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PROPERTIES AND SYNTHESIS OF THE RIBULOSE 1,5 BISPHOSPHATE CARBOXYLASE LARGE SUBUNIT BINDING PROTEIN OF HORDEUM VULGARE

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SUMMARY

Previous published data have demonstrated that the large subunit of RuBP carboxylase (LSU) synthesised in isolated *Pisum sativum* (pea) chloroplasts, is associated with a soluble, nuclear-encoded protein. This protein is termed the LSU binding protein (BP) and this observation was confirmed in the present work.

The pea BP has previously been shown to consist of two subunits and work presented in this research project has confirmed this observation. Further research on the pea BP revealed that the BP subunits have different isoelectric points, partial proteolytic digestion products and that both subunits are released upon dissociation by ATP and Mg^{2+} ions. This dissociation is specific to ATP; ADP did not have the same effect.

Analysis of soluble *Hordeum vulgare* (barley) and *Triticum aestivum* (wheat) chloroplast extracts revealed that proteins with a similar molecular mass to the pea BP cross-reacted immunogenically with antiserum raised against the pea BP. This protein was shown to bind newly-synthesised LSU in isolated barley chloroplasts. Antiserum raised against the barley BP cross-reacted monospecifically with both the barley and pea BP.

The barley BP was purified to homogeneity and its physical and chemical characteristics determined. The barley BP was similar to the pea BP in that it consisted of two dissimilar subunits present in equal staining intensities. The barley BP also dissociated to its constituent subunits in the presence of ATP and Mg^{2+} ions. The physical properties of the barley BP differed in several aspects from those of the pea BP. The native and subunit molecular masses of the barley BP were greater than the pea BP and the isoelectric points and partial proteolytic digestion patterns also differ.

The accumulation of the BP during normal leaf development was assayed by rocket immunoelectrophoresis and compared to the accumulation of RuBP carboxylase. Radiolabelling experiments *in vivo* were also carried out using isolated mesophyll protoplasts and these results suggest that the barley BP subunits (as in pea) are nuclear-encoded.

The results presented in this research project are discussed with particular reference to the possible role of the BP in the assembly of RuBP carboxylase.

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Finally a big thanks to Marlies, without whose help I would still be typing this thesis now.

I dedicate this thesis to Wendy and to my family for all their support during my academic career.

DECLARATION

I declare that all of the work in this thesis, except where specifically stated, was original research performed by myself under the supervision of Prof. R.J. Ellis and Dr. A.J. Keys and that none of this work has previously been submitted for any degree. All sources of information have been acknowledged by means of reference.

R. Johnson.

RICHARD JOHNSON

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BP	Large subunit binding protein
BSA	Bovine serum albumin
CTP	Cytidine triphosphate
cpm	Counts per minute
Da	Daltons
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic acid
E	Einstein
EC	Enzyme commission number
EDTA	Ethylenediaminetetraacetic acid
GTP	Guanosine triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
kDa	Kilodaltons
LSU	Large subunit of RuBP carboxylase
mA	Milliampere
MES	4-Morpholine-ethanesulphonic acid
Mr	Relative molecular mass
mRNA	Messenger RNA
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PMSF	Phenylmethylsulphonyl fluoride

RIE	Rocket immunoelectrophoresis
RNA	Ribonucleic acid
RuBP	Ribulose-1,5-bisphosphate
SDS	Sodium dodecyl sulphate
SSU	Small subunit of RuBP carboxylase
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylenediamine
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
UTP	Uridine triphosphate

SECTION ONE INTRODUCTION

1.1. PROPERTIES AND SYNTHESIS OF RuBP CARBOXYLASE

1.1.1. Historical background and significance

The unique function which sets apart the chloroplast from other cellular organelles is its ability to carry out photosynthesis. The term photosynthesis literally means assembly by light and describes the process by which plants synthesise organic compounds from inorganic raw materials in the presence of sunlight.

The major chemical pathway in photosynthesis is the conversion of CO_2 and water to carbohydrate and oxygen. The reaction can be represented very simply by the equation:

$$CO_2 + H_2O \longrightarrow \underset{\text{carbohydrate}}{[CH_2O]} + O_2$$

The fixation pathway of CO_2 to the level of carbohydrate was elucidated from 1946 onwards by Calvin and his co-workers. The reaction mechanisms and experimental techniques are some of the most basic and most important findings in modern plant biochemistry. For his work in elucidating the path of carbon fixation in photosynthesis, Calvin received the Nobel prize for Chemistry in 1961 (for a review of these experiments see Hall and Rao, 1978).

The first step in Calvin's reductive pentose phosphate cycle is catalysed by the enzyme ribulose-1, 5-bisphosphate carboxylase (RuBP carboxylase) EC 4.1.1.39. This enzyme is by far the major soluble protein in plants. The RuBP carboxylase protein is situated in the

chloroplast of higher plants (Ellis, 1981) and has been the subject of more research papers than any other plant enzyme. Two reasons for this interest are (1) its properties determine the relative rates of photosynthetic carbon fixation (Lorimer, 1981; Ellis and Gatenby, 1984) and (2) its synthesis and assembly involves the co-ordination of two distinct genetic systems to produce a multi-subunit enzyme (for review, see Ellis, 1985).

The research work carried out during this research project presented in Section 3 concerns the presence and characterisation of the Hordeum vulgare (barley) RuBP carboxylase large subunit binding protein (BP). The working hypothesis used during this research work was that the BP is involved in the synthesis and assembly of the RuBP carboxylase holoenzyme. Therefore, before discussing the published data concerning the BP, a review of the synthesis, catalytic properties and assembly of the RuBP carboxylase holoenzyme is presented in the remainder of Section 1.1.

The RuBP carboxylase enzyme was first identified by Wildman and Bonner in 1947 who termed it Fraction-1-protein, due primarily to its electrophoretic homogeneity. The RuBP carboxylase enzyme was purified in 1956 by Weissbach et al (1956) who demonstrated that the enzyme catalysed the first step in the Calvin cycle. The enzyme has since been purified to homogeneity from a variety of higher plants (Miziorko and Lorimer, 1983), algae (Iwanij et al, 1974) and cyanobacteria (Torres-Ruiz and McFadden, 1985).

One of the most stricking features of RuBP carboxylase is its sheer abundance. Up to 65% of the soluble proteins in extracts from photosynthetic cells can be accounted for by this single enzyme (Ellis and Gatenby, 1984). The reason for the abundance of RuBP carboxylase may be due to the fact that the enzyme is a sluggish catalyst. At saturating substrate concentrations, the turnover number for carboxylase activity is about 1000 mol of carbon dioxide fixed per mol of enzyme per minute (Ellis and Gatenby, 1984). Under normal atmospheric conditions the turnover drops to about 200 since the enzyme is not saturated at atmospheric concentrations of carbon dioxide (Ellis and Gatenby, 1984). The same low specific activity is found for RuBP carboxylase of both procaryotic and eucaryotic cells, perhaps indicating that constraints on the chemistry of the reaction has prevented the evolution of a more efficient catalyst. A more detailed review of the catalytic properties of RuBP carboxylase is presented in Section 1.1.2.

The carboxylation reaction, producing two molecules of 3-phosphoglycerate from CO_2 and ribulose bisphosphate is interlocked with the first step in the seemingly metabolically wasteful photorespiratory pathway (for review see Keys, 1983). The oxygenase activity of the RuBP carboxylase was first proposed by Ogren and Bowes (1971) based on the observation that oxygen inhibited CO_2 fixation. Photorespiration involves the net loss of carbon as CO_2 from the C2 cycle as well as the subsequent use of energy required for the assimilation of released ammonia.

Evidence is now available to indicate that the net loss of carbon by this cycle is a result of the competition of O_2 with CO_2 at the active site of the RuBP carboxylase enzyme (Lorimer, 1981) and this is reviewed in more detail in Section 1.1.2.

The ratio of carboxylation to oxygenation thus controls the the overall net rate of carbon fixation. The RuBP carboxylase enzyme is thus becoming the focal point for research groups attempting to improve crop yield by increasing the ratio of carboxylation to oxygenation (Somerville and Ogren, 1982; Gutteridge et al, 1984)

Much academic interest in RuBP carboxylase has centered on the synthesis and assembly of the multisubunit RuBP carboxylase holoenzyme. The RuBP carboxylase in higher plants consists of two types of subunit, the large subunit (LSU) which is chloroplast-encoded (Ellis, 1981) and the small subunit (SSU) which is nuclear-encoded (Berry-Lowe et al, 1982). The synthesis and assembly of the RuBP carboxylase holoenzyme has thus provided research workers with an excellent system in which to study the coordinated interaction of two distinct genetic systems to produce a single multisubunit enzyme. The remainder of Section 1.1 will attempt to analyse, in more detail, some of the many research articles covering the enzymic properties, subunit synthesis and the assembly of the RuBP carboxylase holoenzyme.

1.1.2 Enzymic properties.

The enzyme RuBP carboxylase catalyses the first step in the photosynthetic reduction (Calvin) cycle. This involves the carboxylation of ribulose bisphosphate by atmospheric CO_2 to give two molecules of 3-phosphoglycerate (Lorimer, 1981), the first product of the Calvin cycle.

RuBP carboxylase is present in the chloroplasts of higher plants, algae and photosynthetic bacteria. The enzyme usually exists in these groups as a multisubunit holoenzyme consisting of 8 LSU and 8 SSU (Miziorko and Lorimer, 1983) although other combinations do exist in some photosynthetic bacteria (see Section 1.1.3).

The LSU has been shown to contain the catalytic site of carbon fixation. This has been demonstrated by the observation that the catalytically-active *Rhodospirillium rubrum* enzyme contains only LSU (Tabita and McFadden, 1974) and still possesses the ability to fix CO_2. The *Rhodospir llium* LSU also bears the ribulose bisphosphate binding sites and the site of carbamate formation, essential for activity (Christeller and Laing, 1978).

Early attempts to assay the purified RuBP carboxylase enzyme produced *in vitro* rates of carboxylation much too low to account for the rates of photosynthetic carbon fixation observed *in vivo* (Badger and Andrews, 1974). Much of this kinetic data did not take into account the requirement for CO_2 and Mg^{2+} ions to act as activators of both the carboxylase and oxygenase reactions (Jensen and Bahr, 1977). When this activation by CO_2 and Mg^{2+} ions was

taken into account, rates of carboxlation observed *in vitro* could account for rates observed *in vivo* (Jensen and Bahr, 1977).

The activation of the RuBP carboxylase enzyme requires the formation of a carbamate on the ε amino group on lysine 201 of the LSU (Lorimer, 1981). This reaction is followed by the binding of a divalent metal ion, probably to acidic amino acids adjacent to lysine 201, to form a ternary complex that catalyses both carboxylation and oxygenation. The activating CO_2 molecule has been shown not to be the same CO_2 molecule fixed during the carboxylation of ribulose bisphosphate (Lorimer, 1979). Interestingly the sequence of amino acids around lysine 201 has been highly conserved between such species as *Spinacia* (Zurawski *et al*, 1981) through green algae such as *Chlamydomonas* (Dron *et al*, 1982) to the cyanobacterium *Synechococcus* (Reichelt and Delaney, 1983).

Although the mechanism of activation is largely understood, the role it plays in regulating the activity of the enzyme *in vivo* is less clear. The activation of RuBP carboxylase *in vivo* increases upon illumination due to changes in pH and Mg^{2+} ion concentration (Miziorko and Lorimer, 1983). Other physiological factors are also known to regulate the activation step. A report by Salvucci *et al* (1985) identifies a protein in *Arabidopsis thaliana* that may be involved in the activation of RuBP carboxylase. This protein was termed rubisco activase and the partially purified protein was found to restore activation to a mutant plant that lacked light-induced

activation. The diurnal changes in activity of RuBP carboxylase in the leaves of *Phaseolus vulgare* are partially regulated by an endogenous inhibitor that binds tightly to the activated RuBP carboxylase (Berry *et al*, 1987). The diurnal inhibitor has been purified by HPLC and shown to be a phosphorylated compound 2-carboxyarabinitol-1-phosphate, which was shown to bind to RuBP carboxylase *in vitro*.

In all RuBP carboxylase enzymes studied to date the active site has been shown to also catalyse the first step of the photorespiratory pathway. Thus CO_2 and O_2 are both competitive inhibitors and competing substrates, at the same active site, for ribulose bisphosphate. Following activation the ribulose bisphosphate is bound by the RuBP carboxylase enzyme in a form that is susceptible to attack by either CO_2 or O_2. The oxygenation of ribulose bisphosphate produces one molecule each of PGA and 2-phosphoglycollate. The 2-phosphoglycollate is metabolised by the glycollate pathway to yield CO_2 that is released during photorespiration and 3-phosphoglycerate that re-enters the cycle (for review, see Keys 1983). During this pathway ammonia is released and ATP is required during the subsequent reassimilation (Keys, 1983). It has been estimated that up to 50% of the CO_2 fixed may be lost through photorespiration.

The ratio of carboxylation to oxygenation is critical in determining the net rate of photosynthetic carbon fixation (Ogren, 1984). Altering this ratio has been the aim of several research groups interested ultimately in increasing agricultural output (Gutteridge *et al* 1984,

Hartman et al, 1987). It is not known whether the oxygenation pathway is necessary or avoidable. It may be unlikely that the oxygenation reaction could be removed altogether. In all RuBP carboxylase enzymes studied to date the oxygenase reaction has been present, including that of bacterial RuBP carboxylases that are under the most stringent selective pressures (Ellis and Gatenby, 1984). Plants with C4 metabolism, and some algae (Badger et al, 1980), have only managed to reduce losses due to photorespiration by increasing the reaction kinetics to favour carboxylation using CO_2 concentrating mechanisms. It may thus be that the oxygenation reaction is an inevitable consequence of active-site chemistry.

The specificity factor (measuring the ratio of carboxylation to oxygenation) can vary widely between species; if small changes can therefore be engineered by genetic means then the overall effects on crop yield could be significant. To date all attempts to increase the ratio of carboxylation to oxygenation by both mutagenising cells (Somerville and Ogren, 1982) and by site-directed mutagenesis (Gutteridge et al, 1984 Hartman et al, 1987) have failed.

The role of the SSU in the enzymic reaction is not clear. The RuBP carboxylase of Rhodospirillum can exist as an L_2 and in Chromatium vinosum as an L_8 form (Torres-Ruiz and McFadden, 1985); hence the presence of the SSU with the LSU is not strictly obligatory for RuBP carboxylase activity. The removal of SSU of the Synechococcus RuBP carboxylase by mild acid treatment

leaves an L_8 core that is inactive yet soluble. The reconstitution of carboxylase was found to be linear with SSU addition to the L_8 core (Andrews and Ballment, 1983) until almost fully reconstituted activity was recovered. It was therefore postulated that only LSU-SSU pairs were catalytically active.

It has been shown that in the absence of the SSU the formation of carbamate at the activation site and the binding of the reaction state intermediate 2-carboxyarabinitol bisphosphate (2-CABP) still occurs on the L_8 core (Andrews and Ballment, 1984). The effects of the SSU on the activation site may therefore be subtle.

The role of the SSU on the catalytic properties of RuBP carboxylase was studied by Andrews and Lorimer (1985) by analysing the properties of hybrid RuBP carboxylase enzymes. They analysed the catalytic properties of a hybrid RuBP carboxylase assembled in E coli from Synechococcus LSU and Spinacia SSU. The SSU bound an order of magnitude less tightly than the Synechococcus SSU and the specific activity for the heterologous enzyme was about half that of the wild-type enzyme. In addition the Km (CO_2) was twice as high. The degree of partitioning between carboxylation and oxygenation was identical for both the hybrid and wild-type enzymes. Andrews and Lorimer therefore concluded that the catalytic activity was specifically exclusive to the LSU. Experiments with hybrid RuBP carboxylase have also been carried out by van der Vies et al, (1986) who analysed heterologous RuBP carboxylases consisting of the Synechococcus LSU and the

Triticum SSU. The Triticum SSU was found to restore only 10% of the wild-type activity. Again the partitioning between carboxylation and oxygenation was unchanged.

A more specific study on the role of the SSU on RuBP carboxylase catalytic activity was attempted using site-directed mutagenesis on the SSU of Anacystis nidulans expressed with the LSU and assembled in E coli (Voordouw et al. 1987). A single Trp residue at position 54 and 57 of the SSU was replaced with a Phe residue. The Trp residue is highly conserved between species (Voordouw et al, 1987) and the result of these changes was a reduction by 2.5 fold in the Vmax for carboxylation. This demonstrated for the first time that a single amino acid replacement in the non-catalytic SSU influences the catalytic rate of the enzyme. The ratio of carboxylation to oxygenation was not altered however. From these results Voordouw concluded that the SSU was an activating rather than a regulating subunit.

The study of the effects of alterations of the SSU have to date been confined to enzymes containing the LSU of procaryotic RuBP carboxylases. This is because to date all attempts to remove the SSU of higher plant RuBP carboxylase has resulted in the irreversible denaturation of the enzyme (see Section 1.1.4). Attempts to assemble higher plant RuBP carboxylases in E coli have also failed (Bradley et al, 1986) and as a result the effects of site-specific mutations cannot be assayed.

1.1.3 Subunit synthesis

The enzyme RuBP carboxylase is the most widely researched plant protein, indeed it has been referred to as the haemoglobin of plant biochemistry (Ellis and Gatenby 1984). One of the reasons for this interest in the protein is related to the site of synthesis of the higher plant subunits. The RuBP carboxylase of higher plants consists of two types of subunits, the LSU and the SSU. The LSU has been shown to be chloroplast-encoded (Ellis, 1981) whilst the SSU has been shown to be nuclear-encoded (Berry-Lowe *et al*, 1982). The synthesis of RuBP carboxylase thus provides an excellent system in which to study the interaction of two distinct genomes to produce a multisubunit enzyme complex. This section reviews the evidence suggesting the site of synthesis of the two subunits and the control of subunit synthesis.

Since it was first reported in 1962 that the chloroplasts of higher plants contain DNA (Ris and Plaut, 1962) and ribosomes (Lyttleton, 1962) it has become clear that chloroplasts contain all the necessary factors required for protein synthesis (for review see Kirk and Tillney-Bassett, 1978). Much work has since been carried out to determine the products of chloroplast protein synthesis (for review see Ellis, 1981). Several approaches have been used to determine the products, but in some cases the results have failed to be conclusive.

The use of selective inhibitors of protein synthesis *in vivo* has been utilised by several research groups. The observation of the susceptibility of the 70S chloroplast

ribosomes and 80S cytoplasmic ribosomes to chloramphenicol and cycloheximide respectively (Criddle et al, 1970), and the observation that this inhibition is specific, provided the first evidence that the RuBP carboxylase subunits were synthesised on different ribosomes.

The first evidence showing the separate site of synthesis for the two subunits of RuBP carboxylase was shown using greening barley shoots (Criddle et al, 1970). Their results demonstrated that the inhibition of chloroplast protein synthesis by chloramphenicol resulted in an inhibition of the synthesis of the LSU. This inhibition was not observed when the inhibitor of cytoplasmic protein synthesis, cycloheximide, was fed to the young leaves. The reverse result was found with the SSU, therefore Criddle et al concluded that the LSU was synthesised in the chloroplast and the SSU was cytoplasmically-synthesised. These observations were extended to other higher plants including *Pisum sativum* (pea) shoots (Cashmore, 1976), *Glycine* cells (Barraclough and Ellis, 1979) and *Nicotiana* protoplasts (Hirai and Wildman, 1977). In all cases LSU synthesis was inhibited by inhibitors of chloroplast protein synthesis. The use of selectiva inhibitors was not possible using *Spinacia* protoplasts (Nishimura and Akazawa, 1978) and *Chlamydomonas* (Iwanij et al, 1975). In both of these cases the synthesis of LSU and SSU are tightly coupled; inhibition of either of the subunits results in the inhibition of synthesis of both subunits.

The use of inhibitors in these studies has limitations, especially since inhibitors such as chloramphenicol may have a variety of other effects on cellular processes such as ion uptake, oxidative phosphorylation and photophosphorylation (Ellis, 1969). Only if a protein is inhibited at the site of synthesis by one inhibitor but not by inhibitors active at the other site of synthesis is it possible to establish the site of synthesis. The advantage of the use of these inhibitors is that these studies are carried out in vivo so that controls present in whole cells should still be functioning.

Further evidence that the LSU was synthesised on chloroplast ribosomes came from the use of heat-treated plants. The growth of Secale cereale in the light at 32°C produces chlorotic leaves containing plastids that are deficient in plastid ribosomes (Feierabend and Schrader-Reichardt, 1976) which results in the inhibition of chloroplast protein synthesis. Under these conditions the SSU was still synthesised but the LSU was not.

Genetic approaches have also revealed that the LSU is inherited through the maternal line in Nicotiana (Sakano et al. 1974) and Chlamydomonas (Spreitzer and Mets, 1980) indicating that it is cytoplasmically (and not nuclear) encoded. The SSU is inherited in a normal Mendelian fashion, as demonstrated by Kawashima and Wildman (1972) using the inheritance of SSU tryptic peptides. This is evidence that the SSU is nuclear-encoded.

The first direct evidence that the LSU is synthesised and encoded in the chloroplast came from in vitro chloroplast labelling experiments. The first report that the large subunit was synthesised in isolated chloroplasts came from Blair and Ellis (1973) who showed that the LSU was the major product of in vitro chloroplast protein synthesis in pea. The synthesis of the large subunit has also been demonstrated in chloroplasts isolated from Spinacia (Bottomly et al 1974), Euglena (Vasconcelos 1976), Sorghum vulgare (Geetha et al, 1980) and Acetabularia (Green, 1980). The LSU is also synthesised on free ribosomes isolated from pea chloroplasts (Ellis, 1977).

More direct evidence that the LSU is translated from chloroplast RNA has come from in vitro translation of chloroplast RNA in cell-free E.coli systems. The LSU has been synthesised from the chloroplast RNA of Spinacia (Hartley et al, 1975) and Zea (Link and Bogorad, 1980).

Evidence that the LSU is actually encoded on chloroplast DNA has been shown for several species. This was first shown by the linked transcription-translation of Zea chloroplast DNA by E.coli RNA polymerase and rabbit reticulocyte lysate (Coen et al, 1977; Bedbrook et al, 1979). The LSU gene has now been cloned from chloroplast DNA from a wide variety of species including pea (Oishi and Tewari, 1983), Zea (Link et al, 1978), Chlamydomonas (Gelvin et al. 1977) and Euglena (Steigler et al, 1982).

The SSU of RuBP carboxylase is inherited in a normal Mendelian fashion (Kawashima and Wildman, 1972) and its

synthesis _in vivo_ is inhibited by cycloheximide but not chloramphenicol (Criddle _et al_, 1970; Cashmore, 1976; Barraclough and Ellis, 1979). This is evidence that the SSU is nuclear-encoded and synthesised on cytoplasmic ribosomes. Further evidence that the SSU is nuclear-encoded has come from isolated pea nuclei which produce SSU transcripts (Gallagher and Ellis, 1982). These transcripts are polyadenylated and are translated on 80S cytoplasmic ribosomes (Roy _et al_, 1976).

The site of synthesis of the LSU and SSU is less complex in procaryotes due to the lack of compartmentalisation in these organisms. The LSU and SSU of the _Synechococcus_ RuBP carboxylase are cotranscribed as a single transcript, as the two genes are closely linked (Shinozaki and Sugiura, 1985).

The protozoan _Cyanophora paradoxa_ contains intracellular photosynthetic cyanelles containing physiological similarities to higher plant and algal chloroplasts. The RuBP carboxylase of _Cyanophora paradoxa_ is composed of 8 LSU and 8 SSU (Codd and Stewart, 1977) and both the genes for the LSU and SSU are encoded in the cyanelle genome and are thought to be in close proximity (Heinhorst and Shivelly 1983). The cyanelles of _Cyanophora paradoxa_ may thus represent an intermediate stage between procaryotic endosymbionts and photosynthetic cell organelles. A recent report by Reith and Cattalico (1986) has revealed that the chloroplast DNA of the chromophytic alga _Olithodiscus luteus_ contains the sequences for both the LSU, SSU and also the 32 kDa Q_B protein.

The separate site of synthesis of the two subunits of RuBP carboyylase and the imbalance in gene copy number results in the necessity for some method of coordinating the synthesis of the two subunits.

Boffey and Leech (1982) estimated that there are aproximately 150 chloroplasts per cell in Triticum, containing a total of approximately 10,000 copies of the LSU gene per cell. The SSU gene is present in the nucleus as a multigene family. The copy number varies but it is normally between 6-12 copies per cell (Berry-Lowe et al, 1982). A similar calculation for mature pea leaves estimates that there are about 7000 LSU gene copies per cell while the SSU gene is present about 10 copies per cell (Sasaki et al, 1985). Because of this imbalance, and the limiting number of SSU gene copies, it seems likely that control is at the nuclear level. Evidence for nuclear control of RuBP carboxylase synthesis can be seen in pea (Ellis, 1975) where inhibition of 80S ribosomes affects LSU synthesis. The amounts of RuBP carboxylase can also be seen to increase with nuclear ploidy in a series of Triticum (Dean and Leech, 1982c).

The coordinated synthesis of LSU and SSU may be uncoupled over a short period of time. Barraclough and Ellis (1979) demonstrated that LSU synthesis can be uncoupled from SSU synthesis over a few hours in isolated Glycine max cells and Roy et al (1978) demonstrated the presence of unassembled pools of SSU in pea leaves. The effect of α-amanitin on greening pea leaves results in a reduction of SSU mRNA but not of LSU mRNA, indicating that

there is no direct link between nuclear transcription and chloroplast transcription (Sasaki, 1986). There is however a reduction in the accumulation of the LSU; therefore the expression of the LSU may be partly controlled at the post-transcriptional level in pea leaves. Heat shock in Glycine max (Vierling and Kay, 1985) results in a huge drop in SSU synthesis (and drop in SSU mRNA) but there is little change in LSU message level.

These results are in contrast to those observed in Triticum (Dean and Leech, 1982b), barley (Nivison and Stocking, 1983) and Chlamydomonas (Iwanij et al, 1975) where changes in LSU and SSU synthesis are tightly coupled. The SSU of Chlamydomonas, if not assembled, is rapidly degraded (Schmidt and Mishkind, 1983). The protease responsible is nuclear-encoded, therefore nuclear control may still be operating in this case. Pools of SSU have been detected in pea leaves (Roy et al, 1978) but these have a short half-life and are quickly degraded by a protease. Nuclear control in pea leaves may therefore be operating in a similar way as in Chlamydomonas. This result is in contrast to those of Sasaki et al (1985) who reported that pools of SSU are present in pea leaves and are relatively stable.

1.1.4 Assembly of RuBP carboxylase

The assembly of RuBP carboxylase in higher plants requires the coordinated synthesis and assembly of two subunits from different genetic systems. Because of the compartmentalisation of the chloroplast, assembly requires

the synthesis and post-translational transport of one of the subunits (the SSU) into the chloroplast before assembly can take place. This Section reviews the events following the translation of the two subunits up until the assembly of the RuBP carboxylase holoenzyme.

The SSU of RuBP carboxylase is nuclear-encoded and the gene is present in the nuclear genome as a multigene family (Berry-Lowe et al, 1982; Corruzi et al, 1983). The SSU is present in the cytoplasm as a higher molecular weight precursor in Chlamydomonas (Schmidt et al 1979), Glycine max (Berry-Lowe et al 1982), pea (Highfield and Ellis, 1978), Spinacia (Chua and Schmidt, 1978) and Triticum (Broglie et al, 1983). The length of the aminoterminal extension varies, with 55 residues in Glycine, 44 in Chlamydomonas and 47 in Triticum (although a 46 residue aminoterminal precursor was also cloned).

The aminoterminal extension is strongly positively charged (Mishkind et al, 1985) and this may be important for interaction with the strongly negatively charged chloroplast surface (Ishiye et al, 1981). Removal of the aminoterminal extension results in the SSU not being transported into the chloroplast (Ellis and Robinson, 1985). The aminoterminal extension of the SSU can be interchanged between species. Both pea and Spinacia chloroplasts will take up the SSU precursor of Chlamydomonas (Mishkind et al, 1985) although full processing required the presence of the algal peptidase.

The transport of the SSU precursor into the chloroplast occurs post translationally and is different

to the cotranslational transport involving bound ribosomes described by Blobel and Dobberstein (1975). The SSU precursor binds to the chloroplast envelope and specific receptors may be involved in this interaction since the treatment of chloroplast with proteases results in the inhibition of SSU uptake (Chua and Schmidt, 1978; Cline et al, 1985). The import of the SSU precursor into chloroplasts is energy-dependent (Grossman et al, 1980), ATP can be shown to increase SSU precursor uptake by chloroplast suspensions. The same effect is also seen by light stimulation. If chloroplast are pretreated with the uncoupler nigericin, import of the SSU does not occur (Cline et al, 1985) but the SSU precursor does bind to the outer envelope. Uptake can then proceed by adding ATP to the chloroplasts.

Following transport into the chloroplast the SSU precursor is processed in the stromal compartment. The enzymic processing of the SSU precursor was first shown by Dobberstein et al (1977) who demonstrated that processing activity was present in whole cell extracts of Chlamydomonas. Highfield and Ellis (1978) were the first to demonstrate that the SSU precursor could be transported into the intact chloroplasts of pea and was subsequently processed. The processing activity was shown to be present in the soluble stromal compartment, and not bound to the envelope of the chloroplast, by Smith and Ellis (1979).

Attempts have been made to purify the SSU processing activity, column elution results indicating that the processing activity has a Mr of 180, 000 and a pH optimum near 9.0 (Robinson and Ellis, 1984b). As first shown by Grossmann et al (1980)the partially purified processing enzyme does not require ATP to process the SSU precursor. This perhaps indicates that the processing activity is not the energy-dependent step in SSU uptake/processing. The processing event appears to take place in a two step process. The 20 kDa precursor of the pea SSU appears to proceed to the 14 kDa mature product via an 18 kDa intermediate (Robinson and Ellis, 1984a).

The SSU precursor sequence can also direct the transport of foreign polypeptides into the chloroplast. The SSU precursor sequence was used to form a chimeric gene with bacterial neomycin phosphotransferase II (NPT-II) (Van der Brock et al, 1985). The chimeric gene was used to transform Nicotiana cells, and bacterial NPT-II was found to be present in the chloroplasts of these transformed cells. The SSU precursor had been correctly cleaved from the translation product to produce the mature NPT-II polypeptide. In the absence of the SSU transit sequence the NPT-II was shown to be present in the cytoplasm of the transformed cells. By fusing the promoter, first exon and intron, as well as part of the second exon, of the SSU to the aminoterminal end of the NPT-II gene Schrier et al (1985) demonstrated that Nicotiana cells transformed with the chimeric gene not only contained the processed NPT-II within the chloroplast

but that the NPT-II expression was light-inducible.

There is evidence that the LSU of RuBP carboxylase may also be synthesisd as a precursor. Comparison of the amino acid sequence at the amino terminus of the mature barley and *Zea* LSU with the predicted amino acid sequence derived from the the LSU gene indicates that a 13 amino acid sequence is present after the initiation codon that is not present in the mature LSU (Mcintosh *et al*, 1980; Gatenby, 1984). Langridge (1981) has demonstrated that the *in vitro* translation of *Spinacia* chloroplast RNA yields a form of LSU that appears to have a Mr 1-2,000 larger than the mature LSU synthesised in isolated chloroplasts. This precursor was processed to the mature sized LSU by incubating the putative precursor with soluble chloroplast extracts. The role, if any, of the LSU aminoterminal extension in the assembly of RuBP carboxylase is unknown.

The assembly of the RuBP carboxylase holoenzyme has been studied in a variety of systems, from whole leaves (Roy *et al*, 1978) to chloroplast extracts (Milos and Roy, 1984) and in *E. coli* from the expression of cloned gene sequences (Bradley *et al*, 1986).

The study of the assembly of RuBP carboxylase in whole green leaves can only be assayed by radiolabelling techniques. This is because it is not possible to assay changes in RuBP carboxylase amounts over periods of a few hours. Following labelling, the assembly of RuBP carboxylase can be assayed by the co-migration of the labelled RuBP carboxylase with the unlabelled pool of RuBP

carboxylase present in the leaf. This co-migration may be assayed by sucrose gradients (Roy et al, 1978) or by non-denaturing PAGE (Barraclough and Ellis, 1980). Alternatively the assembly of the newly-synthesised LSU and SSU can be assayed by immunoprecipitation with specific antibodies (Sasaki et al, 1985).

The synthesis and assembly of RuBP carboxylase in pea leaves was investigated by Roy et al (1978). Using sucrose gradients to analyse the radiolabelled products of pea seedlings they concluded that pools of unassembled LSU and SSU were present in young pea leaves. These unassembled subunits were quickly assembled into the holoenzyme in pulse-chase experiments. Further work by Sasaki et al (1986) has shown that the amounts of these free subunits are tightly coupled during the development of etiolated pea seedlings upon greening. However the SSU pool was present slightly in excess of the LSU pool.

