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# The Regulation of Maize Leaf Phosphoenolpyruvate Carboxylase

by

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Thesis submitted for the degree of doctor of philosophy

Department of Biochemistry

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## Abbreviations

The abbreviations used in this thesis are those recommended by the Biochemical Society, London, except for those listed below :

| | |
|---|---|
| BSA | Bovine serum albumin |
| CAM | Crassulacean acid metabolism |
| DCMU | 3(3,4-dichlorophenyl)-1,1-dimethyl urea |
| DTT | Dithiothreitol |
| FPLC | Fast protein liquid chromatography |
| G6P | Glucose 6-phosphate |
| MDH | Malate dehydrogenase |
| OAA | Oxaloacetate |
| PEP | Phosphoenolpyruvate |
| $P_i$ | Ortophosphate, inorganic phosphate |
| PPDK | Pyruvate, phosphate dikinase |
| PMSF | Phenylmethylsulphonyl fluoride |
| RuBP | Ribulose 1,5-bisphosphate |
| SDS | Sodium dodecyl sulphate |
| TCA | Trichloroacetic acid |
| TEMED | N,N,N',N'-tetramethylethylenediamine |

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## SUMMARY

When this project started, it had been shown that the PEP carboxylase of CAM plants was regulated by a reversible phosphorylation. The main aim of this work was therefore to examine the hypothesis that PEP carboxylase from maize leaves was regulated by phosphorylation in response to light or darkness.

The kinetic properties of the enzyme in crude extracts was studied. Using a pH value of 7.0 and a concentration of PEP of 0.5 mM in the assay mixture a difference in the malate sensitivity was found between extracts prepared from darkened and illuminated (1000 $\mu$mol $m^{-2}s^{-1}$) leaves. The enzyme in extracts prepared from darkened leaves had an apparent $K_i$ for malate of approx. 0.32 mM, and the enzyme in extracts prepared from illuminated leaves had an apparent $K_i$ of approx. 0.98 mM. This increase in apparent $K_i$ for malate on illumination was seen using either blue light or red light and when white light was used. Further, the electron transport inhibitor 3-(3',4'-dichlorophenyl)-1,1-methylurea (DCMU) prevented the change in the malate sensitivity on illumination from occurring. The latter two findings suggest that the light stimulus needed for the activation of PEP carboxylase is perceived by the photosynthetic pigments of the chloroplast.

*In vivo* labelling of maize leaves with $^{32}P_i$ showed that when they were illuminated, the PEP carboxylase contained much more $^{32}P$ label as phosphoserine and had a higher apparent $K_i$ for malate (1.2 mM) than similar leaves which were held in darkness before extraction (apparent $K_i$ for malate 0.5 mM). This suggested that maize leaf PEP carboxylase is regulated by phosphorylation.

A method for the purification of PEP carboxylase from darkened leaves was developed. The procedure involved ammonium sulphate precipitation followed by hydroxylapatite and Mono Q chromatography and depended on the inclusion of chymostatin in the buffers to prevent the proteolysis and loss in malate sensitivity of the enzyme during the purification. After the final purification step the enzyme appeared to be pure as judged by SDS/polyacrylamide-gel electrophoresis. It was indistinguishable in its behaviour from PEP carboxylase in extracts prepared from darkened leaves as judged by native polyacrylamide-gel electrophoresis, SDS/polyacrylamide-gel electrophoresis and Superose 6 gel filtration studies and had a similar low apparent $K_i$ for malate (approx. 0.3 mM).

Studies aimed at investigating the possiblity that changes in the oligomeric state of PEP carboxylase are involved in the regulatory mechanism of the enzyme were carried out using the purified enzyme and PEP carboxylase in extracts prepared from illuminated and darkened leaves. Superose 6 gel filtration was used to test this hypothesis. The purified enzyme and the enzyme in extracts of darkened or illuminated leaves showed a concentration-dependent dissociation of tetramers into dimers. Purified PEP carboxylase and enzyme in extracts prepared from darkened leaves were equally sensitive to malate (apparent $K_i$ approx. 0.30 mM) under conditions where they existed either as tetramers or dimers. However, the enzyme in extracts prepared from illuminated leaves was less sensitive to malate inhibition (apparent $K_i$ approx. 0.95 mM) whether it was present as a tetramer or as a dimer. It therefore seems that changes in the oligomerization state of PEP carboxylase are not directly involved in its regulation by light.

Purified PEP carboxylase was used as a substrate for phosphorylation in *in vitro* studies directed at investigating the activity of the putative protein kinase responsible for phosphorylating PEP carboxylase *in vivo* and at finding the stoichiometry of the phosphorylation. When the *in vitro* phosphorylation of PEP carboxylase by leaf extracts was examined, it was found that extracts prepared from illuminated leaves were able to phosphorylate the purified enzyme to a much greater degree that extracts prepared from darkened leaves, especially if the extracts were desalted before use. This suggests that the kinase is much more active in illuminated leaves than in darkened leaves. A partial purification of the kinase from illuminated leaves was achieved by using ammonium sulphate fractionation, blue dextran-agarose chromatography and Mono Q chromatography. The partially purified kinase still contained many contaminating peptides. Its activity remained stable for several weeks when stored in 20% glycerol at -20 °C. This kinase sample was used to examine the stoichiometry of phosphorylation. The highest value attained was 0.76 moles of $P_i$ incorporated per mole of PEP carboxylase subunit. This value might have been greater but for the evident proteolysis of the PEP carboxylase substrate on prolonged incubation with the kinase preparation. The use of protease inhibitors slowed but did not completely prevent this proteolysis. A range of compounds were tested as possible effectors of PEP carboxylase phosphorylation. Of these, malate showed the greatest effect, reducing the phosphorylation of the enzyme by about 40% when added to a concentration of 5 mM.

The addition of the mammalian protein phosphatase PP-2A to PEP carboxylase which had been

phosphorylated *in vitro*, caused a decrease in the phosphorylation state and in the apparent $K_i$ for malate of the enzyme, while the addition of mammalian protein phosphatase PP-1 had no effect. Using specific substrates and inhibitors of mammalian PP-1 and PP-2A activity, it was found that both PP-1- and PP-2A-like activities were present in extracts of maize leaves. These findings suggest that a phosphatase similar to mammalian PP-2A could be responsible for the dephosphorylation of PEP carboxylase *in vivo*.

Overall the work provided firm evidence that maize leaf PEP carboxylase is regulated by phosphorylation, and revealed some details about the protein kinase and protein phosphatase involved.

