A CHARACTERISATION OF RUBISCO ACTIVASE FROM *SPINACIA OLERACEA*

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Abstract

Rubisco activase undergoes ATP hydrolysis to activate Rubisco which has generated an inhibitor. Recent crystallographic evidence has proposed several models however the correct model is disputed. The point of contention involves the active oligomeric state of Rubisco activase. This study indicates that the oligomeric state of spinach Rubisco activase is dependent on concentration, temperature and ligand binding. Rubisco activase appears to exist in a number of oligomeric states, and in polydispersive solutions, of which all appear active. Activity was recorded in concentrations ranging from 0.01 to 0.1 $mg.ml^{-1}$. SEC, SEC-MALS and DLS indicated that the prominent oligomer present in solution changed through these concentrations from dimers to hexamers. Nucleotide supplementation was shown to cause an increase in molecular weight and thermal stability while Mg^{2+} was shown to have a minor destabilising effect on both. This data indicates that the oligomeric state of spinach Rubisco activase is dynamic, largely dependent on concentration and ligand association. Comparison of data indicated that a dimer-tetramer is required for activity with a tetramer giving full activity. Critical oligomeric states were also seen at a hexamer and an octamer for thermal stability.

Abbreviations

°C: Degrees Celsius

3-PG: 3-phosphoglycerate

AAA+: ATPases associated with diverse cellular activities

ADP: Adenosine di-phosphate

Arg: Arginine

ATP: Adenosine tri-phosphate

AU: Absorbance unit

AUC: Analytical ultracentrifugation

CBB: Calvin-Benson-Bassham

CO_2: Carbon dioxide

Da: Dalton

DLS: Dynamic light scattering

DSF: Differential scanning fluorimetry

EDTA: Ethylenediaminetetraacetic acid

Gln: Glutamine

H_2O: Water

KCl: Potassium chloride

Kd: Dissociation constant

kDa: Kilo Dalton

LB: Lysogeny broth

LSU: Large sub-unit

Lys: Lysine

MDa: Mega Dalton

mg: Milligram

Mg^{2+}: Magnesium

$MgCl_2$: Magnesium chloride

min: Minute

ml: Millilitre

mM: Millimolar

mRNA: Messenger ribose nucleic acid

NADH: Nicotinamide adenine dinucleotide

NanoESI-MS: Nano-electrospray ionisation mass spectrometry

NBD: Nucleotide binding domain

nm: Nanometre

O_2: Oxygen

OD: Optical density

PCR: Polymerase chain reaction

PEG: Polyethylene glycol

Pi: Inorganic phosphate

Pro: Proline

PVPP: Polyvinylpolypyrrolidone

r.nm: Radius in nanometres

Rca: Rubisco activase

RI: Refractive index

Rubisco: Ribulose^{-1},5-bisphosphate carboxylase/oxygenase

RuBP: Ribulose bis-phosphate

SEC: Size exclusion chromatography

SEC-MALS: Size exclusion chromatography multi angle light scattering

Tm: Apparent melting temperature

TRP: Tryptophan

UV: Ultra violet

Val: Valine

µg: Microgram

µl: Microlitre

µmol: Micromolar

Chapter 1) Introduction

1.1) Photosynthesis

Photosynthesis is the process in which energy from the sun is harnessed by photosynthetic organisms in order to produce sugar phosphates which can be used to power cellular processes. It is hard to understate the importance of this process to life as energy production is constantly required to allow for the survival of higher organisms in the food web. Photosynthesis is also vital to our social and economic growth as a species. As the human population grows, demand for food increases. A sprawling population also means that more land is being converted to farmland. It is these mitigating social effects which make enzymes involved in photosynthesis economically and socially important targets for research. If this process was able to be improved upon it may allow for larger crop yields and may allow for the growth of crops in unfavourable conditions such as drought and high heat.

1.2) Rubisco Activase

Ribulose^{-1},5-bisphosphate carboxylase/oxygenase, or Rubisco, is an extremely important, yet highly inefficient enzyme. Part of this efficiency problem is the readiness at which Rubisco will incorporate oxygen as a substrate, rather than CO_2, creating deleterious by-products which are able to inhibit the active site (Tabita *et al.* 2008). This caused the need for a system in which these deleterious by-products can be released from Rubisco, allowing for the continuation of carbon assimilation within the organism. This problem was overcome through the evolution of an enzyme now known as Rubisco activase (Rca). The enzyme belongs to the AAA+ (ATPases Associated with diverse cellular Activities) family of enzymes and utilises ATP hydrolysis to power conformational change which is then transferred through

Rubisco, allowing for the freeing of the inhibiting by-product (Neuwald *et al.* 1999). Rubisco activase consists of three major domains, the AAA+ domain, required for ATP hydrolysis, the N-terminal domain, involved in species specificity and association with Rubisco and the C-terminal domain which is involved in the regulation of the enzyme (Esau et.al 1996).

1.3) Discovery of Rubisco Activase

Rca was first isolated in 1982 from *Arabidopsis thaliana*, after it was found that mutation of a specific gene prevented the activation of Rubisco in the mutants, while not affecting any other chloroplast or photosynthetic enzymes. It was also found that high concentrations of CO_2 were able to overcome the difficulties experienced by the mutant (Somerville *et al.* 1982). The activity of Rubisco was observed to decrease over time. Rca, once identified, was able to be purified and it was found that addition of activase allowed continued activity of Rubisco while at physiological CO_2 levels (Portis *et al.* 1986). These experiments gave an initial insight into the requirement of Rca for carbon assimilation via the Calvin-Benson-Bassham (CBB) pathway. The CBB pathway describes several redox reactions which occur within chloroplasts and are responsible for the production of 3-phosphoglycerate which can be utilised as an energy source for cellular activities (Bassham *et al.* 1950).

Two isoforms of Rca have been elucidated. Research performed on spinach and *Arabidopsis thaliana* revealed one approximately 45 kDa isoform (α isoform) and a truncated 42 kDa isoform (β isoform) (Werneke *et al.* 1989, To et.al 1999). In these species the two isoforms were shown to be created via alternative splicing of the Rca mRNA (Werneke *et al.* 1989). *Solanaceae* plants have been shown to only produce

the β isoform, whereas non-*Solanaceae* plants produce both the β and the α isoform (Wang *et al.* 1993). Evidence indicates that the α isoform is involved in the regulation of the Rca and is able to inhibit the activity of the β isoform (Zhang and Portis 1999). This inhibition is able to occur due to the finding that Rca forms an oligomer, allowing the opportunity for the large isoform to form a heterooligomer with the small isoform (Wang *et al.* 1993). Regulation of Rca is light dependant and is also affected by ATP/ADP ratios within the biological system, utilising thioredoxin-f as a redox switch (Buchanan 1991). Once Rca had been purified, experiments were set up to determine whether this newly isolated enzyme was indeed the cause for the activation of Rubisco. Initial experimentation indicated that the addition of Rca *in vitro* did not cause activation of inhibited Rubisco. A vital clue to Rca's mechanism was revealed when an activity assay, exploring the activation of inhibited Rubisco by Rca, was contaminated by adenine triphosphate (ATP) (Streusand and Portis 1987). This occurred due to a reaction scheme in which ATP was utilised to produce the inhibitor (Portis and Salvucci 2002). This gave the first evidence that Rca dependent Rubisco activation required ATP. These experiments opened the doors for researchers to begin elucidating the structure and mechanism of Rca.

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In non-*Solanaceae* plants it has been found that the larger α-isoform contains two conserved C-terminal cysteine residues found on the C-terminus that are believed to be involved in the regulation of ATP hydrolysis of Rca (Zhang *et al.* 2001). Once it was determined the large isoform was responsible for the activity state, it was soon found that redox regulation was responsible for the activity of the large isoform (Zhang and Portis 1999). This lead to the hypothesis that reduction of the C-terminal cysteines may result in a conformational change, allowing for easier binding of ATP

to the α-isoform (Wang and Portis 2006). This was supported by the mutation of the cysteine residues C-411 and C-392, of *A. thaliana* β-isoform Rca, which resulted in the loss of the α-isoforms increased sensitivity to inhibition (Zhang and Portis 1999).

1.4) Mechanism of Action

In order for Rca to confer a conformational change through Rubisco the two proteins must interact with each other, however the exact nature of this interaction has been difficult to elucidate (Blayney *et al.* 2011). Some data was gained from the use of chemical cross linking of the large subunit of Rubisco coupled with co-immunoprecipitation (Salvucci and Crafts-Brandner 2004). These results were used to create an electron micrograph which indicated an Rca oligomer may encircle Rubisco (Buchen-Osmond *et al.* 1992).

As previously discussed Rca in non-*Solanaceae* plants differs from Rca from *Solanaceae* plants. This divergence has led to incompatibility between the Rca of one family and the Rubisco of the other (Wang *et al.* 1992). Several different species were tested for cross species compatibility of Rubisco and Rca (Zhu and Spreitzer 1994). It was found that non-*Solanaceae* plants (including spinach, barley, whey and maize) showed significant compatibility between the Rubisco and Rca of different species, as between *Solanaceae* species (including tomato, tobacco and petunias). It was also found that Rca from spinach was able to activate the Rubisco found in *Chlamydomonas reinhardtii.* Primary residues were found to be phylogenetically distinct between the *Solanaceae* and non-*Solanaceae* families, three of which were at the C-terminus. This was an attractive target for site directed mutagenesis as the C-

terminus, while in its native state, is close to Rubisco's loop six which is involved, along with the carboxyl terminal itself, in the closing of the Rubisco active site (Zhu and Spreitzer 1994). This was later supported by experimentation done by Esau *et al.* which used chimeric activases to exemplify the role of the C-terminal in specificity. This study created several chimeras which showed a switch in family specificity upon the splicing of a new C-terminus (Esau *et al.* 1998).

In the first major site directed mutagenesis experiments two residues of the Rubisco enzyme from *C. reinhardtii* were mutated in order to determine the effects on the native protein. A lysine at position 356 was changed to glutamine and a Proline at position 89 was altered to an Arginine. The K356Q mutant showed no change in specificity and slightly reduced activation potential. In contrast to this the P89R mutant caused an inversion in the specificity from non-*Solanaceae* to *Solanaceae* (Larson *et al.* 1997). This substitution introduces a larger and more positively charged residue which is thought to prevent the association of spinach Rca with the Rubisco from *C. reinhardtii,* however it is these conditions that favour the association of Rca from *Solanaceae* species.

Much research has followed searching for more residues involved in the activation of Rubisco and the species specificity that is observed. As discussed Rca belongs to the AAA+ family of enzymes, many of which contain a 'sensor 2' domain which has been shown to be responsible for substrate recognition. In the case of Rca, the substrate is Rubisco. This provided an attractive region for mutagenesis as it had the potential to impart a higher understanding of substrate specificity. It was found that

altering amino acids within the Box VII and sensor 2 region could cause a change in the selectivity of activase for Rubisco. This was due to the role of two critical residues, lys 313 and val 316. These residues were shown to interact with two residues, Rubisco 89 and Rubisco 84, positioned on the N-terminal of the LSU of Rubisco (Li *et al.* 2005).

Recently Henderson *et al.* (2011) showed a crystal structure of Rca in which the two residues identified by Li *et al.* (2005), K-313 and V-316, resided on the tip of a protrusion created by a β-turn connecting helix 3 to the irregular strands (Henderson *et al.* 2011). This gives a potential interaction mechanism as the identified residues on the protrusion may act as the point of binding or association between activase and Rubisco, allowing for the transfer of the conformational change.

1.5) Crystallography

In order to better understand the mechanism of Rca crystallography has been utilised in order to define the protein's atomic structure. The first such data to be published was by Henderson *et al.* in 2011, which defined 97 amino acids (residues 248-346) of the C-terminal domain of Rca from the higher order plant *Larrea tridentate* (creosote). This 1.9 Å resolution structure indicated a four α-helical bundle, which forms a 50 Å long elongated "paddle". This structure was then superimposed upon several AAA+ proteins showing high homology to spastin, VPS4 and FtsH, all of which are members of the family to which activase belongs. Within the structure it was seen that helix 3 contains a nine revolution extended paddle formation. The paddle is thought to be maintained by three trans-proline residues which provide

rigidity to the structure. The paddle arranges its residues so that the hydrophobic residues, including W-301 face inward whilst the hydrophilic residues are faced outward. These residues are thought to be involved in Rca self-association in that the activase molecules are able to associate their hydrophobic patches with one another allowing for the spiralled motif observed. The tip of the paddle was also shown to contain the residues K-313 316 which have been shown previously to be involved in Rubisco species recognition (Li *et al.* 2005, Henderson *et al.* 2011).

Another structure was elucidated for the 35 kDa AAA+ protein CbbX, which is a red type Rca from *Rhodobacter sphaeroides.* A 3 Å resolution structure indicated structural similarities between the red and green type activases (Muellar-Cajar *et al.* 2011). This red type activase was shown to activate red type Rubisco through fall-over assays. In contrast to green type red type activase will only undergo ATP hydrolysis in the presence of RuBP or Rubisco, and it is thought that RuBP binds allosterically to red type activase. This allosteric binding was also shown to stimulate oligomerization of CbbX, forming species from 0.6 – 10 MDa. It is thought that the necessity of RuBP for ATP hydrolysis allows for regulation of the pathway, as when the substrate is low it prevents the oligomerization and activity of CbbX, allowing for the retention of ATP stores. However upon introduction of the substrate higher forms are encouraged, allowing for the formation of the hexameric protein.

The most recently published structure proposed a stable 6 member ring formation. The 2.95 Å structure depicts Rca from *Nicotiana tabacum* (tobacco) without the N-terminal (it is believed flexibility in the N- or C-terminal caused disassociation during crystallisation). This structure indicated that activase held the classic AAA+ protein

domains including the walker and sensor motifs. The structure indicated an oligomer which consisted of six turns. This helical shape was however proposed to be a result of crystal packing and not representative of the species in solution. A mutant (R294V/A) caused stabilisation of higher order species when combined with hydrolysable ATP analogues (Li *et al.* 2006). This was inferred as Rca being unable to form stable species above hexamers (Stotz *et al.* 2011).

The hypothesis that Rca forms a six membered ring is supported by structural information of other AAA+ proteins which commonly form hexameric proteins. An important example of this was the structure obtained for the CbbX protein which showed that it formed six-fold symmetrical rings(Mueller-Cajar *et al.* 2011). This is seen for several different AAA+ proteins such as Fidgetin-like 1 protein and FtsH (Peng *et al.* 2013). Some AAA+ proteins have however been shown to have multiple active oligomeric sizes in support of the hypothesis that Rca exists in a dynamic oligomeric state. One such protein is ClpB, which was shown to exist in solution as monomers, dimers, hexamers and heptamers with low ionic strength solutions causing a shift towards the hexamer and high ionic strength solutions causing a shift towards heptamers and several intermediate sized oligomers. This dynamic oligomerization was also shown to be affected by nucleotide binding (Akoev *et al.* 2004).

1.6) Concentration and Oligomeric state

It was soon found that the addition of ATP and Mg^{2+} to spinach Rca resulted in an increase in oligomeric state (Wang *et al.* 1993). However this aggregation was dynamic in that heterogeneous apparent molecular masses were detected within a single sample by gel filtration. This aggregation was also shown to be influenced by

temperature and protein concentration. This caused a range of molecular masses ranging from 290 kDa to >600 kDa (Wang *et al.* 1993). The effects of additives on the molecular weight of Rca will be explored further in chapter 5.

A concentration dependant study was carried out which exemplified the effects of concentration on oligomeric state. A concentration series was explored in which Rca concentrations, in the presence of ATP, ranging from 1 $mg.ml^{-1}$ to 10 $mg.ml^{-1}$ were examined for their molecular weight. This showed a range of molecular masses from 280 kDa to 660 kDa. It was also observed that over a period of 4 hours the 660 kDa species was able to form a soluble, inactive oligomer of > 2 MDa when incubated at 25 °C without nucleotides (Barta *et al.* 2010).

More recently nanoelectrospray ionization mass spectrometry (nano ESI-MS) has been utilised to gain a better understanding of the oligomeric species of Rca. This showed the presence of species from monomers to hexamers. It was stated that larger oligomers may have existed but could have dissociated as a result of the method. This prompted the thought that the major biologically active unit would exist as a hexamer (Blayney *et al.* 2011). This is a promising model due to the observation that many other AAA+ proteins exist as hexamers in their native active form (White and Lauring 2007).

1.7) Thermal Stability

Evidence indicates that thermal instability of Rca is responsible for a loss in the photosynthetic potential of plants under moderate heat stress (Feller *et al.* 1998). It has long been understood that net photosynthesis is inhibited by heat stress so the

effects of temperature on gas exchange and Rubisco activation in leaves was studied and compared to the effects of temperature on the isolated enzymes. It was found that a loss in net photosynthesis was a result of a higher rate of Rubisco deactivation and occurred when leaf temperature exceeded 35 °C. This increase in deactivated Rubisco was thought to be attributed less to an increase in deactivation and more to a decrease in the reactivation ability of Rca. This was evidenced by the observation that isolated Rubisco activity was highest at >50 °*C* whereas Rca had a lower optimum temperature of 42 °*C*. Isolated activase was also shown to lose its Rubisco activation potential at higher temperatures *in vitro* (Crafts-Brandner and Salvucci 2000).

The drop in activity of Rca at high temperatures is a result of the creation of non-specific, inactive aggregates. Experiments performed on *Arabidopsis thaliana* β-isoform Rca showed that incubation at room temperature (approximately 21 °*C*) of an active 660 kDa oligomer resulted in the formation of a soluble yet inactive molecule with a molecular weight of approximately 2 MDa. Aggregation caused by heat stress was shown to be reduced by supplementation of ATP and ADP, whereas Mg^{2+} supplementation was shown to encourage aggregation of Rca. It was found that the aggregation caused a loss of activity. This activity loss was therefore promoted by Mg^{2+} while nucleotide supplementation was able to reduce the loss of activity as a result of aggregation (Barta *et al.* 2010). The heat stability of Rca will be further discussed in chapter 4.

The effect of Mg^{2+} and nucleotide supplementation has been explored for several species. Henderson *et al.* (2013) studied the effect of nucleotides, free phosphate and

Mg^{2+} on the thermal stability of cotton β-Rca. They found similar results to those determined for *A. thaliana.* Nucleotide supplementation caused an increase in apparent melting temperature (Tm), as measured by a thermoflour assay. ADP caused an increase in Tm of up to 10 °*C*, with a less pronounced yet similar effect given by ATP supplementation. Free phosphate (Pi) was also tested and was shown to cause an increase in thermal stability. Mg^{2+} also caused a decrease in thermal stability, up to 1.3 °*C*, and reduced the stabilising effect of ADP. This is in contrast to an increase in the thermal stability provided by ATP when in the presence of Mg^{2+}. It is thought that the increased thermal stability provided by nucleotide supplementation is caused by a decrease in conformational flexibility upon ligand binding (Celej *et al.* 2003). The observation that Mg^{2+} increases the thermal stability provided by ATP may be caused by Mg^{2+} creating a more positively charged environment within the active site which would suit ATP, which is more negatively charged than ADP due to the additional phosphate group, more favourably allowing for tighter binding and therefore a reduction in conformational flexibility. This however cannot be the sole reason as ATP will be consumed by Rca at a relatively rapid rate. This is supported by the observation that ADP, Mg2+ and Pi supplementation gives a similar Tm to ATP and Mg^{2+} supplementation. This is not surprising as ATP and Mg^{2+} supplementation of an active Rca will cause ATP hydrolysis resulting in a ratio of ADP and ATP with Pi generated during hydrolysis (Henderson *et al.* 2013).

It has also been indicated that the α-isoform of spinach Rca has greater thermal stability than the smaller β-isoform. The α-isoform was shown to retain ATPase activity for a significantly longer period when incubated at 40 °*C* as compared to the

β-isoform. The difference in thermal stability is thought to be a result of a difference in subunit association strength between the two isoforms (Crafts-Brandner *et al.* 1997).

1.8) Rubisco

Rubisco or ribulose^{-1},5-bisphosphate carboxylase/oxygenase is an enzyme responsible for the assimilation of CO_2 into the Calvin-Benson-Bassham (CBB) cycle. The enzyme is responsible for catalysing the reduction and bonding of CO_2 to ribulose bisphosphate (RuBP), forming two molecules of 3-phosphoglycerate (3-PG). This process allows biologically available carbon to re-enter the biosphere, allowing for energy to be produced within the food web. Although this enzyme is vitally important to the development of photosynthetic organisms, and life in general, it has been shown to exhibit poor catalysis and substrate specificity (often utilising oxygen rather than CO_2). In order to have a sufficient turnover rate to satisfy the energy demands of photosynthetic organisms Rubisco has been found to account for up to 50% of an individual's soluble protein to this enzyme.

It has been postulated that to improve the poor efficiency of Rubisco would improve the growth rate of many plants, due to Rubisco being the rate limiting enzyme of the CBB cycle. The poor catalytic rate of Rubisco is partly attributed to its potential for self-inhibition and its requirement for reactivation by activase.

Rubisco's function is to catalyse the carboxylation of RuBP forming 2, 3 – enediol(ate), which decomposes to form two molecules of 3-phophoglycerate (3-PG) which can be passed into other cellular processes such as glycolysis. The

carbamylation of a specific lysine residue, K-201(in spinach), along with the co-ordination of a Mg^{2+} ion, is first required in order to create an activated active site (Taylor and Andersson 1996). Once activated, a favourable environment exists for the co-ordination of ribulose^{-1},5-bisphosphate (RuBP) which binds to the Mg^{2+} ion. This binding results in a conformational change which causes the loop 6 region to change from the open to the closed conformation after a 12 Å movement (Taylor and Andersson 1996). Once this flexible 'lid' domain has shut the reaction is able to commence. The reaction requires the binding of a CO_2 molecule to RuBP, as CO_2 is a relatively unreactive species a proton is first abstracted from RuBP, creating the 2,3 enediol of RuBP, allowing for the binding of the CO_2 molecule (Gutteridge *et al.* 1984) This allows the creation of a six carbon sugar from a five carbon sugar and represents the acquisition of carbon into the food web. Once the CO_2 molecule has bound the addition of H_2O to the third carbon results in molecular scission producing two molecules of 3-PG from RuBP (Pierce *et al.* 1986).

One of the reasons for its poor catalytic efficiency is due to its catalytic bifunctionality and potential to undergo a different reaction. Other than the carboxylation of RuBP the enzyme is also capable of utilising O_2 in place of CO_2. This results in the creation of one of several deleterious by-products formed upon oxidation, such as 3-ketoarabinitol (Andrews 1996). General catalytic misfire can also create several deleterious five-carbon sugars, such as D-glycero-2,3-bisphosphate and 2-carboxytetritol^{-1},4-bisphosphate, and the six carbon sugar 2-carboxy-D-arabinitol 1-phosphate. Creation of these by-products causes unfavourable interactions preventing the loop 6 of Rubisco from being released, effectively inhibiting the enzyme by blocking the active site. These deleterious reactions have been shown to occur during

around 1% of Rubisco's reactions (Pearce 2006). It has been postulated that these by-products may play a regulatory role as during the dark cycle activase does not function as well so may allow for the inactivation of Rubisco while energy stocks are low within the cells (Parry *et al.* 2008).

1.9) Implications of Rubisco and Rubisco activase

The elucidation and understanding of Rubisco and Rca could aid us in our fight against starvation and climate change. As climate change worsens we will find conditions that push the limits of the natural systems currently in place. It has been shown that heat stress causes a drop in photosynthetic activity, due to the thermal instability of Rca, which could cause a large reduction in global plant biomass generation as temperatures begin to move outside of optimal growing conditions. As this change is thought to be occurring at a rate beyond what would occur in nature natural evolution may not be able to compensate for this shift in global weather conditions and temperature. Engineering of Rubisco or Rca may become vital in our efforts to curb the effects of these changes. The conversion of CO_2 into O_2 also helps to reduce the greenhouse effects thought to be causing the climate shifts. If Rubisco can be engineered to be a more efficient enzyme it could help to reduce the abundance of CO_2 in the atmosphere. With increased Rubisco efficiency a rise in net photosynthesis would also be seen which would translate into an increase in plant biomass and would allow for the growth of plants in environments which had low CO_2 concentrations/ light levels or other unfavourable conditions. It would also allow for an increase in food production in fertile soils. With an exploding population and increasing economic, social and environmental pressures finding a large enough food source to sustain the global population will become a more pertinent issue.

Engineering of Rca to improve its thermal stability could have a large effect on the ability of plants to undergo photosynthesis while under heat stress. Solutions could involve the creation of higher α-form/β-form ratios or the altering of the charge within the active site to encourage stability in the presence of nucleotides. Rubisco efficiency could also be a target for engineering, the creation of a Rubisco with a higher specificity or lower affinity for deleterious bi-products could also help increase photosynthetic rate. Potential sites for the engineering of Rubisco and Rca have been discovered through the elucidation of the structure of the two enzymes and their interactions with physiologically relevant molecules and each other. Although much research will be required before this knowledge can be applied to the creation of genetically superior plants the pool of knowledge about the two enzymes is increasing, being made ever easier by the continued development and refinement of technology and techniques which can be used to determine protein properties.