The assembly of RuBP carboxylase in isolated chloroplasts and chloroplast extracts has been analysed by the radiolabelling of isolated chloroplasts (Blair and Ellis, 1973; Barraclough and Ellis 1980; Milos and Roy 1984) These experiments have led to the observation that the newly synthesised LSU was not immediately assembled into the RuBP carboxylase holoenzyme but was first associated to a protein named the large subunit binding protein (BP). The associated LSU could be released from the BP by the addition of ATP and Mg^{2+} ions and was subsequently assembled into the RuBP carboxylase holoenzyme in soluble chloroplast extracts (Milos and Roy,

1984). The properties of the BP and its possible role in the assembly of RuBP carboxylase is reviewed in Section 1.3.

Attempts to assemble RuBP carboxylase from the dissociated subunits of the higher plant enzyme have failed due to the insolubility of the LSU when it is released from the SSU in the L_8S_8 oligomer (Voordouw et al. 1984). An earlier report by Nishimura and Akazawa (1974) claims that the LSU and SSU separated from the holoenzyme by p-mercuribenzoate will partially reassociate (with up to 37% recovery) by adding 2-mercaptoethanol. Since this result was published this observation has been disputed (Voordouw et al, 1984) and has not been repeated.

The most detailed experimental results concerning the assembly of RuBP carboxylase have been obtained from studying the assembly of RuBP carboxylase subunits from the expression of the cloned gene sequences in E coli. Much of this experimental data has been obtained from the expression of the LSU and SSU of Synechococcus. The results obtained have produced a useful model to predict the steps in the assembly of the active cyanobacterial enzyme.

The LSU of Synechococcus 6301 was expressed in E.coli by Van der Vies et al (1986). In the absence of the expression of the SSU the LSU was seen to form a soluble L_8 core that was enzymatically inactive. However approximately 20% of the LSU was present in the E.coli in an L_1 or L_2 form with the remainder in the L_8 form. When the SSU is also expressed in E.coli on a separate M13

phage no L_1 or L_2 forms were seen in the E.coli extracts and a range of L_8S_{2-5} oligomers were formed. The degree of saturation of the oligomer with SSU was dependant upon the rate of expression of the SSU vector. When the LSU and the SSU were cotranscribed from the same plasmid active L_8S_8 was formed (Gatenby et al, 1985).

Experiments by Guervitz et al (1985) demonstrated that the LSU of Anabaena was expressed in E.coli but in an excess to the SSU due to premature termination within the intergenic region. From these results they published a model describing the assembly of RuBP carboxylase where the excess LSU was insoluble, the role of the SSU thus being to keep the LSU in a soluble form and to initiate assembly via heterodimers of LSU and SSU. These results were not supported by the findings of Van der Vies et al (1986) who demonstrated that the L_8 core formed in the absence of the SSU was soluble. The result of Van der Vies et al (1986) demonstrates that the Synechococcus LSU and SSU do not need to be cotranscribed for assembly to occcur. The SSU is also not needed for the formation of soluble LSU since both the L_{1-2} and the L_8 molecules are soluble. The SSU was however absolutely necessary for RuBP carboxylase activity in E.coli.

The assembly of RuBP carboxylase in E.coli provides an excellent system in which to study the effects of site-specific mutation on the enzymic properties of the enzyme. These types of experiment have previously been carried out on the L_2 Rhodospirill um RuBP carboxylase expressed in E.coli (Gutteridge et al, 1984). The effects of the site-

specific amino acid change were a reduction in the rate of carboxylation although the ratio of carboxylation to oxygenation remained constant. Other approaches to the study of RuBP carboxylase activity in E.coli have centred on the role of the SSU in catalysis. Site-directed mutagenesis of the conserved amino acids of the SSU of the Synechococcus SSU expressed in E.coli with the LSU (Voordouw et al, 1987) produced an RuBP carboxylase enzyme with lowered catalytic activity. The effects of these site-specific mutations and also the assembly of RuBP carboxylase from subunits of different species are reviewed in Section 1.1.2.

To date attempts to assemble the RuBP carboxylase of higher plants such as Triticum (Bradley et al, 1986) in E.coli have failed. The LSU of Zea is synthesised in E.coli along with the SSU but was found to be insoluble and as a result was neither assembled nor showed any catalytic activity. The expression of the LSU and the SSU of Triticum in E.coli produced a LSU that was present in the soluble fraction of the cell but was inactive. Closer examination revealed that the LSU was present as a high molecular weight aggregate that was soluble but possessed no associated SSU.

The assembly process in Synechococcus is likely to be much more simple than in higher plants mainly due to the lack of compartmentalisation within these cells. The SSU gene is located downstream from the LSU gene and it appears that the two subunits are cotranscribed as a single mRNA (Shinozaki and Siguira, 1985). This solves

the problem associated with the coordination of synthesis of the two subunits. Another simplification in this system is the lack of the SSU processing pathway. The SSU of Synechococcus is not synthesised as a precursor since it does not have to cross the chloroplast envelope. The assembly of the higher plant RuBP carboxylase is much more complex however. As reviewed earlier in this section the two subunits are encoded on different genetic systems and one of the subunits (the SSU) is synthesised as a precursor which is subsequently processed in the soluble compartment of the chloroplast. There is also evidence that the LSU may also be synthesised as a precursor (Langridge, 1981).

The complexity of the assembly process in higher plant cells may mean that simply expressing the two genes for the RuBP carboxylase enzyme in E.coli will not produce a correctly assembled RuBP carboxylase. Another reason for this lack of assembly may be the requirement for another protein in the assembly process. This protein is the large subunit binding protein (BP) and its properties and suggested role in the assembly of the RuBP carboxylase of higher plants is discussed in Section 1.3. To date there is no direct evidence available to prove that the BP is a necessary factor in the assembly of higher plant RuBP carboxylase. If future experiments show the necessity of the BP in the assembly of the higher plant RuBP carboxylase then it will prove necessary to jointly express these genes in E.coli if the assembly of RuBP carboxylase is to occur.

1.2 THE DEVELOPMENT OF MONOCOTYLEDONOUS LEAVES

1.2.1 The development of photosynthetic capacity in monocotyledonous leaves

The study of the development of photosynthetic capacity during normal leaf development is experimentally difficult in dicotyledonous leaves. This is because the dicotyledonous leaf consists of a mosaic of cells that are both morphologically and functionally different and are also at different stages of cellular development. An example of the extremely complex cellular growth pattern in dicotyledonous leaves is the developing Xanthium leaf (Maksymowch, 1973) whose development is both complex and heterogenous.

In an attempt to synchronise the events of cellular development several groups have studied the greening of etiolated seedlings (for review see Tobin and Silverthorne, 1985). One species that has been used in particular during these studies are etiolated pea seedlings which have been utilised by many research groups. This is because etiolated pea seedlings show a large developmental response upon illumination (Lennox and Ellis 1986) One problem with using such a system however is that the results obtained may not correctly mirror the events that take place during normal seedling development. For example the route of development of proplastids during normal leaf development may be completely different from the developmental pathway of etioplasts. These etioplasts develop in the dark and prior to illumination already

possess a complex internal membrane system (Leech and Baker, 1983).

Studies on the greening of etiolated monocotyledonous plants have been carried out but in many cases the changes in cellular components are not as marked as those observed in dicotyledonous plants such as pea. For example, the growth of barley seedlings is only marginally inhibited by darkness and the leaf elongation continues in the dark (Klein and Mullet, 1987). As a result of this relatively small photoresponse the use of graminaceous seedlings such as barley in these greening studies does not provide such a good system to work with.

Although light has only a limited effect on the accumulation of RuBP carboxylase in barley leaves a phytochrome-type response has been demonstrated for a limited set of polypeptides. Work with isolated barley nuclei has shown that the synthesis of mRNA encoding the light-harvesting chlorophyll a/b protein (LHCP) shows a classical phytochrome response to illumination with red and far red light (Mosinger et al, 1985). The effect of red light on transcription in isolated barley nuclei can also have a negative effect. For example the synthesis of mRNA encoding the protochlorophyllide oxidoreductase is inhibited by red light and enhanced by far-red light (Mosinger et al, 1985).

The leaves of monocotyledonous plants, due to their morphology, present a useful system in which to study the development of photosynthetic capacity under normal growth conditions. In monocotyledonous leaves such as barley,

Triticum, and Zea, cell division is restricted to a basal meristem; hence each leaf provides a gradient of cellular development with the youngest cells at the base of the leaf and the oldest cells at the tip of the leaf (Boffey et al, 1979). Slices of these leaves, sectioned at right angles to the long axis, contain cells and organelles of increasing age and maturity so that development can be investigated and related to cell age. Because of this morphology developing leaves have been studied from Triticum (Dean and Leech, 1982abc), barley (Viro and Kloppstech, 1980) and Zea (Mayfield and Taylor, 1984).

The remainder of Section 1.2 will be divided into two sections. This section will review the development of cellular and plastid morphology in monocotyledonous leaves. Section 1.2.2 will focus in particular on the accumulation of RuBP carboxylase during leaf development and also review the synthesis of RuBP carboxylase in isolated leaf cells and protoplasts.

In young meristematic leaf cells of monocotyledonous plants proplastids which will eventually form chloroplasts are present but are tiny, with a diameter of 1µm. They are pleiomorphic but often spherical and are bounded by a double membrane. These proplastids possess few internal membranes and only traces of chlorophyll. The ultrastructural changes which occur during chloroplast development have been studied in several developing monocotyledonous leaves but by far the most thorough research has been carried out on developing Triticum leaves (Dean and Leech, 1982abc).

All the major changes that occur during the development of proplastids occur after cell division has ceased. The developing plastids accumulate several round starch grains which they lose as they pass through the amoeboid stage. The thylakoid system in developing chloroplasts is formed by the extensive formation of new membranes and subsequent foldback/stacking to form the characteristic granal fretwork system. This stacking requires continuos illumination (Strasser and Butler, 1976).

The later stages of chloroplast development involve large increases in plastid size and in the size and number of the grana. At a stage in Triticum development (in cells that are two days old) when the number of 70S ribosomes per chloroplast is decreasing, the number of 70S ribosomes per cell is actually increasing. This is due to a second phase of plastid replication that is occurring at this time. During this phase the plastid complement per cell increases at least 3-4 fold (Possingham, 1980), although the chloroplasts have only grown to half their final size. The number of plastid division cycles is the major factor in determining chloroplast number per cell in the the mature leaf.

The mean number of chloroplasts per cell appears to be species-specific and is probably under nuclear control. For example Triticum hybrids show chloroplast numbers that reflect the ploidy level of the nucleus (Dean and Leech, 1982c).

During the structural development of the chloroplast in monocotyledonous leaves there is a concomitant development of photosynthetic function. This development of photosynthetic activity requires not only the synthesis of constituent molecules of the photosynthetic apparatus but also posttranslational modification and assembly of soluble and membrane-bound macromolecular complexes. The following section reviews in detail the accumulation of the major soluble chloroplast protein, the enzyme RuBP carboxylase.

1.2.2 The synthesis and accumulation of RuBP carboxylase during monocotyledonous leaf development

The synthesis and accumulation of RuBP carboxylase has been studied in a variety of plant species during normal leaf development and during the greening of etiolated tissue (for review see Tobin and Silverthorne, 1985).

As reviewed in the previous section the greening of etiolated graminaceous seedlings does not present an ideal system in which to study the accumulation of RuBP carboxylase. For example the etioplasts of *Hordeum* synthesise and accumulate nearly all of the soluble polypeptides of mature chloroplasts of light-grown seedlings (Klein and Mullet,1987). The morphology of monocotyledonous leaves does however provide a useful system in which to study the accumulation of RuBP carboxylase during normal leaf development.

The accumulation of RuBP carboxylase on a per cell basis appears to be under nuclear control. This has been demonstrated by analysing the effects of nuclear ploidy on RuBP carboxylase content of mesophyll cells in a non-isogenic polyploid series of Triticum (Dean and Leech, 1982c). The most striking relationship was the constant ratio found between the nuclear DNA content per mesophyll cell and the RuBP carboxylase content per mesophyll cell. The RuBP carboxylase content per cell was seen to increase in step with each increase in nuclear ploidy. These results suggest that the effect of nuclear ploidy may be to increase the dosage of the SSU gene which in turn leads to increased RuBP carboxylase accumulation. It is equally possible however that an increase in copy number of some other nuclear-encoded protein is causing the observed increase in RuBP carboxylase accumulation.

The accumulation of RuBP carboxylase has been analysed in great detail in developing Triticum leaves (Dean and Leech, 1982b). Changes in genome expression during normal cellular and plastid development were investigated by analysing homogeneous populations of leaf cells and plastids in serial sections. The results were expressed in relation to the age of the cells in the leaf section. The mesophyll cell number was estimated by counting cells after chromate treatment of leaf sections. Plastid number was also estimated microscopically in these preparations. By expressing the amounts of RuBP carboxylase on a per cell or per plastid basis the changing characteristics of the cell and chloroplast can

be taken into account. The accumulation of RuBP carboxylase per cell was found to increase 20-fold from 15 to 60 hours after cell division but the most rapid phase of RuBP carboxylase accumulation occured after chloroplast division had ceased 36 hours after cell division. Whilst there is an increase in RuBP carboxylase in serial sections from the base to the tip of the Triticum leaf, not all soluble proteins show this type of developmental pattern. For example the PEP carboxylase involved in C4 metabolism is present in small amounts in the base of the leaf and hardly changes in amounts from the base to the tip of the leaf (Aoyagi and Bassham, 1986).

In both Triticum leaves (Dean and Leech, 1982b) and barley leaves (Viro and Kloppstech, 1980), RuBP carboxylase is present at the base of the leaf and increases during cellular development. The accumulation of RuBP carboxylase in serial sections of the developing Zea leaf follows a different pattern. This is related to the development of the Kranz anatomy in the Zea leaf which undertakes C4 metabolism. In the early regions of the third leaf there are no detectable amounts of RuBP carboxylase as detected by immunoblotting (Mayfield ad Taylor, 1984). These immunoblots revealed that RuBP carboxylase was present only in leaf sections that had developed bundle sheath cells (ie Kranz anatomy), as was PEP carboxylase (Mayfield and Taylor, 1984). Although RuBP carboxylase was not present in these young leaf sections, LHCP and ATP-generating proteins were present, indicating that mesophyll cells develop light-harvesting

capacity prior to the onset of CO_2 fixation. The results of Mayfield and Taylor were in contrast to those of Williams and Kennedy (1978) who found detectable levels of RuBP carboxylase and PEP carboxylase activity in basal leaf sections. The reasons for this difference may be that at low levels of accumulation the enzymic activity can be detected but the immunoblot fails to detect these low levels.

Further work by Martineau and Taylor (1985) analysed the nature of mRNA in serial leaf sections of Zea as used by Mayfield and Taylor. By hybridisation of cDNA probes they demonstated that mRNA to the LSU and SSU accumulated in young leaf sections prior to bundle sheath formation. Although up to 60% of the maximum mRNA levels was detected in these young leaf sections neither polypeptide was detected. This lack of synthesis was not due to failure of mRNA to form polysomal complexes; hence Martineau and Taylor concluded that some other posttranscriptional control was operating to control RuBP carboxylase accumulation in developing Zea leaves. They postulated that a similar posttranslational control may be operating as observed in Chlamydomonas inwhich unassembled SSU where rapidly degraded (Schmidt and Mishkind, 1983).

Experiments analysing the synthesis of the LSU and the SSU during the development of monocotyledonous leaves were reported by Dean and Leech (1982a). The first leaves of 7-day-old Triticum seedlings were radiolabelled with [^{35}S]-methionine. The incorporation into the LSU and SSU was assayed in serial leaf sections and correlated with

the presence of translatable mRNA for the two subunits in identical leaf sections. Their results demonstrated that the synthesis of the LSU and SSU throughout leaf development in _Triticum_ was tightly coupled. This included a reproducible burst in synthesis in cells that were 35 hours old. Translation of mRNA from identical sections in reticulocyte-lysates suggested that changes in the synthesis of the two subunits could be accounted for by changes in the mRNA levels.

Similar results were found by Nivison and Stocking (1983) who performed similar experiments in developing barley leaves. Rather than radiolabelling whole leaves however they incubated leaf discs taken from serial leaf sections above the leaf base. They observed that the synthesis of the LSU and SSU reached a peak in the mid leaf region and then declined. During these fluctuations however the rates of synthesis of the LSU and SSU remained tightly coupled.

The remainder of this section will now review some of the published data obtained on the synthesis of RuBP carboxylase in isolated cells and protoplasts. The use of isolated cells and protoplasts in these studies has several advantages and some disadvantages. The main disadvantages are that the isolation of cells and protoplasts requires the complete disruption of cellular tissue. Subsequent labelling experiments are carried out in media that provide an environment that is foreign to the cells. Under such conditions the normal cellular controls affecting the synthesis and accumulation of RuBP

carboxylase may be disrupted. The use of isolated cells and protoplasts does have advantages over whole leaves. For example samples can be taken during time course experiments and these cells rapidly take up both radio labelled amino acids and inhibitors of protein synthesis. Due to the ability to pipette isolated cells and protoplasts, identical, homogeneous samples can be taken at different time points.

The synthesis of RuBP carboxylase in isolated cells has been studied by Barraclough and Ellis (1979) who analysed the accumulation of radioabelled LSU and SSU in isolated Glycine cells. Newly-synthesised LSU and SSU was assembled into RuBP carboxylase, assembly being monitored by the co-migration of radiolabelled subunits with the RuBP carboxylase on non-denaturing polyacrylamide gels. The assembly of LSU and SSU into RuBP carboxylase was estimated by separating the two subunits in a second dimension of SDS PAGE followed by scintillation counting of the excised bands. Their results indicated that the synthesis of the two subunits were coordinated during the synthesis of RuBP carboxylase in isolated Glycine cells over a period of four hours. In the presence of cycloheximide the synthesis of the SSU was inhibited, as expected. The LSU however was synthesised for up to four hours after SSU synthesis had ceased. This result demonstrates that uncoupling of synthesis of the LSU and SSU can occur in isolated Glycine cells over a few hours. This result is in contrast to those of Nivison and Stocking (1983) who observed that the synthesis of the LSU

and SSU in barley leaf discs is tightly coupled.

A tight coupling of LSU and SSU synthesis has been observed in isolated protoplasts. Experiments by Nishimura and Akazawa (1978) on the uptake of [^{14}C]-leucine by isolated Spinacea protoplasts revealed that these protoplasts synthesised both the LSU and the SSU. In the presence of either cycloheximide (inhibitor of 80S ribosomes) or chloramphenicol (inhibitor of 70S ribosomes) the synthesis of both subunits of RuBP carboxylase was inhibited. From these results they concluded that the synthesis of the subunits of RuBP carboxylase was tightly coupled in vivo. Inhibition of synthesis of either subunit resulted in the non-synthesis of the other subunit.

Similar results were obtained by Hirai and Wildman (1977) working with Nicotiana protoplasts, but the synthesis of the two subunits was not as tightly coupled. Their results demonstrated that following inhibition of synthesis of the SSU with cycloheximide there was a lag of 90 minutes prior to inhibition of RuBP carboxylase synthesis. This compares to a 30 minute lag before inhibition of synthesis by chloramphenicol. From these results Hirai and Wildman postulated that although a pool of SSU existed in isolated protoplasts, a pool of unassembled LSU did not exist.

1.3 THE RuBP CARBOXYLASE LARGE SUBUNIT BINDING PROTEIN

1.3.1 Historical background and significance

Analysis of the products of *in vitro* chloroplast protein synthesis has shown that by far the major labelled soluble product is the LSU of RuBP carboxylase (Ellis, 1981). Early attempts to analyse the fate of the newly-synthesised LSU in isolated pea chloroplasts by non-denaturing PAGE revealed that the LSU was not assembled into the RuBP carboxylase holoenzyme but migrated with a different mobility (Blair and Ellis, 1973). Bottomley *et al* (1974), working with isolated *Spinacia* chloroplasts, reported that the newly synthesised LSU was assembled into RuBP carboxylase. This report was disputed by Ellis (1977) who repeated these experiments under a range of non denaturing gel concentrations and demonstrated that the co-migration of the LSU with the RuBP carboxylase was fortuitous. It was originally thought that this slowly-migrating radiolabelled band on the 4% non-denaturing gel (which co-migrated with a major-staining band) represented an aggregated form of unassembled LSU (Ellis, 1977).

When isolated, intact *Pisum* chloroplasts were incubated in a medium containing 0.33 M sorbitol as osmoticum rather than the high KCl osmoticum, used by Blair and Ellis (1973), some of the newly-synthesised LSU comigrated with the pool of unlabelled RuBP carboxylase holoenzyme (Barraclough and Ellis, 1980). The majority of the newly synthesised LSU still migrated on the 5% non-denaturing gel with a slower mobility than the RuBP

carboxylase. This pool of newly-synthesised LSU was not precipitated by antiserum to the RuBP carboxylase holoenzyme (Barraclough and Ellis, 1980). With prolonged incubation periods, after protein synthesis had diminished, more of the labelled LSU was precipitated by antiserum to the RuBP carboxylase. This increased comigration of labelled LSU with RuBP carboxylase was coupled with a corrresponding decrease in the slowly migrating pool of labelled LSU. It was not possible to determine whether this was a precursor-product type relationship over time-course experiments because other pools of newly-synthesised LSU may exist that were not resolved on the gel. For example large pools of radioactive material were seen to be trapped at the top of the gel (Barraclough and Ellis, 1980).

When the slowly-migrating band, containing the radiolabelled pool of unassembled LSU, was excised from the non-denaturing gel and analysed on an SDS-denaturing gel an interesting observation was made (Barraclough and Ellis, 1980). It was observed that the radiolabelled band migrated with the same mobility as the LSU of RuBP carboxylase as expected. The labelled band did not however migrate with the Coomassie-stained band that made up the majority of the slowly-migrating aggregate. It was thus concluded by Barraclough and Ellis that the majority of the newly-synthesised LSU resolved on the 5% non denaturing gel is associated with another protein with a Mr of 720, 000. This protein was not radiolabelled in isolated pea chloroplasts and was present in much greater

quantities than the pool of LSU associated with the protein. Barraclough and Ellis postulated that the protein may be involved in the assembly of RuBP carboxylase and because of its properties it was named the large subunit binding protein (BP) (Ellis et al, 1980).

The first observations made by Barraclough and Ellis on the BP present in pea chloroplasts were made by analysing the products of in vitro chloroplast protein synthesis on single-concentration non-denaturing gels. Another approach that led to confirmation that a pool of LSU was associated with the BP in both isolated chloroplasts and whole seedlings of pea was used by Roy et al. (1982). The radiolabelled products of both isolated chloroplasts and whole leaves of pea were analysed on sucrose density gradients. These results demonstrated that after a 30 minute radiolabelling period isolated chloroplasts contained two pools of newly-synthesised LSU. These were identified as a pool of free LSU sedimenting near the top of the gradient and a pool of LSU sedimenting faster through the gradient than the RuBP carboxylase. This faster sedimenting pool of newly-synthesised LSU was associated with the BP, first identified by Barraclough and Ellis (1980) on non denaturing gels. This pool of radiolabelled LSU associated with the BP was also found to be present in chloroplasts isolated from radiolabelled whole leaves.

It was first suggested in 1980 that the BP may play a role in the synthesis and assembly of RuBP carboxylase (Barraclough and Ellis, 1980). As reviewed earlier in

Section 1.1 the catalytic properties of RuBP carboxylase are central to determining the rate of photosynthetic carbon fixation. Much work is now being carried out in an attempt to alter the kinetic properties of RuBP carboxylase in order to increase the net rate of carbon fixation. If the BP of higher plants is involved in the synthesis and assembly of RuBP carboxylase then it will be necessary to express the BP in any system designed to assay the catalytic activity of assembled higher plant RuBP carboxylase. To date all attempts to assemble the higher plant RuBP carboxylase in the absence of the BP have failed (Bradley et al, 1986).

The remainder of Section 1.3 will review the published data concerning the properties and synthesis of the BP and further review the available data suggesting a possible role for the BP in RuBP carboxylase assembly.

1.3.2 Properties and synthesis of the RuBP carboxylase large subunit binding protein.

The observation that the newly-synthesised LSU was not immediately assembled into RuBP carboxylase in isolated chloroplasts was first made by Blair and Ellis (1973). It was demonstrated by Barraclough and Ellis in 1980 that the newly-synthesised LSU was associated with an abundant chloroplast protein, the BP. This section reviews the physical and chemical properties of the BP. All the published data to date is produced for the BP of pea.

The BP was first reported to have a subunit molecular mass of 60 kDa by Barraclough and Ellis (1980). Analysis of the BP by SDS PAGE with a low bisacrylamide to acrylamide ratio results in a greater resolution of higher molecular mass proteins. These polyacrylamide gels revealed that the BP was composed of two subunits (Hemmingsen and Ellis, 1986). The slowest migrating subunit was termed the α subunit and has a molecular mass of 61 kDa. The fastest migrating subunit, the β subunit, has a molecular mass of 60 kDa (Hemmingsen and Ellis, 1986).

The native molecular mass of the BP was estimated at 720 kDa by gel filtration (Hemmingsen and Ellis, 1986). Because of the equal staining intensities of the two bands and their co-purification it was estimated that the native BP had the subunit composition $\alpha_6\beta_6$ (Musgrove and Ellis, 1986). This estimated molecular mass does not take into account the possibility of one or more LSU being associated with the BP and affecting the native molecular mass.

The observation that the BP was not labelled during *in vitro* labelling experiments with isolated chloroplasts (Barraclough and Ellis, 1980) was the first published evidence that the BP may be cytoplasmically-synthesised. It is not conclusive evidence however. Another interpretation could be that the BP is chloroplast-encoded but is not synthesised (or is rapidly degraded) in the absence of cytoplasmic protein synthesis. A more unlikely explanation is that neither of the BP subunits contain a

methionine residue which is used in the radiolabelling experiment.

More direct evidence that the BP subunits are cytoplasmically-synthesised was obtained by the immunoprecipitation of the *in vitro* translated products of pea polysomes (Hemmingsen and Ellis, 1986). Antiserum raised against the two BP subunits immunoprecipitated a single polypeptide synthesised in a wheatgerm translation system. The polypeptide had a larger molecular mass than the mature BP subunits suggesting that the BP subunits are synthesised in the cytoplasm as a larger precursor prior to post translational transport. Because the two BP subunits are known to exist as distinct polypeptides that have different amino terminal extensions it was suggested by Musgrove *et al* (1987) that the two BP subunits are synthesised as precursors with the same molecular mass.

The synthesis of the BP has been shown to be stimulated by light during the greening of etiolated pea seedlings (Lennox and Ellis, 1986). The synthesis of RuBP carboxylase in pea seedlings has previously been shown to be greatly stimulated by light acting on the level of transcription (Gallagher and Ellis, 1982). Rocket immunoelectrophoresis revealed that etiolated pea seedlings contained low but detectable amounts of both RuBP carboxylase and BP. When the etiolated seedlings were exposed to light the increase in RuBP carboxylase was greater than the increase in accumulation of the BP. Comparison of etiolated seedlings with seedlings grown under a 12 hour photoperiod shows that the content of RuBP

carboxylase is increased 30-fold in the light compared to a 7-fold increase in the BP (Lennox and Ellis, 1986). The expression of the genes for the BP are thus not subject to the same degree of photoregulation as the genes for RuBP carboxylase.

The only published chemical property of the BP to date is its reversible dissociation by ATP and Mg^{2+} ions. The dissociation of the oligomeric BP was first reported by Bloom *et al* (1983) who demonstrated that both ATP and Mg^{2+} ions were required for the dissociation of the BP oligomer and the subsequent release of the newly-synthesised LSU. These results were obtained by analysing radiolabelled chloroplast extracts on sucrose density gradients. In the presence of ATP and Mg^{2+} ions the dissociated BP and newly-synthesised LSU migrated near the top of the gradient.

Analysis of radiolabelled chloroplast extracts on 5% non-denaturing polyacrylamide gels reveals that after dissociation of the BP by ATP the BP is no longer resolved on the gel. This problem was alleviated by analysing the dissociated BP subunits on gradient non-denaturing polyacrylamide gels (Lennox and Ellis, 1986). When standard molecular mass markers are also run on the same gradient gels it was possible to estimate the molecular mass of the dissociated BP subunits. It was estimated that the dissociated BP subunits migrate with an estimated molecular mass of 60,000 (Lennox and Ellis, 1986). This suggested that the dissociated BP subunits were migrating as monomers.

It was noted by Lennox and Ellis (1986), using immunoblotting techniques, that the degree of dissociation of the BP to its subunits was dependent upon the *in vitro* ATP concentration. In the absence of ATP however, even after dialysis of the chloroplast stromal extract, some of the BP remained in the monomeric form. With increasing ATP concentrations up to 5 mM the degree of dissociation of the BP increased, but even at 5 mM ATP not all of the BP oligomer had dissociated (Lennox and Ellis, 1986).
The dissociation of BP by ATP was found to be specific to ATP; other nucleotides such as GTP and UTP would not dissociate the BP although Ca^{2+} ions could replace Mg^{2+} ions. During the dissociation the BP subunits have been shown to be neither phosphorylated nor adenylated (Hemmingsen and Ellis, 1986).

The dissociation of the BP by ATP and Mg^{2+} ions has been shown to be reversible (Hemmingsen and Ellis, 1986). Under conditions where the ATP used to dissociate the BP in soluble chloroplast extracts is depleted by *in vitro* chloroplast protein synthesis, the BP is reassociated to the oligomeric form. This dissociation/reassociation could be repeated several times.

The dissociation of the BP also results in the release of the newly-synthesised LSU associated with the BP. During the reassociation of the BP in the absence of ATP the the released LSU is reassociated with the BP (Hemmingsen and Ellis, 1986). This demonstrates that the newly-synthesised LSU is still soluble when released from the BP oligomer although it is possible that the BP may

still be bound to the BP monomer. It also demonstrates that the BP has a high binding affinity for the LSU. In labelled chloroplast extracts the number of newly synthesised LSU must be extremely small in relation to the the total soluble protein pool yet the BP still binds specifically to the newly-synthesised LSU.

From the results reviewed above, Lennox and Ellis (1986)postulated the following equation to describe the reversible dissociation of the BP:

$$\alpha_6\beta_6 \xrightleftharpoons{Mg^{2+}\ ATP} 6\alpha + 6\beta$$

This equation does not take into account the fate of the associated LSU. To date no experimental evidence has been published to determine whether the newly synthesised LSU remains associated with the BP monomers or is released as some other form.

The results of Lennox and Ellis (1986) indicate that at *in vitro* ATP concentrations as low as 0.5 mM ATP the BP is to some extent dissociated. This initially suggests that under normal chloroplast ATP concentrations observed *in vivo* at 1-3 mM (Krause and Heber, 1976) the BP would exist primarily in the subunit form. The results of *in vitro* experiments do not describe the conditions that exist *in vivo* however. In soluble stromal extracts the concentration of BP has been estimated to be 50-fold less than that present in the in the intact chloroplast prior to lysis (Musgrove *et al*, 1987). If the equation presented above, to describe the dissociation of the BP,

is freely reversible, then the effect of this greatly increased *in vivo* BP concentration will be to favour the reverse reaction. The *in vivo* form of the BP therefore cannot be determined by these *in vitro* observations.

The most interesting physical and chemical properties of the BP are its affinity to bind the newly-synthesised LSU and its reversible dissociation by ATP. These observations have been reviewed in detail in this section. The following section takes these and other experimental findings and discusses them in rel ation to a possible role for the BP in the assembly of RuBP carboxylase.

1.3.3 Possible roles of the large subunit binding protein

The presence of the BP in pea chloroplasts was first reported by Barraclough and Ellis (1980) who also postulated that the BP may be involved in the assembly of RuBP carboxylase. This basic hypothesis was used in the design and execution of many of the experiments reported in Section 3.

During the prolonged incubation of isolated pea chloroplasts Barraclough and Ellis (1980) demonstrated that the pool of newly-synthesised LSU associated with the BP decreased in amount whilst there was a subsequent increase in assembly of newly-synthesised LSU into RuBP carboxylase. Barraclough and Ellis suggested that this could not be definitively interpreted as a precursor-product type rel ationship between the LSU associated with the BP and subsequently assemhled into RuBP carboxylase. This is because it was possible that other pools of LSU

may be present in chloroplast extracts but these pools may not be resolved on the non denaturing gel. For example a large radioactive smear can be seen to be trapped at the top of these gels and this may contain a pool of unassembled LSU.