Chapter 1

# INTRODUCTION

## 1.1. $C_4$ photosynthesis

### 1.1.1. Types of photosynthetic $CO_2$ assimilation in higher plants

Most organisms which carry out photosynthetic $CO_2$ assimilation, including the majority of higher plants, use the enzyme ribulose-1,5-bisphosphate (RuBP) carboxylase as their primary $CO_2$ fixing enzyme in the photosynthetic carbon reduction (PCR) cycle. This pathway, which occurs in the chloroplast, is also known as the Calvin cycle, the $C_3$ pathway or the reductive pentose phosphate pathway (Figure 1.1.). Plants which possess only the PCR cycle as their means of $CO_2$ fixation are commonly referred to as $C_3$ species. The pathway has four principal features (Edwards and Walker, 1983) :

1. Carboxylation, in which $CO_2$, which has been taken into the leaf from the atmosphere, reacts with RuBP to form two molecules of 3-phosphoglycerate (3-PGA). This reaction is catalysed by RuBP carboxylase which has a high affinity for $CO_2$.

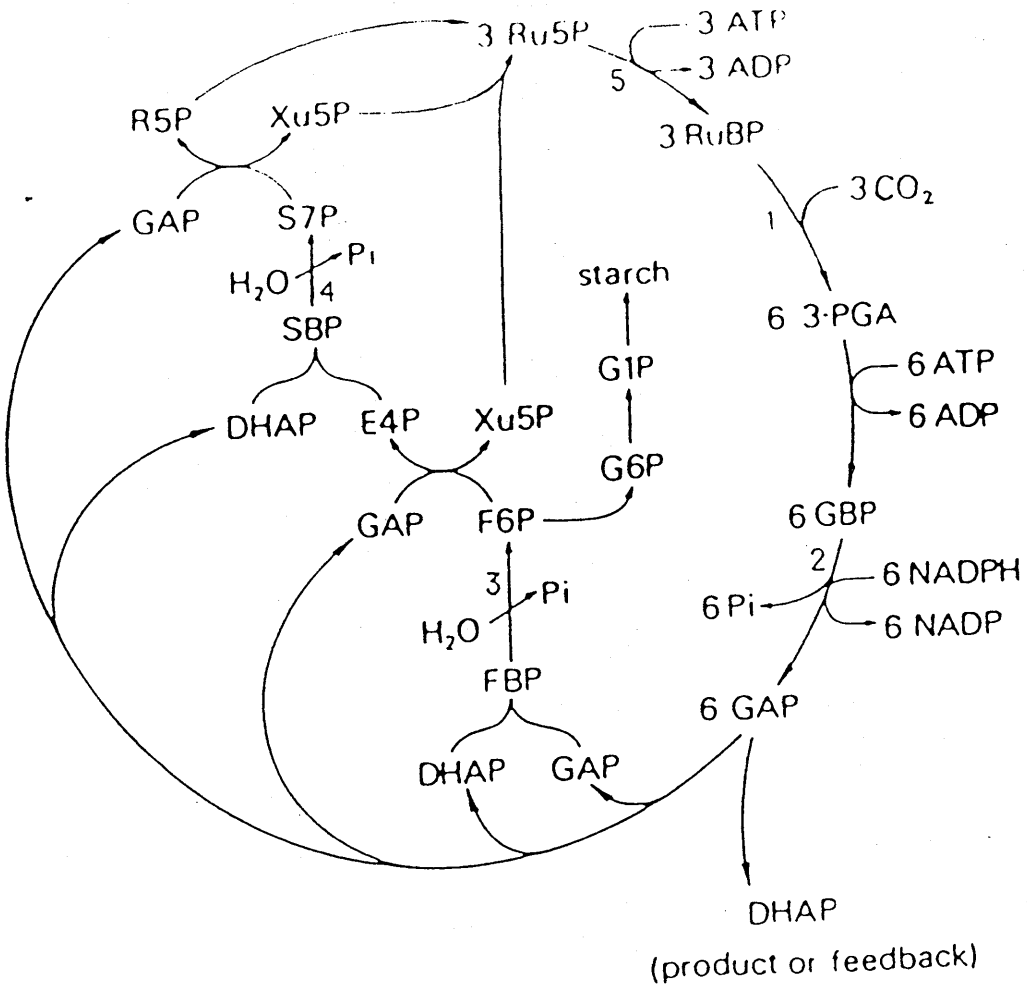
2. Reduction of the 3-PGA to triose phosphate (glyceraldehyde 3-phosphate and dihydroxyacetone phosphate) which requires ATP and NADPH.

3. Regeneration, in which five molecules of triose phosphate are rearranged to form three molecules of RuBP to complete the cycle.

4. Autocatalysis. One molecule of triose phosphate may be formed as the product for every three molecules of $CO_2$ which enter the PCR cycle. This 3-carbon compound may be converted to starch or sucrose or, alternatively, it may be used in the regenerative part of the cycle in order to build up intermediates. This can lead to an increase in the capacity to assimilate $CO_2$. Without this autocatalytic aspect, there cannot be any increase in the capacity for carboxylation and hence no capacity for plant growth (Edwards and Walker, 1983). The need for intermediates to build up is thought to be the reason why there is a short induction period when leaves which have been kept in the dark are brightly illuminated. The photochemical apparatus which carries out the processes of photosynthetic electron transport and photophosphorylation (see Section 1.2.1.) can respond immediately, but photosynthetic

FIGURE 1.1. **The photosynthetic carbon reduction cycle.** (From Leegood *et al.*, 1985). The abbreviations are : RuBP, ribulose 1,5-bisphosphate; 3-PGA, 3-phosphoglycerate; GBP, glycerate 1,3-bisphosphate; GAP, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate, FBP, fructose 1,6-bisphosphate; G6P, glucose 6-phosphate; G1P, glucose 1-phosphate; Xu5P, xylulose 5-phosphate; E4P, erythrose 4-phosphate; SBP, sedoheptulose 1,7-bisphosphate; S7P, sedoheptulose 7-phosphate; R5P, ribose 5-phosphate; Ru5P, ribulose 5-phosphate. The light regulated enzymes in the pathway are numbered as follows : 1, RuBP carboxylase; 2, NADP-glyceraldehydephosphate dehydrogenase; 3, fructose-1,6-bisphosphatase; 4, sedoheptulose-1,7-bisphosphatase, 5, ribulose-5-phosphate kinase.