Chapter 2) Materials and Methods

2.1) Rca Purification

BL21 DE3 star cells were transformed using a plasmid containing the spinach Rca β-isoform. These cells were grown in sterilised LB broth. Cells were grown at 30 °*C* for 4 hours then cooled, induced with 50 mM IPTG and grown at 24 °*C* for 12 hours.

Spinach Rubisco β-isoform was purified from BL21 star DE3 *E. coli* cells as described by Barta *et al.* (Barta *et al.* 2011) however the anion exchange step was performed before the gel filtration step. Spinach Rubisco was extracted from spinach as described by Barta *et al.* (Barta *et al.* 2011) however this protein was found to form large aggregates. The anion exchange column used was a and the gel filtration column used was a Hiload 16/600 superdex^{TM} 200 pg connected to an Akta FPLC. Samples were kept on ice or at 4 °*C* at all stages of purification to prevent proteolysis.

2.2) Activity Assays

ATP hydrolysis and Rubisco activation potential was determined by measuring NADH oxidation via coupled assays as described by Barta *et al.* (Barta *et al.* 2011).The assays were run at 4 °*C* using a Cary 100bio Uv-visible spectrophotometer excitation wavelength of 340 nm.

2.3) Thermal Stability

Differential scanning flourimetry (DSF):Thermal stability assays were carried out on a Biorad IQ-5 multicolor real time PCR detection system. Measurements were taken at 0.5 °C increments starting at 20 °C and raised to 70 °C. Samples were prepared in

100 µl aliquots containing 20 µl of sypro orange, diluted from stock 1 in 1000. 50 µl of a solution containing the appropriate concentration of protein was then added and the sample was made up to 100 µl using either water or a solution containing nucleotides or magnesium dependent on the experiment run. 25 µl samples were then placed, in triplicate, in a PCR plate. The plate was then spun and degassed before being placed in an IQ-5 multicolor real time PCR detection system for thermal stability determination.

2.4) Determination of Molecular Weight.

Size-exclusion chromatography (SEC): SEC was performed at 4 °C using a _____column. 100µl of enzyme at 0.5 $mg.ml^{-1}$ (unless otherwise stated) was loaded onto the column and eluted with 25 mM HEPES, 150mM KCl, 0.2 mM EDTA, 10 mM β-meRcaptoethanol (unless otherwise stated), pH 7.5 at 1 $ml.min^{-1}$. A UV detection unit was used to record UV absorption at 280 nm. Bio-rad Gel filtration standard was used to calibrate the instrument

Size-exclusion Chromatography multiangle light scattering(SEC-MALS): SEC-MALS was carried out at 28 °C using a Superdex 200 10/300 GL column (GE Healthcare). 200µl of enzyme at the appropriate concentration was loaded onto the column and eluted with 25 mM HEPES propane, 150 mM KCl, 0.2 mM EDTA, 0.5 mM ADP, pH 7.5, at 0.5 $ml.min^{-1}$. A Viscotek TDA unit was used to measure the refractive index, low angle and right angle light scattering, and viscosity. BSA was used as a standard to calibrate the instrument.

Dynamic light scattering (DLS): DLS was carried out at 4 °C unless otherwise noted using a Zetasizer nano series, Nano-Zs. Samples were prepared in 1 ml disposable cuvettes in a buffer containing 25 mM HEPES, 150 mM KCl, 0.2 mM EDTA, 10 mM β-meRcaptoethanol, pH 7.6. Results were obtained using a backscatter angle of 176°

Chapter 3) Purification and Activity

3.1) Introduction

As previously described Rca is an AAA+ protein which utilises ATP hydrolysis in order to undertake enzymatic work. This work is required to unlock Rubisco which has been blocked due to aberrant reactions. A great deal of work has been performed in determining the active properties of Rca.

It has been shown that the activity of Rca is dependent on concentration. The specific activity of Rca increases with concentration, indicating a self-associative nature. This has since been ratified through experiments showing increasing oligomerisation with concentration. This can be seen in work performed by Wang *et al.* (1993) which shows a concentration dependant activity profile for ATP hydrolysis by Rca. At 10 $\mu g.ml^{-1}$ Rca specific activity, of ATP hydrolysis, was recorded at 0.3 $\mu mol.min^{-1}.mg^{-1}$. The specific activity is seen to rise to 1.2 $\mu mol.min^{-1}.mg^{-1}$ at 120 $\mu g.ml^{-1}$. A hyperbolic increase in specific activity is seen which begins to plateau at 80 $\mu g.ml^{-1}$. This was thought to be caused by increased self-association as concentration increases (Wang *et al.* 1993).

A similar pattern to that of ATP hydrolysis by Rca, was found for Rubisco activation. It was shown by Salvucci (1992) that a similar hyperbolic relationship between Rubisco activation and Rca concentration existed. It was found that large increases in Rubisco activation by Rca occurred upon increasing concentrations up to 0.2 $mg.ml^{-1}$. The increase was shown to plateau at around 0.3 $mg.ml^{-1}$. This indicates a

concentration dependant increase in activity until a plateau is reached which is likely caused by saturation of Rca (Salvucci 1992).

Since these initial experiments were carried out more evidence was found to support the requirement of ATP in the activation of Rubisco. Robinson and Portis discovered that not only was ATP and Rca required for Rubisco activation in physiological conditions but they also found that Rca was inhibited in the presence of high ADP concentrations. This inhibition gave evidence to indicate that Rubisco activity could be controlled by the energy levels of the chloroplast (Robinson and Portis 1989). As Rca requires ATP hydrolysis in order to activate Rubisco as shown by the fact that no activation of Rubisco occurred upon the addition of a non-hydrolysable nucleotide (Salvucci *et al.* 1993). Rca utilises ATP hydrolysis in order to produce a conformational change (Salvucci and Ogren 1991). This is achieved via the conserved AAA domain or nucleotide binding domain (NBD) found in all AAA+ proteins. This domain is a conserved sequence around 220 amino acids long which is contains several motifs which allow for the utilisation of high energy nucleotides. Among these motifs is the walker A motif (GXXXXGK), required for the binding of nucleotides and the walker B motif ((R/K)XXXXGXXXXLhhhhD), required for the hydrolysis of nucleotides (Dougan et.al 2002). This domain allows for the energy gained through the hydrolysis of ATP to be converted into a conformational change which can be passed onto Rubisco, however the exact mechanism is unknown.

Rca requires ATP hydrolysis in order to activate Rubisco however it does not require Rubisco to undergo ATP hydrolysis (Robinson and Portis 1989). This causes a problem for the cell in that valuable energy stocks could be easily depleted if

regulatory measures did not exist within the protein. Evidence for this was given in a set of experiments in which it was found that the small isoform had significant ATPase activity at an ADP: ATP ratio of 1:1, indicative of stroma ratios while in the dark. Conversely it was found that the large isoform, added in equimolar amounts to the small isoform, was able to completely inhibit activity while in the presence of a 1:3 ADP: ATP ratio (typical of stromal ratios when in light conditions) (Zhang and Portis 1999). This indicated that the large isoform was responsible for the activation state of activase.

As discussed in the introduction two isoforms of Rca exist, the α and β isoform. These isoforms differ in that the α isoform has an extended C terminus which holds a regulatory region (Zhang *et al.* 2001). In search of a mechanism of how the α-isoform was regulated a series of site directed mutagenesis experiments were conducted based on speculation that reduction of the cysteine residues may cause a conformational change in the NBD allowing for easier docking of ATP into the ATPase domain due to more favourable positioning of negatively charged residues (Zhang *et al.* 2001). An alanine replacement experiment showed the importance of several negatively charged residues within the C-terminal (Zhang and Portis 1999). Amongst several alanine replacement mutants the mutants E390A, D394A and D401A displayed a significantly higher rate of ATP hydrolysis as compared to the wild type. Two double mutants were then created to test for a synergistic response. It was found that the double mutants E390A/D401A and D394A/E398A caused up to a 4.6 fold increase in ATP hydrolysis at a physiologically relevant ATP: ADP (Zhang and Portis 1999). This value was far more similar to the small isoform than the oxidised wild type, as was the rate of hydrolysis for the C411A mutant, which prevented redox regulation of the α-isoform.

The affinity for ADP was not significantly changed however. These results showed a necessity for both the presence of a di-sulphide bridge between two specific cysteine residues and the positioning of sufficient negatively charged residues within the binding pocket to prevent the association of ATP with activase (Portis *et al.* 2008).

In order for the α-isoform to cause the regulation of the β-isoform, the two must be able to interact. This is explained by the observed variable oligomeric states in which activase exists. It has been shown that several conditions, such as protein concentration, temperature and the presence or absence of different compounds can influence the oligomeric state of activase.

It was found that upon the addition of several detergents (PEG, PVPP and ficoll) caused an increase in specific ATPase activity of Rca from both spinach and tobacco. The activation of Rubisco was also found to be higher in the presence of PEG. It was found that this was likely due to an increase in oligomeric size which was found, via rate zonal sedimentation and gel filtration, to be 2 – 4 fold higher in the presence of PEG (Salvucci 1992).

The research described has helped to elucidate the activity profile of Rca however now it remains to elucidate the mechanism behind the activity and how Rca associates with itself and with Rubisco so the activity can be better manipulated in the search for a more efficient Rubisco enzyme.

3.2) Purification

Multiple cell types and incubation temperatures were used initially to determine growth rates and protein production after transformation. BL21 (DE3) pLys S and BL21 star (DE3) cells were transformed with either a his-tagged α isoform, a his tagged β isoform or an untagged β isoform. Initially a His-tagged spinach Rca construct was transformed into BL21 star(DE3) cells. Protein was able to be purified from this source however it rapidly formed large aggregates, as detected by dynamic light scattering (DLS), which held minimal activity. SDS page gels indicated the presence of spinach Rca and minimal activity was seen under ATP hydrolysis assays. This activity was far below values previously described within the literature and showed no dependence on concentration which has also been previously reported. DLS however showed molecular weights slightly under 1 MDa indicating large inactive aggregates. The protein would also precipitate after approximately 24 hours if stored at 4 °C or once thawed after being stored at -80 °C. It was determined that optimal active protein production and cell growth occurred with BL21 star (DE3) cells with the untagged Rca construct. Rca was also extracted from spinach leaves however difficulties with purity made this a less ideal purification method.

3.3) Activity

Rca was tested for both ATPase activity and for its Rubisco activation potential. These were both measured using coupled assay systems as described in the methods section. Both allow for the activity of Rca to be seen as a consumption of NADH which can be measured using a spectrophotometer. These tests were run in order to

determine that the enzyme was active and to determine the activity in comparison to data collected by other groups.

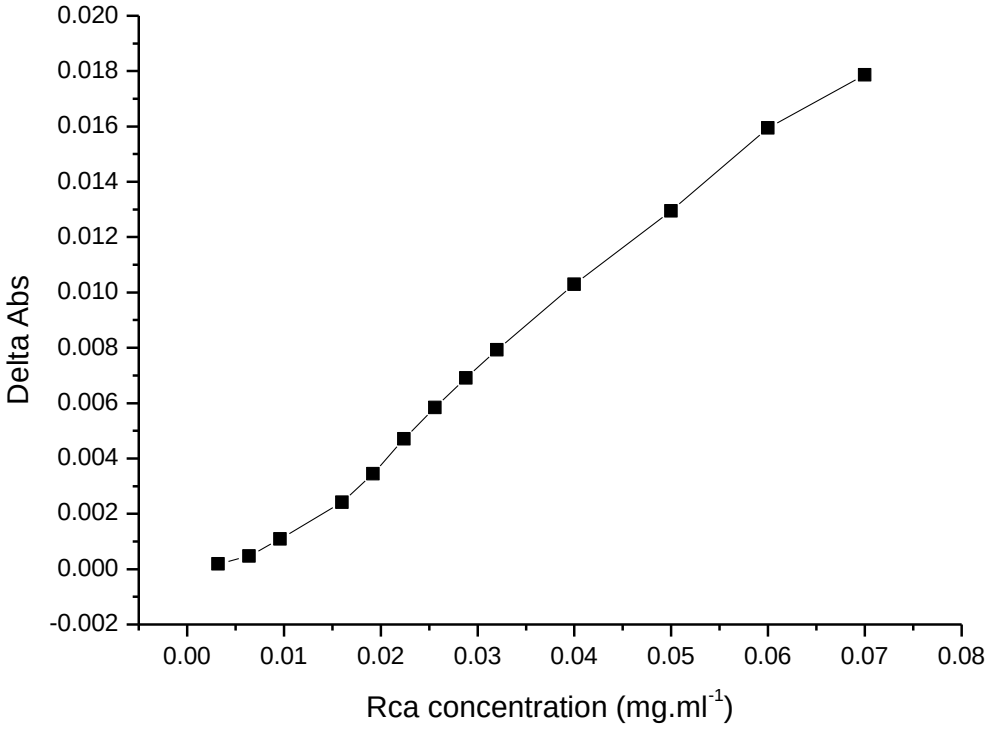

Fig. 3.1) Rate of ATP hydrolysis of spinach Rca isoform

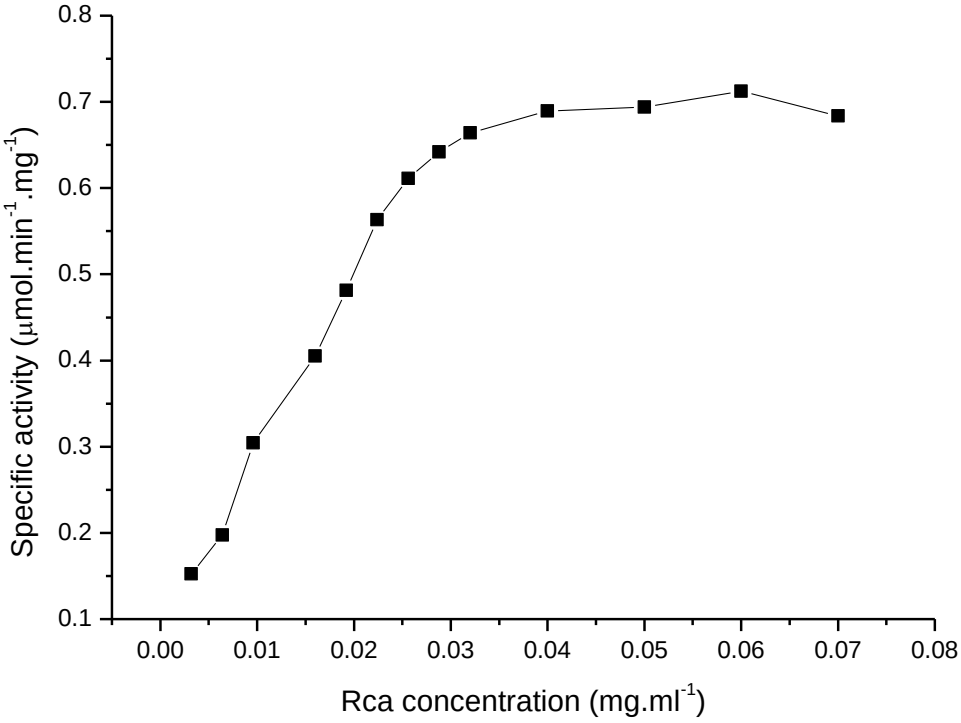

Fig. 3.2) Specific activity of ATP hydrolysis for spinach Rca

The above data shows both the absorbance against time and the specific activity for the ATPase activity of spinach Rca. The specific activity shows that ATPase activity of Rca is concentration dependant. Specific activity increases until the Rca concentration reaches 0.04 $mg.ml^{-1}$, with 50% activity at approximately 0.02 $mg.ml^{-1}$. This would indicate that the ATPase activity of Rca is concentration dependant. These results show a similar trend and value set as shown to previously reported results (Wang *et al.* 1993). It should also be noted that the coupled assay system used to measure the rate of ATP hydrolysis contains no Rubisco meaning that Rca retains hydrolytic activity in the absence of Rubisco.

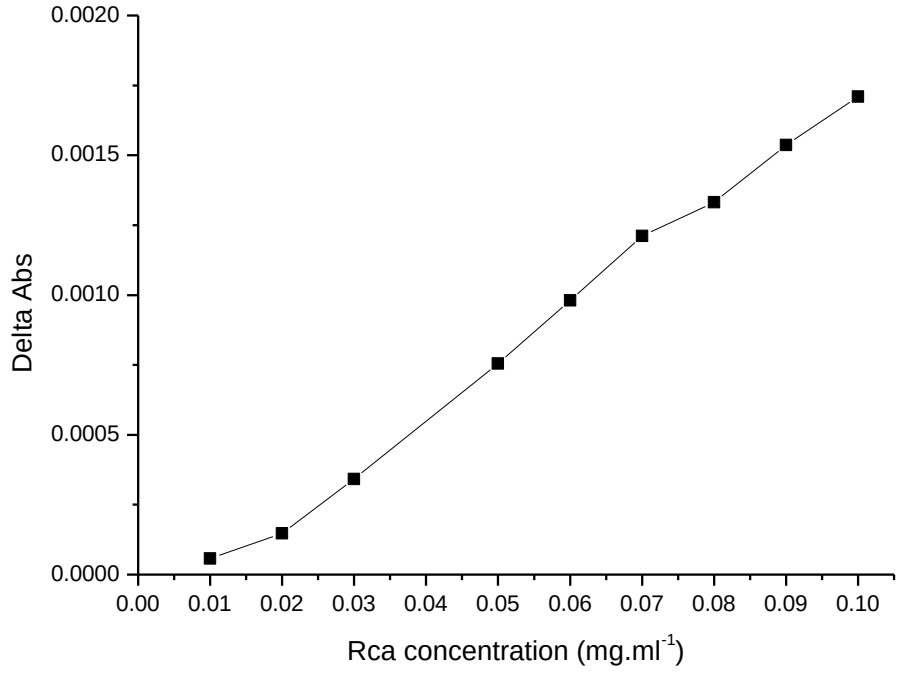

Fig. 3.3) Rate of Rubisco activation by spinach Rca

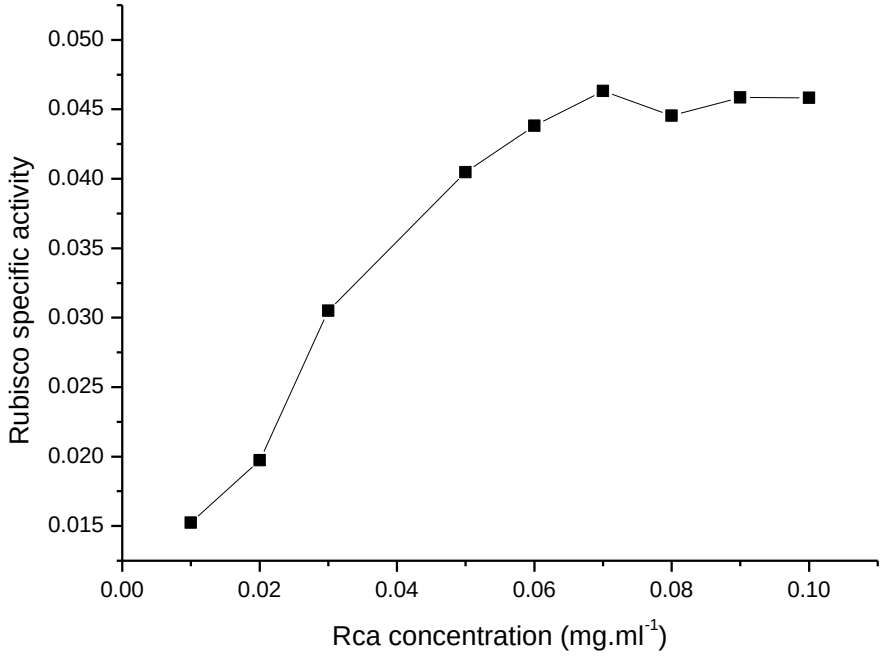

Fig. 3.4) Specific activity of Rubisco activation by spinach Rca

The above data shows both the absorbance against time and the specific activity for the Rubisco activating potential of spinach Rca. Specific activity can be seen to rise until 0.07 mg.ml^{-1} with 50% activation activity seen at 0.03 mg.ml^{-1}. This indicates

that the activity of Rca is concentration dependant. This is seen as the hyperbolic pattern observed in which activity increases rapidly with concentration at lower concentrations until a saturation point is reached. This is again slightly different compared to values seen in the literature which shows a plateau for Rubisco activation at around 0.03 mg.ml^{-1}. However a similar trend and value set is seen between the observed results and those previously reported by Salvucci (Salvucci 1992).

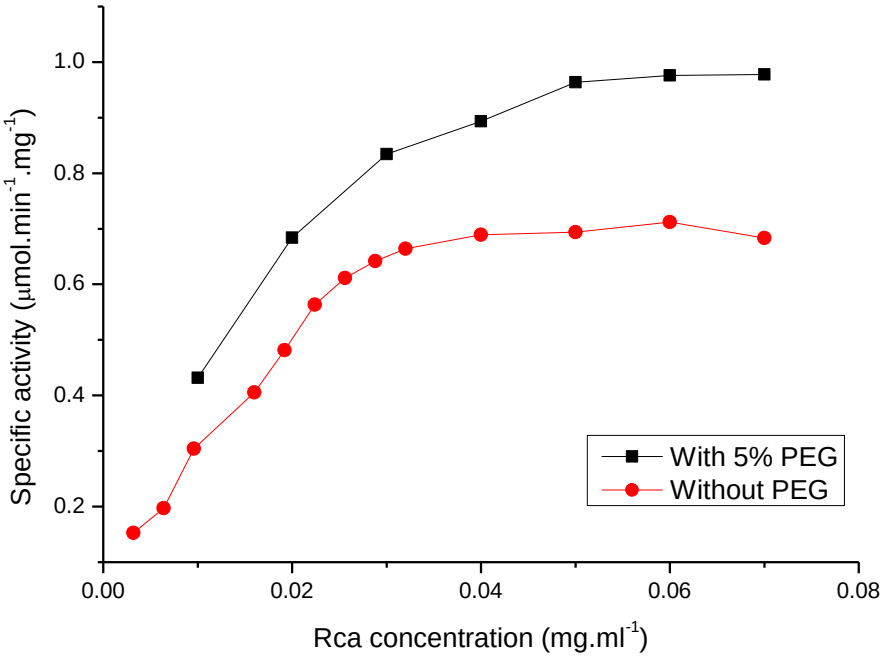

Fig. 3.5) Rate of ATP hydrolysis of spinach Rca isoform with and without the addition of 5% polyethol glycol.

It has been previously shown that the addition of crowding agents can cause an increase in the enzymatic rate of Rca. This experiment was repeated using spinach Rca. Upon the addition of PEG a 70% increase in activity was observed, this is lower than the 80-120% increase seen in previously described work (Salvucci 1992). Although an increase in activity was seen the same trend was observed, showing an

increase in specific activity as concentration increased and indicating a self-associative nature.

3.4) Chapter Conclusion

The experiments and procedures described above have all been previously tested however these experiments were necessary to determine the activity of the purified Rca in comparison to previously described literature and to ensure the Rca was active and behaving normally to ensure the validity of the other experiments.

The research performed on the activity of Rca indicates that it has a concentration dependant activity profile. This has been shown to be due to self-association which is backed up by evidence showing the addition of PEG causes an increase in activity. The addition of PEG the concentration remains the same although as PEG is a crowding agent it encourages Rca to from higher oligomeric species which in turn causes the observed increase in activity.

It should be noted that the observed rates of activity for both ATP hydrolysis and Rubisco activation were slightly lower than those shown in previous literature. Similar trends are seen and the slightly lower activity may be as a result to a lower purity of enzyme. This shows that the the enzyme was functioning correctly for later experiments done on sizing and temperature stability.

Chapter 4) Thermal Stability

4.1) Introduction

Denaturation of Rca has been shown to be the major source for the loss in photosynthetic activity due to heat stress upon plants (Salvucci and Crafts-Brandner 2004). This is of importance as global temperatures rise and as global population rises as it means that more biomass production will be required and crop plants will be grown in less ideal conditions. High temperatures have been shown to have a negative impact on the activation of Rubisco (Kobza and Edwards 1987). The reduction in activation potential increases until it becomes irreversible, generally 10 °C higher than when a loss in activity is first seen. The temperature range in which an Rca is active is dependent on the species being tested (Salvucci and Crafts-Brandner 2004). The reduction in activating potential is thought to be a result of the creation of large inactive aggregates which prevents the association of Rca with Rubisco (Feller *et al.* 1998). Nucleotide and Mg^{2+} supplementation has been shown to affect the thermal stability of Rca. ATP, ATP-γ-S and ADP have been shown to increase the thermal stability of Rca while Mg^{2+} seems to have a destabilizing effect under certain condition (Henderson *et al.* 2013, Wang *et al.* 1993, Barta *et al.* 2010, Kim and Portis 2006). A difference in the effects of nucleotide supplementation has also been seen across species and even between Rca isoforms of the same species (Crafts- Brandner *et al.* 1997).