During the experiments of Barraclough and Ellis and also in subsequent experiments, the assembly of newly-synthesised LSU into RuBP carboxylase was assayed by the comigration of label with the RuBP carboxylase holoenzyme on non-denaturing gels. There is the possibility that the comigration of newly-synthesised LSU with the RuBP carboxylase holoenzyme does not represent correct assembly since the LSU may be simply sticking to pre-existing RuBP carboxylase. The only definitive method of determining whether RuBP carboxylase assembly is occuring is by assaying an increase in RuBP carboxylase activity. It is not possible however to assay changes in RuBP carboxylase activity in chloroplast extracts over a few hours. This is because a large pool of RuBP carboxylase already exists masking any small changes over a few hours.

Since the first report by Barraclogh and Ellis there have been several reports published attempting to quantify the postulated transfer of LSU from the BP into RuBP carboxylase. These results have produced evidence that supports the hypothesis that the the LSU associated with the BP is assembled into RuBP carboxylase. These results do not provide evidence that the BP is a necessary factor in the assembly of RuBP carboxylase.

The BP present in chloroplast extracts can be

dissociated into its constituent subunits by ATP and Mg_{2+} ions as first reported by Bloom *et al* (1983). During this dissociation the LSU is also released from the 720 kDa BP. By analysing labelled chloroplast extracts on sucrose gradients followed by SDS PAGE it was demonstrated that the released LSU no longer sedimented ahead of the RuBP carboxylase as it did when associated with the BP. The LSU migrates nearer the top of the gradient with a sedimentation coefficient equivalent to a LSU dimer or a LSU-BP heterodimer (Cannon *et al*, 1986).

Th ATP-mediated dissociation of the BP and its subsequent release of associated LSU has been shown to result in an increase in assembly of RuBP carboxylase as judged by an increase in radiolabel comigrating with the RuBP carboxylase on non-denaturing gels (Milos and Roy, 1984). These results confirmed that the LSU associated with the BP is competent to assemble into RuBP carboxylase assuming that the comigrating LSU is correctly assembled. It does not however prove that the BP is a necessary factor in RuBP carboxylase assembly.

Further experiments on the assembly of RuBP carboxylase in chloroplast extracts revealed that although ATP is required for the initial dissociation of the BP, it actually inhibits the assembly of released LSU into RuBP carboxylase. If the endogenous ATP levels in the chloroplast extracts, following dissociation, were reduced by adding glucose and hexokinase the assembly process was enhanced (Cannon *et al*, 1986).

Attempts have been made to study the assembly of RuBP carboxylase in chloroplast extracts in the absence of the BP. This was achieved by adding specific antiserum to the BP to chloroplast extracts and assessing the effects on assembly by non-denaturing PAGE. The results of these experiments showed that the addition of ATP to radiolabelled chloroplast extracts resulted in the dissociation of the BP as expected. If antiserum to the BP was subsequently added to the chloroplast extract, the assembly of RuBP carboxylase was inhibited (Cannon et al, 1986). From these results Cannon et al postulated that in the presence of ATP the newly-synthesised LSU was associated with the BP. The next step in the assembly process was the formation of a LSU-BP-SSU heterotrimer prior to assembly. The initial processes in the assembly of RuBP carboxylase ie the association of newly-synthesised LSU with the BP and its subsequent release and assembly into RuBP carboxylase, were both regulated by ATP levels.

A rigorous interpretation of these results is difficult however, since the BP antiserum used by Cannon et al also cross-reacts with the subunits of RuBP carboxylase. This was suggested to have an insignificant effect on the released LSU and free SSU pool in the chloroplast extracts since this cross-reactivity would be swamped by the endogenous RuBP carboxylase pool. This assumption does not take into account the relative cross-reactivity of the antiserum against free subunits and the assembled RuBP carboxylase holoenzyme. If the BP

antiserum cross-reacts much more strongly with free subunits than assembled subunits the inhibition of assembly by the BP antiserum could be explained by the removal of free RuBP carboxylase subunits by this cross-reactivity.

The hypothesis that the BP is involved in the assembly of RuBP carboxylase is still valid because as reviewed in Section 1.4, attempts to assemble the higher plant RuBP carboxylase in E.coli have failed (Bradley et al, 1986). Assembly of RuBP carboxylase from the procaryotic Synechococcus subunits in E.coli have succeeded without the co-expression of the BP in these transformants. The assembly of the Synechococcus RuBP carboxylase is much more simple than in higher plants however (see Section 1.4 for review). The complexity of the higher plant assembly pathway(involving the synthesis of two subunits from different genetic origins and the subsequent post-translational transport and processing of one of the subunits) may thus require an additional factor, the BP.

There have been several postulated roles for the BP in the assembly process and these will be reviewed in Section 4.3.

1.4 AIMS OF PROJECT

The aims of this project can be divided into two sections. The first section aims to confirm and extend published data on the pea BP. This characterisation will focus in particular on the dissociation of the pea BP into dissimilar subunits.

The second section of the project aims to extend the initial work on the BP to the cereal crops of barley and *Triticum*. This area of research work was carried out as part of the CASE studentship, and was jointly-sponsored by the Rothamsted Experimental Station in Harpenden. Under the terms of the Studentship, research into the BP was carried out on these economically-important cereal crops. This research aims to first determine whether the newly-synthesised LSU in chloroplasts isolated from these species is bound to the BP. Following confirmation of this basic observation, experiments aimed at characterising the barley and *Triticum* BP will be carried out. These experiments include the purification and characterisation of the BP, the study of its synthesis *in vivo* and its accumulation during leaf development.

Throughout this work the results obtained will be compared and contrasted with those obtained for the pea BP. These results obtained will be discussed in relation to the working hypothesis used throughout this research project, namely that the BP may be involved in the assembly of RuBP carboxylase.

SECTION TWO MATERIALS AND METHODS

2.1 GROWTH OF PLANTS

Hordeum vulgare seeds (var. Apex) were obtained from the Rothamsted Experimental Station in Harpenden. *Pisum sativum* seeds (var. Feltham First) were obtained from Charles Sharpe and Co. Ltd., Sleaford.

The seeds were grown in compost (Fisons Levington Compost, John Astley and Son Ltd.) under white fluorescent lights (Phillips) with a photofluence rate of photosynthetically active radiation of 45 $\mu E\ m^{-2}\ s^{-1}$. During growth the photoperiod was 12 hours and the temperature was maintained at 20±2°C. The seedlings were watered when required and the age of the seedlings taken from time of sowing.

Alternatively seedlings were grown as above in total darkness at 20°C for 7 days before harvesting.

2.2 CHLOROPLAST TECHNIQUES

2.2.1 Isolation of chloroplasts

Chloroplasts from *Pisum sativum* (pea) were isolated from 8 to 9-day-old seedlings as described by Ellis and Hartley (1982). The seedlings were harvested using scissors and gloves and dropped into a polycarbonate vessel, pre-chilled to a temperature of 0°C. Approximately 40 g of peas were harvested and 200 ml of sterile

chloroplast isolation medium (CIM) poured onto the peas as a frozen slurry. The composition of CIM was:-

0.35 M sucrose
25 mM Hepes-NaOH
2 mM EDTA
2 mM sodium isoascorbate
pH 7.6

The sodium isoascorbate was added to the CIM just prior to the homogenisation. Homogenisation was for 3 x 6 second bursts.The homogenate was quickly filtered through 8 layers of muslin into a 500 ml beaker on ice and the filtrate centrifuged at 3,200 x g, 4°C for 1 minute. The supernatant was then discarded and the pellet resuspended in 10 ml of fresh CIM using a cotton bud and centrifuged as above. This yielded a washed crude chloroplast preparation.

Barley chloroplasts were prepared exactly as above using 7-day-old seedlings.

<u>2.2.2 Radiolabelling of chloroplast proteins in vitro</u>

The washed chloroplast preparation (2.2.1) was gently resuspended in 1 ml of either of the following sterile incubation media, in a 15 ml Corex tube:-
sorbitol resuspension medium (SRM):

0.33 M sorbitol

50 mM Tricine-KOH

pH 8.4

KCl resuspension medium(KRM)

0.2 M KCl

66 mM Tricine-KOH

6.6 mM $MgCl_2$

pH 8.3

250 μCi of [^{35}S]-methionine (1000 Ci/mmol) was added to the chloroplast suspension and the mixture incubated (as in Blair and Ellis (1973)) at 20°C in a water bath, illuminated from the bottom at a light intensity of 100 μE m^{-2} s^{-1} by a Phillips 500W Photoflood. The chloroplasts were incubated for a time period of up to 30 minutes.

Samples were removed from the suspension and centrifuged for 1 minute at 10,000 x g at 4°C in an Eppendorf 5412 microfuge. This centrifugation yielded a washed chloroplast pellet which was lysed in a small volume of 10 mM Tris-HCl (pH 7.6). The lysate was then centrifuged for 5 minutes as before to yield a supernatant consisting of a soluble chloroplast stromal extract.

The incorporation of [^{35}S]-methionine into chloroplast proteins was estimated by assaying the incorporation into the acid-insoluble fraction, using the method of Siddell and Ellis (1975). Separate samples (5

μl) from the chloroplast suspension were removed at various time points and dropped onto a 1 cm square piece of Whatman filter paper and dried. The filters were then placed in 20% (w/v) trichloroacetic acid (TCA) at 100°C for 20 minutes. The solution was replaced with fresh TCA and the filters washed for a further 5 minutes at room temperature. The filters were then washed twice in ethanol, once in ether and then dried in a stream of nitrogen. The dried filters were placed in 4 ml of Beckman NA scintillant in plastic scintillation vials and the radioactivity measured using an LKB Minibeta 1212 scintillation counter.

2.3 ISOLATION OF SOLUBLE LEAF PROTEINS

The soluble leaf proteins were extracted from sections of barley leaves using a mortar and pestle, by the method of Smith and Ellis (1981). Five barley seedlings of equal height were chosen and the first leaves cut at their bases and placed on a glass plate on ice. The leaves were cut into 1 cm sections and the sets of 5 sections from corresponding positions were collected in a mortar on ice. The sections were ground for 1 minute in 1 ml of sterile homogenisation buffer which consisted of:-

50 mM Tris-HCl

1 mM EDTA

10 mM 2-mercaptoethanol

2 mM PMSF

pH 8.0

The homogenate was centrifuged for 20 minutes in an Eppendorf centrifuge at 18,000 x g at 4°C. The supernatants were removed and each made up to a volume of 1 ml with ice-cold extraction buffer and stored at -20°C.

2.4 PURIFICATION OF PROTEINS

2.4.1 Purification of RuBP carboxylase and the large subunit binding protein

The large subunit binding protein and RuBP carboxylase were both purified from soluble extracts of whole leaves using a method based on that of Hemmingsen and Ellis (1986). All the procedures during the purification were carried out at 4°C.

Approximately 50 g of 7-day-old seedlings were harvested and placed in a polycarbonate vessel. The leaves were then covered in 300 ml of sterile homogenisation buffer consisting of:-

10 mM Tris-HCl

1 mM EDTA

1 mM PMSF

10 mM 2-mercaptoethanol

pH 8.0

and homogenised with a Polytron for 3 x 6 seconds bursts at the maximum speed setting. The homogenate was filtered through 8 layers of muslin and the filtrate centrifuged at 18,000 x g at 4°C for 20 minutes. The pellets were discarded and the supernatants made to 40% saturation with solid ammonium sulphate. After 30 minutes the precipitated protein was removed by centrifugation as above and the supernatant decanted. The supernatant was then made to 70% saturation with solid ammonium sulphate and the precipitate removed after 30 minutes by centrifugation as above.

The precipitate was resuspended in 10 mls of sterile buffer A consisting of:-

10 mM Tris-HCl
0.5 mM EDTA
10 mM 2-mercaptoethanol
pH 8.0

and desalted by passing through a G-25 Sephadex (Pharmacia) column equilibrated in buffer A (diameter 2 cm, height 40 cm). The protein was eluted by gravity and 2 minute fractions collected using an LKB Redirac fraction collector. The amount of protein in the fractions was measured by their absorbance at 280 nm and those containing protein were pooled.

The pooled fractions were loaded onto a DEAE-Sephacel (Pharmacia) ion-exchange column (diameter 3 cm, height 10 cm) equilibrated in buffer A and the column was eluted

under gravity. Fractions were collected on an LKB fraction collector. The column was washed in increasing concentrations of KCl dissolved in buffer A at a concentration of 100, 200, 300, and 400 mM KCl. Each step was continued until no further protein was eluted from the column (as determined by absorbance at 280 nm).

The 300-400 mM eluate containing the large subunit binding protein was dialysed against 1 litre of buffer A for 1 hour and then made to 70% saturation with solid ammonium sulphate. The precipitate was removed by centrifugation and redissolved in 2 ml of buffer A. Solid sucrose was added to make a final concentration of 5% and the solution was loaded onto a Sephacryl S300 superfine (Pharmacia) column (diameter 3 cm, height 90 cm) equilibrated in 10 mM Tris-HCl pH 7.6. The column was connected to an LKB Microperpex peristaltic pump and 10 mM Tris elution buffer passed through at a flow rate of 10 mls per hour. Fractions were collected at 20 minute intervals using an LKB Redirac fraction collector.

The absorbance at 280 nm was used to determine the elution of protein and samples were taken from the fractions to find those containing the large subunit binding protein by both SDS-denaturing and non-denaturing gel electrophoresis.

The fractions containing the large subunit binding protein were pooled and stored at -20°C.

The 100-300 mM KCl eluate from the DEAE column, containing the RuBP carboxylase, was made to 70% saturation with ammonium sulphate and the precipitate

recovered by centrifugation. The precipitate was redissolved in 2 ml of buffer A and loaded onto the Sephacryl S300 superfine column as above. The fractions containing protein as determined by absorbance at 280 nm were analysed by SDS-denaturing and non-denaturing gel electrophoresis. Those fractions containing RuBP carboxylase were stored at -20°C.

The RuBP carboxylase and large subunit binding protein were identified by their mobilities on SDS-denaturing and non-denaturing gel electrophoresis.

2.4.2. Purification of proteins for raising antibodies

Fifty grams of light-grown barley leaves were used to make a chloroplast stromal extract as described in section 2.2.2. The extract was loaded onto a 5% (w/v)non-denaturing gel and electrophoresed overnight as described in section 2.7.2 except that no gel comb was inserted into the gel. The gel was stained and destained (section 2.7.4) and the band containing the large subunit binding protein was excised using a razor blade and boiled in SDS sample buffer. The treated gel was layered onto a 15% (w/v) SDS polyacrylamide gel and electrophoresed overnight. This gel also had no comb inserted into it, but otherwise it was made as described in section 2.7.1. The binding protein band was excised with a razor blade after staining/destaining (section 2.7.4) and the large subunit binding protein electroeluted from the gel as described in section 2.11.3.

The purity of the large subunit binding protein

preparation was determined by subjecting the samples to SDS-denaturing gel electrophoresis (section 2.7.1) and then silver staining (section 2.7.4). The remaining sample was used to raise antibodies as described in section 2.6.1.

2.4.3 Purification of ATPase subunits

The subunits of the ATPase (coupling factor) from barley thylakoids were prepared using a modified method of Strotmann et al (1973). Approximately 40 g of leaves from 7-day-old barley seedlings were used to prepare chloroplasts as described in section 2.2.1 and the chloroplasts were then lysed in 10 ml of 10 mM Tris pH 7.6 . The thylakoids were sedimented by centrifugation of the lysate at 18,000 x g for 15 minutes. The pellet was resuspended in 50 ml of freshly prepared 10 mM sodium pyrophosphate-HCl pH 7.5 by using a vortex mixer. The thylakoids were pelleted again by centrifugation at 30, 000 x g for 10 minutes at 0°C and the pellet washed twice more in sodium pyrophosphate as described above

The washed thylakoid pellet was resuspended in 10 ml of 2 mM Tricine-KOH pH 7.8 and re-pelleted by centrifugation at 200,000 x g for 45 minutes. The supernatant liquid containing the ATPase subunits was carefully decanted and stored on ice. The thylakoid pellet was washed twice more in 2 mM Tricine-KOH as above and the supernatants pooled. The supernatant was decreased in volume to approximately 2 ml by treatment with aquacide and after concentration boiled in SDS sample

buffer (section 2.7.1) and stored at -20°C.

2.5 ESTIMATION OF MOLECULAR WEIGHT BY COLUMN CHROMATOGRAPHY

The relative molecular mass of the barley large subunit binding protein was estimated using gel filtration.

Sephacryl S400 superfine was supplied by Pharmacia. A glass column was packed according to the manufacturer's specifications with Sephacryl S400 (1.7 cm x 95 cm) at a flow rate of 53 ml per hour. The column was equilibrated at 4°C with sterile 20 mM Tris-HCl pH 8.0.

The column was eluted at a flow rate of 30 ml per hour using a Microperpex peristaltic pump and 1 ml fractions were collected.

High molecular weight standards (Sigma), including blue dextran, were reconstituted following instructions and run separately down the column.

The elution of each standard was detected by its absorbance at 280 nm. A calibration curve was drawn using the volume of each buffer needed to elute each standard plotted against the log of its molecular mass (see Figure 16).

A barley chloroplast extract containing 6 mg ml^{-1} protein was prepared as in section 2.2.2 and was loaded onto the column and eluted exactly as for the protein markers. Samples from the fractions containing protein were seperated by non-denaturing gel electrophoresis (see

section 2.7.2) and the gel was stained as in section 2.7.4. The large subunit binding protein bands from each fraction were excised and the Coomassie stain eluted for 1 hour at 20°C in 1 ml of 1% (w/v) SDS. The absorbance of the eluted Coomassie solution was assayed at 595 nm and used to plot an elution profile of the large subunit binding protein. Using the calibration curve the relative molecular mass was estimated.

2.6 IMMUNOLOGICAL TECHNIQUES

2.6.1 Production of antibodies

The large subunit binding protein of barley leaves was purified by gel electrophoresis as described in section 2.4.2.

One New Zealand White rabbit was injected subcutaneously with 60 µg of the protein per injection with a total of 5 injections. The first injection contained 1 ml of the large subunit binding protein mixed with 1 ml of Freunds complete adjuvant (Grand Island Biological Co. Ltd.). All subsequent injections contained incomplete adjuvant.

Two weeks after each injection blood was removed from the ear of the rabbit and allowed to clot at room temperature. Serum was isolated by centrifugation at 3, 000 x g for 30 minutes at 4°C, and stored at -20°C. The serum was analysed for antibody activity by immunoblotting as described in section 2.6.3.

2.6.2 Preparation of iodinated protein A

A 1 mg ml^{-1} stock solution of *Staphylococcus aureus* protein A was prepared by dissolving the protein in phosphate-buffered saline (PBS) which had the following composition:-

138 mM NaCl

2.8 mM KCl

7.37 mM Na_2HPO_4

1.46 mM KH_2PO_4

pH 7.8

25 µl was added to 1 mCi $Na[^{125}I]$ (Amersham, 13.5 mCi ug^{-1}) together with 10 ul of freshly prepared chloramine T (2 mg ml^{-1}) dissolved in PBS.

These solutions were mixed and left at room temperature for 2 minutes before adding 25 µl of tyrosine solution (2 mg ml^{-1}), 50 µl of 10% (w/v) bovine serum albumin (BSA) and 200 µl of PBS.

The iodinated protein A was separated from free iodine by gel filtration on a 5 ml column of Sephadex G-25 medium grade (Pharmacia). The column was pre-washed by adding 10 ml of 10% (w/v) BSA in PBS.

Fractions of approximately 0.2 ml were collected and radioactivity present determined by an LKB Ultrogamma counter and those fractions containing iodinated protein were stored at 4°C.

2.6.3 Immunoblotting of proteins

Protein samples to be analysed by immunoblotting were first subjected to polyacrylamide gel electrophoresis under either denaturing (section 2.7.1) or non-denaturing (section 2.7.2) conditions.

After electrophoresis gels were quickly washed in a transfer buffer consisting of:-

192 mM glycine
25 mM Tris base
20% (v/v) methanol

The gel was placed on a "Scotch-Brite" pad and overlaid with a piece of nitrocellulose paper (Schleicher and Schull; pore size 0.45 µm) cut to the same dimensions of the gel and previously soaked in transfer buffer. All bubbles between the gel and filter were removed. Gloves were worn throughout the procedure. A second "Scotch-Brite" pad was placed on top of the nitrocellulose and the sandwich was transferred to a Trans-Blot cell (Biorad) containing 3 litres of the transfer buffer.

The transfer was performed at room temperature for 2 hours at 60 V. After transfer the filters were incubated for 1 hour at room temperature in 100 ml of PBS containing 4% (w/v) BSA. The antibody was then added and the filter incubated at room temperature overnight. After washing in 100 ml of PBS for 10 minutes the filter was placed in 100 ml of 4% (w/v) BSA containing 10^6 cpm [^{125}I]-protein A prepared as described in section 2.6.2. The filter was incubated for 2 hours at room temperature, washed in PBS

containing 1% (v/v) Triton X-100 and then dried and autoradiographed at room temperature using an intensifying screen as described in section 2.11.5.

2.6.4 Analysis of proteins by rocket immunoelectrophoresis

Rocket immunoelectrophoresis (RIE) was performed using a Shandon 600 electrophoresis tank as described by Laurell (1966) and Plumley and Schmidt (1983).

A 1% (w/v) agarose solution Sigma; type 1 low EEO) in barbital buffer (Sigma) containing 1% (v/v) Triton X-100 was prepared by heating to 100°C. This solution was incubated at 50°C in a water bath.

Antisera (300 µl) to either the large subunit binding protein or RuBP carboxylase was mixed with warm agarose solution and 18 ml of the agarose-antibody solution was poured onto a 8 cm x 20 cm glass plate and allowed to set.

The antibody to the pea large subunit binding protein was used to assay the barley large subunit binding protein. The barley RuBP carboxylase was assayed using antibodies raised against wheat RuBP carboxylase.

The agarose was left to set and holes, 4 mm in diameter, punched along the longest edge of the gel. The plate was supported in the electrophoresis tank and the electrode vessels filled with barbital buffer containing 1% (v/v) Triton X-100. Strips of Whatman 3 MM filter paper, soaked in the same buffer, were used to connect the gel plate and the buffer in the reservoirs. To each well 10 µl of each test sample was added and the plate subjected to electrophoresis for 18 hours at room

temperature at 80 V.

The gel on its supporting plate was removed from the electrophoresis cell, washed in PBS and blotted with 6 layers of Whatman 3MM filter paper. The gel was finally dried with a hair dryer and stained with Coomassie blue for 30 minutes and destained as described in section 2.7.4. The heights of the rockets were measured and recorded.

2.7. POLYACRYLAMIDE GEL ELECTROPHORESIS

2.7.1 Denaturing polyacrylamide gel electrophoresis

Proteins were analysed by SDS-denaturing polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (1970). A 15% slab gel was cast between two 15 cm x 15 cm glass plates at a thickness of 0.15 cm. The gel solution consisted of:-

30% (w/v) acrylamide + 0.3% (w/v) bisacrylamide	24 ml
3 M Tris-HCl (pH 8.8)	6 ml
H_2O	17.4 ml
10% (w/v) SDS	0.48 ml
TEMED	20 µl

This solution was mixed with 200 µl of freshly prepared 10% (w/v) ammonium persulphate (APS). The solution was poured to within 3 cm of the top of the plate and overlaid with water-saturated butan-1-ol. The gels were left to polymerise for 1 hour.

Linear gradient gels were prepared using an MSE gradient maker. The following solutions were prepared:-

	10%	30%
60% (w/v) acrylamide containing 0.3% (w/v) bisacrylamide	-	11 ml
60% (w/v)acrylamide containing 1.6% (w/v) bisacrylamide	3.65 ml	-
75% (v/v) glycerol	-	8.03 ml
H_2O	15.32 ml	-
3M Tris-HCl (pH8.8)	2.75 ml	2.75 ml
10% (w/v) SDS	0.22 ml	0.22 ml
TEMED	7 µl	9 µl
10% (w/v) APS	53 µl	16 µl

18 ml of each of the two solutions was placed in the gradient maker chambers and the gradient generated between the plates. The mixture was overlaid with butan-1-ol as above.

After 1 hour to allow the gel to set, the butan-1-ol was washed off with water and a stacking gel prepared on top of the resolving gel by adding the following solutions:-

30% (w/v) acrylamide + 0.3% (w/v) bisacrylamide	4 ml
0.5 M Tris-HCl (pH 6.8)	5 ml
H_2O	10.8 ml
10% (w/v) SDS	0.2 ml
TEMED	15 µl
APS	100 µl

A slot-former was inserted into the gel and the stacking gel left to polymerise. The gel was mounted in the electrophoresis tank and the tank filled with running buffer consisting of :-

25 mM Tris base

192 mM glycine

0.1% (w/v) SDS

pH = 8.3

Solutions of the protein samples were added to an equal volume of sample buffer and were then boiled for 5 minutes. The sample buffer consisted of:-

0.5 M Tris-HCl (pH 6.8)	2.5 ml
glycerol	2.0 ml
10% (w/v) SDS	4.0 ml
2-mercaptoethanol	1.0 ml
Bromophenol blue	0.01 g
H_2O	0.5 ml

The sample solutions were loaded onto the gel and the gel was run at 9 mA constant current for 17 hours at room temperature.

2.7.2 Electrophoresis under non denaturing conditions

Non-denaturing PAGE was carried out using the method of Hedrick and Smith (1968). A gel with a final concentration of 5% (w/v) acrylamide was cast between 2 glass plates as described above by adding a solution containing:-

25% (w/v) acrylamide + 1% (w/v) bisacrylamide	12 ml
3 M Tris-HCl (pH 8.8)	7.5 ml
H_2O	40 ml
TEMED	40 µl
APS (fresh)	420 µl

The gel was cast between the glass plates and a slot former inserted into the gel. The gel was left to polymerise for 1 hour.

Linear gradient gels (4-30% acrylamide) were prepared using an MSE gradient maker, with the following solutions:-

	4%	30%
60% (w/v) acrylamide containing 0.3% (w/v) bisacrylamide	-	14.4 ml
25% (w/v) acrylamide containing 1% (w/v) bisacrylamide	4.6 ml	-
30% (v/v) glycerol	-	10.6 ml
3 M Tris-HCl (pH 8.8)	3.75 ml	3.75 ml
H_2O	20.4 ml	-
10% (w/v) APS	160 µl	75 µl

An equal volume of each solution (22.5 ml) was poured into the gradient maker and after the gel was poured a slot-former was inserted into the gel and left to polymerise for 1 hour.

Both linear gradient gels and single concentration gels were mounted in the electrophoresis apparatus. The

gels were pre-electrophoresed at 14 mA constant current for 2 hours with running buffer consisting of:-

50 mM Tris base
384 mM glycine
(pH 8.5)

L-cysteine (8 mM final concentration) was put into the upper reservoir prior to electrophoresis in order to ensure reducing conditions in the gel. A solution of 50% (v/v) glycerol 0.01% (w/v) bromophenol blue was mixed with the sample to give a final concentration of 10% (w/v) glycerol. The samples were then loaded onto the gel; gradient gels were run for 20 hours at 18 mA constant current and 5% gels were run for 17 hours at 14 mA constant current.

2.7.3 Isoelectric focussing.

Soluble proteins from both barley and pea leaves were analysed using two-dimensional gel electrophoresis as described by Roscoe and Ellis (1982).

The following solutions were stored at -20°C:-

Solution A

9.5 M urea

2% (v/v) Nonidet P40

5% (v/v) 2-mercaptoethanol

2% (v/v) Ampholines containing; 1.6% pH range 3.5-10; 0.2% pH range 9.0-11.0; 0.1% pH range 4.0-6.0; 0.1% pH range 5.0-7.0.

Solution B

5 M urea

1% Ampholines, at half the concentration of those in solution A. Solution C

10% (v/v) glycerol

2.3% (w/v) SDS

62.5 mM Tris-HCl pH 6.8

5% (v/v) 2-mercaptoethanol

The soluble protein extract, containing approximately 300 µg of protein, was lyophilised and stored at -20°C.

The following gel solution was prepared:-

urea	5.5 g
Nonidet P40	20 ml of 10% (v/v)
28.38% (v/v) acrylamide +	
1.62% (v/v) bisacrylamide	1.33 ml
H_2O	1.97 ml
Ampholines	2% (v/v) (as in A)
TEMED	10 µl

The solutions were mixed and 10 µl of freshly prepared APS (10%, v/v) added to the solution. The gel solution was poured into cylindrical glass rods (120 mm x 1.5 mm) sealed at one end with Parafilm and kept at 35°C in a water bath. The gel solution was poured to within 10 mm of the top of the rod and overlaid with 40 µl of 8 M urea and allowed to polymerise for 60 minutes. The overlay was removed and replaced with 40 µl of solution A which was in turn overlaid with water and left a further 60 minutes. The entire overlay was then removed and the gels transferred to a Shandon Southern Analytical Gel Apparatus. Solution A (40 µl) was added to the top of the gels and overlaid with degassed 0.02 M NaOH. The upper reservoir was filled with degassed 0.02 M NaOH and the lower with 0.01 M H_3PO_4. The gels were pre-electrophoresed for 15 minutes at 200 V, 30 minutes at 300 V and 30 minutes at 325 V. The upper reservoir electrolyte and the overlay were then removed.

The freeze-dried samples were dissolved in 40 µl of solution A and loaded above the gels. The samples were in turn overlaid with 40 µl of solution B and the upper reservoir filled with degassed 0.02 M NaOH. Electrophoresis was carried out for 18 hours at 325 V at room temperature before the gels were removed from the tubes by injection of distilled water from a 1 ml syringe. The gels were washed in 2 changes of buffer C for 30 minutes before being laid on top of an SDS-denaturing gel prepared as described in section 2.7.1 and cemented in place with 1% agarose made up in buffer C. The proteins in

the tube gel were separated in the second dimension onto the gel as in section 2.7.1 and the gel was stained and destained as in section 2.7.4.

The apparent isoelectric point of the proteins were estimated using an additional tube gel loaded with the same amount of the protein as the sample being analysed. The tube gel was sliced at 0.5 cm intervals and the slices each soaked for 1 hour at room temperature in 1.5 ml of freshly autoclaved distilled water. The pH of each solution was measured.

2.7.4 Staining of polyacrylamide gels

The gels were stained in a solution of:-

0.5% (w/v) Coomassie brilliant blue R
50% (v/v) methanol
7% (v/v) acetic acid

The gels were destained with several changes of the above solvent mixture minus the Coomassie. Gels were then photographed and dried.

If a greater sensitivity of protein detection was required silver staining was used as described by Wray *et al* (1981). The gels were washed in 4 changes of 50% (v/v) methanol for 4 hours prior to staining and then washed for 5 minutes in distilled water. The staining solution consisted of 0.8 g of silver nitrate dissolved in H_2O which was then added to 1.4 ml of 14.8 M ammonia in 21 ml of 0.36% NaOH. The two solutions were mixed by stirring

rapidly and made up to 100 ml. The gels were then stained for 15 minutes, washed for 5 minutes in distilled water, and the silver developed by adding freshly made developing solution consisting of 2.5 ml 1% (w/v) citric acid and 0.25 ml 38% formaldehyde made up to 500 ml with water. Stain development took approximately 30 minutes and was stopped by adding 50% (v/v) methanol containing 7% (v/v) acetic acid. The gels were photographed and dried.

2.8 PARTIAL PROTEOLYTIC DIGESTION OF PROTEINS

The limited proteolytic digestion of proteins excised from gels was carried out using the method of Bottomley (1982).

The protein to be digested was first run into an SDS-denaturing polyacrylamide gel as described in section 2.7.1 and the appropriate band excised.

The excised bands were incubated in digestion buffer consisting of:-

125 mM Tris-HCl pH 6.8
0.5% (w/v) SDS
10% (v/v) glycerol
0.01% (w/v) bromophenol blue
1 mM EDTA

Each slice was equilibrated in 1 ml of this buffer for a total of 30 minutes with 3 changes.

A 10-30% SDS-denaturing polyacrylamide gel was prepared as described in section 2.7.1, but with a 5 cm

stacking gel. The gel was mounted in a tank and reservoirs filled with buffer as described in section 2.7.1. The wells were filled with digestion buffer and each protein-containing gel slice pushed to the bottom of the well.

A 5 mg ml^{-1} stock solution of Staphylococcus aureus V8 protease (Sigma) was made up previously in water and was subsequently diluted with digestion buffer to give an appropriate concentration to load on the gel. A known amount of protease was layered on top of the gel slices and the current switched on the gel at 18 mA and the gel run until the bromophenol blue had almost reached the resolving gel. The gel was then switched off for 10 minutes to allow proteolysis to proceed. The current was then switched on and the gel run as described in section 2.7.1. The gel was then silver stained as described in section 2.7.4.