In the absence of photorespiration the initial carboxylation of three RuBP molecules ($C_5$) yields six molecules of 3-PGA ($C_3$). These are then converted to DHAP and GAP (triose phosphate) using six molecules of ATP and six of NADPH and releasing six molecules of $P_i$ in the process. Under steady-state conditions of photosynthesis, five of these $C_3$ molecules are used to produce three molecules of Ru5P ($C_5$) with two further $P_i$ molecules being released by the two bisphosphatase reactions. The Ru5P can then be reconverted to RuBP at the expense of three further molecules of ATP. One molecule of triose phosphate, the product, can be converted to sucrose in the cytosol or starch in the chloroplast. When necessary, all of the triose phosphate can be fed back into the cycle to build up intermediates and no product is formed (autocatalysis).


3 Ru5P
3 ATP
3 ADP
5
R5P
Xu5P
3 RuBP
3 CO2
1
GAP
S7P
Pi
H2O
4
starch
6 3-PGA
SBP
G1P
6 ATP
6 ADP
DHAP
E4P
Xu5P
G6P
6 GBP
GAP
F6P
2
6 NADPH
3
Pi
6 Pi
6 NADP
H2O
FBP
6 GAP
DHAP
GAP
DHAP
(product or feedback)

$CO_2$ fixation exhibits a lag while PCR cycle intermediates build up (Robinson and Walker, 1981).

RuBP carboxylase also possesses an alternative, oxygenase function which allows $O_2$ to compete with $CO_2$ for the active site of the enzyme. The enzyme is therefore often known as RuBP carboxylase/oxygenase (often abbreviated to rubisco). This oxygenase function of the enzyme is the key initial reaction in the process of photorespiration. Photorespiration is the uptake of $O_2$ and the formation of $CO_2$ in the light during metabolic reactions associated with photosynthesis (Tolbert, 1980). In the process, $O_2$ and RuBP react to form P-glycolate which is then ultimately converted to 3-PGA (the product of the carboxylase reaction) at the expense of ATP and NADH (Lorimer and Andrews, 1981). The relative activity of the carboxylase and oxygenase functions is dependent on a variety of environmental parameters especially $CO_2$ and $O_2$ concentration, pH and temperature. It has been postulated, however, that the carboxylase activity may be generally three to five times that of the oxygenase activity under *in vivo* conditions (Robinson and Walker, 1981). While the exact role in photosynthesis of the oxygenase action and photorespiration is a matter of some conjecture, it is clear that this other function of RuBP carboxylase markedly reduces its capacity for $CO_2$ fixation, uses energy and can result in a substantial loss of carbon ($CO_2$) assimilated by photosynthesis.

In order to avoid the loss in the catalytic efficiency of RuBP carboxylase which occurs through its oxygenase activity, some plant species have evolved modifications of the PCR cycle. These plants fall into two main groups with completely different leaf anatomies; (i) species which use the $C_4$ photosynthetic pathway (discussed in Section 1.1.2.) and (ii) those which have crassulacean acid metabolism (CAM). Both groups use the enzyme phosphoenolpyruvate (PEP) carboxylase, which forms oxaloacetate (OAA) from $HCO_3^-$ and PEP, to catalyse the initial fixation of atmospheric $CO_2$ ($CO_2$ is first converted to $HCO_3^-$ by carbonic anhydrase in the leaf), rather than RuBP carboxylase. In both $C_4$ and CAM plants, carbon flow must be efficiently channelled through two different carboxylase enzymes.

CAM plants generally grow in arid environments and are considered to be an adaptation of $CO_2$ fixation to water stress and conservation (Ting, 1985). At night they fix atmospheric $CO_2$ using PEP carboxylase, which forms OAA, and malate dehydrogenase (MDH) which then rapidly converts the OAA to malate which is accumulated in large vacuoles. The malate is stored in the vacuoles until it is exported during the day-time for decarboxylation by malic enzyme with the $CO_2$ released being formed

into carbohydrate by the PCR cycle. This enables the the plant to keep its stomata shut during the hot day-time and have them open only during the much cooler night-time, thus permitting the necessary conservation of water by reducing transpiration. There is a considerable fluctuation in the concentration of malate in cells performing CAM; nocturnal 'acidification' occurring when the stomata are open and diurnal 'deacidification' occurring when they are closed. This pathway permits maximum possible carbon gain while allowing minimal water loss by having temporal separation of $CO_2$ fixation from the atmosphere by PEP carboxylase ($CO_2$ uptake overnight) from light driven carbon reduction (recycling of $CO_2$ supplied by endogenous malate breakdown during the day). CAM has been reviewed by Kluge and Ting (1978), Kluge (1979), Osmond and Holtum (1981) and Ting (1985).

### 1.1.2. The $C_4$ pathway : mechanism and function

The $C_4$ pathway is an adaptation of the PCR cycle which is believed to have evolved independently in several families of flowering plants. While the number of species which use $C_4$ metabolism is relatively small in comparison with the number of $C_3$ plants, several important crop plants use the $C_4$ pathway for $CO_2$ fixation, for example maize (which was used as the representative $C_4$ species in this study), sugar cane, sorghum and millet. Many major weeds (especially amongst the grasses) are also $C_4$ plants (Edwards and Walker, 1983).

The $C_4$ pathway does not replace the PCR cycle but rather acts as an appendage to it that basically serves to concentrate $CO_2$ (to perhaps ten times atmospheric concentrations) at the active site of RuBP carboxylase. This effectively abolishes the wasteful oxygenase activity of the enzyme and the process of photorespiration (Canvin, 1979). Plants which use the $C_4$ pathway have distinct physiological and biochemical characteristics. The primary products of $CO_2$ fixation are 4-carbon acids (hence the name $C_4$ photosynthesis) whereas $C_3$ plants have the 3-carbon acid 3-PGA as their initial product. $C_4$ plants have a unique leaf structure (Kranz anatomy) essential to the process of $CO_2$ fixation, which occurs in the light unlike in CAM plants (see Section 1.1.1.). The process is partitioned between two cell types arranged in concentric layers around the vascular tissue. These are the mesophyll cells and the bundle sheath cells which only occur in $C_4$ plants. The use of two cell types by $C_4$ species in the $CO_2$ fixation process allows the spatial separation of the two carboxylation reactions which both take place in the light. In the mesophyll cells, which lie outside the bundle sheath cells and are closer to the epidermis, PEP carboxylase catalyses the initial fixation of $CO_2$ from the atmosphere while the bundle sheath cells,

