Biochemical investigation of carbamylated proteins from Arabidopsis thaliana

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Abstract

Carbon dioxide (CO_2) plays a vital role in biological processes including photosynthesis, respiration, and multiple signalling pathways. CO_2 is able to interact with proteins by forming carbamates on neutral amine groups. However, due to the labile nature of this post-translational modification, carbamates are extremely difficult to observe and have therefore gone under-researched. The recent development of a novel technique to trap carbamates on proteins has led to identification of a range of carbamylated proteins from *Arabidopsis thaliana*. This thesis describes an investigation into the effect of carbamylation on the activity of two such proteins.

Fructose 1,6 bisphosphate aldolase (FBA1) is a metabolic enzyme that contains a carbamate modification on lysine 293. Activity assays conducted in the presence and absence of CO_2 indicated that the cleavage activity of FBA1 is not affected by CO_2 concentration. Similarly, analysis of subunit conformation indicated that the proportion of protein present as a tetramer was not affected by increasing the CO_2 concentration. However, investigation of a mutant that cannot form a carbamate, FBA1-K293A, suggested that the carbamylated lysine residue may important for protein function. Mutating residue 293 to glutamate also produced a protein with reduced activity compared with the WT. It was not possible to conclude from trapping experiments and mass spectrometry analysis whether the carbamate modification may still have been present on the WT protein during earlier experiments in the absence of CO_2.

Three putative carbamates have previously been identified on the non-specific lipid binding protein LTP1. The lipid binding affinity of LTP1 was investigated using the fluorescent probe TNS. This was found to be significantly reduced in the absence of CO_2. The mutant protein LTP1-K65A, which is unable to form one of the three putative carbamates identified on this protein, also had reduced binding in the absence of CO_2. Preliminary dose response experiments indicated that this protein does have a lower affinity for TNS than the WT, however the effect observed in the absence of CO_2 is likely to be due to the action of one of the other carbamates.

Although the effect of carbamate modifications on the function of these two proteins was not fully determined in this investigation, the results suggest that the recently

developed carbamate trapping technique provides an effective method for identifying functionally relevant novel carbamates.

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Declaration

The work presented in this thesis was carried out at the Durham University between October 2016 and September 2017. The work is my own original research unless otherwise indicated by statement or citation, and has not been submitted for any other qualification.

Statement of copyright

Acknowledgements

Firstly I would like to thank my supervisor Professor Martin Cann for his support throughout this project and his endless optimism that science will eventually work. I would like to thank Dr Phil Townsend for sharing his vast molecular biology expertise and Dr Victoria Linthwaite for all her guidance and encouragement even on my slowest of days. Thanks also to the other members of labs 234 and 229 for making this year so enjoyable, and to Mitchell Gillies, without whom I might still be lost in a tangle of formatting.

Abbreviations

ABA	Abscisic acid
Arabidopsis	*Arabidopsis thaliana*
Ca^{2+}	Calcium ions
Cl	Chlorine
CO_2	Carbon dioxide
CCS	Carbon capture and storage
Da	Daltons
DHAP	Dehydroxyacetone-phosphate
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionisation
FACE	Free-air CO_2 enrichment
FBA	Fructose 1,6 bisphosphate aldolase
FBP	Fructose 1,6 bisphosphate
GCA2	Controlled by ABA 2
GDH	α-glycerophosphate dehydrogenase
G3P	Glyceraldehyde-3-phosphate
HCO^{3-}	Bicarbonate ion
IPTG:	Isopropyl β-D-1-thiogalactopyranoside
K^+	Potassium ion
LTP	Lipid transfer protein
MS	Mass spectrometry
MW	Molecular weight
NaCl	Sodium Chloride
NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
$NaHCO_3$	Sodium bicarbonate
OC	Oleoyl coA
PC	Phosphatidylcholine
PCR	Polymerase chain reaction

PE	Phosphatidylethanolamine
RuBisCo	Ribulose bisphosphate carboxylase oxygenase
RuBP	Ribulose 1,5 bisphosphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SuBP	Sedoheptulose-1,7-biphosphate
SLAC1	Slow anion channel-associated 1
TBE	Tris/Borate/EDTA buffer
TEO	Triethyloxonium tetrafluoroborate
TNS	6-(*p*-Toluidino)-2-naphthalenesulfonic acid
TPI	Triosephosphate isomerase
UV	Ultraviolet
WT	Wild type

Chapter 1: Introduction

1.1 Overview

The research presented in this thesis is an investigation into the effect of carbon dioxide (CO_2) on the function of two proteins from *Arabidopsis thaliana*. These two proteins, Fructose 1,6-bisphosphate aldolase (FBA1) and non-specific lipid transfer protein 1 (LTP1), are both thought to interact with CO_2 by forming carbamates on specific lysine residues. Carbamates are post-translational modifications that have been shown to have a regulatory function for certain proteins. However, due to their labile nature, very little research has been undertaken investigating the role of carbamates in the function of proteins. This project aimed to elucidate the role of carbamates in the function of these two proteins, with the hope that this would increase our knowledge of how carbamates may function more generally within biological systems.

This chapter will include a general introduction to what is already known about the role of CO_2 in biological systems and will discuss why it is important for us to fully understand how rising CO_2 may affect plant growth. Finally, it will cover the process by which carbamates are able to form and which proteins are known to contain these post-translational modifications.

1.2 Rising atmospheric carbon dioxide

Atmospheric CO_2 is rising due to deforestation and the burning of fossil fuels. By 2100 the concentration of CO_2 in air may rise to as much as 1000 μmol mol^{-1} from pre-industrial levels of just 280 μmol mol^{-1}. This large increase in greenhouse gasses means that global temperatures will rise, precipitation will be more variable and extreme weather events will increase both in terms of frequency and severity (IPCC, 2014). All of these effects are likely to have a detrimental effect on crop yield across the globe. Understanding the effect of

this elevated CO_2 on plant growth is therefore of vital importance for predicting the future of crop security.

1.3 Carbon dioxide in biological systems

CO_2 is an essential substrate for photosynthesis, is produced as a by-product of respiration and is involved in acid-base homeostasis (Raines, 2003; Hetherington and Raven, 2005; Gutknecht *et al.* 1977). It is also known to play an important signalling role in many biological processes (Hetherington and Raven, 2005).

Inorganic carbon exists in solution in a pH - dependant equilibrium. Below pH 6.4, CO_2 is the dominant form of inorganic carbon, whereas above this pH bicarbonate (HCO_3) is more prevalent. At even higher pH values, above 10.3, CO_3^{-2} dominates. Carbonic anhydrases are important enzymes in biological systems including plants as they increase the rate at which these forms of inorganic carbon are interconverted (Moroney *et al*. 2001).

$$\mathbf{CO_2 + H_2O \leftarrow\rightarrow HCO_3^- + H + \leftarrow\rightarrow CO_3^{-2} + 2H^+}$$

Changes in CO_2 concentration are known to regulate multiple signalling processes in a variety of organisms (Taylor and Cummins, 2011; Hall *et al.* 2010; Sitt and Krapp, 1999). However, it is usually assumed that it is either the action of bicarbonate or of changing pH that has a physiologically relevant effect on protein function. Considerably less investigation has been done in to the direct effect of CO_2 itself on protein function.

1.3.1 Photosynthesis

The role of CO_2 as a substrate for photosynthesis is perhaps the most obvious use for this molecule in all of biological science. The primary mechanism for this process of carbon fixation in plants is the Calvin Cycle

(Raines, 2003) (Figure 1.2). Ribulose bisphosphate carboxylase oxygenase (RuBisCo) is the first enzyme to act in this pathway and catalyses the formation of two molecules of 3-phosphoglycerate by combining CO_2 and ribulose 1,5 bisphosphate (RuBP) (Andersson, 2008). This light-dependant cycle involves a total of 11 enzymes, resulting in regeneration of RuBP and the formation of compounds that are essential for plant growth and development (Raines, 2003).

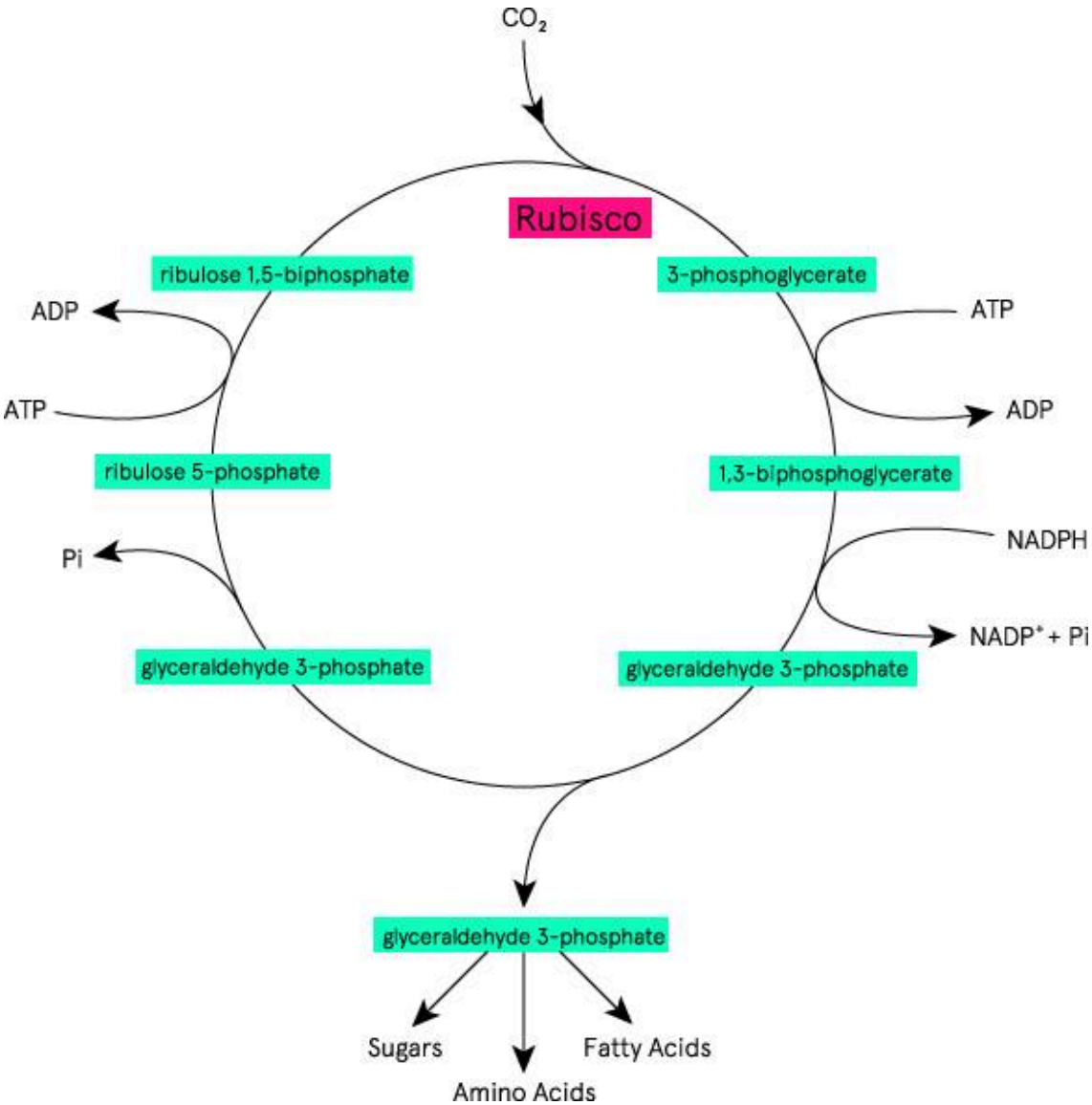


Figure 1-1: The Calvin Cycle. Rubisco initiates the cycle by catalysing carboxylation of RuBP to form 3-phosphoglycerate. As the cycle progresses RuBP is regenerated and glyceraldehyde 3-phosphate is produced, which goes on to form molecules important for growth.

1.3.2 Effect of CO_2 concentration on crop yield

As CO_2 is a primary substrate for photosynthesis, the process necessary for production of sugars required for growth, it could be expected that an increase in atmospheric CO_2 concentration would lead to an increase in carbon fixation, plant growth, and consequently crop yield. Early studies investigating impact of rising atmospheric CO_2 on crop species did indeed indicate that elevated CO_2 causes a considerable increase in yield (Kimball, 1983). However, these studies were conducted in controlled chambers and may not be representative of how plants would respond to increased CO_2 in the field. The development of free-air CO_2 enrichment (FACE) technology has allowed the effect of elevated CO2 to be observed in a more natural growing environment. This allows elevated levels of CO_2 to be maintained in open sections of field throughout entire growing seasons. Large scale meta–analysis of FACE experiments suggests that rising CO_2 may indeed increase agricultural yield, as well as increasing canopy temperature and reducing evapotranspiration (Kimball, 2016).

It is notable that C4 and C3 plants have been found to respond differently to rising CO_2, with C4 plants generally only showing a significant increase in yield when water was limiting. This is likely to be due the location of the Calvin cycle enzyme RuBisCo. In C3 plants such as wheat, RuBisCo is in cells in direct contact with the atmosphere and is not saturated with CO_2. However, in C4 plants such as maize, RuBisCo is located in bundle sheath cells in which localised concentrations of CO_2 are more than three times higher than atmospheric conditions (Long *et al.* 2006, Kimball, 2016). Therefore conditions of high atmospheric CO_2 such as those predicted for the end of this century may have a negligible effect on the yield of C4 plants, while leading to an increase in yield for C3 plants.

However, despite these comforting predictions that elevated CO_2 will increase crop yield, there is some controversy over the figures. Long *et al*. (2006)

argue that from their analysis, FACE experiments show less than half the additional yield from elevated CO_2 than was expected from the results of chamber experiments. They also state that FACE experiments don't show any increase in yield for the grain crops of Sub-Saharan Africa with increasing CO_2. As this area is likely to be one of those worst hit by the negative effects of climate change, the lack of any compensating increase in yield due to higher CO_2 levels could lead to serious challenges for food security. Additionally, meta-analysis studies of FACE experiments have been criticised as they may be significantly affected by reporting bias. Statistical analysis of these studies by Haworth *et al*. (2016) suggests that yield increases may have been overestimated. This could suggest that yield increase due to elevated CO_2 will not be sufficient to compensate for the detrimental effects of other aspects of climate change on crop survival. Additionally, research in to the nutrient content of plants grown under conditions of elevated CO_2 suggests that when the yield is increased, the concentration of nutrients in crops decreases (Loladze, 2014). Gaining a clearer understanding of how crops will be affected by elevated CO_2 is therefore extremely important.

1.3.3 CO_2 detection and signalling in plants

Elevated CO_2 has been shown to have numerous effects in plants. These include increasing the rate of photosynthesis, decreasing the amount of RuBisCo protein present in cells and decreasing plant nitrogen concentration (Sitt and Krapp, 1999).

CO_2 enters plant cells both by diffusion across the plasma membrane and via aquaporins such as NtAQP1. Expression of this aquaporin has been shown to increase both the permeability of the membrane to CO_2 and the rate of photosynthesis, indicating a physiologically significant role in CO_2 transport in to cells (Uehlein *et al.* 2003).

A particularly important role for CO_2 in plant cell signalling is in the regulation of stomatal aperture (Kim *et al*. 2010). Stomata are pores through which the CO_2 required for photosynthesis enters the plant. The size of the stomatal opening is regulated by a pair of guard cells in order to prevent excessive transpirational water loss (Esau, 1977). CO_2 and abscisic acid (ABA) signalling converge in guard cells leading to stomatal closure. This complex signalling pathway leads to transport of ions such as K+, malate and Cl^- across guard cell membranes, changing the osmotic potential and thus regulating stomatal aperture (Kim *et al*. 2010). Only a few mutants have been identified which show an altered stomatal response to CO_2. The *slac1* mutant is completely insensitive to CO_2, as well as to ABA and ozone. SLAC1 is therefore though to be a positive mediator of CO_2-induced stomatal closure (Vahisalue *et al*. 2008). SLACK1 is a subunit of anion channels and controls ion homeostasis in guard cells (Kim *et al*. 2010, Negi *et al*. 2008). The SLAC1 anion channel has been shown to be activated by bicarbonate, however the bicarbonate-binding proteins responsible for this activation have not been identified (Xue *et al.* 2011). HT1 kinase is the only known negative regulator of CO_2 induced stomatal closure*,* with *ht1-2* mutants showing a constitutive CO_2 response in stomatal aperture (Hashimoto, 2006) (Figure 1-2).

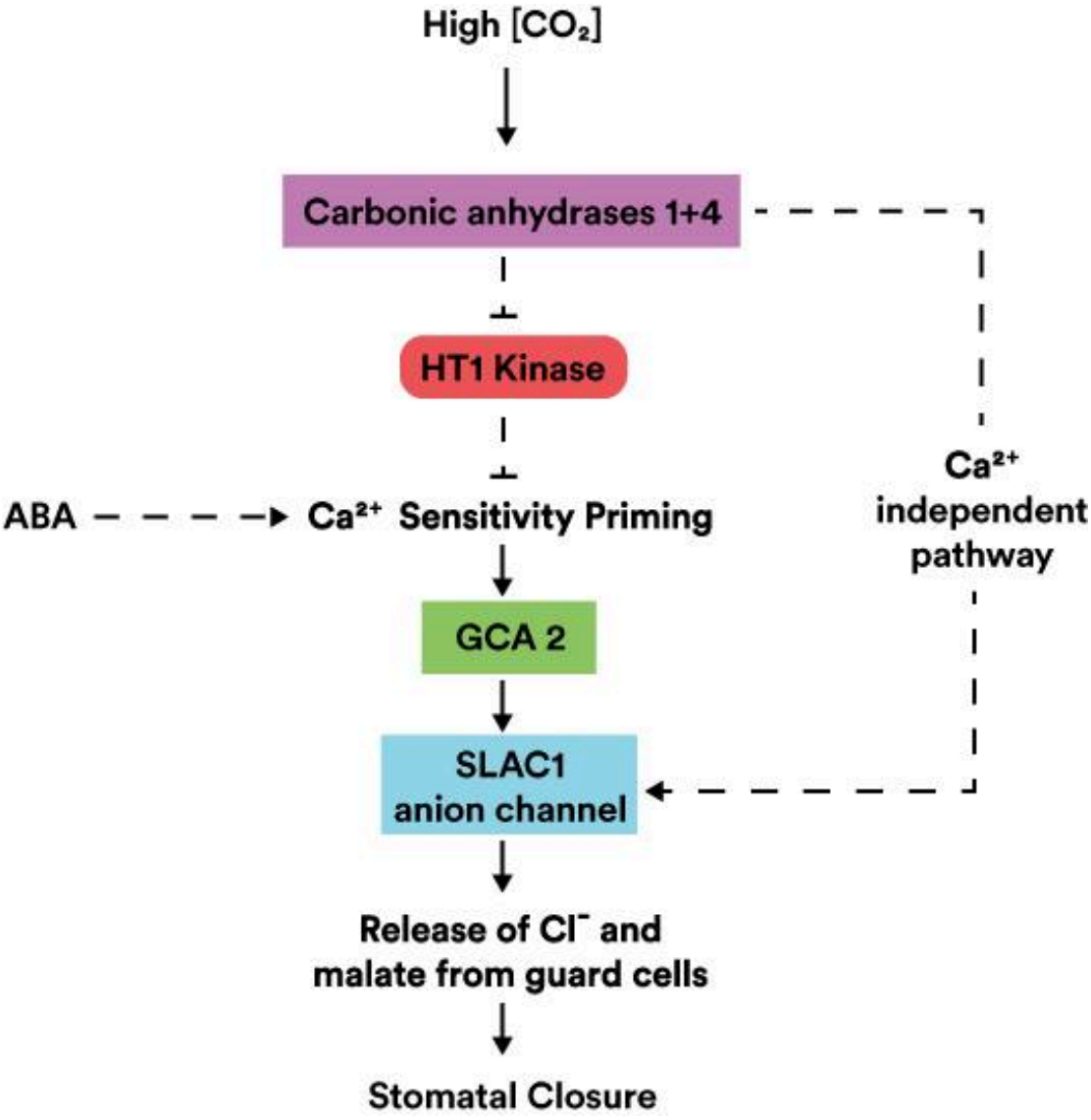


Figure 1-2: Simplified model showing convergence of CO_2 and ABA signalling leading to stomatal closure. Here carbonic anhydrases 1 and 4, GCA2 (Controlled by ABA 2) and the anion channel SLAC 1 are positive regulators of stomatal closure, while the HT1 kinase acts as a negative regulator of this process.