2.9 RADIOLABELLING OF LEAVES

One 6-day-old barley leaf was excised just below the leaf base and immediately put into an Eppendorf tube containing 50 µCi of [^{35}S]-methionine (100 Ci/mmol) in 50 µl of distilled water. Care was taken to ensure that the cut end of the leaf stayed submerged in water throughout the incubation. The leaf was placed under an illuminated light bank under the same conditions used during growth as described in Section 2.1, and air was blown over the leaf by a fan to increase transpiration. When nearly all the solution had been taken up by the leaf

a further 200 µl of water was added to avoid wilting. The leaf was incubated for a total of 6 hours before soluble protein was extracted in 1 ml of extraction buffer as described in section 2.3.

Leaves to be incubated in the presence of cycloheximide were first supplied with a 10 µg ml^{-1} solution of cycloheximide in water through the cut end for 15 minutes before labelling as above. Throughout the labelling period cycloheximide was also present at this concentration.

2.10 PROTOPLAST TECHNIQUES

2.10.1 Isolation of intact protoplasts

Isolated intact protoplasts were prepared using the method of Edwards *et al* (1978). Seven-day-old barley seedlings had their lower epidermis removed by making a cut near the tip of the leaf and stripping off the epidermal layer. The stripped leaves were floated on the surface of an incubation medium consisting of:-

0.5 M sorbitol
5 mM MES-KOH pH 5.5
50 mM $MgSO_4$
100 mM KH_2PO_4

to which 1% (w/v) Cellulysin (Calbiochem) and 0.5% (w/v) macerase (Calbiochem) had been added. The leaves were incubated in this incubation medium for 3 hours at 22°C

after which most of the protoplasts could be removed from the leaves by gentle agitation. The protoplasts were harvested by centrifugation at 200 x g for 2 minutes at 4°C and all subsequent steps were performed on ice. The protoplasts were gently resuspended in 6 ml of buffer B, consisting of:-

0.5 M sucrose
5 mM MES-KOH pH 6.0
50 mM $MgSO_4$
100 mM KH_2PO_4

The resuspended protoplasts were very gently pipetted into a 15 ml Corex tube and overlaid with 2 ml of buffer C consisting of:-

0.1 M sorbitol
0.4 M sucrose
5 mM MES-KOH pH 6.0
50 mM $MgSO_4$
100 mM KH_2PO_4

and this buffer was in turn overlaid with 2 ml of incubation medium without the enzymes and adjusted to pH 6.0. The tube was centrifuged at 200 x g for 10 minutes during which intact protoplasts migrated up the gradient to form a band between layers C and A. The protoplasts were removed with a 1 ml pipette and stored on ice. The protoplasts were shown to be intact by microscopical analysis at 200 x magnification.

2.10.2 Radiolabelling of protoplasts

The isolated intact protoplasts were resuspended in incubation medium. Protoplasts containing 300 µg of chlorophyll were resuspended in 1 ml of:-

0.5 M sorbitol

5 mM MES-KOH pH 5.8

1 mM $MgCl_2$

1 mM KH_2PO_4

1 mM $NaHCO_3$

2 mM $Ca(NO_3)_2$

The protoplast suspension was incubated in a 25 ml glass conical flask suspended in a water bath. The temperature was maintained at 26°C and the incubation started by adding 250 µCi of [^{35}S]-methionine (1000 Ci/mM) to the medium. The flask was bottom-illuminated throughout using a tungsten bulb giving a light intensity of 52 µE m^{-2} s^{-1}. Protoplasts incubated in the presence of cycloheximide had the inhibitor present at a concentration of 10 µg ml^{-1} throughout the labelling period.

At various time points 5 µl aliquots were removed and incorporation of [^{35}S]-methionine into TCA-insoluble material assayed as described in section 2.2.2. Soluble proteins were extracted from aliquots of protoplasts by lysing the protoplasts in 10 mM Tris-HCl pH 8.0, 1mM PMSF as described for chloroplasts in section 2.2.2.

2.11. GENERAL TECHNIQUES

2.11.1 Sucrose density centrifugation

Purified large subunit binding protein was analysed on a four-step sucrose gradient both in the presence and absence of ATP and Mg^{2+} ions. The gradients consisted of 4 x 3ml steps at the following sucrose concentrations (w/v); 5%, 20%, 35%, and 50%, the gradients being hand-layered using a pipette. The sucrose solutions were dissolved in:-

50 mM Tris-HCl
7 mM 2-mercaptoethanol
1 mM PMSF
pH 7.6

Gradients containing ATP and $MgCl_2$ contained 10 mM of each compound. The large subunit binding protein was layered onto the top of the gradient (200 µg of the protein in 1 ml of 10 mM Tris-HCl, pH 8.0) The gradients were centrifuged at 88,000 x g for 18 hours at 4°C in a Beckman SW40 Ti rotor. The gradients were then fractionated into 500 µl aliquots and stored at -20°C.

2.11.2 Estimation of chlorophyll

Chlorophyll concentrations were determined using the method of Arnon (1949). Aliquots of chloroplast preparations were made to 80% (v/v) with acetone and incubated in the dark at room temperature for 10 minutes. Precipitated material was removed by centrifugation in an

Eppendorf microfuge for 5 minutes and the supernatant removed.

The absorbance of the supernatant at 645 nm and 663 nm was measured on a Shimadzu spectrophotometer against a blank of 80% acetone and the chlorophyll concentration calculated using the following formula:

$$\mu g\ ml^{-1}\ \text{chlorophyll} = (20.2 \times A_{645}) + (8.02 \times A_{663})$$

2.11.3 Electroelution of proteins

Proteins to be eluted from the gel were first visualised by Coomassie blue staining as described in section 2.7.4. The protein was then excised from the gel and placed in a glass tube (diameter 7 mm, length 120 mm) with 0.5 ml of stacking gel set in the bottom (section 2.7.1)

A piece of dialysis tubing sealed by a clip at the bottom was attached to the bottom of the tube. The tube was filled with SDS running buffer (section 2.7.1) and the tube and dialysis bag placed in an electrophoresis tank and the gel slices electrophoresed for 17 hours at 90 V constant voltage.

After electrophoresis the current direction was reversed for 30 seconds and the dialysis bag containing the electroeluted protein removed and dialysed for 3 hours against 1 litre of 10 mM Tris-HCl pH 8.0.

2.11.4 Protein assay

Protein assays were carried out as described by

Bradford (1976). The protein solutions containing between 20 µg and 100 µg of protein in 100 µl volume were pipetted into chromic acid-washed test tubes. To this solution was added 5 ml of assay dye reagent (Biorad) prepared by diluting the stock reagent five-fold with distilled water.

A range of standard protein concentrations were prepared using gamma globulin (Sigma) between 0 and 140 µg per 100 µl, and 5 ml of the same stock reagent was added to each of these.

All the samples were vortexed, left at room temperature for 10 minutes and the absorbance at 595 nm measured using a Shimadzu spectrophotometer against a blank containing 100 µl of water and 5 ml of stock reagent.

A calibration curve was drawn and the amounts of protein in the test samples measured from this curve.

2.11.5 Autoradiography

Gels containing [^{35}S]-methionine-labelled proteins and immunoblots were exposed to Fuji X-ray film at -80°C for enough time for bands to be visualised on the film. When immunoblots were autoradiographed an intensifying screen was used to enhance ionisation.

After exposure the film was developed in Kodak FX-40 developer and fixed in Kodak Unifix. The film was washed and dried.

2.11.6 Photography

All photographs were taken on Kodak Panatomic-X film and the film developed in Ilford FF Contrast developer prior to fixing with Kodak Unifix. All solutions were used as directed by the manufacturer. The negatives were washed and dried and prints made on Kodak F4 professional quality paper. The paper was developed and fixed using the above reagents and then washed and dried.

2.11.7 Scintillation counting of polyacrylamide gel slices

The method used for counting gel slices was developed from that of Barraclough and Ellis (1978). The bands to be counted were excised from the gel with a razor blade. If the gel had previously been dried down it was reswollen for 15 minutes in distilled water before excising the required bands. Two bands were also cut from the blank region of the gel to give a background count.

Each piece of gel was transferred to a plastic scintillation vial and covered with 200 µl of hydrogen peroxide solution (100 vol.) and the gel slices digested at 50°C for 16 hours. Any traces of gel remaining were removed by a further 1 hour incubation at 80°C. The samples were then cooled and 4 ml of Beckman Readysolve scintillation fluid added to each. The samples were shaken until clear and counted on an LKB Minibeta 1212 scintillation counter.

2.12 CHEMICALS

All chemicals used were of the highest grade available. Company suppliers were:-

Amersham International plc, Amersham, Bucks.

L-[^{35}S]-methionine (1000 Ci/mmol^{-1}), Na[^{125}I] (13.5 mCi/μg^{-1})

BDH Chemicals Ltd, Poole, Dorset.

Acrylamide, ammonium persulphate, sodium dodecyl sulphate.

Calbiochem-Behring, Cambridge.

Cellulysin, Macerase.

Eastman Kodak, New York, USA.

N,N'-methylene bisacrylamide,N,N,N,N'-tetramethylene diamine.

Pharmacia (GB) Ltd, London

Sephacryl S300 superfine, DEAE Sephacel, low molecular weight protein kit for SDS gels

Sigma Chemical Co. Ltd, Poole, Dorset.

Adenosine triphosphate (ATP), phenylmethylsulphonyl fluoride (PMSF), Coomassie brilliant blue R, agarose, barbital buffer, gamma globulins (bovine), bovine serum albumin (BSA), *Staphylococcus aureus* protein A, chloramine T, L-cysteine, *Staphylococcus aureus* V8 protease.

SECTION THREE   RESULTS AND DISCUSSION

3.1 CHARACTERISATION OF THE RuBP CARBOXYLASE LARGE SUBUNIT BINDING PROTEIN OF PISUM SATIVUM.

3.1.1 Radiolabelling of *Pisum sativum* chloroplast proteins *in vitro*.

It was first reported in 1962 that chloroplasts contain both DNA (Ris and Plaut, 1962) and ribosomes (Lyttleton, 1962) and since these first observations were made it has become apparent that all the necessary factors required for the synthesis of proteins are present within the chloroplast (Kirk and Tilney-Bassett, 1978). Much work has now been carried out in an attempt to identify the many protein products encoded by the circular DNA within the chloroplast (for review, see Ellis, 1981).

It is possible to analyse the products of chloroplast protein synthesis by first isolating chloroplasts in a suitable medium and then labelling the products of *in vitro* protein synthesis using radiolabelled amino acids, preferably of a high specific activity. The synthesis of proteins in the *in vitro* system requires a source of energy and this can be supplied by either adding ATP to lysed chloroplasts (Bottomley *et al*, 1974) or by using light to drive protein synthesis in intact chloroplasts by photophosphorylation (Blair and Ellis, 1973). The advantage in using intact chloroplasts to synthesise proteins is that quite crude preparations of chloroplasts can be quickly isolated using mechanical extraction (Ellis and Hartley, 1982) and upon illumination only intact chloroplasts will have the ability to generate ATP

necessary for protein synthesis to proceed (Bottomley *et al*, 1974). Since most experiments of this type have been carried out with non-cereal chloroplasts, initial experiments were carried out with pea chloroplasts to gain experience of *in vitro* chloroplast labelling.

Figure 1(A) shows the rate of incorporation of [^{35}S]-methionine into TCA-insoluble protein by isolated pea chloroplasts incubated in a sorbitol resuspension medium. The rate of incorporation reaches a plateau after 10 minutes under these conditions although the chloroplasts are still intact after 30 minutes, as judged by phase contrast microscopy. The fall in the rate of protein synthesis may be due to photodamage by the high light intensity in the incubation chamber or because of gradual leakage of nucleotides from the chloroplasts (Nivison and Jagendorf, 1984). The incorporation of methionine into TCA-insoluble protein can be shown to be taking place in the chloroplast, driven by photophosphorylation, since in the dark control there is very little incorporation, indicating that bacterial contamination is minimal due to the use of sterile solutions.

Analysis of the soluble stromal proteins by SDS denaturing PAGE in Figure 1(B) reveals that by far the most abundant proteins are the large subunit (LSU) and small subunit (SSU) of RuBP carboxylase, which can amount up to 80% of the total soluble leaf proteins in some plant species (Huffaker, 1982).

The major labelled product, after 30 minutes incubation, is shown on the autoradiograph in Figure 1 (C)

Figure 1 Radiolabelling of *Pisum sativum* chloroplast proteins *in vitro*

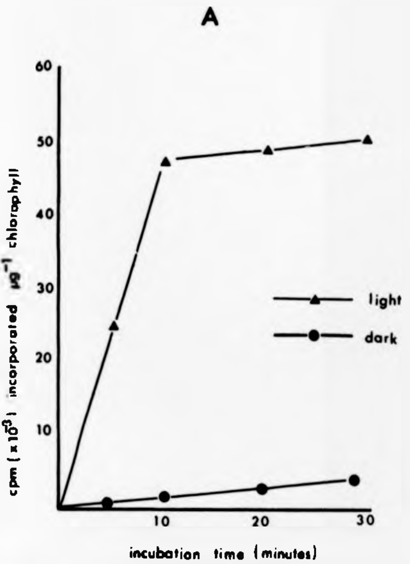
Chloroplasts were isolated from 50 g of pea seedlings as described in Section 2.2.1 and chloroplasts containing 65 µg of chlorophyll resuspended in 200 µl of SRM as described in Section 2.2.2. The suspension was incubated for 30 minutes with 50 µCi of [^{35}S]-methionine as described in Section 2.2.2 in an illuminated water bath and 5 µl aliquots were removed at time points to assay TCA-insoluble incorporation as described in Section 2.2.2. A duplicate sample was treated exactly as above except that all the light had been excluded from the chloroplast by means of aluminium foil.

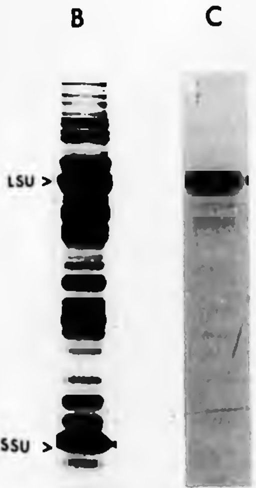
After 30 minutes, 50 µl of the light-incubated chloroplasts were removed and used to make a soluble extract as described in Section 2.2.2. This extract was subjected to SDS PAGE as described in Section 2.7.1.

(A) shows the TCA-insoluble incorporation expressed as cpm ug^{-1} of chlorophyll $x10^{-3}$

(B) shows a stained SDS PAGE analysis of soluble chloroplast proteins

(C) shows an autoradiograph of (B) LSU and SSU represent the large subunit and small subunit of RuBP carboxylase respectively.


A
60
50
40
30
20
10
cpm (x 10^{-3}) incorporated µg^{-1} chlorophyll
light
dark
10
20
30
incubation time (minutes)


B
C
LSU >
SSU >

and migrates with the LSU of RuBP carboxylase. This observation is as expected since the LSU is encoded on the chloroplast DNA of all higher plants that have been studied (Ellis, 1981) and represents the majority (>90%) of the labelled methionine incorporated into soluble protein by isolated pea chloroplasts (Ellis, 1977). The SSU of RuBP carboxylase, which is present in equimolar amounts in the assembled holoenzyme, is not labelled in this experiment since it is synthesised in the cytoplasm of the plant cell and is nuclear-encoded (see Ellis, 1985).

Experiments were then performed to confirm the observation made by Barraclough and Ellis (1980) that newly synthesised LSU made *in vitro* is non-covalently bound to another stromal protein prior to incorporation into the holenzyme.

3.1.2 Assembly of RuBP carboxylase in isolated *Pisum sativum* chloroplasts.

Figure 1(B) illustrates the large number of soluble polypeptides that are found within the the chloroplast stromal compartment. Because of the nature of SDS denaturing PAGE however, it is not possible to analyse proteins in their native form since boiling proteins in SDS will result in their denaturation into constituent polypeptides. However it is possible to analyse soluble proteins without destroying their native stucture by using non-denaturing PAGE in the absence of SDS (Hedrick and Smith, 1968). This technique can be used to study the assembly of newly-synthesised LSU into the RuBP

carboxylase holoenzyme in isolated chloroplasts.

Figure 2(A) shows the result of a time course experiment where soluble stromal extracts have been subjected to non-denaturing PAGE at specific time points during the labelling of chloroplasts in vitro The major staining band on the gel is the RuBP carboxylase holoenzyme (Barraclough and Ellis, 1980) and is shown to consist of LSU and SSU (see Figure 2(C)).

An autoradiograph of Figure 2(A) detects the major labelled soluble products of pea chloroplast protein synthesis and is shown in Figure 2(B). It can be seen that no visible signs of labelled LSU can be seen migrating with the RuBP carboxylase holoenzyme until 60 minutes after the start of the incubation, even though the majority of the LSU had been made by 10 minutes (Figure 1). The major labelled band on the autoradiograph representing the newly-synthesised LSU is migrating more slowly than the RuBP carboxylase on the gel and exactly superimposes the major stained band that migrates above the RuBP carboxylase holoenzyme. This result confirms the original observations made by Barraclough and Ellis (1980) using isolated pea chloroplasts.

It was thought initially that this slowly migrating stained band on the gel was an aggregate composed of unassembled LSU within the chloroplast (Ellis, 1977). This interpretation was shown not to be correct by Barraclough and Ellis (1980) and their finding that the stained band is not LSU is confirmed in Figure 2(C) and (D). The RuBP carboxylase holoenzyme was excised from the non-denaturing

Figure 2 Association of newly-synthesis ed large subunit with the large subunit binding protein.

Pea chloroplasts containing 325 µg of chlorophyll were isolated as in Section 2.2.1. The chloroplasts were resuspended in 1 ml of SRM and incubated with 250 µCi of [35S]-methionine as described in Section 2.2.2 for 120 minutes. At 30 minute intervals 100 µl al iquots were removed and a soluble chloroplast extract isolated as in Section 2.2.2. The soluble extracts were analysed on a 5% non-denaturing polyacrylamide gel as described in section 2.7.2.

After 120 minutes incubation an extra 100 ul sample was taken and subjected to the above treatment. After staining with Coomassie blue the band corresponding to the RuBP carboxylase and the BP were excised from the gel and equilibrated in SDS sample buffer. The slices were boiled for 5 minutes and then analysed by SDS PAGE on a gel with a high bisacrylamide content as described in Section 2.7.1.

(A) shows a 5% stained non-denaturing polyacrylamide gel
used to analysed the soluble chloroplast extracts.

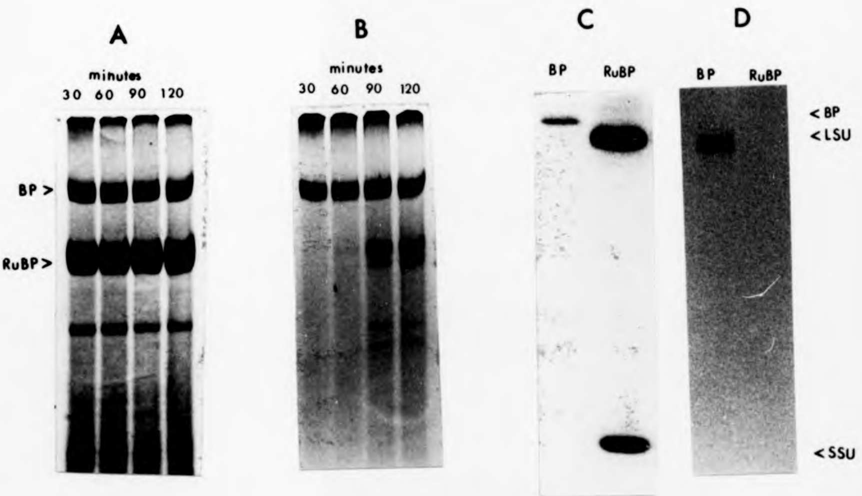
(B) is an autoradiograph of (A)

(C) shows the staining products of SDS PAGE when the BP and
RuBP carboxylase holoenzyme are excised from a 5% non-denaturing gel and subjected to SDS PAGE.

(D) is an autoradiograph of (C)

BP represents the large subunit binding protein

LSU and SSU represent the large and small subunits of RuBP carboxylase.


A
minutes
30 60 90 120
BP >
RuBP >
B
minutes
30 60 90 120
C
BP RuBP
D
BP RuBP
< BP
< LSU
< SSU

gel, after 120 minutes incubation, and analysed on an SDS gel. The two stained bands represent the LSU and SSU as expected. The autoradiograph of Figure 2(C) reveals that no visible signs of the labelled LSU can be seen in the holoenzyme although some labelled LSU comigrates with the holoenzyme, as shown in Figure 2(B) after 120 minutes incubation. This failure to detect labelled LSU in the holoenzyme is perhaps due to insufficient exposure.

The major labelled band was also subjected to this treatment to check the findings of Barraclough and Ellis (1980). The autoradiograph of the excised band, after SDS PAGE, reveals that the labelled band has the same M_r as the LSU. However the major staining band on the SDS gel does not migrate with the LSU but is a protein with a higher molecular mass of 60 KDa. This protein is much more abundant than the labelled LSU since no stained band can be seen on the gel corresponding to the LSU. Because of the properties of this 60 KDa protein it was named the LSU binding protein (BP) by Ellis et al (1980) and postulated to be required for the assembly of the RuBP carboxylase holoenzyme.

The BP binds to the newly-synthesised LSU, and does not simply co-migrate fortuitously with the LSU, since the labelled band on the autoradiograph exactly matches the stained band on the gel when extracts are electrophoresed at different polyacrylamide concentrations (Barraclough and Ellis, 1980) while dissociation of the BP with ATP and Mg^{2+} ions results in the release of associated LSU (see section 3.3.4). The BP must also possess a high affinity

for the labelled LSU since in a stromal extract the labelled LSU can be released from BP with ATP and Mg^{2+} but when the ATP is subsequently removed the relatively minute quantities of labelled LSU reassociate with the BP (Hemmingsen and Ellis, 1985).

The *in vivo* role, if any, of the BP in the assembly of RuBP carboxylase has not yet been determined. Quantitative analysis of autoradiographs of the type shown in Figure 2 (B) reveals that most of the labelled LSU is associated with the BP (Musgrove and Ellis, 1986). A possible role for the BP may thus be to act as a store of LSU that can be released when all the factors required for the assembly of the holoenzyme are present. The LSU associated with the BP has been reported to migrate with the RuBP carboxylase holoenzyme after addition of ATP and Mg^{2+} ions to lysed chloroplast extracts (Milos and Roy, 1984). This report suggests that the binding of LSU with the BP does not occur because of the absence of SSU necessary for the assembly of RuBP carboxylase , since a pool of SSU must already exist within the chloroplast if assembly of holoenzyme occurs subsequently. However whether the comigration of labelled LSU with the holoenzyme results from assembly or binding is not clear.

It is not known if the binding of the LSU to the BP is an obligatory step in assembly of the RuBP carboxylase holoenzyme either *in vitro* or *in vivo* but to date all attempts to assemble the higher plant RuBP carboxylase holoenzyme from cloned gene products in *E.coli* have failed (Gatenby, 1984). A reasonable working hypothesis is thus

that the assembly of higher plant RuBP carboxylase requires the presence of the BP.

The remaining work described in this section (3.1) describes other features of the BP from Pisum sativum and serves to provide a basis for comparison with BP isolated from Hordeum vulgare .

3.1.3 The subunit composition of the Pisum sativum large subunit binding protein

The BP of pea consists of subunits of molecular mass of 60 KDa , as originally reported by Hemmingsen and Ellis (1986) and confirmed in Figure 2(C). This Mr has been calculated from a 15% acrylamide gel with an acrylamide to bisacrylamide ratio of 37:1.

Analysis of the BP on a 15% SDS-denaturing gel with a ratio of acrylamide to bisacrylamide of 100:1 reveals that the BP consists of two subunits . This observation is shown in Figure 4(A) and confirms the original finding of Hemmingsen and Ellis (1986). The slower migrating subunit was termed the α subunit, and the faster migrating subunit the β subunit , by Musgrove and Ellis (1986). Estimation of the molecular mass of the two subunits by SDS PAGE gives values of 61.5 KDa and 59.5 KDa for the α and β subunits respectively (Figure 3). These values compare closely to those obtained by Hemmingsen and Ellis (1986) who reported values of 60.7 KDa and 59.5 KDa .

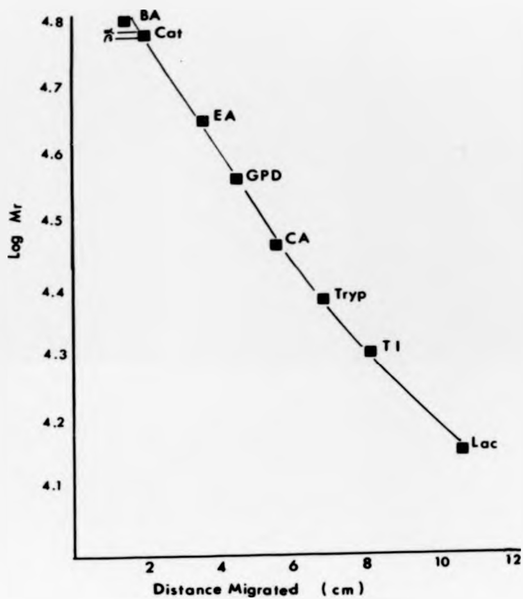
Both subunits co-purify and are present with equal staining intensities when analysed by densitometry (Musgrove and Ellis, 1986). It has thus been suggested

Figure 3 Determination of the subunit relative molecular mass of the large suunit binding protein of *Pisum sativum*.

The apparent molecular mass of the two subunits was determined by the method of Weber and Osborn (1969). Purified BP was analysed on a 15% SDS polyacrylamide gel as described in Section 2.7.1 with molecular weight markers (see Figure 4 A). After electrophoresis the gel was visualised by Coomassie blue staining and the distance migrated by each protein measured from the top of the gel and compared with the molecular weight markers.

The relative molecular masses of the protein of the protein markers x 10^{-3} were ; lactalbumin (Lac) 14.2, trypsin inhibitor (TI) 20, trypsinogen (Tryp) 24, carbonic anhydrase (CA) 29, glyceraldehyde-3-phosphate dehydrogenase (GPD) 36, egg albumin (EA) 45, catalase (Cat) 60, bovine albumin (BA) 66.

Distances migrated: α subunit 21 mm (61.5 KDa)
β subunit 22.5 mm (59.5 KDa)



BA
Cat
EA
GPD
CA
Tryp
TI
Lac
4.8
4.7
4.6
4.5
4.4
4.3
4.2
4.1
Log Mr
2
4
6
8
10
12
Distance Migrated (cm)

Figure 4 Subunit composition of the large subunit binding protein of *Pisum sativum*.

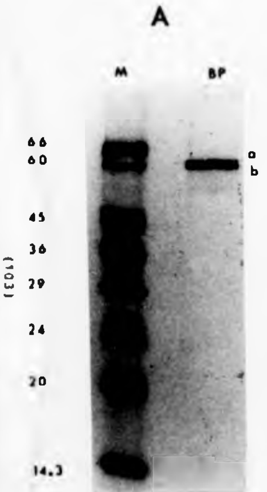
Purified pea BP was kindly provided by Janet Musgrove. Figure 4(A) shows the purified BP subjected to SDS PAGE as described in section 2.7.1. The relative molecular mass of the two subunits is estimated in Figure 3.

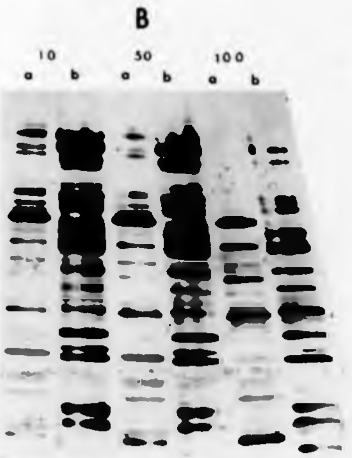
The separate subunits were analysed by partial proteolytic digestion as described in section 2.8. The subunits were isolated by subjecting 3x10 µg samples of the purified BP to 15% PAGE as described in section 2.7.1. The two subunits were excised separately from the gel and analysed on a 10-30% SDS polyacrylamide gel after proteolytic digestion. Three different amounts of V8 protease were used as indicated on the silver-stained gel in (B), where the numbers are µg of protease used.

The difference in isoelectric points of the two BP subunits are shown in (C) after 100 µg of the purified BP was subjected to 2-D analysis as described in Section 2.7.3. The α subunit has an apparent isoelectric point of 5.5 while the value for the β subunit is 6.0 .

BP represents the large subunit binding protein.

a and b represents the two subunits of the large subunit binding protein.


A
M
BP
a
b
66
60
45
36
29
24
20
14.3
(103)


B
10
50
100
a
b
a
b
a
b

C
Basic
Acidic

that the BP, with a molecular mass of 720 KDa , has a subunit composition of α_6 β_6 (Musgrove and Ellis ,1986).

Further evidence that the α and β subunits are different can be seen in Figure 4(B) showing that the partial proteolytic products of the two separate polypeptides are different. Of the 42 proteolytic products only 6 have the same Mr suggesting the two subunits are different and originate from seperate genes (Musgrove et al, 1986). The two subunits also have different apparent isoelectric points as shown in Figure 4(C).

These findings, indicating that the two BP subunits are different, are confirmed by sequence analysis of the first 20 amino acids of each subunit which are seen to be different (Musgrove et al, 1986).

3.1.4 The dissociation of the Pisum sativum large subunit binding protein by ATP

The dissociation of the pea BP by ATP and Mg^{2+} ions was first demonstrated by Bloom et al (1983). Further analysis of stromal extracts by immunoblotting (Lennox and Ellis, 1985) revealed that in the presence of ATP and Mg^{2+} ions the 720 KDa BP is dissociated to its 60 KDa subunits while subsequent removal of ATP results in the reassociation of the 720 KDa BP. The dissociation of the BP is specific to ATP since other nucleotides such as CTP,UTP,GTP,AMP and cyclic AMP will not cause dissociation; Ca^{2+} ions can replace the necessary Mg^{2+} ions however.

Experiments were carried out to further characterise

this ATP-mediated dissociation and to produce data to compare with the barley BP. The first question was to ask whether one or both subunits are produced on dissociation. To determine whether one or both subunits of the BP are released upon dissociation it is necessary to dissociate the BP on a sucrose gradient and analyse the products by SDS-denaturing PAGE, since the two subunits cannot be distinguished on a non-denaturing gel. Figure 5(A) shows the sedimentation of BP in a sucrose gradient in the absence of ATP and Mg^{2+} ions. The BP remains in its oligomeric form and sediments in a sucrose gradient with a sedimentation coefficient reported to be 29S by Milos and Roy (1984). Both subunits of the BP cosediment under these conditions. In Figure 5(B) pure BP was analysed on a sucrose gradient in the presence of 10 mM ATP and Mg^{2+} ions. It can be seen that the oligomeric form of the BP has been completely dissociated by the ATP and Mg^{2+} ions and that both the α and β subunits have been released from the oligomeric form and sediment near the top of the gradient.

Analysis of the dissociation of the BP by ATP on non-denaturing gels revealed that even at an ATP concentration of 5 mM not all of the BP had dissociated (Lennox and Ellis, 1985). This is not the case on the sucrose gradient in Figure 5(B) where dissociation of the BP is complete. The reason for this may be that during the electrophoresis of the BP on the non-denaturing gel the ATP and Mg^{2+} ions will electrophorese out of the sample before full dissociation can occur, or allow some reassociation.

Figure 5 Dissociation of the *Pisum sativum* large subunit binding protein by ATP.

Two 5-50% (w/v) sucrose gradients were prepared as described in Section 2.11.1 with one of the gradients containing 10 mM ATP and 10 mM $MgCl_2$. Equal amounts of purified pea BP (200 µg) were layered onto the top of the gradients; the sample layered onto the top of the gradient containing ATP and $MgCl_2$ was also made to this concentration before layering.

The gradients were centrifuged as described in Section 2.11.1 and fractionated into 24 x 500 µl fractions. The first 10 fractions (100 µl of each fraction) from the top of the gradient were analysed on a 15% SDS polyacrylamide gel as described in section 2.7.1. Below fraction 10 in the gradient no protein was present.

(A) shows the sedimentation profile of the purified BP in the absence of ATP and $MgCl_2$

(B) shows the sedimentation profile of the purified BP in the presence of ATP and $MgCl_2$.

α and β represent the two subunits of the BP.
The track labelled BP contains the purified pea BP and the track labelled M contains molecular weight markers at 66, 45, 36, 29 and 24 KDa (see Figure 3 for details).

