ]glutamate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP, 20 mM glucose , 0.15 U/ml hexokinase and 5 µM fluorocitrate to block the TCA cycle at aconitase.

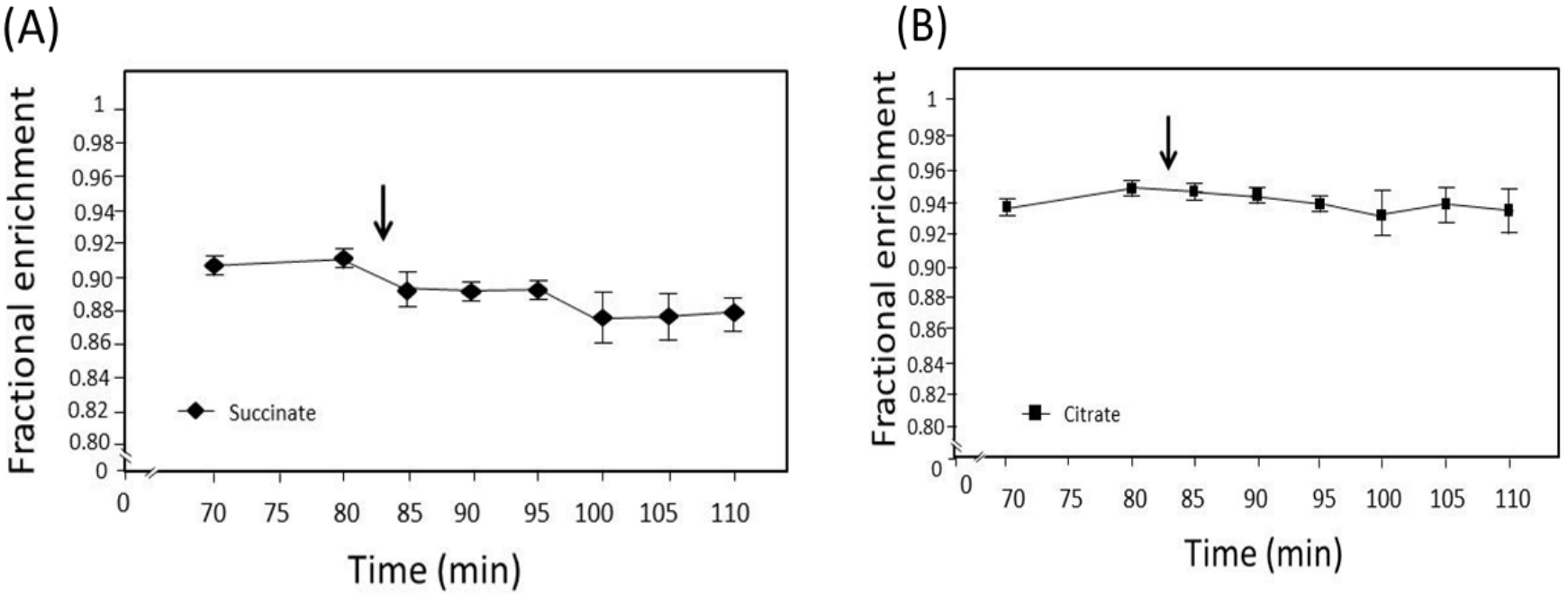


**Figure 4.4 Isotope dilution after addition of unlabelled 2OG or malate in isolated *S. tuberosum* mitochondria metabolising [3-13C]pyruvate or [3-13C]glutamate respectively.** (A) Average fractional enrichment of succinate before and after the addition of 0.1mM unlabelled 2OG. The arrow denotes the addition of unlabelled intermediate. Isolated mitochondria were incubated with 10 mM [3-13C]pyruvate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP, 0.1 5U/ml hexokinase and 10 mM malonate to block the TCA cycle at SDH. (B) Average fractional enrichment of citrate before and after the addition of 0.1mM unlabelled malate. In both cases *n*=2, error bars denote standard deviations . The arrow denotes the addition of unlabelled intermediate. Isolated mitochondria were incubated with 10mM [3-$^{13}$C ]glutamate, 0.3 M $NAD^+$, 0.2 mM ADP, 0.1 mM TPP, 20 mM glucose , 0.15 U/ml hexokinase and 5 µM fluorocitrate to block the TCA cycle at aconitase.

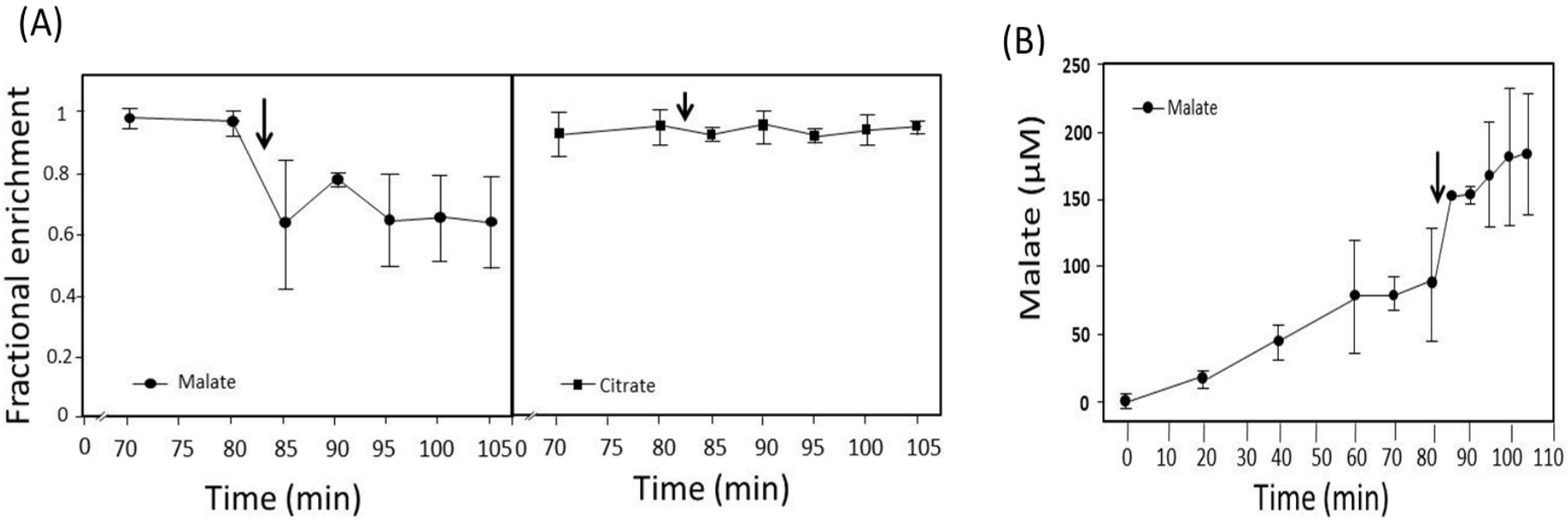


**Figure 4.5 Measuring isotope dilution after addition of unlabelled fumarate in isolated *A. thaliana* mitochondria metabolising [3-$^{13}$C]glutamate.** (A) Average fractional enrichment of malate and citrate before and after the addition of 0.1mM unlabelled fumarate; (B)Average concentration of malate over the course of the experiment. In both cases *n*=2, error bars denote standard deviations. The arrow denotes the addition of unlabelled fumarate. Isolated mitochondria were incubated with 10mM [3-$^{13}$C]glutamate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP, 20 mM glucose , 0.15 U/ml hexokinase and 5 µM fluorocitrate to block the TCA cycle at aconitase.

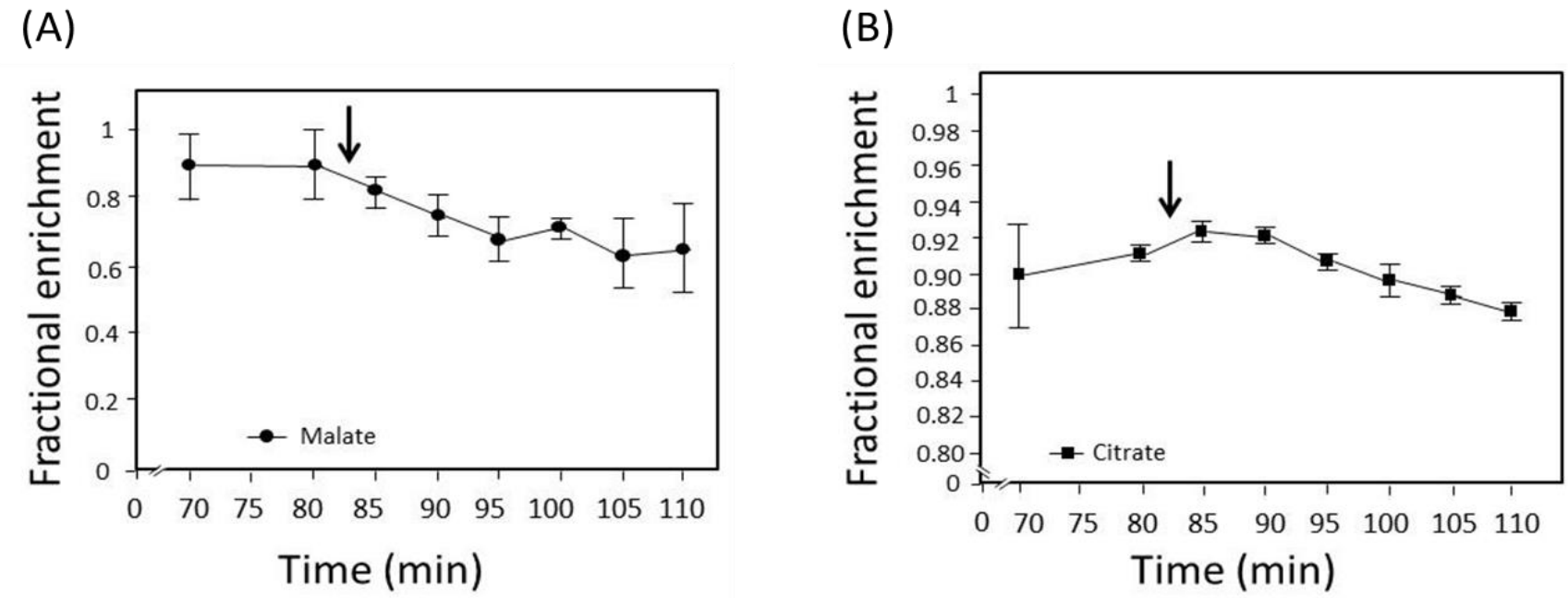


**Figure 4.6 Measuring isotope dilution after addition of unlabelled succinate in isolated *A. thaliana* mitochondria metabolising [3-$^{13}$C]glutamate.** (A) average fractional enrichment of malate and citrate before and after the addition of 0.1 mM unlabelled succiante; (B) Average fractional enrichment of citrate over the course of the experiment. In both cases *n*=2, error bars denote standard deviations. The arrow denotes the addition of unlabelled succinate. Isolated mitochondria were incubated with 10mM [3-$^{13}$C]glutamate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP, 20 mM glucose , 0.15 U/ml hexokinase and 5 µM fluorocitrate to block the TCA cycle at aconitase.

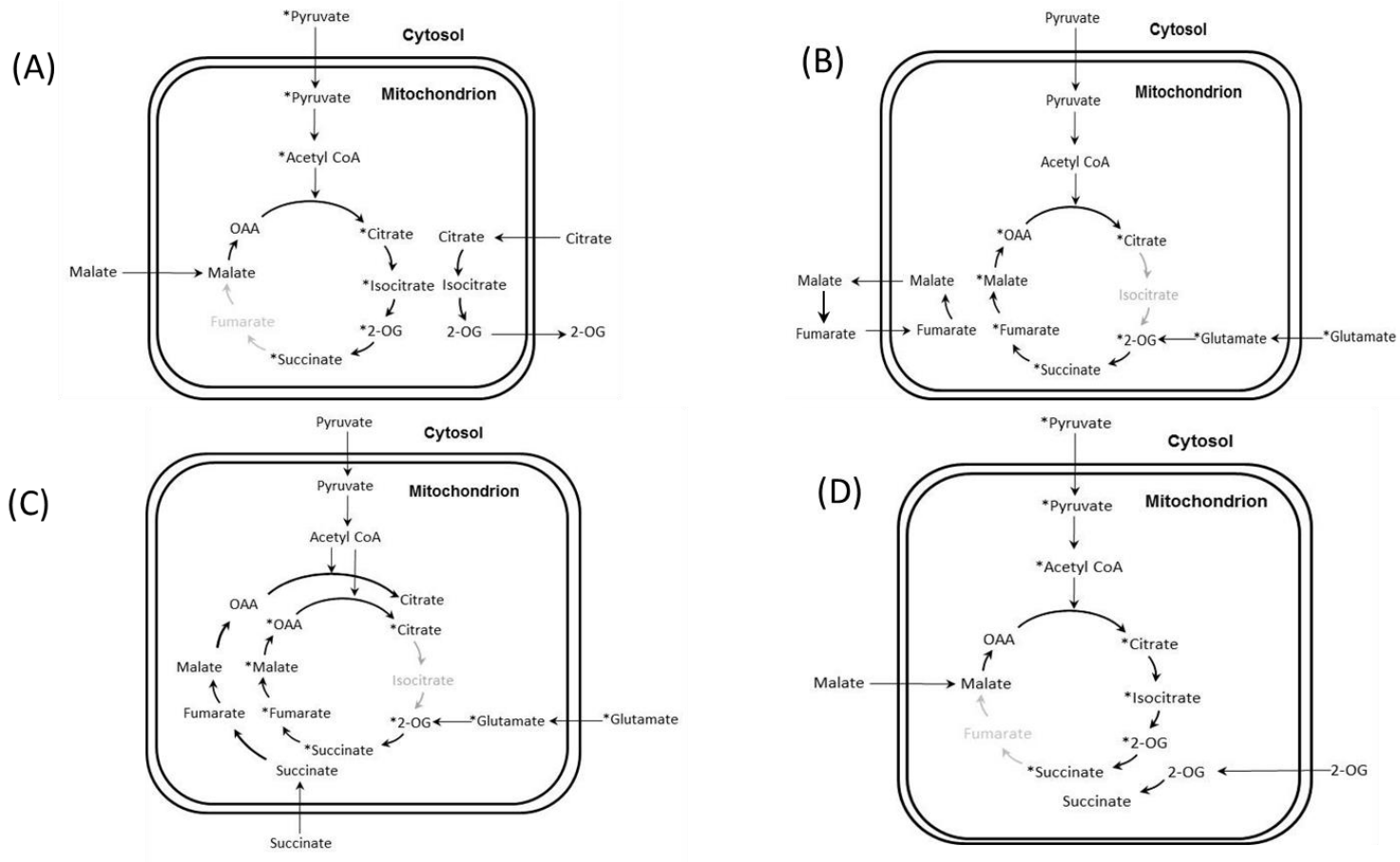


**Figure 4.7 Possible pathways of unlabelled intermediate metabolism in mitochondria.** (A) After addition of citrate in mitochondria metabolising labelled pyruvate; (B) After addition of unlabelled fumarate in mitochondria metabolising labelled glutamate; (C) after addition of unlabelled succinate; (D) after addition of unlabelled 2OG. * denotes $^{13}C$ labelled metabolite.

## Discussion

### Metabolite channelling is present in the TCA cycle

The aim of this chapter was to investigate whether metabolite channelling occurred in the plant TCA cycle. If the enzymes of the TCA cycle do not channel metabolites then metabolic intermediates should have free access to all the TCA cycle enzymes in the mitochondria. As a result, addition of unlabelled intermediates would dilute the labelled pools of all the other TCA cycle metabolites. However, when unlabelled citrate was added to *S. tuberosum* mitochondria, the fractional enrichment of succinate remained constant, although there was a fall in the fractional enrichment of 2OG. Similarly, when unlabelled fumarate was added to *S. tuberosum* or *A. thaliana* mitochondria, a fall in fractional enrichment was measured in malate but not in citrate. This suggests that the unlabelled intermediates only have access to some enzymes of the TCA cycle, and that metabolite channelling occurs between citrate synthase and aconitase, and succinate dehydrogenase and fumarase. Furthermore the results imply that labelled citrate and succinate are produced from a separate pool of organic acids to the malate and 2OG pools which are diluted.

One way this could be achieved is shown in Figure 4.7 a and b. Labelled pyruvate or glutamate is consumed by the TCA cycle and converted into succinate or citrate respectively. Unlabelled citrate and fumarate have access to the mitochondrial matrix via transporters, where they are metabolised into organic acids by a second set of TCA cycle enzymes. These unlabelled intermediates may then be exported into the external medium, preventing them from being metabolised further. Either or both of these pathways could be channelled.

## Channelled and unchannelled pathways could occur in separate mitochondria

Results suggest that there are two separate pools of TCA cycle enzymes with different degrees of channelling. This could be due to metabolon formation in the mitochondria, or if there are two pools of mitochondria: one which does not take up certain unlabelled intermediates, and the other taking in intermediates and catalysing a sub set of reactions.

There is plenty of evidence that mitochondria in eukaryotes are not a homogenous population, but differ in both their composition and activity. For example, within developing seedlings, mitochondria can be separated into different populations based on their density. Dai *et al.* (1998) showed that mitochondria isolated from mung bean seedlings could be separated into four classes based on their weight: normal sized, actively respiring mitochondria, smaller, poorly respiring mitochondria, and two further classes of lower density, smaller mitochondria which did not respire. All four classes of mitochondria contained DNA and some mitochondrial proteins, including MnSOD, COXII and the alpha and beta subunits of ATPase. However, only the former two classes contained PDH and were impermeable to DNAase, suggesting that the latter two populations were structurally and functionally immature. Similarly, Logan *et al.* (2001) were able to separate two populations from developing maize embryos; a 'heavy' and a 'light' population. After imbibition, the heavy population developed into "normal" mitochondria, with a high matrix protein concentration and internal cristae. In contrast the light population appeared to have a much lower matrix protein concentration and few internal cristae. The light mitochondria also lacked mtHSP70, CS, PDC E1a subunit and contained very small amounts of F1-F0ATPase. While both

mitochondrial populations had a functioning electron transport chain, only the heavy fraction was able to catalyse reactions through the TCA cycle. It is though that these classes represent different stages in the development of mitochondria, although they could also represent different classes of mitochondria which go on to have separate roles in the cell. There is also evidence that the protein composition of mitochondria is different in tissues with different metabolic conditions. Thompson *et al.* (1992) showed that the protein content of mitochondria changes during leaf development in barley. Barley leaves change from heterotrophic cells at the base of the leaf though to mature, photosynthetic cells at the leaf tip. Mitochondrial proteins were measured at different stages along the leaf as the cells matured. Group A proteins included the E1b subunit of PDC, AOX, COX II and III, malic enzyme and a and b ATPase. These peaked around 20 nm from the base of the leaf. Group B proteins included E1a PDC, PDC and IDH, and were at their maximum 50 nm from the base, when the cells were around 45 hours old. Group C proteins included GDC, formate dehydrogenase and fumarase, and were at their maximum from 50 nm to the leaf tip. These changes in protein content correlate with the development of a heterotrophic to an autotrophic environment.

There is evidence that mitochondria are different within the same cell. Mitochondrial-targeted GFP has been used to examine mitochondria in *Arabidopsis* seedling hypocotyls and roots (Logan and Leaver 2000). These experiments have shown that in a single tissue, and even in single cells, mitochondria differ in their size and shape, and appear to be unevenly distributed thoughout the cytoplasm. There is some evidence that the shape and form of mitochondria is related to metabolic state, as mitochondria from mouse liver alternated between a condensed form (more inter-cristal membrane

connections, with dilated inter-cristal spaces) in state 3 and an “orthodox” state (fewer cristal junctions and a more compressed inter-cristal space) in state 4 (Hackenbrock 1968).

Some experiments have suggested that mitochondria in individual cells might also differ in their metabolism. Most of these experiments are in neurones, where the cells are large enough to carry out microscopy or metabolite analysis on individual cells or groups of cells. In neurones, mitochondria can be separated into those of synaptic and peripheral origin and show slight differences in their enzyme content, as well as the relative activity of GDH and AAT (McKenna *et al.* 2000). Similarly calcium uptake by mitochondria in ganglion neurones was shown to be different in mitochondria located near the plasma membrane and those located near the nuclear, reflecting the different influx rates of calcium within the cell (Pivovarova *et al.* 1999). Waagpeterson *et al.* (2005) showed that αKGDH was not distributed evenly between mitochondria within a single astrocyte. This was true in cells originating in both the cortical and cerebellar regions of the brain. Margineantu *et al.* (2002) found that pyruvate dehydrogenase tagged with GFP was concentrated at spots in certain mitochondria in human skin fibroblasts. In contrast, both COX1 and RFP (used as a control) were evenly distributed throughout the mitochondria. Labelling studies also suggest that there are different populations of mitochondria within a single mouse neuronal cell. Studies comparing labelling when glucose or acetate was used as a precursor suggested that citrate and glutamate released from the mitochondria are made in different TCA cycles (Sonnewald *et al.* 1993a). Unlabelled glutamine is able to dilute labelled pools of aspartate and glutamate to a greater degree than GABA, suggesting that glutamine

only had access to a subset of mitochondrial enzymes (Waagpeterson *et al.* 1998). Experiments suggest that compartmentation of acetyl coA exists in liver mitochondria, with acetyl coA produced from oxidation of palmitate preferentially used for production of ketone bodies, while acetyl coA produced via pyruvate dehydrogenase preferentially enters the TCA cycle (Glutz *et al.* 1975).

### Metabolite channelling could be achieved through separate pools of enzymes within the same mitochondria.

An alternative explanation is that two sets of enzymes exist within the same mitochondria, with citrate and fumarate having access to only one set. The two pools could be physically separated by one or both sets of enzymes forming a metabolon. There is some evidence that separate pools of TCA cycle enzymes exist in the same mitochondria. Robinson and Srere (1985) found that half of the citrate synthase activity measured in mitochondria after sonication was associated with the membrane and half was free in the matrix. This could be because the enzymes which are associated with the membrane are disrupted or released during sonic oscillation. However it could also reflect the organisation in the mitochondria *in vivo*. Further evidence for two pools of mitochondria was found in plants. Wiskich *et al.* (1990) found evidence for separate pools of enzymes within the same matrix in mitochondria isolated from in pea leaves. Malate dehydrogenase was found to be able to operate in opposite directions simultaneously when oxidising malate and glycine. After using an inhibitor (phthalonate)which blocks OAA transport across the inner mitochondrial membrane, they suggested that the reactions occurred in the same mitochondrial matrix, rather than in separate mitochondria.

It is not possible to tell from our experiments whether TCA cycle enzymes are physically separated within the same mitochondrial matrix, or whether our results reflect differences between separate mitochondria. To investigate this we would need to have a more detailed picture of how enzymes interact with each other in the mitochondrial matrix, as well as a better understanding about the distribution of transporters between mitochondrial populations.

### Succinate has access to more enzymes of the TCA cycle

When unlabelled succinate was added to *A. thaliana* mitochondria it diluted both the citrate and the malate pool suggesting that, unlike fumarate, succinate has access to enzymes which produce citrate. This also appears to be the case in *S. tuberosum* mitochondria, although the dilution is much smaller, and may be partly due to noise in the spectra. Unlabelled succinate enters the mitochondria, and is converted into citrate through the TCA cycle. It is important to point out that it is not possible to tell whether the dilution seen in citrate is the result of one un-channelled pathway or a channelled and an un-channelled pathway (illustrated in Figure 4.7c).