This chapter examines the effects of proteinconcentration and nucleotide supplementation on the thermal stability of spinach Rca. The thermal stability of spinach Rca is shown to be dependent on protein concentration. Further increases in thermal stability were seen upon the addition of ADP, ATP and ATP-γ-S.

4.1.1) Plant Growth at High Temperatures Restricts Plant Growth

Inhibition of plant growth at high temperatures is thought to be initially due to a change in Rubisco activation state (Kobza and Edwards 1987). It was shown that Rubisco activation was inhibited at temperatures exceeding 30 °C and 35 °C for wheat and cotton respectively. This inhibition of activity was found to be reversible up to 40 °C after which large aggregates of Rca were observed which remained upon a reduction in temperature (Feller *et al.* 1998). The drop in plant viability is thought to be initially caused by a reduced rate of photosynthesis (Eckardt and Portis 1997). As temperature increases above the optimal temperature the ability for Rca to activate Rubisco decreases significantly as does the rate of Rubisco deactivation. As temperature rises, the ability for Rca to activate Rubisco begins to be overtaken by the rate at which Rubisco is deactivated, significantly reducing net photosynthesis (Crafts-Brandner and Salvucci 2000).

Instability due to temperature may be tempered by using Rca's from different species. It was found that the temperature optimum for the Rca's of different species was dependent on the environment in which they are cultivated. Salvucci and Crafts-Brandner looked at the effects of heat stress on several different species from different environments, the main two which were compared were the creosote bush (*Larrea tidentata),* a desert plant, and Antarctic hairgrass (*Deschampsia antractica*). These plants exist in very different climates with creosote existing in temperatures above 45 °C while Antarctic hairgrass exists at an average temperature of 0 °C. These two plants were shown to have a difference of 10 °C in optimum temperature with Rca activity dropping after 20 °C for Antarctic hairgrass while Rca activity was reduced

over 30 °C for creosote. It should be noted that the purified enzyme would appear to have different thermal tolerances compared to the enzyme while in the plant. Although the optimum temperatures were different for each plant similar trends were seen for the effects of heat stress on ATPase and net photosynthesis. It would also be of importance to determine the source of the increased thermal stability from plants such as creosote as it may give understanding to the genetic component associated heat stability (Salvucci and Crafts-Brandner 2004).

It has proved to be difficult to create a Rubisco enzyme with increased activity or substrate specificity and a viable, improved enzyme has not been engineered. This lead to work by Kurek *et al.* who were able to use gene shuffling techniques combined with high throughput screening to generate several *Arabidopsis thaliana* Rca mutants with increased thermal stability compared to wild type. *Arabidopsis* plants were then transformed with the modified enzymes and compared with native plants and plants transformed using the wild type enzyme. The plants were then grown under moderate heat stress, 30 °C, and were tested for photosynthetic rate, biomass production and seed yield. The plants carrying the modified genes showed increases in all tested areas compared to both the native plants and the plants transformed with the wild type enzyme (Kurek *et al.* 2007). It should be noted that plants transformed with the wild type enzyme showed a lower photosynthetic rate than the native plants. This may be a result of inefficiencies caused by the transformation process. If this is the case then it is likely having an effect on the growth of the plants transformed with the modified enzymes. This means that more significant increases in growth could be achieved if these inefficiencies in the transformation process were reduced.

4.1.2) The α Isoform is more Stable than the β Isoform

It has been shown that these different isoforms of spinach hold different thermal properties (Crafts- Brandner *et al.* 1997). The experiments on spinach show a significantly higher thermal stability in the α isoform as compared to the β when in the presence of ATP-γ-S. This showed a significantly higher temperature could be reached before a loss of activity was seen in the α form (Salvucci *et al.* 2001). A smaller increase in stability is seen for the β isoform when in the presence of ADP however. This indicates that nucleotide supplementation increases the thermal stability of Rca although it also shows that a far greater effect on stability can be seen when utilising the α isoform and ATP-γ-S in certain species. Mixtures of the α and β from spinach were shown to hold thermal tolerance similar to the α isoform (Crafts-Brandner *et al.* 1997). As the increase in thermal stability seen from the α isoform is found only in certain species, the extended region may be of great importance as a replacement for the β isoform or α isoforms which do not provide the additional stability (Crafts- Brandner *et al.* 1997).

4.1.3) Nucleotides Stabilise Rubisco Activase

Ligand binding has been shown to increase thermal tolerance in many different proteins. Rca has been shown through several experiments to gain an increase in thermal tolerance upon the binding of nucleotides.

Salvucci *et al.* showed that the temperature at which spinach Rca would aggregate was raised when in the presence of ATP and ATP-γ-S (Salvucci *et al.* 2001). This was compared to results showing that thermal inactivation of Rca occurred at higher temperatures when supplemented with adenine nucleotides (Crafts-Brandner *et al.*

1997). These results together caused the conclusion to be drawn that the loss of activity caused by heat stress is a result of denaturation of Rca.

Barta *et al.* ran experiments on the degree of thermal inactivation of Arabidopsis β Rca in the presence of different nucleotides. It was shown that ATP, ATP-γ-S and ADP increased the thermal stability of Rca. Mg^{2+} was also shown to have a destabilising effect on Rca in the presence and absence of nucleotide (Barta *et al.* 2010). Although low Mg^{2+} concentrations were shown to destabilise Rca it was noted that this also results in a higher rate of Rubisco catalytic misfire which would offset the gain in stability (Kim and Portis 2006).

A paper was recently released by Henderson *et al.* which explored the effects of supplementation on the thermal stability of cotton β Rca. The core of this paper explored the effects of nucleotide, magnesium and phosphate supplementation on the Tm of cotton Rca utilising the thermoflour assay. This paper indicated that both ATP and ADP had a stabilising effect on Tm, with ADP giving greater protection. Mg^{2+} caused minor destabilisation alone and reduced the stabilising effects of ADP supplementation. In contrast however it increased the stabilisation caused by ATP supplementation. Free phosphate (P_i) was also tested in conjunction with ADP which increased the protection provided by ADP. The increases in Tm observed were attributed to a reduction in dynamic motions of the protein fold caused by ligand binding. It was postulated that the increase in stability given by Mg^{2+} when in the presence of ATP is due to the balancing of the β and γ phosphates of ATP while in the binding pocket as Mg^{2+} is required to properly co-ordinate the nucleotide. P_i, in the presence of ADP, was thought to increase stability by creating the ADP-P_i complex

generated after ATP hydrolysis has occurred and may help to balance charges within the nucleotide binding site (Henderson *et al.* 2013).

Although the association of Rca and ligands is not completely understood we can look at other, similar proteins to help our understanding of Rca. An example is that of F_0F_1 ATP synthase which, like Rca, is a member of the AAA+ protein family. F_0F_1 ATP synthase is responsible for the generation of ATP from ADP utilising a trans-membrane proton gradient to power the reaction. DSC was utilised to determine the thermal stability of the protein and again hydrogen/deuterium exchange was used in order to determine the flexibility of the protein. It was found that $Mg^{2+} \cdot$ ADP caused a reproducible increase in thermal stability while a reduction in thermal stability was observed in the presence of Mg^{2+}. ATP and ADP supplementation were also shown to increase and decrease the conformational flexibility of F_0F_1 ATP synthase respectively. This again provides support for the idea that ligand binding causes an increase in thermal stability as a result of reduced flexibility. It is also of interest to note that $Mg^{2+} \cdot$ ATP causes a reduction in thermal stability. It was found that this was caused by the binding of ATP to the active site resulting in a more open conformation which caused the exposure of several reactive peptide groups. ADP supplementation on the other hand was shown to reduce the number of exposed peptide groups, aiding in stabilisation (Villaverde *et al.* 1997).

The results generated by this previous work has indicated that ligand binding can increase thermal stability however this relationship still needs to be explored. For this reason the effects of different concentrations of nucleotide and protein on the thermal stability of spinach Rca has been explored.

4.2) Results and Discussion

4.2.1) Thermal Stability Assays

In order to explore the thermal stability of spinach Rca differential scanning flourometry (DSF) was used. DSF is a rapid way to test the effects of ligand binding on protein stability (Niesen *et al.* 2007). As the temperature increases proteins begin to unfold, the temperature at which this occurs is indicative of their thermal stability. A fluorescent dye, Sypro orange, was used which has an affinity for hydrophobic regions. As the protein unfolds more hydrophobic regions become exposed and increase the fluorescence intensity. The temperature is raised by a predetermined value (0.5 °C increments were used in my experimentation) at a time and the fluorescence is measured over the course. This means that the results are accurate to within half a degree. By measuring this change in fluorescence as temperature increases the melting or unfolding temperature of the protein can be found and can be compared to the Tm in the presence of different ligands. For my experiments I have used the point of maximum inflection from the derivative of the absorbance/melting profile. The transition midpoint of the melting profile can also be used to determine the binding affinity for small molecules and their ligands. This paper will focus on the apparent Tm (Niesen *et al.* 2007).

4.2.2) Thermal stability Depends on Protein Concentration.

The thermal stability of spinach Rca was tested to determine the effects of concentration and supplementation. This can give us insight into the self-associative properties of Rca. However this technique does not give data as to the specific denaturation temperature. This allows for the comparison of the apparent Tm of a

protein under different conditions. Other groups have used the peak absorbance to measure the apparent Tm such as Henderson *et al.*

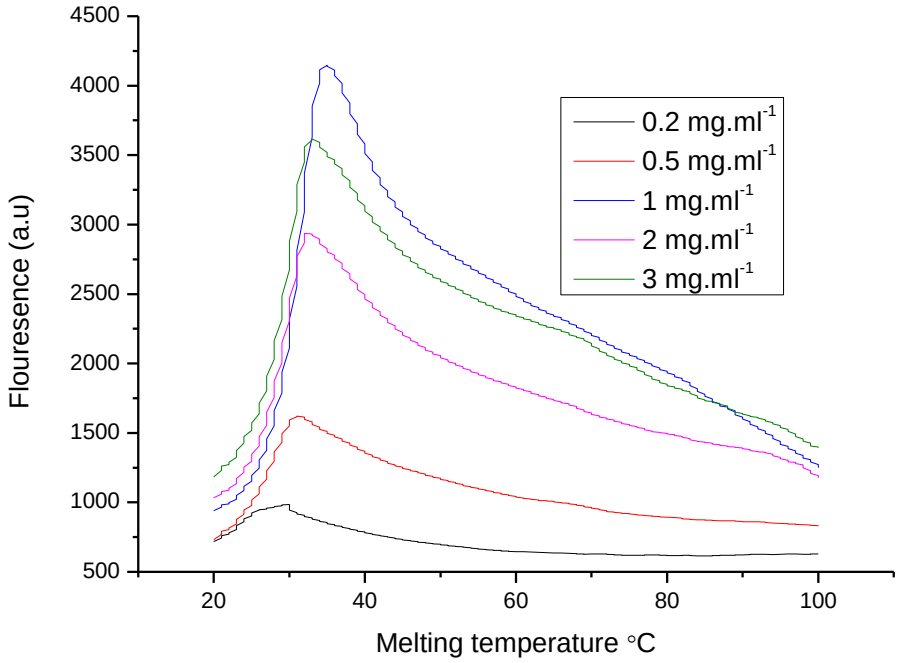

Fig. 4.1) Trace of select concentrations from DSF of Rca showing the absorbance profile (trace of data from fig. 4. 3)

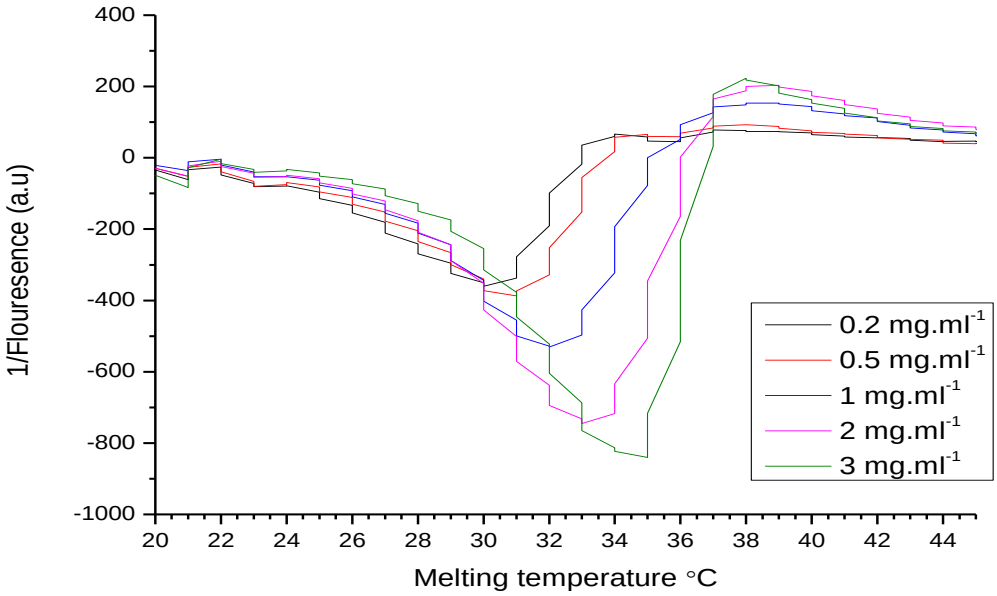

Fig. 4.2): Trace of select concentrations from DSF of Rca showing the reciprocal of the absorbance profile (trace of data from fig. 4.3)

Rca exhibits an increase in thermal stability as protein concentration increases. As larger oligomers of Rca are generated the potential for dynamic motion is reduced, due to the creation of a more structured molecule, and a higher thermal stability can be achieved (Henderson *et al.* 2013). As concentration increases so does the oligomeric state of tobacco Rca (Keown *et al.* 2013). This may provide an increase in thermal stability due to a decrease in protein flexibility which helps prevents non-specific aggregation/binding at higher temperatures.

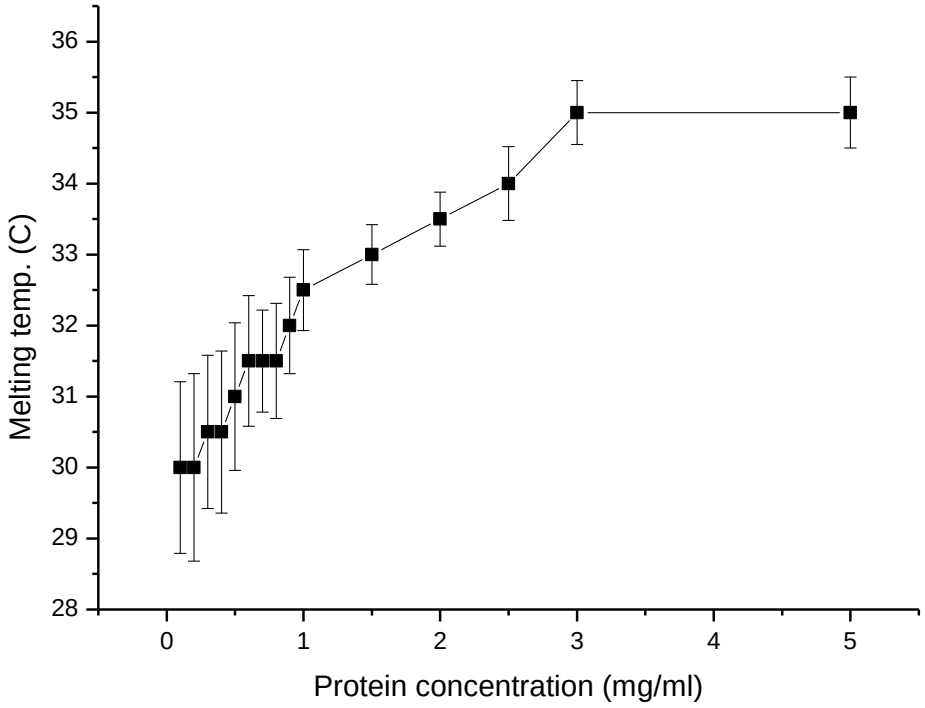

Fig. 4.3) The effect of protein concentration on apparent Tm of spinach Rca as measured by DSF

It can be seen that a hyperbolic trend exists between melting temperature and protein concentration. The midpoint is seen at 1 $mg.ml^{-1}$ with a total increase of 5 °C. This trend appears to plateau at around 3 $mg.ml^{-1}$ however concentrations higher than 5 $mg.ml^{-1}$ were not tested. It was found over several runs that the Tm at a given concentration could differ by up to 2 °C and the results shown is was representative of several runs. This error margin appeared to reduce as concentration increased.

4.2.3) Nucleotide Supplementation

Rca requires ATP in order to activate Rubisco (Robinson and Portis 1989). This requires association with Mg^{2+} which is able to co-ordinate ATP into the active site of the enzyme (Knight *et al.* 1990). Activation was shown by Zhang and Portis to be influenced by ATP/ADP ratios (Zhang and Portis 1999). ATP, ADP and Mg^{2+} are therefore commonly used to characterise Rca. Originally Rca was tested in the presence of ATP or ADP in the absence of Mg^{2+}. The addition of Mg^{2+} causes Rca to become catalytically competent and the ATP in solution is rapidly converted to ADP and free phosphate. By completing these tests in the absence of Mg^{2+} we can view the effects of ATP binding without being hydrolysed. It should be noted that Mg^{2+} has been shown to affect nucleotide binding. Mg^{2+} supplementation of spinach α Rca by Wang and Portis caused a five-fold decrease in the dissociation constant of ATP, from 41 to 8.8 µM, and a two-fold increase in the dissociation constant for ADP from 0.76 to 1.38µM (Wang and Portis 1991).

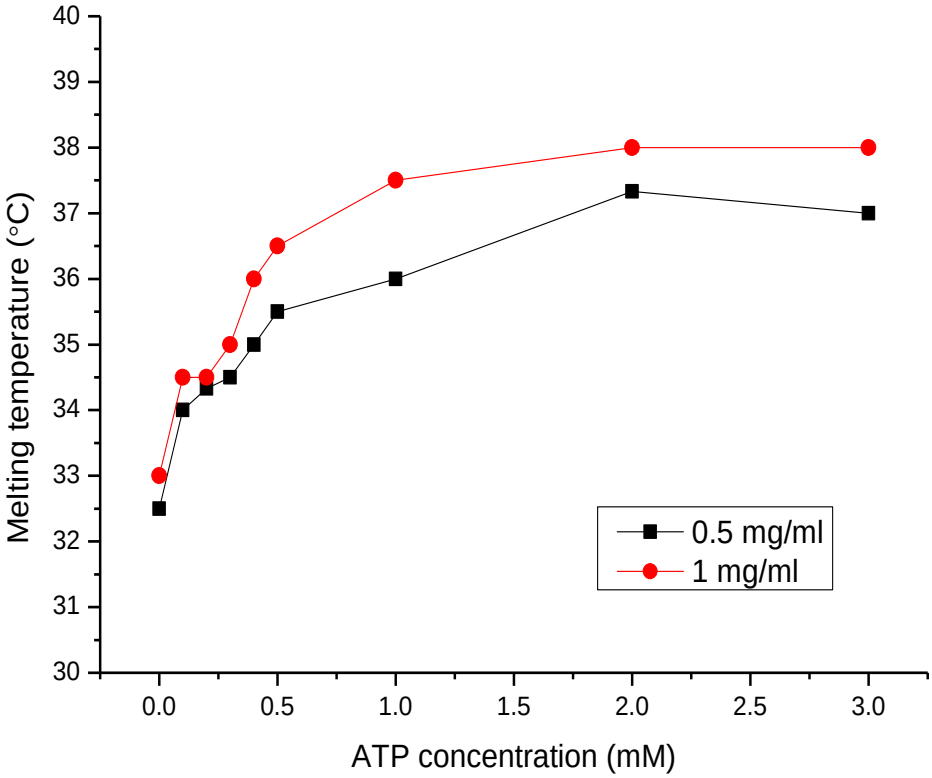

Fig. 4.4): Effect of varying ATP concentrations on the Tm of spinach Rca at varying concentrations as measured by DSF

Both ADP and ATP in the absence of Mg^{2+} caused an increase in Rca stability up to 2 mM (Fig. 4.4, 4.5). A hyperbolic relationship between ATP concentration and Tm can be seen. At 2 mM ATP a rise in Tm of 5 °C is seen for both protein concentrations tested and 50% protection (based on the point at which half maximal temperature increase is gained) is observed at around 0.6 mM ADP. Figure 4.4 and 4.5 indicate that a higher protein concentration also provides thermal protection when in the presence of nucleotides. These experiments were run using the same protein batch as was used for ADP and protein concentration experiments.

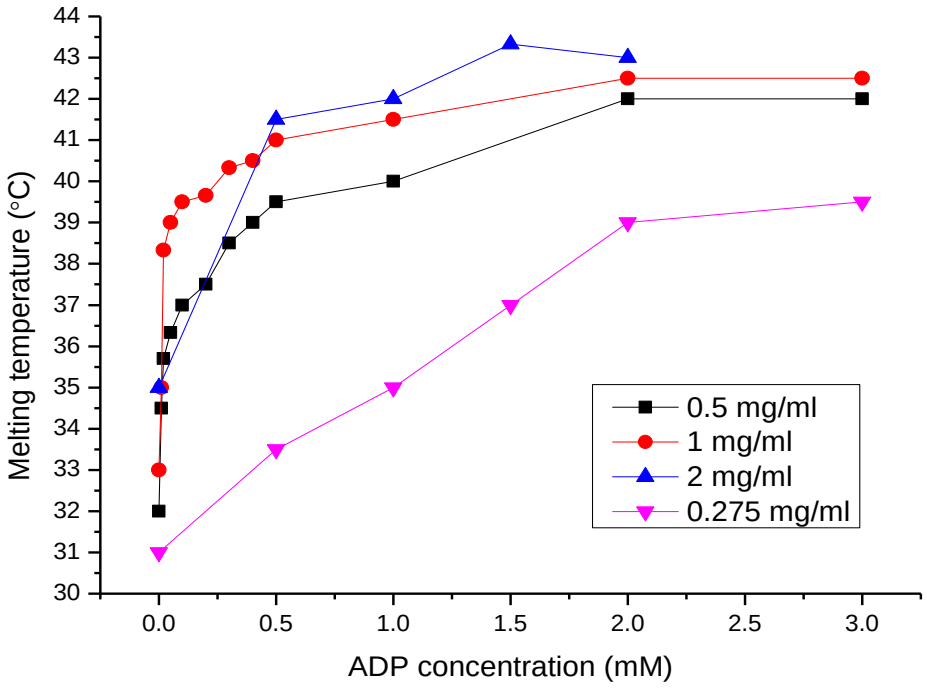

Fig. 4.5) Effect of varying ADP concentrations on the Tm of spinach Rca at varying concentrations as measured by DSF

ADP showed a greater effect on the thermal stability of Rca. 2 mM ADP caused an increase of 10 °C to the melting temperature of 0.5 mg.ml^{-1} Rca and a midpoint at 0.3 mM (Fig. 4.5). A similar pattern was seen using the same ADP concentrations while changing the protein concentration. The exception to this was measurements done the lowest protein concentration measured, 0.275 mg.ml^{-1}, this concentration was repeated once with similar results. At this concentration it was found that a flatter hyperbolic relationship existed between melting temp and ADP concentration as opposed to the hyperbolic relationship seen in the higher protein concentrations. The difference observed may have been caused by a change in the interaction between ADP and Rca at low concentrations. A greater increase in stability by ADP could be expected as ADP has been shown to have a lower Kd for nucleotide binding with Rca compared to ATP resulting in tighter association of the nucleotide in the nucleotide

binding site. For the wild-type Rca β-isoform of Arabidopsis, in the absence of Mg^{2+}, a Kd of 5.9 µM was found for ATP while a Kd of only 0.57 µM was observed for ADP (Wang and Portis 1991, Wang and Portis 2006). Figure 4.6 shows an overlay of ADP and ATP supplementation of 0.5 $mg.ml^{-1}$ Rca. It should be noted that different values are shown for 0 mM ATP and 0 mM ADP, these figures are representative of several runs and the values received between runs of the same protein batch could differ by up to 1.5 °C, shown in the error bars.