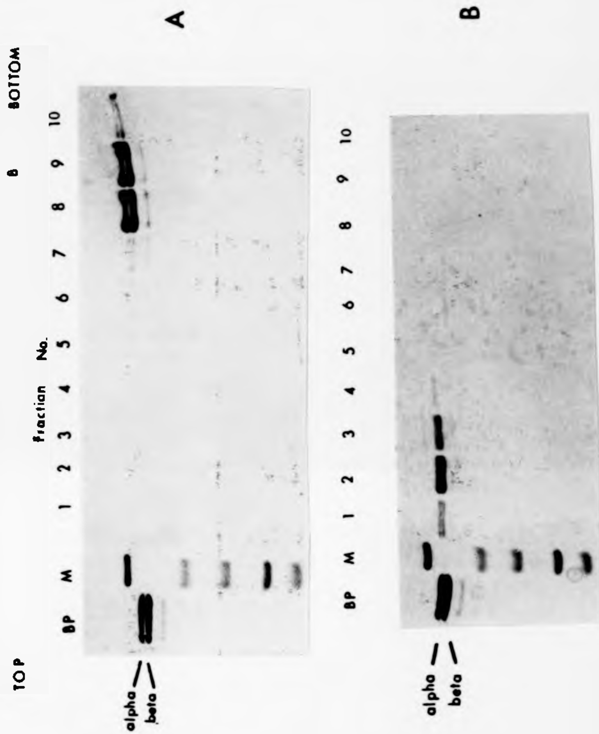

A
B
TOP
BOTTOM
fraction No.
BP
M
1
2
3
4
5
6
7
8
9
10
alpha
beta

Figure 6. Lack of dissociation of the *Pisum sativum* large subunit binding protein by ADP.

Two 5-50% (w/v) sucrose gradients were prepared as described in Section 2.11.1. One gradient contained 10 mM ATP and 10 mM $MgCl_2$ and the other gradient contained 10 mM ADP and 10 mM $MgCl_2$. Equal amounts of purified BP (200 µg) were layered onto the top of the gradients with each sample containing either ADP or ATP at the same concentrations as in the gradients.

The gradients were centrifuged as described in 2.11.1 and fractionated into 24 x 500 µl fractions. The first 10 fractions (100 µl each fraction) from the top of the gradient were analysed on a 15% SDS polyacrylamide gel as described in 2.7.1. Below fraction 10 in the gradient no protein was present.

(A) shows the sedimentation profile of the purified BP in the presence of ADP and $MgCl_2$

(B) shows the sedimentation profile of the purified BP in the presence of ATP and $MgCl_2$.

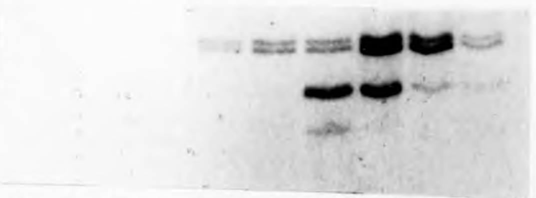
α and β represents the two subunits of the BP and LSU represents the large subunit of RuBP carboxylase. The samples of BP was contaminated by some RuBP carboxylase which acted as an internal marker on the gradient.

TOP BOTTOM

Fraction No.

1 2 3 4 5 6 7 8 9 10

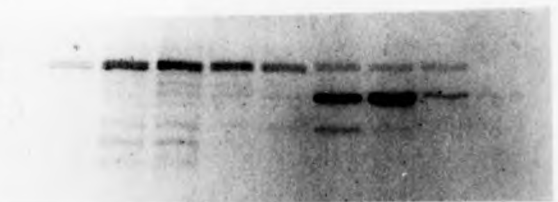
alpha
beta
LSU -



A

1 2 3 4 5 6 7 8 9 10

alpha
beta
LSU -



B

The dissociation of this oligomeric BP into its constituent subunits is specific to ATP; if the ATP in the sucrose gradient is replaced by ADP the BP does not dissociate but remains in its oligomeric form and sediments close to the RuBP carboxylase holoenzyme (Figure 6). The *in vivo* ratio of ATP to ADP may thus be important in determining the proportion of the BP pool that is dissociated into its subunit form with a subsequent release of LSU competent for assembly into the RuBP carboxylase holoenzyme.

Taking all the data from this and published work together, the dissociation of the oligomeric BP into its constituent subunits may thus be represented by the following equilibrium:

$$\alpha_6\beta_6 \xrightleftharpoons{Mg^{2+}\ \ ATP} 6\alpha + 6\beta$$

The position of this equilibrium cannot be determined from these *in vitro* experiments. The concentration of ATP in the chloroplasts has been estimated to be within the range of 1-3 mM (Krause and Heber, 1976) and *in vitro* the BP would be dissociated to some degree at this concentration of ATP since *in vitro* concentrations as low as 0.1 mM ATP results in the partial dissociation of the BP oligomer (Musgrove and Ellis, 1986). Under *in vivo* conditions however the concentration of BP in the chloroplasts is much higher than in the sucrose gradients or in stromal extracts. The concentration of BP in the

chloroplast is estimated at about 10 mg ml^{-1} (Musgrove *et al*, 1986) whilst in the sucrose gradients in Figure 5 and 6 it is approximately 50-fold less concentrated at 200 µg m^{-1} . If the equilibrium represented above is freely reversible, dependent upon ATP concentration, then at higher BP concentrations found in the chloroplast the reverse reaction would be favoured and more of the BP would remain in the oligomeric form than would be observed *in vitro*.

The above equation is probably an over-simplification and it seems likely that other stromal factors may be required in the reassociation of the BP oligomer. Removal of the ATP from a stromal sample containing dissociated BP subunits by dialysis does not lead to the BP reassociating into its oligomeric form (Lennox and Ellis, 1985) perhaps indicating that factors lost during dialysis may be necessary for the BP subunits to reassociate.

3.2 PURIFICATION AND CHARACTERISATION OF THE RuBP CARBOXYLASE LARGE SUBUNIT BINDING PROTEIN OF HORDEUM VULGARE

3.2.1 Association of the newly-synthesised RuBP carboxylase large subunit with the large subunit binding protein in *Hordeum vulgare*.

Experiments have been carried out using chloroplasts from a range of plant species to analyse the products of *in vitro* chloroplast protein synthesis (for review see Ellis, 1981). Much of this work has been carried out using isolated pea chloroplasts which can be isolated mechanically from pea leaves with a high degree of intactness (Ellis and Hartley, 1982). These chloroplasts have the ability to incorporate labelled amino acids into proteins at high rates as confirmed in Section 3.1.

The CASE research award, which forms the basis of this project, involved extending research into the large subunit binding protein (BP) to the important cereal crops of *Hordeum vulgare* (barley) and *Triticum aestivum* (wheat). The initial work on the BP has been carried out using isolated pea chloroplasts in which Barraclough and Ellis (1980) first reported that newly-synthesised LSU was not assembled into the RuBP carboxylase holoenzyme immediately but first bound to the BP. These original experiments by Barraclough and Ellis were repeated in Section 3.1 in order to confirm their findings and to learn the techniques necessary to

extend the work to both barley and wheat. Experiments were thus performed to determine whether newly-synthesised LSU associated with a BP in both wheat and barley chloroplasts.

The first evidence indicating that the BP was present in cereal plants can be seen in Figure 7. Both wheat and barley soluble chloroplast extracts contain a protein of similar subunit molecular mass to the pea BP that cross-reacts immunologically with antiserum raised against the pea BP.

To determine whether this 60 kDa protein has similar properties to the BP present in pea chloroplasts it was necessary to look at the synthesis and assembly of RuBP carboxylase _in vitro_. To analyse the products of barley chloroplast protein synthesis, mechanically-isolated barley chloroplasts were incubated _in vitro_ with [^{35}S]-methionine. The incorporation of labelled methionine into protein is light-dependent as shown in Figure 8(A) and as found with isolated pea chloroplasts in Figure 1(A). The incorporation of [^{35}S]-methionine also reaches a plateau after 15 minutes as found with pea chloroplasts.

The barley chloroplasts were incubated in a KCl osmoticum (KRM) since methionine was not incorporated into protein when barley chloroplasts were incubated in the sorbitol osmoticum (SRM) used for pea chloroplasts confirming an earlier report (Ellis, 1977). Mechanically-isolated wheat chloroplasts did not

Figure 7 Immunological cross-reactivity of the large subunit binding protein of *Hordeum vulgare* and *Triticum aestivum* with antibodies to *Pisum sativum* large subunit binding protein.

Soluble chloroplast stromal extracts were prepared from mechanically-isolated chloroplasts from wheat, barley and pea leaves as described in Section 2.2.2. Equal amounts of stromal protein (100 µg) were subjected to SDS PAGE analysis on a 15% (w/v) acrylamide gel with a high bisacrylamide to acrylamide ratio as described in Section 2.7.1. The gel was then used for immunoblotting as described in Section 2.6.3.

The nitrocellulose filter was probed with 200 µl of antibody raised against the pea BP, supplied by Dr S. Hemmingsen. The bound antibody was detected by $[^{125}I]$-protein A as described in Section 2.6.3 and the exposed autoradiograph is shown opposite.

Track W represents wheat soluble chloroplast proteins

Track B represents barley soluble chloroplast proteins

Track P represents pea soluble chloroplast proteins

W
B
P

synthesise proteins *in vitro* in either SRM or KRM although the chloroplasts used in these experiments appeared to be intact by phase contrast microscopy. As a result of this inability of isolated wheat chloroplasts to incorporate labelled amino acids, all future research into the BP of cereals was carried out on barley.

To determine the major products of *in vitro* chloroplast protein synthesis, the soluble chloroplast proteins were subjected to SDS PAGE after the chloroplasts had been labelled for 30 minutes as shown in Figure 8(B). As found with pea soluble chloroplast proteins, the major staining bands represent the LSU and SSU of RuBP carboxylase. The RuBP carboxylase can represent up to 90% of the soluble leaf protein in cereal plants such as wheat and barley, dependent upon light intensity and nitrogen levels in the soil (Huffaker, 1982).

The labelled products of *in vitro* chloroplast protein synthesis were visualised by autoradiography as shown in Figure 8(C). As with pea chloroplasts (Figure 1(C)) the major labelled product has the same mobility as the LSU of RuBP carboxylase. This is as expected since the barley LSU is encoded within the chloroplast DNA as with all higher plants studied to date (Ellis, 1981).

In order to determine whether this newly-synthesised LSU in barley binds to another protein, it is necessary to analyse the products of *in vitro* barley

Figure 8 Radiolabelling of *Hordeum vulgare* chloroplast proteins *in vitro*

Chloroplasts were isolated from 50 g of 7-day-old barley seedlings as described in Section 2.2.1. Chloroplasts containing 115 µg of chlorophyll were resuspended in 200 µl of KRM as described in Section 2.2.2 and the resuspension incubated for up to 90 minutes in an illuminated water bath as described in Section 2.2.2. At the start of the incubation 50 µCi of [^{35}S]-methionine was added and TCA-insoluble incorporation was assayed at time intervals as described in Section 2.2.2. A duplicate sample was treated exactly as above except that all light was excluded from the chloroplasts by aluminium foil.

After 30 minutes incubation 2 x 50 µl aliquots were removed from the light-incubated chloroplasts and a further 50 µl aliquot was taken after 90 minutes. Soluble chloroplast stromal extracts were made as described in Section 2.2.2. One aliquot, taken after 30 minutes incubation was subjected to SDS PAGE as described in Section 2.7.1. Soluble extracts taken at 30 and 90 minutes were subjected to non-denaturing PAGE as described in Section 2.7.2.

(A) shows the TCA-insoluble incorporation expressed as cpm μg^{-1} of chlorophyll

(B) shows a Coomassie-stained SDS PAGE analysis of soluble barley chloroplast proteins after 30 minutes of labelling

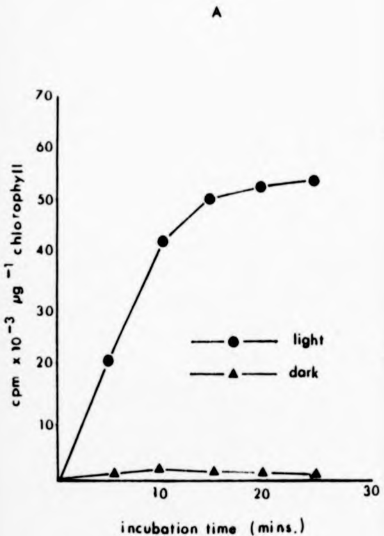
(C) is an autoradiograph of (B)

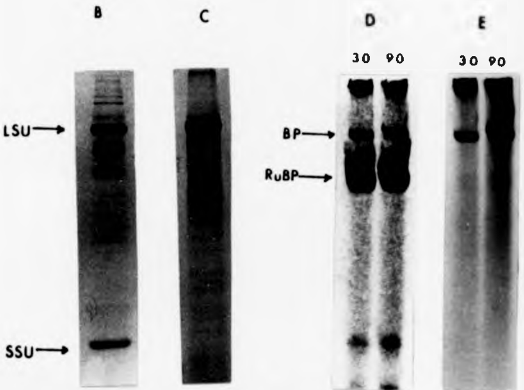
(D) shows a Coomassie-stained 5% non-denaturing gel used to analyse the soluble chloroplast extracts after 30 and 90 minutes incubation

(E) is an autoradiograph of (D)

LSU and SSU represent the large subunit and small subunit of RuBP carboxylase.

RuBP represents the RuBP carboxylase holoenzyme and BP represents the large subunit binding protein.


A
cpm x 10^-3 µg^-1 chlorophyll
70
60
50
40
30
20
10
light
dark
10
20
30
incubation time (mins.)


B
C
D
E
30 90
30 90
LSU
SSU
BP
RuBP

chloroplast protein synthesis under non-denaturing conditions. Figure 8(D) shows that under non-denaturing conditions, as with pea soluble chloroplast proteins, the major staining band is the RuBP carboxylase holoenzyme consisting of large and small subunits. Analysis of the labelled products on non denaturing PAGE (Figure 8(E)) reveals similar results to those obtained with pea chloroplasts. The major labelled band does not migrate with the RuBP carboxylase holoenzyme but with a major staining band migrating more slowly through the gel. This co-migration is exact, with the labelled band on the autoradiograph matching that of the stained band on the gel. This staining protein band that migrates exactly with the major labelled band could thus be the equivalent of the BP in pea chloroplasts. Very little of the newly-synthesised LSU migrates with the RuBP carboxylase holoenzyme after 90 minutes incubation. This may be because the KCl incubation medium does not support RuBP carboxylase assembly in isolated chloroplasts (Barraclough and Ellis, 1980).

To confirm that the major labelled band in Figure 8(E) is LSU, a similar experiment was performed to that described by Barraclough and Ellis (1980) and presented in Figure 2(C). The RuBP carboxylase holoenzyme and the more slowly-migrating band, which is the major labelled product, were excised from the 90 minute track of the non-denaturing gel shown in Figure 8(D). The two bands were subjected to SDS PAGE as shown in Figure 9(A). The

RuBP carboxylase holoenzyme can be seen to consist of both LSU and SSU as expected. The more slowly-migrating BP band consists mainly of a single staining band on the gel with a molecular mass of 60 KDa. There is also a trace of staining LSU which is probably due to contamination of RuBP carboxylase when the BP is excised from the gel.

Analysis of the labelled products of Figure 9(A) by autoradiography in Figure 9(B) reveals that the major labelled product associated with the 60 KDa band has the same molecular mass as the LSU of RuBP carboxylase. There is some labelled LSU assembled into the holoenzyme as shown in Figure 9(B) after prolonged exposure of the autoradiograph.

Although the majority of the labelled LSU visible on the autoradiograph of the non-denaturing gel in Figure 8(E) is associated with the 60 kDa band, it is dif[f]icult to analyse this labelled band as it disappears to a great extent in the SDS dimension. This may be due to protease action and hence PMSF was added to the chloroplast lysis buffer and the excised band was boiled in SDS sample buffer before electrophoresis. Even after this treatment proteolysis of the labelled LSU still occured.

The association of newly-synthesised LSU with a 60 KDa protein in barley chloroplasts is a similar finding to that found by Barraclough and Ellis (1980) for pea chloroplasts. This protein in barley was thus also termed the large subunit binding protein (BP).

Figure 9 Association of the newly-synthesised large subunit with the large subunit binding protein in *Hordeum vulgare*

Barley chloroplast proteins were subjected to non-denaturing PAGE after 90 minutes labelling with [^{35}S]-methionine as described in Figure 8. After staining with Coomassie blue (Section 2.7.4) the RuBP carboxylase and BP bands were excised from the gel and boiled for 5 minutes in 100 µl of SDS sample buffer.

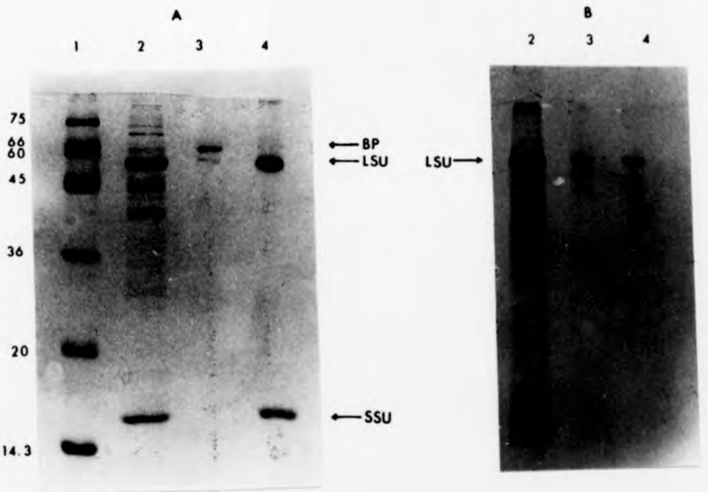
The bands and the sample buffer were transferred to separate tracks on a 15% (w/v) SDS polyacrylamide gel containing a high bisacrylamide to acrylamide ratio. The gel was prepared and electrophoresed as described in Section 2.7.1. After electrophoresis the gel was stained with Coomassie blue as described in Section 2.7.4.

(A) shows the 15% (w/v) polyacrylamide gel stained with Coomassie blue. Track 1 contains molecular weight markers as described in Figure 3. Track 2 contains a soluble barley chloroplast extract after 30 minutes labelling *in vitro* with [^{35}S]-methionine. Track 3 contains the excised BP band. Track 4 contains the excised RuBP carboxylase band.

(B) is an autoradiograph of Tracks 2, 3 and 4.

BP represents the large subunit binding protein.

LSU and SSU represents the large subunit and small subunit of RuBP carboxylase respectively.


A
1
2
3
4
75
66
60
45
36
20
14.3
BP
LSU
SSU
B
2
3
4
LSU

The remaining work in Section 3.2 was undertaken to further characterise the barley BP and to compare and contrast these properties with those of the pea BP.

3.2.2 Production of antibodies to the *Hordeum vulgare* large subunit binding protein.

Work was carried out to raise monospecific antibodies to the BP of barley to use in future experiments, both to identify the BP in soluble protein extracts and to further study the characteristics of the barley BP.

Attempts to purify the barley BP by gel and ion exchange chromatography yielded BP that was contaminated with traces of RuBP carboxylase (see Figure 12). These traces of RuBP carboxylase in the BP preparation produce antibodies in the serum that cross-react immunologically with the LSU and SSU of RuBP carboxylase. This cross-reactivity can be removed by an antigen-affinity column (Morgan, 1986).

To alleviate the problem of RuBP carboxylase contamination, the barley BP was purified by preparative gel electrophoresis as shown in Figure 10(A). The barley BP used to raise antibodies was pure as judged by silver staining after SDS PAGE. After a series of injections into a rabbit a polyclonal antibody was present in the serum that showed an immunological cross-reaction with a 60 kDa protein. This cross-reaction was monospecific in both barley and

Figure 10 Raising monospecific antibodies to the *Hordeum vulgare* large subunit binding protein.

Barley BP was purified by preparative gel electrophoresis and used to raise antibodies by injection into a New Zealand White rabbit as described in Section 2.6.1. Prior to injection the BP was shown to be pure by silver staining after SDS PAGE.

After a series of injections, as described in Section 2.6.1. serum was prepared from the rabbit and tested for anti-BP antibodies by immunoblotting. Equivalent amounts of soluble chloroplast proteins from barley and pea were subjected to SDS PAGE on a 15% gel with a high bisacrylamide to acrylamide ratio, as described in Section 2.7.1. The gel was then immunoblotted as described in Section 2.6.3. using 200 µl of the rabbit serum.

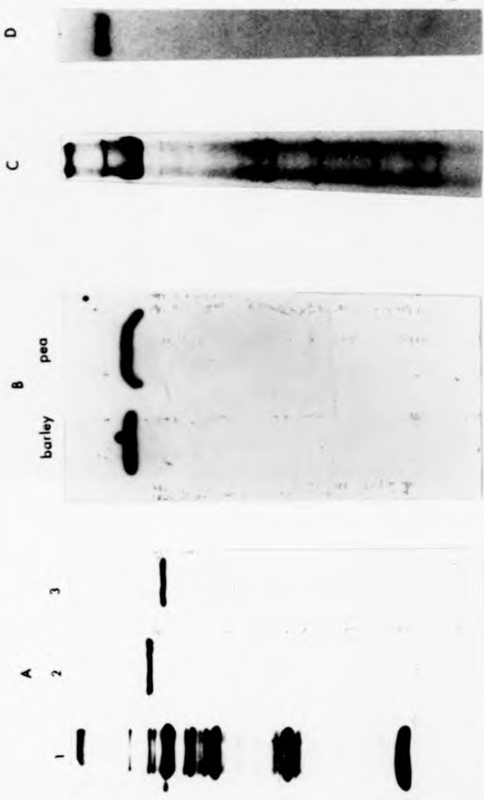
The serum was further tested for cross-reactivity with the barley BP on a non-denaturing gel. A sample of barley soluble chloroplast proteins (200 µg) was subjected to non-denaturing PAGE on a 4-30% non-denaturing gradient gel. The gel was then subjected to immunoblotting as described in Section 2.6.3. using 200 µl of the serum.

(A) is a silver-stained 15% SDS polyacrylamide gel with a high bisacrylamide to acrylamide ratio. Track 1 is loaded with 20 µg of barley soluble stromal protein. Track 2 is loaded with 5 µg of purified barley BP. Track 3 is loaded with 5 µg of barley LSU.

(B) is an immunoblot of barley and pea soluble chloroplast proteins using the serum containing barley anti-BP antibody.

(C) is a 4-30% non-denaturing gel loaded with 200 µg of barley soluble chloroplast proteins.

(D) is an immunoblot of (C) using serum containing the barley anti-BP antibody.


A
1
2
3
B
barley
pea
C
D

pea soluble chloroplast extracts as shown in Figure 10(B).

Further evidence that the protein that cross-reacted with the antibody was the BP can be seen in in Figure 10(D). The antibody cross-reacts with a staining band with exactly the same mobility as the barley BP on a non-denaturing gradient gel; the gel is shown in Figure 10(C). The antibody was stored at -20°C and used in future experiments to characterise the barley BP.

3.2.3 Purification of the *Hordeum vulgare* large subunit binding protein.

The BP of barley was purified to compare and contrast the properties of the protein with the BP of pea. The purification protocol is similar to that used to purify the pea BP by Hemmingsen and Ellis (1985) but with several modifications.

The BP of both pea and barley has no known enzymic activity and the only criterion available for assaying the protein during purification is by its characteristics on gel electrophoresis. This assay is helped by the observation that the BP is the only major staining band from chloroplasts that migrates more slowly than the RuBP carboxylase on a 5% non-denaturing gel. It is also known to have a subunit molecular mass of approximately 60 KDa as shown in Figure 9.

During the course of the research project however, it became clear that the BP of pea is composed of two subunits (Hemmingsen and Ellis, 1986). These subunits

migrate as a single-staining band during SDS-denaturing PAGE, when the polyacrylamide gel contains an acrylamide to bisacrylamide ratio of 37:1. When the pore size of the gel is increased by increasing the acrylamide to bisacrylamide ratio to 100:1 the pea BP can be seen to migrate as two subunits as shown in Figure 14.

The BP of barley was purified from soluble extracts of whole leaves rather than a soluble chlorplast extract as used by Hemmingsen and Ellis (1985). This is because chloroplast preparations made from barley leaves gave very poor recoveries, whilst whole soluble leaf extracts gave larger yields of protein.

The desalted 40-70% ammonium sulphate fraction (see Section 2.4.1 for purification protocol) was eluted from the DEAE-Sephacel ion-exchange column as described. As can be seen in Figure 11(B) the barley BP elutes between 300 and 400 mM KCl. This behavior is different to that of pea BP which elutes between 200 and 300 mM KCl. This difference is an advantage during the purification since a 300 mM KCl elution will remove most of the RuBP carboxylase from the column, and BP can thus be eluted with much of the contaminating RuBP carboxylase removed. Little Coomassie-staining protein could be seen on the gel in fractions eluted with KCl concentrations higher than 400 mM (not shown).

The 300-400 mM KCl eluted fractions, shown to contain BP by gel electrophoresis, were pooled and

subjected to gel filtration on an S300 Sephacryl column as described in Section 2.4.1. To analyse the elution of the BP the column fractions were subjected to non-denaturing and SDS-denaturing PAGE. The gels are shown in Figure 12 . The gel in Figure 12(A) is an SDS-denaturing polyacrylamide gel and has a lower bisacrylamide to acrylamide ratio than used in Figure 11. This results in the BP migrating as two subunits on the gel as found with the pea BP.

Further evidence that the two subunits are derived from the BP can be seen from the non-denaturing gel (Figure 12). The more slowly migrating BP is eluted from the gel filtration column before the RuBP carboxylase holoenzyme confirming that the BP is larger, as in pea.

Fractions 3, 4 and 5 were pooled and stored at -20°C although they contained traces of RuBP carboxylase. A summary of the steps of the purification is shown in Figure 13.

An estimation of the yield of BP during the purification is shown in Table 1.

3.2.4 The molecular mass and subunit composition of the large subunit binding protein of *Hordeum vulgare*

When analysed by SDS PAGE on a gel containing a low bisacrylamide to acrylamide ratio the barley BP migrates as a doublet, as found for pea BP. The slowest-migrating subunit is termed the α subunit and the fastest-migrating subunit the β subunit. The

FIGURE 11 Purification of the *Hordeum vulgare* large subunit binding protein by ion exchange chromatography.

A soluble barley leaf extract was subjected to sequential salt elution after ammonium sulphate fractionation as described in Section 2.4.1. The barley proteins were eluted under gravity with increasing KCl concentration by batch elution.

Fractions containing protein, as determined by absorbance at 280 nm, were pooled and analysed by non-denaturing and SDS PAGE as described in Section 2.7.2. and 2.7.1. respectively.

(A) shows a 15% high bisacrylamide SDS gel. Tracks 1-4 were loaded with 100 µl samples of the 100, 200, 300 and 400 mM KCl elution steps. The proteins were visualised by Coomassie staining.

(B) is a 5% non-denaturing gel loaded with the same samples as above.

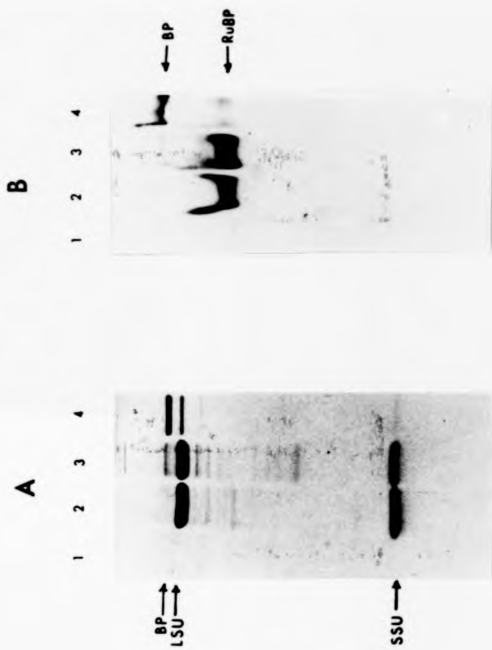

A
B
1 2 3 4
1 2 3 4
BP
LSU
SSU
BP
RuBP

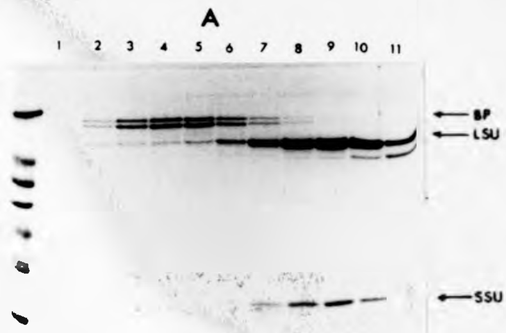
FIGURE 12 Purification of the *Hordeum vulgare* large subunit binding protein by gel filtration.

The 300-400 mM KCl eluate from the ion exchange column shown in Figure 11 was dialysed and concentrated to a final volume of 2 ml as described in Section 2.4.1. The concentrate was loaded onto a Sephacryl S300 column, as described in Section 2.4.1. and eluted at a flow rate of 10 mls per hour.

Fractions containing protein were determined by their absorbance at 280 nm. The peak fractions were analysed by both SDS and non-denaturing PAGE as described in Sections 2.7.1. and 2.7.2. Equivalent fractions (100 µl) were loaded onto both gels.

(A) shows a stained 15% (w/v) polyacrylamide gel containing a low bisacrylamide to acrylamide ratio. Tracks 1-11 are the peak protein-containing fractions from the S300 column. Track M is loaded with molecular weight markers as described in Figure 3 (minus catalase marker).

(B) shows a stained 5% non-denaturing polyacrylamide gel loaded with equivalent fractions as in (A).


A
1
2
3
4
5
6
7
8
9
10
11
BP
LSU
SSU

B
1
2
3
4
5
6
7
8
9
10
11
BP
RuBP

TABLE 1 Summary of the purification of the large subunit binding protein from *Hordeum vulgare*.

50 g of 7-day-old barley seedlings were used to purify BP as described in Section 2.4.1. Protein assays were performed as described in Section 2.11.4.

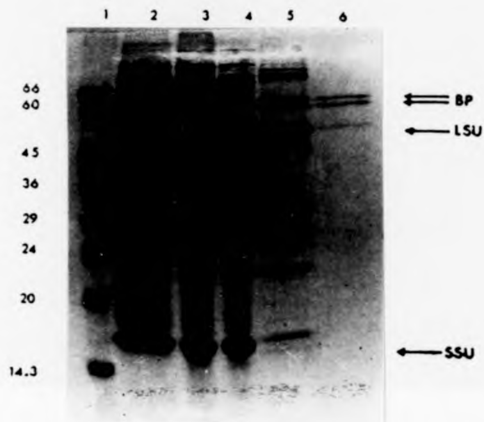
Fraction	Volume (ml)	Protein (mg)
Total soluble leaf extract	164	325
40-70% $(NH_4)_2SO_4$ fraction	10	319
Desalted $(NH_4)_2SO_4$ fraction	45	306
300-400 mm KCl DEAE fraction	123	24.6
S300 pooled BP fractions	34	3.4

% total soluble leaf protein recovered as BP = 1.06%

<u>FIGURE 13</u> Summary of purification of the large subunit binding protein from *Hordeum vulgare*.

Aliquots from each stage of the purification described in Section 2.4.1. were stored at 4°C. A 15% (w/v) SDS gel was loaded with the following samples; The protein concentrations and volumes are shown in Table 1. The gel was stained with Coomassie blue.

Track			
Track	1	molecular weight markers	(as in Figure 3)
	2	Total soluble extract	64 µl
	3	40-70% ammonium sulphate fraction	8 µl
	4	G25 desalting step	15 µl
	5	300-400 mM KCl elution fraction	100 µl
	6	Sephacryl S300 pooled BP fractions	75 µl


1
2
3
4
5
6
66
60
45
36
29
24
20
14.3
BP
LSU
SSU

purified barley BP subunits migrate more slowly through the gel than the pea BP, as shown in Figure 14, with apparent molecular masses of 62 kDa and 61 kDa, calculated from the calibration curve shown in Figure 3. Evidence that the barley BP subunits are distinct polypeptides can be seen from their partial proteolytic digestion products, shown in Figure 15(A). Partial proteolytic digestion of the separate α and β subunits of the barley BP in Figure 15(A) gives different patterns for each of the subunits. This result is similar to that obtained for the two BP subunits of pea, although the products obtained are different between the two species.

Determination of the apparent isoelectric points of the two barley BP subunits is shown in Figure 15(B) The isoelectric points of the two barley subunits are much closer together than the two subunits of the pea BP and also much more acidic. This could explain why the barley BP elutes from a DEAE ion-exchange column at a higher salt concentration than does the pea BP.

To determine the number of subunits in the native BP oligomer, gel filtration was used to estimate the native molecular mass. A Sephacryl S400 column was calibrated with Pharmacia high molecular weight markers as shown in Figure 16. The native molecular mass of the BP was estimated, as shown in Figure 16, to be 758.6 kDa.

This figure is higher than the 720 kDa estimated for the pea BP but this may be due to the slightly

FIGURE 14 The molecular mass of the *Hordeum vulgare* large subunit binding protein subunits.

The BP of *Hordeum vulgare* consists of two subunits; the slowest migrating subunit is termed the α subunit and the fastest the β subunit. Equivalent amounts of pea and barley BP (10 μg) were electrophoresed on a 15% SDS polyacrylamide gel made with a low bisacrylamide to acrylamide ratio (see Section 2.7.1.), which results in the subunits being separated.