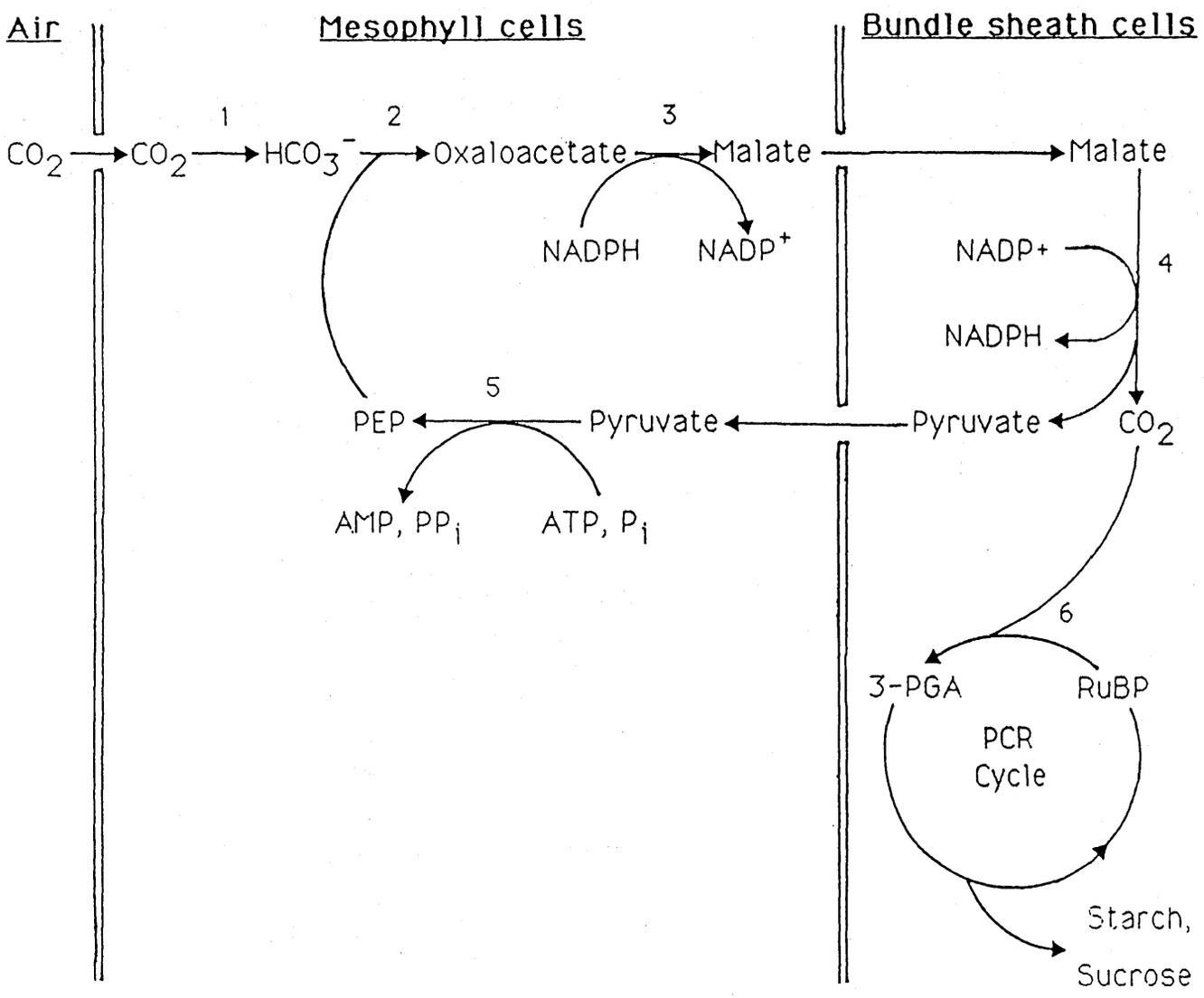
which are closer to the vascular strands, contain RuBP carboxylase and the PCR cycle enzymes.

Figure 1.2. shows the scheme of the $C_4$ pathway in a species such as maize. Carbonic anhydrase converts atmospheric $CO_2$ into $HCO_3^-$, the substrate form of $CO_2$ for PEP carboxylase (Coombs *et al.*, 1975). PEP carboxylase is the first committed step in the pathway and catalyses the conversion of PEP and $HCO_3^-$ into OAA in the cytoplasm of the mesophyll cells. The OAA is then rapidly converted to other 4-carbon acids : either malate by NADP-MDH or aspartate by aspartate aminotransferase. These $C_4$ acids occur in much higher concentrations than OAA and are transferred to the bundle sheath cells, probably by simple diffusion down a concentration gradient (Stitt and Heldt, 1985). After this transfer, the exact decarboxylation mechanism which follows varies amongst different species. In each case, however, the end result is the same, with the $CO_2$ released by decarboxylation being assimilated by RuBP carboxylase and the PCR cycle in the bundle sheath cell chloroplasts. The 3-carbon product which is left after decarboxylation (pyruvate or alanine) is transferred back to the mesophyll cells, again probably by diffusion. In the mesophyll cell chloroplast it is converted to PEP, the primary $CO_2$ acceptor, by the action of pyruvate, phosphate dikinase (PPDK).

The decarboxylation phase of the pathway is carried out in one of three different ways, depending on the species, whereas the carboxylation phase seems to be common to all $C_4$ plants. These three types differ in chloroplast ultrastructure and decarboxylating enzyme type (Hatch, 1977). Some species, such as maize, have an NADP utilising malic enzyme (see FIgure 1.2.), while others have an NAD utilising malic enzyme or have PEP carboxykinase as their major $C_4$ acid decarboxylase. PEP carboxykinase-type species and NAD malic enzyme-type species use aspartate as the major $C_4$ acid in the bundle sheath cell whereas NADP malic enzyme-type species use malate.

The relative capacities of $C_3$ and $C_4$ plants for $CO_2$ assimilation are strongly dependent on environmental factors. Energy is required to drive the $C_4$ pathway (see Figure 1.2.) and this potentially makes $C_4$ plants less efficient at $CO_2$ fixation than plants which use the PCR cycle only. This is the case for plants growing in normal air, moderate temperatures (20-25 °C) and limiting light conditions (Ku and Edwards, 1978). However, photorespiration by $C_3$ plants (see Section 1.1.1.) reduces their efficiency of $CO_2$ fixation to levels similar to those of $C_4$ species under such conditions. $C_4$ plants, however, have an advantage when growing in the conditions of full sunlight (when ATP is not limiting)

FIGURE 1.2. **The $C_4$ pathway in maize.** The enzymes which catalyse the numbered reactions are : 1, carbonic anhydrase; 2, PEP carboxylase; 3, NADP-malate dehydrogenase; 4, malic enzyme; 5, pyruvate, phosphate dikinase; 6, RuBP carboxylase. The mechanism and function of the $C_4$ pathway are discussed in Section 1.1.2.