Additionally, long-term exposure to high CO_2 concentrations leads to a decrease in stomatal density, indicating a regulatory role for CO_2 in stomatal development (Xu *et al*. 2016, Woodward, 1987). The HIC gene has been identified as a negative regulator for stomatal development. Mutants for this gene were shown to have increased stomatal density when the concentration

of CO_2 was doubled (Gray *et al*. 2000). It is not yet known how CO_2 has this effect on this developmental pathway.

Another potentially important effect of elevated CO_2 is its influence on nitrogen metabolism. Numerous studies have shown that the efficiency of nitrogen use is increased in conditions of elevated CO_2. Additionally the concentration of nitrogen within the plant decreases when CO_2 levels are high. Many aspects of plant growth are regulated by nitrate and nitrogen metabolism. Therefore CO_2 may be influencing plant growth by changing nitrogen concentration and use efficiency (Sitt and Krapp, 1999).

As these examples demonstrate, CO_2 is known to be involved in multiple signalling pathways, which are required for various cellular processes. Elevated CO_2 is therefore likely to have a far more complex effect than simply causing an increase in the rate of photosynthesis. As such, gaining a better understanding of the role of CO_2 in plant growth and development at the molecular level is of vital importance for predicting the effect that rising CO_2 levels will have on crop yield.

1.4 Carbamylation

An important mechanism by which CO_2 may directly influence cellular processes is by interacting with proteins. CO_2 is generally an unreactive molecule, however it is able to combine with amine groups under ordinary conditions to form carbamates. These are labile post-translational modifications and are thermally unstable (Hampe *et al*. 2003). Carbamates can form either on the N-terminal amine of proteins or on the side chain of lysine residues. Nucleophilic attack of neutral amines on CO_2 leads to the formation of a carbamate (Figure 1-3). This reaction is in an acid-base equilibrium (Hampe *et al.* 2003). Carbamate formation was first detected over 100 years ago, and was one of the first post-translational modifications to be

identified (Jorgensen and Stiles, 1917). However, due to the labile nature of this modification, very little research has been done in to carbamylation as a means of regulating protein function.

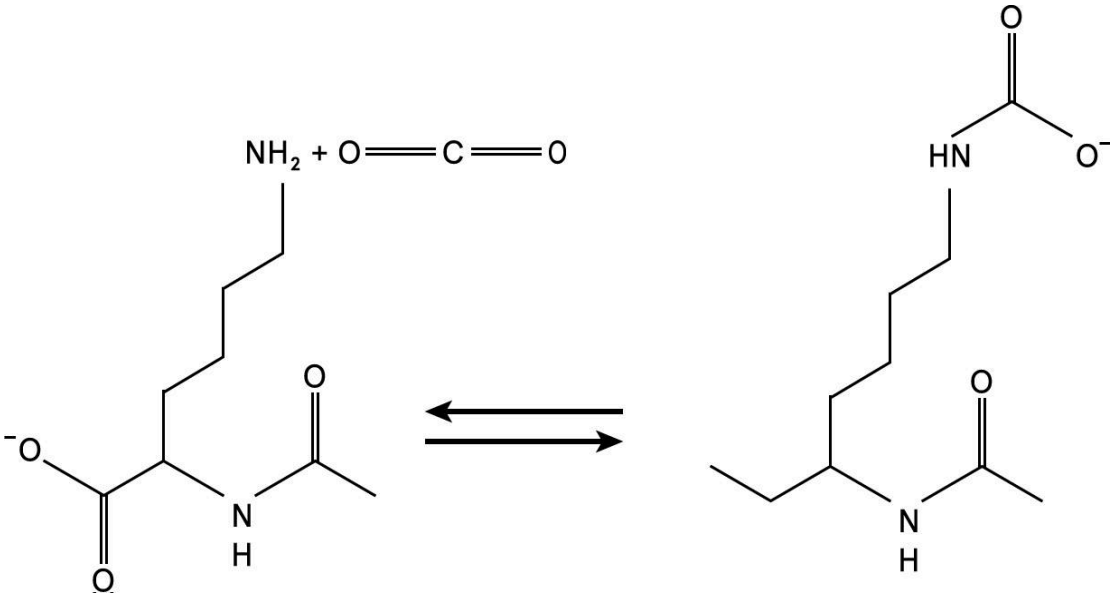


Figure 1.3: Reversible carbamate formation on a lysine residue. Carbamates can only form on uncharged amines.

Carbamylation may alter protein function in multiple ways. Carbamylation of an active site lysine creates an acidic residue, which can play a direct role in enzyme catalysis (Dementin *et al*. 2001). In other cases carbamylation can allow bridging of metal ions, causing conformational changes which are necessary for protein function (Stec, 2012). The first enzyme identified that is activated by carbamate formation was RuBisCo (Lorimer and Miziorko, 1980). In mammalian systems, the best-known functional example of carbamate formation is the interaction of CO_2 with the N-terminal amine group of haemoglobin chains resulting in a change in the oxygen binding properties of this protein (Vandegriff *et al*, 1991).

Basic conditions have been shown to favour carbamate formation, with the proportion of amino acid in the carbamylated form increasing with increasing pH (Stadie and O'Brien, 1935). This is because the amine group on lysine

must be in an uncharged state in order for carbamylation to occur. The pKa of the amine group on lysine varies with environment, averaging from 9-10 (Abraham *et al*. 2009). Therefore under normal conditions within plant cells most amine groups will be charged and thus unable to form carbamates. Nevertheless, carbamates are known to form on certain proteins. This may be due to microenvironments within folded proteins creating conditions more favourable to carbamate formation (Gros and Bauer, 1987).

Carbamylation may be a more common post-translational modification than is generally thought (Morrow *et al.* 1974). Recent computational models predict that at least 1.3% of large proteins may contain carbamylated lysine residues, suggesting that carbamylation may be an important general method for regulating protein activity (Jimenez-Morales *et al*. 2014).

Despite the great potential for carbamylation as a regulatory mechanism, very little work has been done to investigate this post-translational modification. This is mainly due to the challenges involved in observing carbamates. They are labile and the carboxyl group is released in acidic conditions, making this modification extremely hard to detect using mass spectrometry. Proteins containing labile modifications are not easy to crystallize, and identification of particular post-translational modifications in crystal structures is difficult (Jimenez-morales *et al*. 2014). Additionally, the conditions for crystallization may not be physiologically relevant. Because of these difficulties, the development of a method for stabilising carbamates on proteins was essential (Linthwaite, 2017).

1.5 Trapping carbamates

The recent development of soft ionisation techniques for mass spectrometry have made it possible to identify carbamate formation on proteins, however it was not possible to locate these post translational modifications to a specific

amino acid as the high energy required for fragmentation results in release of CO_2 (Terrier and Douglas, 2010). Carbamates have been successfully identified using mass spectrometry, however these occurred at pH values significantly higher than those found in biological systems so may not be physiologically relevant.

Linthwaite (2017) developed a novel method for trapping carbamates on proteins by ethyl esterification with the electrophilic reagent TEO. An ethyl group is transferred to the CO_2 molecule bound to an amine (Figure 1-4). This stabilises the modification and prevents release of CO_2. The trapped carbamate is then stable and can be detected using mass spectrometry. One of the major benefits of this method compared to previous work is that it enables trapping of carbamates to occur in solution under physiologically relevant pH and CO_2 concentration.

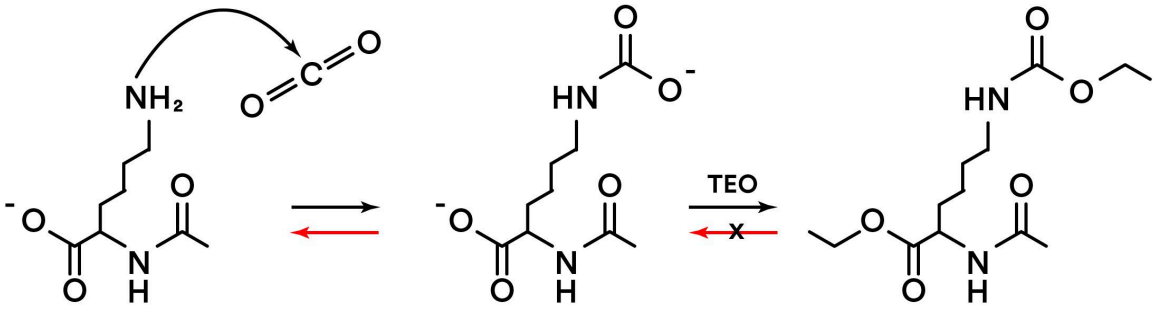


Figure 1-4: Ethyl esterification of a carbamylated lysine residue with the reagent TEO.

The reagent TEO has a half-life of 7.4 min (King *et al*. 1986), allowing the pH of the reaction to be maintained at physiological levels. This was achieved by the controlled addition of NaOH using a pH stat.

This technique was used to successfully trap a carbamate on the N-terminal amine group of haemoglobin, a protein that is already known to form a

carbamate (Perella and Rossi-Bernandi, 1980). It was then possible to observe this carbamate using mass spectrometry, demonstrating that this novel trapping technique provides an effective mechanism for identifying physiologically relevant carbamates. Linthwaite (2017) went on to use this method to screen the Arabidopsis leaf proteome, identifying carbamates on multiple proteins which were previously not known to interact with CO_2.

1.6 Carbamylated plant proteins

1.6.1 RuBisCo

The most abundant protein on earth, RuBisCo, is activated by carbamylation (Stec, 2012). This enzyme is found in almost all autotrophs and is essential for carbon fixation. It is responsible for combining CO_2 with RuBP to form two molecules of 3-phosphoglycerate (Figure 1-1), which is then used to create the molecules that are the building blocks of life (Andersson, 2008). In order to function, RuBisCo must be activated by carbamylation of a lysine residue within the active site. This carbamylated lysine is distinct from the binding site that of the substrate CO_2 (Lorimer, 1979). The carbamate is stabilised by the binding of Mg^{2+}, which is essential for catalysis. This regulatory carbamate was initially identified using $^{13}CO_2$ NMR spectroscopy studies (O'Learly *et al.* 1979). However it was not possible to determine which lysine residue was carbamylated using this method, and the conditions required for these experiments were such that non-specific carbamate formation could occur. X-ray crystallography was used to locate this carbamate to lysine 201 (Miziorko, 1979). The requirement for CO_2 and Mg^{2+} to activate this enzyme allows RuBisCo activity to be light regulated. In the dark, Mg^{2+} leaves the stroma and the pH declines, leading to a reduction in the proportion of RuBisCo that is activated (Andersson, 2008).

1.6.2 Urease

Urease is another common enzyme that is known to be carbamylated. This enzyme is important for nitrogen metabolism in plants and catalyses the hydrolysis of urea to form ammonia and carbamate (Pearson *et al*. 1998). This enzyme is carbamylated on a lysine residue in the active site, allowing the bridging of two nickel ions. This carbamate is vital for urease function and in the absence of CO_2 the enzyme is inactive (Yamaguchi and Hausinger, 1997).

1.6.3 Putative carbamates identified by Linthwaite (2017)

A novel method for trapping carbamates on proteins (described in 1.4) has lead to the identification of carbamates on a wide range of plant proteins following a screen of the *Arabidopsis* proteome (Linthwaite 2017). These proteins include a Lipid Transfer Protein, a peroxidase, Fructose bisphosphate aldolase 1 (FBA1), two chloroplast RNA editing factors and photosystem II subunit Q. The activity of some of these proteins has previously been linked to inorganic carbon, for example photosystem II is only active in the presence of a high concentration of dissolved CO_2. However, many of these proteins were not previously known to have any interaction with CO_2, and the affect that most of these observed carbamates have on protein function is unknown. Further work is required to identify the role of carbamylated lysine residues in the function of these proteins. The research discussed in this thesis investigates the effect of CO_2 on the function of two of these proteins.

1.7 Motivation for investigation

Carbamylation is an important mechanism for protein regulation which may be much more common than previously imagined. Carbamates are known to be vital for the function of two of the most abundant proteins on earth, RuBisCo in plants and haemoglobin in mammals (Stec, 2012; Vandegriff *et al*. 1991). Additionally, carbamates have now been identified on a wide range of proteins from *Arabidopsis* (Linthwaite, 2017). However, due to the labile nature of this post-translational modification, very little research has been undertaken to investigate the role of carbamates in protein function.

Many recently identified proteins which appear to form carbamates were not previously known to have any connection with CO_2 signalling. This project will investigate the role of these carbamates in the function of two such proteins: Fructose 1,6 bisphosphate aldolase 1 (FBA1) and Lipid transfer protein 1 (LTP1).

1.7.1 Project relevance

Although CO_2 is known to play a role in a wide range of biological processes, very little is known about the molecular mechanism involved. Atmospheric CO_2 is rising at an alarming rate and the subsequent effect on plant growth has not yet been fully elucidated. Although it is known that an increase in CO_2 leads to an increase in the rate of photosynthesis, meta-analyses of field studies undertaken in conditions of high CO_2 yield conflicting results about how much this affects overall plant growth (Kimbal, 2016; Long *et al.* 2006). Increased yield produced by conditions of elevated CO_2 has been shown to be accompanied by a decrease in the nutrient content of crop plants, posing a further threat to food security (Giri *et al.* 2016). Additionally, CO_2 is known to have other effects on plant cellular processes. For example, elevated CO_2 has been shown to result in decreased water uptake and changes to nitrogen

metabolism (Leakey *et al*. 2009; Sitt and Krapp, 1999). Gaining a better understanding of how CO_2 can affect plants at the molecular level is therefore of the utmost importance. This is necessary to improve predictions of how elevated atmospheric CO_2 may influence crop security.

Furthermore, in order to combat rising greenhouse gases, experts suggest we will need to develop efficient methods for carbon capture and storage (CCS) (Metz *et al*. 2005). Further investigation into the interaction of CO_2 and plant proteins could increase our understanding of how CO_2 regulates carbon fixation, perhaps allowing for development of more efficient CCS technologies.

Carbamylation is becoming increasingly identified as a method of regulating proteins in other organisms as well as plants. Recent evidence suggests that some mammalian connexins contain carbamylation motifs and may be involved in measurement of the partial pressure of CO_2 within the body (Meigh *et al*. 2013). Multiple bacterial enzymes have also been shown to require carbamate formation for proper function, including alanine racemase, which plays a vital role in synthesis of cell wall components (Morollo *et al*. 1999). Research in to the effect of CO_2 on cellular processes at the molecular level could also have clinical relevance, for example by increasing our knowledge of the mechanisms by which hypercapnia can affect the human body (Azzam *et al*. 2010). Increasing our knowledge of how CO_2 can affect protein function in plants may therefore improve our understanding of protein regulation across a wide variety of biological systems.

1.7.2 Aims and Hypotheses

We hypothesise that carbamates identified by Linthwaite (2017) on the *Arabidopsis* proteins FBA1 and LTP1 are functionally relevant. We therefore

also hypothesise that the activity of these proteins will be altered by changes in CO_2 concentration.

The main aim of this project was to investigate the effect of CO_2 on the known activity of these two proteins and determine whether these carbamates play a role in protein function. A broader aim of this project was to gain a better understanding of how increasing concentrations of CO_2 may affect plants at the molecular level.

Two main approaches will be implemented to achieve these goals. Firstly, the activity of the chosen proteins will be assessed in the presence and absence of CO_2 using appropriate activity assays. Secondly, mutant studies will be employed to further elucidate the effect of carbamylated lysine residues on the function of FBA1 and LTP1.

Chapter 2: Methods

2.1 Transforming cells

Plasmid DNA (1 μL) was added to competent *Escherichia coli (E. coli)* cells (50 μL, various strains) and then incubated on ice for 20 min. The cells were then heat shocked at 42 °C for 30 s and placed immediately back on ice. After 3 min, 1 mL LB broth was added to the cells and the solution was shaken for 1 h at 37 °C. 800 μL of the bacterial culture was spread on to an agar plate containing appropriate selective antibiotics and 200 μL on to another. Plates were incubated overnight at 37 °C.

2.2 Large scale expression of FBA1 protein

Rosetta PLysS cells were transformed with the plasmid *pet14b-FBA1, pet14B-FBA1-K293A* or *pet14B-FBA1-K293E*. LB broth (200 mL) containing ampicillin and chloramphenicol was inoculated with one plate colony of transformed cells and incubated at 30 °C with shaking overnight. 10 mL of overnight culture was added to each of 12 1 L LB preparations (also containing ampicillin and chloramphenicol). These cultures were incubated with shaking at 37 °C until the OD_{600} reached 0.4. 1 mL betaine was added to each culture. Incubation continued until the OD_{600} reached 0.6. Incubator temperature was then reduced to 17 °C for 1 h. 1 mL IPTG (20 mM) was added to each flask of culture to initiate protein expression and the cells were grown for a further 16 h at 17 °C. The cultures were harvested by centrifugation at 4000 g. The pellet resuspended in wash buffer (50 mM Tris, 100 mM Nacl, 1 mM EDTA) and centrifuged again.

2.3 Purification of FBA1 protein

Cells pellets were resuspended in binding buffer (50 mM Tris-Hcl, 100 mM Nacl, 10 mM imidazole). Protease inhibitor cocktail was added. Cells were lysed by sonication at 50 % power for 3 min. Cell lysate was centrifuged at 50,000 g for 30 min. Following centrifugation the supernatant was incubated on rollers at 4 °C with 50 % Ni^{2+} resin. The FBA1 recombinant protein contains a histag at the N-terminus, allowing binding to the Ni^{2+} resin. After 1 h of incubation the cell lysate was centrifuged at 700 g for 10 min and the supernatant discarded. Pellets containing the resin were resuspended in the binding buffer and transferred to a column with the flow through collected. The column was washed with binding buffer until no protein eluted. The sample was then eluted with buffer containing increasing concentrations of imidazole (from 50 mM to 250 mM) and each wash fraction collected and stored at -80 °C. The column fractions were then run on an SDS-PAGE gel and the concentration of protein present in each sample was determined from e_{280} values.

2.4 SDS-PAGE gel

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels were used to determine the molecular weight of proteins. Protein samples were mixed with loading buffer (100 mM Tris-HCl pH 6.8, 200 mM dithiothereitol, 4 % (w/v) SDS, 0.2 % (w/v) bromophenol blue, 20 % (v/v) glycerol) in a 4:1 ratio and incubated at 90 °C for 10 min to allow denaturation to occur. Protein samples and a protein ladder (Pageruler^{TM}Plus pre-stained) were loaded on to the gel. 15-20% resolving gels were used depending on the expected molecular weight of the protein of interest. The gel was then run in running buffer (25 mM Tris-HCl pH 7.5, 192 mM glycine, 0.1 % (w/v) SDS) for 1 h at 180 V. The gel was incubated with Generon Quick Coomassie with rocking for two hours to stain the protein.