Track 1 is loaded with molecular weight makers (see Figure 3 for graphical plot), track 2 with 10 μg of pea BP, track 3 with 10 μg barley BP, track 4 with pea soluble chloroplast proteins, track 5 with barley soluble chloroplast proteins.

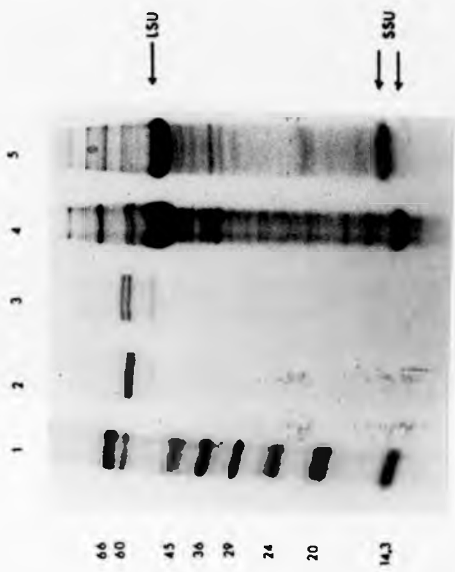

LSU
SSU
1
2
3
4
5
66
60
45
36
29
24
20
14.3

FIGURE 15 Proteolytic analysis of the α and β subunits of the *Hordeum vulgare* large subunit binding protein.

Barley large subunit binding protein was purified as described in Section 2.4.1. and 100 µg of the protein used for isoelectric focussing as described in Section 2.7.3. The focussed subunits were visualised by electrophoresis in a second dimension on a 15% SDS denaturing polyacrylamide gel made with a low bisacrylamide to acrylamide ratio. The gels were stained and destained with Coomassie blue as described in Section 2.7.4. The apparent isoelectric points of the two subunits were obtained as described in Section 2.7.3. The isoelectric point of the α subunit was calculated to be 4.9 and the β subunit to be 5.1.

The partial proteolytic digestion products of the separate α and β BP subunits were obtained as described in Figure 4. The α and β subunits were each digested with either 100 or 50 ng of V8 protease as described in Figure 4 and analysed on a 10% to 30% SDS denaturing polyacrylamide gel.

(A) shows the two subunits of the barley BP analysed, after isoelectric focussing, on a 15% SDS denaturing polyacrylamide gel.

(B) shows a silver-stained SDS-denaturing gradient polyacrylamide gel with the partial proteolytic digestion products of the α and β subunits.

α and β represent the two subunits of the barley large subunit binding protein.

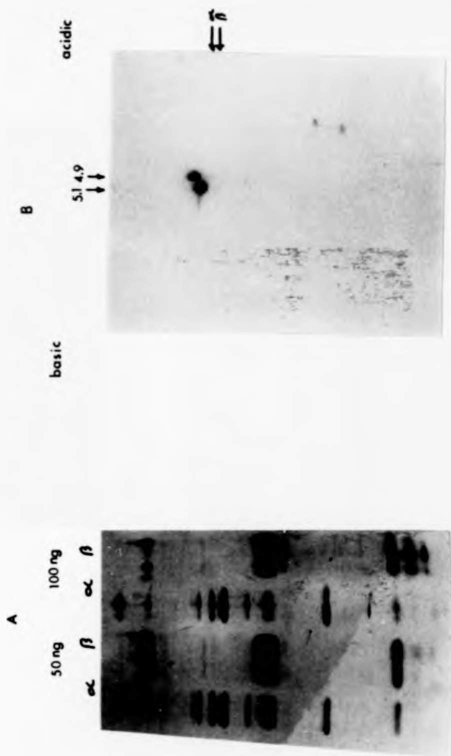

A
50 ng
100 ng
α
β
α
β
B
basic
acidic
5.1 4.9
β

FIGURE 16 The native molecular mass of the *Hordeum vulgare* large subunit binding protein.

The native molecular mass of the barley BP was estimated by gel filtration. A Sephacryl S400 column was packed and calibrated with Sigma high molecular weight standards listed below as described in Section 2.5.

A 6 mg/ml barley chloroplast protein extract (1 ml) was loaded onto the column and the K_{av} of the BP eluted from the column estimated as described in Section 2.5.

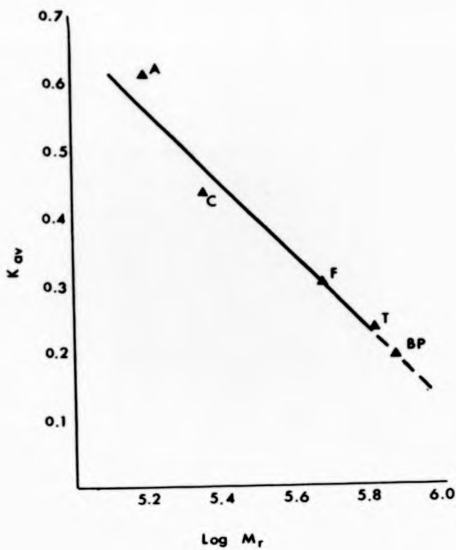
The molecular mass of the barley BP was estimated by extrapolation of the standard curve.

The standards used were: adolase (A) 158 kDa; catalase (C) 232 kDa; ferritin (F) 440 kDa; thyroglobulin (T) 669 kDa.

BP K_{av} = 0.1829

Log M_r = 5.88

Estimated molecular mass = 758.6 kDa



0.7
0.6
0.5
0.4
0.3
0.2
0.1
K_{av}
A
C
F
T
BP
5.2
5.4
5.6
5.8
6.0
Log M_r

larger subunit size of the barley BP subunits. If the barley BP exists as an $\alpha_6\beta_6$ oligomer, as has been suggested for the pea BP (Musgrove and Ellis, 1986), then the expected molecular mass of the barley BP oligomer would be 738 kDa.

The native molecular mass estimated from the column suggests that the BP of barley has 12 subunits. The suggested subunit composition of $\alpha_6\beta_6$ is based on the equal staining intensity of the two subunits when the BP is purified, and on the observation that the two subunits always exactly co-purify. There is no evidence published to date that other α to β ratios cannot exist or indeed that the BP may not consist of α_{12} or β_{12} separate oligomers. This is probably unlikely however, since both subunits always co-purify and both appear in equal amount when the oligomer is dissociated by ATP and Mg^{2+} ions.

3.2.5 Dissociation of the *Hordeum vulgare* large subunit binding protein by ATP

The only chemical property of the pea BP published in the literature, other than its ability to bind the LSU of RuBP carboxylase, is the dissociation of the BP to its monomeric subunits by ATP and Mg^{2+} ions (Bloom, Milos and Roy, 1983). This dissociation was shown to be reversible by Hemmingsen and Ellis (1986), who also demonstrated that the dissociated BP subunits were neither adenylated or phosphorylated during dissociation.

The dissociation of the pea BP by ATP and Mg2+ ions has been confirmed in the present work (see Section 3.1). By sucrose density centrifugation it was shown in addition that both the α and β subunits were released upon dissociation. Figure 6 shows that the BP is not dissociated by ADP, and earlier work by Musgrove and Ellis (1986) reports that other nucleotides such as GTP and UTP will not dissociate the pea BP. Experiments were thus carried out to determine whether the barley BP also showed this dissociation into its individual subunits by ATP and Mg^{2+} ions.

The dissociation of the barley BP was studied _in vitro_ using freshly purified BP, and was assayed by non-denaturing gradient gel electrophoresis. By using gradient gel electrophoresis it is possible to visualize the dissociated subunits of the BP which are not readily visualised on a single 5% non-denaturing gel. On a gradient non-denaturing gel however, it is not possible to distinguish between the α and β subunits of the BP.

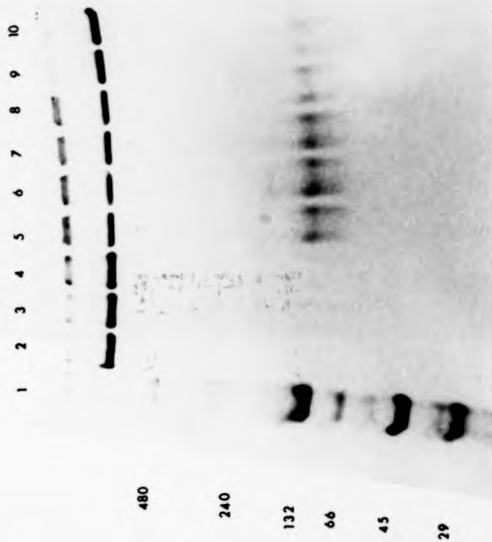
Analysis of the Coomassie-stained gel shown in Figure 17 reveals that, as with the pea BP, the barley BP is also dissociated by ATP and Mg^{2+} ions. This dissociation does not occur when either ATP or Mg^{2+} ions alone are incubated with the BP, while Ca^{2+} ions can replace the Mg^{2+} ions and may increase the degree of dissociation (Figure 17). The incubation conditions used are similar to those used in the original experiments by Milos and Roy (1984) although,

FIGURE 17 Dissocation of the *Hordeum vulgare* large subunit binding protein by ATP.

Freshly purified BP was prepared as described in Section 2.4.1. and stored at 4°C. Nine equal aliquots (80 µl volume) containing 30 µg of barley BP were stored in separate Eppendorf tubes on ice. The following additions were made (see table below) and, following the appropriate incubations, the entire sample was subjected to non-denaturing gel electrophoresis on a 4-30% (w/v) gradient gel as described in Section 2.7.2. After electrophoresis the gel was stained and destained in Coomassie blue as described in Section 2.7.4.

	final concentration			1st incubation	2nd incubation
Track	10 mM ATP	10 mM $MgCl_2$	10 mM $CaCl_2$	(min) 0°C	(min) 24°C
2	-	-	-	0	0
3	-	+	-	30	60
4	+	-	-	30	60
5	+	+	-	30	60
6	+	-	+	30	60
7	+	+	-	0	90
8	+	+	-	90	0
9	+	+	-	0	0
10	-	-	-	0	0

Track 1 was loaded with Sigma molecular weight markers for non-denaturing PAGE. These were:
α lactalbumin 14,200; carbonic anhydrase 29,000; albumin (chicken egg) 45,000; albumin (bovine) monomer 66,000 and dimer 132,000; urease (Jack Bean) dimer 240,000 and tetramer 480,000.


1
2
3
4
5
6
7
8
9
10
480
240
132
66
45
29

as shown in Figure 17, there is no requirement for a 0°C pre-incubation, prior to incubation at 24°C, to dissociate the BP.

The molecular mass of the dissociated BP subunit on the gel is difficult to measure. With the dissociated pea BP the molecular mass is 60 kDa (Lennox and Ellis), but the dissociated subunits from barley BP electrophorese as a smear between the 66 and 132 kDa markers, perhaps indicating that the dissociated BP may in fact be in dimeric form. In all of the tracks where ATP and divalent cations are present the BP has not dissociated to completion. This may be due to the ATP and Mg^{2+} ions electrophoresing out of the sample before dissociation is complete. It is therefore not possible to determine by this method whether both the α and β subunits are released from the 760 kDa BP.

In order to determine whether both subunits are released upon dissociation of the BP it is necessary to analyse the dissociated BP subunits on a sucrose gradient as used for pea BP (see Figure 6).

Barley BP was analysed on a sucrose gradient as described in Figure 18 and the gradient fractions analysed on a 15% SDS polyacrylamide gel made with a low bisacrylamide to acrylamide ratio. The BP used in this experiment was contaminated with RuBP carboxylase which acted as an internal marker on the gradient. As can be seen from Figure 19, the barley BP subunits sediment slightly ahead of the RuBP carboxylase holoenzyme in the absence of ATP. In the gradient

FIGURE 18 Dissociation of the *Hordeum vulgare* large subunit binding protein by ATP on sucrose gradients.

Two 5-50% (w/v) sucrose gradients were prepared as described in Section 2.11.1, with one of the gradients containing 10 mM ATP and 10 mM $MgCl_2$. Equal amounts of purified barley BP (20 µg) were layered onto the top of the gradients; the sample layered onto the top of the gradient containing ATP and $MgCl_2$ was also made to this concentration before loading.

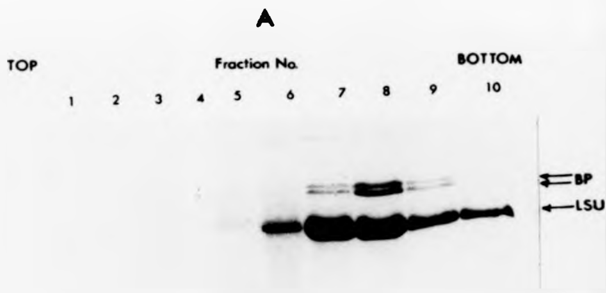
The gradients were centrifuged as described in Section 2.11.1 and fractionated into 24 x 500 µl fractions. The first ten fractions (100 µl of each fraction) from the top of the gradient were analysed on a 15% (w/v) SDS polyacrylamide gel as described in Section 2.7.1. Below fraction 10 in the gradient no protein was present.

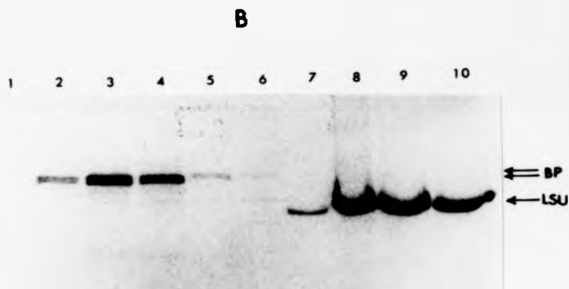
(A) shows a sedimentation profile of barley BP in the absence of ATP and $MgCl_2$.

(B) shows a sedimentation profile of barley BP in the presence of ATP and $MgCl_2$.

α and β represent the two subunits of the barely BP.

LSU represents the large subunit of RuBP carboxylase.


A
TOP
Fraction No.
BOTTOM
1 2 3 4 5 6 7 8 9 10
BP
LSU


B
1 2 3 4 5 6 7 8 9 10
BP
LSU

containing ATP and Mg_{2+} ions the barley BP has dissociated and both the α and β subunits sediment near the top of the gradient. The dissociation, under these conditions,is complete. These subunits migrate either as monomers or dimers as suggested in Figure 17.

The dissociation of the pea BP by ATP and Mg^{2+} into its subunits results in the release of the LSU bound to BP. Experiments by Milos and Roy (1984), using in vitro-labelled chlorplast extracts, have shown that the release of the labelled LSU from the BP by ATP and Mg^{2+} is followed by the subsequent migration of the labelled LSU with the RuBP carboxylase holoenzyme. The BP may therefore be acting as a store for unassembled LSU prior to holoenzyme assembly. Experiments similar to these performed by Milos and Roy were thus carried out using labelled barley chloroplast extracts to determine whether the LSU associated with the BP was released in the presence of ATP and Mg^{2+} ions and whether this led to subsequent comigration of the labelled LSU with the holoenzyme.

A soluble chloroplast extract was made from barley chloroplasts incubated in the presence of [^{35}S]-methionine as described in Section 7. As can be seen from the autoradiograph shown in Figure 19, when the labelled soluble extract is analysed on a 5% non-denaturing polyacrylamide gel, the labelled band containing the unassembled LSU migrates with the barley BP. When the chloroplast extract is incubated with ATP and Mg^{2+} ions, exactly as described by Milos and Roy

FIGURE 19 Dissocciation of the *Hordeum vulgare* large subunit binding protein in *in vitro*-labelled chloroplast extracts.

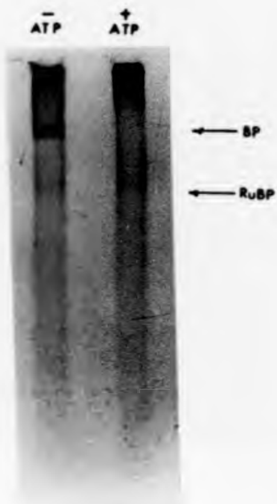
Barley chloroplasts, equivalent to 100 µg of chlorophyll, were prepared as described in Section 2.2.1. The chloroplasts were labelled with [^{35}S]-methionine as described in Figure 7 and the chloroplasts divided into two equal aliquots after 30 minutes labelling.

The chloroplasts were lysed in 100 µl of ice-cold 10 mM Tris-HCl pH7.6. A soluble stromal extract was obtained by centrifugation in an Eppendorf microfuge for 5 minutes at 4°C. To 50 µl of each of the stromal extracts, 50 µl of the following buffer was added:

100 mM HEPES
440 mM KCl
12 mM $MgCl^2$
40 mM DTT
1 mM methionine
pH 7.6

One sample was incubated on ice for 90 minutes whilst the other was made to 5 mM ATP and after 30 minutes on ice was incubated at 24°C for 60 minutes. Both soluble chloroplast extracts were subjected to PAGE on a 5% non-denaturing gel. An autoradiograph of the gel after 48 hours exposure is shown opposite.

RuBP represents the position of the RuBP carboxylase holoenzyme on the gel and BP represents the position of the large subunit binding protein on the gel.



−
ATP
+
ATP
BP
RuBP

(1984), the BP of barley dissociates. The labelled LSU associated with the BP is also released. The autoradiograph reveals that, although incubation with ATP at 24°C results in release of the LSU from the BP, there has been no subsequent increase of label at the RuBP carboxylase holoenzyme position on the native gel, as found by Milos and Roy (1984). The reason for this may be due to the difference in the media used to incubate the chloroplasts or perhaps to the presence of ATP-dependent proteases in the barley stromal extract. These proteases may destroy the unassembled LSU, stopping assembly into the RuBP carboxylase holoenzyme (Liu and Jagendorf, 1984)

3.2.6 Discussion.

The chloroplasts of wheat and barley both contain an abundant, soluble protein that cross-reacts immunologically with antiserum raised against the BP of pea. Further work in Section 3.2 has demonstrated that this protein from barley shows many of the characteristics of the BP isolated from pea chloroplasts although there are some slight differences between the two species. In isolated chloroplasts of both pea and barley, the BP has been shown to bind non-covalently to the newly-synthesised LSU of RuBP carboxylase.

The purified BP of pea and barley chloroplasts consists of two subunits. These have been termed the α

and β subunits (Musgrove and Ellis, 1986) and form an oligomeric protein consisting of 12 subunits. Due to the exact co-purification of the two subunits their equal staining intensities and their appearance together when the BP is treated with ATP and Mg^{2+}, it has been suggested that the 720 kDa BP oligomer has the subunit composition $\alpha_6\beta_6$ (Musgrove and Ellis, 1986). The estimated molecular mass of the oligomeric BP of barley also aproximates to an $\alpha_6\beta_6$ oligomer. The number of newly-synthesised LSU molecules attached to each oligomeric molecule of BP is unclear. It is however unlikely to be more than one or two since a greater number would lead to an altered mobility on native gel electrophoresis, and it has been demonstrated by Musgrove and Ellis (1985) that the labelled LSU band exactly matches the stained BP band from pea on the non-denaturing gel. The same matching was also observed for the BP protein from barley in the present work.

The BP of both pea and barley is dissociated in vitro by ATP and Mg^{2+} ions and this dissociation is reversible (Hemmingsen and Ellis, 1986). Upon dissociation the LSU associated with the BP is released from the BP and sediments in a sucrose gradient with an estimated sedimentation coefficient of 7S (Bloom et al. 1983). It is not clear whether the LSU is still associated with the BP under these conditions but it has been estimated that at a sedimentation coefficient of 7S the LSU would exist either as a LSU-LSU dimer or as a LSU-BP heterodimer (Cannon et al, 1986). Under

conditions where the dissociated BP is reassociated to its oligomeric form by removal of endogenous ATP by protein synthesis (Hemmingsen and Ellis, 1986) the newly-synthesised LSU reassociates with the BP oligomer. This reassociation of LSU with the BP oligomer requires the BP to have an extremely high affinity for unassembled LSU since the concentration of labelled LSU in the chloroplast extracts must be extremely low. This is perhaps further evidence that the binding of LSU to BP is a highly specific event and not merely a non-physiological sticking of LSU to another chloroplast protein.

Throughout the course of this research project experiments have been designed under the working hypothesis that the BP is involved in the synthesis and assembly of the RuBP carboxylase holoenzyme. A more detailed discussion of the possible role of BP in the assembly of RuBP carboxylase is presented in Section 4.

To date all attempts to assemble the RuBP carboxylase holoenzyme of higher plants from the co-expression of cloned LSU and SSU genes in bacterial cells have failed (Gatenby, 1984). If the BP is a necessary factor involved in the assembly of RuBP carboxylase, it will be necessary to express cloned cDNA sequences of the BP subunits in any future attempts to express RuBP carboxylase genes in bacteria. The properties of the BP subunits, and their subsequent cloning and expression, may thus become important in any future attempts to alter the catalytic properties

of the RuBP carboxylase holoenzyme of higher plants by site-directed mutagenesis (Gutteridge *et al*, 1984).

3.3 IN VIVO RADIOLABELLING OF HORDEUM VULGARE PROTEINS

3.3.1 Radiolabelling of *Hordeum vulgare* leaf proteins

Experimental observations made by Barraclough and Ellis (1980), and confirmed in Section 3.1.2, demonstrated that in isolated chloroplasts the newly-synthesised LSU was not immediately assembled into the RuBP carboxylase holoenzyme but first bound to a soluble chloroplast protein, the BP. Experiments described in Section 3.2.1 extended this observation to isolated barley chloroplasts. Experiments were carried out employing the *in vivo* radiolabelling of barley leaves in an attempt to determine the site of synthesis of the barley BP subunits. During the *in vitro* radiolabelling of pea chloroplasts the subunits of the pea BP are not labelled suggesting that the BP is synthesised by cytoplasmic ribosomes and hence nuclear-encoded, although this is not conclusive evidence (see Discussion). Evidence from barley chloroplast labelling experiments also suggests that the barley BP is nuclear-encoded, since the barley BP is not labelled in isolated chloroplasts (see Section 3.2.1). This is not conclusive evidence however; the lack of labelling of the BP during the incubation may be due to the non-physiological nature of the *in vitro* chloroplast labelling experiments (see Discussion). *In vivo* radiolabelling experiments were thus carried out in the presence and absence of specific inhibitors of cytoplasmic protein synthesis in order to determine the site of synthesis of the BP subunits.

The two-dimensional separation of leaf proteins labelled in the absence of inhibitors is shown in the autoradiograph in Figure 20. Under these conditions the LSU and SSU of RuBP carboxylase are both labelled as shown. It can also be seen that both the α and β subunits of the BP are labelled, indicating that both subunits contain methionine.

A barley leaf was radiolabelled in the presence of cycloheximide and soluble leaf proteins separated by two-dimensional gel electrophoresis, as shown in Figure 21. In the presence of cycloheximide protein synthesis on cytoplasmic ribosomes in barley leaves is inhibited (Criddle et al, 1970). Since there is no evidence to suggest that mRNA can cross the chloroplast envelope, all proteins labelled under these conditions will be synthesised by chloroplast ribosomes. The SSU of RuBP carboxylase should not be labelled in the presence of cycloheximide as it is synthesised by cytoplasmic ribosomes. As can be seen in Figure 21, in the presence of cycloheximide the SSU is not labelled. Under these labelling conditions the α and β subunits of the BP are not labelled since the arrowed position of the two subunits on the two-dimensional separation shows no label has been incorporated. This is evidence that the two barley BP subunits are nuclear encoded. It is not conclusive evidence however since the BP subunits may be synthesised in the chloroplast but rapidly degraded in the absence of cytoplasmic protein synthesis (see Discussion). This type of experiment can only give conclusive

Figure 20 *In vivo* radiolabelling of *Hordeum vulgare* leaf proteins.

A single 6-day-old barley leaf was excised at the leaf base (under water) and the cut end of the leaf was immersed in 50 µl of water containing 50 µCi of [^{35}S]-methionine.

The leaf was incubated under the conditions described in Section 2.9 for 6 hours. The soluble leaf proteins were then extracted as described in Section 2.3.

An aliquot of the radiolabelled soluble proteins containing 200 µg of protein was freeze-dried and subjected to isoelectric focussing as described in Section 2.7.3. The gels were then subjected to a second dimension in SDS PAGE as described in Section 2.7.1. A gel with an acrylamide to bisacrylamide ratio of 100:1 was used in order to resolve the α and β subunits of the BP.

After electrophoresis the gel was stained in Coomassie blue and subjected to autoradiography as described in Section 2.7.4 and 2.11.5 respectively. A photograph of the autoradiograph is shown opposite.

LSU and SSU represent the large and small subunits of RuBP carboxylase respectively.
α and β represent the two subunits of the large subunit binding protein.

Acidic
Basic
SDS
LSU
SSU

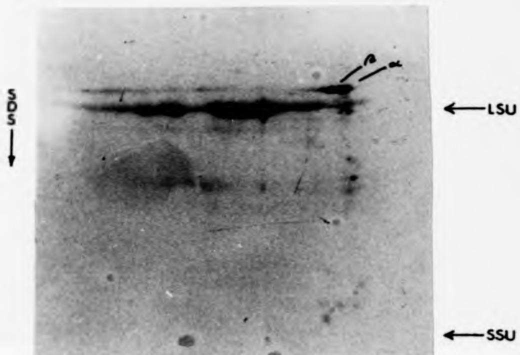
Figure 21 *In vivo* radiolabelling of *Hordeum vulgare* leaf proteins in the presence of cycloheximide.

A single 6-day-old barley leaf was radiolabelled as described in Figure 20 except that for 15 minutes before radiolabelling and throughout the 6 hour incubation cycloheximide was present in the labelling solution at a concentration of 10 $\mu g\ ml^{-1}$.

After 6 hours labelling the soluble leaf proteins were extracted and subjected to isoelectric focussing and SDS PAGE as described in Figure 20. The labelled proteins were visualised by autoradiography as described in Section 2.11.5. A photograph of the autoradiograph after 24 hours exposure is shown opposite.

LSU and SSU represent the position of the large and small subunits of RuBP carboxylase respectively.

α and β represent the two subunits of the large subunit binding protein.


Acidic
Basic
SDS
β
α
LSU
SSU

determination of the site of synthesis of a protein if a particular protein is still synthesised in the presence of one type of inhibitor but not another. Conclusive proof that the BP subunits are cytoplasmically-synthesised would be shown by their continued labelling in the presence of a selective inhibitor of chloroplast protein synthesis such as D-chloramphenicol. Attempts to selectively inhibit chloroplast protein synthesis by feeding D-chloramphenicol to barley leaves produced soluble, labelled leaf extracts in which the LSU was still labelled but at a reduced rate (not shown). This result indicates that chloroplast protein synthesis was still occurring under these conditions.

The use of whole leaves for *in vivo* radiolabelling experiments provides the most physiological conditions in which to study the synthesis and assembly of chloroplast proteins. However there are problems associated with labelling experiments with whole leaves, particularily related to the sampling and extraction of soluble proteins during time-course experiments. In the next section protoplasts were used to overcome these sampling difficulties to analyse the synthesis and assembly of RuBP carboxylase *in vivo*

3.3.2 Radiolabelling of *Hordeum vulgare* protoplasts

The assembly of the LSU of RuBP carboxylase cannot be studied in barley using isolated chloroplasts labelled as described in Section 3.2.1. This is because under the

incubation conditions used the newly-synthesised LSU is not assembled into the RuBP carboxylase holoenzyme. It is thus necessary to study the assembly of RuBP carboxylase in an intact cellular environment.

Experiments in this section were carried out using intact mesophyll protoplasts isolated from young barley leaves to study the synthesis of the LSU in vivo its association with the BP, and its assembly into RuBP carboxylase.

The labelling of protoplasts provides an excellent system to study the in vivo assembly of RuBP carboxylase for several reasons. Homogeneous suspensions of protoplasts can be quickly and evenly labelled by adding [^{35}S]-methionine to the incubation medium. The protoplasts can be sampled during the labelling time course simply by pipetting incubation samples and soluble extracts obtained by lysis in low ionic strength buffers. Specific inhibitors to protein synthesis can also be added to protoplasts to quickly inhibit protein synthesis.

Previous work on protoplast protein synthesis has been carried out by other research groups. Nishimura and Akazawa (1978) studied the radiolabelling of RuBP carboxylase in Spinacia leaf protoplasts with [^{14}C]-leucine and Barraclough and Ellis (1979) analysed the synthesis and assembly of RuBP carboxylase in isolated soybean leaf cells. Wheat protoplasts have been isolated previously by Edwards et al (1978) and used as a source of intact chloroplasts competent in photosynthesis. The procedure of Edwards et al was used to isolate barley

leaf protoplasts, except that the young leaves had the lower epidermis removed prior to enzymatic isolation of protoplasts.

Experiments were carried out to analyse the soluble radiolabelled products of protoplast protein synthesis in the presence and absence of specific inhibitors. Isolated, intact protoplasts were radiolabelled in a sorbitol medium used by Walker et al (1978) to study protoplast photosynthesis. The medium also contained $CaCl_2$ which enhanced incorporation and intactness. The protoplasts were labelled at a light intensity and temperature equivalent to the growth conditions of the seedlings. If protoplasts were labelled at light intensities used for chloroplast protein synthesis they quickly lysed due to photodamage.

The soluble products of protoplast protein synthesis are shown in the autoradiograph in Figure 22(B). Track 4 shows that under the conditions of labelling used many proteins are labelled including the LSU and SSU, showing both cytoplasmically-synthesised and chloroplast-synthesised proteins are labelled in this experiment. In the presence of cycloheximide it was expected that only proteins synthesised in the chloroplast would be labelled (Criddle et al, 1970). As can be seen in Figure 22(B) track 5, in the presence of cycloheximide the SSU (and many other proteins) are not labelled. Under these conditions the LSU is still labelled as expected.

Two other labelled bands in Figure 22(B) track 5, migrating more slowly than the LSU, are also labelled in

Figure 22 Inhibition of cytoplasmic protein synthesis in *Hordeum vulgare* protoplasts by cycloheximide.

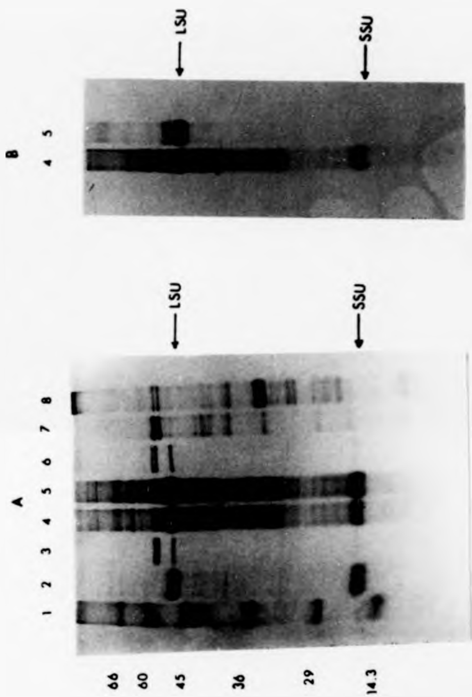
Mesophyll protoplasts from 6-day-old barley seedlings were isolated as described in Section 2.10.1 and resuspended in incubation medium as described in Section 2.10.2.

The protoplasts were divided into two 1 ml aliquots containing 300 µg of chlorophyll each, in a 25 ml conical flask. One of the flasks had cycloheximide added to a final concentration of 10 µg ml^{-1}. The flasks were incubated in a bottom-illuminated light bath as described in Section 2.10.2 and after 15 minutes incubation, 250 µCi of [^{35}S]-methionine was added to each flask.

After labelling for 60 minutes the protoplasts were isolated and soluble protein extracts made by lysing the protoplasts in 1 ml of lysis buffer as described in Section 2.10.2. The two soluble extracts (10 µl of each) were analysed by SDS PAGE as described in Section 2.7.1 and autoradiographed as described in Section 2.11.5.

(A) shows a photograph of the SDS gel loaded as follows: 1) molecular weight markers; 2) RuBP carboxylase; 3) barley BP; 4) radiolabelled protoplast proteins; 5) radiolabelled protoplast proteins in presence of cycloheximide; 6) barley BP; 7) barley ATPase subunits; 8) barley thylakoid proteins.

(B) shows an autoradiograph of (A) 4 and 5 being tracks 4 and 5 of gel (A)



A
1 2 3 4 5 6 7 8
B
4 5
LSU
SSU
LSU
SSU
66
60
45
36
29
14.3

the presence of cycloheximide. The two labelled bands migrate with the same mobility as the α and β subunits of the ATPase coupling factor, shown in Figure 22(A) track 7. The α and β subunits of the ATPase are chloroplast encoded and as can be seen in Figure 22(A) the α subunit of the ATPase coupling factor has exactly the same mobility on the SDS gel as the β subunit of the barley BP. From this reult it is only possible to conclude that the α BP subunit is not synthesised on chloroplast ribosomes.