There are several explanations for this dilution pattern. If metabolite channelling is the result of separate pools of TCA cycle enzymes in different types of mitochondria, then this implies that both sets have a functioning succinate/fumarate carrier, but that one set only transports succinate and not fumarate into the mitochondria. Alternatively enzymes could be separated in the same mitochondria, by metabolite channelling through one or both sets of enzymes. In this scenario, the results suggest that succinate has access to the enzymes which are part of a putative metabolon as well as the free pool of enzymes. However, although there is evidence that enzymes of the

TCA cycle interact with each other (Beeckmans and Kanareck1981, Barnes and Weitzman 1986), succinate dehydrogenase has not been found in any of the proposed metabolons (although Porpaczy *et al.* 1983 found an interaction between 2OGDH and succinate thiokinase). This may be partly due to experimental constraints. In contrast to other TCA cycle enzymes, succinate dehydrogenase also forms part of the mitochondrial oxidative phosphorylation system and remains strongly attached to the membrane after mitochondria are disrupted (D'Souza and Srere 1983). The techniques used to identify metabolons involve disrupting or removing the membrane, and this could explain the absence of SDH from interaction experiments. Other TCA cycle enzymes do bind to the membrane, and this is specific to inner mitochondrial membrane, suggesting they bind to a component in the membrane (Robinson and Srere 1987). There is also evidence that TCA cycle enzymes interact directly with complexes of the oxidative phosphorylation system. Sumegi and Srere (1984) found that pyruvate dehydrogenase, alpha-ketoglutarate dehydrogenase and mitochondrial malate dehydrogenase bound to complex 1, and Popaczy *et al.* (1984) also found an interaction between NAD-dependent isocitrate dehydrogenase, alpha-ketoglutarate dehydrogenase complex and complex 1. Complexes I, III, IV and V are thought to exist as "super-complexes" in the mitochondrial membrane. Typically complex II has not been identified in these complexes (Dudkina *et al.* 2010), although there is some evidence that cytochrome bc1 interacts with complex II in yeast (Bruel *et al.* 1996). There is evidence that cytochrome bc1 also interacts with malate dehydrogenase in *Bos taurus* mitochondria *in vitro* (Wang *et al.* 2010).

**It is not possible to draw conclusions about metabolite channelling between 2-OGDH and SDH, and MDH and CS.**

Experiments with unlabelled citrate suggest that there are two pools of mitochondrial enzymes – one set which is channelled and produces succinate, and another which can metabolise externally added intermediates and produces 2OG. However when unlabelled 2-OG is added, it is able to dilute the succinate pool, suggesting that unlike 2-OG made from unlabelled citrate, it has access to enzymes which produce succinate.

There are at several implications of this result. The first is that external 2-OG imported directly into the mitochondria has access to enzymes which 2-OG made from citrate does not. This could happen if, for example, the transporter by which 2-OG enters the cell interacted with a set of 2-OGDH enzymes that produce succinate. An alternative scenario is that when unlabelled citrate is converted to 2-OG it is directly exported from the mitochondria before it has access to 2-OGDH. This could occur if the transporters preferentially exported 2-OG made via this pathway, for example if the enzymes interacted directly with the transporter. There is some evidence that the enzymes of the TCA cycle interact with transporters in the membrane. Grigorenko *et al.* (1990) reported that immobilised CS1 in yeast interacts with the citrate transporter, and there is also evidence that AAT interacts with TCA cycle enzymes in neurones (McKenna *et al.* 2000). However there is no evidence that this occurs in plants and the interaction of these proteins *in vitro* does not necessarily mean that they interact *in vivo*.

It is not possible to tell whether the dilution in succinate is the result of one set of 2-OGDH enzymes which is unchannelled with respect to externally added 2-OG, or the

result of two pools of 2-OGDH: one of which is channelled and produces labelled succinate, and one of which is unchannelled and produces a mixture of labelled and unlabelled succinate, as in both cases a fall in fractional enrichment would occur. In order to distinguish between these two possibilities we would need to know much more about how enzymes in the TCA cycle interact with each other and with metabolite transporters in the mitochondria.

When malate was added to mitochondria metabolising [3-$^{13}$C]glutamate, the fractional enrichment of citrate fell very slightly (Figure 4.4b), but it is difficult to state with any confidence whether this is a real effect. Even if a dilution effect had been measured, it would be difficult to draw any conclusions as to the nature of metabolite channelling around this part of the TCA cycle, because it is not possible to measure OAA using GS-MS. For this reason it would not be possible to tell whether the dilution was the result of one un-channelled pathway or a channelled and an un-channelled pathway (Figure 4.7d). Malate must have some access to all the enzymes under some conditions because it was used to ensure the TCA cycle was active in experiments using labelled pyruvate. However under these conditions malate was the only source of carbon skeletons, as the TCA cycle had been blocked at SDH. This is not the case in experiments where it is added as an unlabelled intermediate, as glutamate was used as the precursor.

### Metabolite channelling is similar in both *A. thaliana* and *S. tuberosum*

There is very little qualitative difference between the metabolite channelling observed in *S. tuberosum* and *A. thaliana* when unlabelled fumarate was added to the mitochondria. In both systems, a fall in fractional enrichment was seen in malate but

not in citrate. Similarly, when unlabelled succinate was added, a fall in fractional enrichment was measured in both malate and citrate. There was a difference in the size of the dilution, with a much larger fall measured in *A. thaliana*, than in *S. tuberosum*. This may be explained by a smaller pool of citrate in *A. thaliana* experiments, meaning that the effect of the same concentration of succinate was much lower.

The conserved pattern suggests that metabolite channelling in the TCA cycle is conserved between the *A. thaliana* and *S. tuberosum*. This is not altogether surprising, as there is evidence for a TCA cycle metabolon in both eukaryotic and prokaryotic cells, and in multicellular and unicellular organisms (Srere 1999). The same enzymes have been found to interact with each other in *E. coli* (Barnes and Weitzman 1986), *B. subtilis* (Meyer *et al.* 2011) and *S. cerevisiae* (Beeckmans and Kanareck 1981), suggesting that metabolite channelling may also be similar between different species. The TCA cycle is a vital pathway in cells, and is therefore likely to be under strong selection pressure. The result of this is that if metabolite channelling is present and has an important function then it is likely to be conserved across species. The presence of metabolite channelling in both species suggests that it has a functional role. This will be tested in the next chapter.

# Chapter 5: Investigating the role of metabolite channelling in the plant TCA cycle

## Introduction

There is evidence that metabolite channelling exists in the TCA cycle in a variety of organisms including yeast (Sumegi *et al.* 1993), rats (Sherry *et al.* 1994) and other mammals (Wang *et al.* 2010, Shatalin *et al.* 1999). Results from chapter 4 suggest that metabolite channelling also exists in the plant TCA cycle. The existence of metabolite channelling in several eukaryotic kingdoms suggests that it is functionally important. However, currently very little is known about the role that metabolite channelling plays in the TCA cycle. One possibility is that metabolite channelling is important in regulating the TCA cycle, and the aim of this chapter is to investigate this.

### Metabolite channelling may be important for regulating competing demands on the TCA cycle

One possible role for metabolite channelling is that it helps to regulate competing demands on TCA cycle intermediates. The TCA cycle is part of a large network of reactions, many of which utilise intermediates of the TCA cycle for purposes other than respiration. For example, nitrogen assimilation removes 2OG from the TCA cycle for use as a carbon skeleton. Similarly, several amino acid biosynthetic pathways use TCA cycle intermediates: aspartate is produced from OAA, and glutamate is produced from 2OG. Removal of these intermediates from the TCA cycle reduces the availability of organic acids that can support the full cyclic flux. However, most processes which remove intermediates from the TCA cycle also require ATP or NADH, and as a result require a full cyclic flux through the TCA cycle. This may be impeded by the removal of

carbon skeletons, and it is likely that the two need to be finely balanced in order to optimise plant fitness.

It is possible that one of the roles of metabolite channelling is to help regulate these competing demands on the TCA cycle, with the proportion of enzymes which are organised as a metabolon changing as demands from cyclic and biosynthetic pathways also change. This is known to occur in the glycolytic pathway. Gycolytic enzymes are known to interact with the surface of mitochondria in plants to form a metabolon which supports substrate metabolite channelling (Graham *et al.* 2007, Giegé *et al.* 2003). Graham *et al.* (2007) showed that the fraction of glycolytic enzymes associated with the mitochondria increased with increasing respiration rate, and decreased with inhibition of respiration by KCN. This was proposed to constitute a regulatory mechanism to ensure that sufficient pyruvate is provided to support respiration. It also prevented the withdrawal of pyruvate by competing biosynthetic pathways (such as the synthesis of amino acids), and those which withdraw metabolites from upstream in the glycolytic pathway (e.g. the oxidative pentose phosphate pathway). In this way, the proportion of glycolytic enzymes in the channelled fraction can change to regulate competing demands on the glycolytic pathway.

It is possible that metabolite channelling exists in the TCA cycle for a similar reason. In the previous chapter, it was shown that two pools of TCA cycle enzymes exist: a channelled and an unchannelled pool. It is possible that under different metabolic conditions metabolite channelling could change. For example, the proportion of enzymes in each fraction or the 'tightness' of the metabolite channelling could alter in order to support different fluxes. If there was a high demand for ATP, the proportion

of enzymes in the channelled section of the TCA cycle could increase to ensure that the demand for cyclic flux was met, while reducing the number of intermediates withdrawn for biosynthesis. Conversely, if the biosynthetic demand was high, the proportion of channelled enzymes could decrease to allow the demand for carbon skeletons from the TCA cycle was met. In both cases, the maintenance of some enzymes in either a channelled or free pool would ensure that there was always the potential for a cyclic or a biosynthetic flux to be maintained. Another way that metabolite channelling could alter would be through changing the interactions between enzymes, so that different enzymes associate with each other and alter which metabolites are channelled. One way to test whether any of these changes are occurring in mitochondria is to compare metabolite channelling in mitochondria under different metabolic conditions.

Few experiments have directly tested the idea that metabolite channelling is important in regulating the TCA cycle. However, some studies have found that enzyme kinetics change when enzymes are immobilised near to each other. Lindbladh *et al.* (1994) found that the $K_m$ of substrates of MDH was reduced in fusion proteins of MDH and CS compared to free enzymes. Robinson *et al.* (1987) measured the rate of different reactions of the TCA cycle in free enzymes and on disrupted mitochondria which contained a metabolon of TCA cycle enzymes. They showed that the rate of reaction was faster in the metabolon fraction. There is also some evidence that TCA cycle enzymes interact with both complex 1 (Sumegi and Srere 1984) and a citrate transporter (Persson and Srere 1992), although it is not clear whether these interactions have a function. There have been no attempts to investigate whether

metabolite channelling or metabolon composition changes *in vivo* in response to different metabolic conditions.

### Different TCA cycle flux modes operate in leaves in the light and the dark

The first part of this chapter aims to compare metabolite channelling in mitochondria isolated from tissues where the flux modes in the TCA cycle are different. One condition where the operation of the TCA cycle alters drastically is in leaves during the day and the night. In the majority of heterotrophic tissue and autotrophic tissue in the dark, a cyclic flux mode to support ATP production is thought to be the dominant flux (Steuer *et al.* 2007, Williams *et al.* 2008). In contrast, the status of the TCA cycle in autotrophic tissue during the day has been widely debated. It is known that the TCA cycle is down-regulated in the light (Tcherkez *et al.* 2009) and that several TCA cycle enzymes are partially inhibited, including pyruvate dehydrogenase (Budde and Randall 1990) and succinate dehydrogenase (Popov *et al.* 2009). Proteomic studies have also shown that the levels of the de-carboxylating section of the TCA cycle are decreased in the light (Lee *et al.* 2008).

Despite this, genetic experiments have shown that the TCA cycle has an important role in the light (Carrari *et al.* 2003, Nunes-Nesi *et al.* 2005, Nunes-Nesi *et al.* 2007, Nunes-Nesi *et al.* 2008), although in ways that are not fully understood. Similarly, several parts of the TCA cycle are thought to be necessary in the light, including the production of mitochondrial aspartate, OAA and malate which are needed for metabolite shuttles which are important in transferring excess reductant between the cytosol and mitochondria during the light (Dry *et al.* 1987, Noguchi and Yoshida 2008). Some reactions of the carboxylating side of the TCA cycle are also thought to be

necessary for the production of carbon skeletons for nitrogen assimilation and photorespiration, although their exact contribution is still the subject of some debate (Hodges *et al.* 2003; Foyer *et al.* 2010).

There is considerable evidence that parts of the TCA cycle do operate in illuminated photosynthetic tissues (Figure 5.1). Hanning and Heldt (1993) analysed fluxes through the TCA cycle in spinach leaf mitochondria. Their model suggests that the dominant flux is from malate to citrate, with citrate exported to provide 2OG for nitrogen assimilation. Malate was produced via OAA from PEP (Figure 5.1a). The malate-OAA shuttle was also found to be active and involved in redox balance (a quarter of NADH produced was exported though the shuttle). As a result there was a significant reverse flux from OAA to malate (Figure 5.1b). In contrast the cyclic flux was very low. Isotope labelling experiments in *Xanthium strumarium* also showed that cyclic flux through the TCA cycle was low, with a reduction of 75% compared with the cycle in the dark (Tcherkez *et al.* 2009). However large fluxes were measured from malate (produced by PEPcarboxylase) through to fumarate, and from citrate through to 2OG (Tcherkez *et al.* 2009). There is also evidence that 2OG produced through the TCA cycle during the day has its origin in citrate made in the dark, which is stored and then re-transported back into the mitochondria or the cytosol in the day (Figure 5.1c). Labelling studies in *B. napus* leaves found that the majority of amino acids synthesised during the light used carbon skeletons that had been produced and stored the previous night, while the majority of the N had been added during the day (Gauthier *et al.* 2010). It was not clear whether conversion of the stored citrate to 2OG occurs in the cytosol or the

mitochondria (Sweetlove *et al.* 2010). This would result in a TCA cycle where the only major flux in the light is between OAA and fumarate (Figure 5.1d).

These experiments show that different flux modes exist in the TCA cycle in leaves in the day and the night. It is possible that metabolite channelling is involved in regulating these flux modes. For example, evidence suggests that citrate is exported from the TCA cycle in the dark, where it is stored and then used during the light to produce 2OG. Metabolite channelling could separate citrate into two pools: one which contains citrate which is exported from the TCA cycle for storage in the vacuole, and another which is kept in the mitochondria to allow a complete cyclic flux. In the light, metabolite channelling may be important in separating stored citrate which is used to synthesise 2OG from citrate which is needed to provide the small cyclic flux through the TCA cycle. Similarly, metabolite channelling may be able to separate the proposed reversed fluxes between malate and fumarate and the small cyclic flux in illuminated leaves.

### CCCP and KCN can be used to change the flux through the TCA cycle.

If metabolite channelling is important for regulating the TCA cycle, we might expect it to change under conditions where the rate of the TCA cycle is increased or decreased. One way to change this balance is to change the rate of respiration, for example, by uncoupling the mitochondria. In mitochondria, the rate of the TCA cycle is linked to ATP synthesis through the electron transport chain. In the electron transport chain NADH produced from the TCA cycle is oxidised by complex I, which then reduces ubiquinone to ubiquinol in the membrane. Complex II, which oxidises succinate in the TCA cycle, also reduces ubiquinone. Complex III then reduces cytochrome c, in the

process also re-oxidising ubiquinol. Cytochrome c is re-oxidised by complex IV, which then reduces oxygen to water. This process pumps H+ across the membrane through complex I, III and IV, which is used to synthesise ATP. This mechanism links TCA cycle activity to ATP production, as when the rate of ATP synthasis is reduced, the ratio of NADH:NAD increases and inhibits the TCA cycle. When mitochondria are uncoupled by dissipating the proton gradient, this link between the TCA cycle and ATP synthesis is removed. This can result in a higher flux through the TCA cycle, as flux is no longer limited by the demand for ATP. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) is a protonophore which is acts as an uncoupler (Felle and Bentrup 1977). It works by entering the mitochondrial membrane and allowing protons to cross the lipid bilayer. As a result, it is not possible for the proton electrochemical gradient to be maintained. Smith *et al.* (2004) showed that increasing the levels of uncoupling protein in plants led to an increase in TCA cycle flux between pyruvate and citrate, under conditions where the TCA cycle is normally limited by the coupling of the mitochondria. The paper also showed that CCCP had a similar effect. It has been suggested that activation of uncoupling protein acts as a mechanism to provide carbon skeletons for biosynthesis under conditions where the demand for ATP is also high.

A second way to alter the balance between respiration and biosynthesis is by reducing the rate of respiration through the use of inhibitors. For example, KCN acts as a strong inhibitor of complex IV (Villani and Attardi 2007), and so reduces electron transport and ATP synthesis. This may have the effect of reducing the cyclic flux through the TCA cycle.

## Experimental strategy

The aim of this chapter is to investigate the role that metabolite channelling plays in regulating the TCA cycle. This will be done through two approaches. The first will compare mitochondria isolated from *A. thaliana* seedlings at different stages of the diurnal cycle. This will test whether metabolite channelling changes under conditions where flux modes through the TCA cycle are altered. The second will compare mitochondria where the rate of the TCA cycle has been altered through CCCP and KCN.

# Results

## Investigating metabolite channelling in mitochondria isolated from the light and dark

### *Experimental approach*

Mitochondria were isolated from *A. thaliana* seedlings grown as described in materials and methods. For the dark experiments, plants were removed from the growth chamber 7 h into an 8 h night. These were compared with experiments where plants were removed from the chamber 3 h into the light. In both experiments isolated mitochondria were incubated with 10 mM [3-$^{13}$C]glutamate, 0.3 mM $NAD^+$, 0.2 mM ADP and 0.1 mM TPP, 20 mM glucose and 0.15 U/ml hexokinase. The incubation media also contained 5µM fluorocitrate which inhibits aconitase and so blocks the TCA cycle. The experiment was carried out as described in chapter 4, with unlabelled fumarate or succinate added at 82 min to a concentration of 0.1 mM. Samples taken over the time course were prepared and analysed by GC-MS as described previously.

### *Metabolite channelling is qualitatively the same in mitochondria isolated from the light and the dark*

Isotope dilution experiments were carried out in mitochondria isolated from the light or the dark. The fall in fractional enrichment of malate and citrate after the addition of unlabelled succinate or fumarate was compared. In all experiments, metabolite concentration increased over time and was similar to that shown in chapter 4 (data not shown).

Figures 5.2 and 5.3 show that changes in fractional enrichment of malate and citrate after unlabelled fumarate was added to mitochondria metabolising [3-$^{13}$C]glutamate.

The pattern of metabolite channelling was the same in mitochondria isolated in the light and the dark. In both experiments, malate fractional enrichment fell after fumarate was added (Figure 5.2A), while the citrate fractional enrichment remained constant (Figure 5.3A).

There is some evidence that succinate dehydrogenase is inhibited by light in *A. thaliana*, and labelling experiments suggested that very little flux is carried through SDH in mitochondria in the light (Hanning and Heldt 1993, Tcherkez *et al.* 2009). I therefore decided to test whether metabolite channelling was also different between SDH and fumarase in mitochondria isolated from the light and the dark. As before, the pattern of metabolite channelling was the same in mitochondria isolated from the light and the dark: in both experiments the fractional enrichment of malate (Figure 5.4A) and citrate (Figure 5.5A) fell after the addition of succinate. This shows that the overall pattern of dilution was no different in mitochondria isolated in the light and the dark.

## Investigating metabolite channelling in mitochondria with altered respiration rates

### *CCCP and KCN can be used to alter the relative rates of the TCA cycle*

The amount of CCCP which was required to uncouple the mitochondria, and the concentration of KCN required to reduce the rate of oxygen consumption to 50% of its normal value. This was achieved by measuring the rate of oxygen consumption of mitochondria in state III with increasing concentrations of CCCP or KCN. Mitochondria were incubated with 10 mM malate, 10 mM citrate, 0.3 mM $NAD^+$, 0.2 mM ADP and 0.1 mM TPP to stimulate the TCA cycle. CCCP was added in 0.1 µM increments, and

KCN added in 5 µM increments. In both cases the rate of oxygen consumption was measured. This was done until there was no longer a change in rate (i.e. the mitochondria were fully uncoupled or KCN-dependent respiration fully inhibited). The resulting curves were used to identify the appropriate concentration of CCCP or KCN to add to the mitochondria in the isotope dilution experiments (Figure 5.6). This was done for each replicate and corrected for protein content, to ensure differences in mitochondrial activity were taken into account, and the concentration of CCCP or KCN required for each mitochondrial preparation was calculated. The isotope dilution experiment was then carried out as described above in the presence of the CCCP or KCN.

### *Metabolite channelling is qualitatively the same in mitochondria treated with CCCP, KCN and "normal" mitochondria*

As before, the pattern of metabolite channelling was the same in all replicates. Figure 5.7A shows the fractional enrichment in mitochondria when CCCP was added (to a final concentration 0.6µM/mg protein and 0.5µM/mg protein for rep1 and rep2 respectively). In both replicates, malate fractional enrichment fell while citrate remained constant. This was the same pattern seen in mitochondria which were not treated with CCCP (Figure 5.7B). This was also the case in mitochondria treated with KCN (Figure 5.8A): the pattern of metabolite channelling was the same as untreated mitochondria (Figure 5.8B). The final concentration of KCN was 7µM/mg protein and 8µM/mg protein for rep1 and rep2 respectively.

### It is not possible to draw conclusions about the quantitative changes in metabolite channelling in the TCA cycle

Differences in metabolite channelling (such as the proportion of enzymes in the channel) might be reflected in changes in the dilution of malate. However, in all the experiments it is difficult to draw conclusions about this, as the fractional enrichment of malate differed greatly between replicates in the same experiment. This is particularly clear in the experiment where fumarate was added to the mitochondria in the light, where there was very large difference between the fractional enrichment of malate for the two replicates (Figure 5.2). The large difference between replicates means that it is difficult to draw any conclusions as to the quantitative difference in metabolite channelling between the two experiments. The difference between replicates was smaller when succinate was added (Figure 5.4), but there is still considerable variability, particularly later in the time course. Similarly, there was a very large difference between the dilution of malate in mitochondria that had been treated with KCN (Figure 5.8), although the differences between enrichment in mitochondria treated with CCCP was smaller. This means that it is difficult to state with any confidence whether the dilution is quantitatively different between mitochondria isolated in the light and the dark, and between mitochondria treated with CCCP and KCN.

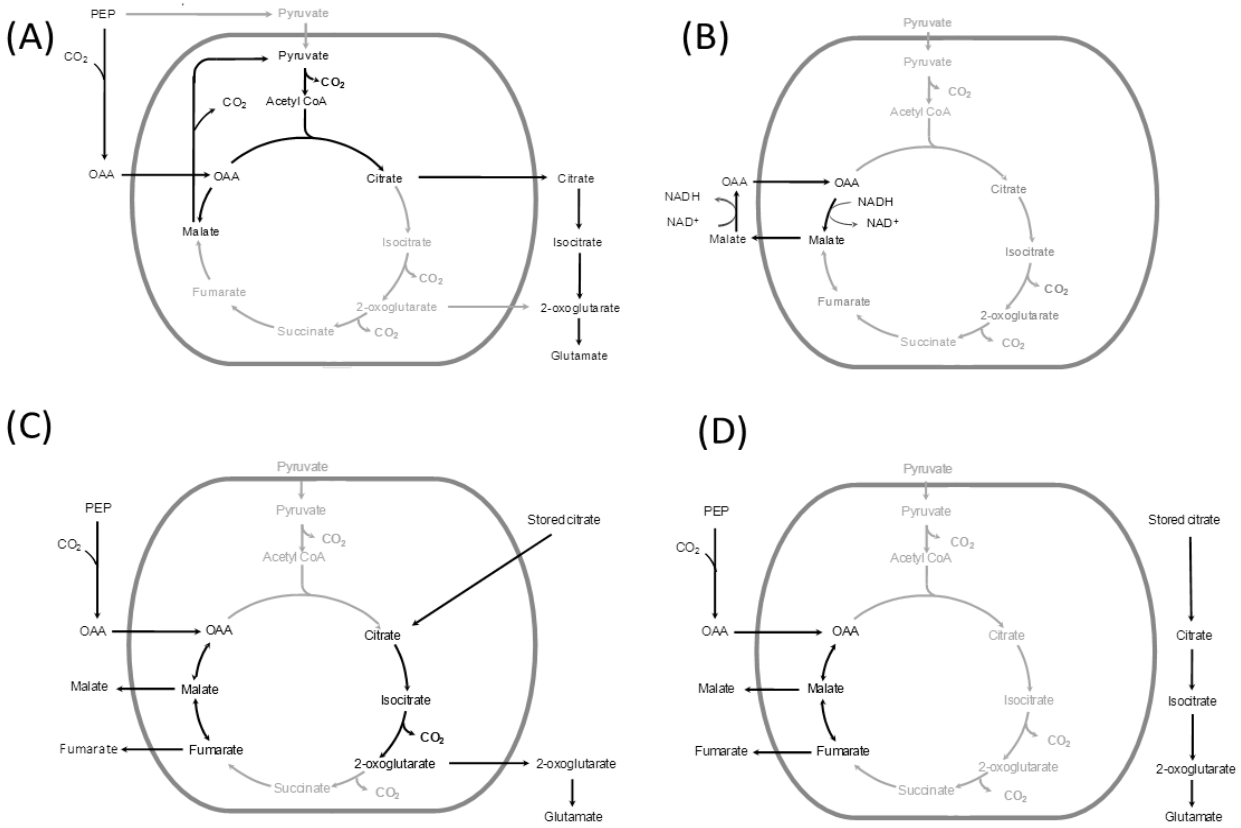


**Figure 5.1**: **Proposed TCA cycle flux modes in leaf mitochondria in the light**. Major fluxes are shown in black, while lesser ones are shown in grey. (a) non-cyclic flux mode and (b) the malate-OAA shuttle, which were both proposed to be active by Hanning and Heldt (1993); (c) the flux mode through the TCA cycle proposed by Tcherkez *et al.* 2009 ; (d) the non-cyclic flux mode that would be active if stored citrate was metabolised through cytosolic enzymes (Sweetlove *et al.* 2010).