2.5 FBA1 cleavage assay

All buffers were degassed and bubbled with nitrogen for 2 h before the reaction mixture was made up. 7 μg of purified FBA1 protein (WT, K293A or K293E) was added to 40 mM TBE buffer (pH 8) containing 0.2 mM B-NADH and 10 units of the coupling enzymes GDH/TPI. The reaction mixture was incubated under a nitrogen atmosphere for 20 min at 4 °C. $NaHCO_3$ (for reaction with 3 mM inorganic carbon) or NaCl (for control reaction without inorganic carbon) dissolved in 200 mM Hepes (pH 8) was added to a final concentration of 3 mM. The substrate FBP (2 mM) was added and the reaction mixed by pipetting. Absorbance at 340 nm was measured every 30 s for 5 min. The observed decrease in absorbance over the course of 5 min was used to calculate the specific activity of the enzyme.

2.6 Native-PAGE gels

Native-PAGE gels were used to assess the molecular weight of protein in its native conformation. Samples of FBA1 WT and K293A protein were mixed with Native Sample buffer in a 2:1 ratio. Protein and a protein ladder (Native Mark) were loaded on to the native page gel and run for 1 h with Tris-Glycine buffer at 140 V. For gels with increased CO_2, $NaHCO_3$ was added to the running buffer to a final concentration of 1 mM. The gel was then stained to allow visualisation of the protein.

2.6.1 polyhistidine stain

Native-PAGE gels were stained with a polyhistidine stain. Before the stain could be applied, gels were set with 10 % acetic acid and 40 % ethanol for 1 h. They were then incubated over night in the dark with NTA-Atto 647N. The

gels were washed with water and imaged using a Fujifilm FLA-3000 scanner with 633 nm excitation and 675 nm emission wavelengths.

2.6.2 Coomassie stain

Native-PAGE gels were incubated with Generon Quick Coomassie stain for 2 h. They were then washed with water and allowed to de-stain for 2 h before imaging.

2.7 One step PCR mutagenesis

Forward and reverse mutagenic primers were designed including the required mutation at the target site. PCR mixture was made up with the components listed in Table 1 and PCR performed according to the conditions described in Table 2. 5 μL of the PCR mixture was run on an agarose gel to confirm that amplification had occurred. 50 μL of competent Mac1T1 cells were transformed with 5 μL PCR product and grown over night at 37 °C on agar plates containing ampicillin. Colony PCR was then performed on seven of the colonies that grew on this plate to confirm the presence of the plasmid of interest.

Table 1. PCR reaction components

Component	Amount
5X HF buffer	5 μL
DMSO	1 μL
Forward primer	2 pmol
Reverse primer	2 pmol
2mM dNTPs	2.5 μL
Template DNA	1 ng
dH_2O	to total volume 25 μL

Table 2. PCR reaction conditions

Temperature °C	Time
98	120 s
18 cycles with the following settings:	
98	30 s
57	30 s
72	75 kb/s
Final extension:	
72	10 m

2.8 Colony PCR

PCR mixture was assembled as in Table 3. Half of a bacterial colony was removed from an agar plate, added to the PCR mixture and mixed well. PJET forward primer was used with the reverse mutagenic primer and PJET reverse primer was used with the forward mutagenic primer. 18 cycles of PCR were performed with the conditions described in Table 4. 5 μL of PCR product was then run on an agarose gel to confirm the presence of the plasmid of interest. Colonies containing the plasmid were grown over night in 5 mL LB broth. The plasmid was purified by mini-prep and stored at -20 °C.

Table 3. Colony PCR mixture components

Component	Amount
5x buffer	5 μL
25mM Magnesium Chloride	1.5 μL
Forward primer (25 pmol/μL)	1 μL
Reverse primer (25 pmol/μL)	1 μL
2mM dNTPs	2.5 μL
Gotaq G2 flexi polymerase	0.3 μL
dH_2O	13.7 μL

Table 4. Colony PCR reaction conditions

Temperature °C	Time
95	30 s
55	30 s
72	1 min/kb

2.9 Agarose gel

1.5 g of agarose was added to 100 mL TAE buffer and microwaved for 1 min. 0.1 mg ethidium bromide was added and mixed with buffer. The gel was poured and allowed to cool. 25 μL of samples containing DNA were added to wells in the cooled gel. The gel was run in TAE buffer for 1 h at 120 V. DNA was then visualised under UV transillumination.

2.10 Plasmid purification by mini-prep

A mini-prep spin kit (QIAGEN) was used for plasmid purification. Cells were grown over night in 5 mL LB containing appropriate selective antibiotics. The culture was centrifuged at 4000 g for 5 min. The supernatant was discarded and the cell pellet was resuspended in 250 μL Resuspension buffer. The sample was transferred to a mirocentrifuge tube and 250 μL Lysis buffer was added. The tube was inverted 4 times to mix. 350 μL Neutralisation buffer was added and the sample mixed by inverting the tube 6 times. The tube was centrifuged at 14,000 g for 5 min and the supernatant transferred to a spin column. The spin column was centrifuged at 14, 000 g for 1 min. 500 μL wash solution was added and the column was centrifuged for 1 min at 14,000 g.

The flow through was discarded and the column was centrifuged once more for 1 min to ensure residual solution was removed. The column was moved to a clean microcentrifuge tube and 50 μL of dH_2O added. After incubation for 2 min the column was centrifuged for a further 2 min to collect the DNA.

2.11 Test expression of LTP1

BL21 and *Tuner-Rosetta E.coli* cells were transformed with *pet14b-LTP1* and grown overnight on plates containing 0.4 % (w/v) glucose and appropriate selective antibiotics. One colony of each cell type was transferred to 5 mL LB, again containing 0.4 % (w/v) glucose and antibiotics, and grown overnight with shaking at 37 °C. 250 mL of LB (containing 0.4 % (w/v) glucose and antibiotics) was inoculated with 3 mL overnight culture. Cells were then incubated at 37 °C with shaking until the cell density reached an OD_{600} of 0.5. Protein expression was then induced with either 2 mM or 0.2 mM IPTG. The culture was grown for a further 3 h at 37 degrees C. 1 mL samples were taken before induction, after 1 h and after 3 h. These samples were centrifuged at 6000 g for 5 min and the supernatant discarded. The cells were resuspended in 100 μL wash buffer (50 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.4) and sonicated for 5 s at 25 % power. The samples were centrifuged once more at 6000 g and the supernatant and pellet were separated. Both the pellet and supernatant from all samples were run on a SDS-PAGE gel.

2.12 Large scale expression of LTP1

Tuner-Rosetta cells were transformed with *pet14B-LTP1* or *pet14B-LTP1-K65A*. LB broth (250 mL) containing 0.4 % (w/v) glucose and the antibiotics ampicillin and chloramphenicol was inoculated with one colony of transformed cells and grown over night at 37 °C with shaking. These cells were then used to inoculate 12 1L LB preparations containing the above antibiotics and 20 mM glucose. Cells were grown at 37 °C with shaking until they reached an

OD_{600} of 0.7. Protein expression was induced with the addition of 0.2 mM IPTG. The cultures were grown for a further 3 hours at 37 °C. Cells were harvested by centrifugation at 4000 g. The pellets were resuspended in wash buffer (50 mM Tris, 100 mM NaCl, 0.1 mM EDTA, pH 7.5) and centrifuged once more at 4000 g to wash. Supernatant was discarded and the pellets were stored at -80 °C.

2.13 Refolding LTP1 protein from inclusion bodies

Cell pellets were resuspended in lysis buffer (PBS containing 1 % Triton X 100 and 350 μg/mL Lysozyme, pH 7.3) and sonicated at 20 % power for 4 min. The cell lysate was then centrifuged at 20,000 g for 25 min at 4 °C. The supernatant was discarded and the pellet resuspended by sonication in PBS containing 1% Triton X 100 (v/v). Lysate was centrifuged again using the above conditions. This was repeated a further two times. In the final sonication step the pellet was resuspended in PBS without Triton X 100. After the final centrifugation, pellets were resuspended in PBS containing 6 M urea and incubated over night on rollers at 4 °C to resolubalise the protein. The lysate was then diluted 1:10 with PBS to allow refolding of the protein.

2.14 Purification LTP1

Refolded protein in PBS was centrifuged at 20,000 g for 25 min. Following centrifugation the supernatant was incubated on rollers with 50 % Ni^{2+} resin for 2 h. This was then centrifuged at 700 g for 10 min and the supernatant discarded. The resin was resuspended in binding buffer (50 mM Tris, 100 mM Nacl, 10 mM imidazole) and transferred to a column. The column was washed with binding buffer until no protein eluted. The sample was then eluted with buffer containing increasing concentrations of imidazole (from 50 mM to 1 M)

and each wash fraction collected. The column fractions were then run on a 20 % SDS-PAGE gel.

2.15 LTP1 TNS binding assay

Measurement buffer was prepared (175 mM mannitol, 0.5 mM K_2SO_4, 0.5 mM $CaCl_2$, 5 mM MES at pH 7). TNS dissolved in DMSO was added to the measurement buffer to a final concentration of 10 μM and mixed by shaking for 1 min. 4 μM LTP1 protein (WT or K65A) was added to the mixture and mixed by pipetting. The sample was transferred to a 96 well plate and florescence was measured (excitation wavelength 320 nm, emission wavelength 437 nm). This was compared to a control sample containing no protein. This assay was performed both on the bench at atmospheric CO_2 concentration and in the absence of CO_2 in a nitrogen atmosphere cabinet and the results were compared.

2.16 LTP1 TNS binding dose response curve

An assay was performed as above with varying concentrations of TNS from 0.625 μM to 160 μM. Results were plotted on a curve and the K_d of LTP1 calculated.

2.17 LTP1 lipid binding assay

4 μM WT LTP1 was added to measurement buffer containing 10 μM TNS. Biological lipid (Oleoyl coA, phosphatidylcholine or phosphatidylethanolamine) was added to a final concentration of 18 μM and the sample was shaken for 1 min to mix. This assay was adapted from Bogdanov *et al.* 2016, and concentrations of substrates used were the same as in this paper. The

fluorescence of the sample (excitation wavelength 320 nm, emission wavelength 437 nm) was compared with the fluorescence of a sample with no added lipid and the decrease in florescence calculated. A greater decrease in florescence indicated a greater decrease in TNS binding to LTP1 and thus greater binding of the biological lipid to the protein. Biological lipids used in this assay were of bovine origin and are all commercially available from Sigma Aldrich.

2.18 Carbamate trapping on FBA1

FBA1 protein (WT) was dialysed overnight in to 50 mM phosphate buffer (pH 7.4) and then concentrated to 0.5 mg/mL. 350 mg of the reagent TEO was measured out. A third of this was dissolved in 0.33 mL phosphate buffer (50 mM, pH 7.4) and added to the protein. Once the pH of the mixture stabilised, the remaining two thirds of the reagent were added one by one in the same way. The reaction was incubated at room temperature with stirring for 30 min. A pH stat was used to slowly add 1 M NaOH to the mixture during the course of the reaction ensure a stable pH of 7.4 was maintained. Once the reaction was complete the protein sample was dialysed over night in to dH_2O. An aliquot of this sample was digested using trypsin and analysed using mass spectrometry.

2.19 Protein Digestion

Protein was digested using the FASP protein digestion kit (Expedeon) and all materials were provided with this kit. 30 μL of trapped protein sample and 200 μL of Urea Sample Solution were added to the spin filter provided and centrifuged at 14,000 g for 15 min. 200 μL of Urea Sample Solution was added and the sample centrifuged again. 10 μL of 10X Iodoacetamide Solution and 90 μL of Urea Sample solution were added to the spin filter and mixed by shaking for 1 min. The spin filter was then incubated in the dark for

20 min before centrifugation for 10 min. 100 μL of Urea Sample Solution was added and the spin filter centrifuged for 15 min. This step was repeated a further two times. 100 μL of Ammonium Bicarbonate Solution (50 mM) was added and the sample centrifuged for 10 min. This step was also repeated twice. 75 μL Digestion Solution (4 μg trypsin in 75 μL of Ammonium Bicarbonate Solution) was added to the sample and mixed by shaking for 1 min. The top of the spin filter was wrapped in parafilm to prevent evaporation and the sample was incubated over night at 37 °C. The spin filter was then transferred to a new collection tube. 40 μL Ammonium Bicarbonate Solution was added and the spin filter was centrifuged for 10 min. This step was repeated once more. 50 μL Sodium Chloride Solution (0.5 M) was added and the sample centrifuged for 10 min. The filtrate contained the digested protein.

2.20 ESI-MS

Protein samples were prepared in an MS compatible ABC buffer and run with a 2 h gradient of acetonitrile from 2-80 % containing 1 % formic acid.

2.21 Mass-spectrometry data analysis

Data from ESI-MS was analysed using the GPM database X!Tandem. The Arabidopsis peptides present in the sample data were searched for the transfer of an ethyl group (MW 28.0313) and a trapped carbamate on a lysine residue (MW 72.0211).

Chapter 3: FBA1

3.1 Overview

Fructose 1,6 bisphosphate aldolases (FBAs) are ubiquitous plant enzymes involved in multiple metabolic processes. FBAs catalyse the reversible cleavage of fructose 1,6 bisphosphate (FBP) to form dehydroxyacetone-phosphate (DHAP) and glyceraldehyde-3-phosphate (G3P). This cleavage reaction takes place in the cytoplasm as part of both glycolysis and glucogenesis (Lu *et al*. 2012). In the stroma of chloroplasts, FBA acts as part of the Calvin cycle catalysing the condensation of both FBP and sedoheptulose-1,7-bisphostphate (SuBP) (Fleshner *et al*. 1999). These enzymes are also thought to play a role in sugar signaling, the response to abiotic stresses such as drought and high salinity, and processes in plant development (Lu *et al*. 2012; Geng-Yin *et al*. 2017). FBAs can be divided in to two classes based on their catalytic mechanism. Class I aldolases act as tetramers and require an active site lysine which is essential for catalysis and stabilizes a reaction intermediate, whereas class II aldolases form dimers and require the presence of a metal ion for catalytic activity (Perham, 1990). The *Arabidopsis* genome contains eight FBA genes. FBA1 is a class I aldolase expressed in the shoots of *Arabidopsis*. The amino acid sequence for this enzyme contains a plastid location signal peptide indicating that it is likely to function within the chloroplast. The expression of this enzyme has been shown to be affected by various abiotic stresses indicating a potential role for FBA1 in stress responses (Lu *et al*. 2012).

A previous screen of the *Arabidopsis* proteome identified a carbamate on the lysine residue at position 293 in FBA1 (Linthwaite, 2017). Following ESI mass spectrometry this carbamate was confidently located using two different data analysis software. Its presence was confirmed using purified and active FBA1 protein in the presence of 1 mM inorganic carbon. This results in approximately the same CO_2 concentration as is found in *Arabidopsis* cells at physiologically relevant pH. Preliminary experiments indicated that this

carbamate may have an effect on the activity of this enzyme (Linthwaite, 2017).

The formation of a carbamate on FBA1 is of particular interest, not only because of the biological significance of this enzyme, but also because this enzyme plays a role in the same process as RuBisCo. RuBisCo is known to require activation by carbamylation to perform its role in the Calvin cycle (Stec, 2012). The presence of a carbamate on another Calvin cycle enzyme could potentially indicate that carbamylation is a more common method of regulating the function of photosynthetic enzymes than previously thought. Additionally, from a more practical point of view, FBA1 is a good choice of carbamylated protein for further investigation as it has previously been expressed and purified in a soluble active form and its activity is relatively straightforward to assay (Linthwaite, 2017).

This chapter will discuss the expression and purification of recombinant FBA1, assaying the activity of this protein and investigation of its subunit conformation.

3.2 Expression of FBA1

The amino acid sequence for WT FBA1 is shown below with the carbamylated lysine residue and plastid-targeting signal highlighted (Figure 3-1). A truncated version of this protein with the plastid-targeting signal removed has increased solubility and was therefore used for all experiments (Linthwaite, 2017).

[MASSTATMLKASPVKSDWVKGQSLLLRQPSSVSAIRSHV
APSALTVRA]ASAYADELVKTAKTIASPGHGIMAMDESNAT
CGKRLASIGLENTEANRQAYRTLLVSAPGLGQYISGAILFE
ETLYQSTTDGKKMVDVLVEQNIVPGIKVDKGLVPLVGSYD
ESWCQGLDGLASRTAAYYQQGARFAKWRTVVSIPNGPS
ALAVKEAAWGLARYAAISQDSGLVPIVEPEIMLDGEHGIDR
TYDVAEKVWAEVFFYLAQNNVMFEGILLKPSMVTPGAEAT
DRATPEQVASYTL**K**LLRNRIPPAVPGIMFLSGGQSELEATL
NLNAMNQAPNPWHVSFSYARALQNTCLKTW

Figure 3-1: FBA1 protein sequence. The plastid targeting sequence is highlighted in grey and the site of carbamate formation in red.

FBA1-WT was expressed and purified according to the protocol developed by Linthwaite (2017) (2.1-2.3). The recombinant protein contains a polyhistidine tag allowing purification by nickel affinity chromatography. Wash fractions produced by the purification process were run on an SDS-PAGE gel to confirm the presence of FBA1 (Figure 3-2). This protein can be seen in all fractions at its predicted MW of 43 kDa. From analysis of the bands on the gel it was clear that the greatest concentration of FBA1 protein was present in the fraction eluted with 100 mM imidazole. This fraction was not very pure and contained a considerable amount of contaminant. The fraction eluted with 250 mM imidazole appeared to contain pure FBA1, however this was present only at a low concentration.

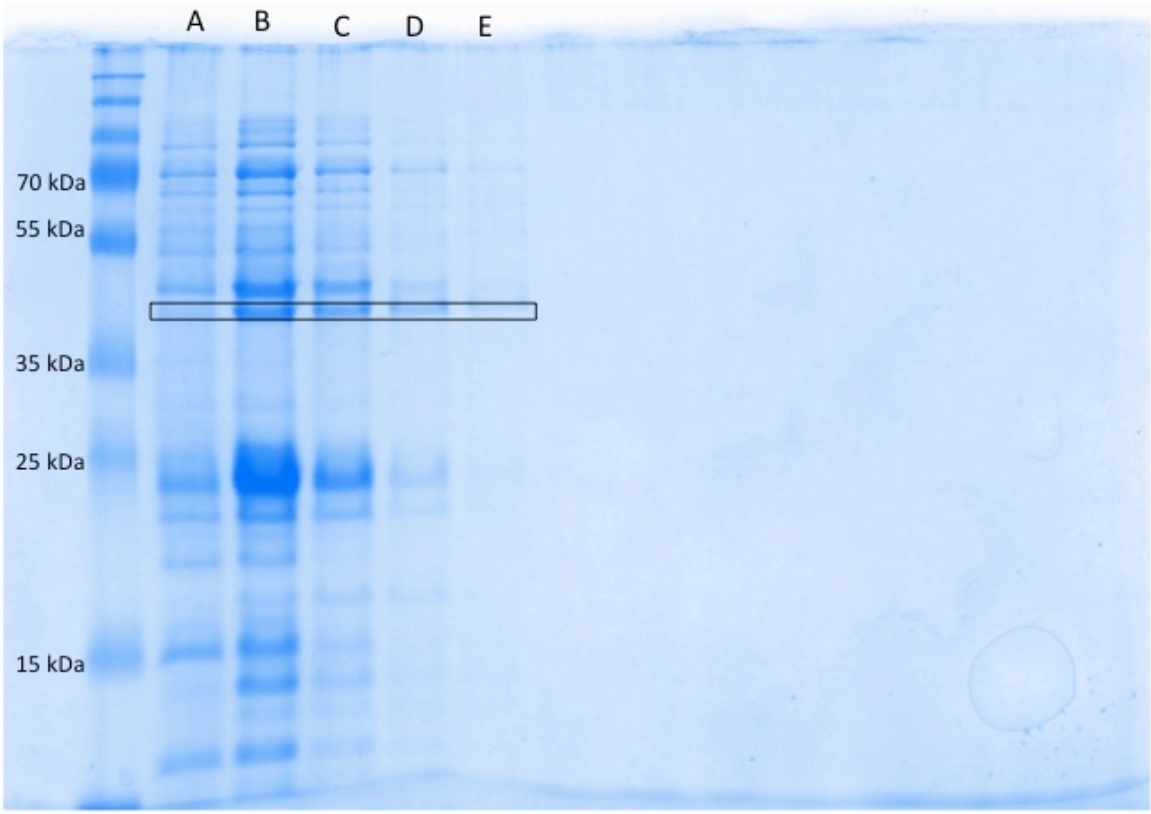


Figure 3-2: SDS-PAGE gel of wash fractions collected from FBA1-WT purification. Fraction A was eluted with a buffer containing 50 mM imidazole, B with 100 mM, C with 150 mM, D with 200 mM and E with 250 mM. FBA1 can be seen at the predicted MW of 43 kDa.