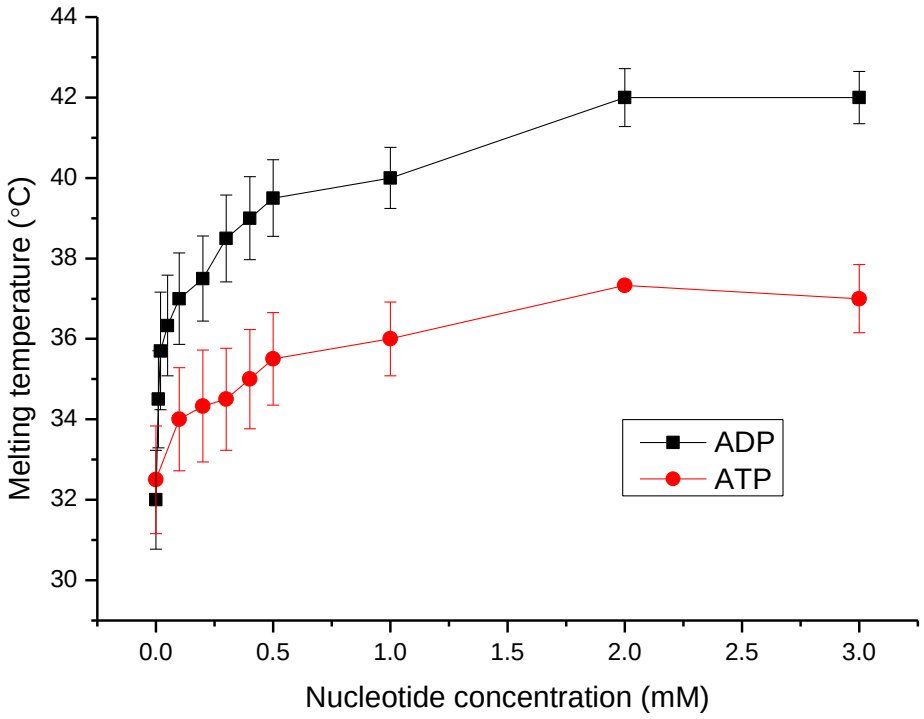

Fig. 4.6) Comparison of the effect of nucleotide supplementation of Rca at 0.5 $mg.ml^{-1}$ Rca concentration

4.2.4) The Effect of Magnesium

Magnesium has been previously shown by others (such as Henderson *et al.*) to affect the stabilising effects of nucleotide supplementation. As previously discussed Mg^{2+} was shown to have a minor destabilising effect on cotton Rca when by itself or in the presence of ADP (Henderson *et al.* 2013). In research presented by Henderson *et al.* the effect on thermal stability was measured by use of the thermoflour assay. When alone Mg^{2+} was shown to affect a 1.3 °C drop in Tm at 9 mM, while a 0.6 °C decrease was observed at 3 mM Mg^{2+} caused a similar decrease in stability when in the presence of ADP. 9 mM Mg^{2+} was shown to cause a significant increase of 5.5 °C to the thermal stability when in conjunction with ATP (Henderson *et al.* 2013).

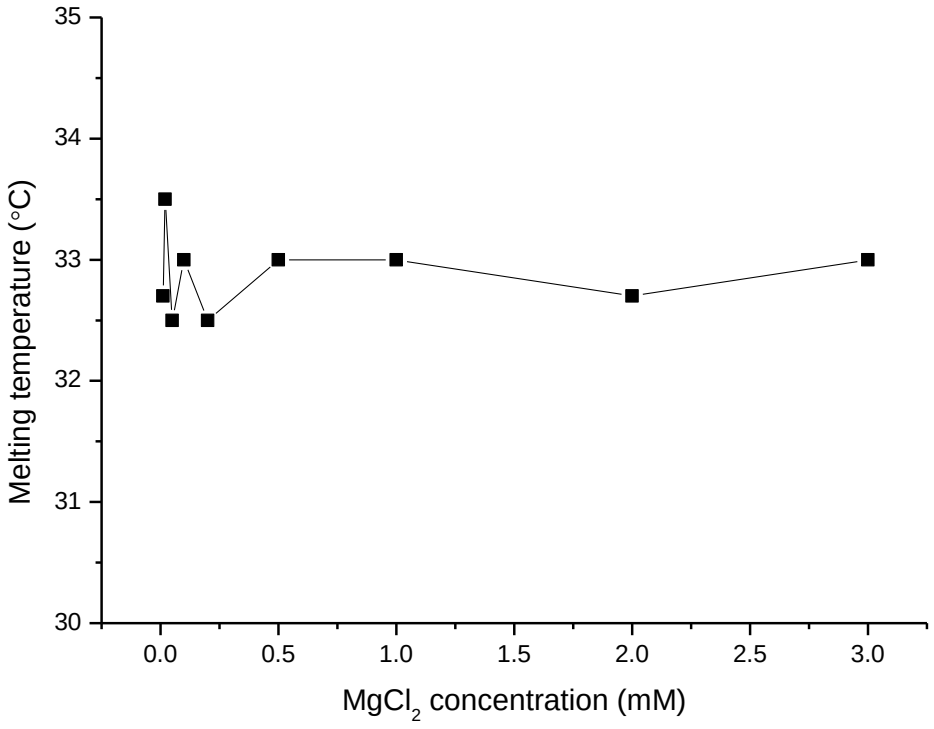

Fig. 4.7) Effect of varying $MgCl_2$ concentrations on the Tm of spinach Rca as measured by DSF

The effect of Mg^{2+}, in the form of $MgCl_2$, on the melting temperature of Rca was explored. In comparison to the work done on cotton Mg^{2+} by Henderson *et al.* by itself shows no statistical change in melting temperature from 0.01 to 3 mM (Fig. 4.7)

(Henderson *et al.* 2013). Although slight variation was observed between the concentrations this is assumed to be due to the noise of the technique. Several runs of the same protein batch indicated no effect and the variation seen was within 1 °C. As Mg^{2+} is responsible for the co-ordination of nucleotides within the active site its effects on melting temperature in the presence of its substrate and product (ADP and ATP) was explored. Concentrations up to 3 mM were tested as it was within physiological relevant concentrations. Ishijima *et al.* measured free Mg^{2+} concentrations within spinach chloroplasts. It was found that free Mg^{2+} concentrations went from 0.5 mM during the dark cycle up to 2 mM in the light cycle. Although it was stated that Mg^{2+} concentration exists within the tens of mM it was also noted that the pool of Mg^{2+} utilised for intracellular processes is the free Mg^{2+} present. It should also be noted that some Mg^{2+} may remain bound to Rca as Mg^{2+} is used during the purification process (Ishijima *et al.* 2003).

The previous chapter showed that Rca will undergo ATP hydrolysis in the absence of Rubisco. As shown in the previous chapter the specific activity for Rca, in the presence of ATP, plateaus at approximately 0.045 $umol.min^{-1}.mg^{-1}$. This indicates that at the protein concentrations tested rapid hydrolysis of the ATP present will occur, resulting in the generation of ADP and free phosphate. This could lead to an assumption that ATP in the presence of Mg^{2+} should yield similar results to that ADP with or without Mg^{2+}. However Mg^{2+} appears to have a destabilising effect on Rca when in the presence of nucleotides. Mg^{2+} has been shown to have a destabilising effect on other species Rca's in the absence of adenine nucleotide (Barta *et al.* 2010). Although I found no change in thermal stability upon the addition of Mg^{2+} alone it can

be assumed the lower thermal stability of Rca seen in the presence of nucleotide and Mg^{2+} is caused by a similar mechanism.

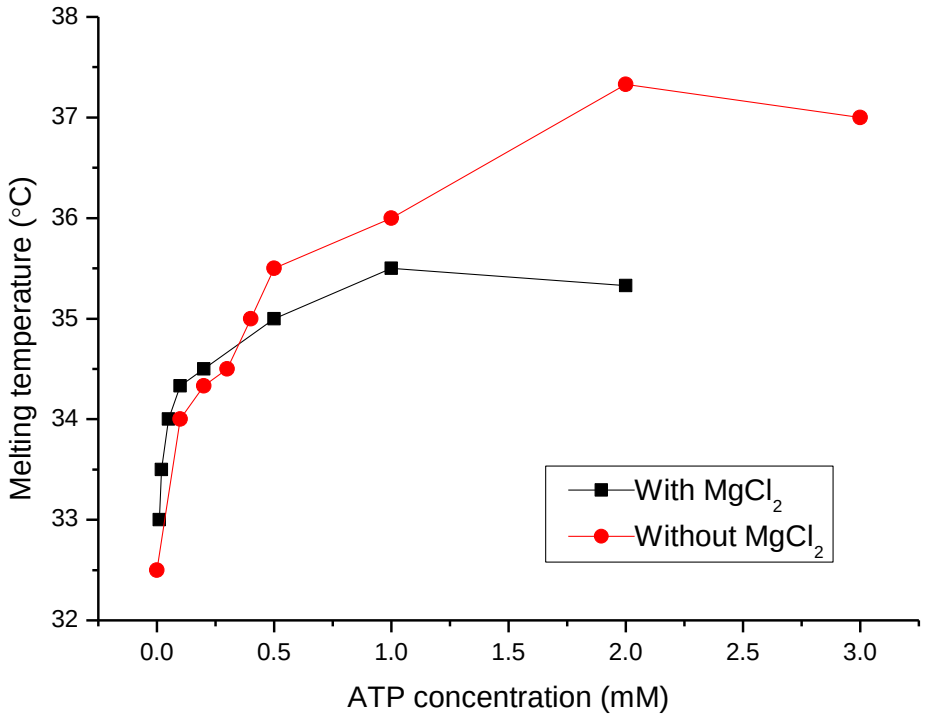

Fig. 4.8) Effect of varying ATP concentrations on the Tm of spinach Rca in the presence of 5 mM $MgCl_2$ as measured by DSF

$MgCl_2$ was used at a concentration of 5 mM to ensure activation of the Rca and an excess in relation to the nucleotide. Similar results were seen for ATP supplementation over several runs, shown is a representation of these runs. ATP with Mg^{2+} was run twice with consistent results and those shown are representative of a single batch of protein. Varying nucleotide concentrations were then explored in the presence of magnesium. Mg^{2+} was found to reduce the stabilising effect of ATP on Rca. Little difference is seen between supplementation with and without Mg^{2+} however a larger difference can be seen at higher ATP concentrations. Although both showed a similar hyperbolic trend higher apparent Tm were seen for ATP in the absence of Mg^{2+} at ATP concentrations above 0.5 mM (fig. 4.8). This is in contrast to work done on cotton β-Rca which showed an increase in the stabilising effect of ATP

in the presence of Mg^{2+} (Henderson *et al.* 2013). It has also been previously shown that phosphate is able to cause minor increases in stabilisation. As ATP is hydrolysed free phosphate and ADP is released. However the effect of ADP supplementation seen is different from hydrolysed ATP.

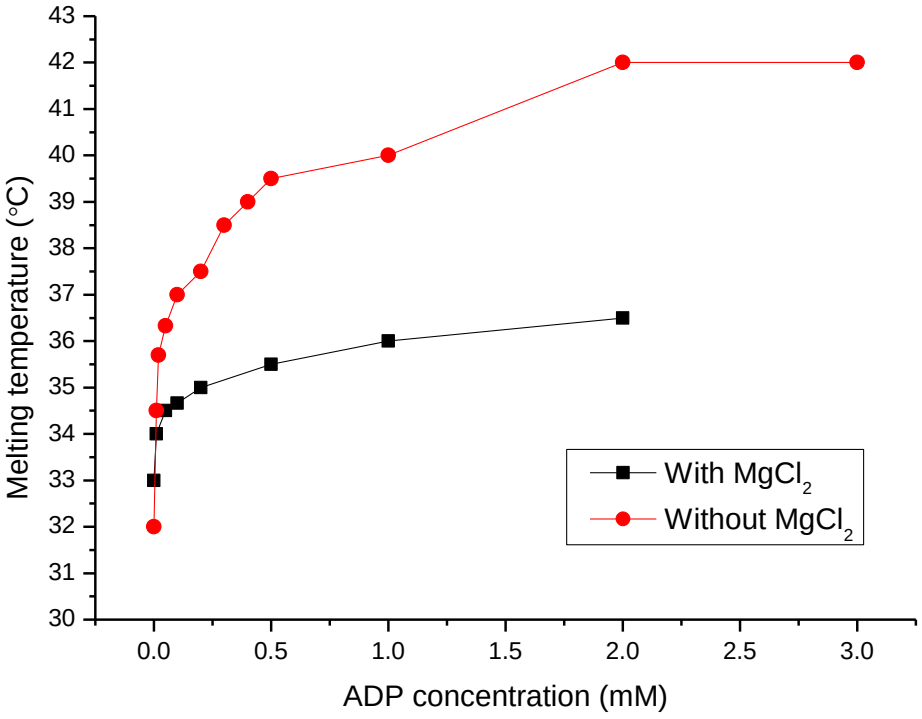

Fig.4.9) Effect of varying ATP concentrations on the Tm of spinach Rca in the presence and absence of 5 mM $MgCl_2$ as measured by DSF

$MgCl_2$ was shown to have a more profound effect on the stabilising properties of ADP. It was found that ADP in the presence of $MgCl_2$ shows a similar melting profile to ATP in the presence of $MgCl_2$. ADP and Mg^{2+} supplementation can be seen to provide thermal protection to Rca. In the presence and absence of Mg^{2+} a hyperbolic trend was found. It also shows that both show 50% protection at approximately 0.5 mM ADP. ADP supplementation in the presence of Mg^{2+} however offers significantly less protection at all nucleotide concentrations. In the presence Mg^{2+} shows a higher Tm for 0 mM ADP however this difference is likely due to the difference in Tm seen between runs.

$MgCl_2$ appears to decrease the stabilising effect of Rca by nucleotides, with a more significant effect on ADP stabilisation. It also appears that the melting temperature of Rca in the presence of ADP with $MgCl_2$ is very similar to the melting temperature of Rca in the presence of ATP. This is likely due to the hydrolysis of ATP to ADP by Rca due to the activation of Rca's active site by Mg^{2+}. ATP hydrolysis is a relatively rapid reaction consuming most of the ATP within several minutes as shown by activity assays performed. As DSF has a set up time of around 30 minutes and a run time of around 60 minutes it can be assumed that most of the ATP present in solution has been converted to ADP by the time the experiment is run.

ATP-γ-S is a non-hydrolysable analogue of ATP in which one of the gamma-phosphate oxygens is replaced by a sulphur atom. This causes a significant decrease in the rate of hydrolysis and can be used to inhibit ATP dependant reactions (Wang *et al.* 1993). This allows for exploration of the protein while in its transient bound state which would not be held under standard conditions.

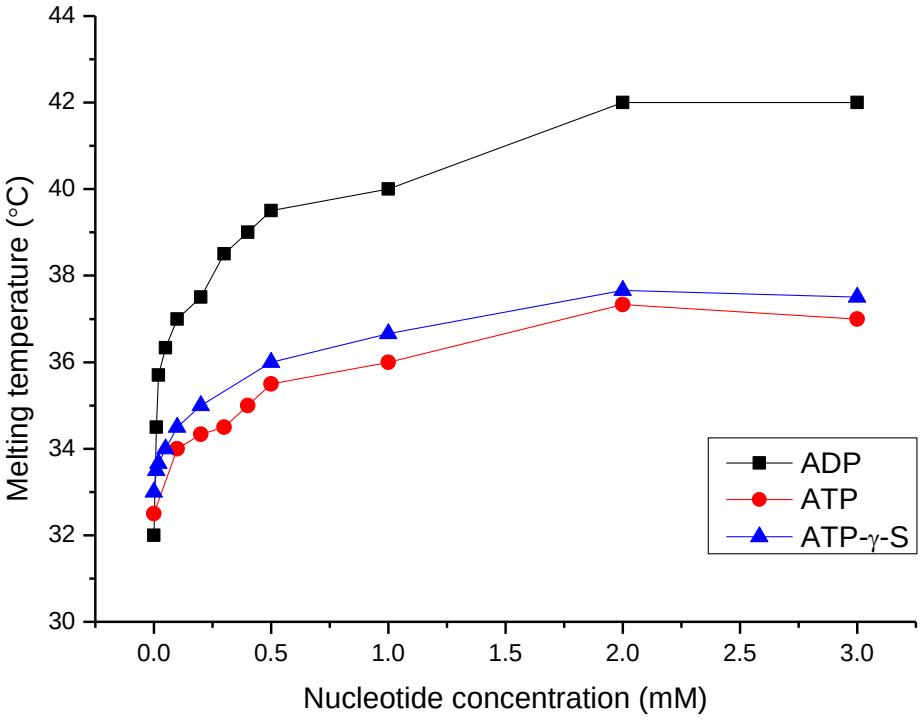

Fig. 4.10) Effect of varying ATP-γ-S concentrations on the Tm of spinach Rca as measured by DSF

ATP-γ-S in the absence of Mg^{2+} showed a similar profile to ATP in the absence of Mg^{2+}. The results shown is representative of two runs for ATP-γ-S. An increase of 0.5 °C was seen at 0.01 mM ATP-γ-S (fig. 4.10). The increase in Tm plateaued at an increase of 4.5 °C. This is unsurprising as ATP-γ-S should act similarly to ATP in the absence of Mg^{2+} as neither will be able to be hydrolysed due to the lack of activation of the active site.

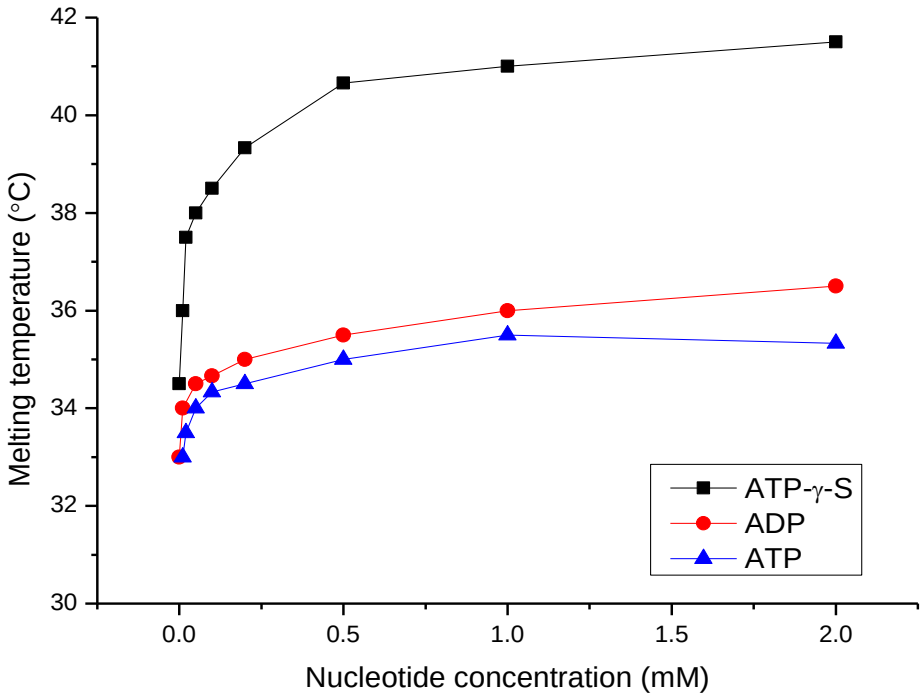

Fig 4.11) Effect of varying ATP-γ-S concentrations on the Tm of spinach Rca in the presence of 5 mM $MgCl_2$

ATP-γ-S in the presence of $MgCl_2$ caused an increase in stability as compared to ATP-γ-S in the absence of $MgCl_2$. In the presence of 5 mM $MgCl_2$ an addition of 0.01 mM ATP-γ-S yielded an increase in Tm of 0.5 °C up to an increase of 7 °C at 2 mM ATP-γ-S. This increase in stability may be due to electrostatic charge balancing. ATP has a large negative charge due to the number of phosphate groups attached, this large negative charge may be why ADP is able to bind more tightly and cause a greater increase in stability. However on the addition of $MgCl_2$, Mg^{2+} enters the active site of Rca, and the positive charge of Mg^{2+} will aid in the balancing of the electrostatic charges within the active site allowing for tighter binding of ATP. This same effect is not seen in ATP in the presence of Mg^{2+} as it is likely hydrolysed to ADP giving it a similar profile to ADP with Mg^{2+}.

ADP has a higher affinity for the binding site of Rca in the absence of Mg^{2+} indicating that it has a more appropriate electrostatic environment for ADP compared with ATP. The introduction of a positive charge from Mg^{2+} may cause a shift in the electrostatic charge of the active site to favour ATP rather than ADP. This could cause the observed effect of $MgCl_2$ addition on solutions of Rca containing ATP-γ-S and ADP. It has also been postulated that within the ADP bound state non-specific aggregation may result from the presence of cations which can interact with surface residues of Rca.

4.2.5) Aggregation due to Heat Stress is Reduced by Nucleotide Supplementation

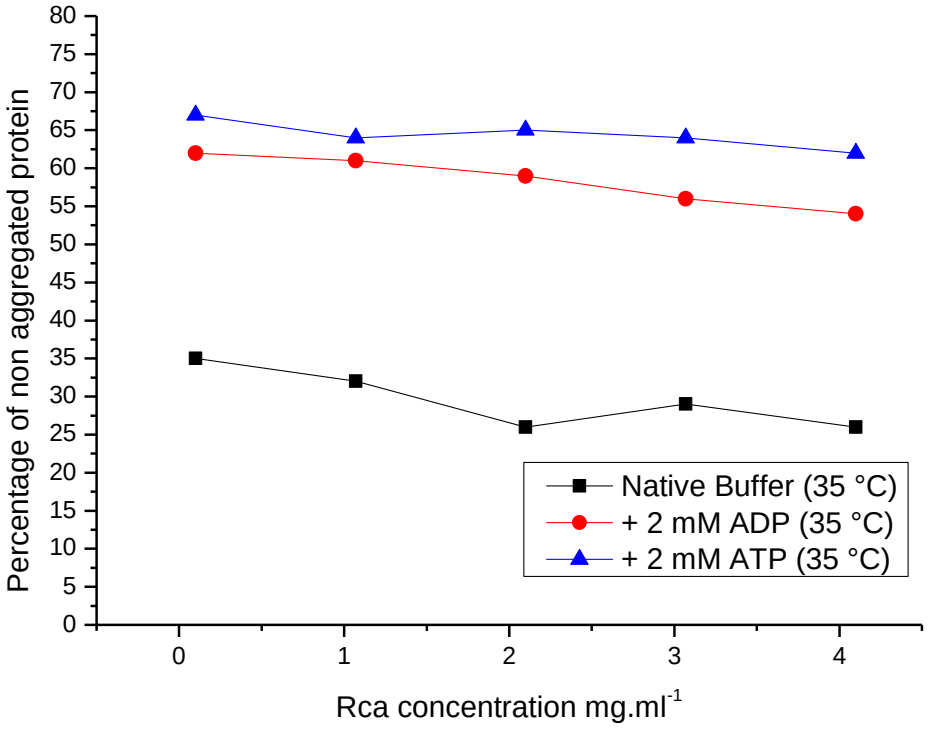

Fig. 4.12) Percentage of non-aggregated protein under varying conditions and Rca concentrations as measured by DLS

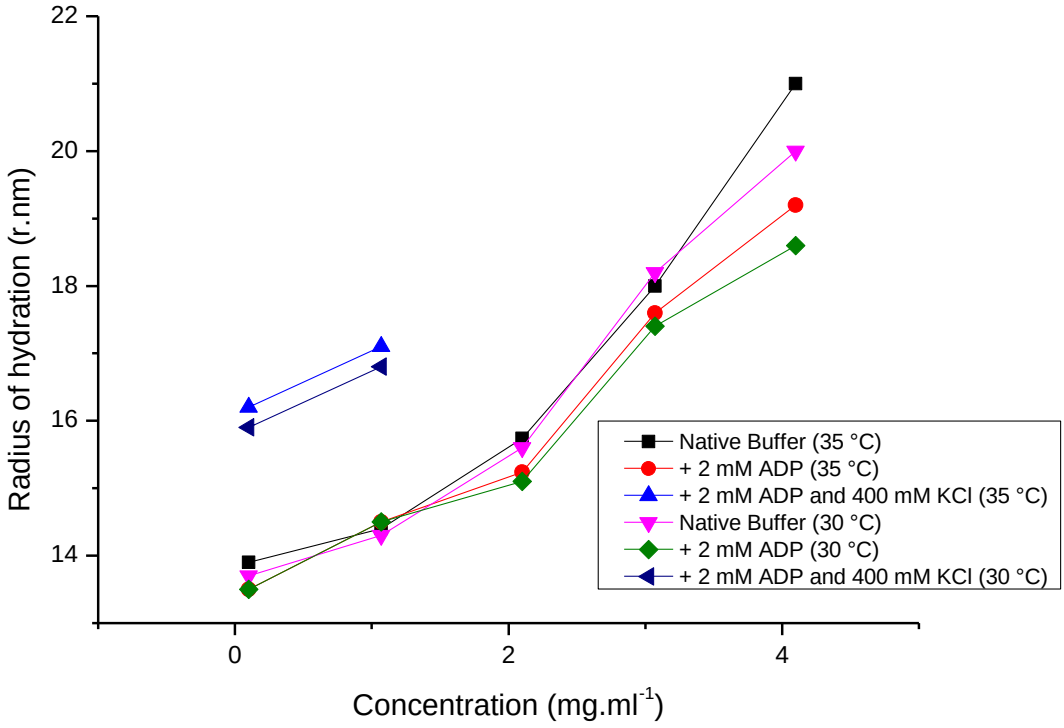

Fig. 4.13) Radius of hydration (r.nm) of varying concentrations of spinach Rca at different temperatures and with different supplemention

The stabilisation seen upon the binding of substrates and ligands is attributed to a reduction in steric flexibility of the protein which can lead to non-specific aggregation which can be seen using DLS. As shown in figure 4.12 and 4.13 ADP and ATP both caused a reduction in non-specific aggregation as seen by the change in the relative sizes of different molecular weight peaks. In the absence of ligands more large aggregates, over 10 r.nm, were observed at 30 and 35 °C. At 35 °C it was seen that in the absence of nucleotide approximately 65% to 75% of the signal, dependant on concentration, came from non-specific aggregates. The addition of nucleotide significantly reduced the percentage of non-specific aggregate within the solution. An addition of 2 mM ADP caused a decrease of around 30% of observed non-specific aggregate while 2 mM ATP caused a decrease of around 25%. This indicates that

nucleotide binding allows for greater thermal stability due to inhibition of large non-specific aggregates which is likely a result of increased steric hindrance while bound to substrate or product.