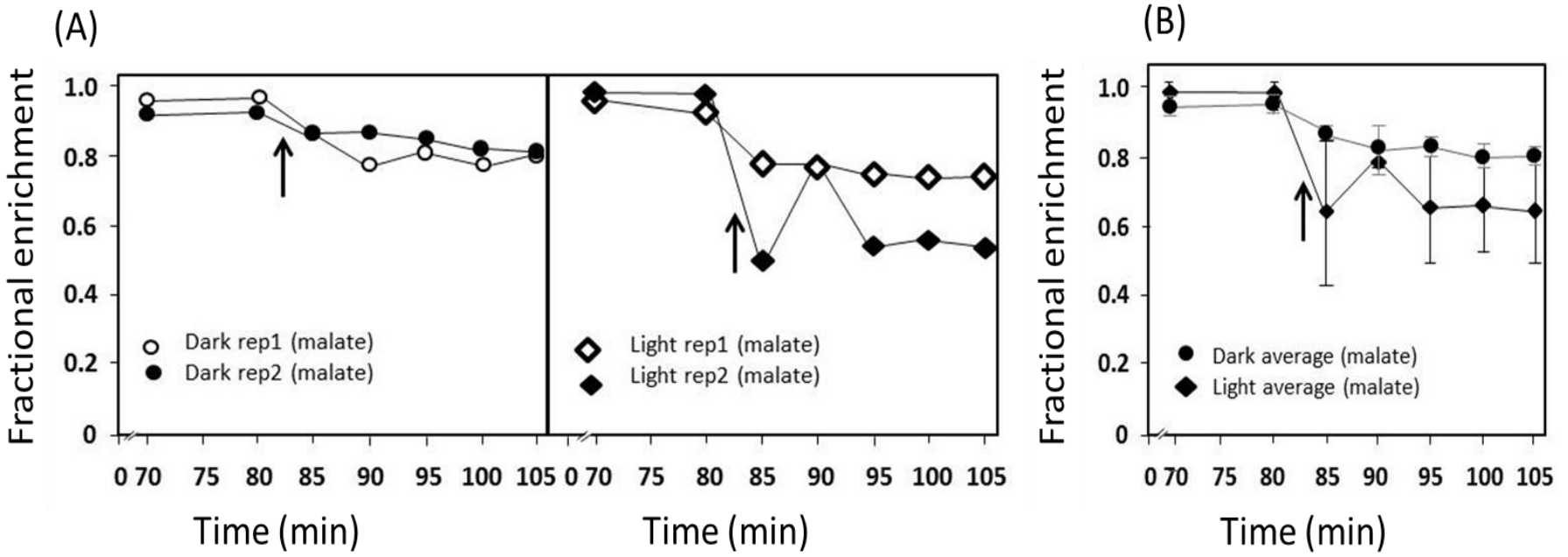


**Figure 5.2: Effect of addition of unlabelled fumarate on the fractional enrichment of malate in *A. thaliana* mitochondria isolated in the night compared with mitochondria isolated in the light.** (A) fractional enrichment of malate pool and (B) comparison of the average fractional enrichment of malate pool. Arrow denotes addition of 0.1mM unlabelled fumarate. Mitochondria were incubated with 10 mM [3-$^{13}$C]glutamate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP, 20 mM glucose , 0.15 U/ml hexokinase and 5 µM fluorocitrate to block the TCA cycle at aconitase

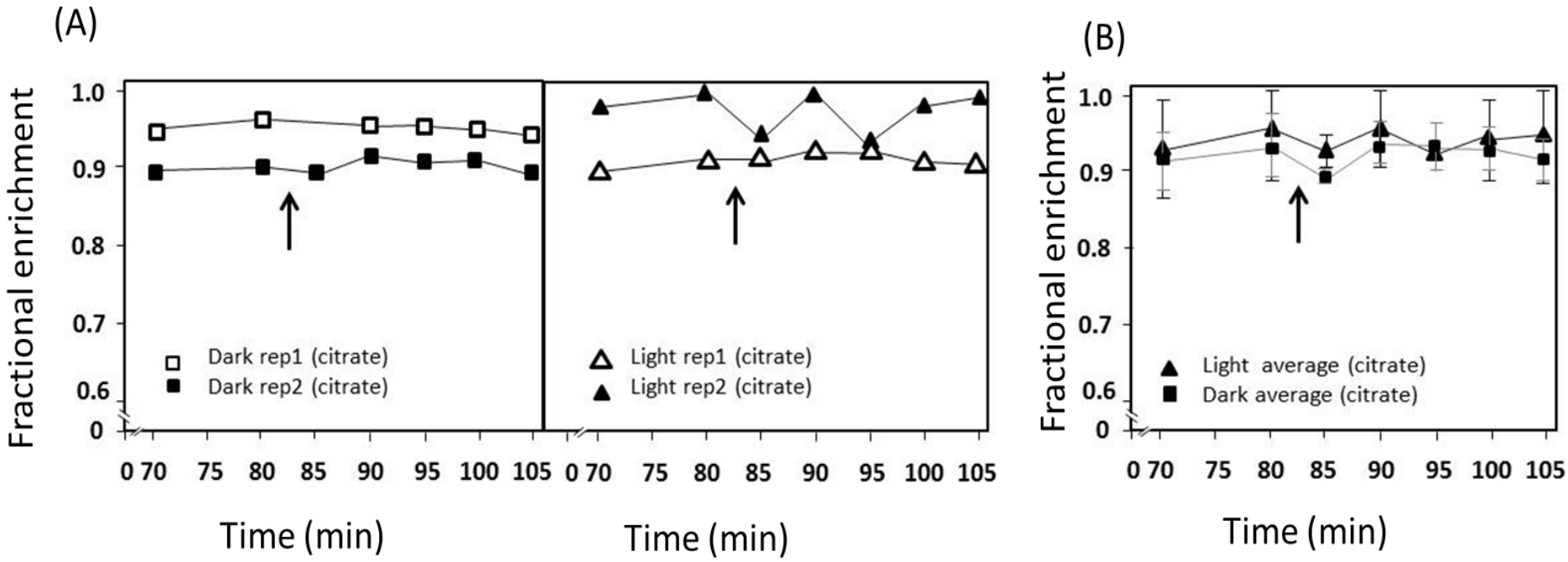


**Figure 5.3: Effect of addition of unlabelled fumarate on the fractional enrichment of citrate in *A. thaliana* mitochondria isolated in the night compared with mitochondria isolated in the light.** (A) fractional enrichment of malate pool and (B) comparison of the average fractional enrichment of malate pool. Arrow denotes addition of 0.1mM unlabelled fumarate. Mitochondria were incubated with 10 mM [3-$^{13}$C]glutamate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP, 20 mM glucose , 0.15 U/ml hexokinase and 5 µM fluorocitrate to block the TCA cycle at aconitase

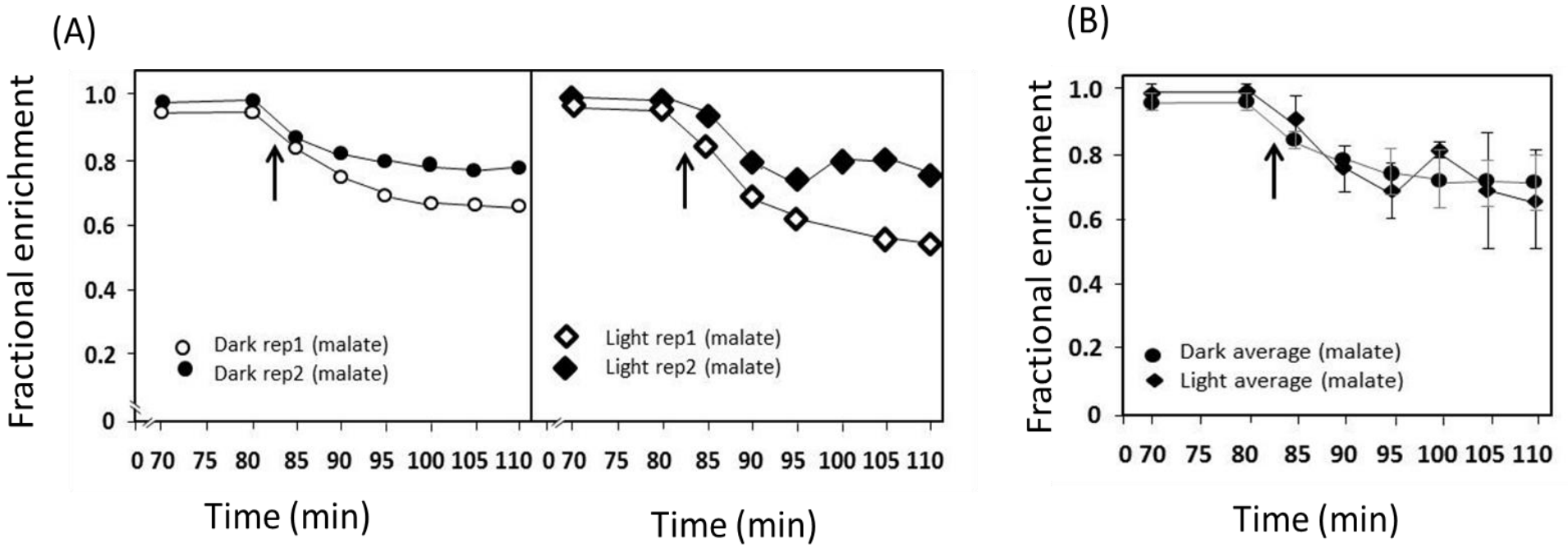


**Figure 5.4: Effect of addition of unlabelled succinate on the fractional enrichment of malate in *A. thaliana* mitochondria isolated in the night compared with mitochondria isolated in the light.** (A) fractional enrichment of malate pool and (B) comparison of the average fractional enrichment of malate pool. Arrow denotes addition of 0.1mM unlabelled succinate. Mitochondria were incubated with 10 mM [3-$^{13}$C]glutamate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP, 20 mM glucose , 0.15 U/ml hexokinase and 5 µM fluorocitrate to block the TCA cycle at aconitase

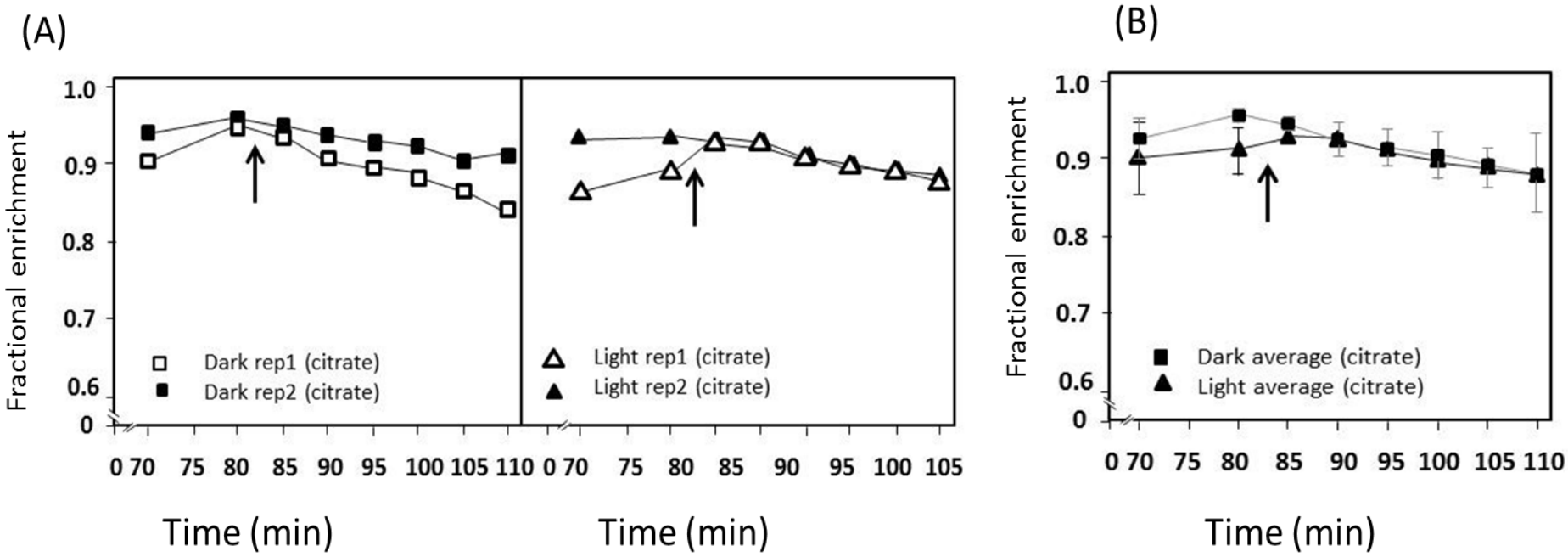


**Figure 5.5: Effect of addition of unlabelled succinate on the fractional enrichment of citrate in *A. thaliana* mitochondria isolated in the night compared with mitochondria isolated in the light.** (A) fractional enrichment of malate pool and (B) comparison of the average fractional enrichment of malate pool. Arrow denotes addition of 0.1mM unlabelled succinate. Mitochondria were incubated with 10 mM [3-$^{13}$C]glutamate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP, 20 mM glucose , 0.15 U/ml hexokinase and 5 µM fluorocitrate to block the TCA cycle at aconitase

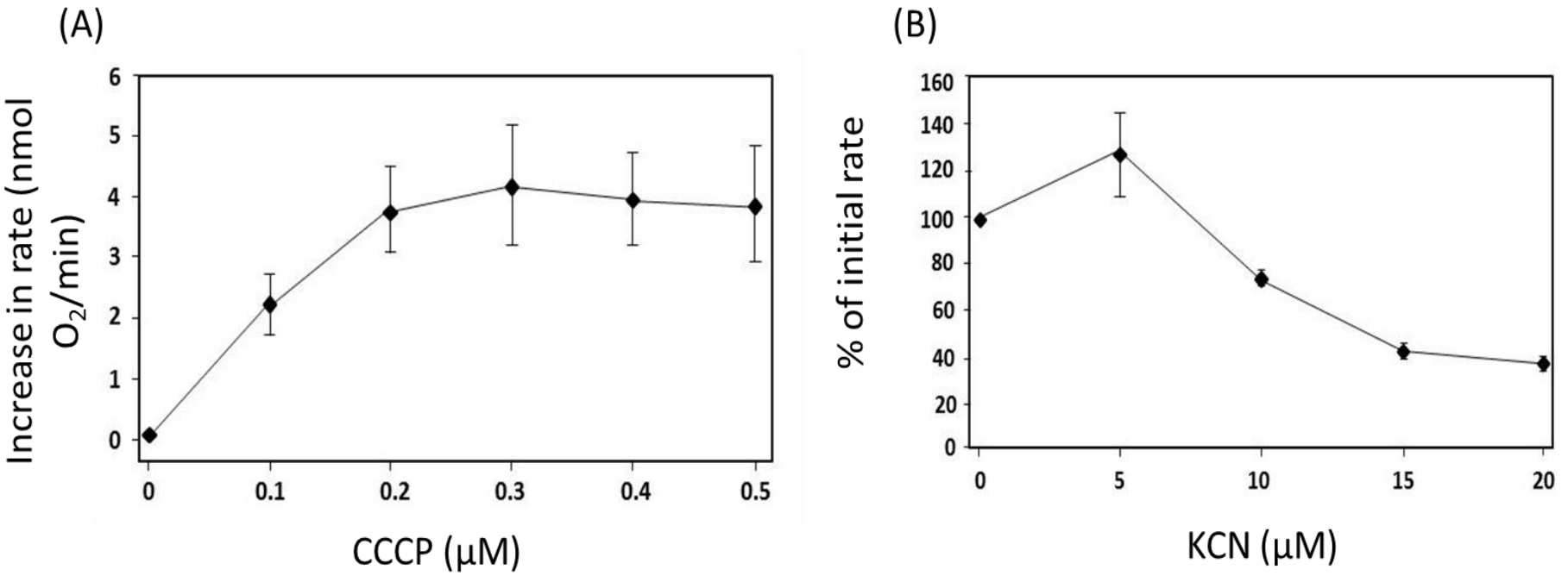


**Figure 5.6: Effect of CCCP and KCN on the rate of oxygen consumption of *A. thaliana* mitochondria.** (A) Average increase in rate (nmol $O_2$ $min^{-1}$) of mitochondria treated with increasing concentrations of CCCP. B) Average % of initial rate of mitochondria treated with increasing concentration of KCN. The starting rate was treated as 100%, at 40% subsequent additions of KCN had no effect on the rate. In both cases *n*=3.

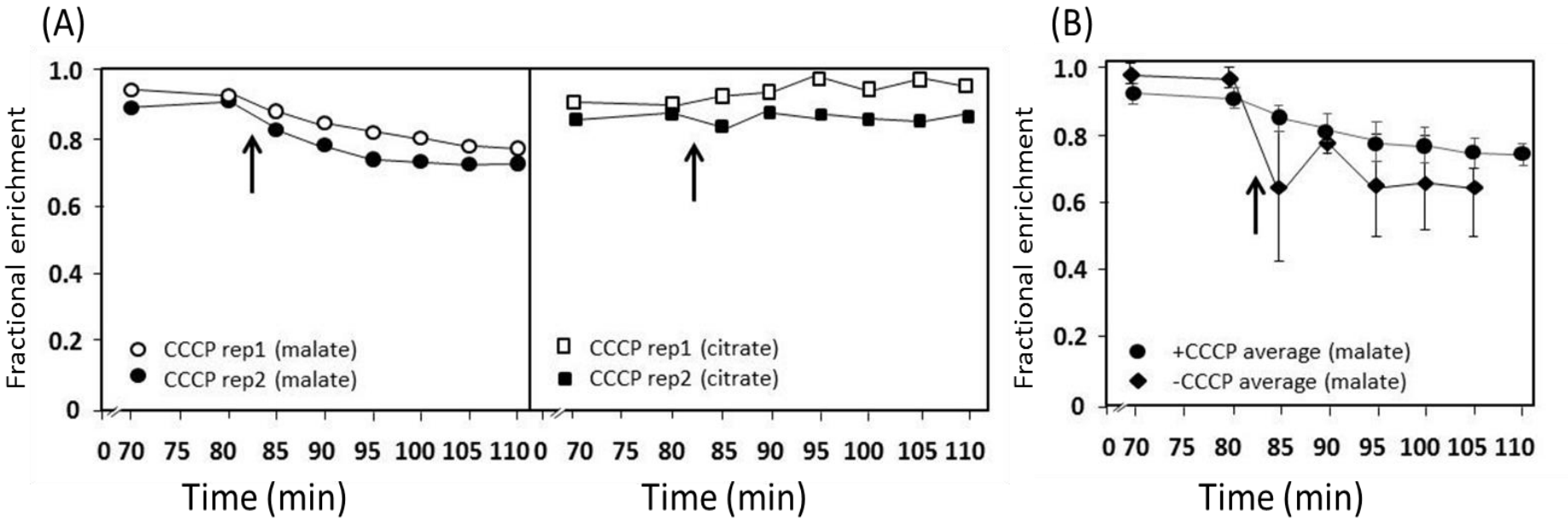


**Figure 5.7: Effect of addition of unlabelled fumarate on the fractional enrichment of malate in *A. thaliana* mitochondria in the presence of CCCP.** (A) Fractional enrichment of malate and citrate pool and (B) comparison of the average fractional enrichment of malate pool in untreated mitochondria and mitochondria treated with CCCP. Arrow denotes addition of 0.1mM unlabelled fumarate. Mitochondria were incubated with 10 mM [3-$^{13}$C]glutamate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP, 20 mM glucose , 0.15 U/ml hexokinase and 5 µM fluorocitrate to block the TCA cycle at aconitase. The final concentration of CCCP was 0.6µM/mg protein and 0.5µM/mg protein for rep1 and rep2 respectively.

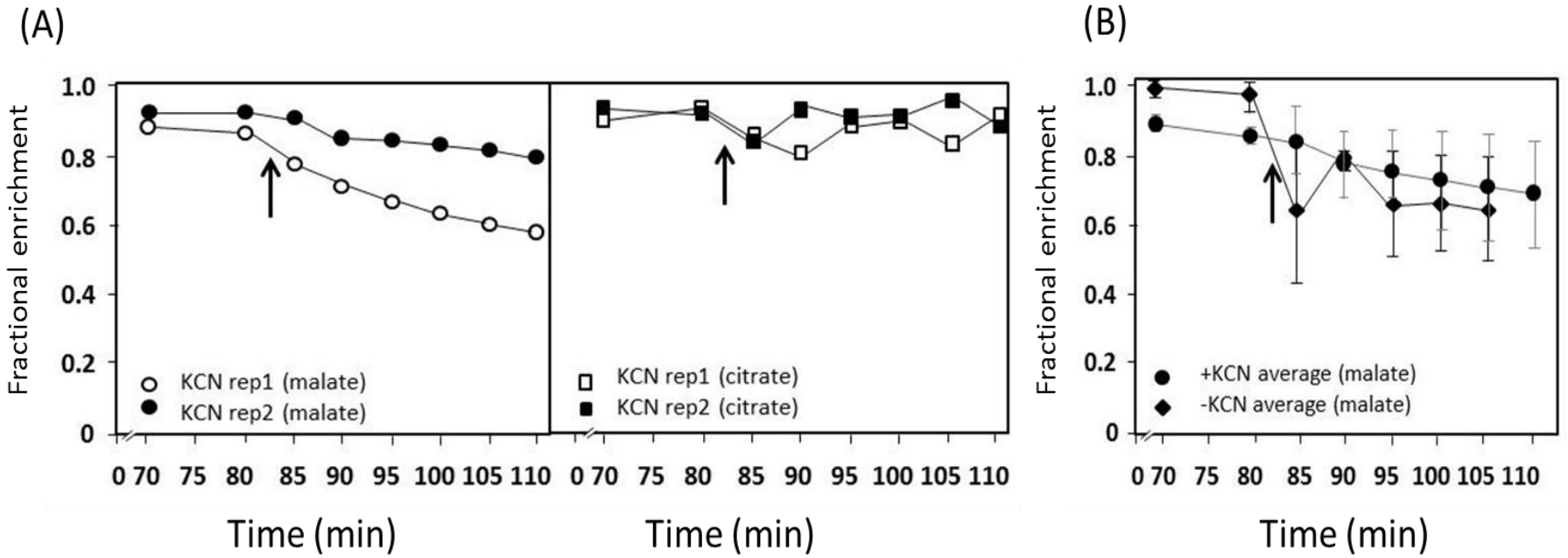


**Figure 5.8: Effect of addition of unlabelled fumarate on the fractional enrichment of malate in *A. thaliana* mitochondria in the presence of KCN.** (A) Fractional enrichment of malate and citrate pool and (B) comparison of the average fractional enrichment of malate pool in untreated mitochondria and mitochondria treated with KCN. Arrow denotes addition of 0.1mM unlabelled fumarate. Mitochondria were incubated with 10 mM [3-$^{13}$C]glutamate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP, 20 mM glucose , 0.15 U/ml hexokinase and 5 µM fluorocitrate to block the TCA cycle at aconitase. The final concentration of KCN was 7µM/mg protein and 8µM/mg protein for rep1 and rep2 respectively.