A mutant enzyme, in which the carbamylated lysine residue was mutated to an alanine (FBA1-K293A), was expressed and purified simultaneously according to the same protocol. An alanine residue was chosen to replace the lysine as this amino acid is inert, so should not have unwanted effects on protein function.

3.3 FBA1 Cleavage activity assay

It was hypothesised that the presence of a carbamate on lysine 293 of FBA1-WT would affect the specific activity of this enzyme. Therefore it was expected that the specific activity of the aldolase would be higher in the presence than in the absence of CO_2. Additionally, it was hypothesised that the specific

activity of the mutant protein FBA1-K293A remain constant with changing CO_2 concentration and would be comparable with that of FBA1-WT in the absence of CO_2.

In order to test these hypotheses, an enzyme assay was developed to allow the cleavage activity of FBA1 to be measured.

3.3.1 Mechanism

The cleavage reaction catalysed by FBA1 (Figure 3-3) occurs as part of glycolysis and glucogenesis in all organisms (Lu *et al*. 2012). The substrate FBP interacts with catalytically important lysine and cysteine residues in the active site of the enzyme leading to the release of G3P. A histidine residue then interacts with the remaining chain to form DHAP.

Figure 3-3: FBA1 cleavage reaction

In order to assay the cleavage activity of FBA1 this reaction was coupled to oxidation of reduced nicotinamide adenine dinucleotide (NADH) to form NAD^+ using the coupling enzymes α-glycerophosphate dehydrogenase (GDH) and triosephosphate isomerase (TPI) (Figure 3-4). NADH absorbs light at 340 nm whereas NAD^+ does not (Dawson, 1985). Therefore a decrease in absorbance at 340 nm can be measured and used to calculate the rate of the cleavage reaction.

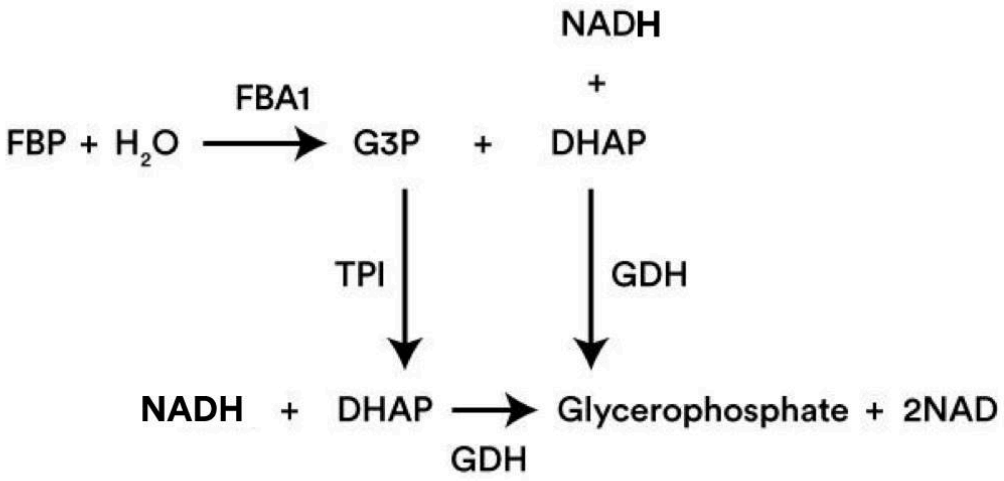


Figure 3-4: Cleavage reaction coupled to oxidation of NADH

3.3.2 Testing activity of purified protein wash fractions

In order to confirm that the purified FBA1 protein produced in 3.2 was active, the above cleavage assay was used to determine the activity of the FBA1 enzyme present in each of the wash fractions produced during purification. WT or K293A protein from each of the wash fractions was mixed with appropriate buffers and the substrate FBP was added to start the reaction. A control sample containing the same concentrations of buffer and substrate but no aldolase was also prepared for comparison. The absorbance of the reaction mixture was read every 30 s for 20 min. The results of these experiments are shown in Figure 3-5.

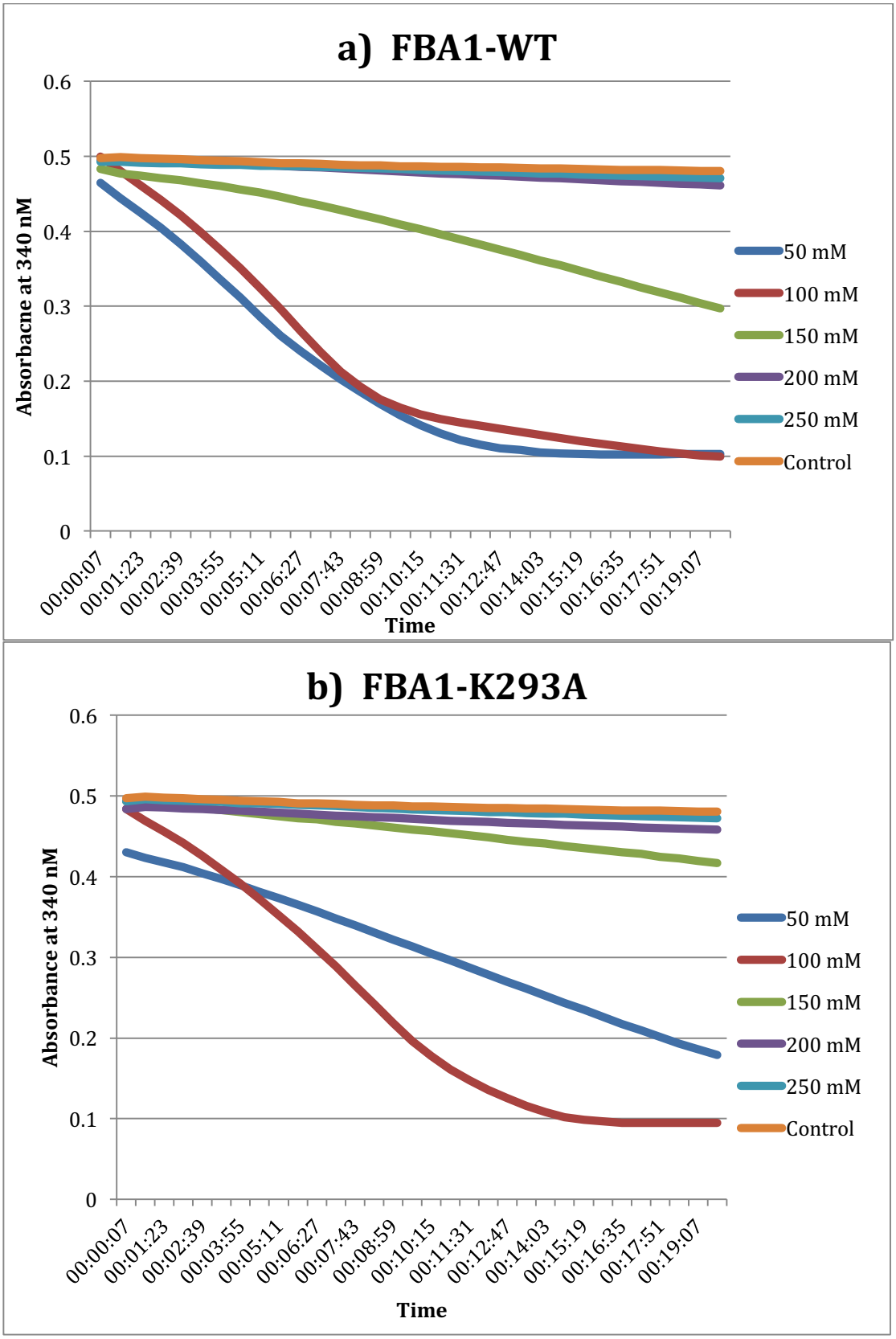


Figure 3-5: Activity assay of FBA1-WT (a) and FBA1-K293A (b) wash fractions produced by purification compared with a control sample containing no aldolase protein

Figure 3-5 demonstrates the change in the absorbance of each sample over the course of the cleavage reaction. A greater decrease in absorbance indicates higher cleavage activity of the enzyme. Some aldolase activity was observed in all fractions for both the mutant and the WT enzyme when compared with the control which contained no aldolase protein. This indicated that the purified protein was active and in the correct conformation. For the WT protein, the highest cleavage activity was observed in the wash fraction eluted with 50 mM imidazole, closely followed by 100 mM imidazole (figure 3-5). For the K293A mutant protein, the fraction eluted with 100 mM imidazole showed the highest activity (figure 3-6). The purest fraction (eluted with 250 mM imidazole) showed almost negligible activity for both proteins. Due to the high cleavage activity observed in this fraction for both FBA1-WT and FBA1-K293A, the 100 mM fraction was chosen for future cleavage assays.

3.3.3 Assaying activity in the presence and absence of CO_2

In order to investigate the effect of carbamate formation on the cleavage activity of FBA1, both the WT and K293A proteins were assayed in the presence and absence of CO_2 (2.5). This was achieved by the removal of CO_2 from the reaction mixture followed by the addition of either 3 mM inorganic carbon in the form of $NaHCO_3$ or 3 mM NaCl as a control. Complete removal of CO_2 from the system presented a challenge to this investigation. All buffers were degassed and bubbled with nitrogen for 1 h prior to assembly of the reaction mixture. The reaction mixture, including the FBA1 protein, was incubated within a dessicator containing nitrogen gas for 30 min before the addition of the substrate to ensure any CO_2 already bound to the protein dissociated. The reaction mixture was kept at 4 °C throughout this process to ensure the enzyme remained active. $NaHCO_3$ was added to reintroduce CO_2 to the system. This was added immediately before the reaction was started in order to reduce CO_2 loss by degassing. For control experiments in the absence of inorganic carbon, 3 mM NaCl was added instead. The assay was

started with the addition of 40 μM of the substrate FBP. This low concentration was chosen as it allows the effect of CO_2 concentration on the K_m of the enzyme to be investigated (Linthwaite, 2017).

The reduction in absorbance of the reaction mixture was measured over the course of 5 min. This was compared to a standard curve of NADH absorbance to allow calculation of the amount of NAD^+ (mol) produced during the course of the reaction. The amount of product (mol) formed during the cleavage reaction is known to be equal to half of the amount of NAD^+ produced. The amount of product formed was then used to calculate the specific activity (the amount of product produced per min per mg total protein) of each enzyme under the conditions tested. The mean specific activities are shown in figure 3-6 below.

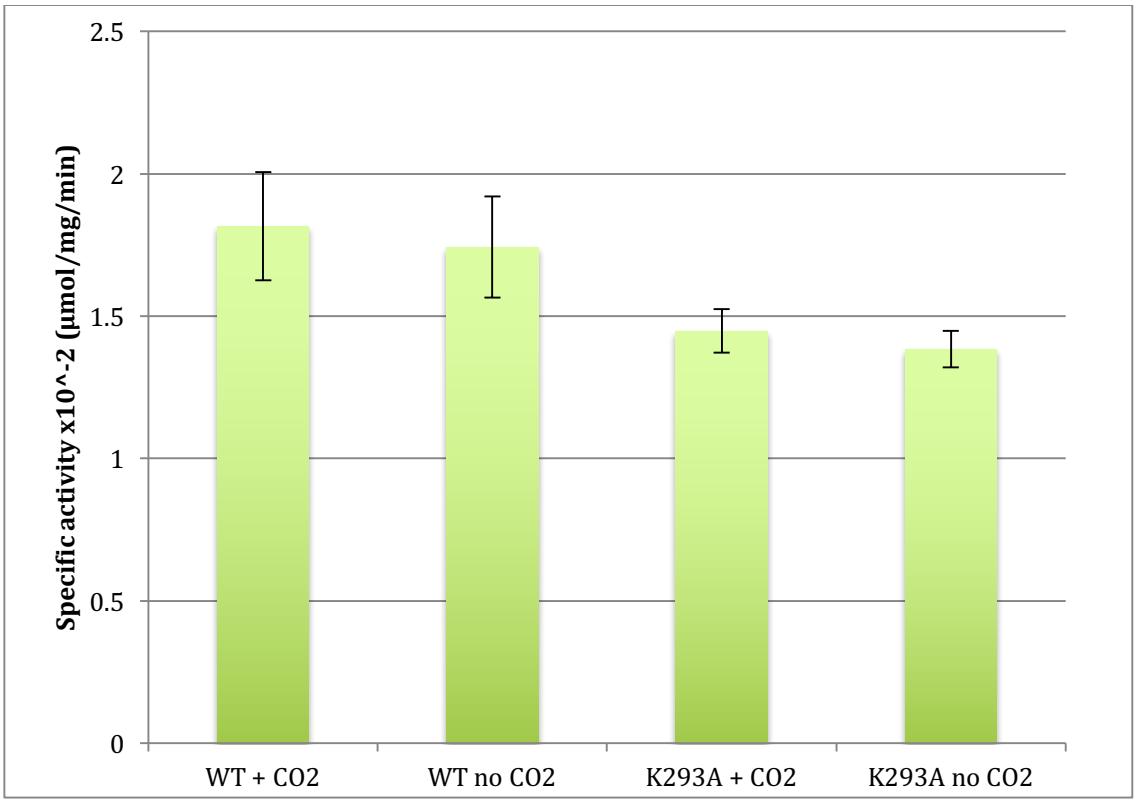


Figure 3-6: Specific activity of WT and K293A FBA1 in the presence and absence of CO_2. Mean specific activities are presented here (n = 8) and error bars represent the standard error.

No significant difference in activity was observed with the addition of CO_2 for either the WT or the K293A mutant enzyme. However, the mutant FBA1 had reduced activity compared to the WT. A Shapiro-Wilk normality test indicated that not all data sets were normally distributed. A log(y) = y transformation was applied to all data. Following this transformation, all data was found to be normally distributed. A one-way ANOVA confirmed that there is no significant difference in the activity of either the WT or K293A protein with the addition or removal of CO_2 ($P > 0.005$). However, the difference in specific activity between the WT and K293A enzyme is statistically significant ($P < 0.005$).

One limitation of this experiment was the difficulty in ensuring all CO_2 was successfully removed from the experimental system. It is therefore possible that some CO_2 was still present in the samples prepared in the absence of inorganic carbon. There is also the potential that even if CO_2 was successfully removed from the reaction mixture, the carbamate may be particularly stable on this residue and thus not effectively removed by incubation of the protein under a nitrogen atmosphere. Later, mass spectrometry analysis of this residue indicated that this is not the case and this carbamate is not stable at atmospheric CO_2 conditions (3.6).

An additional limitation of this investigation was the presence of protein contaminants in the samples assayed. Because of this it is possible that there was a difference in the concentration of FBA1-WT and FBA1-K293A present in the reaction mixture. Both proteins were expressed and purified simultaneously under the same conditions to reduce the chances of this. Impurities in the samples assayed may also interfere with carbamate formation.

The results of this investigation indicate that CO_2 concentration does not affect the cleavage activity of FBA1. Therefore the carbamate observed on lysine 293 may not play a role in this aspect of protein function. However, the mutant protein lacking this lysine residue has lower activity than the WT. This

could indicate that although the carbamate is not required for the cleavage function of this protein, the lysine residue on which it is found may be important for enzyme activity. However, it is possible that the carbamate is particularly stable and is not removed by incubation of the protein in a CO_2-free atmosphere. If this is the case then the observed difference in specific activity between the WT and mutant enzyme could indeed be due to the presence of this carbamate.

3.4 Structure

In order to account for the observed difference in activity between the WT and K293A mutant enzymes, the structure of FBA1 must be considered. As a class I FBA, FBA1 is expected to act as a tetramer made up of four identical polypeptide chains (Perham, 1990). The subunits of this tetramer are thought to be joined at two different types of subunit interface (Geng-Yin *et al*. 2017). Our lysine of interest, K293, is found at one of these interfaces between subunits (Figure 3-7). It is solvent exposed and is conserved in FBA proteins across multiple plant species. It was hypothesised that this residue is required for quaternary structure formation.

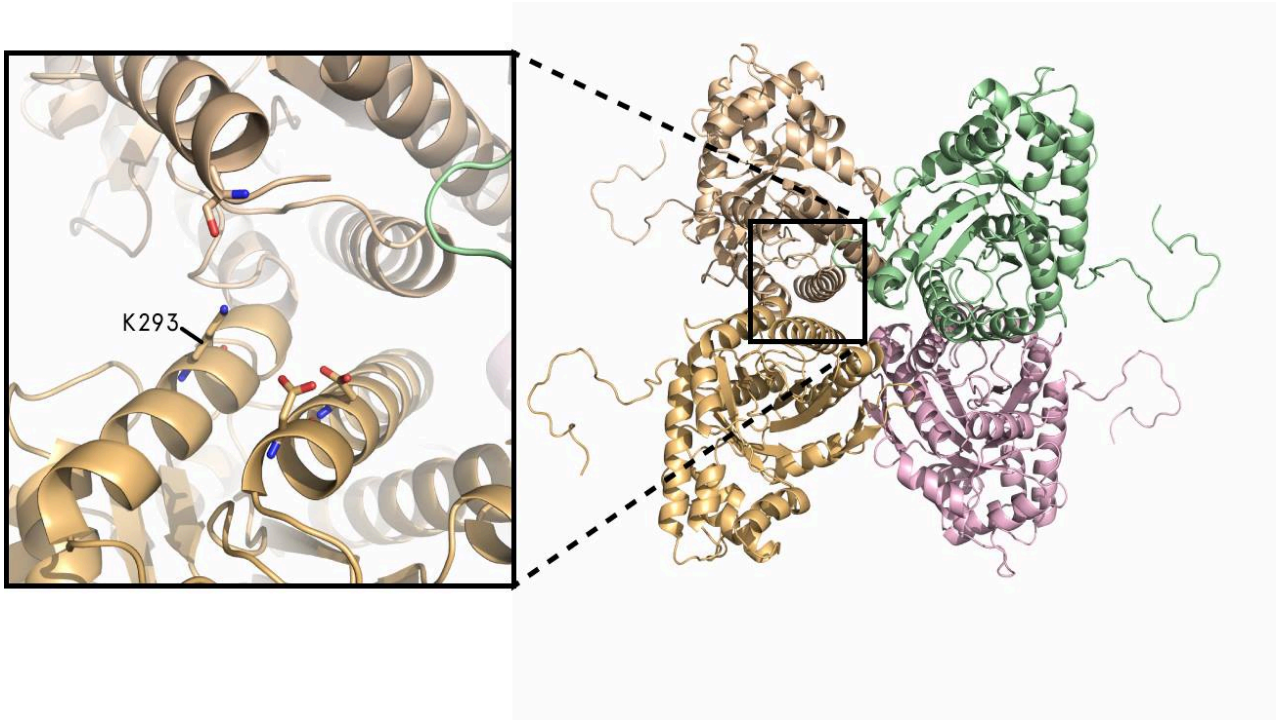


Figure 3-7: Quaternary structure of FBA1 with K293 shown at the border between subunits (PBD ID: 4D2J)

3.4.1 Native-PAGE gels

Native-PAGE gels were used to investigate the effect of lysine 293 on the quaternary structure of FBA1 the protein (2.6). These gels separate active protein by mass, allowing the subunit composition of protein in its native state to be observed. The conformation of a protein may affect the rate at which it travels through the gel or even prevent the protein running through the gel at all, meaning direct measurement of protein molecular weight is not possible. However despite this potential limitation it is possible to gain a good approximation of the subunit composition of many active proteins (Wittig *et al.* 2006).