Air
Mesophyll cells
Bundle sheath cells
$CO_2$
$CO_2$
$HCO_3^-$
Oxaloacetate
Malate
Malate
NADPH
$NADP^+$
$NADP^+$
NADPH
PEP
Pyruvate
Pyruvate
$CO_2$
AMP, $PP_i$
ATP, $P_i$
3-PGA
RuBP
PCR
Cycle
Starch,
Sucrose
1
2
3
4
5
6

and high temperatures found in the habitat areas of most $C_4$ species. This is because under such arid conditions $C_4$ plants may have a better water use efficiency than $C_3$ plants by keeping their stomata relatively closed and therefore saving water (Osmond *et al.*, 1982). The disadvantage of this in inhibiting $CO_2$ uptake is compensated for by the avoidance of photorespiration. If there is sufficient water available to allow the stomata to remain fully open then the lack of photorespiration in $C_4$ plants permits them to have much higher $CO_2$ fixation rates and thus higher productivity than $C_3$ plants. $C_4$ photosynthesis has been reviewed by e.g. Hatch (1977), Ray and Black (1979), Edwards and Huber (1981) and Edwards and Walker (1983).

## 1.2. The role of light in the control of enzyme activity in higher plants

### 1.2.1. Methods of photocontrol of enzyme activity

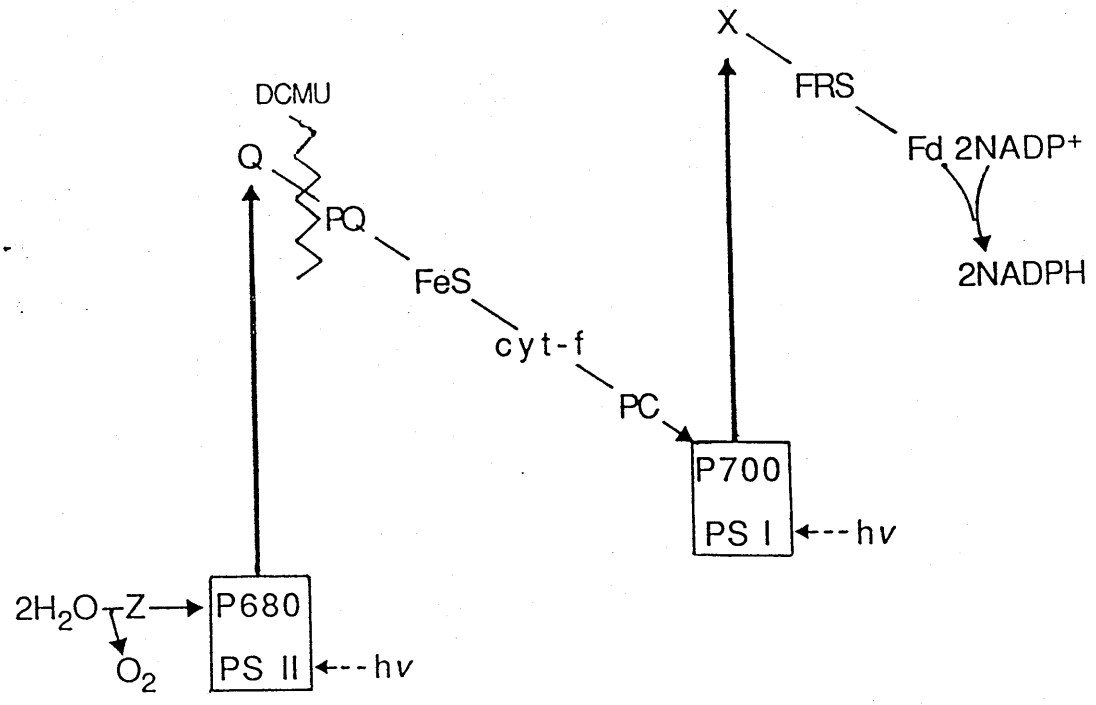
The complete process of photosynthesis in green plants is achieved through two complex sets of reactions. In the first of these, comprising photosynthetic electron transport and oxidative phosphorylation (Figure 1.3.), radiant energy is used to produce $O_2$, NADPH and ATP in the chloroplast (see Edwards and Walker, 1983). The reducing power and energy can then be used in the second process, photosynthetic $CO_2$ fixation (described in Section 1.1.), to convert atmospheric $CO_2$ into carbohydrates. These carbohydrates can then be broken down to provide a variety of organic molecules either for use by the cell or they may be exported for utilisation by other tissues in the plants.

Light can affect the activity of enzymes in the central metabolic pathways by direct and indirect methods. This is essential in order to direct metabolism towards the synthesis of sugars and storage products in the light and towards the breakdown of these compounds in the dark. For example, there is good evidence to suggest that key enzymes in the process of starch mobilisation, ADPglucose pyrophosphorylase and fructose-1,6-bisphosphatase, are under some form of light control in order to avoid futile cycling, which would be wasteful of energy, and to permit effective functioning of the pathway. Other pathways are similarly modulated by light and dark.

Light regulates the expression of a large number of genes that are involved in metabolism. This form of photocontrol of enzymes can exert its effect at several levels from transcription to post-translational turnover and has been shown to occur for several genes encoding enzymes involved in photosynthesis (e.g. Jenkins, 1988).

FIGURE 1.3. **'Z Scheme' of non-cyclic electron transport in higher plants.** P680 is the reaction centre of PS II, P700 is the reaction centre of PS I; Z, unknown intermediate; Q, quinone (the initial electron acceptor from PS II); PQ, plastoquinone; FeS, iron sulphur centre; cyt-f, cytochrome-f; PC, plastocyanin; X, bound iron sulphur centre; FRS, ferredoxin-reducing substance; Fd, ferredoxin.

In the scheme, PS II accepts four electrons from $2H_2O$ on illumination. These electrons are then passed through a series of electron acceptors to $NADP^+$ to form NADPH. Light energy ($h\nu$) may be harvested by both PS II, which preferentially uses shorter wavelength light, and PS I which preferentially uses longer wavelength light.