4.2.6) Tobacco Rubisco Activase

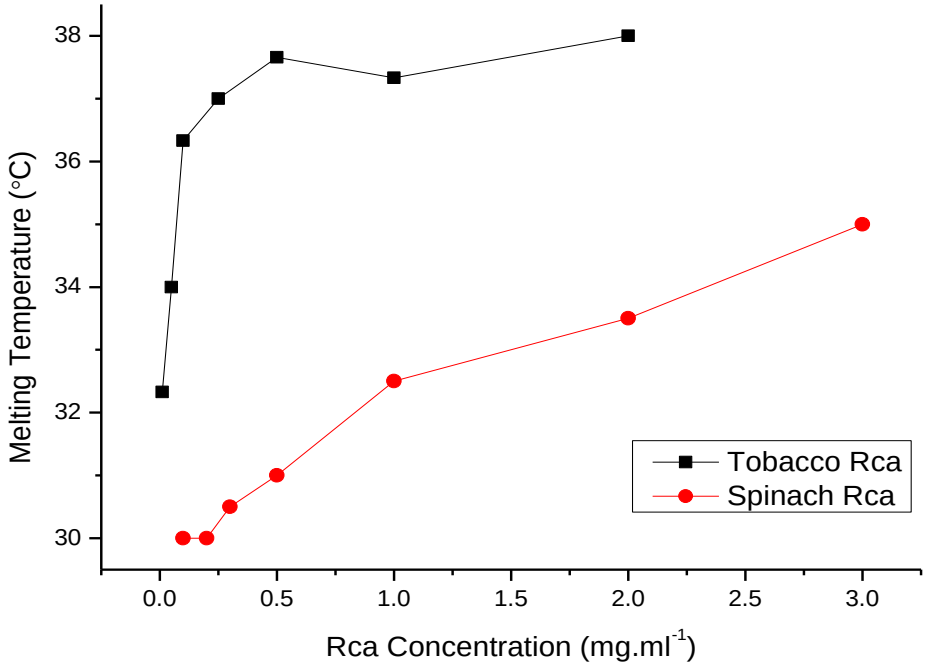

Fig.4.13) Comparison of the melting temperatures of Tobacco and spinach Rca at varying temperatures as measured by DSF

The effects of concentration on the thermal stability of tobacco Rca was also tested for comparison. Tobacco Rca was chosen as it is being worked on within the same laboratory so was easily obtained. As shown the thermal stability of tobacco increases as concentration increases up to 0.5 $mg.ml^{-1}$ after which no significant increase in stability was observed. A maximum increase in melting temperature of 6 °C was observed from the increase in concentration. This is in contrast to spinach, which showed a similar increase in melting temperature of 5 °C yet a different trend and thermal stability were observed. Spinach showed a more linear relationship between

melting temperature and concentration at low concentrations. Tobacco also exhibited a higher melting temperature at all concentrations compared to spinach, reinforcing the differences in thermal stability between Rcas of different species.

4.3) Chapter Conclusions

In conclusion it appears that the thermal stability of Rca is influenced by the presence of substrate or product and by the concentration of protein. The increase in thermal stability attributed to the increase in protein concentration may indicate a dynamic oligomeric state based on concentration. As Rca concentration increases the apparent Tm rises, likely due to a reduction in protein flexibility caused by larger Rca oligomers. Mg^{2+} has been shown to be necessary for the increase in thermal stability caused by nucleotide binding.

The effect of nucleotides on the thermal stability of spinach Rca observed is similar to observations made on Rca from different species. It has also shown similar trends to previous work done on spinach Rca as discussed in the introduction for this chapter (Salvucci *et al.* 2001, Wang *et al.* 1993). Rca from several species has been isolated and tested for various properties. Tobacco and cotton are two plants which are commonly used in order to determine the properties of Rubisco and Rca as these are common and economically relevant plants (Henderson *et al.* 2013, Wang *et al.* 1993).

Henderson *et al.* 2013 recently released a paper which explored the effects of substrate and product on the thermal stability of the β-isoform of cotton Rca as discussed in the introduction. Cotton Rca showed a higher basal Tm than my experiments showed for spinach Rca. Although different basal Tm's were observed

similar Tm trends upon the addition of substrate/product were seen for spinach and cotton. Similar effects of ADP in the absence of Mg^{2+}, 2 mM ADP caused a significant rise in cotton Rca as tested by Henderson and a smaller, but still significant, rise in the Tm for spinach observed in the current study for spinach Rca. The effects of nucleotide in the presence of Mg^{2+} on cotton Rca showed a reduction in the stabilising effects of ADP. It was also seen upon the addition of Mg^{2+} although at 2 mM a decrease of 2 °C was observed for cotton Rca compared to a reduction of 6.5 °C for spinach Rca. It was observed from my experimentation that the addition of Mg^{2+} and ATP provides less thermal protection as compared with ATP supplementation alone. This is in contrast to results observed for cotton Rca where ATP and Mg^{2+} supplementation showed a slight increase in temperature stability as compared to ATP alone. This increase in stability was attributed to more favourable electrostatic interaction within the active site, allowed by the additional positive charge from the Mg^{2+}. This was similar to the effects of ATP-γ-S in the presence of Mg^{2+} for spinach Rca. The differences observed between the species may be due to the native growing/optimisation temperatures for the different plants (Henderson *et al.* 2013).

A definitive model of how nucleotides provide thermal stability has not yet been published however conclusions can be drawn from the gathered data and from knowledge of the interactions of similar proteins. When ATP is bound Rca may be in a more open conformation as was seen with F_1F_0. This conformational change could lead to the exposure of hydrophobic residues when ATP is present which may account for the reduction in stability caused by ATP in the presence of Mg^{2+}. The reduction in stability of ADP in the presence of Mg^{2+} may be caused by a change in the charge of

the active site creating a less favourable binding site for ADP. Mg^{2+} caused an increase in stability for ATP-γ-S which may be a result of a shift in the electrostatic environment of the active site to favour ATP without the conversion of ATP to ADP. These results have also shown similarities to the effects of nucleotide and concentration of Rca's from different species including *Solanaceae* plants.

Chapter 5) Sizing

5.1.1) The Active Oligomer of Rubisco Activase

The main focus of this thesis is to determine the active oligomeric state/s of spinach Rca. A recent paper was published which gave a crystal structure for tobacco Rca and suggested that the minimum active state was a hexamer (Stotz *et al.* 2011). This may have been reinforced by the elucidation of the structure for the activase of red-type Rubisco, CbbX. CbbX was shown to function as a hexamer however has a very different mechanism of action (Mueller-Cajar *et al.* 2011).

This is in comparison to data which shows that activase exists in a dynamic oligomeric state of which most are active. Early reports indicated that the molecular weight of Rca was extremely variable and was thought to be a result of differential aggregation (Portis 1990).Under gel-filtration chromatography spinach Rca was always seen as an asymmetrical peak which was skewed towards smaller molecular weights. A peak of 340 kDa was taken as the molecular weight although it was noted that a heterogeneous mixture of molecular weights was observed (Wang *et al.* 1993). Nano-electrospray ionisation mass spectrometry (NanoESI-MS) was also used to determine the oligomeric state of tobacco Rca. The mass to charge ratio observed indicated the existence of species from monomers to hexamers (Blayney *et al.* 2011). Although the largest oligomer seen by Nano-ESI-MS was a hexamer larger oligomers have been observed up to a hexadecamer (Wang *et al.* 1993). Far larger oligomers up to 2 MDa have also been observed but this was seen as a result of errant aggregation resulting from heat-induced stress (Barta *et al.* 2010).

5.1.2) The Effect of Concentration on Molecular Weight

The oligomeric state of Rca from several species has been shown to be affected by protein concentration and nucleotide supplementation. The effects of protein concentration on molecular weight were examined for cotton β-isoform Rca. When in the presence of ADP, cotton Rca was shown to exist predominantly as a monomer when Rca concentrations were below 0.5 μM. As the concentration was raised a shift was seen in the dominance of different oligomers. Trimers were predominantly seen at ~5 μM, hexamers at ~25 μM and a 24 subunit species was observed at ~100 μM. It was thought that this increase in species size would increase indefinitely as concentration rises (Chakraborty *et al.* 2012).

A recent study was also looked at the distribution of tobacco Rca oligomers in solution. Using analytical ultracentrifugation (AUC) it was found that a range of Rca species existed within a solution. The distribution of species present was influenced by concentration. At the lowest concentration tested, 0.6 μM, monomers and dimmers were present amongst the species. As the protein concentration was increased a broader spectrum of species was seen. At concentrations above 10 μM large supramolecular complexes were observed up to a 12 unit oligomer (Keown *et al.* 2013). This showed that within solution activase exists in a series a oligomeric forms, smaller and larger than the proposed hexameric active form.

Size exclusion chromatography showed that the molecular weight of Arabidopsis Rca increased with concentration. Concentrations from 1 $mg.ml^{-1}$ to 10 $mg.ml^{-1}$ were tested, in the presence of Mg^{2+} and ADP, which showed a range of molecular weights from 220 kDa to 660 kDa respectively (Barta *et al.* 2010). This was supported again by work done which showed that Rca aggregation represented a dynamic equilibrium

(Wang *et al.* 1993). The addition of crowding agents will also cause an increase in oligomeric size. The addition of polyethylene glycol is well known to cause an increase in Rca specific activity. The increase in activity is attributed to the increase in molecular mass of Rca (Salvucci 1993).

5.1.3) Effect of Nucleotide and Magnesium Supplementation on Molecular Weight

The effect of nucleotides on molecular weight is a key point of research for Rca. It has been shown that nucleotide supplementation influences molecular weight which likely causes the changes in thermal stability seen upon supplementation.

Early experiments indicated that the addition of ATP or ATP-γ-S to a solution of spinach Rca caused an increase in intrinsic fluorescence when in the presence of Mg^{2+}. This increase indicates that a structural change has occurred, in the form of excited tryptophan residues. The increase in fluorescence occurred over a period of ~2 minutes and gradually decreased over time. The decline in fluorescence was thought to be due to a build-up of ADP which competes for binding sites with ATP, again causing a structural change. ATP or ATP-γ-S alone caused no change in fluorescence however indicating the need for Mg^{2+} for this structural change to occur (Wang *et al.* 1993). These findings lead to the determination of molecular weight under these conditions using gel-filtration chromatography. At 25 °C spinach Rca was shown to have a molecular weight of 340 kDa in the presence of ATP alone or ADP and Mg^{2+}. When in the presence of ATP and Mg^{2+} a significant increase in molecular weight was observed reaching around 600 kDa. Molecular weight was also shown to be affected by temperature. At 4 °C ATP or ADP and Mg^{2+} supplementation gave a

molecular mass of 500 kDa while ATP and Mg^{2+} gave a molecular weight of over 600 kDa. This shows a strong relationship between nucleotide/magnesium supplementation and spinach Rca molecular weight (Wang *et al.* 1993).

Further experiments were done on the apparent molecular mass of tobacco and spinach Rca in the presence of nucleotides. These experiments indicated a species dependant reaction to nucleotide supplementation. At 2 $mg.ml^{-1}$ spinach Rca was shown to exist as a 200 kDa oligomer in the presence of 0.1 mM ADP and Mg^{2+}, while existed as a 400 kDa oligomer in the presence of 0.2 mM ATP and Mg^{2+}. This reinforced the findings that ATP causes greater self-association of spinach activase. In contrast tobacco Rca at 2 $mg.ml^{-1}$ was shown to have a molecular weight of 250 kDa in the presence of Mg^{2+} and ADP or ATP (Li *et al.* 2006).

These studies showed that Mg^{2+} also plays a significant role in the behaviour of Rca. Studies run on Arabidopsis Rca showed that Mg^{2+} alone can encourage aggregation of Rca. A series of experiments were run which looked at the effect of varying concentrations of Mg^{2+} at varying temperatures on the oligomeric state of 3 $mg.ml^{-1}$ Rca. Aggregation was examined by monitoring the change in light scattering by use of a spectroflourometer. This experiment was run at 30, 35 and 40 °C and used Mg^{2+} concentrations of 0, 2 and 5 mM. At 30 °C little difference was seen between the samples with different Mg^{2+} concentrations. At 35 °C and 40 °C significant increases are seen in light scattering as the Mg^{2+} concentration is increased. This shows the propensity for Mg^{2+} to alone have a significant effect on oligomeric state of the enzyme. The effects of this aggregation could however be inhibited by the addition of ADP. 0.5 mM ADP supplementation caused prevented any increase in scattering at 30

°C and significantly reduced light scattering at higher temperatures (Barta *et al.* 2010).

Size exclusion chromatography was also used to determine the effects of supplementation on molecular mass during heat stress. Incubation of Arabidopsis Rca at 25 °C caused a slow decline in activity. This decline in activity was coupled with the formation of an inactive 2 MDa aggregate along with the active 660 kDa peak previously found. ADP supplementation however was able to curb the loss of activity and prevent the creation of the large molecular weight compound. This shows that although nucleotide supplementation has been shown to increase the molecular weight of Rca it also helps to prevent erroneously large aggregate formation (Barta *et al.* 2010).

5.2) Determination of Molecular Weight by DLS

Several other AAA+ proteins have been characterised using a variety of techniques and have been shown to often form large oligomeric species, often hexameric rings. The oligomeric state has been shown to be influenced by nucleotide and magnesium supplementation, crowding agents, protein concentration and temperature (Henderson *et al* 2013). Maximal Spinach Rca activity has been previously shown to occur at approximately 0.1 $mg.ml^{-1}$ however activity can be seen at concentrations of 0.005 $mg.ml^{-1}$ and below (Wang 1993). As concentration has been shown to influence the oligomeric state of Rca various concentrations were first tested for molecular weights in order to determine if the molecular weight was static over the range of known active concentrations. These were followed by experiments in order to determine the

effects of nucleotide and magnesium supplementation on molecular weight over a rage of concentrations.

Initial sizing tests for spinach activase were achieved via dynamic light scattering (DLS) using a zetasizer. Light scattering at 173° was used in order to provide the scattering patterns of molecules as they tumble in solution. These scattering patterns are able to be used to determine the lengths of the different dimensions of the molecule in question as it tumbles, giving an average diameter of the molecule. This diameter is known at the hydrodynamic diameter as it includes the size of the solvent shell around the molecule. One limitation of this technique is that DLS assumes the unit being measured is spherical, which we know spinach activase is not. DLS takes an average of measured sides as the protein tumbles in solution to give an average particle radius (r.nm), measured in nanometres, which can be converted to an estimated molecular weight, measured in kilo Daltons (kDa). Rca has been shown to have an elongated region by crystal structures. As it is not a spherical protein the assumptions made by the technique cause an inaccuracy in the estimated hydrodynamic diameter. Although it is an inaccurate technique for a non-spherical protein it does show a trending increase in molecular weight and average molecular size.

5.2.1) Effect of Concentration on Size

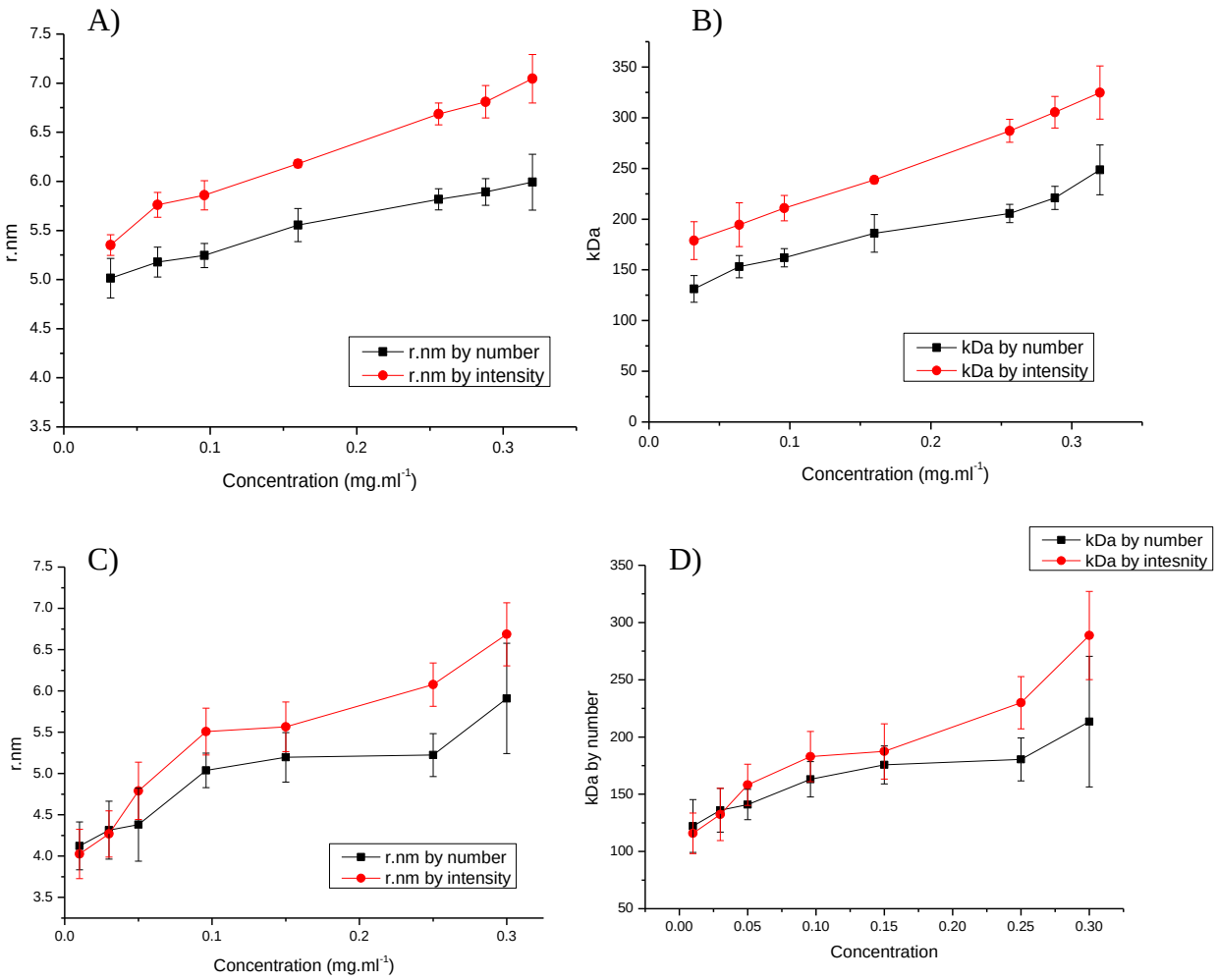

Fig.5.1 A) Radius of hydration (r.nm) of Rca at varying concentrations at 20 °C. B) Molecular weight (kDa) of Rca at varying concentrations at 20 °C. C) Radius of hydration (r.nm) of Rca at varying concentrations at 8 °C. D) Molecular weight (kDa) of Rca at varying concentrations at 8 °C.

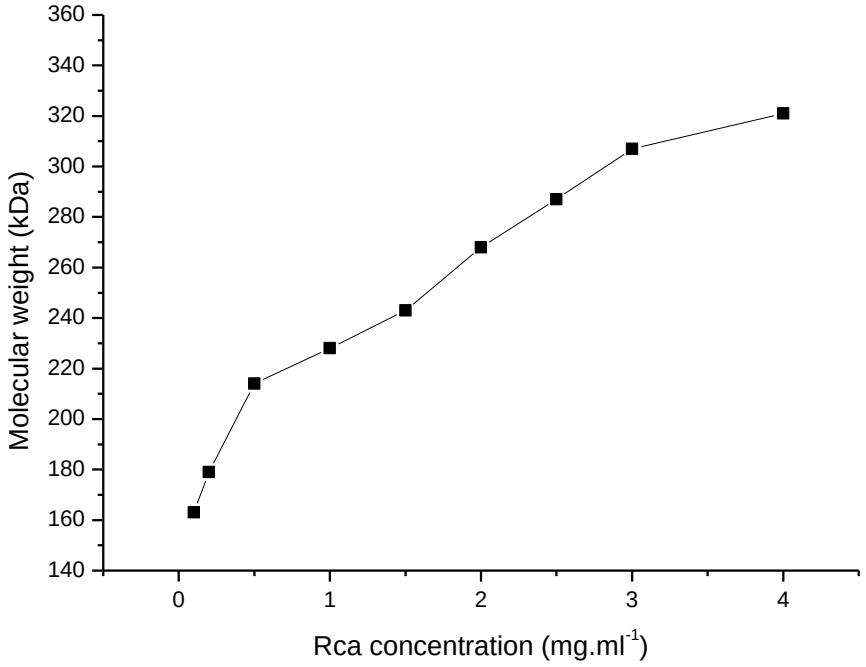

Fig. 5.2) Molecular weight of Rca at varying concentrations at 20 °C

DLS of spinach Rca indicated that the average molecular radius was influenced by protein concentration and temperature. We have proposed that Rca exhibits activity in a range of oligomeric sizes, influenced primarily by protein concentration. The information shown above is representative of 3 separate runs. Higher concentrations were run however these runs gave multiple peaks indicating the presence of large aggregates which gave inaccurate readings from the scattering. The average radius of particles in solutions of varying spinach Rca concentrations was tested by DLS at 8 °C and 20 °C. At both temperatures an increase in r.nm was observed, correlating to an increase in Rca concentration. At 8 °C a change in r.nm of 1.6 (4.3-5.9) by number and 2.4 (4.2-6.6) by intensity was observed over a concentration range of 0.01-0.1 $mg.ml^{-1}$. 20 °C showed a similar, yet less profound effect on r.nm by concentration. At 20 °C an increase in r.nm of 0.9 (5.0-5.9) by number and 1.7 (5.3-7.0) by intensity was observed over a concentration range of 0.03-0.3. Lower concentrations for each

were attempted however the signal to noise ratio became too high at concentrations below 0.01 $mg.ml^{-1}$ for 8 °C and 0.03 $mg.ml^{-1}$ for 20 °C.

From the r.nm it is possible to approximate a molecular weight of the protein in question. At 8 °C an increase in the kDa of Rca of 187 (100-288) was observed by intensity against an increase of 87 (126-213) by number. The observed r.nm for Rca at 20 °C gave a change in kDa of 117 (131-248) by number and 146 (178-324) by intensity. As indicated by crystal structures Rca has been shown to have a molecular weight of approximately 45 kDa. This would indicate that at 8 °C spinach Rca predominantly exists as a dimer when at a concentration of 0.01 $mg.ml^{-1}$ while at 0.3 $mg.ml^{-1}$ spinach Rca exists predominantly as a hexamer, as measured by intensity. An increase in stoichiometry is seen at 20 °C, 0.03 $mg.ml^{-1}$ indicated the predominance of a tetramer (as opposed to a trimer at 8 °C) while molecular weights corresponding up to a septamer was observed at 0.3 $mg.ml^{-1}$.

Although this technique lacks accuracy for the measurement of Rca it does show that Rca exhibits dynamism within its molecular size correlating to concentration. Significantly larger sizes were also seen at higher temperatures and concentrations, up to 2 MDa, however it is assumed that these oligomeric states were a result of non-specific aggregation and were unlikely to be active macromolecules. This technique is also unable to indicate whether different species sized species exist within the solution as it gives an average particle size.

5.2.2) Effect of Supplementation on Molecular Weight

Nucleotide supplementation has been shown to cause an increase in thermal stability, thought to be caused by an increase in subunit association. This means that the average molecular weight of a solution of Rca should increase upon nucleotide supplementation. Varying concentrations of ADP, ATP and magnesium were tested for their effect on the molecular weight on 0.5 mg.ml^{-1} Rca at 20 °C. Both ADP and ATP supplementation caused an increase in molecular weight of Rca. Both showed significant increases over the concentration range 0.01 to 0.5 mM, with the effects levelling off at around 2 mM. This supports the thermal stability data gained by DSF which showed large changes in thermal stability upon nucleotide supplementation up to 0.3 mM, with 2 mM showing a levelling of the effects.