Active FBA1 protein was mixed with loading buffer before being loaded on to a native page gel. The gel was then run at 140 V to prevent overheating causing denaturation of the protein. The purest wash fraction of protein

(eluted with 250 mM imidazole) was used for these experiments to prevent contaminants from confusing the analysis. This fraction contained considerably less impurities than the fraction used for the activity assays detailed above.

Two different types of gel stain were tested for visualisation of the protein. First a polyhistidine-tag stain was used. As this only binds to proteins with a polyhistidine-tag, it would be expected that only the FBA1 protein would be visible using this stain. Although FBA1 was observed in all its subunit conformations using this staining technique, images produced were of poor resolution due to limitations of the machine used for imaging. As this stain fluoresces and is not visible with the naked eye, it was not possible to use another technique to image gels stained in this manner. As the protein in this sample contains very little contaminant, Generon Quick Coomassie stain was also used to stain gels. Although this stain is not specific to purified FBA1, this technique produced higher resolution images in which the protein of interest was clearly visible. Gels stained with Coomassie were therefore used for quantification and analysis of protein bands. An image of one such gel can be seen in Figure 3-8.

The effect of CO_2 concentration on tetramer formation was also investigated. For practical reasons it was not possible to perform this experiment in the absence of CO_2. Instead, the CO_2 concentration of the system was increased by the addition of 10 mM $NaHCO_3$ to the gel running buffer.

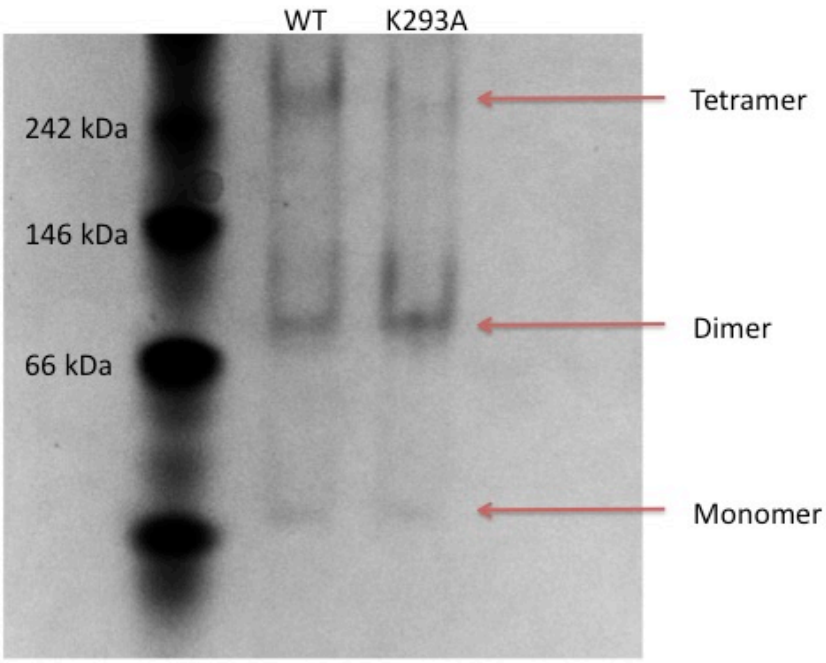


Figure 3-8: Native-PAGE gel stained with Instant Blue. The tetramer, dimer and monomer forms of WT and K293A FBA1 can be seen.

Figure 3-8 shows a native-PAGE gel run at atmospheric CO_2 concentration and stained with Quick Coomassie. Both the FBA1-WT and FBA1-K293A proteins can be seen in tetrameric, dimeric and monomeric formation. The proportion of WT protein found as a tetramer appears to be higher than for the mutant, with most of the FBA1-K293A protein present as a dimer.

The protein bands observed on the gel were analysed using Image J and the relative amount of protein present in each band quantified. Only a minimal amount of either the WT or mutant protein was found as a monomer. The amount of tetramer relative to the amount of dimer present in each sample was calculated and the results can be seen in figure 3-9.

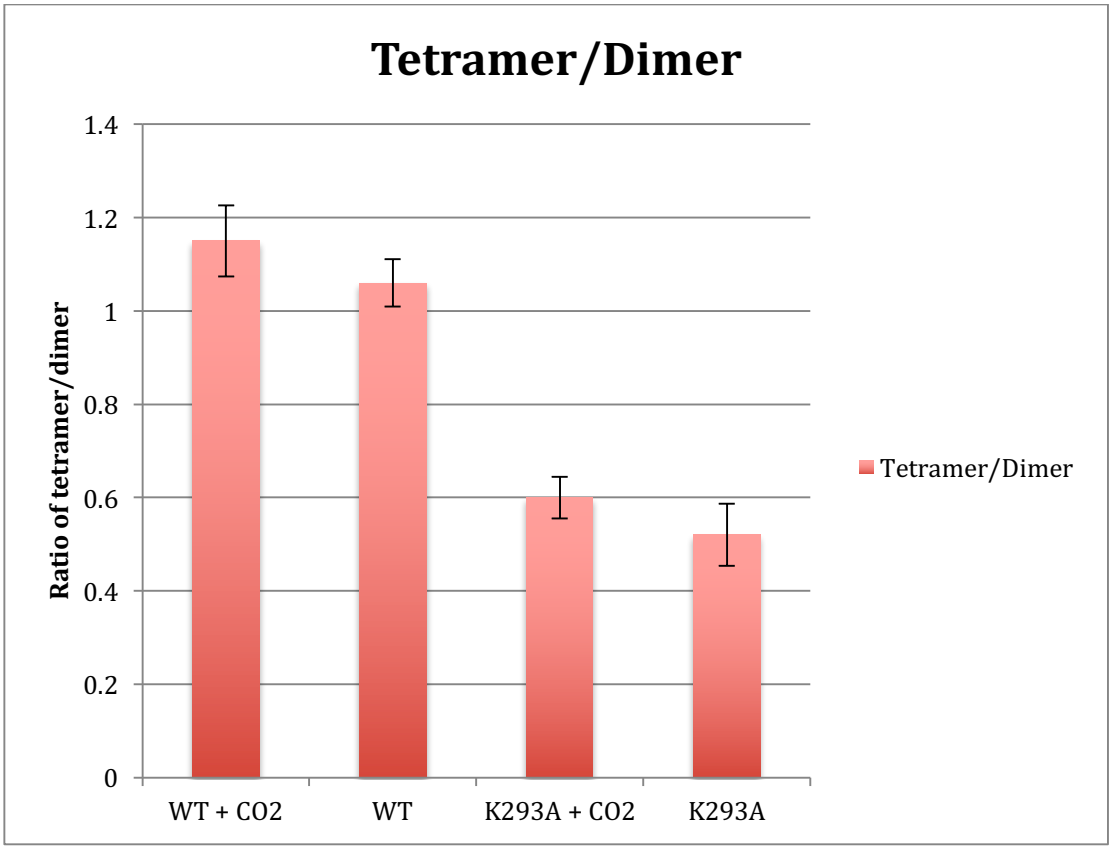


Figure 3-9: Results of Native page gel experiments. Mean values (n = 7) are shown here and error bars represent the standard error.

These results confirm the initial observation that a higher proportion of the WT protein is present as a tetramer than the K293A mutant protein. A similar amount of tetramer and dimer are found in the WT sample. In contrast, most of the K293A mutant protein observed was in the dimer formation, with almost twice as much dimer than tetramer present in the sample. The addition of 10 mM inorganic carbon to the experiment did not significantly affect the ratio of tetramer to dimer. A Shapiro-Wilke normality test indicated that all data sets from these experiments were normally distributed. A one-way ANOVA was used to compare mean values. This analysis confirmed that the difference between the value for tetramer/dimer for the WT and K293A protein was statistically significant ($P < 0.005$), and that there is no statistically significant difference for either protein with the addition of 10 mM inorganic carbon ($P > 0.005$).

Overall, the results of this investigation indicate that lysine 293 has an important role in tetramer formation. As two different types of interface are present within the FBA1 tetramer, the dimer is still able to form normally in the K293A mutant, whereas tetramer formation is impaired. Increasing the concentration of inorganic carbon from atmospheric to 10 mM did not affect the proportion of protein present as a tetramer, indicating that it may not be the carbamate on lysine 293 which is responsible for this effect on quaternary structure. However, it is not possible to conclude from these results that CO_2 concentration does not affect tetramer formation, as a significant limitation of this experiment was that it was not possible to remove atmospheric CO_2 from the system. If the carbamate is already stable on this residue at atmospheric concentrations of CO_2 then this carbamate would be present on the WT protein for all experiments and no difference in tetramer formation could be expected with the addition of more inorganic carbon. Therefore it is possible that this carbamate is responsible for the observed difference in tetramer formation between the WT and K293A mutant.

As FBA1 is thought to act as a tetramer within plant cells it is likely that the observed reduction in tetramer formation for K293A may be responsible for the reduced cleavage activity displayed by this mutant enzyme.

3.5 K293E

The amino acid glutamate shares properties with those of a carbamylated lysine and it has previously been demonstrated that this residue is able to perform the function of a carbamylated lysine residue under certain circumstances (Meigh *et al*. 2013). The replacement of a carbamylated lysine residue with glutamate may therefore lead to creation of a protein in which the effect of the carbamate on protein function is constitutively present. Such a mutant would be invaluable for further investigation of the effect of carbamates on protein function.

In order to mimic the presence of a stable carbamate modification on FBA1, a mutant was therefore made in which lysine 293 was mutated to a glutamate (FBA1-K293E). It was hypothesised that FBA1-K293E would have the same specific activity as the WT enzyme with the carbamate modification present.

3.5.1 PCR mutagenesis

The mutant K293E was made by one step PCR mutagenesis using the sense oligo: GTTGCGAGCTACACCCTGGAGCTGCTGCGTAACCGTATC and the antisense oligo: GATACGGTTACGCAGCAGCTCCAGGGTGTAGCTCGCAACPCR as primers to amplify the FBA1-WT plasmid, incorporating the K293E point mutation (2.7). The WT template DNA plasmid was then digested with the restriction enzyme Dpn1. This enzyme is only able to digest methylated template DNA and not the newly made mutant plasmid (Liu and Naismith, 2008). The presence of the point mutation at position 293 was confirmed by DNA sequencing.

3.5.2 Expression

The mutant plasmid was transformed in to competent *Rosetta PLysS* cells. FBA1-K293E was the expressed and purified according to the same protocol used to make the WT enzyme (2.1-2.3). FBA1-WT was expressed and purified simultaneously to ensure comparable conditions during this process. Wash fractions from purification were run on SDS-PAGE gels (Figure 3-10). Wash fractions from purification were tested for cleavage activity and once again the fraction eluted with 100 mM imidazole showed the highest activity.

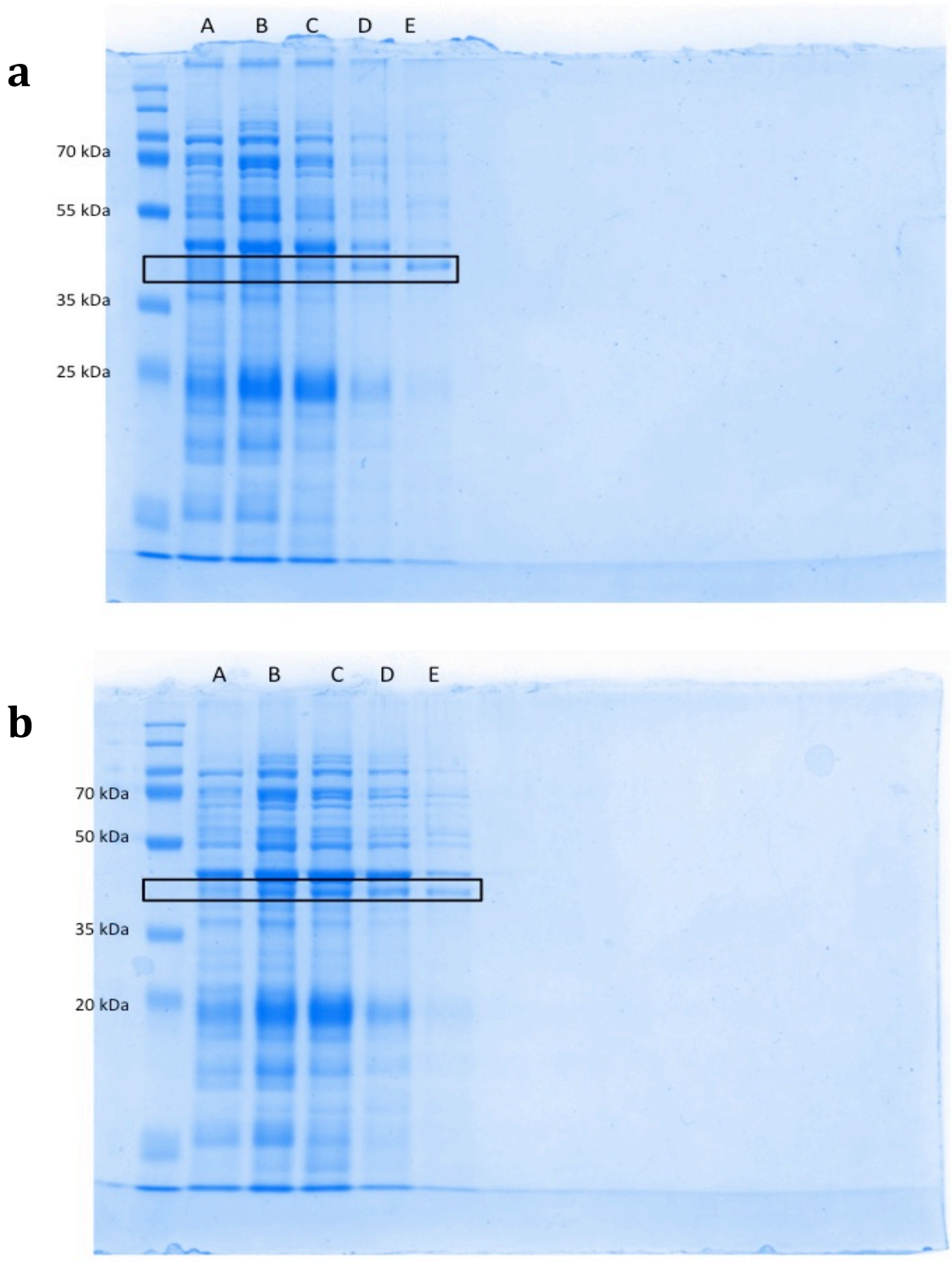


Figure 3-10: SDS-PAGE gel showing wash fractions from purification of FBA1-K293E (a) and FBA1-WT (b). Fraction A was eluted with a buffer containing 50 mM imidazole, B with 100 mM, C with 150 mM, D with 200 mM and E with 250 mM. FBA1 can be seen at the predicted MW of 43 kDa

Analysis of the SDS-PAGE gel images indicate that wash fractions contained a higher level of contaminant compared with FBA1 protein produced in earlier purifications.

3.5.3 Cleavage assays

It was hypothesised that FBA1-K293E would have the same specific activity as FBA1-WT in the presence of CO_2. In order to test this hypothesis, cleavage assays in the absence of CO_2 with the addition of 3 mM $NaHCO_3$ or NaCl were performed as above for both the WT and K293E proteins (2.5). The specific activities of the WT and mutant enzymes were calculated and are shown in figure 3-11.

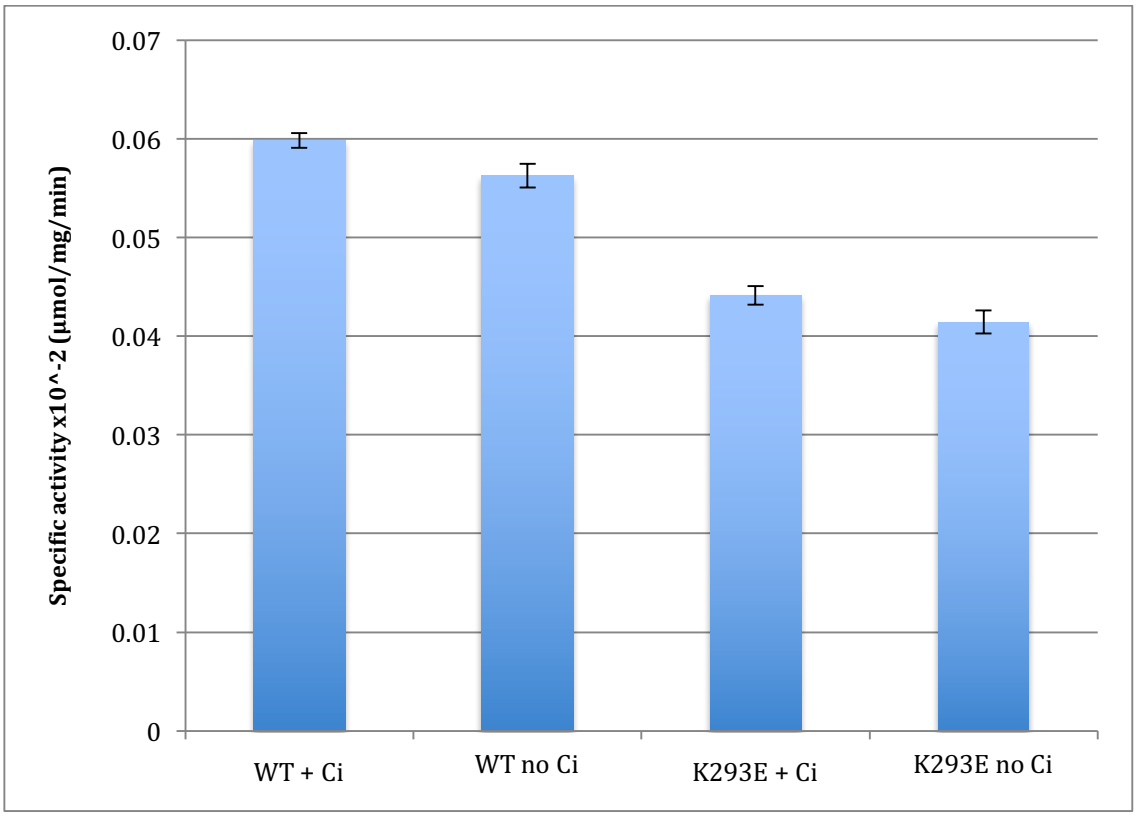


Figure 3-11: Specific activity of WT and K293E FBA1 in the absence of CO_2 and with the addition of 3 mM inorganic carbon. Mean values are shown here (n = 8) and error bars represent the standard error.

As in previous experiments, no significant difference in activity was observed for either enzyme with the addition of 3 mM inorganic carbon. However, the K293E mutant consistently showed reduced activity compared with the WT. These results were confirmed with statistical analysis. All data was found to be normally distributed using a Shapiro-Wilk normality test. A one-way ANOVA verified that there is no significant difference between the specific activity of the enzyme with and without CO_2 for either the WT or the mutant ($P > 0.005$). The difference in activity between the WT and K293E is statistically significant ($P < 0.005$).

The specific activity of the WT FBA1 calculated here is considerably lower than in earlier experiments. This may be due to less efficient expression of the aldolase. The protein samples produced following purification contained a high proportion of contaminants compared with in earlier assays. This could possibly be due to circumstantial differences in the purification process. However, since the WT and K293E protein used in these assays were expressed and purified simultaneously, assay data produced using these proteins is still comparable.