X
FRS
Fd
2NADP+
2NADPH
DCMU
Q
PQ
FeS
cyt-f
PC
P700
PS I
hν
2H2O
Z
O2
P680
PS II
hν

The activity of certain enzymes is extremely sensitive to pH and $Mg^{2+}$ concentration, parameters which alter markedly on illumination in the chloroplast. In spinach, the stromal pH has been shown to increase by about one pH unit and the $Mg^{2+}$ concentration rises by as much as 2 mM on illumination (Heldt, 1979), although pH is believed to be maintained at a value close to neutrality in the cytosol (Kurkdjian and Guern, 1989). This can allow enzymes to be in an optimal environment for catalysis. However, more direct effects of light on enzyme activity also occur via post-translational covalent modification. This is a further important regulatory mechanism which may activate or inactivate an enzyme and may also reinforce or sometimes override its allosteric regulation (Stadtman, 1970).

The reduction/oxidation (redox) of enzymes has been found to be involved in the light modulation of the enzymes in several metabolic pathways (Anderson, 1986). This is mediated *in vivo* by reductants generated from photosystem 1 (PS 1) in the chloroplast, and can be mimicked *in vitro* by the addition of thiol reducing compounds such as DTT (Anderson, 1979; Buchanan 1980).

Two models have been propounded to explain this phenomenon. Anderson (1979) has proposed that a vicinal dithiol of a membrane bound protein, the light effect mediator (LEM), is reduced by the electron transport chain and can then cause the reduction of a soluble protein modulase which may in turn reduce the target enzyme by thiol-disulphide interchange. Buchanan (1980) suggested a different scheme in which a protein called ferredoxin/thioredoxin reductase catalyses the reduction of a soluble protein, thioredoxin, by ferredoxin, a component of the electron transport chain, in the light. Reduced thioredoxin can then act as a reductant for the conversion of a disulphide to a dithiol at the regulatory site of the target enzyme. Reversal of this process would occur when the sulphydryl groups were re-oxidised by a component normally reduced in the light.

One fundamental difference between the schemes is that in the ferredoxin-thioredoxin system the reductant that reacts with the target enzyme is a soluble protein whereas, by contrast, the LEM is tightly bound to the membrane and remains reduced. It is not known whether both these systems operate in the chloroplast, perhaps on different enzymes, or whether they could be related despite the observed differences between the systems and act on the same enzymes. Buchanan (1980) suggested that the differences may be partly due to differences in experimental procedure. However, further work is clearly necessary in order to establish the exact method of light activation and also to find some mechanism of dark deactivation.

It has recently become evident that phosphorylation is probably as important a form of enzyme control in higher plants as it is in animals. Phosphorylation is probably the most widespread example of energy-dependent metabolic control and plays a central role in the regulation and integration of many metabolic processes in both prokaryotic and eukaryotic organisms. An important feature of this integration is that, in general, enzymes which catalyse antagonistic reactions are oppositely affected by phosphorylation. For example, many enzymes which are "rate limiting" in biodegradative pathways are activated by phosphorylation while those in biosynthetic pathways are mostly inhibited by phosphorylation. This has led to the suggestion that different pathways may be regulated by the same protein kinases (which phosphorylate proteins) and protein phosphatases (which dephosphorylate proteins) or by different ones which are affected in the same way by effector molecules. This would allow a coordinated response of metabolic pathways to extracellular signals (Cohen, 1985). A generalized scheme of this regulatory mechanism is :

$$\text{Enzyme} + n\text{X-O-P} \longrightarrow \text{Enzyme-P}_n + \text{X} \qquad 1.$$

$$\text{Enzyme-P}_n + \text{H}_2\text{O} \longrightarrow \text{Enzyme} + n\text{P}_i \qquad 2.$$

The phosphoryl donor (X-O-P) is usually ATP but may be GTP, ADP or PEP (Budde and Chollet, 1988). Reaction 1 is catalysed by a protein kinase and reaction 2 by a protein phosphatase. The dephosphorylation is usually a simple hydrolysis requiring $H_2O$ and liberating orthophosphate ($P_i$) although the unusual case of the plant enzyme PPDK which requires $P_i$ for the dephosphorylation reaction is discussed in Section 1.2.2. Phosphorylation by protein kinases may occur on serine, threonine or tyrosine residues of the target enzyme with multiple phosphorylation sites being commonplace.

Such a reversible reaction may form part of a cascade system for signal amplification in response to a stimulus (e.g. hormonal or neuronal stimuli in animals or light in plants). These cascade systems may amplify a signal by several orders of magnitude (Schacter *et al.*, 1984) and can sense fluctuations in the concentration of metabolites, therefore acting to integrate metabolism. Perhaps the best example of this type of system is the case of mammalian glycogen metabolism where a cascade sequence of several phosphorylation/dephosphorylation reactions activates glycogen breakdown in response to a hormonal signal (e.g. Cohen, 1988).

Many proteins are phosphorylated in crude plant extracts and to date at least eight proteins have been

reported to be regulated by reversible phosphorylation; several of these show light-dependent phosphorylation. Of the second messenger systems which are known to affect the phosporylation/ dephosphorylation of proteins in animals, $Ca^{2+}$ and calmodulin-dependent protein kinases have been found in plants (Ranjeva and Boudet, 1987) while Martiny-Baron and Scherer (1989) have found evidence to suggest the existence of a phospholipid/$Ca^{2+}$ dependent protein kinase, although no role for cyclic nucleotides (cyclic AMP and cyclic GMP) has been established (Budde and Chollet, 1988). As yet, there is little understanding of the dephosphorylation mechanism by protein phosphatases in higher plants and few reports have been published on these enzymes (MacKintosh and Cohen, 1989). However, MacKintosh and Cohen (1989) have successfully used mammalian substrates and inhibitors to investigate protein phosphatases in *Brassica napus* seeds (see also Section 6.2.3.).