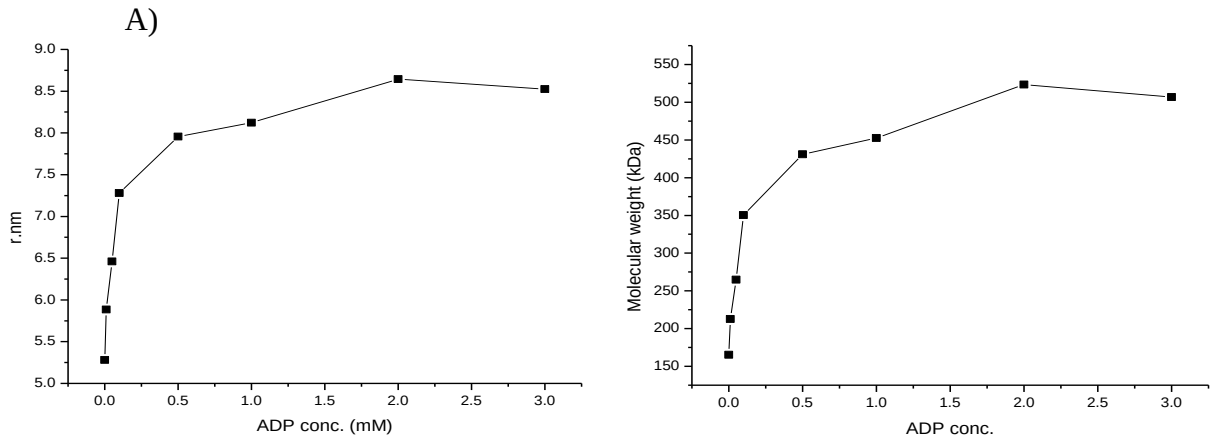

Fig.5.3 A) Radius of hydration of 0.5 mg.ml^{-1} Rca in the presence of varying ADP concentrations and the absence of Mg^{2+}as measured by number. B) Molecular weight of 0.5 mg.ml^{-1} Rca in the presence of varying ADP concentrations and the absence of Mg^{2+}as measured by number.

ADP showed a greater effect on the molecular weight of Rca. A concentration dependant increase of 265 kDa (165 kDa-431 kDa) was observed over the first 0.5 mM of ADP supplementation, with a further increase of 92 kDa (431 kDa-523 kDa) seen from 0.5 mM to 3 mM.

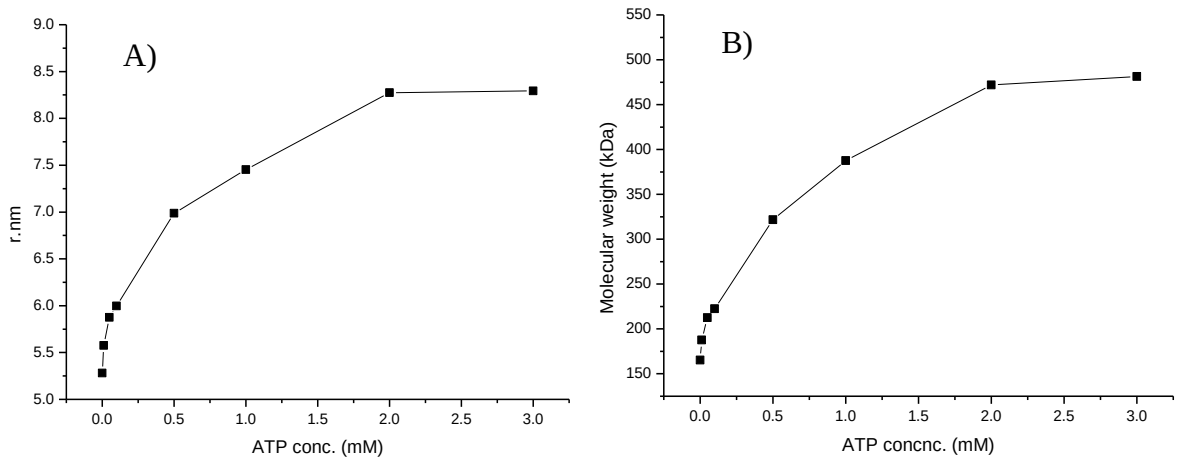

Fig.5. A) Radius of hydration of 0.5 mg.ml^{-1} Rca in the presence of varying ATP concentrations and the absence of Mg^{2+}as measured by number. B) Molecular weight of 0.5 mg.ml^{-1} Rca in the presence of varying ATP concentrations and the absence of Mg^{2+}as measured by number.

ATP showed lesser effects, causing an increase in molecular weight of 156 kDa (165 kDa-321 kDa) up to 0.5 mM ATP, with a further increase of 159 (321 kDa-481 kDa) reaching a plateau at 2 mM ATP. This equals a maximum increase of 316 kDa for ATP supplementation and 358 for ADP supplementation. It should be noted that these runs are representative of a single run.

As seen, ADP provides a greater increase in molecular weight. It would seem that ADP offers a greater molecular weight increase at lower concentrations than ATP however in the presence of higher concentrations the effect ATP appears to come closer to the effect provided by ADP. This is evidenced by the fact that ADP supplementation gave its highest molecular weight at 523 while ATP gave a molecular weight of 481 kDa, a difference of only 42 kDa. This is in contrast to supplementation at 0.5 mM with ADP giving a size of 431 kDa and ATP giving a molecular weight of 321.6 kDa, a difference of 109.4 kDa. This is equivalent to the difference of approximately 1 and 3 subunits for 0.5 mM and 3 mM ATP/ADP supplementation. If the theory that changes in the thermal stability of Rca are caused

by changes in oligomeric state then it should be expected that ADP would cause a greater increase in molecular weight as it causes a greater increase in thermal stability.

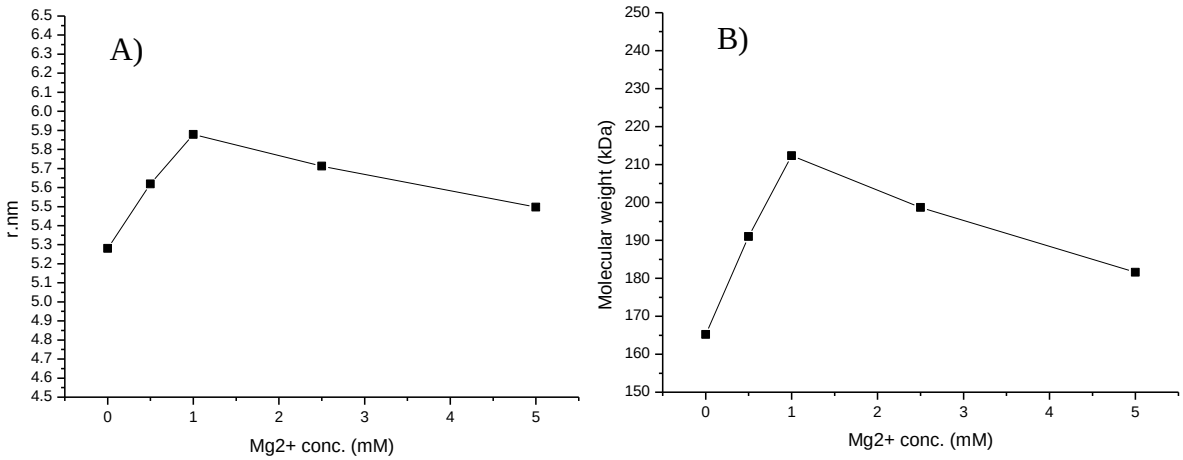

Fig.5.5 A) Radius of hydration of 0.5 mg.ml^{-1} Rca in the presence of varying MgCl concentrations. B) Molecular weight of 0.5 mg.ml^{-1} Rca in the presence of varying MgCl concentrations.

Mg^{2+} was also tested by DLS for its effects on the molecular weight of Rca. A very small increase in molecular weight was seen upon the addition of Mg^{2+} to Rca however this increase did not appear to be concentration dependant and was small enough to potentially be outside of the accuracy of the equipment. Several concentrations were tested from 0.5 mM to 5 mM MgCl. The results showed no trend, with sizes ranging from 181 kDa to 212 kDa, sizes within the error of the technique. This was opposed to the molecular weight of Rca with no supplementation, 165 kDa. This indicates a small increase in size which may be caused by the activation of the Rca, by Mg^{2+}, allowing a slight conformational change to occur which may slightly favour self-association.

Rca has been shown to be sensitive to temperature. It has been shown that Rca instability at high temperatures causes a loss of photosynthetic activity in plants. This was apparent in samples incubated at 30 °C and 35 °C. Significantly higher particle sizes were seen at these temperatures, a r.nm of 7 seen at 0.1 mg.ml^{-1} for 35 °C. High

temperatures and protein concentrations also led to the creation of very large aggregates, 4.1 $mg.ml^{-1}$ Rca gave a r.nm of 10.5 at 35 °C. This corresponds to a molecular weight of over 600 kDa

5.3) Determination of Molecular Weight by Size Exclusion chromatography

Size exclusion chromatography (SEC) was also used to explore the effects of protein concentration and buffer conditions on molecular weight. SEC utilises a column packed with a porous resin which allows for the separation of molecules within a solution based on their size. Large molecules fall through the porous resin and are eluted earlier while small molecules get caught within the resin and are eluted later. This allows for proteins to be separated based on size over a certain volume. These elution volumes can be compared against a known standard of proteins of known molecular weight to determine the molecular weight of the molecules within a solution.

In order to determine the molecular weight of Rca a protein standard was used in order to create a standard curve to match obtained elution volumes to the elution volumes of other known proteins. A Bio-rad gel filtration standard was used containinig thyroglobulin (670,000 kDa), γ-globulin (158,000 kDa), ovalbumin(44,000 kDa), myoglobulin (17,000) and vitamin B_{12} (1,350 kDa). These were run before each SEC a standard was run and a standard curve was created in order to determine the observed molecular weights of Rca. Below is an elution profile for the standards used and the standard curve produced from it. It should be noted that generally the final peak from the standard (vitamin B_{12}) was omitted as it prevented

an accurate line of best fit and was outside of the molecular weight range being tested for.

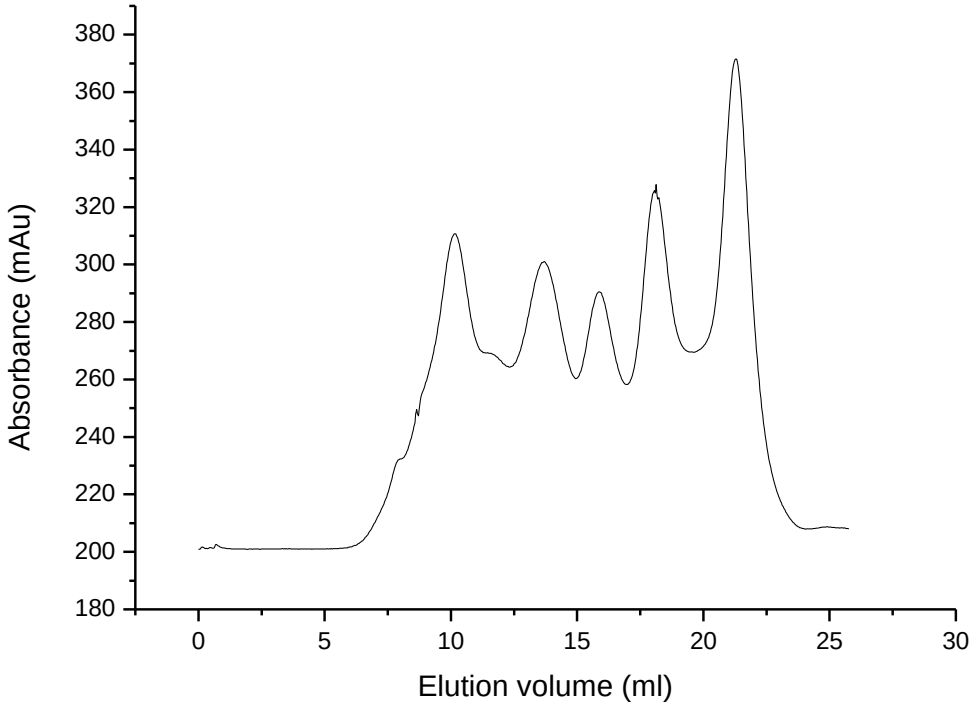

Fig. 5.6) Elution profile of SEC protein standard

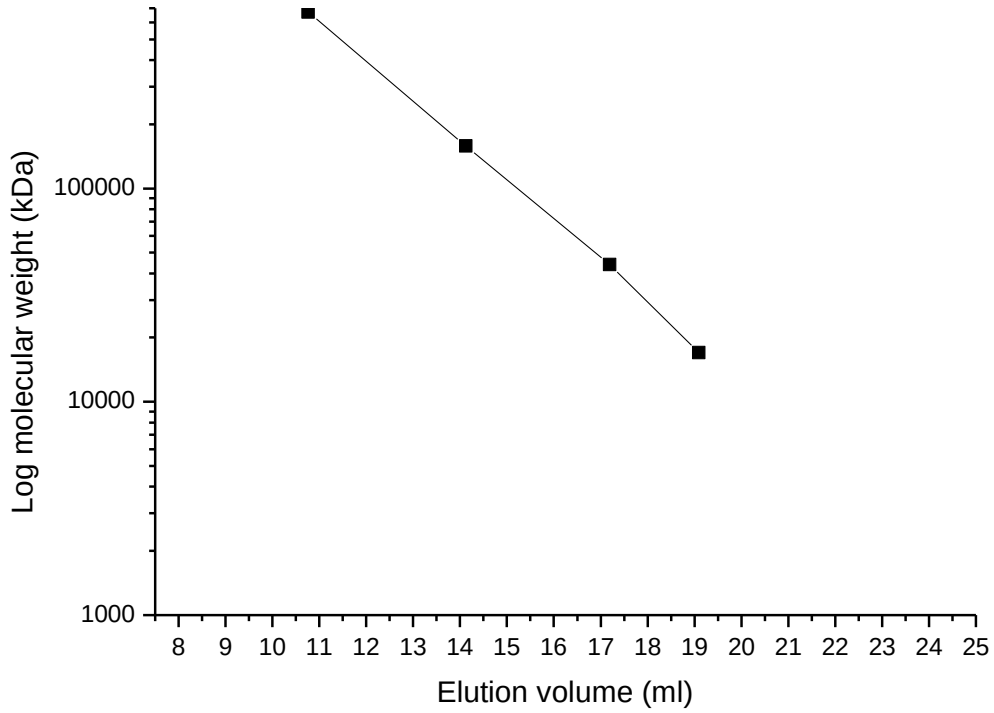

Fig.5.7) Calibration curve for the SEC standard

5.3.1) Effect of concentration on molecular weight

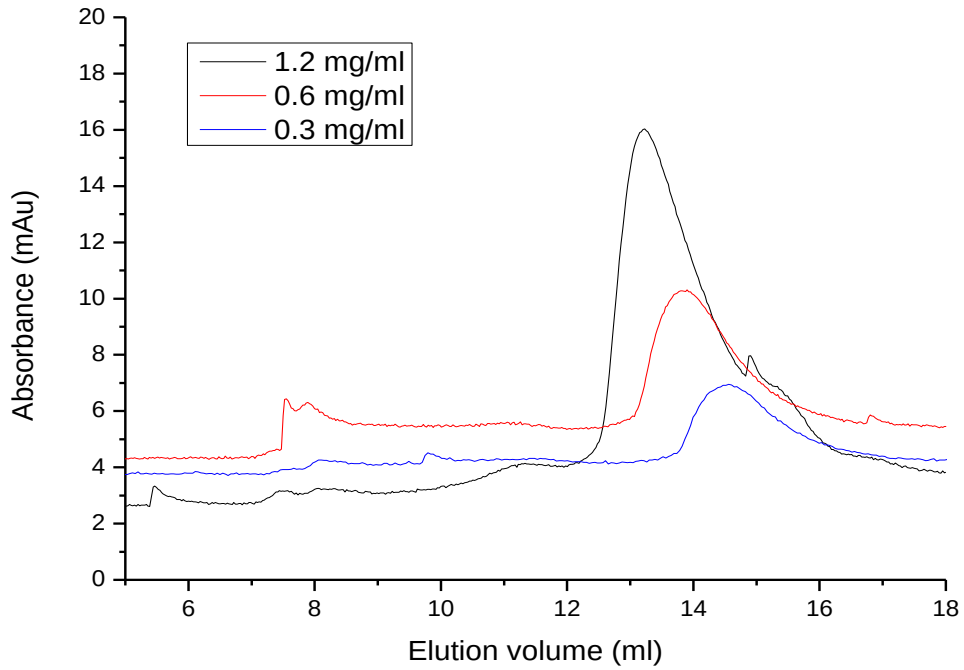

Fig. 5.8) SEC traces of Rca at varying concentrations.

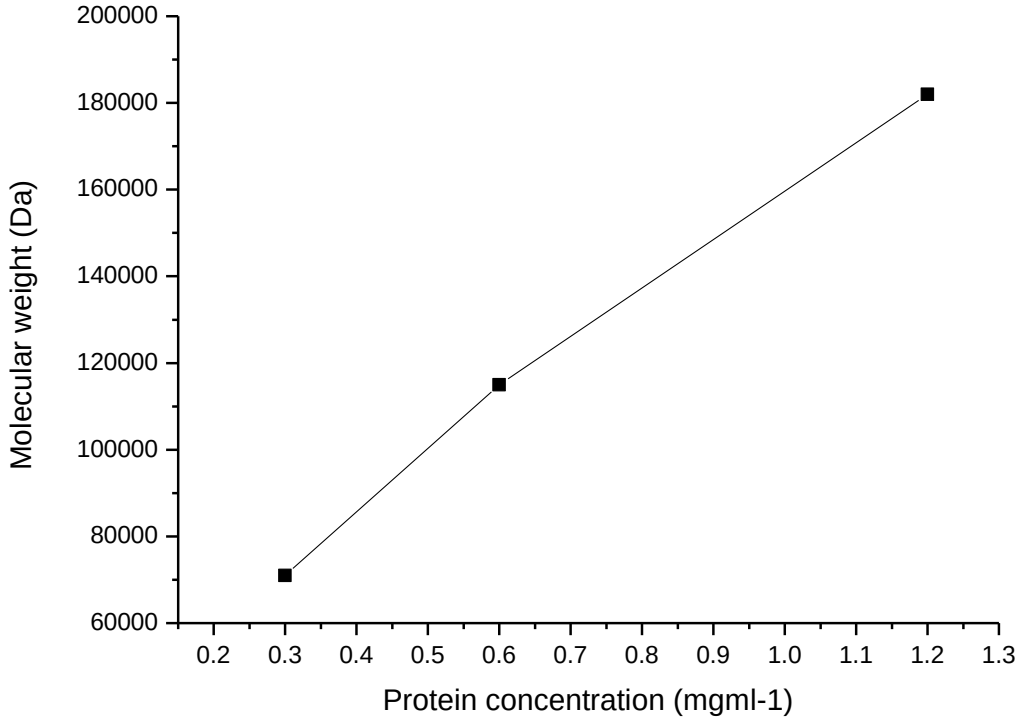

Fig. 5.9. Effect of Rca concentration, in the absence of Mg^{2+}, on molecular weight as measured by SEC

The effects of concentration on molecular weight were explored initially. A concentration dependant effect on molecular weight was observed by SEC. A range of loading concentrations were tested from 0.3 $mg.ml^{-1}$ to 1.2 $mg.ml^{-1}$ and a change in molecular weight of 71 kDa to 182 kDa, respectively, was observed. This clearly indicates that as Rca concentration increases so does its apparent molecular weight. Within the traces for the different concentrations a trailing peak is observed. This would indicate that species of multiple molecular weights exist within the solution, the kDa was taken from the peak of the curve. The observation that concentration affects molecular weight and that different molecular weights exist within a single solution of Rca indicates that Rca exhibits dynamism in terms of its molecular weight.

5.3.2) Effect of supplementation on molecular weight

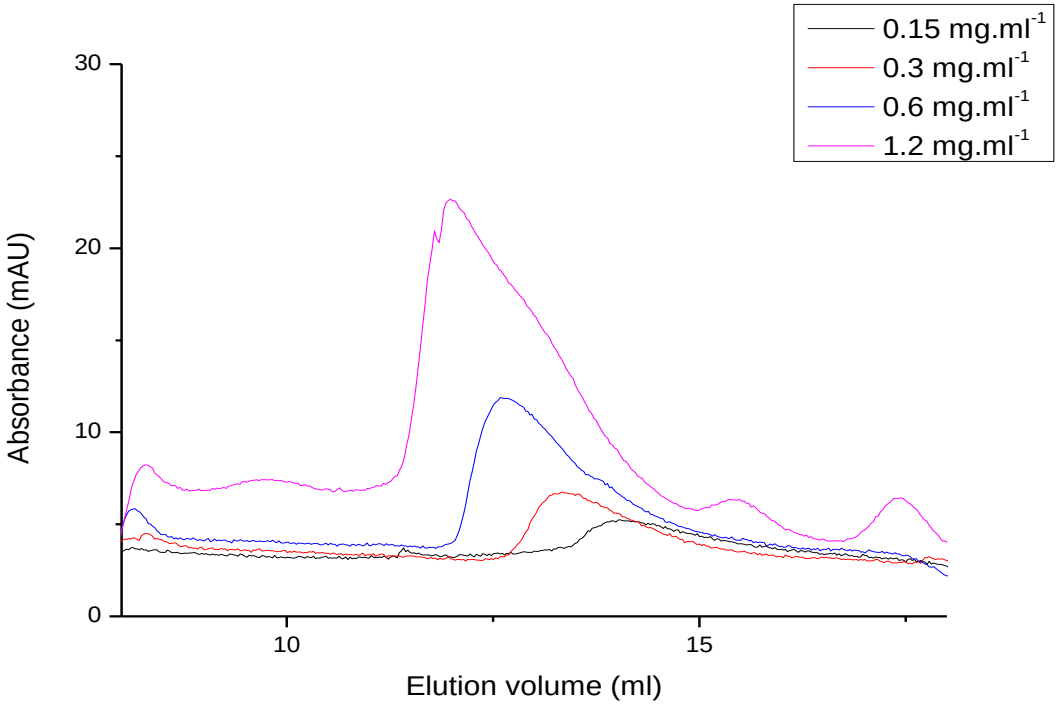

Fig. 5.10. SEC traces of 0.5 mM ADP on the molecular weight of varying Rca concentrations

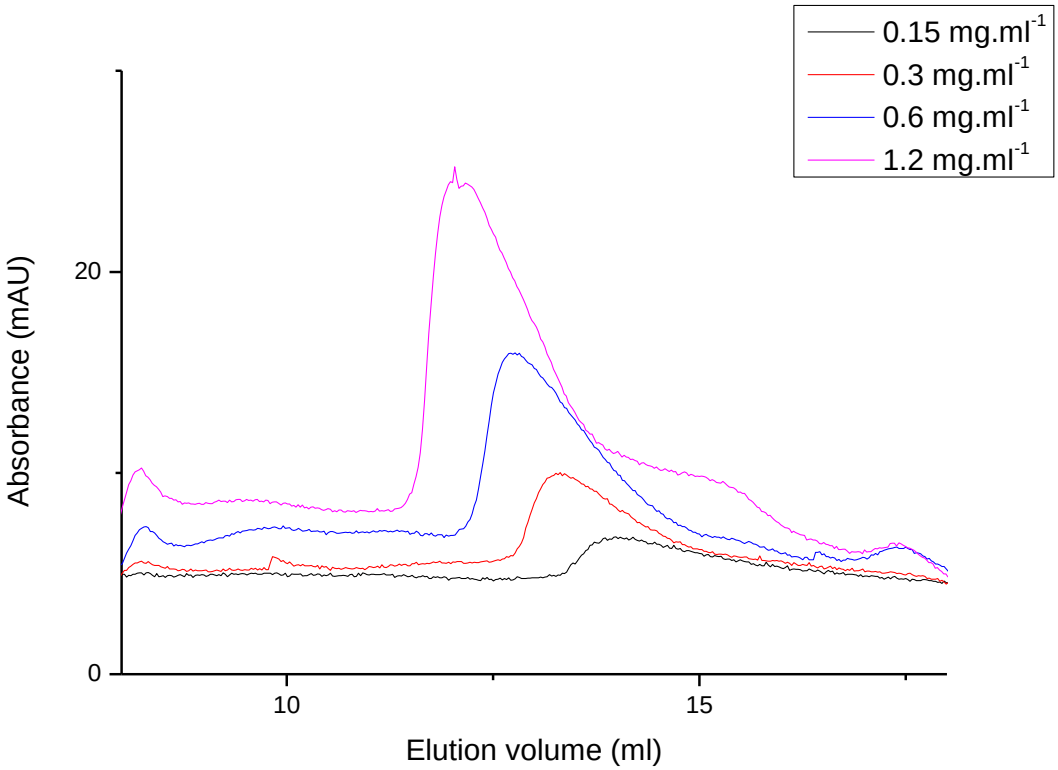

Fig. 11. SEC traces of 0.5 mM ATP on the molecular weight of varying Rca concentrations

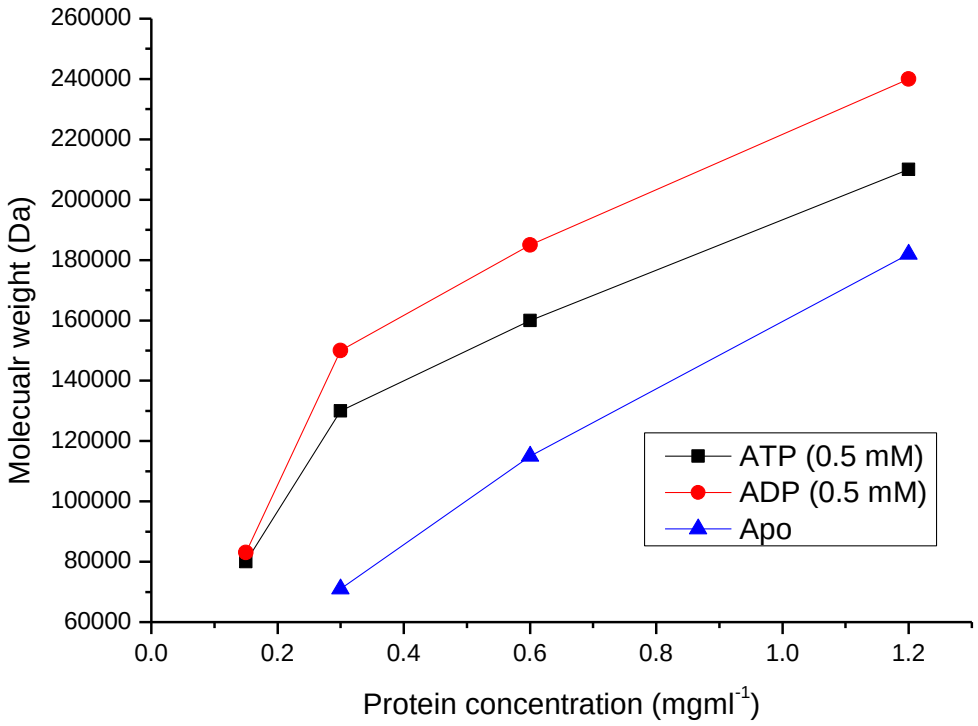

Fig. 5.12) Effect of nucleotide supplementation,in the absence of Mg^{2+}, on the molecular weight of Rca at varying concentrations as measured by SEC

The effect of ADP and ATP on molecular weight were also explored by SEC. These nucleotides were each tested at 0.5 mM, in the presence of a range of Rca concentrations, under SEC. Both ADP and ATP caused an increase in molecular weight with ADP giving a larger effect. As protein concentration increased the effect on molecular weight was observed to decrease as compared to apo. This is likely a result of the increase in size as a result of concentration reducing the potential effect of the nucleotides. This is in contrast to an observed increase in the difference in molecular weights between ATP and ADP as Rca concentration increases.