## Discussion

It is clear that some aspects of metabolite channelling remain unchanged by treatments. In all the experiments, the pattern of metabolite channelling remained the same: i.e. unlabelled fumarate was converted into malate but not into citrate, and this did not change in mitochondria isolated from the dark or the light, or in mitochondria with increased or decreased rates of the TCA cycle. Similarly, unlabelled succinate had access to all enzymes of the TCA cycle, regardless of whether the mitochondria were isolated from the light or the dark. One way that metabolite channelling is thought to regulate metabolic pathways involves changes in the composition of metabolons, to direct flux through one pathway or another. The fact that the pattern of metabolite channelling remains the same across the experiments suggest that this does not occur in the TCA cycle in the conditions tested.

### It is unclear whether the proportion of enzymes involved in metabolite channelling changes in response to changes in the TCA cycle

A different way that metabolite channelling might change is that the proportion of enzymes in the metabolon might change. This could result in a change in the dilution of TCA cycle intermediates. For example, if fewer enzymes were involved in channelling metabolites, then we might expect the dilution of malate to be greater, as unlabelled fumarate would have access to more enzymes of the TCA cycle. However, it was not possible to tell from the results whether this occurred, as the variation between fractional enrichments was very large, and as a result masked any differences between the treatments. Thus it is not possible to tell

whether the proportion of enzymes which are present in a TCA cycle metabolon in the mitochondria changed under different treatments. This means that only limited conclusions can be drawn about metabolite channelling in the TCA cycle under different conditions.

### It may not be possible to detect changes in metabolite channelling using this method

It is important to consider the limitations of this method for detecting changes in metabolite channelling in the TCA cycle. For example, it is possible that the metabolon composition does change *in vivo* in response to changes in conditions, but that it could not be detected by this experimental method. One possibility is that the experimental conditions did not provide the correct environment for changes in metabolite channelling to occur. For example, the same concentrations of substrates and co-factors were used for the comparison of metabolite channelling in mitochondria isolated from plants in the day and the night. *In vivo*, these would be likely to be very different. For example, there is evidence that NADH levels are higher in the light (Hanning and Heldt 1993) and that glutamate is produced rather than consumed (Tcherkez *et al.* 2009). In addition the use of high substrate concentration was necessary to measure changes in the fractional enrichment of organic acids, but the concentration is higher than would be expected in the plant cell. The result of this is that the conditions of the experiment might be constraining the system to behave in a certain way, and this might mask or prevent any changes in metabolite channelling.

Another complicating factor is that mitochondria were isolated from *A. thaliana* seedlings, which are made up from several tissue types. Although most of the mitochondria will be from cotyledons, there will also be mitochondria from root and shoot tissue. There is evidence to suggest that fluxes though the TCA cycle are different in different tissues (see general introduction and Schwender *et al.* 2006, Alonso *et al.* 2007, Allen *et al.* 2009, Rocha *et al.* 2010) and as a result metabolite channelling might also be different. Changes in metabolite channelling in mitochondria from photosynthetic tissue might be too small to detect once the contribution from the other tissues have been added.

Another factor which is important is the extent to which changes in the metabolon are lost on isolation. Metabolons vary in their stability, from very stable (such as tryptophan synthase (Hyde *et al.* 1998)) to more transient metabolons which change in response to changes metabolic state such as the glycolytic metabolon (Graham *et al.* 2007). It has been proposed that the TCA cycle metabolon is likely to be dynamic and therefore stabilised by relatively weak forces (Ovadi and Srere 2000). While this instability could be useful in facilitating changes in metabolite channelling in response to changes in metabolic conditions, it also means that it is likely to be susceptible to disruption during mitochondrial isolation. In addition, metabolite channelling may require the presence of small molecules or ligands to stabilise the complex in a certain configuration, and these could be lost during isolation (it is already known, for example, that the majority of organic acids leak out of the mitochondria during the isolation process). The difficulty of isolating intact metabolons of TCA cycle

enzymes (Robinson and Srere 1985) supports that idea that the interactions are unstable outside the cellular environment.

This could also explain why no difference in metabolite channelling was seen when mitochondria were treated with KCN or CCCP. Metabolite channelling may be able to change in response to changes in the rate of respiration, but it may only be able to occur before isolation, particularly if changes in metabolite channelling require small molecules or ligands to stabilise the metabolon. In addition, TCA cycle enzymes are nuclear encoded and changes in metabolon formation may require new enzymes to be made. If the mitochondria are treated after they have been isolated from the cell, then any changes which would normally happen *in vivo* may not occur.

### In order to understand the role metabolite channelling plays it might be necessary to use a different method

The limitations of the methods used in the chapter mean that it may be necessary to use a different method to uncover the role of metabolite channelling in the TCA cycle. One possibility would be to try and match the experimental conditions to those experienced by the mitochondria in the cell. However this presents considerable technical challenges. It is difficult to measure fluxes and concentrations in different cellular compartments (Wahrheit *et al.* 2011), and there are considerable gaps in our understanding of how conditions and fluxes differ between mitochondria in different tissues and cells (Sweetlove *et al.* 2010). As a result it is difficult to replicate cellular conditions *in vitro*.

A different approach would be to try and measure metabolite channelling *in vivo,* and compare how it changes in different tissues or under different conditions. One way to do this would be through modelling approaches such as those described in Williams *et al.* (2011). Recent improvements in modelling reactions and fluxes through different compartments and at the individual cell level (Wahrheit *et al.* 2011, Kovarik and Allbritton 2011) may make this easier.

Understanding more about the structure of the TCA cycle metabolon may also help elucidate any role that it has in regulating the TCA cycle. For example, if there were differences in the composition or interaction strength of the metabolon under different metabolic conditions, this might suggest metabolite channelling is involved in regulating fluxes through the TCA cycle. Similarly, understanding what regulates the composition of the metabolon might provide some clues as to the role of metabolite channelling in the TCA cycle. For example, if the metabolon interacted with a transporter, this could suggest it was involved in regulating the metabolism of the transported metabolite (McKenna *et al.* 2000). There is some evidence citrate synthase binds with a citrate transporter (Persson and Srere 1992) but this has not been shown in plants. Currently very little is known about what regulates the interactions of the TCA cycle enzymes, although there is some evidence that interaction with the membrane may be required for stability (Robinson and Srere 1985, Srere 2000).

### Metabolite channelling may regulate the TCA cycle under conditions other than those tested

It is also possible that metabolite channelling is involved in regulating other processes than those investigated in this chapter. In theory, any metabolic processes where there are competing demands on the TCA cycle may be regulated by metabolite channelling. For example, amino acid synthesis requires carbon skeletons from the TCA cycle at various points, including 2OG to make glutamate, and OAA to make aspartate (Mifflin and Lea 1977). Such a demand could occur both in photosynthetic tissues in the light, and in heterotrophic tissues where there is also a strong requirement for a cyclic TCA cycle (Williams *et al.* 2008, Steuer *et al.* 2007). In these conditions, metabolite channelling may be important in ensuring that some of the carbon skeletons remain in the mitochondria despite the high activity of the transporters to remove carbon skeletons.

Metabolite channelling may also change in response to changes in the concentration or type of external substrate. There is some evidence for this in neural astrocytes in mammals, where the interaction of TCA cycle enzyme with the membrane is thought to regulate glutamate metabolism (McKenna *et al.* 2000). Glutamate that enters astrocyte mitochondria has two main origins: from glutamine, which is converted to glutamate by the enzyme glutaminase in the inner mitochondrial membrane (Roberg *et al.* 1995), or glutamate that is taken up from the synaptic cleft, and which enters the mitochondria through the glutamate aspartate translocator. There is evidence to show that glutamate is metabolised differently, depending on its origin (Schousboe *et al*. 1993,

Westergaard *et al.* 1996). Glutamate that is taken up directly from outside the mitochondria is metabolised rapidly through GDH and the TCA cycle. This is necessary to rapidly remove glutamate from the synaptic cleft and facilitate normal neuronal function (McKenna *et al.* 2000). In contrast, glutamate made from glutamine is primarily oxidised for energy in astrocytes via aspartate aminotransferase (AAT) (McKenna *et al.* 1993). There is evidence that complexes of enzymes interacting with the membrane help to regulate this process. Both AAT and MDH, in a complex with CS and 2OGDH, can bind the inner mitochondrial membrane (AAT via a binding protein that transfer it onto lipids, and MDH with complex I) (Teller *et al.* 1990). However, AAT and MDH cannot bind to the membrane at the same time, as the binding of AAT inactivates the binding of MDH (Teller *et al.* 1990). It has been suggested that this binding helps to channel glutamate either into the TCA cycle, or through AAT, depending on its origin and the metabolic state of the mitochondria. When the glutamate concentration in the synapse is high, MDH binds to the membrane and facilitates its metabolism through the TCA cycle. When exogenous glutamate levels are lower, AAT could bind to the membrane and promote the oxidation of glutamate from glutamine for energy (McKenna *et al.* 2000). This model explains how the metabolism of glutamate from different sources can be regulated. It also shows that the role of metabolite channelling in regulating the TCA cycle may be complex, and depend on the activity of transporters and source of substrate, as well as the state of the mitochondria.

## Metabolite channelling may not be important in regulating the TCA cycle

It is also conceivable that metabolite channelling may not be important in regulating the TCA cycle at all. Instead metabolite channelling could be an inevitable outcome of the crowded environment of the mitochondrial matrix, be important in freeing up space in the matrix to facilitate more rapid diffusion of small molecules, or be important in increasing the rate of reaction through the TCA cycle at lower free substrate concentrations. This has been discussed in more detail in the general introduction. The main argument against this proposal is that metabolite channelling has been found to be important in regulating flux in a great number of the pathways where it has been identified.

## Summary

The aim of this chapter was to investigate whether metabolite channelling changed in mitochondria in the light and the dark, and in mitochondria where the rate of the TCA cycle was altered. In fact the pattern of metabolite channelling did not alter under different treatments, suggesting that the composition of the putative TCA cycle metabolon did not change. This could be because metabolite channelling does not change under these conditions, or because to the method was not sufficiently sensitive to detect the changes. It was also not possible to draw any conclusions as to whether the proportion of enzymes involved in metabolite channelling changed under different treatments, as the variation between the replicates was too great. These results suggest that a different approach might be needed to investigate the role of metabolite channelling in the TCA cycle more fully.

# Chapter 6: Investigating orientation conserved transfer of label in the plant TCA cycle

## Introduction

Previous chapters have shown that metabolic channelling of TCA cycle metabolites exists in both *S. tuberosum* and *A. thaliana*. The aim of this chapter is to further investigate metabolic channelling in the plant TCA cycle using a method which investigates whether the position of labelled carbons is conserved within symmetrical molecules (orientation conserved transfer, or OCT). This method has previously been used to investigate metabolic channelling in several eukaryotes, including *S. cerevisiae* (Sumegi *et al.* 1993) and rats (Sherry *et al.* 1994), and so will allow a direct comparison of metabolic channelling between plants and other organisms with a channelled TCA cycle

### Orientation conserved transfer: a special case of metabolic channelling

This method works on the assumption that metabolic channelling will restrict the rotation of molecules as they are passed between enzymes. It takes advantage of the fact that two sequential TCA cycle intermediates, succinate and fumarate, are symmetrical. Labelled glutamate is converted into 2OG, which is unequally labelled. It is then converted into succinate via 2OGDH and succinyl CoA ligase. Because succinate is symmetrical it is able to enter the active site of succinate dehydrogenase in either the left-right or right-left configuration (Figure 5.1). This means that the C2 in succinate can become either the C2 or C3 in fumarate. Fumarate is also a symmetrical molecule, meaning that the C2 in fumarate can also become the C2 or C3 in malate. As a result, if C2 in succinate is labelled the

label should become randomised between C2 and C3 in fumarate and in malate. In this case, the ratio of label in malate C2 and C3 should be equal.

In order for this randomisation of label to occur, succinate and fumarate must be free to rotate between the active sites of succinyl CoA ligase and succinate dehydrogenase, and succinate dehydrogenase and fumarase respectively. This is easily achieved if the substrates diffuse freely between the enzymes. However, metabolic channelling may restrict this diffusion, which in turn is likely to restrict the ability of the molecules to rotate. In this case, the orientation of the molecules is more likely to be conserved. As a result, the C2 of succinate is more likely to become the C2 of fumarate than the C3, and the C2 of fumarate is more likely to become the C2 of malate than the C3. As a result, if C2 in succinate is labelled using $^{13}C$ the labelling pattern would be conserved, and more C2 than C3 would be labelled in fumarate and malate (Figure 5.1).

In a completely unchannelled system, we would expect the ratio of C2/C3 to be 1, as the molecules are free to rotate between enzymes. A pathway which was fully channelled and where rotation was completely restricted would see no labelling in C3 and the ratio would tend towards infinity, or 1/0. An intermediate ratio suggests that a proportion of the molecules are being channelled.

There have been several attempts to investigate metabolic channelling between succinate dehydrogenase and fumarase using this method (Figure 5.2). Sumegi *et al.* (1990) investigated labelling in alanine in *S. cerevisiae* cells which were fed with [3-$^{13}C$]propionate. They found that when [3-$^{13}C$] propionate was used as a substrate, the ratio of label in C3/C2 was 2.35. A similar result was found when

*S. cerevisiae* was fed with [4-$^{13}$C]glutamate. In this case, the ratio of label in C2/C3 of aspartate, which is produced via succinate and fumarate, was 1.78 (Sumegi *et al.* 1993). Unequal labelling of TCA cycle intermediates has also been shown to occur in perfused rat liver (Sherry *et al.* 1994). Carbon from [2-$^{13}$C]propionate was preferentially incorporated in the C2 of lactate, and that C3 was preferentially labelled when [3-$^{13}$C]propionate was used as the substrate, although the average ratio of C2/C3 when [2-$^{13}$C]propionate used as a substrate was lower than in yeast, at 1.32. In a similar experiment, labelling in lactate from 2-$^{13}$C propionate in islet cells from rats was measured (Malaisse *et al.* 1996). The average enrichment of C2/C3 was 1.47, suggesting that metabolic channelling also occurs in this system. When the same experiment was done in colon carcinoma cells the average C2/C3 in lactate was lower, at 1.18 although the C2/C3 in lactate which was released into the incubation medium was higher at 1.53 (Malaisse *et al.* 1996 b). Together these results suggest that the rotation of succinate and fumarate is restricted in both yeast and mammalian cells. The best explanation for this is that the molecules are being channelled between succinate dehydrogenase and fumarase and between succinyl coA ligase and succinate dehydrogenase.

## Orientation conserved transfer experiments can be used to compare the extent of metabolic channelling across different species.

Previous chapters used an isotope dilution approach to demonstrate that metabolic channelling exists between succinate dehydrogenase and fumarase in both *S. tuberosum* and *A. thaliana*. There have been no attempts to use isotope dilution to measure metabolic channelling in the TCA cycle in other organisms:

the best direct evidence for metabolic channelling comes from orientation conserved transfer (OCT) experiments. By repeating these experiments in plants we will be able to directly compare metabolic channelling between different species. OCT experiments provide quantitative information on the proportion of the TCA cycle which is channelled. A large C2/C3 ratio of malate or aspartate, which are made from fumarate and succinate, demonstrates that a greater degree of metabolite channelling, while a smaller ratio suggests a lower proportion. As a result, comparing the ratio allows us to compare the proportion of channelled and unchannelled enzymes in the different species.

## Orientation conserved transfer experiments can be used to make inferences about the mechanism of metabolic channelling in the plant TCA cycle.

A further factor which affects the ratio of C2/C3 is how metabolic channelling is achieved. OCT experiments assume that metabolic channelling will restrict the rotation of molecules. However, there are some ways that metabolic channelling could be achieved while still allowing the molecules to rotate between enzymes. The first is that enzymes interact physically with each other to form a complex, called a metabolon (Srere 1985). Metabolic intermediates are transferred directly between active sites within the complex, without diffusing into the bulk phase. For example, tryptophan synthase catalyses the final two steps in L-tryptophan synthesis. The two reactions are catalysed by separate alpha and beta subunits which combine to form a stable multi-enzyme complex (Hyde *et al.* 1988). During synthesis, indole (the intermediate) is transferred between the two active sites through a hydrophobic tunnel within the enzyme complex, which

is long enough to accommodate four indole molecules simultaneously (Hyde *et al.* 1988). A similar system occurs in carbamoyl phosphatate synthase (CPS) complex, which catalyses four steps in the synthesis of carbamoyl phosphate (Anderson and Meister 1966). Here ammonia and carbomate are transferred between the three active sites of the complex through a hydrophobic tunnel (Thoden *et al.* 1997, Raushel *et al.* 1998). In both cases the tunnels are narrow (~3.3A in the case of CPS) (Raushel *et al.* 1998) and probably require the binding of ligands or substrates in order to function (Miles *et al.* 1999). In these cases the rotation of molecules could be restricted, as it is likely that the tunnel would be too small to allow the intermediate to rotate. In order to travel between active sites, the molecule may also need to travel in a specific conformation, which would affect rotation.

However, there are some situations where intermediates are not able to diffuse into the bulk phase, but may still be able to rotate. The best example is dihydrofolate reductase, a complex of two enzymes: thymidilate synthase (TS) and dihydrofolate reductase (DHFR). In protozoa and some plants, this enzyme consists of a dimer of two proteins (TS and DHFR) connected by a short linker sequence (Ferone and Roland 1980). It is known that the protein channels the $H_2$folate intermediate between the two enzymes (Knighton *et al.* 1994). However, the active sites of TS and DHFR are 40 Å apart and the protein cannot undergo conformational changes to bring them closer together. Instead, it is thought that the intermediate molecule 'tracks' along the surface of the enzyme from one active site to the next (Knighton *et al.* 1994, Stroud 1994). Analysis of the surface of the complex shows a track of positively charged residues in a

generally negatively charged protein surface (Knighton *et al.* 1994). This forms an "electrostatic highway" to link the two sites, along which the negatively charged folate molecule moves by a combination of attraction and repulsion (Stroud 1994). In this situation, it could be possible for the molecule to rotate during the transfer between active sites, but not to diffuse away from the surface of the protein.

Another way that metabolic channelling could occur would be if the enzymes were immobilised on a surface in the cell, for example through being anchored to a membrane (Srere *et al.* 1973). A property of fluids is the presence of an unstirred layer at the interface between a surface (such as a membrane) and a solution (Barry and Diamond 1984). This in turn limits the movement of molecules located near the membrane: molecules can become "trapped" in the unstirred layer. This is known to have an effect on reactions which take place on a membrane. A theoretical study (Goldman and Katchalski 1971) compared the behaviour of two sequential enzymes which were attached to a membrane to the same enzymes which were free in solution. They found that when they assumed that an unstirred layer existed which limited diffusion, the product of enzyme one was more likely to be consumed by the second enzyme than escape into the bulk solution: i.e. the reaction was channelled. The result of this was that the rate of production of the end product was much higher in the immobilised system than with free enzymes. This effect has been shown to be important in several metabolic pathways (Conrado *et al.* 2008). The artificial immobilisation of enzymes onto membranes or protein scaffolds has been used

to provide control over flux through pathways in cells (Dueber *et al.* 2009), and is an important target for metabolic engineering (Lee *et al.* 2012). It is possible that this type of metabolic channelling exists in the TCA cycle, as there is evidence that some enzymes associate with the inner mitochondrial membrane. Srere *et al.* (1973) showed that metabolic channelling could be observed between malate dehydrogenase and citrate synthase when they were immobilised in gels and that this increased the rate 100 times more than the equivalent free enzyme system. Several studies have also found that TCA cycle enzymes associate with the inner mitochondrial membrane in *S. cerevisiae* (D'Souze and Srere 1983, Robinson and Srere 1985). There is also evidence that MDH can interact with the cytochrome bc (1) complex (complex III) in bovine mitochondria (Wang *et al.* 2010). Although metabolic channelling can occur by this method, there is nothing to suggest that the rotation of the molecules would be restricted during diffusion between enzymes.

## Experimental strategy

NMR is a well-established technique used for analysing flux through metabolic pathways (Ratcliffe 1994), and can be used to measure labelled carbons in metabolites. NMR is the most appropriate choice for OCT experiments as it is a technique which is able to distinguish between labelled carbons within a molecule. NMR is only capable of measuring nuclei which have a magnetic moment, or a spin number. This means that it will only measure labelled carbons, and unlabelled carbons will not be measured. NMR is also able to distinguish between different labelled carbon nuclei within a molecule. This is because the chemical shift of the carbon can be influenced by its molecular

environment. Unless the molecule is completely symmetrical, individual carbons will experience different magnetic fields as a result of their position within the molecule, and as a result will have a different chemical shifts. It is therefore possible to measure the relative labelling in different carbons within a molecule.

In order to measure OCT successfully, it is necessary to ensure that the metabolite being investigated cannot be made through an alternative pathway which does not use succinate or fumarate, as this could produce an additional labelled signal which would interfere with the accuracy of the results (Evans *et al.* 1993). The more cell and tissue types present in an experimental system the more likely it is that alternative metabolic pathways will be operating. For this reason the experiments will use isolated mitochondria, where the supplied substrate can only be converted into malate via the TCA cycle. Previous work has shown that it is possible to measure labelling in TCA cycle acids in isolated mitochondria when they are supplied with labelled pyruvate (Smith *et al.* 2004) or glutamate (Aubert *et al.* 2001). In both cases it was possible to distinguish between the carbons of most TCA cycle intermediates. It is also important to make sure that label is not randomised as the molecules are metabolised around several turns of the cycle. In this case, if there is incomplete metabolic channelling, malate metabolism will cause label to be present in 2OG, produced through the TCA cycle as well as the label in 2OG that comes from C3 glutamate that is being fed into the mitochondria. This means that any difference in the labelling in C2 and C3 will eventually be lost. This can be prevented by using inhibitors which block the cycle, or by using a very short time frame. Sumegi *et*

*al.* (1990) used fluorocitrate, which is metabolised in the mitochondria to mono fluorocitrate, binds irreversibly to aconitase and inhibits its activity (Elliott and Kalnitsky 1950). Fluorocitrate has been shown to fully inhibit aconitase in isolated plant mitochondria (Smith *et al.* 2004). This both eliminates any scrambling of label, and also has the advantage that it allows large quantities of malate to build up in the medium. This increases the accuracy with which we can measure the labelling in the different carbons.

# Results

## Method development

### *NMR can be used to measure organic acid production in isolated mitochondria*

Isolated mitochondria from *S. tuberosum* were incubated with 10 mM [3-$^{13}$C]pyruvate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP and 10% $^2H_2O$. 20mM glucose and 0.15U/ml hexokinase was also included to form an ATP regeneration system (Smith *et al.* 2004). The incubation medium also contained cytosolic concentrations of organic acids: 2.1 mM citrate, 1.3 mM succinate, 0.6 mM malate, 0.02 mM fumarate and 0.02 mM isocitrate (Farré *et al.* 2001, Smith et al. 2004). An airlift system was used to ensure complete oxygenation of the medium. Spectra were taken every 15 min for 4 h under the conditions described in materials and methods. $^{13}$C labelling in organic acids was measured over the time course. The peaks were integrated relative to a constant internal mannitol signal. Labelled pyruvate was consumed throughout the course of the experiment, and the amount of labelled citrate, succinate, 2OG, fumarate and malate increased, showing that a fully functioning TCA cycle was operating. Glucose was consumed and glucose-6-phosphate was produced over the time course, showing that the hexokinase system was active and the mitochondria were active and producing ATP (Figure 5.3)

### *Label in malate can be measured in mitochondria incubated with [3-$^{13}$C]glutamate*

Mitochondria are known to be able to metabolise glutamate through the TCA cycle (Aubert *et al.* 2001), but it was necessary to investigate whether isolated

mitochondria are able to produce labelled malate in sufficient quantities to measure using NMR. Isolated mitochondria from *S. tuberosum* were incubated with 10 mM [3-$^{13}$C]glutamate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP, 20 mM glucose and 0.15U/ml hexokinase. As before, an airlift system was used to ensure complete oxygenation of the medium (Smith *et al.* 2004) and spectra were taken every 15 min for 4 h. Labelling in malate was measured over the time course, and peaks were integrated relative to a constant internal mannitol signal. Glutamate was consumed throughout the course of the experiment, and label could be measured in malate and fumarate, showing that this section of the TCA cycle was active. Enough labelled malate was produced for accurate measurements over the time course (Figure 5.4).