Experiments were performed, using intact protoplasts, to study the synthesis and assembly of RuBP carboxylase in the absence of SSU synthesis. These experiments were performed to determine whether the LSU would bind to the barley BP _in vivo_ particularily in the absence of SSU synthesis.

Cycloheximide was used to inhibit SSU synthesis; the inhibitor was present throughout the three hour isolation procedure and throughout the labelling experiment, at a concentration of 10 μg ml^{-1}, in order to deplete any internal pools of SSU prior to labelling.

The incorporation of [^{35}S]-methionine into TCA-insoluble protein by protoplasts (labelled in the presence and absence of cycloheximide) is represented graphically in Figure 23. The label incorporated by the protoplasts increases over the 150 minute labelling period. Protoplasts that have been labelled in the presence of cycloheximide do not incorporate [^{35}S]-methionine at as high a rate as in its absence.

Analysis of the soluble extracts by SDS PAGE followed

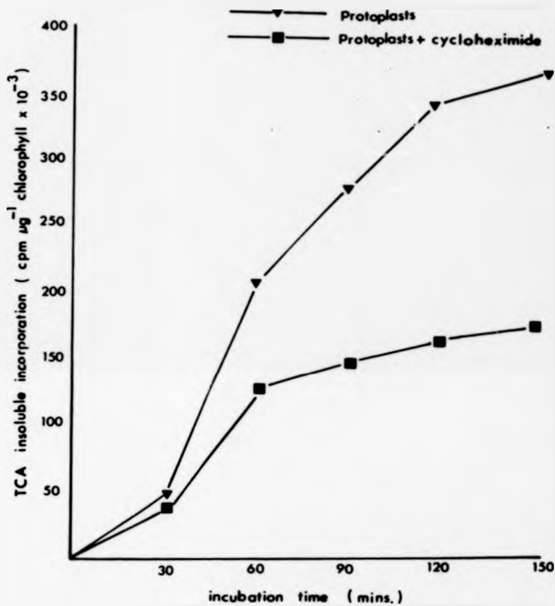
Figure 23 TCA-insoluble incorporation of [^{35}S]-methionine by isolated *Hordeum vulgare* protoplasts in the presence and absence of cycloheximide.

Mesophyll protoplasts from 6-day-old barley seedlings were isolated as described in Section 2.10.1 and resuspended in incubation media as described in Section 2.10.2. Half of the protoplasts were isolated, during the three hour digestive extraction, in the presence of 10 µg ml^{-1} cycloheximide.

Both protoplast preparations were incubated in 1 ml of incubation medium as described in Section 2.10.2 in two 25 ml conical flasks at a concentration of 300 µg ml^{-1} chlorophyll. The protoplasts isolated in the presence of cycloheximide were also incubated in the presence of cycloheximide at the same concentration.

The two conical flasks were placed in a bottom-illuminated light bath as described in Section 2.10.2 and the labelling time-course started by the addition of 250 µCi of [^{35}S]-methionine to each flask.

At various time points shown opposite 5 µl of the protoplast suspensions was removed and TCA-insoluble incorporation assayed as described in Section 2.2.2. The results, in the presence and absence of cycloheximide, are shown opposite.


Protoplasts
Protoplasts + cycloheximide
TCA insoluble incorporation (cpm µg⁻¹ chlorophyll x 10⁻³)
400
350
300
250
200
150
100
50
30
60
90
120
150
incubation time (mins.)

by autoradiography revealed that cytoplasmic protein synthesis had been inhibited as shown by the lack of SSU synthesis (not shown).

During the incubation period aliquots of protoplasts were removed from the incubation flasks and soluble extracts analysed by gradient non-denaturing PAGE as described in Figure 24. The autoradiograph, showing the labelled products, is shown in this Figure.

In the absence of cycloheximide the labelling of RuBP carboxylase and BP in Figure 24 shows that the BP contains only a small proportion of the label in relation to the RuBP carboxylase holoenzyme. If the BP is acting as an intermediate store for the LSU prior to the assembly of RuBP carboxylase then the assembly process must be taking place much more quickly than in isolated chloroplasts (see Figure 2). It is not possible to assess the proportion of newly-synthesised LSU associated with the BP or assembled into RuBP carboxylase from the non-denaturing gel alone. This is because the BP subunits and the SSU are also labelled under these conditions. In order to assess the amounts of LSU in each pool it would be necessary to excise the bands from the gel and separate the LSU in a second dimension by SDS PAGE prior to assessing incorporation. Attempts to assay the newly-synthesised LSU in this way were unsuccessful.

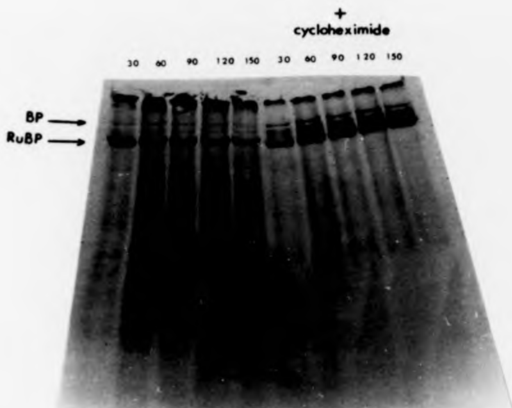
In the presence of cycloheximide the synthesis of LSU and its association with BP or assembly into the RuBP carboxylase holoenzyme can be assayed by non-denaturing PAGE. Under these conditions the SSU and the BP subunits

Figure 24 Inhibition of cytoplasmic protein synthesis in *Hordeum vulgare* protoplasts by cycloheximide.

Hordeum vulgare protoplasts were isolated and radiolabelled as described in Figure 23. At 30 minute time intervals 100 µl of the protoplasts were removed, lysed in 100 µl of lysis buffer, and the soluble protoplast proteins extracted as described in Section 2.10.2. The soluble extracts were subjected to gradient non-denaturing PAGE as described in Section 2.7.2 at time points shown opposite. At each time point (shown opposite in minutes) 25 µl of the soluble protoplast extracts was loaded onto the gel. A photograph of the autoradiograph of the gel after 48 hours exposure is shown opposite.

BP represents the position of the large subunit binding protein.

RuBP represents the position of the RuBP carboxylase holoenzyme.


+
cycloheximide
30
60
90
120
150
30
60
90
120
150
BP
RuBP

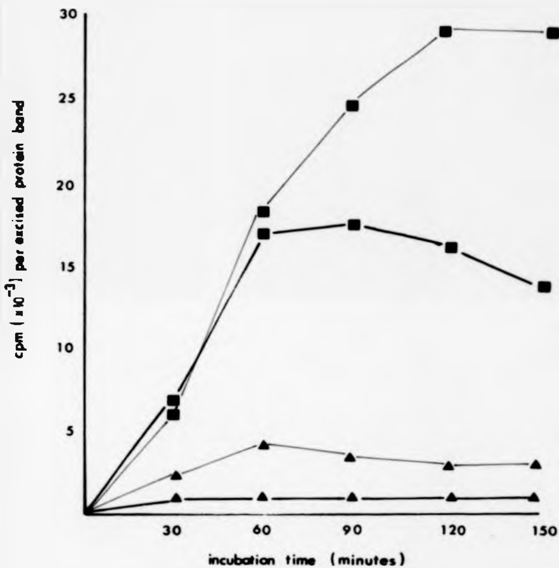
are not labelled (see Figure 21). As can be seen in Figure 24, even after over three hours of inhibition of SSU synthesis, the majority of the newly-synthesised LSU visible on the autoradiograph still migrates with the RuBP carboxylase holoenzyme indicating that it is still being assembled. It was hoped that in the absence of SSU synthesis the LSU would not be assembled, and pools of LSU would be present in the chloroplast either associated with the BP or present in other migrating forms shown on the autoradiograph.

Estimation of the amounts of LSU migrating with the BP and the RuBP carboxylase were obtained by excising the bands from the non-denaturing gel and scintillation counting the solublised gel by scintillation as described in Section 2.11.7. The results are expressed graphically in Figure 25. After 150 minutes labelling in the absence of SSU synthesis 9% of the LSU is associated with the the BP when compared to the LSU migrating with the RuBP carboxylase holoenzyme. This observation does not allow for other pools of newly-synthesised LSU that may exist in soluble protoplast extracts. The only other major labelled band on the autoradiograph runs as a smear between the BP and RuBP carboxylase position. This band is much more strongly labelled in the presence of cycloheximide. The identity of this labelled band is not known. The labelled band may represent another pool of unassembled LSU in the chloroplast or perhaps represents the labelled subunits of the ATPase coupling factor.

Figure 25 Incorporation of [^{35}S]-methionine into soluble protoplast proteins separated by gel electrophoresis.

Isolated barley mesophyll protoplasts were radiolabelled in the presence and absence of cycloheximide as described in Figure 24. The soluble protoplast extracts were subjected to gradient non-denaturing PAGE as described in Figure 24 and subjected to autoradiography.

Following autoradiography the RuBP carboxylase and BP bands were excised from the gel and the amount of [^{35}S]-methionine incorporated into each band assayed by scintillation counting as described in Section 2.11.7. The cpm incorporated into each band over the course of the incubation is shown opposite.



BP
RuBP carboxylase
BP
RuBP carboxylase
+ cycloheximide
30
25
20
15
10
5
30
60
90
120
150
cpm (x10⁻³) per excised protein band
incubation time (minutes)

An interesting observation is that in the absence of cytoplasmic protein synthesis the labelling of the RuBP carboxylase holoenzyme is much greater than in the absence of the inhibitor. This may be due the synthesis of the LSU increasing due to the inhibition of SSU synthesis (or some other cytoplasmic factor) resulting in an over production of the LSU due to a lack of control. A more simple explanation is that in the absence of cytoplasmic protein synthesis more labelled methionine is available to be incorporated into the LSU.

3.3.3 Discussion

The first reported evidence of the existence of BP in pea chloroplasts by Barraclough and Ellis (1980) revealed that under *in vitro* labelling conditions the 60 kDa BP was not labelled. This was the first evidence that the BP was a nuclear-encoded protein, synthesised in the cytoplasm. From this result however it is not possible to definitively determine the site of synthesis of the pea BP. It could be interpretated from this result that the BP of pea is chloroplast-encoded and synthesised in the chloroplast but its synthesis is inhibited in the absence of cytoplasmic protein synthesis. If its synthesis is not inhibited then perhaps its turnover may be greatly increased. Another possible explanation is that the BP subunits do not contain a methionine residue; therefore they could be synthesised in the chloroplast and not labelled (although this would appear unlikely for peptides of this size). Since the first report by Barraclough and

Ellis, further evidence that the BP is nuclear-encoded has been presented by Hemmingsen and Ellis (1986). Immunoprecipitation of the _in vitro_-labelled products of polyadenylated mRNA from pea leaves has revealed that a protein of slightly higher molecular mass is immunoprecipitated by antiserum to the BP. Because of the presence of only one precursor polypeptide and the differences between the partial digestion patterns of the individual subunits it has been proposed that the α and β subunits of the pea BP were products of different nuclear genes. These genes are transcribed to give precursors with identical mobilities on SDS PAGE (Musgrove _et al._ 1987).

Experiments in Section 3.3.1 were performed to determine whether the barley BP is also synthesised in the cytoplasm. The BP has already been shown not to be labelled during _in vitro_ chloroplast labelling experiments (see Figure 9). In the absence of specific inhibitors of protein synthesis both the subunits of the barley BP were labelled with methionine, but in the presence of cycloheximide the two subunits are not labelled. This is suggestive evidence that the barley BP subunits are cytoplasmically-synthesised.

Experiments were also carried out in this Section to analyse the _in vivo_ synthesis and assembly of LSU into the RuBP carboxylase holoenzyme. In an assembly system such as in protoplasts, where a large pool of assembled RuBP carboxylase already exists, it is not possible to conclusively show that assembly is occurring because it is not possible to assay changes in the amounts of RuBP

carboxylase over a few hours. The only criterion available to show that the newly-synthesised subunits are being assembled is the co-migration of labelled LSU (and SSU) with the already existing pool of RuBP carboxylase on a non-denaturing gel. Another interpretation for this observation could be that the newly-synthesised subunits of the RuBP carboxylase are merely sticking to the already existing pool of assembled enzyme. This interpretation is probably unlikely however, since if this was the case the holoenzyme with the labelled subunits attached to it would have an altered mobility on the native gradient gel.

Protoplasts were used to analyse the assembly of the RuBP carboxylase both in the presence and absence of SSU synthesis because of several advantages over using whole leaves. It was observed that the LSU was synthesised in the absence of SSU synthesis for at least three hours. This result is in agreement with those of Barraclough and Ellis (1978) using isolated soybean leaf cells but is a different result to that obtained by Nivison and Stocking (1983). Their work with barley leaf discs showed that in the absence of SSU synthesis LSU synthesis was also inhibited. This may indicate that in protoplasts (and isolated soybean leaf cells) the dependance of LSU on cytoplasmic protein synthesis is not so strict.

An interesting observation from this experiment was that in the absence of cytoplasmic protein synthesis the newly-synthesised LSU still migrates with the RuBP carboxylase holoenzyme, suggesting that assembly was still occurring for at least three hours after SSU synthesis had

been inhibited. These experiments were designed to study the patterns of accumulation of LSU in the absence of SSU synthesis to determine whether unassembled LSU formed separate pools in the chloroplast *in vivo* or whether this unassembled LSU associated with the BP. In this experiment most of the labelled LSU migrated with the RuBP carboxylase holoenzyme with only 9% associated with the BP after 150 minutes of labelling. The assembly of newly-synthesised LSU into RuBP carboxylase in isolated protoplasts is in contrast to results obtained with isolated chloroplasts (see Figure 9; and Barraclough and Ellis, 1980) in which little of the newly-synthesised LSU is immediately assembled into the RuBP carboxylase holoenzyme. It seems likely that pools of SSU exist in isolated chloroplasts (Milos and Roy,1984). The lack of assembly in isolated barley chloroplasts may be due to the chloroplasts being incubated in the wrong ionic conditions for assembly to occur (Ellis,1977).

It is not possible to determine from the non-denaturing gel whether other pools of unassembled LSU may exist. To fully determine all the LSU pools present in the absence of SSU synthesis it would be necessary to analyse the protoplast extracts by sucrose density gradients followed by SDS PAGE as described by Roy *et al* (1978).

Experiments carried out in this section analysed the synthesis of RuBP carboxylase over periods of a few hours. The following section analyses the accumulation of the BP and RuBP carboxylase during leaf development over several days.

3.4 THE ACCUMULATION OF THE HORDEUM VULGARE LARGE SUBUNIT BINDING PROTEIN DURING LEAF DEVELOPMENT.

3.4.1 The accumulation of the large subunit binding protein during leaf development

The investigation of chloroplast differentiation in dicotyledons is hampered during normal leaf development due chiefly to the lack of synchrony in cellular development. In order to synchronise the light-induced events of cellular differentiation in dicotyledonous plants many groups have studied the development of plastids during the greening of dark-grown seedlings.

Large changes in synthesis of soluble proteins upon greening of etiolated monocotyledonous seedlings does not occur in many species however. The amount of RuBP carboxylase upon greening of dark-grown barley seedlings shows only a doubling (Kleinkopf et al, 1970) as opposed to a 30-old increase in RuBP carboxylase over 48 hours upon the greening of etiolated pea seedlings (Lennox and Ellis, 1985).

The mode of growth of the young barley leaf does, however, provide an excellent system in which to study the characteristics and control of gene expression during normal leaf development. Cell division in the barley leaf occurs from a single basal intercalary meristem (Robertson et al, 1974), and above the meristem developmental changes can be studied in the absence of the cell cycle. Sections of tissue cut from similar distances from the leaf base provide cells and plastids of uniform

age and developmental stage. The developing barley leaf can thus be viewed as consisting of a gradient of cellular and plastid development with the oldest cells at the tip of the leaf and the youngest cells at the leaf base (Dean and Leech, 1982). This uniform developmental pattern has been utilised by many workers to study changes in the composition and structure of plastids during leaf development in several graminaceous species (for examples see Leech, 1985).

Experiments were carried out in this section to study the accumulation of RuBP carboxylase during leaf development and to compare and contrast its accumulation with that of the BP. It was thus envisaged that patterns in the accumulation of BP during leaf development may correlate with or discount any possible involvement of BP in the assembly of the RuBP carboxylase holoenzyme.

The accumulation of the RuBP carboxylase holoenzyme and the BP during leaf development was assayed immunologically using rocket immunoelectrophoresis (RIE). Rocket immunoelectrophoresis is a powerful analytical technique with great resolving power enabling the amounts of a single protein in a complex mixture to be analysed. The agarose gels used have a uniform concentration of the antibody and are of uniform thickness. On electrophoresis the antigens behave as anions and migrate into the gel whilst the antibodies are cations at the pH used. Soluble antibody-antigen complexes form, as there is an antigen excess, but eventually during electrophoresis an equivalence point is reached and the staining rocket is formed.

The RuBP carboxylase holoenzyme was assayed using antiserum raised against the RuBP carboxylase of wheat which cross-reacted immunologically with the barley RuBP carboxylase. The barley BP was assayed using antiserum raised against the pea BP which cross-reacts immunologically with the barley BP as shown in Figure 8. The antiserum raised against the barley BP, although producing a positive immunoblotting response, would not produce rockets and was not used in this study.

The accumulation of RuBP carboxylase and BP during barley leaf development was assayed by RIE of soluble extracts taken from serial leaf sections of identical developing barley seedlings. Five identical 7-day-old barley leaves were cut into 12 x 1 cm sections and the five equivalent sections used to extract soluble protein as described in Figure 26. The soluble proteins were subjected to SDS-denaturing PAGE and, as can be seen in Figure 26, there is a big increase in the amounts of the two subunits of RuBP carboxylase through leaf development. The position of the α and β subunits of the BP are also shown; detectable amounts of BP are present throughout the whole leaf although the increase of BP is not as large as that of the LSU and SSU. The two subunits of the BP are present in equimolar amounts when purified (see Section 3.2.3), but in the leaf extracts the β subunit appears to stain more heavily than the α subunit. This may be because the β subunit of the BP is migrating on the SDS gel with exactly the same mobility as the α subunit of the barley ATPase (see Figure 22) .

Figure 26 Accumulation of soluble leaf proteins during *Hordeum vulgare* leaf development.

Five 7-day-old barley leaves were cut into 12x1 cm sections from the leaf base to the tip of the leaf. Equivalent sections from the 5 leaves were used to extract soluble proteins as described in Section 2.3. The five 1 cm sections were extracted in 1 ml of extraction buffer and the soluble proteins separated from insoluble leaf tissue by centrifugation as described in Section 2.3.

From each of the 12 leaf section extracts, 50 µl of the extract was removed and analysed on a 15% SDS-denaturing polyacrylamide gel as described in Section 2.7.1. A photograph of the Coomassie blue-stained gel is shown opposite.

Track A is purified BP

Track B contains purified BP and RuBP carboxylase

Tracks 1-12 were loaded with soluble proteins from the serial sections of the barley leaf with track 1 being the first section above the leaf base.

LSU and SSU represent the large subunit and small subunit of RuBP carboxylase.

α and β represent the two subunits of the barley binding protein.

A
1
2
3
4
5
6
7
8
9
10
11
12
B
a
b
LSU
SSU

The estimation of actual amounts of each protein in soluble leaf extracts was achieved by using standard protein controls. Each RIE plate was accompanied with a set of internal standards in order to avoid discrepancies due to variation between plates. The barley RuBP carboxylase and BP were purified previously as described in Section 2.4.1. and protein concentration was determined by the Bradford method (Section 2.11.4.). Examples of calibration curves used to assay both the RuBP carboxylase and BP in soluble barley leaf extracts are shown in Figure 27.

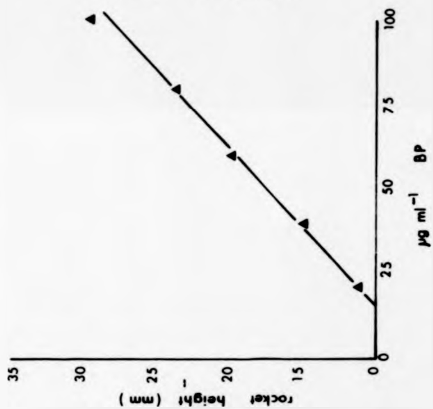
The soluble barley leaf extracts used for SDS-PAGE analysis in Figure 26 were subjected to RIE on the same plate as described in Section 2.6.4. and the amounts of BP and RuBP carboxylase in each leaf section were calculated. The calculated figures are the means obtained from two dilutions and are expressed as µg of protein per cm leaf section. The calculated results are shown in Table 2 and presented graphically in Figure 28.

The increase in RuBP carboxylase from the base to the tip of the barley leaf is much greater than that of the BP. From the base of the leaf to the region of highest RuBP carboxylase concentration (leaf section 9-10) there is a 32-fold increase in amounts of RuBP carboxylase compared to a 6-fold increase in BP over the same region. After the 10 cm section of the leaf the amounts of RuBP carboxylase and BP per section fall, but this is probably due to a reduction in leaf surface area as the leaf narrows to a point at the tip.

Figure 27 Quantitative assay of the *Hordeum vulgare* large subunit binding protein and RuBP carboxylase by rocket immunoelectrophoresis (RIE).

Antiserum raised against the wheat RuBP carboxylase and the pea BP were used for RIE to quantify amounts of barley RuBP carboxylase and BP. RIE was carried out as described in Section 2.6.4. Each RIE plate was accompanied by a set of standards obtained by assaying known amounts of barley BP and RuBP carboxylase, purified as described in Section 2.4.1. After electrophoresis, the rocket height for each standard protein dilution was measured as described in Section 2.6.4. The rocket height was plotted graphically against protein concentration, determined by Bradford protein determination (see Section 2.11.4).

The calibration curves obtained for both the barley BP (A) and RuBP carboxylase (B) are shown opposite.


A
rocket height (mm)
0
15
20
25
30
35
0
25
50
75
100
µg ml⁻¹ BP

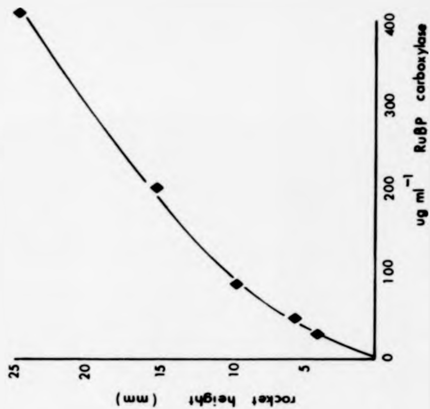

B
rocket height (mm)
5
10
15
20
25
0
100
200
300
400
ug ml⁻¹ RuBP carboxylase

Table 2 Estimation of amounts of large subunit binding protein and RuBP carboxylase in serial leaf sections of *Hordeum vulgare* by rocket immunoelectrophoresis.

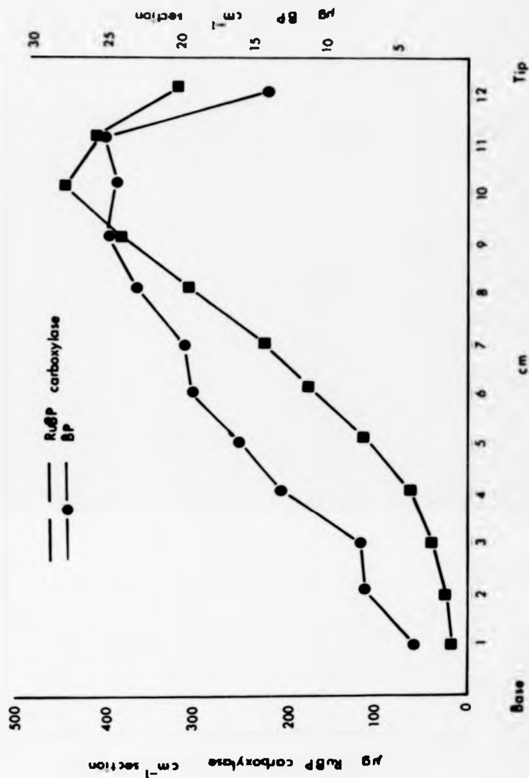
Soluble leaf extracts were made from serial sections from 7-day-old barley leaves as described in Figure 26. The leaf extracts were diluted with extraction buffer to give rockets that could be read from the standard curves in Figure 27. Each leaf section had two dilutions made to give two estimations for each section. The diluted extracts were subjected to RIE as described in Section 2.6.4. The amounts of BP and RuBP carboxylase in each section was estimated by measuring rocket height and reading the actual amount of each protein present from the standard curve in Figure 27. The standards were run on the same plates as the sample dilutions. The estimated amounts of the RuBP carboxylase and the BP in each cm leaf section are shown opposite and are the mean figures for two dilutions.

The amount of chlorophyll in each cm section was also estimated. These figures were obtained by dividing an identical 7-day-old leaf into 12x1 cm sections. The chlorophyll in each leaf section was extracted by incubating the individual sections in 1 ml of 80% acetone in the dark at 20°C for 30 minutes. The amount of chlorophyll was estimated spectrophotometrically as described in Section 2.11.2.

Leaf section cm	Chlorophyll µg cm^{-1}	RuBP carboxylase µg cm^{-1} section	Binding protein µg cm^{-1} section
0-1	0.77	14.0	4.0
1-2	1.94	22.0	7.0
2-3	3.71	39.0	7.5
3-4	6.13	63.0	13.0
4-5	9.40	118.0	16.0
5-6	12.47	174.0	19.0
6-7	14.39	222.0	19.5
7-8	16.77	300.0	23.5
8-9	20.80	389.0	25.0
9-10	18.50	452.0	24.1
10-11	18.10	400.0	25.0
11-12	16.00	322.0	14.0

Figure 28 Graphical representation of the accumulation of the large subunit binding protein and RuBP carboxylase in serial sections of *Hordeum vulgare* leaves.

The amounts of BP and RuBP carboxylase in serial barley leaf sections were estimated as described in Table 2. These values are expressed graphically opposite.



RuBP carboxylase
BP
µg RuBP carboxylase cm⁻¹ section
µg BP cm⁻¹ section
cm
Base
Tip
0
100
200
300
400
500
5
10
15
20
25
30
1
2
3
4
5
6
7
8
9
10
11
12

Similar results to those presented here were reported by Viro and Kloppstech (1980). Their results showed that the amount of RuBP carboxylase in developing barley leaf sections increased up the leaf with increasing cell age. The increase between sections was greatest in sections taken over the expanding region of the leaf.

The accumulation of BP in cereal leaf sections shows a similar pattern to that of RuBP carboxylase, but the increase is not as large as for the RuBP carboxylase. If the BP is involved in the assembly of RuBP carboxylase then an expected result would perhaps be that the amount of BP per section would be at a peak when the rate of synthesis of RuBP carboxylase is at its highest, in the expanding region of the leaf (Nivison and Stocking, 1983). However, RuBP carboxylase is being synthesised and assembled throughout the sections of the leaf at similar rates (as judged by autoradiography as shown in Figure 29 and 30), even at the tip of the leaf where the amount of RuBP carboxylase per section is falling. If the BP is involved in the assembly of RuBP carboxylase then the BP should be present in all sections of the leaf where synthesis of RuBP carboxylase is occurring. These results are in contrast to those of Nivison and Stocking (1983) who showed with radiolabelling experiments, using leaf discs from 7-day-old barley seedlings, that the rate of RuBP carboxylase synthesis reached a peak in the mid-leaf region between the 4 and 6 cm leaf sections. There does not seem to be an area of maximum RuBP carboxylase synthesis when the synthesis is assayed by radiolabelling

and scintillation counting of gel slices, as shown in Figures 29 and 30. The rate of accumulation of radiolabelled RuBP carboxylase appears fairly constant throughout the sections of the barley leaf as shown graphically in Figure 30. This result gives a measurement of the accumulation of radiolabelled RuBP carboxylase. It does not give a measurement of the rate of labelling of individual RuBP carboxylase subunits. Results reported by Nivison and Stocking (1983) show that the synthesis of LSU and SSU are tightly coupled during barley leaf development.

The assay of incorporation of [^{35}S]-methionine into RuBP carboxylase using scintillation counting of excised gel slices, as described in Figure 30, assumes that all the counts assayed are due to incorporation into the LSU and SSU of the assembled RuBP carboxylase holoenzyme. The comigration of label with the RuBP carboxylase could also be due to the comigration of other proteins at this position on the gel or the comigration of unassembled (or partially assembled) RuBP carboxylase subunits. These factors could affect the assay of radiolabelling of the RuBP carboxylase.

Figure 29 Radiolabelling of *Hordeum vulgare* soluble leaf proteins in serial leaf sections.

A single 7-day-old barley leaf was excised under water at the leaf base and the cut end of the leaf was immersed in 50 µl of water containing 50 µCi of [^{35}S]-methionine.

The leaf was incubated under the conditions described in Section 2.9 for 6 hours. The leaf was then divided into 12x1 cm sections from the base to the tip of the leaf. The soluble leaf proteins were extracted as described in Section 2.3.

An aliquot of the radiolabelled leaf proteins (100 µl) from each section was then subjected to non denaturing PAGE on a 4-30% gradient gel as described in Section 2.7.2. After electrophoresis the gel was Coomassie blue-stained as described in Section 2.7.4 and subjected to autoradiography for 48 hours as described in Section 2.11.5.

A photograph of the autoradiograph is shown opposite. RuBP carboxylase represents the position of the RuBP carboxylase holoenzyme. BP represents the position of the large subunit binding protein. Numbers 1-12 represent the twelve sections of the barley leaf, with section 1 being the first cm section above the leaf base.

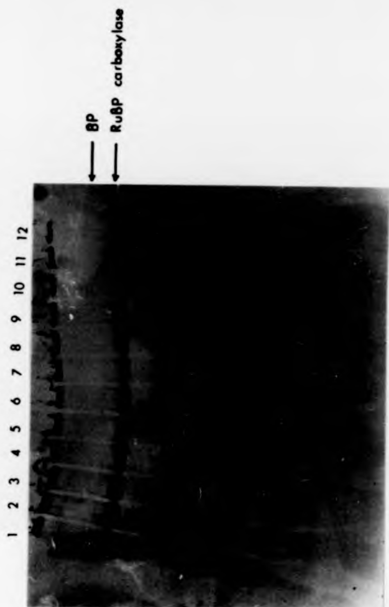
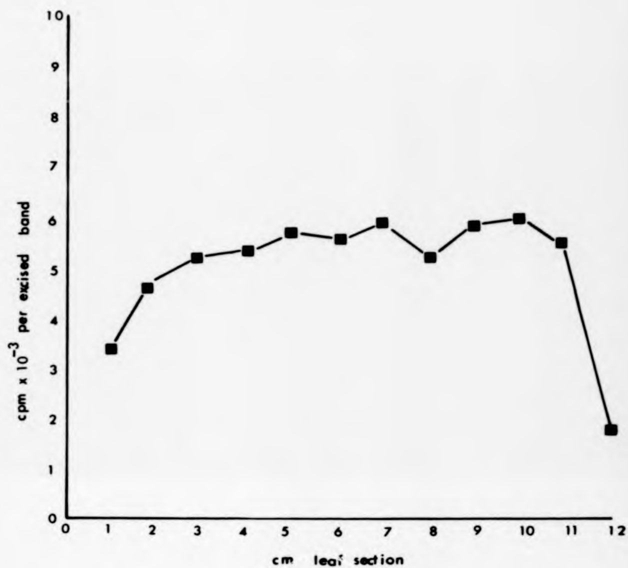

1 2 3 4 5 6 7 8 9 10 11 12
BP
RuBP carboxylase

Figure 30 Incorporation of [^{35}S]-methionine into RuBP carboxylase in serial leaf sections of *Hordeum vulgare*.

A single 7-day-old leaf was radiolabelled with [^{35}S]-methionine as described in Figure 29. The labelled barley leaf was cut into 12x1 cm sections, the soluble proteins were extracted and subjected to gradient non-denaturing PAGE as described in Figure 29. The stained RuBP carboxylase bands from the polyacrylamide gel used to produce the autoradiograph in Figure 29 were excised and used for scintillation counting as described in Section 2.11.7.

The number of counts incorporated into RuBP carboxylase in each leaf section is represented graphically opposite.


cpm x 10^{-3} per excised band
10
9
8
7
6
5
4
3
2
1
0
0
1
2
3
4
5
6
7
8
9
10
11
12
cm leaf section

3.4.2. Discussion

Much work on the study of plastid development has been carried out on the greening of etiolated dicotyledonous seedlings. Etiolated seedlings offer the easiest method of obtaining synchronous populations of cells in order to study the nuclear-chloroplast interactions involved in dicotylodenous plastid differentiation. The gross changes in protein and mRNA synthesis observed when etiolated pea seedlings are exposed to light has resulted in this system in particular being utlilised by many groups of workers (for review see Tobin and Silverthorne, 1985). The disadvantage of this system when trying to correlate these changes with normal plastid development is that the pathway of etioplast differentiation may be completely different to that of normal plastid development.