These results indicate that FBA1-K293E does not have the same activity as the WT protein with lysine 293 carbamylated. Instead, the K293E mutant has significantly lower activity than the WT under both CO_2 concentration conditions tested. There are potentially three main reasons why this result may have been observed. Firstly, it is possible that due to the specific structure of this protein, the glutamate residue at position 293 of FBA1-K293E is not able to successfully mimic the properties of a carbamylated lysine residue within this particular protein environment. Secondly, a major limitation of this investigation is the difficulty in producing pure active aldolase protein. Due to the high level of protein contaminant present in each sample it is not possible to verify whether the concentration of WT and mutant FBA1 added to assays was the same. This difference in activity could be due to a difference in concentration of active enzyme. Finally, it could be that the presence of the

lysine residue of interest itself rather than the carbamate modification is important for the activity of the protein. This lysine could contribute to protein function either by promoting tetramer formation or by another unknown mechanism. If this were the case, replacing it with another residue would decrease the activity of the protein as is observed here.

Taken together, the results of these investigations suggest that either the K293E mutation may not be able to accurately mimic the carbamylated lysine residue, or this lysine residue itself rather than the carbamate modification may be important for enzyme activity. This mutant protein is therefore not effective for investigating the effect of carbamate formation on the function of FBA1. It would be interesting to analyse the subunit composition of FBA1-K293E to determine whether this mutant is present as a tetramer. However, due to time constraints this was not investigated in this project.

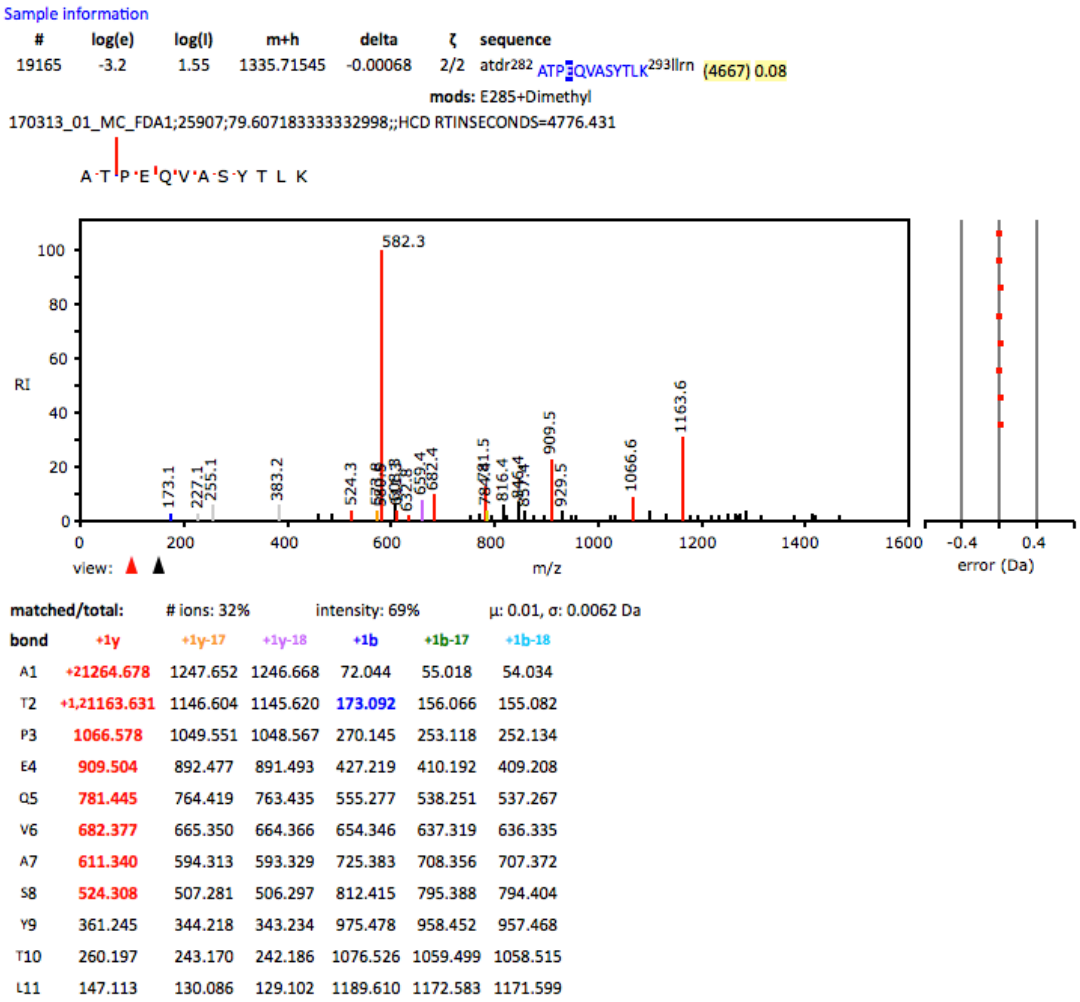
3.6 Trapping FBA1 at atmospheric CO2

Earlier investigations indicated that the cleavage activity of FBA1 is not affected by the removal of CO_2 from the experimental system. It was therefore hypothesized that the carbamate previously observed on lysine 293 may be stable on the protein even in the absence of atmospheric CO_2.

Linthwaite (2017) effectively demonstrated that a carbamate is able to form on FBA1 on this at a CO_2 concentration of 73.5 μM. This is approximately the concentration of CO_2 found within plant cells and is thus physiologically relevant (Portis *et al*. 1986). However, as carbamate formation was not investigated with any lower concentrations of CO_2, it is possible that this carbamate may be extremely stable on this lysine residue and not easily removed. If this is the case it could explain why no change in FBA1 activity was observed with the addition or removal of CO_2 in earlier experiments.

Additionally, limitations with the equipment may have prevented complete removal of CO_2 from the system during cleavage assays. It was therefore important to determine whether this carbamate is found on FBA1 at atmospheric CO_2 concentrations. In order to test this a trapping experiment was performed as described by Linthwaite (2017), however without the addition of any inorganic carbon.

FBA1 WT protein was concentrated to 1 mg/mL by spin filtration. The trapping reagent TEO reacts quickly with amines, therefore the protein was dialysed in to phosphate buffer which does not contain any amines before the trapping reaction was performed. The reagent was added to the protein under atmospheric CO_2 conditions and a stable pH of 7.4 was maintained throughout the reaction using a pH stat. After the reaction was complete, precipitated protein was visible in the sample. TEO causes the addition of ethyl groups to many residues within the protein leading to precipitation (Linthwaite, 2017). This can therefore be used as an indication of whether the reaction has been successful. Following trapping, the protein was dialysed in to water and digested with trypsin. ESI-MS was performed and the resultant data analysed using the database GPM-X!Tandem.



bond	+1y	+1y-17	+1y-18	+1b	+1b-17	+1b-18
A1	+21264.678	1247.652	1246.668	72.044	55.018	54.034
T2	+1,21163.631	1146.604	1145.620	173.092	156.066	155.082
P3	1066.578	1049.551	1048.567	270.145	253.118	252.134
E4	909.504	892.477	891.493	427.219	410.192	409.208
Q5	781.445	764.419	763.435	555.277	538.251	537.267
V6	682.377	665.350	664.366	654.346	637.319	636.335
A7	611.340	594.313	593.329	725.383	708.356	707.372
S8	524.308	507.281	506.297	812.415	795.388	794.404
Y9	361.245	344.218	343.234	975.478	958.452	957.468
T10	260.197	243.170	242.186	1076.526	1059.499	1058.515
L11	147.113	130.086	129.102	1189.610	1172.583	1171.599

Figure 3-12: Mass spectrometry data generated using GPM X!Tandem showing the FBA1 peptide containing lysine 293

The peptide containing lysine 293 was identified in this data (Figure 3-12). The peptide was ethylated, indicating a successful reaction with the reagent TEO. However, no carbamate was found on lysine 293. In earlier work by Linthwaite (2017), the peptide was not cleaved directly following lysine 293 as the carbamate prevented protein cleavage at this site. In the data presented here there is no missed cleavage site as lysine 293 is found at the end of the peptide, indicating that the carbamate is not present. Due to the low quality of this data few b ions are present. However this peptide has a convincing confidence score of – 3.2 indicating that it is likely to have been correctly identified.

The main limitation with this experiment is that although this peptide was found without the carbamate modification, it is still possible that other peptides containing a carbamylated lysine 293 were present in the sample but were not detected. Therefore, although this data confirms that the carbamate on this residue is not completely stable under these CO_2 conditions, it cannot be inferred that this carbamate does not form at all. Additionally, there was not time to perform the trapping experiment in the presence of additional CO_2, so the presence of the carbamate identified in this manner by Linthwaite *et al*. 2017 could not be confirmed. Moreover, this trapping experiment was not performed on full-length FBA1 due to the difficulties in expressing and purifying this protein (Linthwaite *et al.* 2017).

The absence of carbamate on lysine 293 under these conditions indicates that this carbamate is not stable at atmospheric CO_2 concentrations. It is therefore likely that the carbamate was successfully removed by incubation under a nitrogen atmosphere in cleavage experiments described earlier, and this carbamate may not have been present in the protein run on native page gels without the addition inorganic carbon.

3.7 Discussion and conclusions

FBA1 was previously shown to form a carbamate on lysine 293 at physiologically relevant CO_2 concentrations (Linthwaite, 2017). As another Calvin cycle enzyme, RuBisCo, is known to be activated by carbamylation (Stec, 2012), it was hypothesised that the carbamate on present FBA1 may have a role in regulating the catalytic function of this protein. However, the results of the experiments described in this chapter suggest that the concentration of CO_2 does not affect the cleavage activity of this enzyme. When the cleavage activity of FBA1-WT was assayed in the absence of CO_2 and with the addition of at 3 mM inorganic carbon, no significant difference in specific activity was observed. Similarly, analysis of subunit conformation

using native page gels indicated that increasing the concentration of CO_2 does not increase the amount of FBA1 found as an active tetramer. An experiment to trap the carbamate modification on this protein suggested that the carbamate is not stable under atmospheric CO_2 conditions. It is therefore likely that this carbamate was not present in samples in the absence of additional CO_2 in earlier experiments.

Somewhat surprisingly, although the presence and absence of the carbamate on lysine 293 did not appear to alter protein function, mutating this residue was shown to cause a significant reduction in aldolase activity. Replacing this residue with either an alanine (to mimic this lysine residue without a carbamate) or a glutamate (to mimic the lysine with an attached carbamate) resulted in a significant reduction in cleavage activity compared with the WT enzyme. Additionally, FBA1-K293A appears to be deficient in tetramer formation, which may be responsible for this observed reduction in specific activity. Therefore this lysine residue appears to be important for quaternary structure formation and efficient cleavage activity. However, the results of the above experiments suggest that the carbamate identified on this residue may not be required for these functions.

Although the investigations described in this chapter were unable to identify a function for the carbamate modification on FBA1, this carbamate may still be functionally relevant. Sequence comparison reveals that the lysine residue found at position 293 of FBA1 is conserved across FBA proteins in multiple plant species. Linthwaite (2017) also identified a carbamate on the corresponding lysine residue of *Arabidopsis* FBA2. The conserved nature of this carbamylated lysine residue suggests that it may be required for some aspect of protein function. FBAs are also known to catalyse the condensation of DHAP and G3P to form FPB (Fleshner *et al.* 1999) and it is therefore still possible that this carbamate might play a role in this reaction. Additionally, within the cellular environment this carbamate may affect the interaction of FBA1 with other molecules such as those involved in regulation of this

enzyme. Investigation in to the effect of CO_2 on the condensation activity of FBA1 as well as its interaction with other molecules may further elucidate the function of the carbamate modification observed on this protein.

Chapter 4: Lipid Transfer Protein 1

4.1 Overview

Non-specific lipid transfer proteins (LTPs) are small cationic proteins found in all higher plants and are characterised by their ability to bind and transport lipids (Bogdanove *et al*. 2016). They can be subdivided in to two large protein families: LTP1s which have a molecular weight of ~9 kDa and LTP2s are slightly smaller with a molecular weight of ~7 kDa (Carvalo and Gomes, 2008). Many plant species possess a large number of LTP isoforms and these have been shown to have diversity of structure and lipid binding affinity (Guerbette *et al*. 1999). These proteins all contain eight conserved cysteine residues, which form four disulphide bridges necessary for protein function. They contain a hydrophobic internal lipid-binding cavity which has flexible volume, allowing non-specific binding of many different lipids (Carvalo and Gomes, 2008). LTPs were first identified for their ability to transfer lipids between membranes *in vitro* in a non-specific manner. Because of this they were initially predicted to play a role in intracellular lipid trafficking (Kader, 1997). However, these proteins contain an N-terminal signal peptide sequence targeting them for secretion and thus are unlikely to be found within the cell (Carvalo and Gomes, 2007). Additionally, immunolocalisation experiments suggest that LTPs are located at the cell wall (Thoma *et al*. 1993). Therefore despite their ability to transport lipids between membranes, it is unlikely that LTPs play a role in lipid trafficking within the cell.

The actual function of LTPs has not been fully elucidated, with proposed functions including roles in somatic embryogenesis, cuticle deposition,

defence against pathogens and response to abiotic stresses (Salcedo *et al*. 2007). LTPs have been shown to bind specifically to elicitin receptors in the plasma membrane (Buhot *et al*. 2001). These receptors detect elicitins released by phytopathogens and initiate a defensive response to infection. When bound to jasmonic acid, a tobacco LTP was shown to have a significantly increased interaction with elicitin receptors compared with LTP alone, and treatment of plants with LTP-jasmonic acid complex increased resistance to phytopathogen infection (Buhot *et al*. 2004). This provides evidence for a role for LTPs in cell signalling and defence against pathogens. Additionally, expression of different LTPs is differentially enhanced by exposure to pathogens, as well as by abiotic stresses such as drought, salinity and cold (Jung *et al*. 2003). The large amount of duplication and diversification seen within the LTP gene family is also characteristic of proteins involved in defence mechanisms (Bogdanove *et al*. 2016).

The *Arabidopsis* LTP family contains at least 15 genes (Arondel *et al*. 2000). One of these, LTP1, was identified in the screen of the *Arabidopsis* leaf proteome and found to contain carbamates on multiple lysine residues. There was a convincing confidence score for the presence of a carbamate on lysines 65 and 115. A third putative carbamate was identified on lysine 98, however the data for this peptide was of poor quality so this is less certain (Linthwaite 2017, unpublished data). LTP1 is known to be expressed in the epidermal cells of the leaves and stem, as well as in the flower and the protoderm of the embryo. This expression pattern is consistent with a potential role for this protein in both defence against pathogens and cuticle deposition (Thoma *et al*. 1994).

The presence of carbamate modifications on *Arabidposis* LTP1 indicate that CO_2 concentration may influence the activity of this protein. LTP1 was selected for further investigation in to the effect of carbamate formation on the function of proteins for various reasons. Two of the three carbamates potentially present on this protein were confidently identified by mass

spectrometry data analysis software. Additionally, homologous proteins from other plant species have previously been successfully expressed and purified and assaying the lipid binding activity of LTPs is relatively straightforward (Bogdanove *et al.* 2016).

This chapter will cover expression and purification of *Arabidopsis* LTP1 and the use of the fluorescent probe TNS to investigate the effect of CO_2 on the lipid binding activity of this protein. It will also examine the effect of CO_2 on the activity of a mutant LTP1 in which lysine 65 has been mutated to an alanine residue and discuss further experiments which may help fully elucidate the effect of carbamate formation on the function of this protein.

4.2 Expression

The amino acid sequence for Arabidopsis LTP1 is shown below with the lysines containing putative carbamates highlighted (Figure 4-1).

MAGVMKLACLLLACMIVAGPITSNAALSCGSVNSN**L**AACIGYVLQGGVI
PPACCSGVKNL**N**SI**A****K**TTPD**R**QQACNCI**Q**GAARALGSGLNAGR**A**AGIP**K**
ACGVN**I****P****Y**K**I**STSTNC**K**TVR

Figure 4-1: Amino acid sequence of LTP1. Lysines 65 and 115, which are thought to be carbamylated, are highlighted in red. Lysine 98 which may also be carbamylated is highlighted in purple. Residues highlighted in blue are involved in lipid binding.

4.2.1 Test expression

The *E.coli* strain Tuner Rosetta was investigated for the expression of recombinant LTP1. The LTP1 (Figure 4-1) with an attached polyhistidine tag was purchased from Genscript in the plasmid vector *pET14b*. Tuner Rosetta cells were transformed with this plasmid and a small-scale test expression was performed (2.11). Expression of LTP1 was induced with either 0.2 mM or

2 mM IPTG and samples were taken before induction, 1 h after induction and 3 h after induction. Cell lysate from each sample was centrifuged to pellet any insoluble protein and the resultant supernatant and pellet were run on SDS-PAGE gels.

Successfully expressed LTP1 was shown to be present in the pellet from samples 1 h and 3 h after induction, induced with either 2 mM or 0.2 mM IPTG. Some protein was also observed at the correct molecular weight for LTP1 in the pellet of the un-induced samples, suggesting a degree of leaky expression. No LTP1 was observed in the supernatant of any of the samples. This indicates that LTP1 is likely to be found in insoluble protein aggregates called inclusion bodies, which form due to misfolding of proteins during expression in bacteria (Koppito, 2000). This protein therefore needed to be solubilised and refolded correctly following expression. Analysis of the SDS-PAGE gel indicated that most protein was located in the pellet 3 h after induction with 0.2 mM IPTG. Therefore these conditions were used for large-scale expression of LTP1.

4.2.2 Large-scale expression

Large-scale expression of LTP1 was carried out to produce enough protein to assay binding activity (2.12). Following expression, LTP1 is found in an insoluble form within inclusion bodies. Therefore solubilisation and refolding of this protein were required before purification was possible. Inclusion bodies were washed with a series of centrifugation and sonication steps. They were then resuspended in 6 M urea and incubated over night to solubilise the aggregated protein. The protein was refolded by diluting the solution with PBS in a ratio of 1: 10 and incubating for 30 min.

The polyhistidine tag present on the refolded LTP1 allowed purification by nickel affinity chromatography. This protein was found to have high affinity for

the nickel resin. Elution buffers containing concentrations of imidazole from 50 mM to 1 M were required to elute all the protein from the resin. Wash fractions produced by this purification process were run on an SDS-PAGE gel and the results can be seen in Figure 4-2.