### *Fluorocitrate inhibits aconitase and blocks the TCA cycle*

In order to prevent scrambling of label in fumarate and succinate it was necessary to block the TCA cycle at aconitase. To do this, fluorocitrate, an inhibitor of aconitase, was added to the mitochondria. Fluorocitrate is metabolised in the mitochondria to mono fluorocitrate, which binds irreversibly to aconitase and inhibits its activity (Elliott and Kalnitsky 1950). Fluorocitrate was added to a concentration of 5µM, which has been shown to fully inhibit aconitase in isolated plant mitochondria (Smith *et al.* 2004). In this system inhibition of aconitase was complete, as label could be measured in C2 and C3 of malate but not in C1 or C4, which would be expected if the full TCA cycle was active (Figure 5.5)

## Orientation conserved transfer experiments

### *Malate and aspartate C2 and C3 do not have the same labelling pattern in S. tuberosum*

To investigate whether the orientation of labelled molecules is conserved in the plant TCA cycle, the experiment was repeated under the same conditions as described above, and peaks corresponding to malate C2 and C3 were integrated with reference to a constant mannitol peak (present in the buffer). The labelling in each carbon was compared, and the ratio of C2/C3 was calculated at each time point. This was used to calculate an average C2/C3 ratio for each replicate.

It was shown that C2 was consistently more labelled than C3 in malate (Figure 5.6; Table 5.1), although there was some variation between the replicates. The average C2/C3 was 1.4 ±0.21. If there was no metabolic channelling and the molecules were free to rotate between enzymes then we would expect the ratio of C2/C3 to be 1. To test whether these values were different from unity, the 95% confidence interval (CI) of the mean was measured, using a one tailed t-distribution to test whether the ratios were greater than 1.0. Log transformed data was used for the calculation. If the lower CI was less than 1.0, this suggests that the mean of the ratio was not statistically different from equal labelling. The lower CI was 1.18, suggesting that the ratio was statistically greater than 1.

Experiments in *S. cerevisiae* measured the relative label in C2 and C3 in aspartate, rather than malate. As aspartate is synthesised from OAA, which in turn is made from malate through the TCA cycle, if metabolic channelling is present asymmetrical labelling in succinate, fumarate and malate will also be

reflected in aspartate (Figure 5.2*). In S. cerevisiae* the ratio of label in C2/C3 was found to be 1.78 (Sumegi *et al.* 1990) suggesting that metabolic channelling was occurring. In order to compare metabolic channelling in the plant TCA cycle with other organisms, the ratio of label in C2 and C3 in aspartate was also measured. In all replicates there was very little difference between the labelling in C2 and C3 (Figure 5.7; Table 5.1). The average C2/C3 was 1.05 ± 0.02. The lower CI was 1.03, suggesting that the data was just significantly different to 1.0. However, the much lower ratio in aspartate suggests that some of the difference in labelling in malate is not transferred to aspartate.

### *Labelling patterns are similar in S. tuberosum and A. thaliana*

The TCA cycle exists in a different metabolic context in different species, and as a result the metabolic channelling might be different in different plant species. In order to investigate any differences in metabolic channelling in the plant TCA cycle, experiments were also carried out in *A. thaliana*. Mitochondria were isolated from *A. thaliana* seedlings and incubated with [3-$^{13}$C]glutamate as previously described. In all replicates labelling was higher in C2 than C3 in malate (Figure 5.8; Table 5.1) although the ratio was lower than in potato, at 1.24 ± 0.12. The lower CI was 1.06, showing that the data was statistically greater than unity.

The labelling in C2 and C3 in aspartate was also measured. As in *S. tuberosum*, there was no difference between the labelling in C2 and C3 in aspartate (Figure 5.9; Table 5.1). The average difference in labelling was 1.01 ± 0.01. This was not significantly different to unity (lower CI of 0.89). This showed that the labelling difference in malate is not transferred to aspartate.

These results show that the pattern of labelling is similar in *S. tuberosum* and *A. thaliana*, although the ratio of label in C2 and C3 was lower in *A. thaliana* mitochondria. In both cases, the difference in label was not transferred to aspartate. There are two explanations for the differences in labelling in malate and aspartate. One possibility is that there is a genuine difference in the label in malate, suggesting that in plants, aspartate is made from a separate pool of unchannelled malate. An alternative explanation is that the difference in labelling in malate carbons is the result of an experimental artefact, and it was necessary to investigate whether this was artificially increasing the C2/C3 ratio.

### *Changing the acquisition conditions did not alter the relative intensity of the signals*

There are several reasons why the amount of labelling may appear to be different in malate C2 and C3. One is that the rotation of molecules is restricted, but there are also several effects arising from the experimental system which may artificially alter the ratio of label C2/C3. One such effect could arise from a difference in spin-lattice relaxation time for the two carbon atoms.

Incomplete relaxation of the carbons will mean that the signal is inaccurate: if one carbon is fully relaxed and the other isn't there will be a difference between the signal intensities, which may affect the overall ratio of label in carbon in the molecule. To test that this was not affecting the measured ratio, the signal from the four malate carbons was measured under different relaxation delays (ie. the time that elapsed between 90 degree pulses), ranging from 2-12 s. 10 mM malate was dissolved in the standard NMR buffer with 10% $^{2}H_2O$. The signal

intensity of each carbon was measured and expressed as a proportion of malate C3. The signal intensity of C2 and C3 was different when the delay was less than 6 s, but if it was 6 s or longer then the signals were equal (C2/C3 = 0.97, Figure 5.10). As the NMR method used in the original experiment allows 6 s for relaxation, the results suggest that unequal relaxation was unlikely to be altering the ratio of label in malate carbons.

### *EDTA reduces the difference in labelling in malate C2 and C3*

A further possible explanation for the difference in labelling in malate C2 and C3 could be the presence of divalent cations such as magnesium in the incubation medium. These ions can cause line-broadening in the NMR spectra of organic acids which could make it more difficult to integrate the signals correctly. Moreover since the effect is unlikely to be the same for C2 and C3, divalent cation binding could alter the apparent intensity ratio. One way to test whether this effect is altering the ratio is to use a chelator, such as EDTA, which can sequester divalent ions such as $Mg^{2+}$ and $Ca^{2+}$ away from the malate molecule. This means that the line broadening effect of the cations is removed. As a result the broader peaks become narrower, reducing errors in the estimation of their intensity.

To test this, samples from previous OCT experiments were freeze dried and re-suspended in $D_2O$. The peak areas of malate and aspartate C2 and C3 were measured before and after the addition of EDTA (Figure 5.11). EDTA was added until the peak widths were equal: a concentration range of 10 to 15 mM was used depending on the sample. The ratio of C2/C3 was calculated and compared

with the ratio before EDTA was included (Figure 5.12). EDTA reduced the ratio of labelled C2/C3 in malate: the average ratio fell to 1.12 ± 0.06 after EDTA in *A. thaliana*, and to 1.14 ± 0.12 in *S. tuberosum*. One of the *A. thaliana* samples was very poor quality after it had been re-dissolved in D2O, meaning that *n*=2 for this experiment. The ratio of aspartate C2/C3 was also calculated, and fell to 1.01± 0.03 in *S. tuberosum* and 1.01 ± 0.01 in *A. thaliana*. Of all of these, only the ratio in malate in *S. tuberosum* was found to be significantly different to unity. This suggests that a proportion of the difference in ratio in malate was due to the line broadening effects of divalent cations in the buffer.

## Comparing orientation conserved transfer in plants and yeast

In contrast to plants, a difference in labelling was measured in aspartate when the experiment was carried out in *S. cerevisiae* (Sumegi *et al.* 1993). Malate labelling was not reported, so it is not clear whether the labelling in malate is similar. However, EDTA was not used to test how much of the ratio was due to the effect of differences in line broadening between the peaks. Interestingly, more label was measured in aspartate C2 than C3, even though [4-$^{13}$C]glutamate was used as the labelled precursor, whereas our experiment used [3-$^{13}$C]glutamate. We therefore wanted to repeat the experiment with *S. cerevisiae* before comparing the results with *A. thaliana* and *S. tuberosum*. Yeast strain BY47419 (MATα, his3Δ1, leu2Δ0, met 15Δ0, ura3Δ0) was plated on standard YPD plates and grown at 28 °C for 72 h. A 50ml starter culture of lactate medium was inoculated with a few colonies from YPD plates. The medium was incubated for 2 days at 30°C with shaking. A growth curve at OD600nm was plotted over 3 days to measure the growth of the culture (data not shown). 250 ml of media was

inoculated with the 50 ml starter culture and incubated at 30 °C. Cells were harvested in log phase as described in materials and methods. Yeast cells were then experimented on as described in Sumegi *et al.* (1993), but [3-$^{13}$C]glutamate was used as a substrate, rather than [4-$^{13}$C]. 1 gram of packed cells was re-suspended in 6 ml minimal media containing 3 g of [3-$^{13}$C]glutamate. Cells were incubated for 30 min at 30 °C with vigorous shaking. 4% perchloric acid was used to stop the reaction. The sample was neutralised and then freeze dried, before being re-suspended in 100 mM potassium phosphate buffer with 100 mM EDTA, 250 mM 1,4 dioxane and 10% $^{2}H_2O$. Spectra were run for 20 h for the conditions described in materials and methods. Additional $^{13}$C signal peaks were seen in NMR spectra when compared with a sample of buffer, suggesting that some label was metabolised. However it was not possible to assign these peaks. It was not possible to identify aspartate or malate signals in the NMR spectra (Figure 5.13).

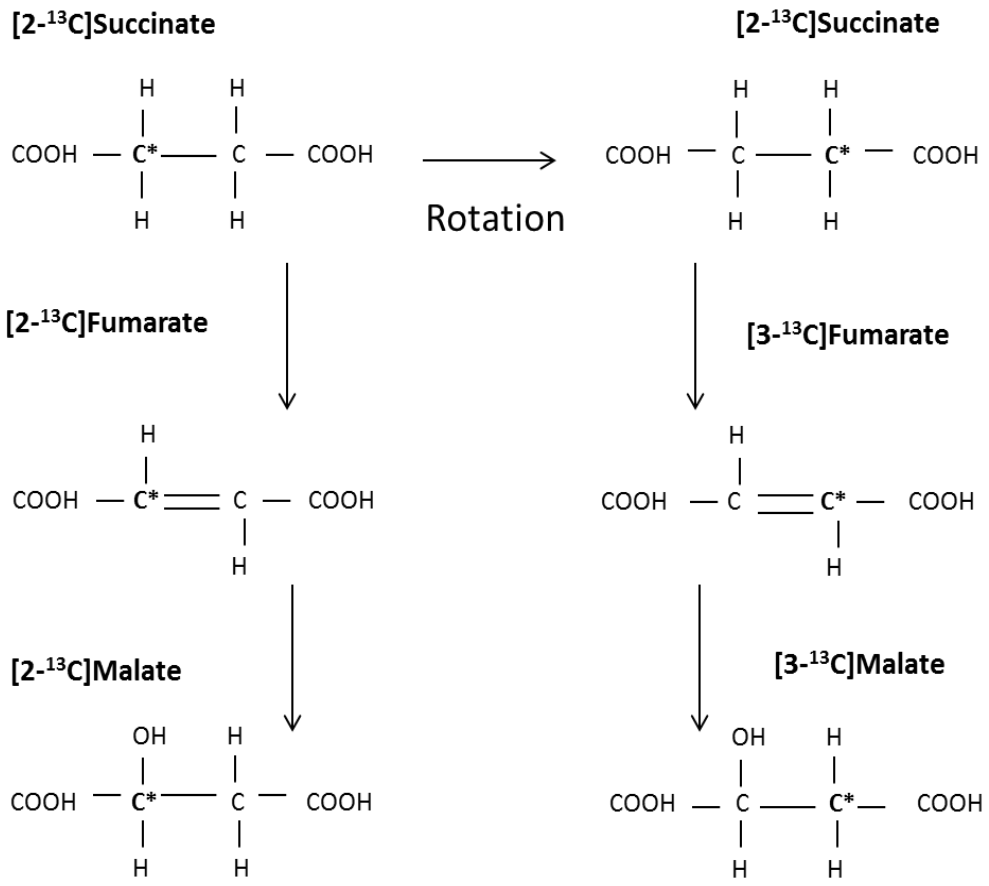


**Figure 5.1 The expected labelling pattern in malate from 3-$^{13}$C glutamate in the absence of channelling**. Labelled carbons are shown in bold. Label from glutamate is transferred to C2 in succinate. Because succinate is a symmetrical molecule the label can be transferred to either C2 or C3 in fumarate. In this case, the amount of label in the two carbons would be equal. However, channelling restricts diffusion and the ability of the molecules to rotate. As a result, labelling in C2 of succinate is more likely to label C2 of fumarate than C3. Therefore, an unequal ratio of label in malate carbons suggests that channelling is present.

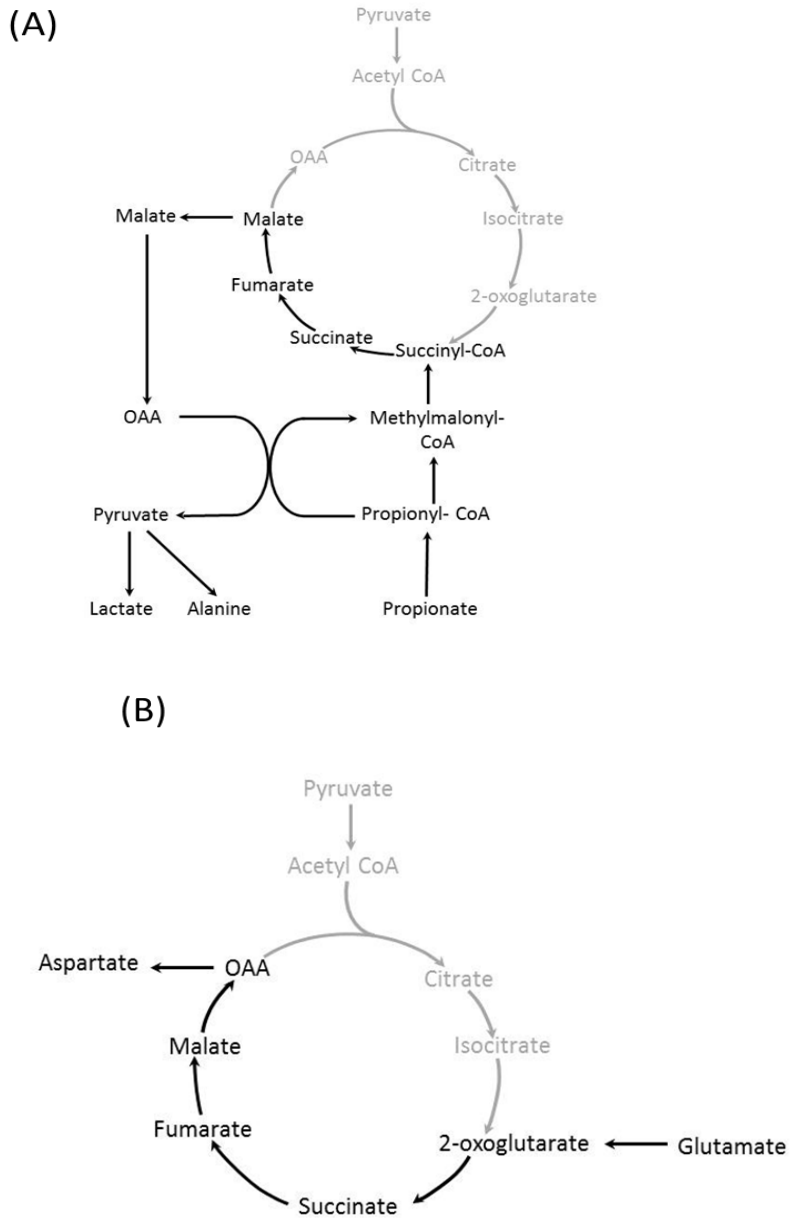


**Figure 5.2 Metabolism of propionate and glutamate in mitochondria.** (A) Pathway of propionate metabolism in mitochondria. Label in propionate enters the TCA cycle at succinyl CoA, and then into succinate. Because succinate is a symmetrical molecule the label can be transferred to either C2 or C3 in fumarate. Fumarate is then converted into malate, where it is converted into pyruvate via OAA. Pyruvate is then converted into either lactate or alanine, where the ratio of label in carbons can be used to assess channelling. (B) Pathway of malate and aspartate production from glutamate. Glutamate is converted to 2OG where it enters the TCA cycle and is converted to malate via succinate and fumarate. Aspartate is then produced from malate via OAA.

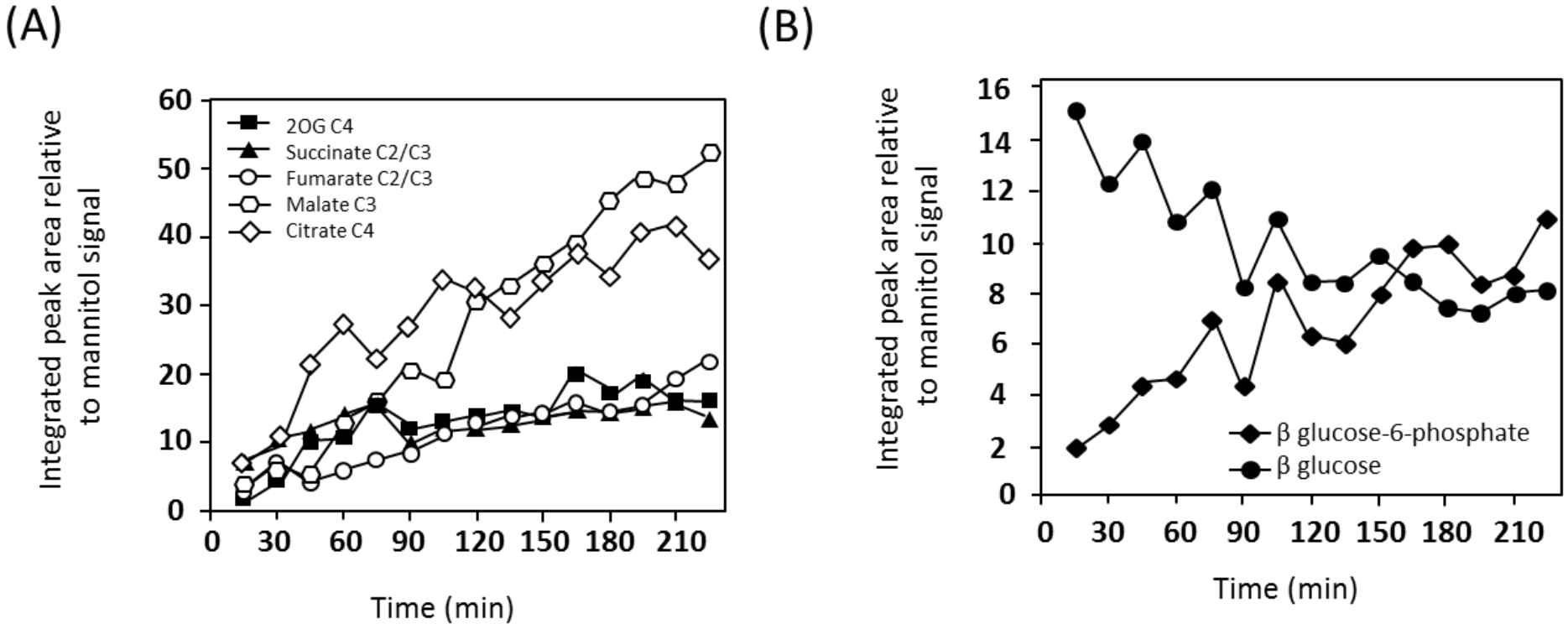


**Figure 5.3 Labelling in metabolic compounds in isolated *S. tuberosum* mitochondria incubated with [3-$^{13}$C]pyruvate.** Mitochondria were isolated from *S. tuberosum* tubers and incubated with 10 mM [3-$^{13}$C]pyruvate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP and 10% $D_2O$, 20 mM glucose and 0.15 U/ml hexokinase. 2.1 mM citrate, 1.3 mM succinate, 0.6 mM malate, 0.02 mM fumarate and 0.02 mM isocitrate was included to stimulate TCA cycle activity (Smith *et al.* 2004). An airlift system was used to ensure continuous oxygenation and complete mixing. NMR spectra were recorded continuously in 15 minute blocks. Peaks were integrated relative to a constant internal mannitol signal. (A) production of labelled organic acids showing that the TCA cycle is active, (B) Glucose consumption and glucose-6-phosphate production shows that the hexokinase system was active.

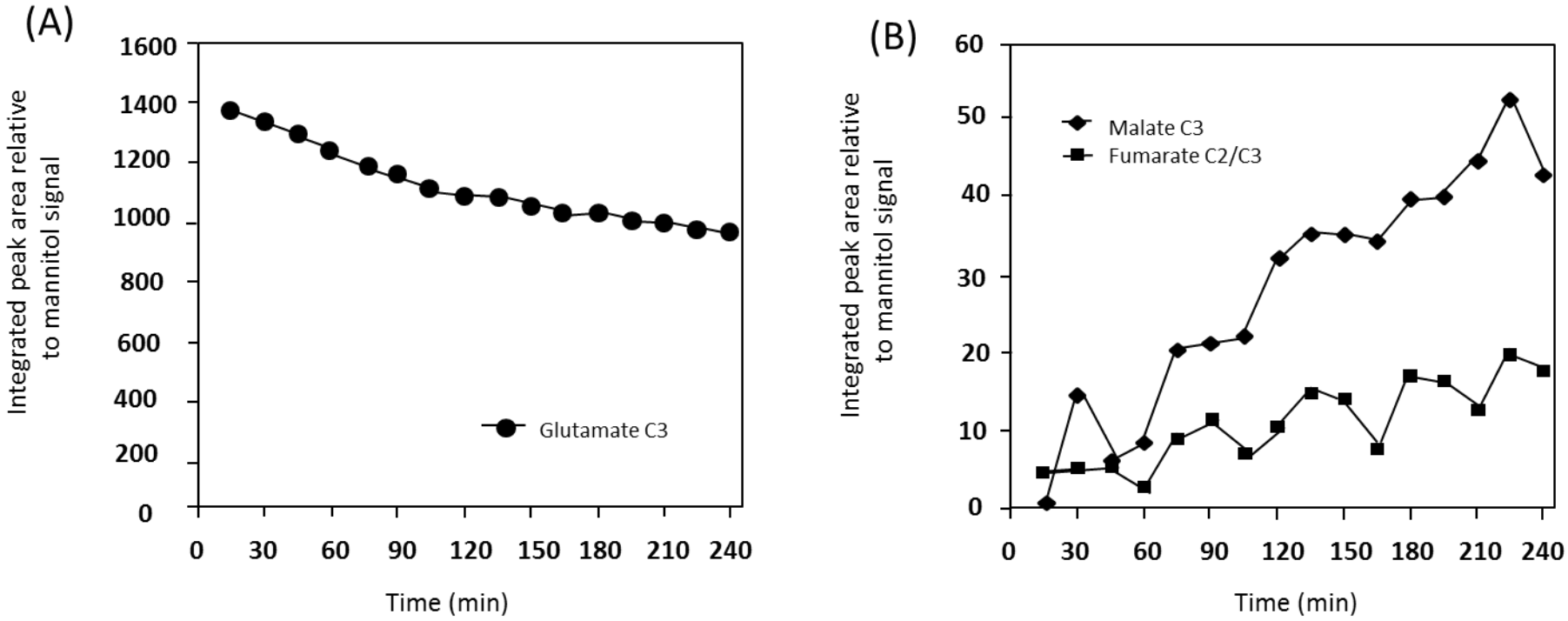


**Figure 5.4 Labelling metabolic compounds in isolated *S. tuberosum* mitochondria incubated with [3-$^{13}$C]glutamate and 5 µM fluorocitrate.** Mitochondria were isolated from *S. tuberosum* tubers and incubated with 10 mM [3-$^{13}$C]glutamate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP and 10% $D_2O$., 20 mM glucose and 0.15 U/ml hexokinase. 5 µM fluorocitrate, which inhibits aconitase, was included to block the TCA cycle. Peaks were integrated relative to a constant internal mannitol signal. (A) Glutamate was consumed over the time course, (B) labelled malate and fumarate built up, showing the TCA cycle was active.