The developmental pattern of monocotylodenous leaves however allows the study of naturally occurring plastid development. Experiments in this section have utilised this developmental pattern to study the accumulation of BP and RuBP carboxylase during normal leaf development.

The results presented here were simply expressed as amounts of protein per cm leaf section. More detailed studies (Dean and Leech, 1982) have expressed proteins on a per cell or per plastid basis to allow for differences in cell size and plastid number between leaf sections. In this study however it was decided that the overall patterns of BP and RuBP carboxylase accumulation were more important than expressing the actual results more

accurately on a per cell basis for example. The main aim of this Section was to determine whether the pattern of BP accumulation during barley leaf development (when compared with the accumulation of RuBP carboxylase) was compatible with the hypothesis that the BP was involved in the synthesis and assembly of RuBP carboxylase.

As could be seen from Figure 26, most of the soluble barley leaf proteins show an increase in accumulation during leaf development, particularly at the region of cell expansion around sections 4-6. The accumulation of RuBP carboxylase follows a similar pattern to most of the other soluble barley leaf proteins in that there is an increase in amount per section during leaf development, particularly around the region of cell elongation, but that the overall increase is much larger than for other soluble proteins.

If the BP is involved in the assembly of RuBP carboxylase then in none of the leaf sections should RuBP carboxylase be present without BP. This is shown to be the case. It would perhaps also be expected that the peak in BP accumulation would also occur in the region of maximum RuBP carboxylase synthesis. This is not the case if the maximum rate of synthesis and assembly of RuBP carboxylase is occurring in section 4-6 as suggested by Nivison and Stocking (1983). As can be seen in Figure 29 and 30 however, the synthesis of RuBP carboxylase is in fact occurring from the base to the tip of the leaf, perhaps explaining why BP is also present from the base to the tip of the leaf.

An interesting finding is that the increase of BP is only 6-fold compared to a 32-fold increase in RuBP carboxylase during leaf development. A reason for this may lie in the actual stability of the BP. Nothing is known about the rate of turnover of BP *in vivo*, whilst the RuBP carboxylase is known to have a very low rate of turnover (Smith et al, 1974).

SECTION FOUR CONCLUSIONS

4.1. PROPERTIES OF THE LARGE SUBUNIT BINDING PROTEIN

The work in this thesis serves both to confirm and extend earlier results reported on the pea BP and also to extend the research on BP to the cereal crops of wheat and barley. The aim of such research is not only to compare and contrast the barley BP with that of pea, but also to obtain results that may provide information on the role of the BP.

The BP of both barley and pea share antigenic sites since antiserum raised against either BP cross-reacts with the BP of the other species. However, the barley BP migrates with an apparently higher Mr than the pea BP and the barley BP subunits have a more acidic isoelectric point. Both the barley and pea BP are dissociated by ATP and Mg^{2+} ions, this dissociation is the only known chemical property of the BP published to date.

The dissociation of the pea BP is reversible although analysis of stromal extracts by non-denaturing PAGE reveals that even at ATP concentrations of 5 mM the BP is not fully dissociated. The BP analysed on sucrose density gradients is completely dissociated by ATP however. The lack of complete dissociation of the BP when analysed by native PAGE may be due to the migration of ATP out of the stromal sample during electrophoresis before complete dissociation has taken place.

The reassociation of the BP following ATP dissociation may be more complex than resulting from simply lowering the ATP concentration. If the ATP in

stromal extracts is removed by dialysis, the BP is not reassociated. The BP does reassociate if the ATP concentration in stromal extracts is depleted during protein synthesis (Hemmingsen and Ellis, 1986). This result may indicate that the reassociation of BP requires other factors which are lost on dialysis.

The dissociation of BP is specific to ATP, although Ca^{2+} ions can replace Mg^{2+} ions, suggesting that the BP does not have a specific binding site for Mg^{2+} ions. The BP of pea is not dissociated by ADP, hence the _in vivo_ chlorplast ATP/ADP ratio may affect the ratio of dissocation to reassociation of the BP and thus affinity for the LSU.

From the results presented in the present work and also earlier reports, it is not possible to determine whether the newly synthesised LSU is released from the BP either as a free subunit (or dimer), or whether the LSU remains as a heterodimer with a BP monomer. From its sedimentation coefficient in sucrose gradients, the released LSU does not exist as a single LSU monomer (Cannon _et al_, 1986).

Future research work on the BP should be carried out on two areas in particular. One area requiring further investigation is the mechanism of ATP-mediated dissociation to determine whether the BP has ATPase activity. Such experiments require BP that is both pure and also shows dissociation by ATP. Another area of BP characterisation which requires further study is the determination of the fate of the LSU upon dissociation by

ATP. From the dissociation/reassociation experiments it is clear that the LSU remains in a soluble form when released from the oligomeric BP. It is not known, however, if the LSU is still associated with the BP. If the LSU released from the BP is present as a free monomer or dimer then this result would discount the hypothesis that the role of the BP is to confer solubility on the unassembled LSU (see Section 4.3.).

4.2. SYNTHESIS OF THE LARGE SUBUNIT BINDING PROTEIN

Experiments were carried out to both analyse the site of synthesis of the BP subunits and also to examine the accumulation of the BP during normal leaf development.

Using *in vivo* radiolabelling techniques it was shown that, as with the pea BP subunits, the barley BP subunits are synthesised in the cytoplasm. As no evidence exists to date to show that mRNA can cross the chloroplast envelope, it can be concluded that the BP subunits are nuclear-encoded. As a result it seems likely that the barley BP subunits (as with the pea BP subunits) are synthesised in the cytoplasm as high molecular mass precursors and post-translationally transported into the chloroplast. Future experiments may thus be carried out to determine the size of the barley BP precursors. Experiments may eventually be carried out to determine whether these precursors are processed by the SSU processing enzyme that has been partially purified from pea chloroplasts.

The synthesis of the BP in the cytoplasm is probably under nuclear control. If the BP is involved in the synthesis and assembly of RuBP carboxylase then it appears likely that the synthesis of BP may be coordinated with the synthesis of RuBP carboxylase. Results obtained from the greening of etiolated pea seedlings has revealed that the accumulation of RuBP carboxylase is strongly photo regulated but the accumulation of BP is not as strongly photoregulated (Lennox and Ellis, 1986). This result does not, however, take into account the possibility that the pea BP may be subject to more rapid turnover than RuBP carboxylase which shows low rates of turnover.

The pattern of accumulation of the BP during normal leaf development was analysed and compared with the accumulation of RuBP carboxylase. If the hypothesis that the BP is involved in the synthesis and assembly of RuBP carboxylase is correct, then in all sections of the leaf that RuBP carboxylase is being synthesised the BP should be present. This was shown to be the case. To date all higher plant tissue containing RuBP carboxylase that has been analysed by immunoblotting, has revealed that the BP is present. This includes the leucoplasts of Ricinus communis endosperm that contains RuBP carboxylase of unknown function.

4.3. ROLE OF THE LARGE SUBUNIT BINDING PROTEIN

The first role postulated for the BP, published by Ellis *et al* (1980), was that the BP acted as a store of unassembled, soluble LSU that would assemble in the presence of the SSU. This role may be necessary, particularly as the LSU is notoriously insoluble when separated from the SSU in higher plants (Voordouw *et al* 1984). The *Zea mays* LSU synthesised in *E.coli* is insoluble (Gatenby, 1984) in the absence of both SSU and the BP but this may not be surprising since many eukaryotic proteins are insoluble when expressed in *E.coli* (Marston, 1986). The wheat LSU, expressed in *E.coli*, was present in a soluble, aggregated form. This does not discount the possibility that the role of the BP is to solubilise the unassembled LSU in the chloroplast since the pH and ionic conditions in *E.coli* are different to those observed in the chlorplast.

If the BP acts as a store of LSU prior to assembly, then perhaps it may be expected that unassembled LSU pools would be present in excess of the SSU pool. This is not the case, however, in pea shoots where free SSU is present in excess of the LSU, and also in this study, where pools of SSU in barley protoplasts are available for assembly for at least 3 hours after SSU synthesis is inhibited.

Another possible role for the BP is that its function is to act as a molecular chaperone. The term `molecular chaperone' was first used by Laskey *et al* (1980) to describe the function of a nuclear protein termed

nucleoplasmin. The role of nucleoplasmin was to act in the correct assembly of nucleosomes from histone proteins and DNA, although nucleoplasmin was not present in the assembled histone. Nucleoplasmin was required however before correct assembly could occur. The BP may be acting as a molecular chaperone in the chloroplast. Thus the role of the BP would be to ensure that the correct molecular interactions occur during the assembly of RuBP carboxylase. In this role BP would be involved in the assembly of RuBP carboxylase but would not be part of the final assembled enzyme.

The role of the BP as a "molecular chaperone" is similar to the function suggested for a group of mammalian proteins that share sequence homology with the 70 kDa heat shock protein (hsp 70). The suggested role for these proteins is in the assembly of newly-synthesised multimeric proteins or other cellular structures in the various cellular compartments where they occur(Munro and Pelham, 1986). One of these proteins, the 78 kDa glucose regulated protein (GRP 78), has a suggested role in the assembly of IgG molecules. The GRP 78 binds non-covalently to immunoglobulin heavy chains in pre-B cells. This interaction is reversible, however, by the addition of ATP. It is suggested that by binding to hydrophobic surfaces of the newly-synthesised heavy chains, GRP 78 prevents these chains from aggregating with each other or sticking non-specifically to other proteins in the endoplasmic reticulum (Munro and Pelham, 1986). The bound GRP 78 would be able to release itself by hydrolysing ATP

and so allow any light chains to gain access to heavy chains in an orderly fashion. Any aggregates of heavy chains (which are notoriously insoluble) that formed may also be disrupted by this protein.

The suggested role of the BP in mediating the assembly of RuBP carboxylase does not seem to apply in the case of prokaryotic RuBP carboxylase. The LSU and SSU of the Synechococcus RuBP carboxylase will assemble in E.coli without the BP (Van der Vies et al. 1986) and its subunits can also be dissociated/reassociated in vitro without an apparent need for the BP (Andrews and Ballment, 1983). The pathway of synthesis of prokaryotic RuBP carboxylase is different to that observed in higher plants, however. The LSU and SSU of Synechococcus are co-transcribed as a single mRNA and therefore the synthesis and assembly is much more simple. In higher plants the LSU and SSU are synthesised in separate cellular compartments and the SSU requires transport across the chloroplast envelope. The BP may therefore be required to mediate correct LSU-SSU interactions and prevent the LSU from binding to other chloroplast proteins particularly if there is a temporary imbalance of LSU to SSU synthesis.

Future research into the role of the BP should initially concentrate on the role of the BP in RuBP carboxylase assembly. This assembly could be carried out in a system such as E.coli or in Saccharomyces which has no RuBP carboxylase. Assembly could therefore be definitively assayed by the appearance of RuBP carboxylase activity in transformants.

Research on the BP has to date mainly centred on the possible role of the BP in the assembly of RuBP carboxylase. If future results show that the BP is not involved in the synthesis and assembly of RuBP carboxylase then data collected to date on the BP will not be without use. Clearly the BP is a large, abundant chloroplast protein which possesses interesting properties. Much research work has been carried out on the 32 kDa `peak D` protein which was fully characterised prior to its function being determined. This protein is now known to be of great importance agriculturally as it contains the major herbicide binding sites for several classes of herbicides (Mattoo and Edelman, 1987). Research into the BP may eventaully prove to be of equal importance, whether the BP is involved in RuBP carboxylase assembly or in some other chloroplast function.

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APPENDIX ONE PUBLICATIONS

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Dissociation of the ribulosebisphosphate-carboxylase large-subunit binding protein into dissimilar subunits

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The ribulosebisphosphate-carboxylase large-subunit binding protein from *Pisum sativum* chloroplasts is an oligomer of two types of subunit with the composition $\alpha_6\beta_6$. These two subunits are immunologically distinct, show different partial protease digestion patterns and have different amino-terminal sequences. Leaves of *Hordeum vulgare* also contain an oligomeric binding protein composed of equal amounts of two types of subunit.

Treatment of either *P. sativum* stromal extracts or purified binding protein with ATP and Mg^{2+} ions causes the dissociation of the oligomeric form of the binding protein to the monomeric subunits. This effect is highly specific for ATP since CTP, UTP, GTP, ADP, AMP, cyclic AMP, NADPH and pyrophosphate do not cause dissociation.

The chloroplast enzyme ribulosebisphosphate carboxylase catalyzes the first steps in the processes of photosynthesis and photorespiration. Since the balance between these two processes controls plant productivity, this enzyme is a major target for alteration by genetic engineering techniques [1]. Ribulosebisphosphate carboxylase from higher plants is an oligomer of eight catalytic large subunits combined with eight small subunits and occurs in soluble form in the chloroplast stroma. The large subunits are synthesized within the chloroplast while the small subunits are imported across the chloroplast envelope after synthesis in the cytoplasm [2].

When isolated intact chloroplasts from higher plants such as pea (*Pisum sativum*) are incubated with labelled amino acids and light as energy source, the major labelled soluble product is the carboxylase large subunit [3, 4]. The majority of these newly synthesized large subunits are not assembled into the holoenzyme in short-term incubations, but are bound non-covalently to another oligomeric chloroplast protein called the large subunit binding protein [5, 6]. Since large subunits isolated from higher plant carboxylase are insoluble in aqueous media, the hypothesis was advanced that the function of the binding protein is to keep the newly synthesized large subunits in a soluble form suitable for assembly with small subunits entering the chloroplasts from the cytoplasm [5, 6]. Consistent with this hypothesis are the reports that large subunits newly synthesized by isolated *P. sativum* chloroplasts will transfer from the binding protein to the carboxylase holoenzyme when stromal extracts are treated with ATP and Mg^{2+} ions [7 – 9]. If this hypothesis is correct it will be necessary to express the binding protein gene(s) in *Escherichia coli* together with the genes for the large and small subunits, so that attempts to produce an improved carboxylase by *in vitro* mutagenesis can proceed [1].

We have purified the binding protein oligomer from chloroplasts of *P. sativum* and shown that it consists of two types of subunit of slightly different apparent M_r [10]. In this paper we report the subunit composition of the oligomer and show that the two subunits are dissimilar. Both subunits are released in monomeric form when the binding protein is treated with ATP and Mg^{2+} ions.

MATERIALS AND METHODS

Materials

Pea plants (*Pisum sativum* var. Feltham First) and barley plants (*Hordeum vulgare* var. Apex) were grown from seed at 20 – 22°C under a 12-h photoperiod for 10 days. Most chemicals were purchased from Sigma (London).

Purification of the binding protein

Binding protein was purified from pea chloroplasts as described [10]. Barley binding protein was purified by the same method, except that extracts of leaves were used as the starting material instead of extracts of chloroplasts, and the fractions eluted by 400 mM KCl from the DEAE-Sephacel column were used instead of fractions eluted by 300 mM KCl.

Preparation of antisera

Antisera to purified pea binding protein oligomer were produced as described [10]. Antisera to separated α and β subunits were made by electrophoresing stromal extracts of pea chloroplasts on non-denaturing 5% (w/v) polyacrylamide gels as described [10] except that gels of 2.5 mm thickness were used to accommodate larger amounts of protein. The gels were stained in Coomassie blue and regions containing the binding protein oligomer excised and boiled for 2 min in 25 mM Tris/glycine, pH 8.8, 0.1% (w/v) SDS, 0.2% (v/v)

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Abbreviations. Large subunit, ribulosebisphosphate-carboxylase large subunit; binding protein, ribulosebisphosphate-carboxylase large-subunit binding protein; SDS, sodium dodecyl sulphate; MgATP, equimolar concentrations of $MgCl_2$ and ATP.

Enzymes. Ribulosebisphosphate carboxylase or 3-phospho-D-glycerate carboxy-lyase (dimerizing) (EC 4.1.1.39).

2-mercaptoethanol. The gel slices were transferred to the top of 15% (w/v) polyacrylamide gels containing SDS [11] and 3 mm in thickness, the ratio of acrylamide to *N,N'*-methylene-bisacrylamide in these gels was 100:1 to ensure separation of the α and β subunits [10]. After electrophoresis and staining, the separated α and β subunits were isolated from the gel by electroelution. The eluted material was dialysed against sterile distilled water, freeze-dried and redissolved in sterile phosphate-buffered saline. Doses of 100 – 150 µg of each subunit were emulsified with one volume of Freund's complete adjuvant and injected subcutaneously into New Zealand white rabbits. Similar amounts of protein emulsified with incomplete adjuvant were injected subcutaneously after 2 and 3 weeks. The animals were bled at 5 weeks and the crude sera stored at −20 °C.

Immunoradiochemical identification of polypeptides

After either non-denaturing gradient or SDS/polyacrylamide gel electrophoresis, polypeptides were immunoblotted as described [10] except that antiserum was used at 50 µl/100 ml.

Partial protease digestion analysis

The α and β subunits were prepared from purified pea binding protein as described above. Slices of SDS/polyacrylamide gel containing the subunits were treated with V8 protease from *Staphylococcus aureus* as described [12] and the products electrophoresed on an SDS/polyacrylamide gel containing a linear gradient (10 – 30% w/v) of polyacrylamide. The gels were stained with silver ions [13].

Amino-terminal sequencing

Lyophilized samples of the pea α and β subunits prepared as described above were subjected to automated solid-phase Edman degradation [14] by the Science and Engineering Research Council protein sequencing unit at the Department of Biochemistry, University of Leeds. Duplicate samples were analysed.

Non-denaturing gradient polyacrylamide gel electrophoresis

Gels were made containing a linear gradient of 4 – 30% (w/v) acrylamide and 0.2 – 0.15% (w/v) bisacrylamide. The gels were pre-electrophoresed with 8 mM cysteine present in the upper reservoir for 2 h to ensure reducing conditions within the gel. After sample loading, gels were run for a further 20 h at 18 mA constant current. When indicated, samples were incubated with ATP and $MgCl_2$ at pH 8.0 for 30 min at 0 °C prior to electrophoresis.

Isolation of chloroplast stromal fraction

Intact chloroplasts wer isolated from pea leaves as described [15] and lysed in 10 mM Tris/HCl, pH 8.0. The mixture was centrifuged at 18000 × *g* for 15 min and the supernatant fraction removed to form the stromal extract.

Sucrose density gradient analysis

Stromal extracts and purified binding protein were analyzed on sucrose step gradients containing 5 – 50% (w/v) sucrose. The gradients were composed of four 3-ml steps (5%, 20%, 35% and 50%) (w/v) sucrose in 50 mM Tris/HCl, 7 mM 2-mercaptoethanol, pH 7.6. If required, ATP (10 mM) and $MgCl_2$ (10 mM) were included in all the steps. The sample (0.5 – 1 ml) was layered on top of the 5% (w/v) solution and the gradients centrifuged at 88000 × *g* for 17 h at 4 °C in a Beckman SW40 Ti rotor. Fractions (500 µl) were removed for analysis on polyacrylamide gels.

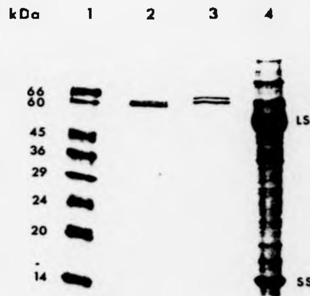

Fig. 1. *Analysis of purified pea and barley binding protein on denaturing polyacrylamide gels.* Binding protein was purified from pea chloroplasts and barley leaves as described in Materials and Methods and denatured by boiling in 2% (w/v) SDS for 2 min. Samples were analysed on a 15% polyacrylamide gel containing SDS and 0.15% bisacrylamide as described [10]. Track 1, molecular mass markers; track 2, pea binding-protein; track 3, barley binding protein; track 4 stromal extract of pea chloroplasts; LS and SS, large and small subunits of pea ribulosebisphosphate carboxylase, respectively

RESULTS

Subunit composition of the binding protein

We have reported that the binding protein purified from pea chloroplasts has an apparent M_r of about 720000 and resolves into two subunits of apparent M_r about 61000 and 60000, respectively, when analyzed by SDS/polyacrylamide gel electrophoresis [10]. Fig. 1 shows that binding protein purified from *Hordeum vulgare* leaves also reveals two closely running subunits on SDS/polyacrylamide gel electrophoresis. The subunits from *H. vulgare* migrate slightly more slowly than those from *Pisum sativum*. The two subunits from both species appear to stain with equal intensity when treated with either Coomassie blue or with silver ions; this visual impression is confirmed by densitometric scanning of the gels (not shown). We propose to name the subunit of lower mobility the α subunit and that of higher mobility the β subunit. The likely subunit composition of the binding protein is thus $\alpha_6\beta_6$.

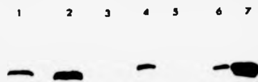

Fig. 2. *Immunoradiochemical analysis of stromal extracts and binding-protein subunits.* Polypeptides were separated on a 15% (w/v) polyacrylamide gel containing SDS and 0.15% (w/v) bisacrylamide and then transferred to nitrocellulose. The nitrocellulose was probed with antiserum and ^{125}I-labelled protein A as described [10]. Tracks were loaded with the following samples: tracks 1 – 3, pea stromal extract; tracks 4 and 6, pea α subunit of the binding protein; tracks 5 and 7, pea β subunit of the binding-protein. Tracks were probed with the following antisera: tracks 1, 4 and 5, antiserum to pea α subunit; tracks 2, 6 and 7, antiserum to pea β subunit; track 3, preimmune serum

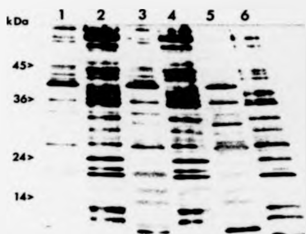

Fig. 3. *Partial protease digestion analysis of α and β pea binding-protein subunits.* Purified pea binding-protein was electrophoresed on 15% (w/v) SDS polyacrylamide gels containing 0.15% (w/v) bisacrylamide and the separated subunit bands excised from the gel. These pieces were treated with V8 protease from *S. aureus* as described [12] and electrophoresed on a 10 – 30% (w/v) SDS/polyacrylamide gel which was stained with silver ions. Tracks 1, 3 and 5 contained α subunit, tracks 2, 4 and 6 contained β subunit. Three concentrations of protease were used: tracks 1 and 2, 0.01 µg/track; tracks 3 and 4, 0.05 µg/track; tracks 5 and 6, 0.1 µg/track

Dissimilarity of subunits

Antisera were raised against the separated α and β subunits of pea binding protein as described in Materials and Methods. Immunoblotting of pea stromal extracts with either antiserum reveals no immunoreactive material other than the subunits themselves (Fig. 2, tracks 1 and 2). This observation suggests that the chloroplast stroma contains no other polypeptide sharing epitopes with the binding protein subunits. Antiserum raised against either subunit shows only a slight cross-reaction with the other subunit (Fig. 2, tracks 4 – 7). We attribute this slight cross-reactivity to contamination of the antigens used to raise the antisera with each other; the maximum separation of the α and β subunits on SDS/polyacrylamide gels is about 2 mm, making it difficult to avoid some cross-contamination. The large difference in reactivity of both antisera towards the two subunits suggests that these subunits are immunologically distinct.

Further evidence that the α and β subunits are dissimilar comes from partial protease digestion analysis. Separated α and β subunits were treated with three concentrations of V8 protease from *S. aureus* and the digestion products visualized by silver staining after separation by SDS/polyacrylamide gel electrophoresis. Fig. 3 shows the digestion patterns obtained with the binding protein subunits from pea. The patterns given by each subunit are clearly different, only about six fragments out of about 42 being shared between the subunits in terms of molecular mass. Similar results were obtained for the subunits from wheat and barley (not shown).

Conclusive evidence that the α and β subunits are dissimilar comes from amino-terminal sequence analysis. Fig. 4 shows the first 20 amino acid residues of the α subunit and the first 30 of the β subunit, both subunits being from pea. The amino-terminal residue of each subunit is alanine, and residue 11 of each sequence is serine, but the remainder of the sequences share no residues in common.

The α and β subunits also differ in their isoelectric points, as judged by two-dimensional gel electrophoresis [16]. The α subunit from pea has an apparent isoelectric point of about 5.5, while the value for the β subunit is about 6.0. The corresponding values for the subunits from barley are 4.9 (α subunit) and 5.1 (β subunit). Examination of the two-dimensional map of pea stromal proteins [16] shows that the binding protein subunits are among the most abundant chloroplast polypeptides after the carboxylase subunits.

ATP-induced dissociation into monomeric subunits

The addition of equimolar concentrations of ATP and Mg^{2+} ions (termed MgATP) to stromal extracts of pea chloroplasts causes complete dissociation of the binding protein, as judged by the disappearance of the staining band from non-denaturing polyacrylamide gels [7, 10]. Subsequent removal of the ATP results in reappearance of the staining of binding protein in its oligomeric form [10]. We investigated this dissociation by means of electrophoresis on non-denaturing gels containing a gradient of polyacrylamide concentration. Such gels allow the resolution of many more protein bands in stromal extracts than the single-concentration polyacrylamide gels used previously and permit the use of immunoblotting technique. A new staining band of protein with M_r of about 60000 appears when pea stromal extracts are treated with MgATP in the range 0.5 – 5 mM (Fig. 5). There is a corresponding decrease in the staining intensity of the binding protein oligomer band running with an apparent M_r of about 720000. The 60000-M_r band reacts strongly with antiserum raised against the binding protein oligomer (Fig. 6). Immunoblotting is more sensitive than staining and reveals the presence of some 60000-M_r protein even in stromal extracts which have been dialyzed to remove endogenous Mg^{2+} ions and ATP (Fig. 6, track 1). Concentrations of MgATP

Fig 4. *Comparison of the amino-terminal sequences of the α and β subunits of pea binding protein.* The sequences were determined by solid-phase Edman degradation [14] of separated pea α and β subunits

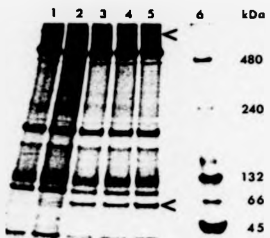

Fig 5. *Dissociation of the pea binding protein to monomer subunits by MgATP.* Stromal extracts of pea chloroplasts were dialyzed for 3 h against 10 mM Tris/HCl pH 8.0 to lower the content of endogenous ATP, and then incubated for 30 min at 0°C with $MgCl_2$ and ATP at the concentrations given. The extracts were electrophoresed on a non-denaturing gradient polyacrylamide gel as described in Materials and Methods and stained with Coomassie blue. The upper arrow indicates the binding-protein oligomer while the lower arrow indicates the binding protein subunits. Track 1, no added MgATP; track 2, 0.1 mM MgATP; track 3, 0.5 mM MgATP; track 4, 1 mM MgATP; track 5, 5 mM MgATP; track 6, molecular mass markers

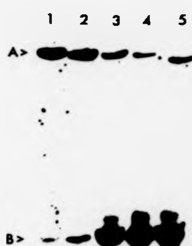

Fig. 6. *Immunoradiochemical identification on monomeric subunits produced by the dissociation of the binding protein by MgATP.* Stromal extracts were prepared from pea chloroplasts and treated with MgATP as described in Fig. 5, but the gel was then immunoblotted with ^{125}I-labelled antibodies to the pea binding protein oligomer. Tracks 1 – 5, as in Fig. 5. Arrows: A, binding protein oligomer; B, binding protein monomers

as high as 5 mM do not cause complete dissociation of the oligomeric form of the binding protein (Figs 5 and 6, track 5). Previous reports [10, 17] that MgATP causes complete dissociation of the binding protein can be explained by the use of non-denaturing gels containing a single concentration of polyacrylamide. Such gels give broader protein bands than those containing a gradient of polyacrylamide concentration, resulting in a lower sensitivity of detection by staining.

The dissociation into monomeric subunits caused by MgATP is highly specific for this nucleotide; CTP, UTP, GTP, ADP, AMP, cyclic AMP, NADPH and sodium pyrophosphate at 1 – 5 mM, with Mg^{2+} ions present at the same concentrations, do not cause dissociation (not shown). Mg^{2+} ions are required for dissociation by ATP but can be replaced by Ca^{2+} ions. We have confirmed the report [7] that the ATP analogue adenosine 5' [β,γ-methylene]triphosphate does not cause dissociation of the pea binding protein.

The data presented in Figs 5 and 6 do not show that both subunits are released when the binding protein is dissociated by MgATP. Antisera raised against the separated α and β subunits do not react with either oligomeric or the monomeric form of binding protein on non-denaturing gels. To determine whether one or both subunits are released on dissociation, sucrose density gradient analysis was used. It has been shown that centrifugation of pea stromal extracts in sucrose density gradients containing MgATP results in the disappearance of binding protein from the lower gradient fractions and its appearance in the upper gradient fractions [10]. This result is expected because MgATP causes dissociation. Fig. 7 illustrates that the purified pea binding protein also shows this behaviour and that both α and β subunits are present in equal amounts in the upper gradient fractions. Thus both subunits are released on dissociation of the binding protein.

It is apparent from Fig. 7 that centrifugation of the binding protein in sucrose density gradients containing MgATP causes complete dissociation since no trace of the oligomeric form can be seen. This behaviour contrasts with that seen when electrophoresis on non-denaturing gradient polyacrylamide gels is used; in this case some oligomeric form can still be detected after treatment with MgATP (Fig. 5). One possible explanation for this difference is that electrophoresis rapidly removes ATP from the extract, allowing some reassociation, while during sedimentation on sucrose density gradients ATP is continuously present.

DISCUSSION

We have shown that the ribulosebisphosphate-carboxylase binding protein consists of equal numbers of two distinct types of subunit termed α and β. The first 20 amino-terminal residues of the α subunit from pea show no homology with the first 30 amino-terminal residues of the β subunit from pea. The estimated difference in M_r between these two subunits is

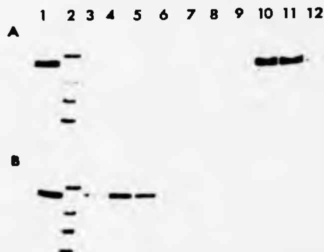

Fig 7. *Dissociation of purified pea binding protein into α and β subunits.* Purified pea binding protein was treated with and without 10 mM MgATP for 30 min at 0 C and the centrifuged on a sucrose density gradient containing 0 or 10 mM MgATP as described in Materials and Methods. Ten fractions (500 μl) were removed from the upper half of the gradient and analysed on 15% (w/v) polyacrylamide gels containing SDS and 0.15% (w/v) bisacrylamide. The gels were stained with Coomassie blue. Track 1, pea binding protein marker; track 2, M_r markers; tracks 3 – 12, gradient fractions with track 3 being the uppermost fraction. Gel A, no MgATP; gel B, 10 mM MgATP. Fractions from the lower half of the gradient contain no protein (not shown).

about 1200 [10] and this difference is about half that predicted if the β subunit were derived from the α subunit by removal of 20 amino-terminal residues. Neither subunit is synthesized by intact isolated pea chloroplasts [18], and immunoprecipitation of the products of *in vitro* translation by pea leaf cytoplasmic ribosomes reveals a single higher-M_r product [10]. All cytoplasmically synthesized chloroplast proteins studied so far are made as larger precursors prior to transport across the chloroplast envelope [2]. Since only a single higher-M_r product is observed, it is possible that a single precursor exists which contains both the α and β subunit sequences. However, calculations indicated that on this hypothesis the α and β subunits sequences would share 95% homoloy and such a high degree of homology is not supported by the partial protease digestion analysis. We therefore propose that the α and β subunits are the products of different nuclear genes which are transcribed to give precursors of identical M_r. Current research aims to clarify this aspect by isolating and sequencing cloned cDNA to both subunits.

The dissociation of the binding protein by MgATP reported earlier [10, 17] has been further investigated. The results are consistent with a model in which the binding protein undergoes a reversible dissociation between the oligomeric form and the monomeric subunits, MgATP causing the equilibrium to shift towards the monomeric subunits. This equilibrium can be represented as follows:

$$\alpha_6\beta_6 \xrightleftharpoons{\text{MgATP}} 6\alpha + 6\beta$$

The reassociation reaction may require stromal factors since removal of ATP by dialysis fails to cause reassociation (not shown), whereas removal of ATP by protein synthesis does cause reassociation [10].

The position of this equilibrium *in vivo* is unknown. The concentration of ATP within the chloroplast is estimated to be in the range 1 – 3 mM [19] which would be expected to favour dissociation. However the concentration of binding protein inside the chloroplast we estimate at about 10 mg/ml, which is 20 – 100-fold higher than that at which it occurs in stromal extracts. The proportion of binding protein occurring in oligomeric form will be increased as the concentration of binding protein increases, since the model above predicts that concentration strongly favours association of subunits. Thus results obtained with stromal extracts cannot be extrapolated directly to the *in vivo* situation and more of the oligomeric form may occur inside the chloroplast than is observed in stromal extracts.

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