The first plant enzyme that was demonstrated to undergo reversible phosporylation was the pyruvate dehydrogenase complex, a key multienzyme complex in mitochondria providing a link between glycolysis and the citric acid cycle. This enzyme can be reversibly phosphorylated on several serine residues by a protein kinase and protein phosphatase associated with the enzyme in a similar fashion to the enzyme in animal mitochondria (Randall *et al.*, 1981). Some evidence has been produced to suggest that three plant proteins are phosphorylated by calcium-calmodulin dependent protein kinases. (i) *Sorghum* leaf PEP carboxylase (Echevarria *et al.*, 1988), (ii) quinate : $NAD^+$ 3-oxidoreductase, an enzyme involved in secondary metabolism, which is activated by phosphorylation of a serine residue (Ranjeva and Boudet, 1987), (iii) the small subunit of RuBP carboxylase where phosphorylation is believed to be important for its transport and the assembly of the holoenzyme (Muto and Shimogawara, 1985). Less well defined phosphorylation systems have been reported for 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, which is involved in isoprenoid biosynthesis (Russell *et al.*, 1985) and 6-phosphofructo-2-kinase (Walker and Huber, 1987) which catalyses the formation of the regulatory metabolite fructose 2,6-bisphosphate implicated in the control of sucrose synthesis and glycolysis. The large subunit of RuBP carboxylase has been shown to be phosphorylated (Guitton and Mache, 1987). However, unlike the kinase responsible for the phosphorylation of the small subunit of this enzyme, the activity of the large subunit kinase was not $Ca^{2+}$ dependent. Recently, evidence has been found to suggest that another enzyme of the sucrose biosynthetic pathway, sucrose phosphate synthase, might

undergo a light-dependent reversible phosphorylation (Huber *et al.*, 1989). The light-dependent reversible phosphorylation of two enzymes of the $C_4$ pathway has also been shown. PPDK undergoes an ADP-dependent phosphorylation and a $P_i$-dependent dephosphorylation (see Section 1.2.2.) while PEP carboxylase has been shown to undergo an ATP-dependent phosphorylation (see Chapters 3 and 6) similar to that observed for the enzyme from CAM plants.

Several proteins of the chloroplast thylakoid are also phosphorylated. One of these has been identified as part of the light harvesting chlorophyll a/b complex (LHC) which is involved in the regulation of light energy distribution between the two photosystems in order to maximise the efficiency of electron transport. This protein is phosphorylated by a soluble light-dependent protein kinase which is activated when the electron transport component plastoquinone is reduced. A light insensitive protein phosphatase, which is membrane bound, hydrolyses the phosphate group from the LHC protein. This comparatively well characterised phosphorylation system in plants is reviewed by Bennett (1984).

### 1.2.2. Photocontrol of the PCR cycle and the $C_4$ pathway

Several of the enzymes in the PCR cycle have been found to be light activated. The light regulation of RuBP carboxylase seems complex, involving the participation of a specific lysine residue on the enzyme and the binding of $Mg^{2+}$ as well as a molecule of $CO_2$ which is not involved in the carboxylase reaction. It has also been found that a soluble chloroplast protein, rubisco activase, and ATP are also involved in the activation process (Portis *et al.*, 1986). The oxygenase activity of the enzyme is activated by light in concert with the carboxylase activity. However, fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, ribulose-5-phosphate kinase and NADP-glyceraldehydephosphate dehydrogenase have all been shown to be under redox regulation (Leegood *et al.*, 1985). These enzymes all catalyse essentially irreversible steps in the cycle. The activity of other enzymes are regulated only through being strongly dependent on product pool size and the concentrations of ADP, ATP, PGA and $P_i$, the latter governing the import and export of photosynthetic products throught the chloroplast envelope (Edwards and Walker, 1983).

While the PCR cycle is clearly regulated by light at several key steps, regulation of the $C_4$ pathway is also needed so that flux through the pathway is prevented during the night and its activity is coordinated with that of the PCR cycle in order to maximise photosynthetic efficiency (Edwards *et al.*,

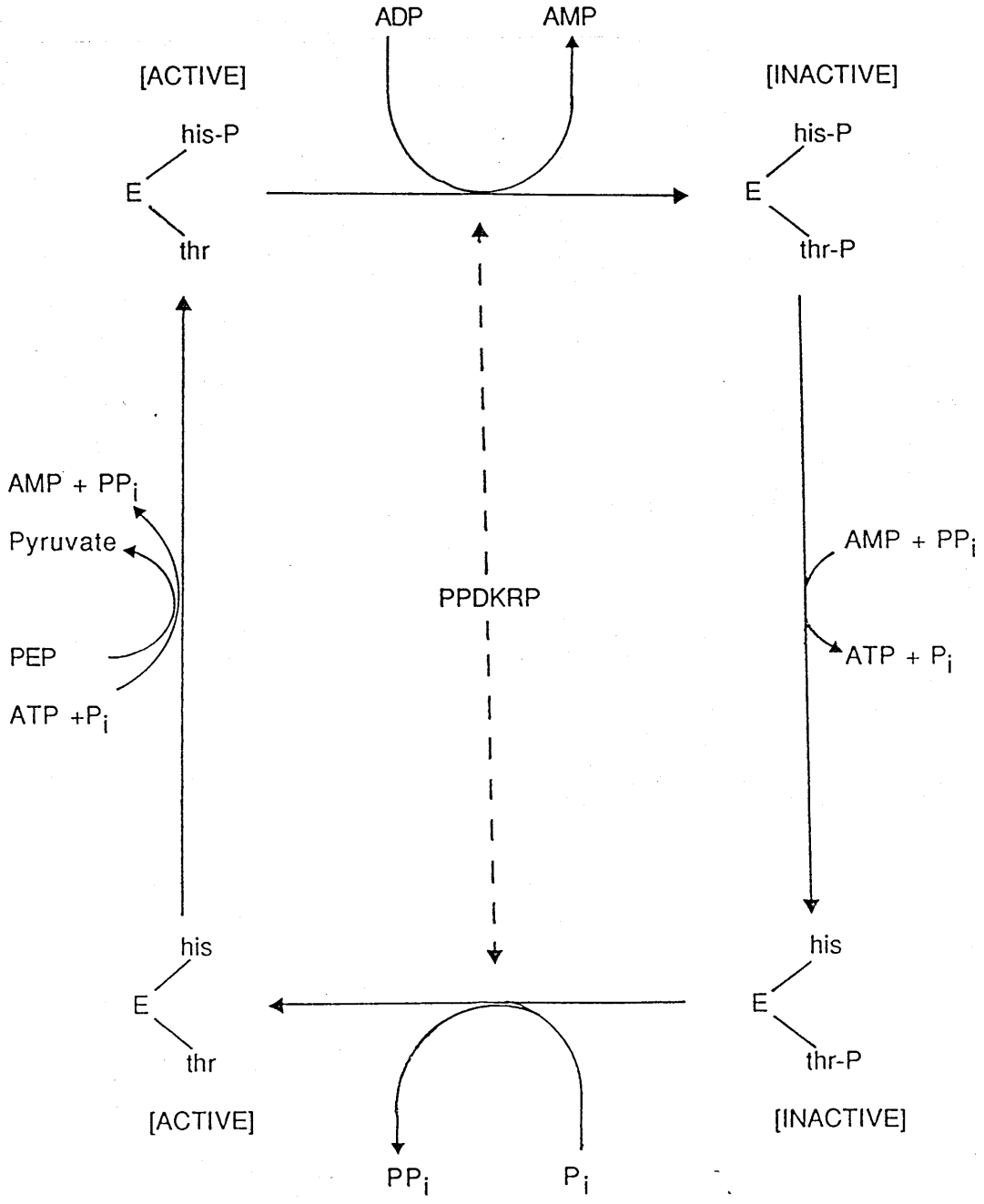
1985). Thus, the modulation of activity of $C_4$ pathway enzymes should occur in concert with the control of PCR cycle enzymes. As with most metabolic processes, enzymes of the $C_4$ pathway show several types of regulation. These include light modulation of enzyme activity, allosteric regulation by effectors and regulation by substrate levels, NADPH/$NADP^+$ ratio, adenine nucleotides as well as pH and divalent cations (Edwards and Walker, 1983).