ATP supplementation of Rca results in an increase in molecular weight over all protein concentrations tested. ATP supplementation of Rca gave molecular weights of 80 kDa at 0.15 $mg.ml^{-1}$ Rca to 210 kDa at 1.2 $mg.ml^{-1}$. This shows an approximate increase of 15 kDa at the lowest protein concentration and an increase of 30 kDa at the highest concentration tested. ADP supplementation caused a larger increase in

molecular weight. ADP supplementation gave a molecular weight for Rca of 83 kDa at 0.125 mg.ml^{-1} and 240 kDa at 1 mg.ml^{-1}. \

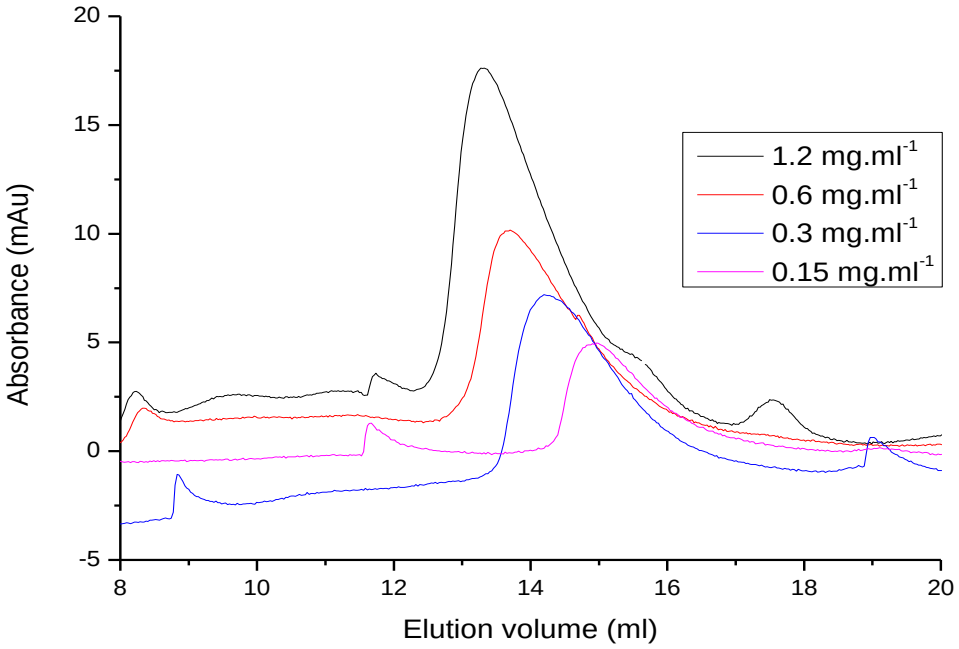

Fig. 5.13) SEC traces of Rca at varying concentrations in the presence of 5 mM MgCl

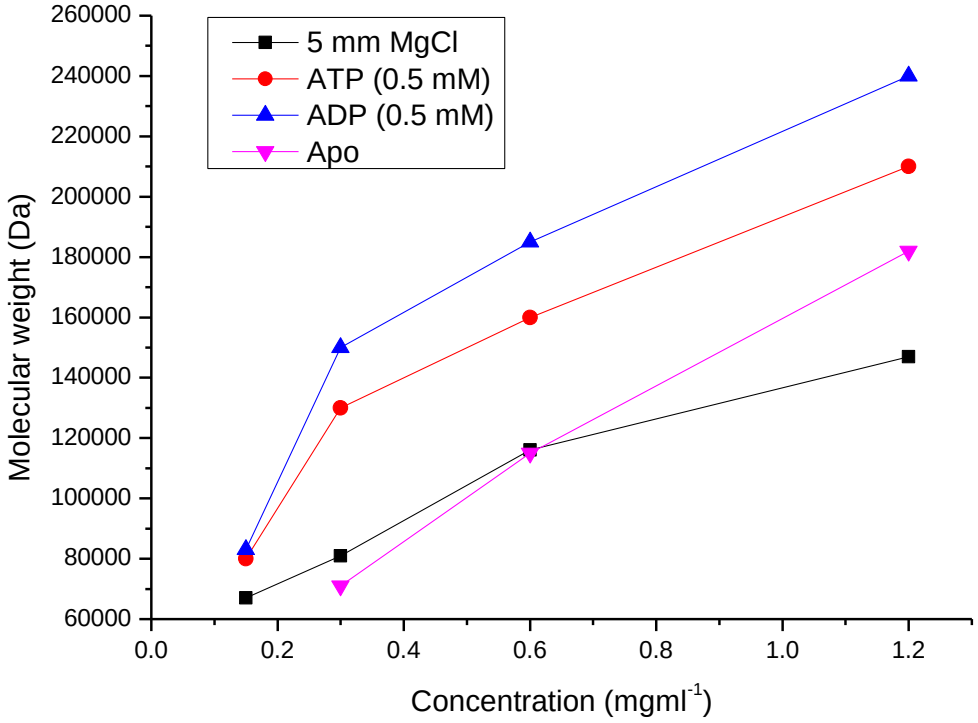

Fig. 5.14 Effect of 5 mM MgCl on the molecular weight of varying Rca concentrations as measured by SEC

The effects of Mg2+ on the molecular weight of Rca was also tested by SEC. The results for the effects of magnesium were gathered over an evening and the following morning over which time the UV detector was reset which is why the 2 sets of profiles do not overlap, each was standardises against an SEC standard run immediately prior to each of the two runs. This profile showed very similar weights to that observed for apo Rca at all concentrations but the highest. At 1.2 mg.ml^{-1} loading concentration it was seen that Mg2+ supplementation caused a significant drop in molecular weight giving a kDa of 147 as compared to 182 for apo Rca. The other weights were similar however with a 1 kDa difference at 0.6 mg.ml^{-1} loading concentration and and 10 kDa difference at 0.3 mg.ml^{-1} loading concentration. Tobacco Rca was also run to compare the effects of concentration on molecular weight of other species. Tobacco Rca showed a similar trend in that increasing concentrations lead to an increase in molecular weight. Tobacco activase did however

show larger molecular weights at similar concentrations. This may be due to more favourable self-association for tobacco Rca as compared to spinach Rca which could also account for the greater thermal stability observed for tobacco Rca.

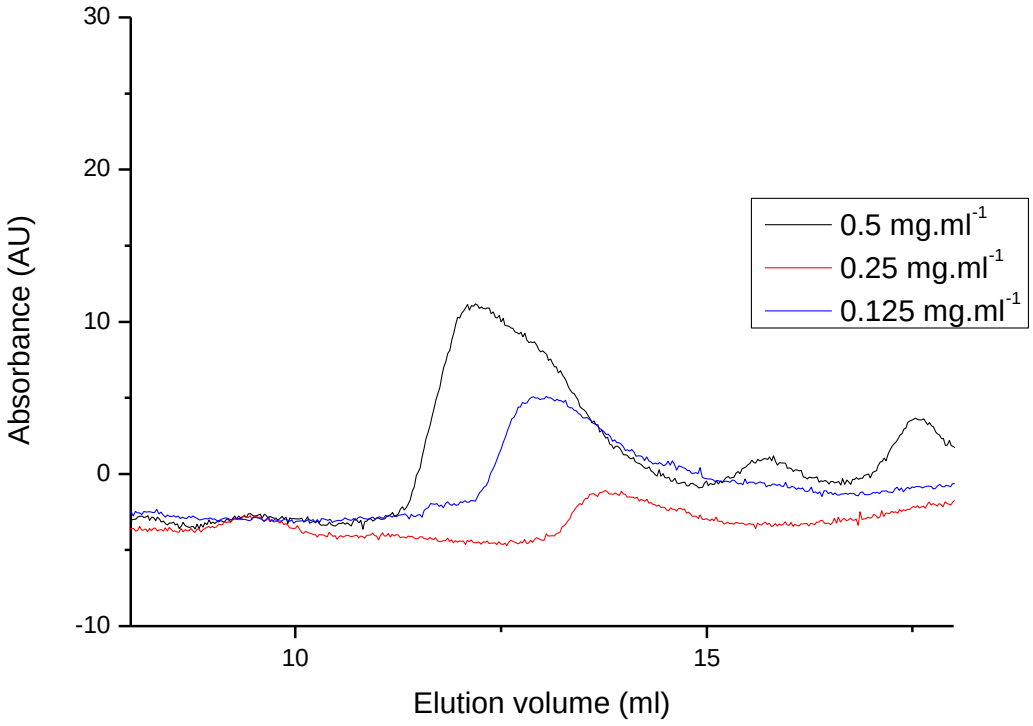

Fig. 5.15) SEC trace of tobacco Rca as various concentrations

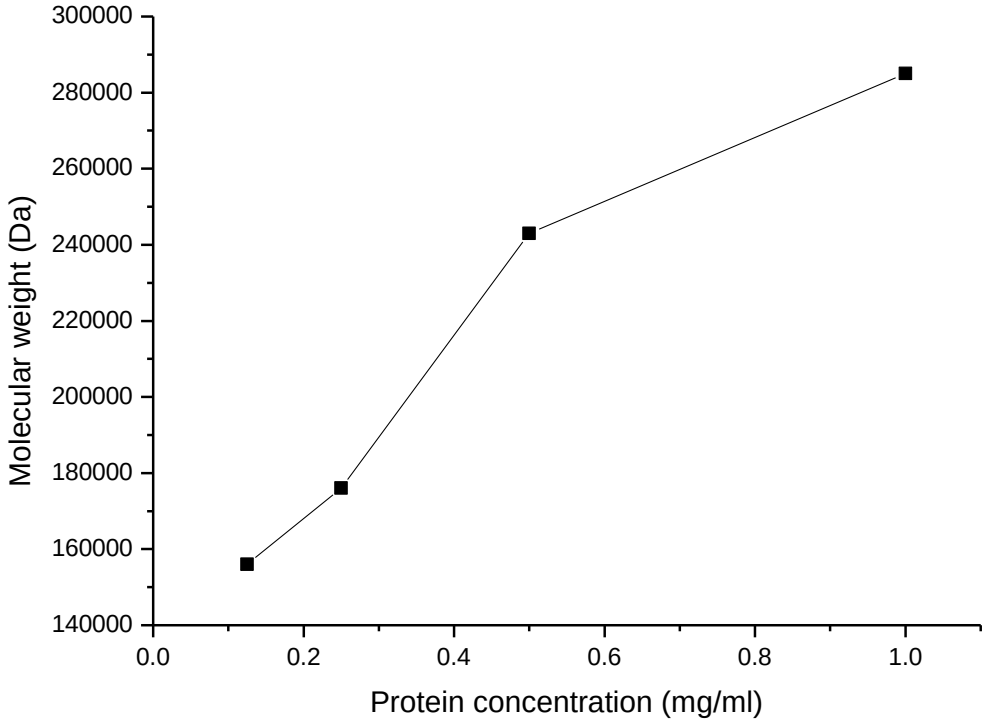

Fig. 5.16) Effect of concentration on the molecular weight of tobacco Rca

As shown tobacco Rca showed an increase in molecular weight from 156 to 285 kDa over the loading concentrations 0.125 $mg.ml^{-1}$ to 1 $mg.ml^{-1}$. This clearly indicates a concentration dependant relationship. It should also be noted that although a very similar trend was observed for tobacco Rca the molecular weight of spinach was observed to be approximately 90 kDa lower than the equivalent concentration for tobacco Rca.

The SEC data obtained has shown the existence of a concentration dependant effect on Rca from both spinach and tobacco, exhibiting the existence of this relationship across species.

5.4) Determination of molecular weight by SEC-MALS

Molecular weight was also determined using a viscotek which can obtain data on molecular size using a technique SEC-MALS, size exclusion chromatography-multiple angle light scattering. A viscotek utilises multiple angles x ray detectors, low angle light scattering (LALS) and right angle light scattering (RALS).

Although spinach Rca appeared to form non-specific aggregates when left at room temperature data was able to be retrieved utilising ADP supplementation, shown to have the greatest stabilising effect on Rca. Unfortunately apo, Mg2+ and ATP buffer conditions were unable to sufficiently prevent protein aggregation. A range of Rca concentrations were tested in the presence of ADP and although the effect of nucleotide supplementation could not be tested it allowed for an insight into the effect of concentration on the molecular weight of Rca.

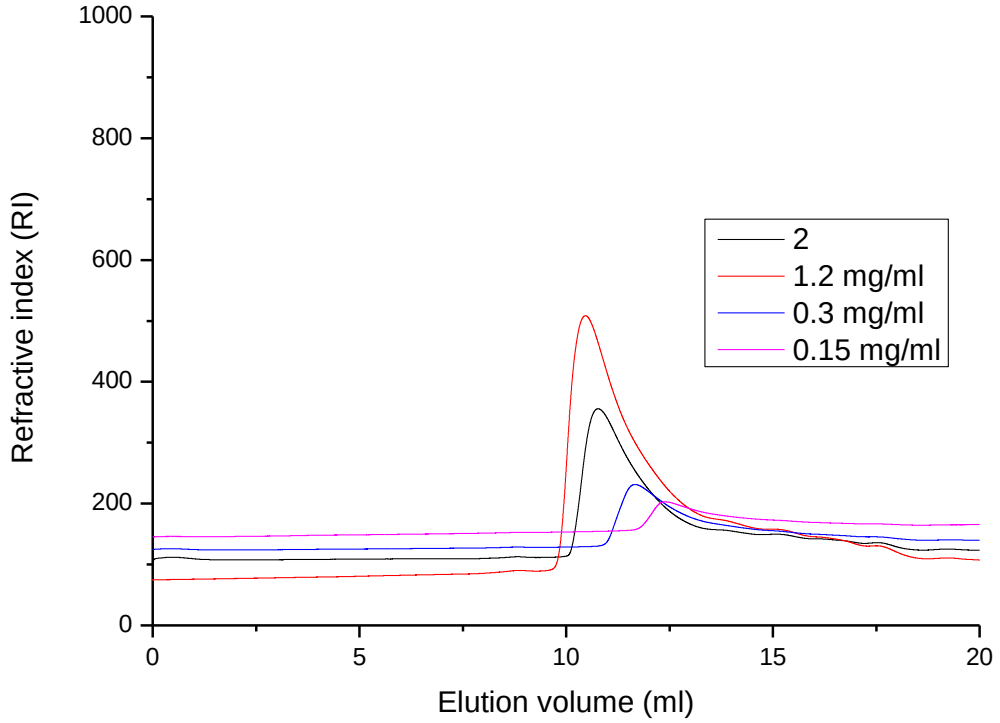

Fig. 5.17) Refractive Index traces of varying Rca concentrations in the prescence of 0.5 mM ADP

For the SEC-MALS a refractive index (RI) detector was used in place of a UV absorbance detector. The RI trace pattern showed significant similarities to that of the UV absorbance pattern obtained through SEC. The RI pattern indicated that the elution volume of the protein increased as the concentration decreased, indicative of a solution whose molecular weight rises as its concentration does.

The viscotek is also able to measure concentration and is able to determine average molecular weights. Using this information we can show how molecular weight changes with concentration. Very high molecular weights were seen in the first few mls of the elution profile. This is either large aggregates in the void volume or due to noise in the receptor. The elution volumes for molecular weight and concentration have been overlayed and the molecular weight is shown to follow the trend of the

concentration. This was seen over the four loading concentrations tested, 4, 2, 1 and 0.5 mg.ml^{-1}.

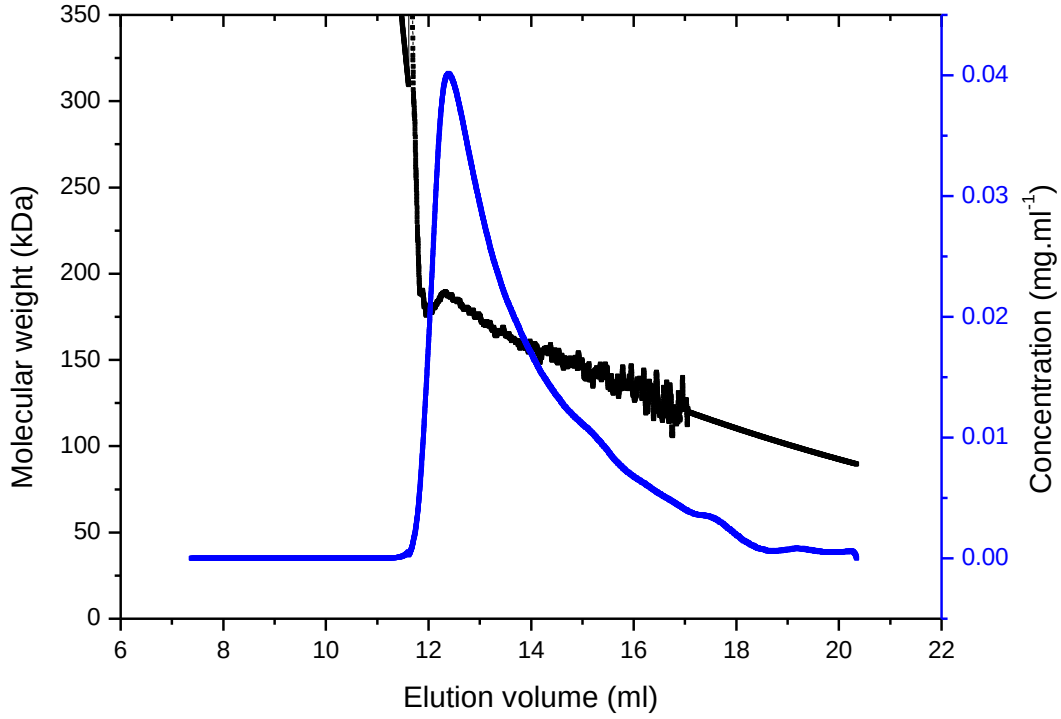

Fig. 5.18) The correlation between molecular weight and concentration of 0.5 mg.ml^{-1}spinach Rca in the presence of 0.5 mM ADP

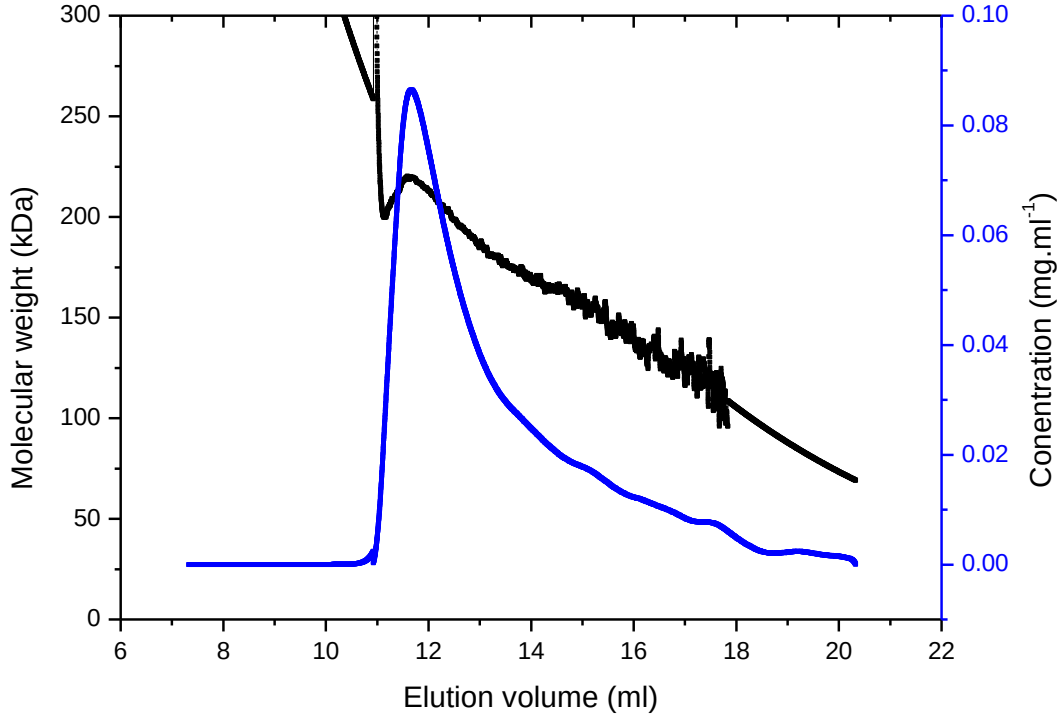

Fig. 5.19) The correlation between molecular weight and concentration of 1 mg.ml^{-1}spinach Rca in the presence of 0.5 mM ADP

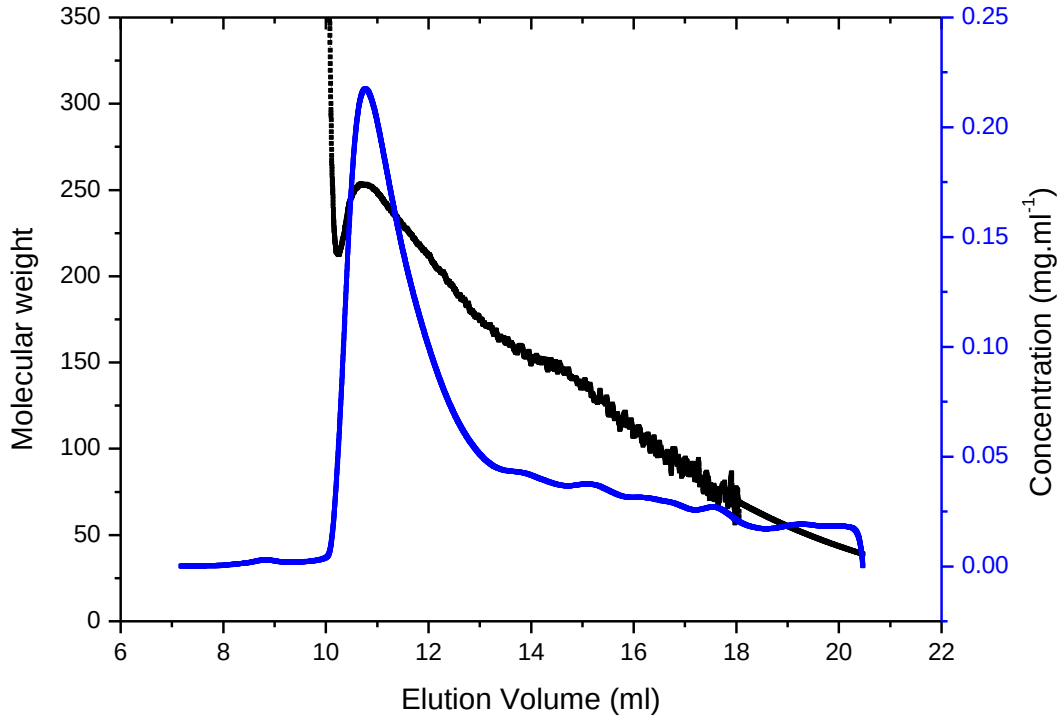

Fig. 5.20) The correlation between molecular weight and concentration of 2 mg.ml^{-1}spinach Rca in the presence of 0.5 mM ADP