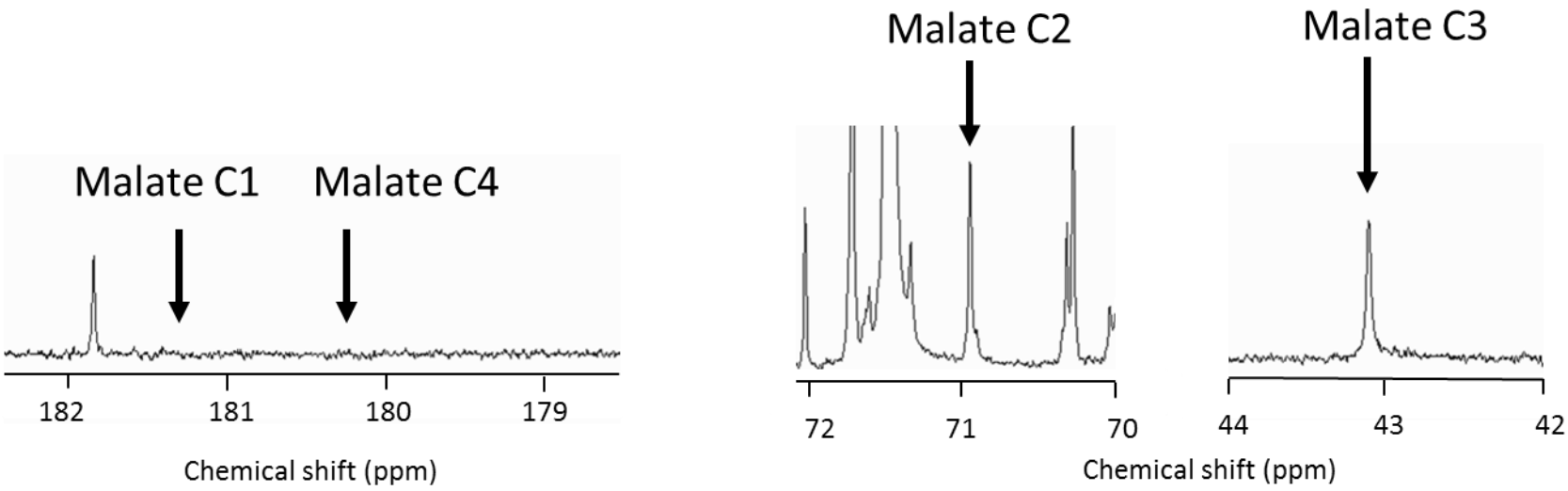


**Figure 5.5 $^{13}$C NMR spectra showing label in malate carbons in isolated *S. tuberosum* mitochondria incubated with [3-$^{13}$]C glutamate and 5 µM fluorocitrate.** Mitochondria were isolated from *S. tuberosum* tubers and incubated with 10 mM [3-$^{13}$C]glutamate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP and 10% $D_2O$., 20 mM glucose and 0.15 U/ml hexokinase. 5 µM fluorocitrate, which inhibits aconitase, was included to block the TCA cycle. NMR spectra were recorded continuously in 15 minute blocks and the spectra summed to show total integration over the time course. However no label was measured in malate C1 or C2, as would be expected if a full TCA cycle was active. This shows that fluorocitrate was effective in blocking the TCA cycle.

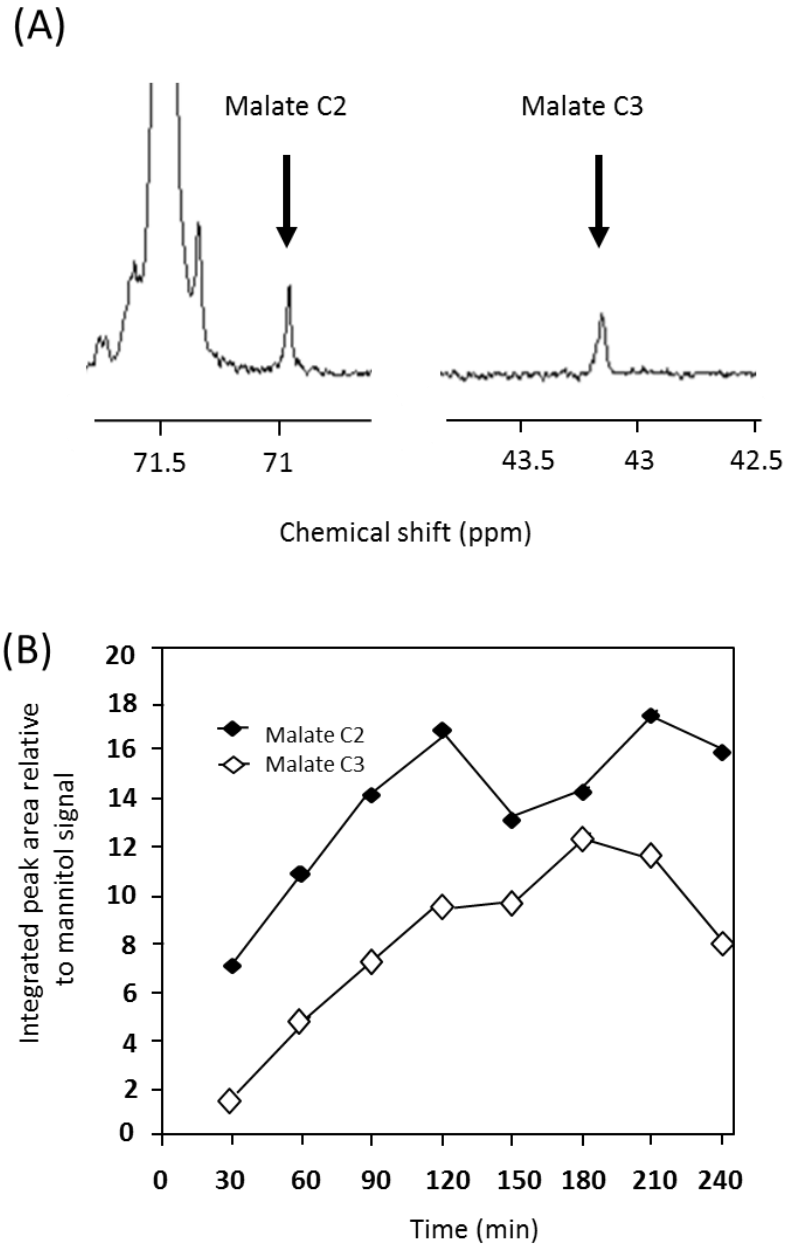


**Figure 5.6 Labelling in malate C2 and C3 in isolated *S. tuberosum* mitochondria incubated with [3-$^{13}$C]glutamate** Mitochondria were isolated from *S. tuberosum* tubers and incubated with 10 mM [3-13C]glutamate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP and 10% $D_2O$, 20 mM glucose and 0.15 U/ml hexokinase. 5 µM fluorocitrate, which inhibits aconitase, was included to block the TCA cycle. NMR spectra were continuously in 15 minute blocks, and peaks were integrated relative to a constant internal mannitol signal. The figures show a representative experiment. (A) C2 was present at 71 ppm and C3 at 43.1ppm, (B) The amount of labelled C2 and C3 of malate increased over the time course. More label was measured in C2 than C3.

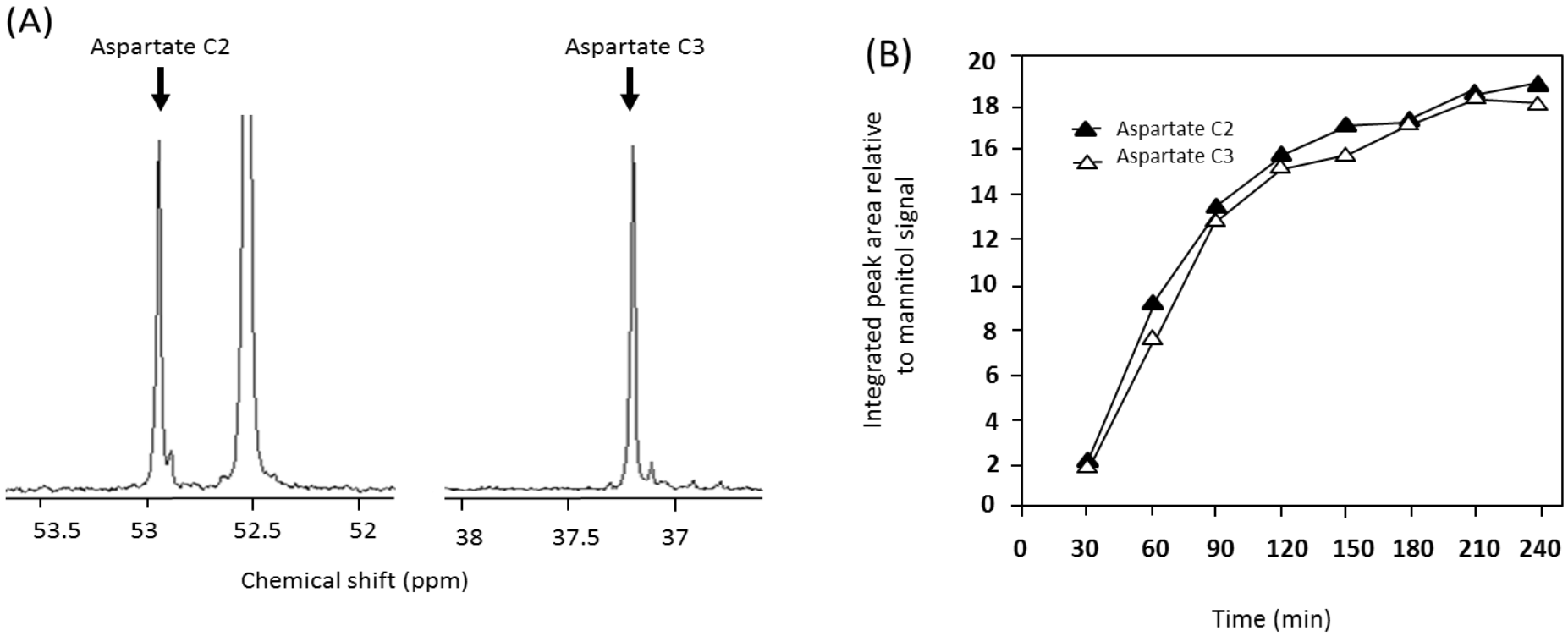


**Figure 5.7 Labelling in aspartate C2 and C3 in isolated *S. tuberosum* mitochondria incubated with [3-$^{13}$C]glutamate** Mitochondria were isolated from *S. tuberosum* tubers and incubated with 10 mM [3-$^{13}$C]glutamate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP and 10% $D_2O$., 20 mM glucose and 0.15 U/ml hexokinase. 5 µM fluorocitrate, which inhibits aconitase, was included to block the TCA cycle. NMR spectra were recorded continuously in 15 minute blocks, and peaks were integrated relative to a constant internal mannitol signal. The figures show a representative experiment. (A) Aspartate C2 was present at 53 ppm and C3 at 37.2ppm, (B) The amount of labelled C2 and C3 of aspartate increased over the time course.

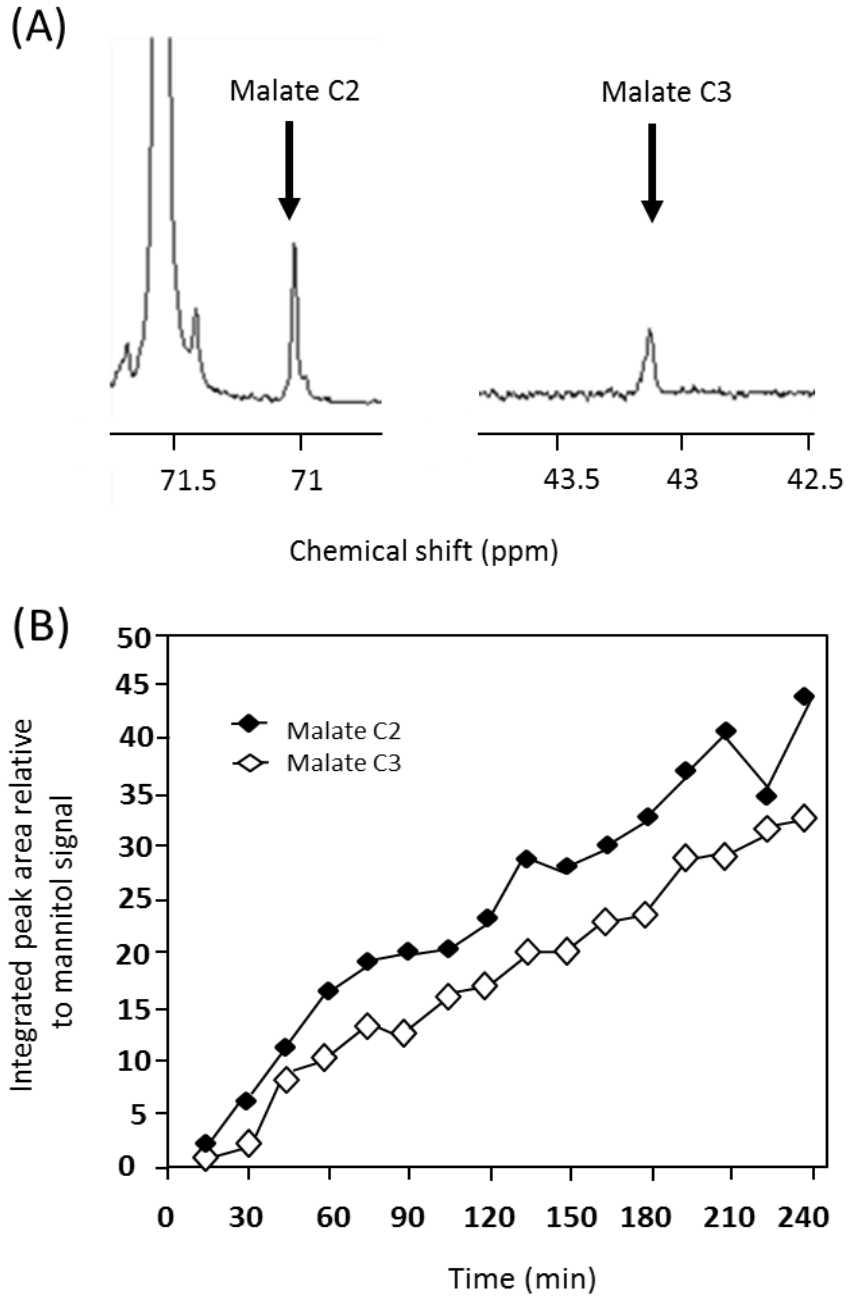


**Figure 5.8 Labelling in malate C2 and C3 in isolated *A. thaliana* mitochondria incubated with [3-$^{13}$C]glutamate** Mitochondria were isolated from 14 day old *A. thaliana* seedlings and incubated with 10 mM [3-$^{13}$C]glutamate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP and 10% $D_2O$, 20 mM glucose, 0.15 U/ml hexokinase and 5 µM fluorocitrate. NMR spectra were recorded continuously in 15 minute blocks, and peaks were integrated relative to a constant internal mannitol signal. The figures show a representative experiment. (A) C2 was present at 71 ppm and C3 at 43.1ppm, (B) The amount of labelled C2 and C3 of malate increased over the time course..

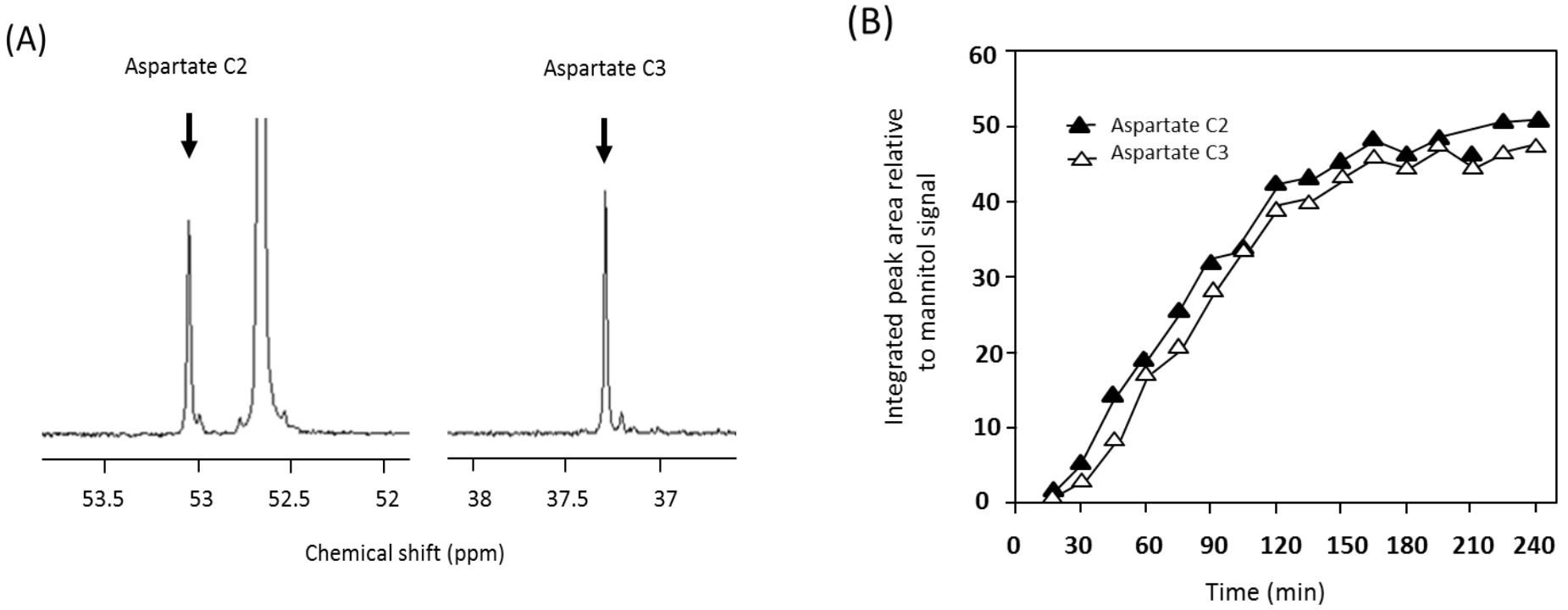


**Figure 5.9 Labelling in Aspartate C2 and C3 in isolated *A. thaliana* mitochondria incubated with [3-$^{13}$C]glutamate** Mitochondria were isolated from 14 day old *A. thaliana* seedlings and incubated with 10 mM [3-$^{13}$C]glutamate, 0.3mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP and 10% $D_2O$, 20 mM glucose and 0.15 U/ml hexokinase. 5 µM fluorocitrate, which inhibits aconitase, was included to block the TCA cycle. NMR spectra were recorded continuously in 15 minute blocks, and peaks were integrated relative to a constant internal mannitol signal. The figures show a representative experiment. (A) Aspartate C2 was present at 53 ppm and C3 at 37.2ppm, (B) The amount of labelled C2 and C3 of aspartate increased over the time course.

| | C2/C3 | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *S.tuberosum* | | | | | | *A.thaliana* | | | | |
| | rep1 | rep2 | rep3 | rep4 | Average | stdev | rep1 | rep2 | rep3 | Average | stdev |
| Malate | 1.32 | 1.27 | 1.71 | 1.3 | 1.4* | 0.21 | 1.36 | 1.23 | 1.13 | 1.24* | 0.12 |
| Aspartate | 1.07 | 1.03 | 1.05 | 1.06 | 1.05* | 0.02 | 1.02 | 1.26 | 1.03 | 1.1 | 0.13 |

**Table 5.1 Label in C2/C3 of malate and aspartate in *S. tuberosum* and *A. thaliana* measured by NMR.** Mitochondria were isolated from *S. tuberosum* tubers or 15 day old *A. thaliana* seedlings and incubated with 10mM [3-$^{13}$C]glutamate, 0.3 mM $NAD^+$, 0.2 mM ADP, 0.1 mM TPP and 10% $D_2O$, 20 mM glucose and 0.15 U/ml hexokinase and 5 µM fluorocitrate. NMR spectra were recorded continuously in 15 minute blocks, and peaks were integrated relative to a constant internal mannitol signal. * denotes an average ratio significantly different to 1.0

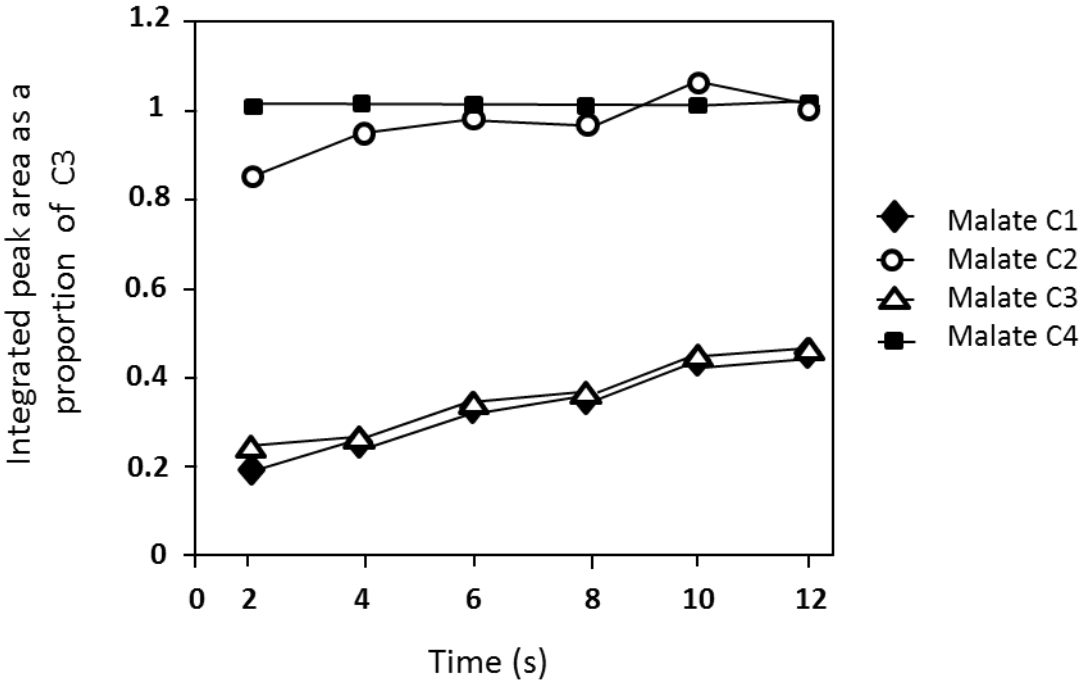


**Fig 5.10 The effect of relaxation delay on the signal intensity of malate carbons**. 100 mM malate was diluted in 10% $D_2O$. Relaxation time was varied between 2 and 12 seconds and 6 separate spectra recorded. Peaks were integrated relative to a constant internal mannitol signal. The signal intensity of each malate carbon was expressed as a proportion of malate C3. The proportion of C2 to C3 at the relaxation time used in the experiment (6 s) was 0.97, suggesting that relaxation is complete.