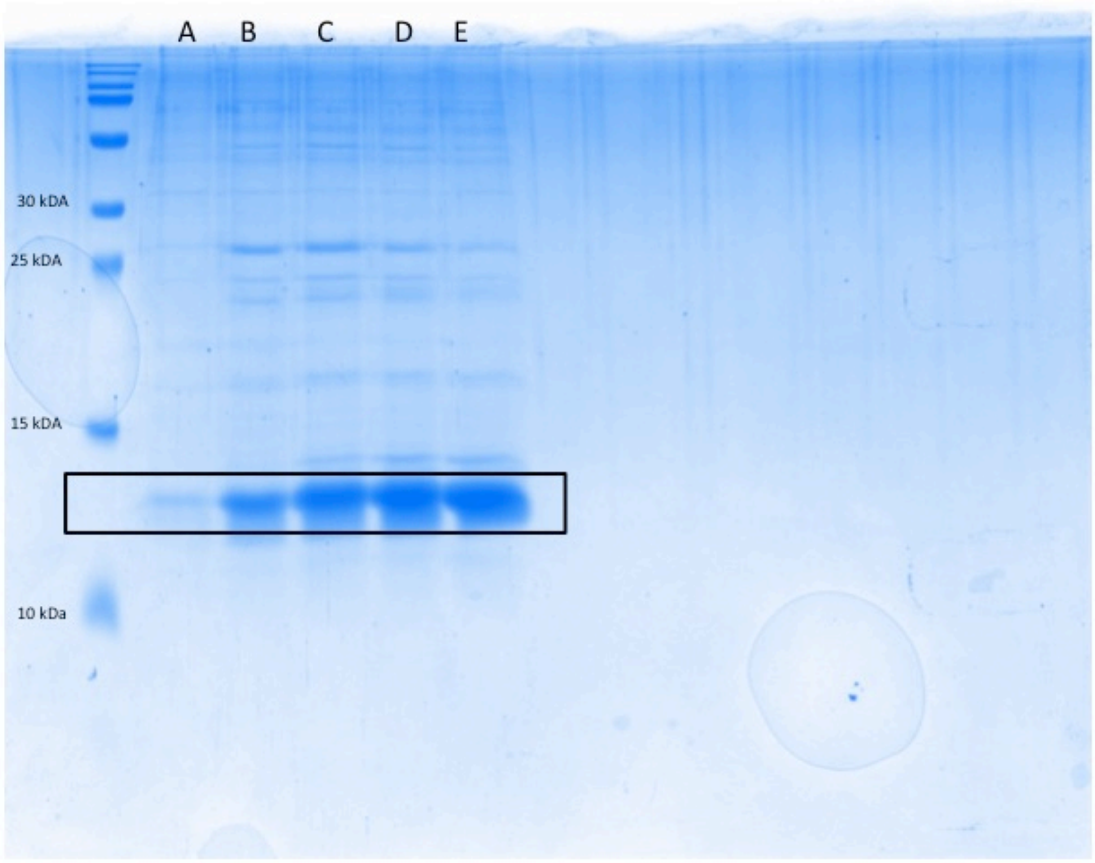


Figure 4-2: SDS page gel of wash fractions produced by purification of LTP1-WT. Protein in lane A was eluted with 50 mM imidazole, lane B with 100 mM, lane C with 200 mM, lane D with 400 mM and lane E with 1 M. LTP1 can be seen between the 15 kDa and 10 kDA protein markers.

LTP1 can clearly be seen on the SDS-PAGE gel at its expected molecular weight of 12 kDa. This protein is very pure in all fractions with very little contamination visible. The fraction eluted with 50 mM imidazole contains very little protein, again indicating that LTP1 has a particularly high affinity for the nickel resin. All fractions were combined for further experiments. Although correct refolding of this protein could not be confirmed, later experiments indicated that an active form of this protein was present in samples.

4.3 Assaying lipid binding of WT LTP1

LTPs are characterised by their ability to bind and transport lipids (Carvalo and Gomes, 2007). It was therefore hypothesised that if the carbamates identified on LTP1 are functionally relevant, it is likely that they may play a role in lipid binding. A lipid-binding assay was developed to investigate the effect of these carbamates on the lipid binding affinity of this protein.

The florescent probe 6-(*p*-Toluidino)-2-naphthalenesulfonic acid (TNS) fluoresces only when bound to hydrophobic lipid binding pockets of proteins. TNS is known to bind competitively to the lipid-binding pocket of LTPs and has previously been used successfully to measure the lipid binding affinity of a pea LTP1 (Bogdanov *et al*. 2016). These studies measured displacement of TNS by biological lipids to determine the affinity of the protein for specific lipids. However, as binding of TNS to LTP1 may also be affected by the concentration of CO_2, we have simplified this assay by directly measuring the binding of the probe to the protein. The fluorescence of TNS was then used to estimate the lipid binding capability of the *Arabidopsis* LTP1 under different conditions.

4.3.1 TNS dose response curves

Changes in the binding affinity of LTP1 for the fluorescent probe TNS will be easier to observe when assays are performed using a concentration of TNS which is close to the dissociation constant (K_d) for this interaction. The K_d is the concentration of TNS at which half of the total molecules of LTP1 protein are associated with the probe (Bisswanger, 2008).

In order to determine the K_d for the interaction of TNS with LTP1, a dose response curve was produced (2.16). Increasing concentrations of TNS from 0.625 μM to 160 μM were combined with LTP1 in measurement buffer and the fluorescence measured. Fluorescence was then plotted against TNS concentration (Figure 4-3).

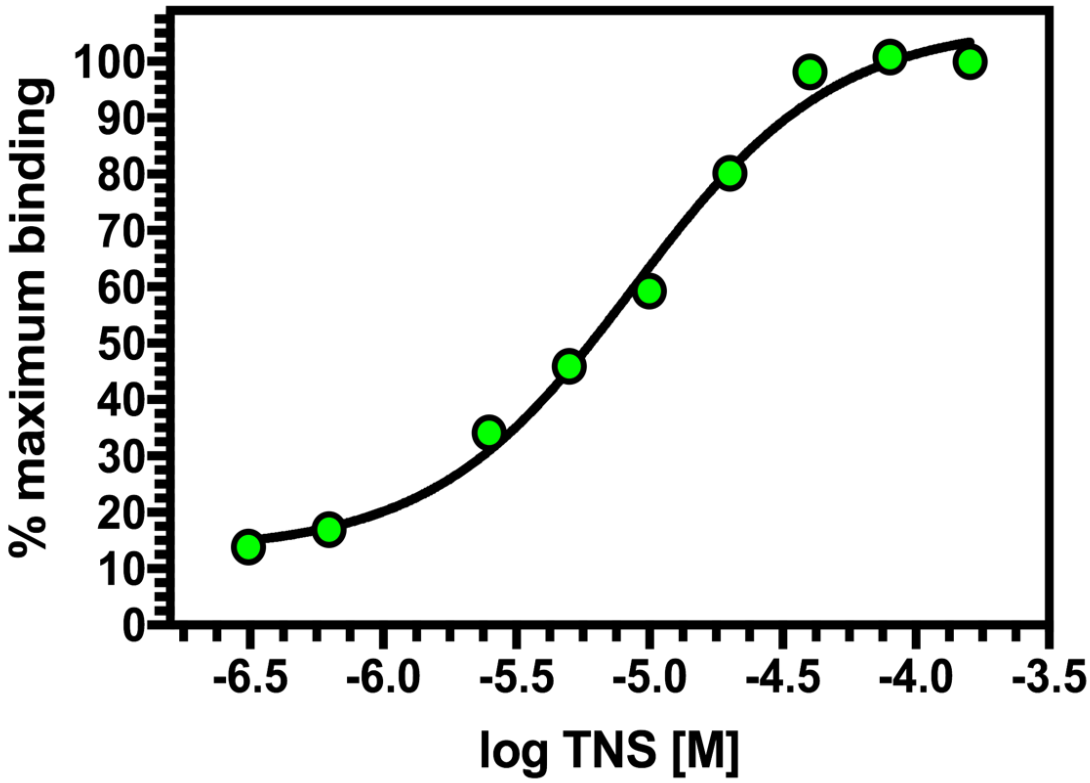


Figure 4-3: Dose response curve showing change in fluorescence with increasing TNS concentration.

From the dose response curve produced (Figure 4-3) it can be seen that maximum fluorescence, when all the protein is bound to TNS, is occurs with approximately 50 μM TNS. The fluorescence is at half the maximum value when around 8.7 μM of TNS is present, indicating that this is the K_d for this interaction.

4.3.2 Assaying lipid binding activity with and without CO_2

In order to investigate whether the carbamates identified on LTP1 contribute to lipid binding activity, the ability of this protein to bind TNS was measured both in the presence and absence of CO_2.

The assay developed above is simple and quick to perform and can therefore be assembled easily within a nitrogen atmosphere chamber. This allows for more efficient and thorough removal of CO_2 than was possible for earlier experiments with FBA1. The measurement buffer for this assay was degassed before being taken in to the chamber and the protein samples and TNS probe were purged for 3 min prior to being taken in to the nitrogen atmosphere.

The assay was assembled both inside the nitrogen atmosphere cabinet in the absence of CO_2 and on the bench at atmospheric CO_2 concentrations. TNS and LTP1 were combined in measurement buffer and mixed well. A control sample containing TNS and measurement buffer but no protein was also prepared. Fluorescence of all samples was measured. For the assay in the absence of CO_2, samples had to be removed from the nitrogen atmosphere cabinet before fluorescence could be measured. This was done to prevent atmospheric CO_2 affecting TNS binding during measurement, however it was not possible to ensure that the protein did not interact at all with CO_2. The fluorescence of the control samples (background fluorescence) were taken away from the values produced for samples containing LTP1. The results of these assays are presented in Figure 4-5.

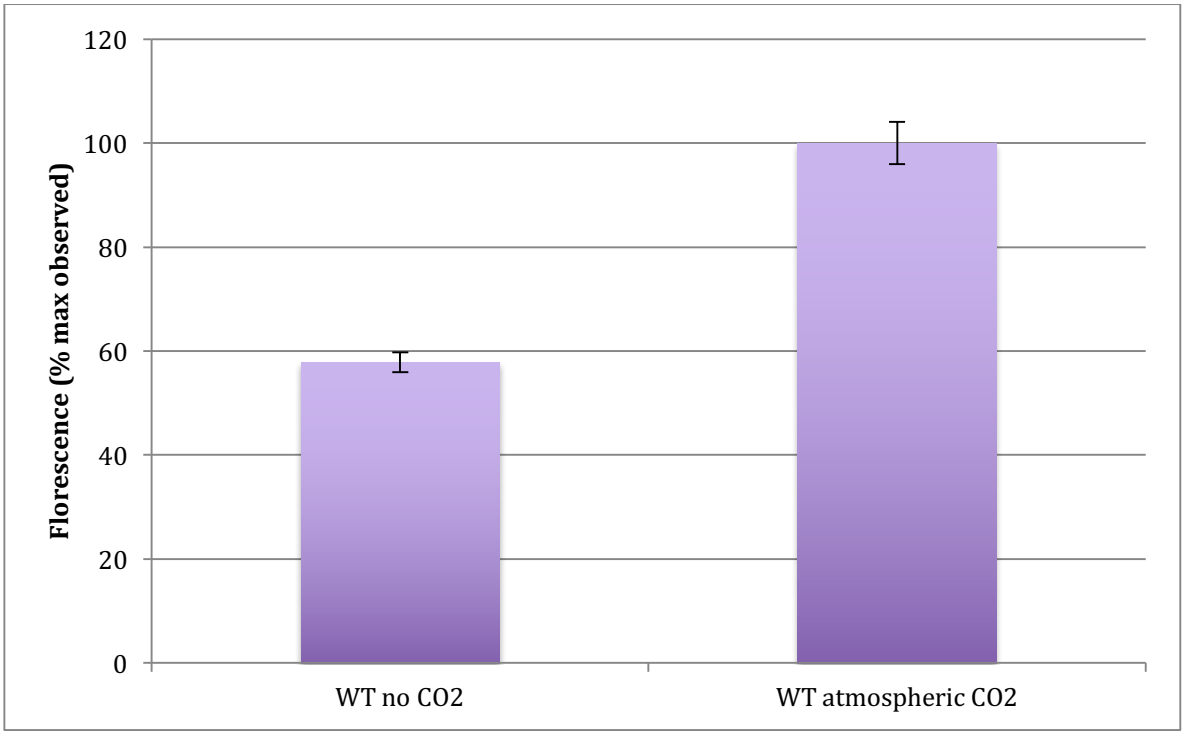


Figure 4-5: Fluorescence of TNS bound to LTP1 in the absence of CO_2 and at atmospheric CO_2 concentrations. Mean values (n = 8) are presented here and error bars represent the standard error.

Figure 4-5 shows that in the absence of CO_2, fluorescence was 58 % lower than when the assay was performed at atmospheric [CO_2]. This indicates a corresponding reduction in binding of TNS to LTP1 when CO_2 was absent. All data from these experiments was analysed using a Shapiro-Wilk normality test and found to be normally distributed. An unpaired T-test indicated that the difference in fluorescence between the assays with and without CO_2 is statistically significant ($P < 0.0001$).

Limitations of this experiment include difficulties in removing CO_2 from the system. Although the TNS and protein were purged before being added to assays, it was not feasible to ensure that all CO_2 was removed from these. Additionally, the samples prepared in the absence of CO_2 had to be measured under atmospheric conditions. It is therefore possible that some inorganic carbon was present in these samples when measurement took place. A further limitation of this experimental set up is that it was not possible to

remove only CO_2 from the system as the nitrogen atmosphere cabinet lacks other components also found in air. For this reason it is not possible to discern whether the observed effect is due only to the absence of CO_2.

However, assuming that the observed effect is due to the presence or absence of CO_2, these results suggest that LTP1 requires CO_2 for efficient TNS binding. It is therefore likely that one or more of the carbamates observed on this protein increase the affinity of the protein for lipids. It should be noted that TNS is not a biological lipid and therefore it can only be inferred that this effect would also occur for binding of LTP1 to biologically relevant lipids. Additionally, from this data alone it is not possible to identify which carbamylated lysine residues play a role in lipid binding. In order to determine which carbamates are responsible for this, the binding activity of mutants lacking these carbamylated lysine residues must be assayed.

4.4 Assaying lipid binding activity of the mutant LTP1-K65A

The presence of CO_2 at atmospheric concentrations appears to increase the lipid binding activity of LTP1, compared with the protein in the absence of CO_2. However, from the results of the experiments performed so far it is not possible to determine which carbamates are responsible for this difference. In order to determine the effect of an individual carbamate on the lipid binding activity of this protein, a mutant was made in which lysine 65 was mutated to an alanine. This residue was chosen for investigation as this carbamate was identified with the best confidence score by Linthwaite (2017). An alanine residue was selected to replace the lysine as this is an inert residue and is therefore unlikely to have unwanted effects on the chemical properties of the protein.

4.4.1 Expression

The plasmid *pET14b-LTP1-K65A* was purchased from Genscript and transformed in to Tuner Rosetta *E. coli* cells. This mutant was expressed, refolded and purified in the same manner as the WT protein (2.12-2.14). Samples from wash fractions produced in the purification process were run on an SDS-PAGE gel (Figure 4-6).

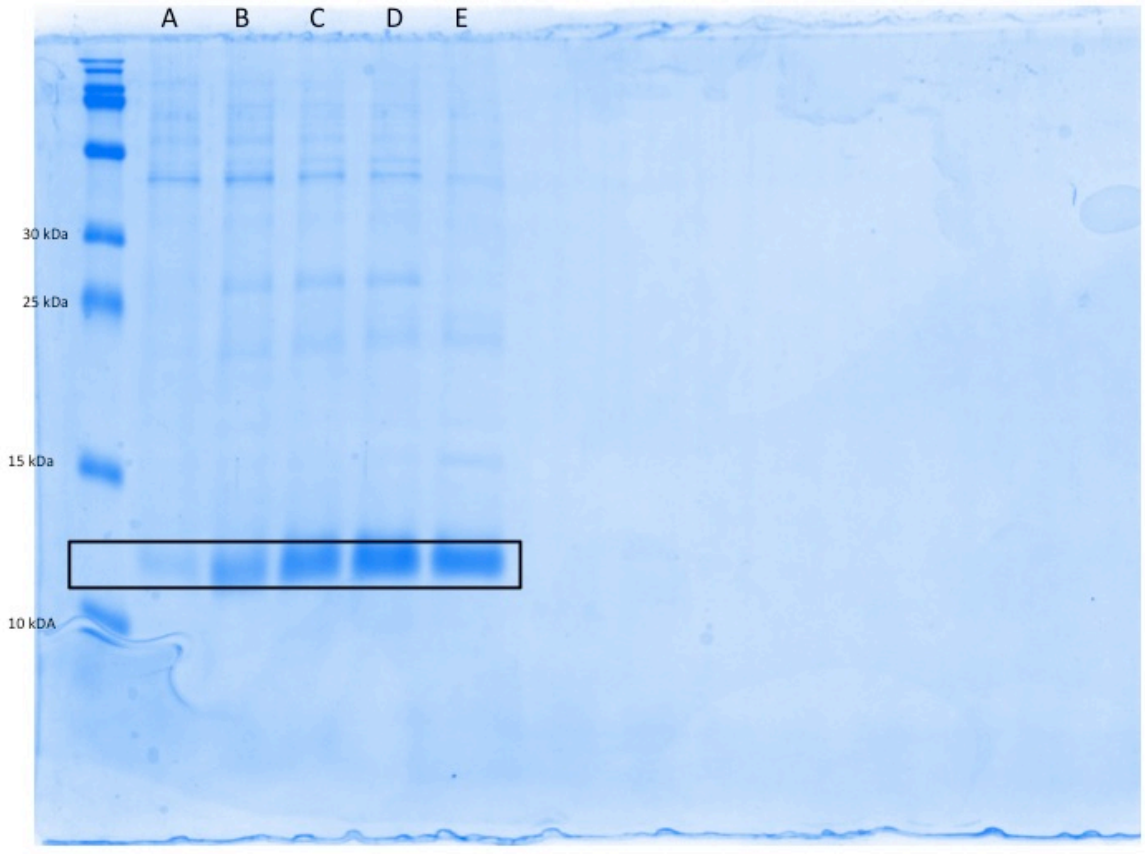


Figure 4-6: SDS-PAGE gel showing wash fractions from purification of LTP1-K65A. Protein in lane A was eluted with 50 mM imidazole, lane B with 100 mM, lane C with 200 mM, lane D with 400 mM and lane E with 1 M. LTP1 can be seen between the 15 kDa and 10 kDA protein markers.

This SDS-PAGE gel (Figure 4-6) indicated that LTP1-K65A was present in all fractions at the predicted molecular weight of 12 kDa. Significantly less protein is present in the fraction eluted with 50 mM imidazole than in other fractions.

All fractions contain relatively pure protein with very little contaminant visible in any of the lanes. This gel closely resembles the SDS-PAGE gel produced following purification of the WT protein (figure 4-2) indicating a similar level of success in expressing and purifying both proteins.

4.4.2 Assaying lipid binding activity of K65A in the presence and absence of CO_2

The affinity of the LTP1-K65A mutant protein for the fluorescent probe TNS was investigated in the presence and absence of CO_2. It was hypothesised that the carbamate located on lysine 65 of the LTP1-WT is responsible for the increase in TNS binding activity observed at atmospheric CO_2 concentrations compared with the absence of CO_2. If this is the case, changes in CO_2 concentration should have no effect on the binding ability of the K65A mutant protein.

This assay was performed both inside a nitrogen atmosphere cabinet and on the bench at atmospheric CO_2 concentrations, in the same manner as for the WT protein (2.15). Control samples containing TNS but no protein were also assembled under both conditions. The limitations of this were the same as for assays with LTP1-WT. The mean fluorescence intensity was calculated and the background fluorescence (mean fluorescence of control samples) was taken away from this value. The results of these assays are shown in Figure 4-7.