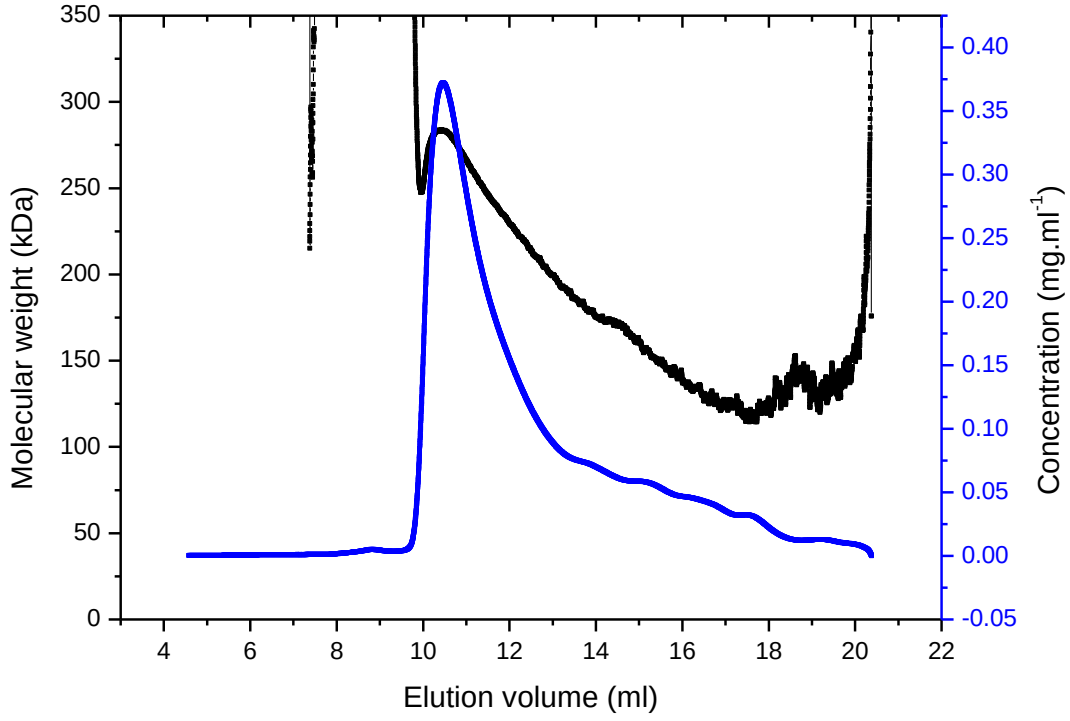

Fig. 5.21) The correlation between molecular weight and concentration of 4 mg.ml^{-1}spinach Rca in the presence of 0.5 mM ADP

It can be seen that the concentration shows a trailing peak and the molecular weight roughly follows the same trend as concentration. This is indicative of the existence of several different oligomeric species with certain oligomers more prevalent than others. It can also be seen that the peak concentration and molecular weight changes between the different loading concentrations. As loading concentration increased so did the apparent concentration as determined by the technique used. The increase in concentration was paralleled by an increase in molecular weight. This is based on a single run as results were difficult to replicate due to the nature of the protein. It can be seen however that a similar trend exists between each loading concentration. An direct relationship can be seen between the concentration and the apparent molecular weight of the protein. A spike in molecular weight can be seen at the same elution volume as the maximum concentration and molecular weight begins to decrease as the

concentration decreases. It can be seen that molecular weights as low as 40 kDa can be observed at lower concentrations indicating the presence of monomers while weights up to 280 kDa, indicating a hexamer, were observed. This clearly indicates a concentration dependent size change in Rca as well as showing that Rca will exist in a series of oligomers within the same solution. The fact that the change in molecular weight is so closely associated with the change in concentration also indicates a very rapid polymerisation/depolymerisation reaction occurring. It should also be noted that although a hexamer was the largest oligomer observed in these tests higher concentrations may yield species with higher oligomeric numbers.

Chapter 6) Discussion

Through the different analytical means used Rca was shown to exist in a series of oligomeric forms, dependant on concentration. This dynamic self-associative property was evident in activity, thermal and sizing experiments. Activity experiments showed that the specific activity of Rca changes based on its concentration ad that Rca is active in a wide range of concentrations. Thermal studies showed that increased concentration and supplementation allowed for increased thermal stability and SEC, DLS and SEC-MALS experiments have shown that the molecular weight of Rca is dependent on concentration and supplementation.

It has been argued that the active oligomer of Rca is a hexamer (Stotz 2011). Concentrations tested during activity experiments can be compared to the concentrations tested under SEC-MALS. This comparison can show the likely prominent oligomer at a particular concentration and determine if activity is seen at the concentration. This indicates the prominent oligomer present at a concentration and whether it is able to undergo work. It is possible that the oligomeric state or stability of Rca may be altered by the column itself.

It is clear that the concentration is closely linked to the molecular weight of the protein and that within a given sample of Rca multiple oligomeric states exist. This can clearly be seen from the data on molecular weight obtained by DLS, SEC and SEC-MALS. Through all SEC experiments a trailing tail was seen on the UV band, typical of Rca. This trailing tail shows that a series of oligomers exist showing a dynamic quaternary structure, based on concentration. Alternatively this may be caused by dilution of the protein, caused by movement through the column, resulting

in rapid loss of monomers to form smaller oligomers. Within individual runs it can be seen that several species of different size exists with a high concentration of a particular molecular weight. The prominent oligomer of Rca can be seen to increase or decrease in respect to the loading concentration. Upon determination of molecular weight in the absence of nucleotide a range of molecular weights from 75 kDa to 170 kDa were observed. This shows that the prominent oligomer is determined by the concentration of the protein and that a series of oligomers exist regardless of the size of the prominent oligomer.

The effect of concentration on oligomeric state is more apparent in the SEC-MALS data. SEC-MALS allows for the determination of several properties, including concentration and molecular weight. This data shows a very close relationship between concentration and molecular weight. The traces show that at the peak concentration of Rca a molecular weight spike is seen. The molecular weight then begins to decrease in relation to the drop in concentration. Again it is difficult to say whether the trailing molecular weights seen is due to the separation of several oligomers in the initial sample or due to a decrease in oligomeric size in response to lowered concentrations after movement through the column. It should be noted that depolymerisation is relatively rapid. This was seen in the DLS work which showed differing molecular weights for different loaded concentration of Rca. As DLS is non-destructive samples were often tested then diluted to a lower concentration. The time in between sample runs was approximately one and a half minutes with time taken to dilute and run system calibrations which allowed enough time for Rca to change its molecular weight based on its new concentration. However the data from SEC-MALS shows that higher loading concentrations caused an increase in molecular weight for a

given concentration, likely due to an increase in the average oligomeric size before injection. It should be noted that the SEC-MALS runs discussed were run with ADP supplementation. Rca is well known to be thermolabile and temperature could not be controlled for SEC-MALS. ADP was shown to cause the greatest increase in thermo-stability and Rca was only able to be explored with this technique under ADP supplementation. Attempts to use this technique without ADP supplementation resulted in the formation of large, inactive aggregates.

DLS gave further evidence for this. DLS allows for the molecular weight to be determined using scattering. This technique assumes that the protein in question is spherical to determine a radial size from which a molecular weight can be calculated. Rca is an a-symmetric non-spherical protein which means that results taken from DLS are not entirely accurate but an approximation. Although the accuracy of the molecular weights given by DLS is not high it does allow for the determination of trends in the molecular weight as the solution is changed. DLS also indicated that molecular weight increased with concentration and the observed trend is similar to that seen by SEC-MALS. Rca is known to be extremely thermolabile with relatively low temperatures causing irreversible aggregation and is thought to be a major factor in the loss of photosynthetic ability due to heat stress (Salvucci and Crafts-Brandner 2004). DLS allows for the effects of temperature to be observed easily. 8 °C, 20 °C and 35 °C were tested for their effects on molecular weight. The data obtained showed an increase in molecular weight from 8 °C to 20 °C. Results obtained from 35 °C showed a polydisperse pattern of high molecular weight aggregates, indicative of aberrant aggregation. This shows the impact of temperature on Rca and the potential for destructive effects of heat on Rca.

The effects of nucleotide and magnesium supplementation on the molecular weight of Rca were explored via SEC and DLS. A significant increase was seen in the molecular weight of Rca upon the addition of both ADP and ATP. Supplementation also allowed testing of lower concentrations .At very low concentration, below 0.3 $mg.ml^{-1}$ the traces obtained by SEC for non-supplemented Rca showed polydisperse patterns with multiple peaks. The addition of nucleotide allowed for more reliable SEC readings, it should be noted that the molecular weight of the lowest supplemented concentration tested, 0.15 $mg.ml^{-1}$, gave similar results to that of non-supplemented Rca at 0.3 $mg.ml^{-1}$. This may have been indicative of a minimum oligomeric state/molecular weight required to obtain reliable results. The results observed by SEC were similar to the results seen by DLS. DLS also indicated that ADP and ATP supplementation caused an increase in molecular weight, with greater increases at lower concentrations. ADP supplementation was also observed to have a greater effect on the oligomeric state of Rca than ATP supplementation, as seen by SEC.

Magnesium supplementation alone showed little effect on the molecular weight of Rca at lower concentrations. ADP was shown to cause a greater increase in molecular weight in comparison to ATP. The difference in molecular weight between the ADP and ATP supplementation increased with concentration with very little difference at 0.15 $mg.ml^{-1}$. This is in contrast to the observation that the difference between the molecular weight of the supplemented Rca and non-supplemented Rca decreases as concentration increases. This may be due to supplementation allowing polymerisation of Rca at low concentrations whereas at higher concentrations polymerisation due to

concentration begins to overshadow the effects from nucleotide supplementation. This means that nucleotide supplementation may be very effective at increasing the molecular weight and activity of Rca at low concentrations yet less effective at concentrations above 0.6 $mg.ml^{-1}$.

Mg^{2+} supplementation in the presence of nucleotide caused a small decrease in molecular weight. This is in contrast to data obtained by Wang *et al.* which showed Mg^{2+} supplementation caused a two fold increase in molecular weight Mg^{2+} has been of spinach Rca when in the presence of ATP. It was also shown that Mg^{2+} had no effect on Rca in the presence of ADP.Mg^{2+} has been previously shown to have a destabilising effect on cottonβ-Rca (Henderson *et al.* 2013).

Activity of Rca was shown to be similar to the results previously reported. The activity observed was slightly lower than previous reports likely due to differences in protein purity. Of particular interest to this thesis is the fact that PEG caused an increase in specific activity. PEG causes an increase in oligomerization which shows that the increase in activity seen upon an increase in concentration is caused by an increase in oligomeric state (Salvucci 1992).

The concentrations tested for activity ranged from 0.01 $mg.ml^{-1}$ to 0.1 $mg.ml^{-1}$. Rca appeared active through all of these concentrations. These concentrations can be compared to those of SEC-MALS to determine a likely molecular weight for a \specific concentration of Rca. At the lowest concentration tested for activity, 0.01 $mg.ml^{-1}$, SEC-Mals shows molecular weights from 75 kDa to125 kDa. This would indicate a dimer or trimer of Rca. At the highest concentration tested for activity, 0.1

$mg.ml^{-1}$, SEC-MALS indicated molecular weights from 130 kDa to around 250 kDa. This would confirm the presence of oligomers from trimers to hexamers at this concentration, dependant on loading concentration. Although it appears the loading concentration of Rca affects the distribution of oligomeric sizes during SEC-MALS it can be seen that a series of oligomers exist. Most importantly at lower concentrations only molecular weights corresponding to dimers and trimers are observed. This would suggest that at the lower tested concentrations the predominant oligomers of Rca present are dimers and trimers yet activity is still observed. It can also be seen that between the highest and lowest concentrations tested by SEC-MALS molecular weights ranging from around 50 kDa to above 300 kDa were observed, indicative of monomers to septamers. Activity was recorded at most of these concentrations, however the higher molecular weights were recorded at concentrations above those tested for activity. Activity was not recorded at the higher concentration due to rapid conversion of the reactants used in the coupled assay which did not allow for an accurate reading of rate. This means that at all concentrations tested by SEC-MALS activity was observed at the corresponding concentrations yet multiple predominant oligomers were observed indicating a dynamic oligomeric state dependant on concentration.

As discussed nucleotide supplementation affects the molecular weight of Rca. Both ADP and ATP supplementation appeared to increase both the molecular weight and thermal stability of Rca, with ADP causing a greater increase in both. The effects of magnesium supplementation on thermal stability were also tested. Magnesium supplementation alone appeared to cause no change in the thermal stability of Rca. The addition of magnesium to Rca supplemented with either ATP or ADP however

caused a destabilising effect. In both cases magnesium caused a significant drop in thermal stability compared to nucleotide supplementation alone. Magnesium and nucleotide supplementation however still caused an increase in thermal stability as compared to non-supplemented Rca.

The effect of ligand binding has been researched for many proteins and as stated the increase in thermal stability of Rca upon nucleotide binding is caused by a restriction of dynamic motions of the protein (Henderson *et al.* 2013, Villaverde *et al.* 1997). It has also been shown that oligomeric state affects both the thermal stability and dynamics of a protein caused by the stiffening of interfacial regions and reduction of dynamics (Marcos *et al.* 2011). This would indicate that the increase in thermal stability of Rca seen upon an increase in concentration is caused by the reduction in protein dynamics due to an increase in oligomeric state. The additional increase in thermal stability after nucleotide supplementation would therefore be caused by an increase in steric hindrance upon the binding of the nucleotides as well as the increase in steric hindrance which is in turn caused by the increased oligomeric state of Rca upon nucleotide binding. Due to the nature of the association it would be difficult to determine how much of the increased thermo-stability was caused by the binding of the nucleotides and how much was caused by the increased oligomeric state due to nucleotide binding.

It has been shown that specific activity of Rca increases with concentration. This is likely due to increased activity upon the creation of higher order oligomers. As nucleotide supplementation has been shown to increase molecular weight it can be assumed that this causes an increase in activity. This is difficult to test as alterations in

the ATP:ADP ratios cause changes in the activity of Rca and increases in activity from oligomerization may be counter acted by decreases in activity due to altered nucleotide ratios (Zhang and Portis 1999). As nucleotide supplementation also increases thermal stability increased nucleotide production could potentially be used to increase the stability of Rca, allowing growth in hotter climates. This could be achieved by increased ATP production however the effects on cellular metabolism would have to be well researched before it could be considered viable.

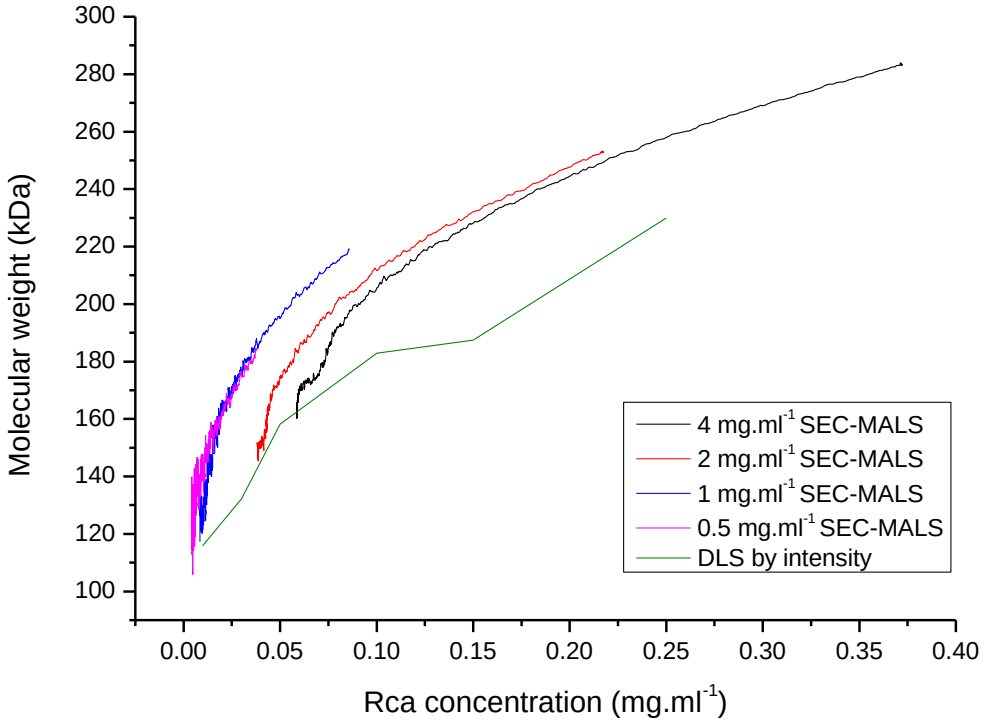

Fig. 6.1) Comparison of molecular weight against concentration from SEC-MALS and DLS

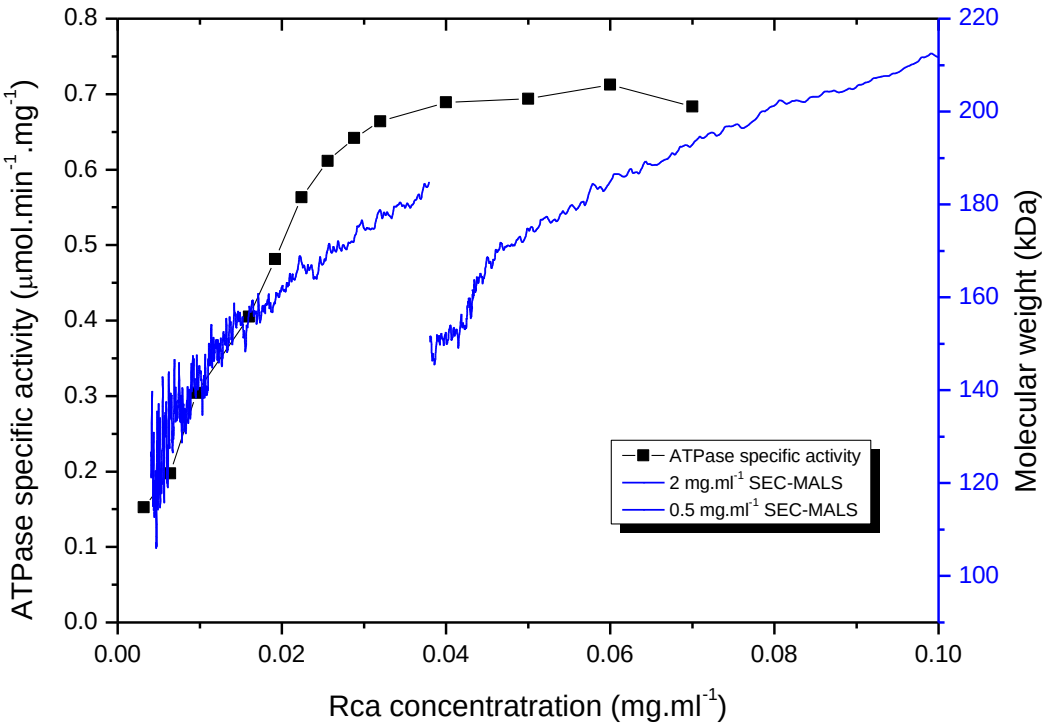

Fig. 6.2) Comparison of molecular weight against concentration from SEC-MALS with ATPase specific activity of spinach Rca

The comparison of sizing data from DLS and SEC-MALS shows good agreement between these numbers up until 0.15 mg.ml^{-1}.Above 0.15 mg.ml^{-1}the techniques show discrepancies in the molecular weight however the concentrations of particular interest are those below 0.1 mg.ml^{-1} as these correspond to concentrations tested for Rca activity. By comparing the activity to the molecular weight data we can determine the oligomeric state required for different levels of activity. 50% activity was observed at 0.02 mg.ml^{-1} with Rca reaching full activity at around 0.03 mg.ml^{-1}. According to SEC-MALS this corresponds to a molecular weight of approximately 140kDa at 50% activity and a molecular weight of approximately 170 kDa at full activity. As β-spinach Rca is a 42 kDa protein it would appear that a dimer-trimer is required for 50% activity while a tetramer is required for full activity. Accuracy of the technique prevents knowing the exact number of subunits required for activity however it would appear that spinach Rca dimers are active. It has been shown that

the active site of Rca exists between two subunits with the arginine finger of one sub-unit interacting with a nucleotide bound to a neighbouring subunit (Wendler *et al.* 2012). As Rca requires two subunits in order to form an active site so a minimum of a dimer for activity is expected. A tetramer may represent the oligomeric state at which maximum cooperation between subunits occurs, causing the plateau in specific activity observed. This is in dispute with recent data suggesting a hexamer is required for Rca activity, however this data was collected from a crystal structure of tobacco Rca (Stotz 2011). The requirement for a 2-4 subunits in order to obtain activity is in agreement with work recently done by Keown et al. (2013) which indicated that tobacco Rca required 2-4 subunits in order to be active (Keown et al. 2013). This again displays inter species similarities between different Rcas.

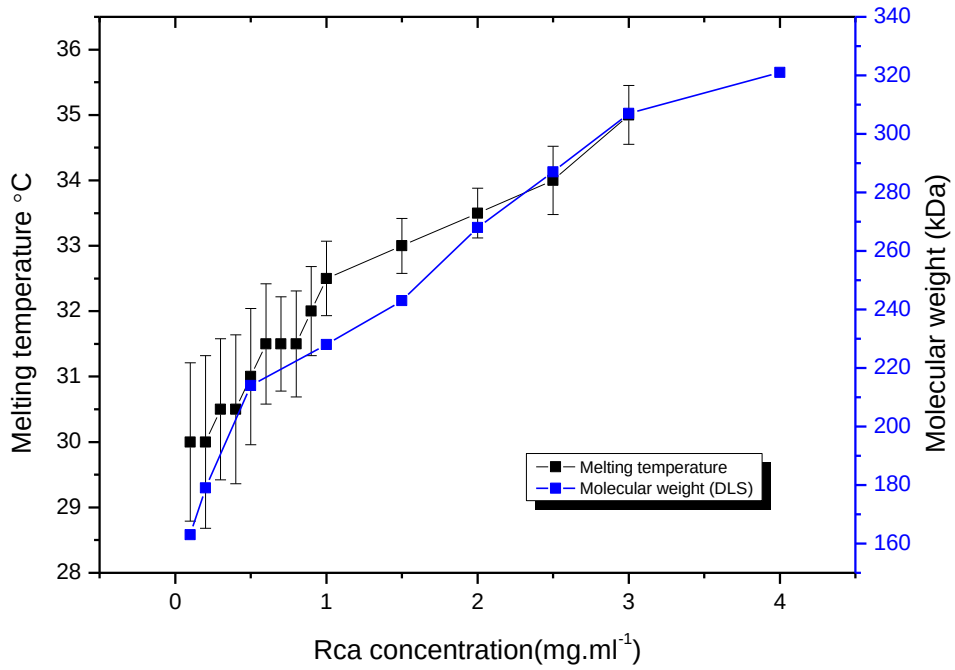

Fig. 6.3) Comparison of thermal stability data from DSF with molecular weight data from DLS

Comparison of thermal stability data to molecular weight data can help gain an insight into how oligomeric state affects thermal stability. It was shown that thermal stability

increased with Rca concentration. The increase in stability was seen to plateau at 3 $mg.ml^{-1}$ with 50% increased stability at 1 $mg.ml^{-1}$. These concentrations can be compared to DLS data which shows molecular weights of 230 kDa at 1 $mg.ml^{-1}$ and 300 kDa at 3 $mg.ml^{-1}$, corresponding to a hexameric and octameric oligomeric state respectively. The pattern of thermal stability observed may be caused by preferential thermal degradation of C- and N-terminal subunits. Increases in oligomeric state have been shown to cause increases I thermal stability due to a reduction in protein flexibility, while this may contribute to the observed stability it does not account for the critical points seen at 1 $mg.ml^{-1}$ and 3 $mg.ml^{-1}$. The likely fully active oligomeric state of spinach Rca has been shown to be a tetramer, this may indicate that the critical oligomeric states for thermal stability observed may be due to the creation of a one/two subunit buffer. Thermal degradation causes increased flexibility in proteins until they lose their tertiary structure. Higher oligomeric states have been shown to cause increased rigidity in proteins due to the creation of bonds (Marcos *et al.* 2011). This means that the likely initial degradation caused by heat would occur at the N- and C- terminal of the protein which lacks the additional rigidity created by oligermisation. The existence of a hexamer and octamer may allow for degradation to occur on the N- and C-terminal while the tetramer, critical for activity, remains relatively protected, indicating a sacrificial system.

This research has shown that Rca most likely exists in a dynamic oligomeric state which shifts with changes in concentration and the presence of ligands. It may allow for exploitation of factors that increase oligomerization and therefore activity and thermal stability which could aid in the growth of plant biomass. SEC-MALS could be used to determine the effects of supplementation on Rca and to determine trends in

molecular weight within different solutions. A temperature controlled environment would have to be used in order to obtain quality data. Further research into how nucleotides are able to confer these advantages would help in establishing a more efficient Rca as more information on the structure and interactions of Rca is elucidated.

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