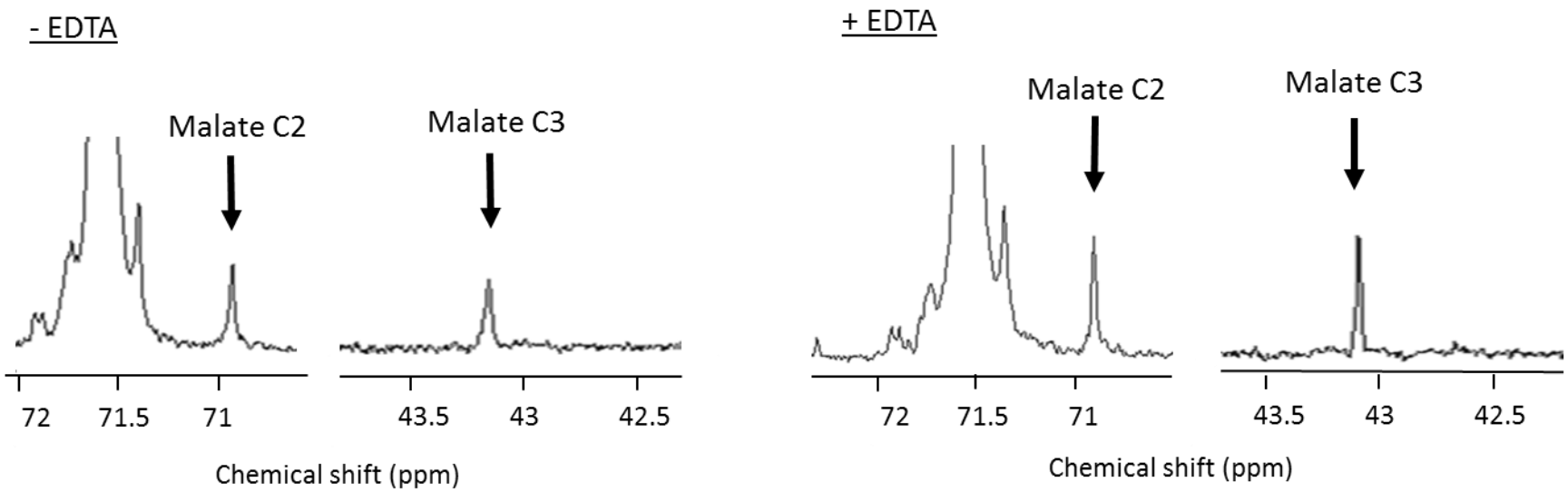


**Figure 5.11 Change in NMR signals before and after EDTA addition.** $^{13}$C NMR spectra showing label in malate C2 and C3 in *S. tuberosum* mitochondria before and after addition of EDTA. Freeze dried samples were re-suspended in $D_2O$. The peak area of malate and aspartate C2 and C3 were measured before and after the addition of EDTA. Peaks were integrated relative to a constant internal mannitol signal.

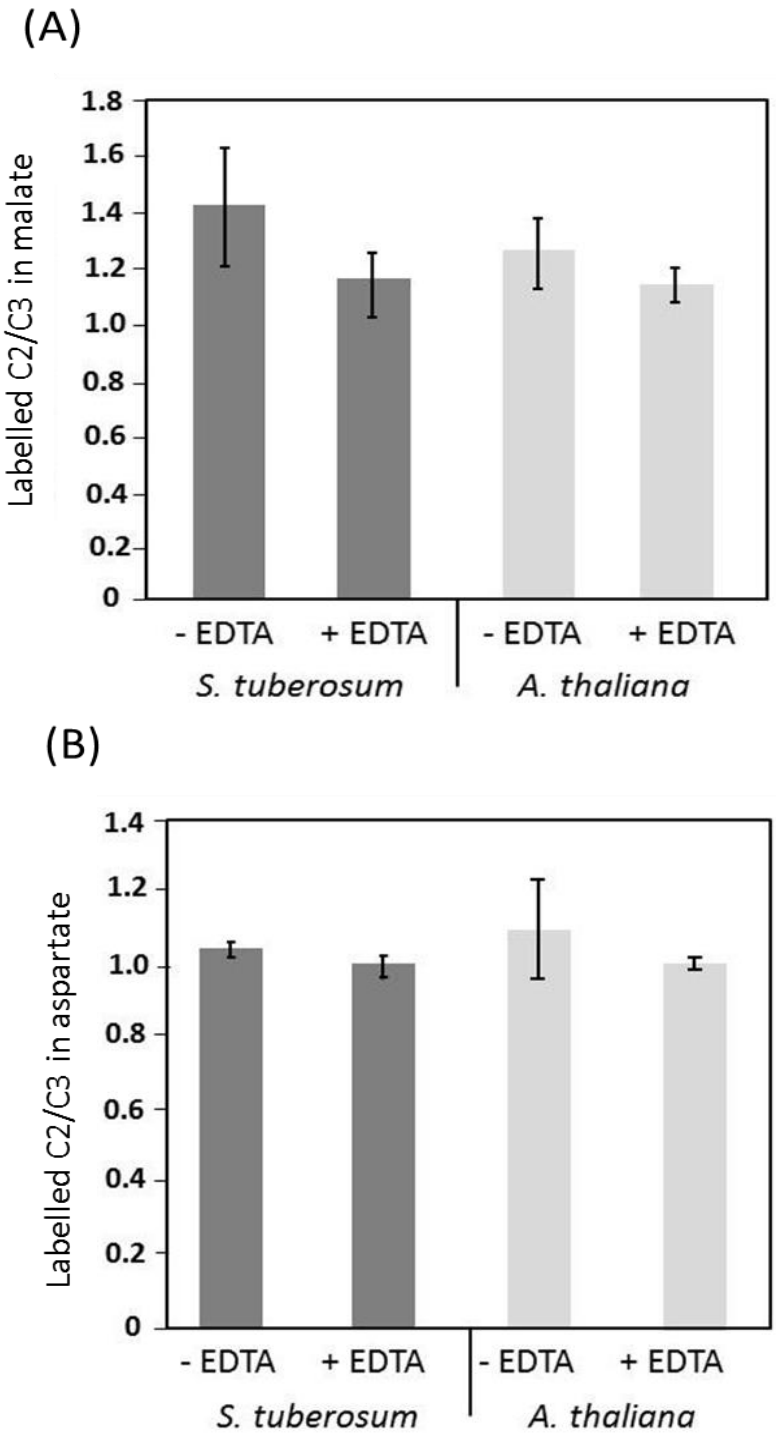


**Figure 5.12 Labelling in malate C2/ C3 and aspartate C2/C3 before and after EDTA addition.** Peaks were integrated relative to a constant internal mannitol signal and the ratio of C2/C3 was calculated. (A) Ratio of label in C2/C3 in malate before and after EDTA was added in *S. tuberosum* and *A. thaliana*, and (B) Ratio of label in C2/C3 in aspartate before and after EDTA was added in *S. tuberosum* and *A. thaliana.*

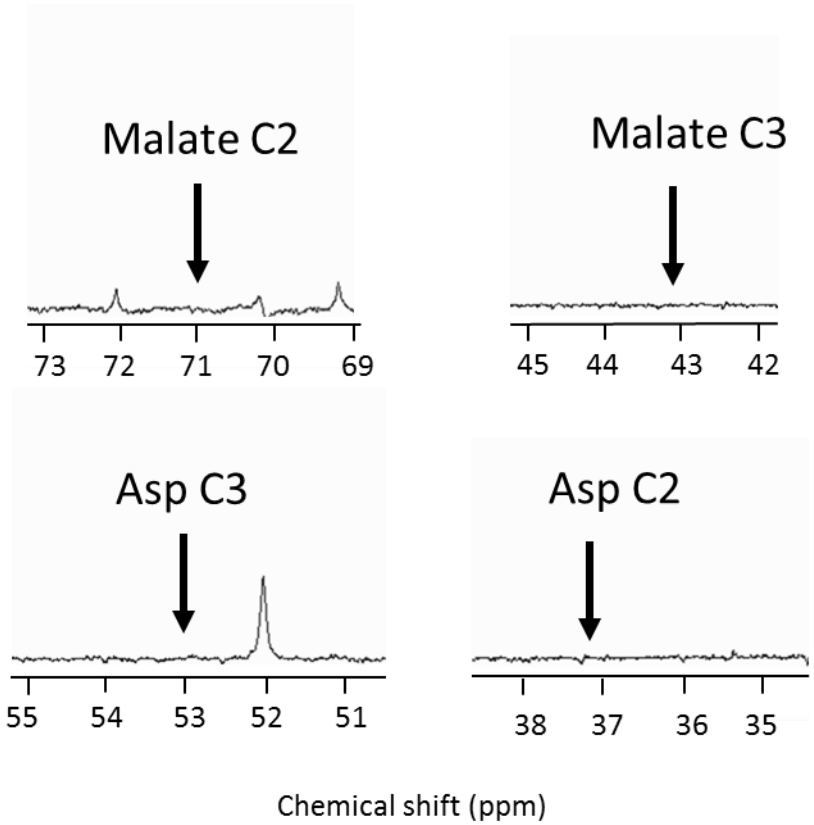


**Figure 5.13 $^{13}$C NMR spectra showing expected position of malate and aspartate carbons in *S. cerevisiae* incubated with [3-$^{13}$C]glutamate.** 1 gram of packed *S. cerevisiae* cells was re-suspended in 6 ml minimal media containing 3g of 3-$^{13}$C glutamate and fluorocitrate. Cells were incubated for 30 minutes at 30 degrees with vigorous shaking. 4% perchloric acid was used to stop the reaction. This was neutralised and then freeze dried, before being re-suspended in 3.3 mL of 10 mM $KH_2PO_4$ buffer with 10 mM EDTA, 25 mM 1,4-dioxane, and 10% $2H_2O$ (pH 7.5) Spectra was collected over 24 hours. It was not possible to identify aspartate or malate signals in the NMR spectra

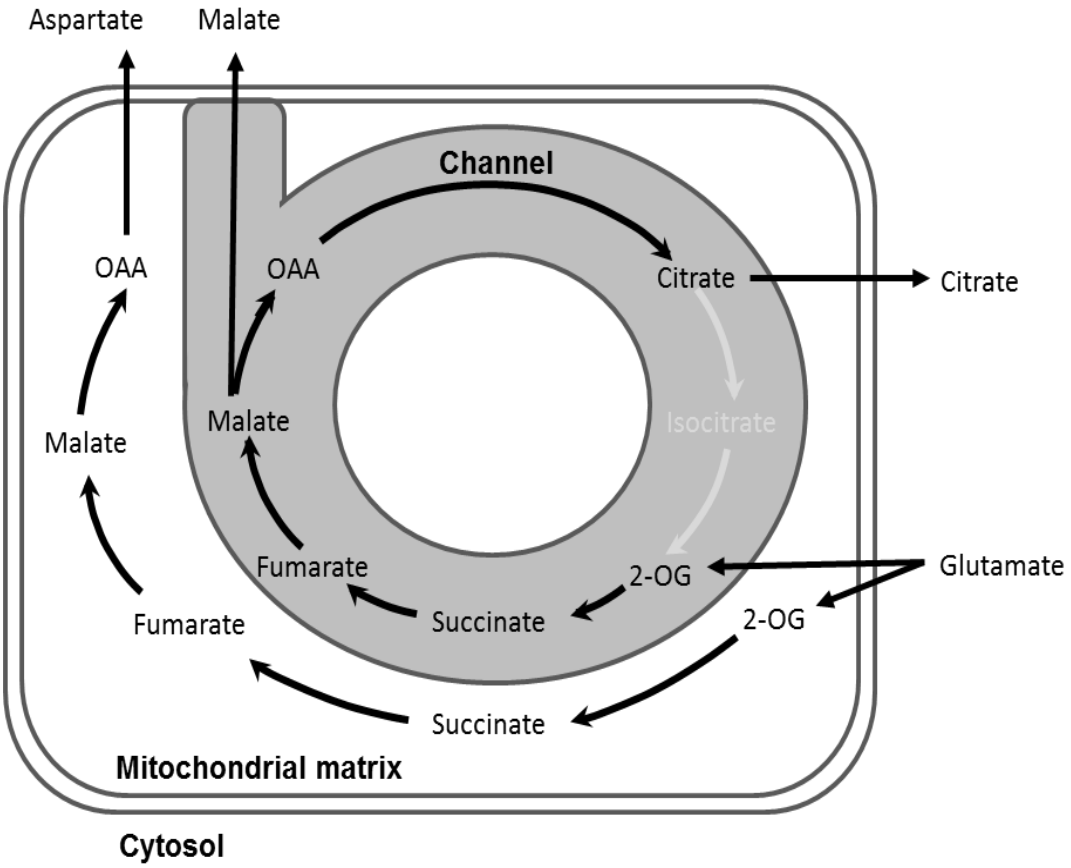


**Figure 5.14 A possible arrangement of channelling which could explain the malate and aspartate labelling patterns in isolated plant mitochondria**. Two separate enzyme pools exist in the mitochondria: one which form a metabolon and is associated with a channelled TCA cycle (in grey circle), and one set which catalyse an unchannelled section of the TCA cycle. In the channelled pathway, rotation of intermediates is restricted, and orientation conserved transfer of label occurs. Malate produced in this pathway is either exported from the mitochondria (where it is measured by NMR) or may go on to produce citrate. In the unchannelled pathway molecules are free to rotate between enzymes, and the malate produced will go on to form aspartate via OAA. This aspartate is then exported and is measured by NMR.

## Discussion

### Orientation conserved transfer of label is measured in malate but not aspartate in *S. tuberosum*

Orientation conserved transfer of label was initially measured in malate and aspartate in *S. tuberosum*. However, after EDTA was included in the medium, only labelling in malate C/C3 remained significantly different to 1. This shows that most of the difference in the labelling of malate and aspartate carbons is due to the presence of divalent cations in the experimental buffer. However, the fact that the effect was not completely removed in malate suggests that a small amount of the malate may be restricted in its rotation. This could occur if a very small proportion of the pathways was channelled.

In contrast to the malate data, there was no difference in the labelling of C2 and C3 in aspartate in *S. tuberosum* after EDTA was included in the experiment. As malate is a precursor of aspartate, the labelling pattern of malate should be conserved in aspartate. The fact that this is not the case suggests that all the aspartate produced from the mitochondria is made from a separate pool of malate which does not have its origin in any channelled pathway.

This evidence is in line with that described in chapter 4, which suggested that there are two sets of enzymes in the TCA cycle: one which forms a metabolon and is associated with a channelled TCA cycle, and one which catalyses an unchannelled section of the TCA cycle. These could be present in the same or separate mitochondria. Figure 5.14 shows how this might be achieved. Glutamate enters the

mitochondria and has one of two fates. It can either enter a channelled pathway, where the rotation of intermediates is restricted, and the malate which is made has more label in C2 than in C3. This malate is then either exported (possibly through a channel in order to avoid mixing with the unchannelled pools) from the mitochondria (where it is measured by NMR) or may go on to produce citrate. Alternatively, glutamate may enter an unchannelled pathway, where the molecules are free to rotate between enzymes. The malate produced in this pathway will have an equal ratio of label in C2 and C3, and go on to form aspartate via OAA. This aspartate is then exported and is measured by NMR.

### Orientation conserved transfer of label is not measured in C2/C3 of malate or aspartate in *A. thaliana*

Before EDTA was included in the experiment, orientation conserved transfer of label was measured in malate carbons in *A. thaliana*. However, after EDTA was included, this difference disappeared, and the ratios were found not to be significantly different to 1. In contrast the ratio of C2/C3 in aspartate was the same, with little difference measured between C2/C3 in both species.

One explanation for this difference is that channelling occurs differently in the two species. For example, in *S. tuberosum* channelling could occur through a method which restricts rotation of malate, while in *A. thaliana* this is not the case. This could result in a difference in the amount of label in C2/C3 in malate in *S. tubersosum*, but not in *A. thaliana*. Another reason for the difference in orientation conserved transfer is that the proportion of enzymes which form a channel may be different in *S. tuberosum* than in *A. thaliana*. This could be the result of different metabolic fluxes through the

TCA cycle in *A. thaliana* seedlings compared with *S. tuberosum* tubers. For example, large fluxes into aspartate production have been measured in developing tubers (Roessner-Tunali *et al.* 2004) to support the synthesis of storage protein. In *A. thaliana* cell cultures, there is also a substantial flux to aspartate to support biosynthesis (Poolman *et al.* 2009, Williams *et al.* 2008). However, in dormant tubers this flux is likely to be low as the rate of growth in tubers will be much slower. In addition, if sucrose levels remain high, the demand for amino acid production may also be low (Roessner-Tunali *et al.* 2003). In *A. thaliana* seedlings there is a much stronger requirement for providing carbon skeletons for biosynthesis to support rapid growth and cell differentiation, and so the demand to withdraw carbon skeletons from the cycle is likely to be greater. This may mean that a higher proportion of TCA cycle enzymes are present in the free pool to provide precursors for aspartate synthesis. This could either remove the need for a separate pool of channelled malate, or could mean that the proportion of enzymes channelling malate is too small to produce a measurable difference in labelling in malate C2/C3 in *A. thaliana*.

### It was not possible to repeat the published OCT experiments in *S. cerevisiae*

In order to compare orientation conserved transfer in plants and yeast more accurately, experiments carried out by Sumegi *et al.* (1993) were repeated. However, it was not possible to detect label in malate C2 and C3, or aspartate C2 and C3. This could be because glutamate was not metabolised, or because label was not metabolised into aspartate and malate via the TCA cycle. Differences in metabolism could be due to the different strain that was used (BY47419 (MATα, his3Δ1, leu2Δ0, met 15Δ0, ura3Δ0 rather than MMY011 (MATα ade 2-1 his 3-11,15 leu 2-3,112 trp 1-1

ura 3-1 can 1-100).) Different strains may have different fluxes through primary metabolism, and this will have an effect on how labelled compounds are metabolised.

### Evidence of orientation conserved transfer of label is found in aspartate in *S. cerevisiae* but not in *S. tuberosum* or *A. thaliana*

*Sumegi et al.* (1993) measured the ratio of label in C2/C3 in aspartate in *S. cerevisiae* under aerobic conditions. The published data showed a C2/C3 ratio of 1.78, which is considerably higher than the value of approximately 1 which was measured in *A. thaliana* and *S. tuberosum.* This suggests that aspartate has its origin in malate that has been channelled. Other experiments in yeast also found unequal labelling of carbons in alanine and lactate, which also originate from the TCA cycle (Figure 5.2). It is important to note that Sumegi *et al.* (1993) did not measure the spectra in the presence of EDTA. It is therefore not clear to what extent the ratio has been affected by divalent cations as seen in the results in this chapter. The result of this is that it is difficult to be completely confident in their published ratio. Unfortunately I was unable to repeat the experiment successfully in *S. cerevisiae*, meaning that I was not able to directly compare the ratio of label under the same conditions.

An alternative explanation for the difference between the labelling patterns in plants and yeast is that, unlike in plants, most of the flux through the TCA cycle is channelled. The difference in metabolic channelling in yeast and plants could be due to different demands on the TCA cycle in these species. In *S. tuberosum*, *A. thaliana* and *S. cerevisiae* the TCA cycle is important in producing both ATP and carbon skeletons for biosynthesis. The result of this is that the cell needs to balance the withdrawal of organic acids for biosynthesis with maintaining a cyclic flux for ATP production.

Because biosynthetic processes also require ATP, competition for TCA cycle intermediates will increase with increasing biosynthetic demand (See Chapter 5).

Separating pathways via metabolite metabolic channelling is one possible way to regulate these competing demands. Another way would be to partition biosynthesis and ATP production between two different pathways: i.e. organic acids from the TCA cycle used for biosynthesis, while the demand for ATP is met from another pathway. There is some evidence that this occurs in *S. cerevisiae.* A feature of yeast is that it is able to grow successfully under both aerobic and anaerobic conditions. Under anaerobic conditions, the majority of ATP is produced via fermenative metabolism (i.e. the production of ethanol from glucose), and the TCA cycle operates in a branched form in order to supply precursors for biosynthesis (Albers *et al.* 1998, Camarasa *et al.* 2003). When yeast is grown under aerobic conditions and at glucose concentrations less than 0.15g/L, the TCA cycle is thought to operate as a cycle. In this case, both growth rates and the rate of biosynthesis are relatively low (Van Hoek *et al.* 1998). However, as growth and biosynthesis increase fluxes through the TCA cycle change. It has been shown that levels of glucose above 0.15 g/L, when relative growth rates are high, fermentative metabolism is triggered (Verduyn *et al.* 1984**)**, and that under these conditions, *S. cerevisiae* produce a large fraction of their ATP through fermentation (Maaheimo *et al.* 2001), while the TCA cycle exists in a branched form (Fiaux *et al.* 2003, Blank and Sauer 2004). As a result, the TCA cycle plays a smaller role in ATP synthesis under these conditions, even though the demand for ATP is high. This effect has important implications for understanding the differences in metabolic channelling between yeast and other organisms. It suggests that yeast regulate these competing

demands on the TCA cycle by using fermentation to produce ATP, while the TCA cycle provides carbon skeletons for biosynthesis.

In plants, there are also some examples where the TCA cycle does not act as a full cycle. These include developing embryos of *Brassica napus* (Schwender *et al.* 2006), roots of *Lotus japonicus* under anoxia (Rocha *et al.* 2010) and the leaves of *Spinacia oleracea* and *Xanthium strumarium* in the light (Hanning and Heldt 1993, Tcherkez *et al.* 2009). However, in all these tissues, the demand for ATP is thought to be low relative to the demand for carbon skeletons for biosynthesis. In contrast, when plants are grown under conditions where there is a high demand for both ATP and biosynthesis, cyclic fluxes through the TCA cycle increase. Similarly, a genome scale model of *A. thaliana* cell suspension cultures showed that when fluxes are constrained by the demand for ATP and carbon skeletons for biosynthesis, the TCA cycle operates in a branched fashion, with the main fluxes providing carbon skeletons for N assimilation and aspartate biosynthesis (Poolman *et al.* 2009). However, if the need to support cell maintenance as well as biosynthesis was used to constrain the model then a conventional TCA cycle was required (Poolman *et al.* 2009). In addition, when *Arabidopsis* cell suspension cultures were grown under elevated oxygen concentrations there was an increase in the cyclic flux through the TCA cycle, which corresponds to an increase in the rate of biomass accumulation and ATP production (Williams *et al.* 2008). In plants regulation of respiration needs to balance both a fully-cyclic TCA cycle and branched reactions which remove intermediates for biosynthesis within the mitochondria. In contrast, there may be less need for two physically separate sets of TCA cycle enzymes to co-exist in the mitochondria of *S. cerevisiae*, as

the respiratory and biosynthetic roles of the TCA cycle are not in direct competition to the same extent.

It is not possible to completely rule out the idea that separate pools of TCA cycle enzymes also exist in yeast. This is because although the ratio of label measured in aspartate was greater than one, it is not possible to say whether the difference is due to a channelled pathway which allows some rotation, or two pathways, one which allows full rotation, and the other where rotation is completely restricted.

Another difference is that in plants malate C2 was found to be labelled to a greater extent than malate C3 when [3-$^{13}$C] glutamate was used as a substrate. In contrast, Sumegi *et al.* (1993) also measured more label in aspartate C2 than in C3, but here [4-$^{13}$C]glutamate was used as a substrate. A similar pattern has been seen between glial cells and yeast; where more label was detected in alanine C3 than C2 when yeast were fed with [3-$^{13}$C] propionate (Sumegi *et al.* 1990), while the reverse was true in glial cells fed with [1-$^{13}$C]glucose (Portais *et al.* 1993). Sherry *et al.* (1994) argued that this was the result of differences in the orientation of the molecules as they are passed between enzyme active sites during metabolic channelling. This could be due to differences in enzyme or metabolon structure between different organisms.

### *There is evidence that TCA cycle enzymes can form a complex in plants*

As outlined in the introduction, there are several ways that metabolic channelling of the TCA cycle could be achieved. The first is that intermediates could be trapped in a physical channel in the metabolon, which prevents diffusion into the bulk phase. Transfer of intermediates through this channel could be due to diffusion, or aided by electrostatic forces. The trapping of intermediates in a physical channel is most likely

to restrict the rotation of intermediates, particularly if the channel is narrow in relation to the substrate molecule (Raushel *et al.* 1998). The second model of metabolic channelling is one where the intermediate travels over the surface of the enzyme. This is achieved by a "track" of charged residues, which direct the intermediate to the correct active site (Knighton *et al.* 1994). In this case, the molecule may be more able to rotate as it moves over the surface of the metabolon, although restricted rotation cannot be ruled out. The third case is that the metabolic channelling effect comes from altered diffusion caused by the presence of an unstirred layer around enzymes which are co-localised to a membrane (Dueber *et al.* 2009). In this situation, we would expect that rotation would be able to occur, as only diffusion, not rotation, of the molecules would be restricted.