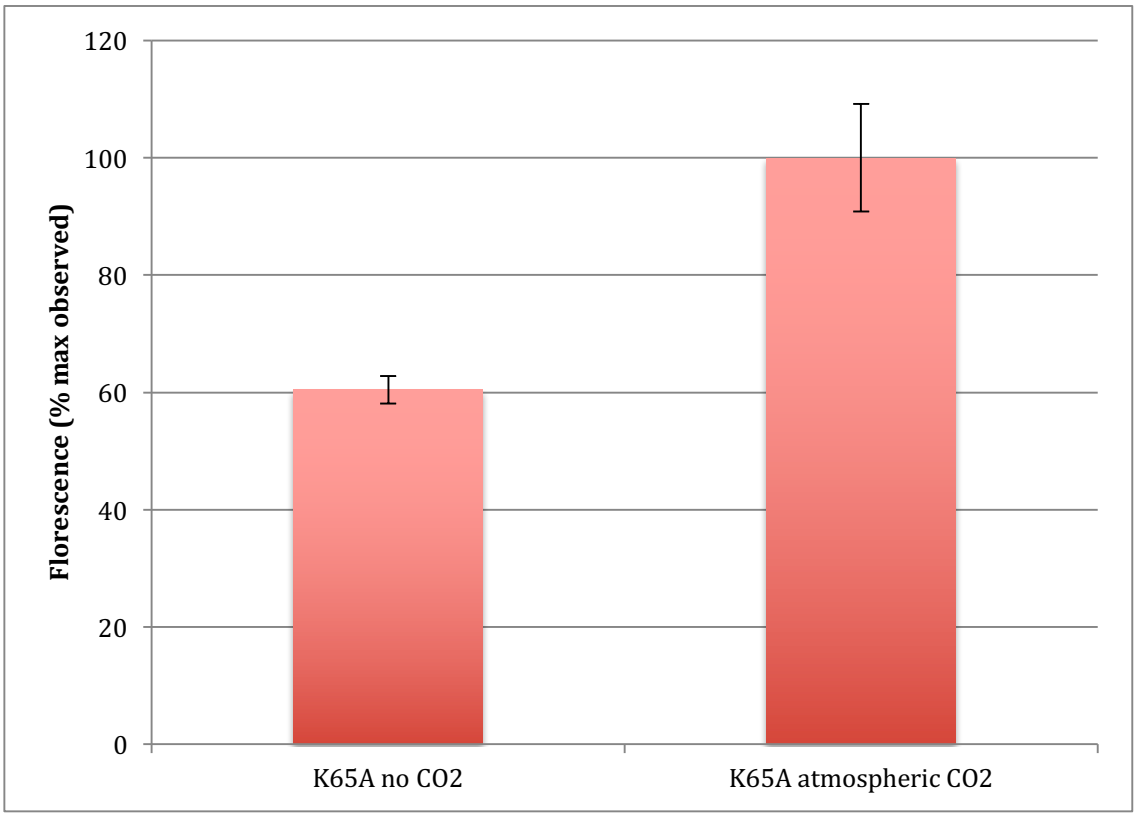


Figure 4-7: Fluorescence of TNS bound to LTP1-K65A in the absence of CO_2 and at atmospheric CO_2 concentrations. Mean values (n = 8) are presented here and error bars represent the standard error

Figure 4-7 clearly shows that the measured fluorescence was lower in the absence of CO_2 than at atmospheric CO_2 concentrations, with fluorescence in absence of CO_2 at 60 % of the fluorescence measured at atmospheric CO_2. This indicates that TNS binding to LTP1 was reduced in the absence of CO_2. The data obtained from these assays did not pass a Shapiro-Wilke normality test, indicating that it is not normally distributed. A parametric statistical test such as an ANOVA was therefore not appropriate to analyse this data. A Mann-Whitney U test was used instead, and showed that the difference in fluorescence observed between the two conditions is statistically significant ($P < 0.05$).

Due to the K65A mutation, no carbamate is present on residue 65 of this protein at any concentration of CO_2. However, a significant difference in

binding activity is still observed between the two conditions tested here. The observed difference in fluorescence for the mutant protein (60 %) is comparable to that observed for the WT protein (58 %) and falls within the standard error for the LTP1-WT results. This suggests that the carbamate observed on lysine 65 of the WT LTP1 is not responsible for increasing the affinity of this protein for TNS in the presence of CO_2.

4.4.3 Dose response curve

The results of the above experiment indicate that the K65A mutation does not affect the affinity of LTP1 for TNS. In order to confirm this, a dose response curve for binding of LTP1-K65A to TNS was produced. It was hypothesized that the K_d for this interaction would be the same as the carbamate on K65A of LP1-WT does not appear to increase the affinity of the protein for the lipid probe, the K_d for the interaction of TNS and the mutant protein will be the same as for the WT.

LTP1-K65A protein was combined with increasing concentrations of TNS and the fluorescence measured (2.16). The results of these assays are presented in Figure 4-8.

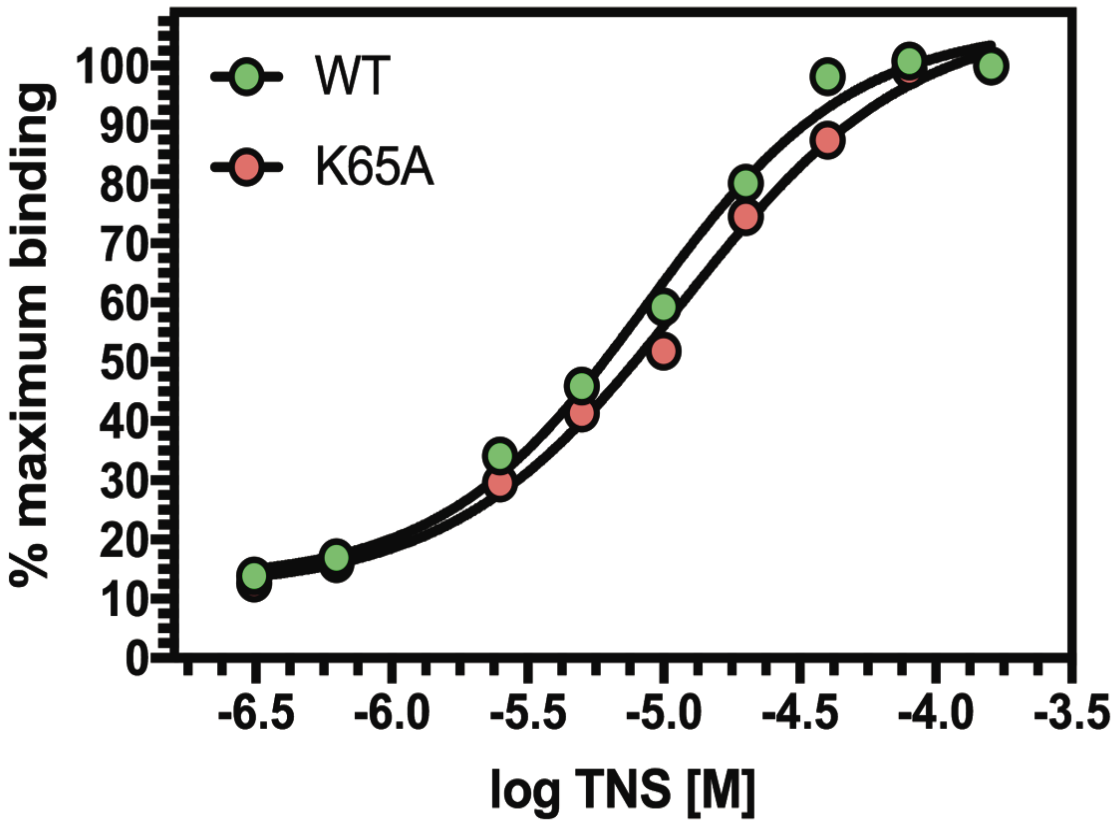


Figure 4-8: Dose response curve for LTP1-K65A and LTP1-WT showing change in fluorescence with increasing TNS concentration.

Figure 4-8 shows dose response curves for both the K65A mutant protein and the WT. It appears from this figure that slightly higher concentrations of TNS are required for the mutant to reach maximum binding than the WT. The fluorescence of the LTP1-K65A assays reached half its maximum value when the concentration of TNS was 11.7 μM. This suggests that the K_d for the interaction of LTP1-K65A and TNS is higher than that of the WT protein and probe. This mutant protein may therefore have a lower binding affinity for the probe than the WT. However, due to time constraints this assay was only repeated twice. It is therefore not possible to determine from this data alone whether this difference in K_d is significant.

4.5 Binding of LTP1 to biologically relevant lipids

All lipid-binding experiments discussed so far have taken advantage of the ability of LTP1 to bind TNS to measure lipid binding affinity. However, this fluorescent probe is artificially made and is not present naturally in plants. It was therefore important to assess the ability of purified LTP1 to bind physiologically relevant lipids. The lipids phosphatidylethanolamine (PE), phosphatidylcholine (PC) and oleoyl coA (OC) were chosen for preliminary investigations as they were readily available and are all present in plant cells. PE and PC are phospholipids found as part of the lipid bilayer (Vance *et al*. 2012). OC is a fatty acid found in both animal and plant oils (Young, 2002).

LTP1-WT protein was combined with TNS and the lipid of interest in measurement buffer. A control sample containing protein and TNS but no biological lipid was also prepared and the fluorescence of all samples was measured. When a lipid binds to LTP1 it will displace TNS, resulting in a decrease in fluorescence (Bogdanove *et al*. 2016). Therefore a lower fluorescence reading indicates that LTP1 has greater affinity for that particular lipid. The results of this investigation are shown in Figure 4-9, with fluorescence expressed as a percentage of the value measured in the absence of biological lipid.

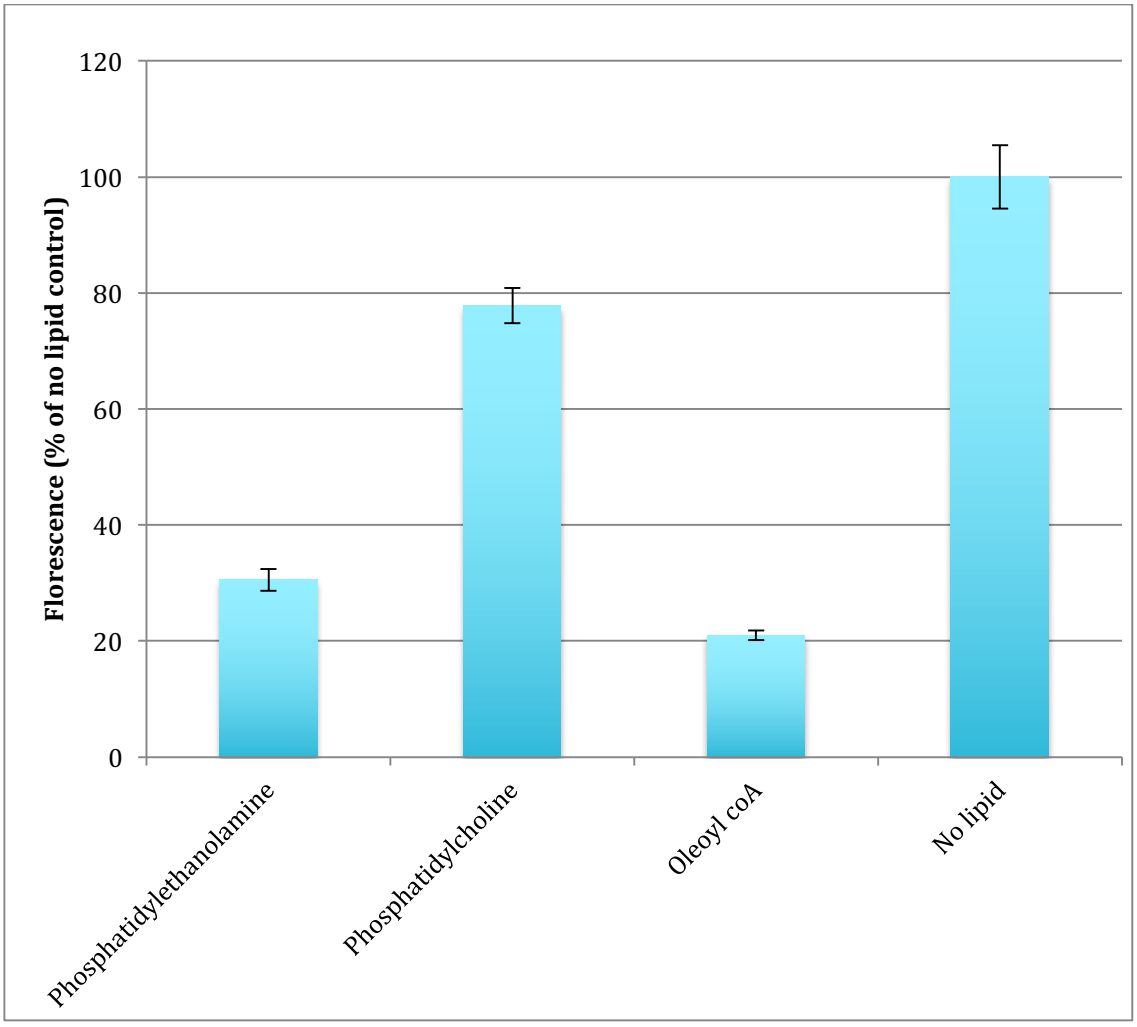


Figure 4-9: Fluorescence of assays with biological lipid present, expressed as a percentage of the fluorescence measured for the no-lipid control. Mean values are presented here (n = 3) and error bars represent the standard error.

All the biological lipids tested here resulted in a decrease in fluorescence, indicating that all are able to bind to LTP1. Oleoyl coA showed the greatest reduction in fluorescence, measuring at only 21 % of the fluorescence of the no-lipid control. The protein also showed considerable affinity for Phosphatidylethanolamine, with fluorescence reduced to 31 % of the control. When phosphatidylcholine was tested, fluorescence decreased to 78 % of the control value. This indicates that LTP1 does bind to this lipid, however with considerably lower affinity than to the others tested.

The results of these preliminary investigations in to binding of LTP1 to

biologically relevant lipids indicate that the purified protein is able to bind multiple plant lipids with varying degrees of affinity. Due to time constraints on this project no other lipids were tested in this manner, however it would be interesting to explore binding of LTP1 to a range of different potential interactors. Additionally, it would be interesting to investigate the effect of CO_2 concentration on binding of LTP1 to biologically relevant lipids to confirm that the presence of CO_2 enhances binding to naturally occurring plant lipids as well as to the fluorescent probe TNS.

4.5 Discussion and Conclusion

Purified LTP1 protein was able to bind to the probe TNS, stimulating production of fluorescence and allowing measurement of binding activity. In the absence of CO_2, the ability of LTP1-WT to bind TNS was significantly reduced, indicating a role for CO_2 in lipid binding. Three putative carbamates have previously been identified on lysine residues in this protein (Linthwaite 2017). One or more of these carbamates may be responsible for increasing lipid-binding affinity in the presence of CO_2. The mutant protein LTP1-K65A, which lacks one of these carbamylated lysine residues, showed the same reduction in TNS binding in the absence of CO_2 as the WT protein. This suggests that the carbamate observed on lysine 65 on LTP1-WT is not required to increase the TNS-binding affinity of this protein. This carbamate may still play a role in another aspect of protein activity, for example it may be involved in the interaction of LTP1 with other proteins or even with more biologically relevant lipids than TNS. A small difference between K_d for the interaction of LTP1-WT and LTP1-K65A with TNS was demonstrated, however not enough data was obtained to determine if this difference was statistically significant. However, as the binding affinity of LTP1-K65A was also reduced in the absence of CO_2, it is likely that one or both of the other carbamates identified (on lysines 98 and 115) may instead be responsible for the observed increase in binding to TNS in the presence of atmospheric CO_2.

Creating mutants which do not contain these lysine residues could further elucidate the role of these carbamates in the lipid binding activity of LTP1.

The screen conducted by Linthwaite (2017) that originally identified the carbamates present on LTP1 was conducted at a higher concentration of CO_2 than the assays performed here. Consequently it is possible that not all the observed carbamates are able to form at atmospheric CO_2 concentrations. If this is indeed the case, it may be that the carbamate on lysine 65 of LTP1-WT was not present at all during the assays discussed here. Therefore, it would be interesting to trap carbamates on purified LTP1-WT both in the absence of CO_2 and atmospheric concentrations to investigate which, if any, of these carbamates are present under these conditions. If these carbamates are present at this concentration of CO_2 it implies that their role in protein function is not regulatory, as they would always be present at the CO_2 concentration found in plant cells. Instead they may be a necessary part of the structure of this protein *in vivo*.

The fluorescent probe TNS was used in all experiments described here to assess lipid binding activity of LTP1. However, as discussed above, this probe is not naturally occurring and therefore may not accurately represent binding of biological lipids to this protein. For this reason, the ability of LTP1 to bind to various naturally occurring plant lipids was investigated. LTP1 was able to bind to all the lipids tested with varying degrees of affinity, indicating that this purified protein has biologically relevant activity. Due to the time scale of this project, the effect of CO_2 on this binding was not investigated, however it would be extremely interesting to investigate whether the results achieved with TNS could be replicated with these plant lipids.

Additionally, LTPs may also interact with molecules other than lipids. For example, they have been shown to bind specifically to elicitin receptors in the plasma membrane, potentially playing a role in the defence against phytopathogens (Buhot *et al*. 2001). Therefore the carbamates present on

LTP1, such as the carbamate on lysine 65 which does not appear to affect lipid binding, may be involved in other aspects of protein function. Further work on this protein could explore the interaction of this particular LTP with elicitin receptors and investigate whether this is affected by CO_2 concentration.

In conclusion, preliminary investigations indicate that the lipid binding activity of LTP1 is increased by the presence of CO_2. Mutant studies demonstrate that this increase in lipid binding affinity is unlikely to be due to the putative carbamate present on lysine 65 of the WT protein. Further studies are required to investigate whether this effect is due to the presence of carbamates on lysines 98 or 115 and to fully elucidate the role of CO_2 in the function of this protein.

Chapter 5: Conclusions and Future Work

This thesis set out to examine the effect of carbamylated lysine residues on the function of two proteins from *Arabidopsis thaliana*.

The metabolic enzyme FBA1 was known to contain a carbamate modification on the lysine residue at position 293. However, no significant difference in the specific activity of this enzyme was observed with the addition or removal of CO_2. Additionally, the ability of FBA1-WT to form a tetramer was not affected by increasing the concentration of CO_2. Nevertheless, the mutant protein FBA1-K293A, which is unable to form a carbamate, had reduced activity compared with the WT and showed impaired tetramer formation. Another mutant, K293E, was found to be unable to mimic the effect of the carbamylated lysine residue. A carbamate trapping experiment confirmed that the carbamate on FBA1-WT is not stable at atmospheric CO_2 concentrations. However, the carbamate may still have been present on some peptides in the sample. Therefore further mass spectrometry analysis is required to determine whether this modification was likely to have been successfully removed for assays in the absence of CO_2. Overall, these results suggest that while lysine 293 is important for tetramer formation and thus efficient cleavage activity, the carbamate modification is not required for this. Further investigation is required to determine whether this carbamate does play role in protein function.

The non-specific lipid binding protein LTP1 contains three putative carbamylation sites. The ability of this protein to bind to the fluorescent probe TNS was shown to be significantly reduced in the absence of CO_2. This effect was also demonstrated for the mutant protein LTP1-K65A, which cannot form a carbamate on lysine 65. Therefore it is likely that the carbamate on lysine 65 is not responsible for the observed effect of CO_2 on the ability of the protein to

bind TNS. Further mutant studies are required to determine the effect of the other observed carbamates on the function of LTP1. Additionally, further investigation is required to find out whether this effect can be replicated using biologically relevant lipids.

Although it was not possible in the time scale of this project to fully elucidate the role of carbamates in the function of these two proteins, the investigations described in this thesis have demonstrated that carbamate modifications identified on *Arabidopsis* proteins by Linthwaite (2017) may be functionally relevant. Therefore the carbamate trapping technique developed by Linthwaite (2017) provides an effective mechanism for identifying novel targets of CO_2 signalling.

The proteins discussed in this thesis were only two from a wide range of *Arabidopsis* proteins that were found to contain carbamate modifications. The methods developed in this project for assaying protein activity in the absence of CO_2 could be used in future experiments to determine the effect of carbamates on the function of other such proteins. In addition, *in vivo* mutant studies could be conducted to examine the physiological effect of these carbamylated proteins on plant growth and development.

The development of a novel technique to identify carbamate modifications on proteins under physiologically relevant conditions has provided us with an invaluable new resource for investigating the biological effect of CO_2 (Linthwaite, 2017). Further proteomic screens could be performed using this method to identify carbamylated proteins in a variety of organisms and cell types. There is huge potential for this technique to be used to greatly increase out understanding of how carbamates contribute to protein function as well as identifying new proteins involved in CO_2 signalling.

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