The experiments in this chapter cannot tell us anything directly about the way that metabolic channelling is occurring in the TCA cycle. However, the presence of a physical channel is supported by several pieces of evidence. There have been several studies looking at how metabolic channelling is achieved in artificially created fusion proteins of malate dehydrogenase, citrate synthase and aconitase in *S. cerevisiae*. These fusion proteins, created by introducing a short sequence of amino acids to link the proteins together, were shown to be able channel OAA in a cell-free system (Lindbladh *et al.* 1994). Elcock and McCammon (1996) used a modelling approach to investigate the possible mechanism of metabolic channelling in a fusion protein of malate dehydrogenase and citrate synthase. They modelled the most likely conformation of the fusion protein, based on the crystal structures of the same proteins in pig heart mitochondria. In their model the active sites of MDH and CS were

60 Å apart with the active sites facing away from each other, which would appear to make metabolic channelling unlikely. However, when simulations using Brownian dynamics were carried out, there was evidence that electrostatic effects were involved in metabolic channelling the substrate between the active sites. The model showed that a negatively charged substrate transferred between the two active sites 100 times more efficiently than a molecule with a positive or a neutral charge. It was suggested that this was due to an area of positive electrostatic potential at the interface between CS and MDH. When this electrostatic region was removed in the model less than 1% of the substrates were transferred successfully between the two active sites. When the reaction was modelled in reverse (so substrates travelled from CS to MDH) all the substrates remain trapped at the CS active site. This suggests that the channel may be unidirectional.

These results support the findings of Velot *et al.* (1997), who used graphic modelling software to investigate electrostatic interactions in a fusion protein of MDH, CS and ACO from porcine mitochondria. Here a positively charged electrostatic region also connected the active sites of CS and ACO in the model. This region also corresponded with a physical "groove" between the enzymes, which was between 12 Å and 29 Å in width: wide enough to accommodate a citrate molecule. Interestingly, when MDH was removed from the model, the electrostatic potential of this region was reduced, suggesting that all three enzymes may be important in maintaining metabolic channelling. In addition, these effects were not seen when cytosolic MDH was used instead of mitochondrial MDH.

Some aspects of these results have been confirmed experimentally (Shatalin *et al.* 1999). Fusion proteins from porcine enzymes (rather than yeast) were created and the metabolic channelling of OAA that occurred between enzymes was measured. It was found that when the ionic strength was increased the amount of metabolic channelling was significantly reduced, suggesting that metabolic channelling does indeed rely to some extent on electrostatic effects. There are several possible criticisms of these approaches. Firstly there is uncertainty around the structure of the fusion protein, which means that it is not clear whether the interaction models suggested by Shatalin *et al.* (1999) are accurate. Secondly as the fusion proteins are an artificial system it is not clear whether they accurately represent the situation *in vivo*. However, they do provide us with a possible model of metabolic channelling in the TCA cycle.

The results in this chapter provide only very little support to the idea that metabolic channelling occurrs through a physical channel. Although there was some difference in labelling in malate in *S. tuberosum* mitohcondria, it was relatively low, and the majority of the difference in labelling appears to be due to the presence of divalent cations in the experimental buffer. However, it does not rule out the possibility that channelling is occurring through a physical channel, as metabolites may still be able to rotate within a physical channel. On the other hand, although there has been no attempt to study the mechanism of metabolic channelling between SDH and MDH, it may not be unreasonable to assume that a similar mechanism to that described by Elcock and McCammon (1996) is present in other parts of the TCA cycle, as all the substrates have a net charge which would make them amenable to metabolic

channelling through electrostatic effects. However, the likelihood of this will be dependent on the structures and the interactions of the individual enzymes, meaning that further modelling or structural analysis would be necessary before it would be possible to draw any further conclusions.

## Summary

The general aim of this chapter was to investigate metabolic channelling in the TCA cycle by investigating the orientation conserved transfer of label between carbons in symmetrical molecules in the TCA cycle. The first aim was to directly compare the extent of metabolic channelling between 2OG, succinate and fumarate in the TCA cycle in *S. tuberosum* and *A. thaliana* using this method. The results show that although unequal labelling was measured in malate and aspartate in *S. tuberosum*, and malate in *A. thaliana*, the majority of this was removed after EDTA was included in the experiment. However, the ratio of label in C2/C3 in malate not reduced to 1.0 after EDTA was added in *S. tuberosum*, suggesting that a very small amount of malate may be restricted in its rotation, and this could be due to metabolic channelling. The results show that the ratio of label in C2/C3 in malate, and therefore the extent of metabolic channelling, was different in malate in *S. tuberosum* and *A. thaliana*, suggesting that the proportion of enzymes associated with a channelled pathway differs between species.

# Chapter 7: General discussion

## Summary of the work in this thesis

The aim of the work in the previous chapters was to investigate whether metabolite channelling occurred in the plant mitochondrial TCA cycle, and attempt to identify whether it had any role in regulating flux through the cycle. Using an isotope dilution experiment, I found that, in isolated mitochondria, citrate, fumarase and succinate did not have access to the active sites of some of the TCA cycle enzymes, suggesting that metabolite channelling was present in the TCA cycle. The results suggested that not all the enzymes of the TCA cycle were involved in metabolite channelling, with some being able to metabolise externally added unlabelled organic acids. We also tested whether the pattern of metabolite channelling changed in mitochondria which had been isolated from plants grown in the light and the dark, and from mitochondria treated with CCCP and KCN to alter the balance between respiration and biosynthesis. There were no substantial changes in the occurrence of channelling but only two replicate sets of experiments were done for each condition and the variation was such that it was not possible to draw quantitative conclusions about the extent to which metabolite channelling was involved in regulating the TCA cycle under these conditions.

## Further work

The experiments in this thesis only investigated metabolite channelling using an isotope dilution method. Given more time, it would have been interesting to complement this work with further experiment. For example, competing enzymes experiments would be helpful to confirm the presence of channelling, although this

may not be possible for all the enzyme pairs if there is not a suitable competing reaction which can be successfully introduced into the mitochondria.

Another useful extension of this work would be to investigate protein interactions in the plant TCA cycle. Interactions between TCA cycle enzymes have been identified in other eukaryotes, but very little published work in plants. A goal of this work was to investigate the role that metabolite channelling plays in regulating the TCA cycle. In this thesis we attempted to do this through measuring metabolite channelling under different conditions. An alternative way would be to disrupt the metabolon and then study the effects that this had on both the TCA cycle and on the metabolic and phenotypic state of the plant. However in order to do this it would be necessary to understand more about how metabolite channelling is achieved in the plant mitochondria. There are several possible ways that protein interactions in the plant mitochondria could be investigated. Pull-down experiments can be used to identify protein interactions between TCA cycle enzymes (Meyer *et al.* 2011). Previous experiments in yeast have isolated an intact TCA cycle metabolon after gentle disruption of the mitochondrial membrane (Robinson and Srere 1985, Barnes and Weitzman 1986). Another option would be to "tag" the enzymes, either with GFP (Haggie and Verkman 2002) or through immuno-gold labelling, which has been used with some success in experiments investigating metabolite channelling in the Calvin cycle (Süss *et al.* 1993). This approach could also be used to investigate whether the enzymes of the TCA cycle associate with the inner mitochondrial membrane in plants. However, it is likely that we would be working at the limit of resolution in both these cases, so success may be limited.

If information was known about the structure of the metabolon, the next useful experiment would be to investigate how the metabolon is held together or stabilised. One option would be to investigate how the metabolon can be disrupted by, for example, disrupting the inner mitochondrial membrane or selectively down-regulating the expression of individual enzymes that are involved in the complex. Another approach would be to model the structure of the metabolon using crystal structures of the enzymes, and disrupt "key" amino acids that might be important in the interaction. We could also test to see whether the enzymes were modified (such as by phosphorylation or acetylation or amino acids) when they were in a metabolon to the "free" state, or if they associated with any smaller proteins or molecules that might help to stabilise the complex. Knowing more about what mediates the formation of any TCA cycle metabolon will help to disrupt it and investigate the effect that it has on the TCA cycle.

The results in this thesis also suggest that there were two pools of enzymes in the mitochondria which supported metabolite channelling to differing extents, but it wasn't possible to tell whether these pools existed in the same or separate mitochondria. It would be interesting to investigate which of these scenarios is the most likely, although this is potentially technically challenging. One possible way would be to use bimolecular fluorescence complementation: if fluorescence was only observed in a sub-set of mitochondria this would suggest that the metabolon was not common to all mitochondria.

It would be interesting to know whether metabolite channelling was the same or different in different tissues either by direct measurement or through modelling.

Currently we are restricted in our ability to do this by direct measurement due to the difficulties of isolating mitochondria in different tissues and by the fact that we still understand relatively little about the different contributions of the TCA cycle in individual cells or tissues, although there have been studies looking at fluxes through the TCA cycle at the level of plant organs such as roots, leaves and embryos (Rocha *et al.* 2010; Tcherkez *et al.* 2009; Alonso *et al.* 2010). A good example of this is the uncertainty surrounding the presence of TCA cycle enzymes in the cytosol in different tissues and species. Several TCA cycle enzymes have been reported in the cytosol as well as the mitochondria in various plant tissues and species (Hodges 2002; Pracharoenwattana *et al*. 2010), but it is not clear whether the enzymes are present in all tissue types. Advances in technology, such as the development of tissue specific cell cultures and better fractionation techniques might help to improve this (Ehrhardt and Frommer 2012).

A different approach would be to use flux modelling to investigate channelling in the TCA cycle. As discussed in chapter 3, Williams *et al.* (2010) have recently developed a way to investigate channelling using steady state metabolic flux analysis. It would be interesting to use this approach to investigate channelling in the TCA cycle more fully and under different metabolic conditions.

Our work suggests that transport in and out of the mitochondria might be important in metabolite channelling. Certainly, if we are expecting metabolite channelling to be a response to changes in demand then we need to know more about transporters, where they are, what they are, how they work and whether enzymes of the TCA cycle interact with them. However currently only relatively few mitochondrial transporters

have been characterised, and our inability to explain the presence of metabolites in certain compartments suggest a major gap in our understanding about metabolite transport in cells (Krueger *et al.* 2011).

## Evolution of metabolite channelling

TCA cycle metabolite channelling has been identified in a number of eukaryotic phyla, including yeast (Sumegi *et al.* 1990; Kispal *et al.* 1989), several mammalian tissues (Sherry *et al.* 1994; Teller *et al.* 1990) and now plants. There is also evidence that it occurs in bacteria, including *B. subtillis* (Meyer *et al.* 2011) and *E. coli* (Barnes and Weitzman 1986). It is present in a variety of cell types within the same phyla – for example, mammalian liver, heart and neurones. Its presence across a range of distantly related phyla raises some questions about the evolution of metabolite channelling. Mitochondria are thought to be the result of a single endosymbiotic event (Sagan 1967), although the exact origin of both the host cell and the symbiont are the subject of considerable debate. There is also some debate as to the origin of the TCA cycle, with some TCA cycle genes thought to be host cell in origin, while others are thought to be more closely related to genes from the symbiont (Martin and Schnarrenberger 2001). It is not clear when or why metabolite channelling evolved in the TCA cycle. It could have been an ancestral state, which was conserved in subsequent lineages after divergence. Alternatively, it could have evolved multiple times in different eukaryotic phyla in response to similar problems or selection pressures.

The presence of metabolite channelling in all these phyla gives some support to the idea that metabolite channelling is of universal importance in the TCA cycle. If

metabolite channelling evolved many times in different organisms then this would suggest that it confers some selective advantage. Similarly, as TCA cycle enzymes are likely to be under very strong selection pressure (because their loss or disruption is usually extremely detrimental) any state which conferred a selective disadvantage is likely to have been lost. Understanding more about the structure of any TCA cycle metabolon, as well as the role that it plays, might help to distinguish between these two scenarios.

## Understanding metabolite channelling might aid successful engineering of the TCA cycle

A major aim of modern plant science is to try and link systems biology to synthetic biology (Smolke and Silver 2011): i.e. to use a model to accurately predict the best ways to engineer metabolic pathways. This knowledge could then be used to engineer plants to increase yields of valuable metabolic products or reduce the production of undesirable ones. The more information about metabolism the model uses (include the presence of metabolite channelling), the better and more predictive it is likely to be.

Understanding metabolite channelling in the TCA cycle may therefore be important in allowing us to successfully engineer metabolism. For example, one important strategic goal in plant biology is to increase the efficiency of N assimilation (Foyer *et al.* 2010). The TCA cycle plays an important role in this process via the production of citrate and 2OG. Ammonium produced in plants is assimilated via the GS/GOGAT cycle, which requires 2OG produced via the TCA cycle. One way to alter N assimilation might therefore be to try and change the flux through the 2OG-producing section of the TCA

cycle. 2OG is made from citrate either via cytosolic NADP-IDH, or in the mitochondrion via NAD-IDH, although it remains unclear which is the predominant route (Galvez *et al.* 1999; Lancien *et al.* 1999; Hodges *et al.* 2003). In either scenario metabolite channelling might be important in creating separate pools of citrate, which are either exported directly in the cytosol, or metabolised into 2OG in mitochondria. It is therefore important to know whether metabolite channelling has any effect on the regulation of flux through this pathway. For example, the two pathways may respond differently to regulation, or carry different fluxes. Because they duplicate the same pathway, it may be necessary to engineer both if one is trying to affect 2OG metabolism. Successful engineering may also need to consider post-translational modifications that affect metabolite channelling or metabolon formation, as well as engineering enzyme kinetics and abundance.

## Understanding metabolite channelling may improve our understanding of human diseases

In a more general way, understanding metabolite channelling may be useful in fields outside of plant science. Recent work has highlighted the important role that the TCA cycle plays in the development of human diseases, and as a result the pathway has become a target for therapies.

Cancer occurs when cells grow and proliferate without responding to the normal checks such as cell death. It has been long known that cancer cells exhibit changes in metabolism compared to normal cells from the same tissues (Warburg 1956). The traditional model of cancer development is that cancers develop from cells which have accumulated mutations in genes which are involved in cell cycle regulation or DNA

repair. These genes are usually referred to as proto-oncogenes or tumour suppressors, and generally encode proteins which are important in signal transduction (Ward *et al.* 2012). In the traditional model, the main role of these genes is thought to be to regulate the cell cycle. The resulting proliferation and un-checked growth of the cells from mutations in these genes leads to alterations in metabolism: for example, the ATP:ADP ratio decreases and the activity of certain enzymes is limited (Ward *et al.* 2012).

However, recent research suggests that this model of cancer development may not be correct in many cancer cells. Instead, the role of tumour suppressors and proto-oncogenes in controlling and regulating metabolism is thought to be crucial in the early stages of cancer (Ward *et al.* 2012). Many tumour suppressors and proto-oncogenes are thought to play an important role in growth factor signal transduction, which leads to changes in metabolism from predominantly catabolic to anabolic. There are theories that many tumour suppressors and proto-oncogenes initially evolved to regulate metabolism, with roles in cell cycle and proliferation evolving as secondary characteristics. The alternative model is as follows. Mutations in tumour suppressors and proto-oncogenes switch on a cascade of signalling that either increases the levels of growth factors, or alters signalling pathways as if growth factor levels had increased. Cells respond to this by altering metabolism to increase production of macromolecules. This in turn causes changes in the regulation of the cell cycle and initiates cell division and proliferation (Boer *et al.* 2010).

Mitochondrial metabolism, and in particular the TCA has been highlighted as playing an important role in this switch to anabolic metabolism. There is increasing evidence

that the PI3K/AKT/mTORC1 signalling pathway is particularly important in altering mitochondria metabolism in cancerous cells (Ward *et al.* 2010). When the pathway is activated glucose uptake into cells increase, Hxk and Pfk1 are activated (ensuring that the extra glucose entering the cell enters glycolysis), and glycolytic flux increases into the TCA cycle (Buzzai *et al.* 2005). Another effect of the up-regulation of the pathway is to increase the amount of citrate that is converted into fatty acids via the activation of ATP citrate lyase (Bauer *et al.* 2005). This may also have the effect of reducing the levels of citrate which build up in the cytosol, therefore ensuring that the negative regulation of glycolysis by citrate is minimised (Ward *et al.* 2010). The pathway is also thought to increase protein synthesis by up-regulating amino acid production through the TCA cycle. This may be particularly useful for therapies, as cancer cells appear to rely on the TCA for the majority of their amino acids, rather than using amino acids which are taken up from the surrounding tissue (Ward *et al.* 2010). Mutations in TCA cycle enzymes are also thought to be important: mutations in SDH and Fumarase have all been identified in various cancers (Ward and Thompson 2010; Selak *et al.* 2005; Eng *et al.* 2003), while mutation of cytosolic NADP-IDH is a strong marker for certain cancers (Yan *et al.* 2009).

TCA cycle metabolite channelling in neurones has been the focus of much research because of the role that it appears to play in sensing and metabolising glutamate. Glutamate metabolism is crucial in ensuring glutamate can work effectively as a neurotransmitter, and in preventing glutamate exito-toxicity which is a feature of strokes, traumatic brain injury and progressive diseases of the CNS (Yi and Hazell

2006). Changes in TCA cycle activities are seen in patients with Alzheimer's disease (Bubber *et al.* 2005) and schizophrenia (Bubber *et al.* 2004).

Regulation of the TCA cycle also appears to be important in insulin resistance, a key hallmark of type 2 diabetes. Skeletal muscle is a key site of insulin resistance in diabetics, and insulin resistance is correlated with incomplete oxidation of fatty acids in these tissues (Gaster *et al.* 2012). It is also characterised by an inability for the cells to switch between lipid and carbohydrate oxidation in response to insulin signalling (Gaster *et al.* 2012). Myotubes established from patients with type 2 diabetes exhibit both incomplete lipid oxidation and a reduced cyclic flux through the TCA cycle (Gaster 2009b). Artificially inhibiting the TCA cycle in normal myotubes has been shown to cause incomplete lipid oxidation (Janssen *et al.* 2006; Gaster, 2009b). The changes in the TCA cycle are thought to be bought about by post-translational modifications, as no difference in the protein levels or degradation rates were observed (Gaster *et al.* 2012). Reduced citrate synthase activity is also a reliable marker for insulin resistance (Ortenblad 2005). These all suggest that regulation of the TCA cycle might be important in the development of insulin resistance.

The above examples highlight the need for a greater understanding of the regulation of the TCA cycle in a variety of different diseases. If metabolite channelling is important in regulating the TCA cycle, then this could make it a target for future therapy.

### Metabolite channelling can be exploited through biotechnology

The previous examples have looked at the important of understanding metabolite channelling in order to more effectively engineer plants metabolism in situ. However

understanding metabolite channelling and metabolons also allows us to exploit it as a tool in biotechnology.

There are several features of metabolons that could be exploited by biotechnology. One of the targets of biotechnology is to introduce metabolic pathways from one organism in to another: for example, putting plant pathways into microbes to increase yield or reduce processing costs and therefore increase commercial viability (Ro *et al.* 2006). One difficulty with this approach is that the host cell may not be able to tolerate the metabolites that are produced. Metabolons may be useful in preventing such unstable or toxic intermediates from leaking out into the cell. For example, dhurrin is synthesised from L-tyrosine via p-hydroxymandelonitrile, which is toxic to most organisms. In *Sorghum bicolor*, 3 membrane bound complexed enzymes are required to produce dhurrin (Winkel 2004. When the full metabolon was introduced in *A. thaliana* (which does not normally produce dhurrin), dhurrin accumulated to 4% dry weight and there was no negative effect on the phenotype, but when a truncated metabolon was introduced into the cells the plants were severely stunted, and high levels of p-hydroxymandelonitrile were detected in the plant (Kristensen *et al.* 2005). Although this pathway naturally occurs as a metabolon, it shows the potential of using artificial metabolons to increase the success of engineering pathways with toxic intermediates into different organisms.

Metabolite channelling may also reduce loss of unstable or reactive intermediates and therefore increase yield or flux through a pathway. One way to achieve high yields it is to increase pathway flux but minimise the levels of intermediates which build up. Traditionally this is done by increasing the activity of individual enzymes in the cell

through enhancing protein expression, expressing recombinant proteins from other sources that have a naturally higher activity than the native enzyme, or by directly engineering enzymes through directed evolution (Zhang 2011). More recently, biotechnologists have started to use metabolons to increase fluxes through a pathway while at the same time ensuring that intermediates do not build up to inhibitory levels (Dueber *et al.* 2009). This can either be done by creating fusion proteins, or by attaching enzymes to molecular scaffolds (often DNA or RNA or proteins) or on cell membranes (Conrado *et al.* 2008). For example Dueber *et al.* (2009) achieved increases in the yield of mevalonate, an intermediate in isoprenoid biosynthesis, by attaching three enzymes of the metabolic pathway to a synthetic protein scaffold. This resulted in a 77 fold increase in yield, even though expression levels were low.

Another advantage of metabolons is that they can be artificially engineered to bring enzymes which do not normally interact together, thereby creating new pathways. For example, Fierobe *et al.* (2001) have published a number of "designer" cellulosomes which contain cellulases from different organism, and are able to lyse a wider variety of substrates than those found in nature.

There are several possible limitations to using metabolons in bioengineering. The first is that a lot of applications rely on a certain amount of modularity between metabolons. This may be true of, for example, certain metabolons of secondary metabolism which are able to switch their enzyme composition to make certain compounds (Crosby *et al.* 2011). However, many enzyme interactions are likely to be specific to particular pairs of enzymes, meaning that they can only interact with a few other enzymes. This may limit the extent to which they can be used in "designer"

metabolons, without undergoing modification by, for example, adding an interaction domain that allows them to be attached to a form of scaffold. The second limitation is that metabolons are likely to be most useful in increasing the rate of reactions in pathways that are diffusion limited, so that bringing the enzymes into closer proximity has an influence on the reaction rate. While this may be useful if reactions are carried out *in vitro*, it may mean metabolite channelling will have little effect in increasing the rate of reaction inside a cell if the reaction is not limited by diffusion *in vivo*.

Metabolite channelling in the TCA cycle has already been investigated as a way of improving the efficiency of biofuel cells. Biofuel cells use living organisms or enzymes to produce electrical energy from chemical reactions (Bullen *et al.* 2006). The field started in the 1960s, when it was recognised that microbes were capable of generating voltage and currents. Such biofuel cells were later developed into enzyme fuel cells, which use enzymes as a catalyst in the fuel cell (Bullen *et al.* 2006). There is interest in fuel cells because they have the potential to produce energy from commonly produced compounds such as sugars or ethanol. They are also candidates to power nanotechnology, particularly technology that may be used in or on living organisms (Halamkova *et al.* 2012). However, currently the technology is still in its infancy.

One of the main constraints on the effectiveness of enzyme biofuel cells is the fact that they tend to have a low power density (or amount of power produced per unit volume). As a result biofuel cells often need to be very large and use high concentrations of enzymes before they work effectively. Traditional enzyme fuel cells also tend to generate much lower oxidation rates than predicted (Moehlenbrock *et al.* 2011). Metabolite channelling has been used to try and improve these aspects of fuel

cells, much of which has been done using TCA cycle enzymes. Arechederra and Minteer (2008) used whole mitochondria to oxidise pyruvate, which resulted in increased levels of oxidation, but only small improvements in the power density of the cell. This was put down to the high volume per catalytic activity of the mitochondria (Moehlenbrock *et al.* 2011). Moehlenbrock *et al.* (2010) attempted to isolate the TCA cycle metabolon from mitochondria by disrupting the membranes, cross linking the enzymes together and then lysing the mitochondria. When the isolated cross linked enzymes were used in fuel cells there was an increase in the current density, but still only a modest increase in the power density of the cell (Moehlenbrock *et al.* 2011). The likely reason for this is that cross linking methods are non-specific, so the reaction mixture likely contained a variety of different enzymes. An aim of this technology would be to isolate pure TCA cycle metabolons, or artificially create them in order to increase the power densities of the cell. Understanding more about metabolite channelling in the TCA cycle, and how the metabolon is maintained and regulated may be beneficial to this technology.

## Summary

The work in this thesis suggests that channelling is present to some degree in the plant TCA cycle. Although it was not possible to say whether channelling is important in regulating the plant TCA cycle, the presence of metabolite channelling in many phyla gives some support to the idea that metabolite channelling is of universal importance in the TCA cycle. Further experiments may enable us to understanding more about any role that metabolite channelling plays in the TCA cycle in plants. Understanding metabolite channelling may be useful in fields outside of plant science, such as the development of human diseases and biotechnology.

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