Before the discovery that PEP carboxylase appears to be light activated, two $C_4$ pathway enzymes had been found to be regulated by light; NADP-MDH and PPDK. These enzymes are both located in the mesophyll cell chloroplasts and are much more active in the light than in the dark, with their activities being extremely responsive to light intensity and temperature.

NADP-MDH, which catalyses the conversion of OAA to malate using NADPH (see Figure 1.2.), is regulated by reduction and oxidation involving thioredoxin in a similar manner to that of other enzymes, including several from the PCR cycle (see Section 1.2.1.). *In vitro* incubation of inactive NADP-MDH from dark leaf tissue with DTT can lead to its activation (Johnson and Hatch, 1970). Similarly, reduced thioredoxin can activate partially purified enzyme (Buchanan, 1980). The photosynthetic electron transport inhibitor 3-(3',4'-dichlorophenyl)-1,1-methylurea (DCMU) (see Figure 1.3. for site of action), prevents NADP-MDH activation. This suggests that the reducing power provided by the electron transport chain, in turn controlled by light intensity, is important in regulating NADP-MDH activity (Edwards *et al.*, 1985). The NADPH/$NADP^+$ ratio is also involved in control of the enzyme's activity, with a high ratio of NADPH to $NADP^+$ necessary for full activation. Thus two different components of the electron transport chain, ferredoxin and NADPH, appear to play a part in NADP-MDH regulation.

PPDK catalyses the reaction which is thought to be closest to "rate limiting" for $C_4$ photosynthesis (Edwards *et al.*, 1985). The enzyme carries out the reversible phosphorylation of pyruvate and $P_i$ to form PEP and $PP_i$ utilising both the $\beta$- and $\gamma$-phosphates of a molecule of ATP in this process. It is regulated by a form of reversible phosphorylation which is so far unique (Figure 1.4.). This regulation is complex and involves phosphorylation on both a histidine (essential for catalysis) and a threonine residue on the enzyme. In its active state neither of these amino acids is phosphorylated. ATP and $P_i$ are used to phosphorylate the histidine residue with AMP and $PP_i$ being released. This is the first step in catalysis and this form of PPDK is still active but may then undergo an ADP-dependent phosphorylation of the

FIGURE 1.4. **Proposed mechanism of the ADP-mediated inactivation and $P_i$-mediated activation of pyruvate, phosphate dikinase.** E represents PPDK; thr, threonine; his, histidine; PPDKRP, PPDK regulatory protein (from Edwards *et al.*, 1985).



threonine residue which inactivates it. For reactivation, the histidine residue must first be dephosphorylated. This requires AMP and $PP_i$ (ATP and $P_i$ are released) and then the phosphothreonine residue can be dephosphorylated in the presence of $P_i$ with $PP_i$ being produced. ADP, AMP and $PP_i$ all inhibit this second dephosphorylation step which leads to the active form of the enzyme being produced. A bifunctional protein termed the PPDK regulatory protein is believed to catalyse both the $P_i$-dependent activation and the ADP-dependent inactivation of PPDK (Edwards *et al.*, 1985; Chollet *et al.*, 1989).

The phosphorylation/dephosphorylation model of PPDK regulation does not show a direct link with light. One possible explanation for this is that the sequence of reactions is strongly affected by the ATP/ADP+AMP ratios with a high ATP to ADP+AMP ratio favouring enzyme activation and a low ratio favouring inactivation. The pyruvate/PEP ratio is also important as a high pyruvate to PEP ratio leads to a need for greater PPDK activity and therefore the degree of phosphorylation is lessened while the reverse is true of a high concentration of PEP. Light is known to affect these ratios and presumably exerts its regulatory effect in such a fashion. However, since concentrations of the metabolites involved in the light activation process have not been found to alter sufficiently to account for the ten-fold change in PPDK activity observed *in vivo* (Chollet *et al.*, 1989) a further control mechanism has been postulated to be needed. One possible level of control could be through allosteric or covalent modification of the bifunctional PPDK regulatory protein affecting its catalytic ability. However, there has been little evidence found for such a mechanism of regulation (Edwards *et al.*, 1985).

The regulation of NADP-MDH and PPDK could provide a high degree of control of the $C_4$ pathway. The redox regulation of NADP-MDH and of four enzymes of the PCR cycle shows that control of these pathways may be coordinated while the activity of PPDK is strongly dependent on light and $CO_2$ (Usuda *et al.*, 1984) and therefore might play an important role in determining the rate of photosynthesis in $C_4$ plants. Until recently there had been scant evidence to suggest that the activity of PEP carboxylase was also regulated by light. However, while photoregulation of NADP-MDH and PPDK might seem to provide efficient control of carbon flow through the $C_4$ pathway, regulation of PEP carboxylase acivity might be beneficial for two main reasons. First, PEP, one of the substrates for PEP carboxylase is an important branchpoint intermediate in intermediary metabolism which may be derived from and utilised by many different routes (see Davies, 1979). Second, because of the position of the enzyme in $C_4$ metabolism (it catalyses the first committed step in the pathway) its regulation could be advantageous in

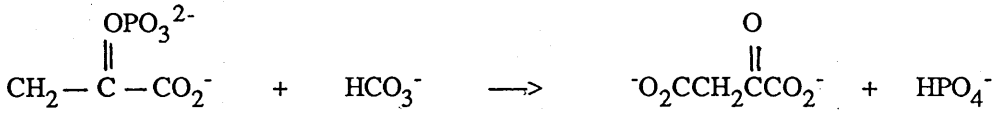
controlling metabolic flux through the pathway and hence in avoiding futile cycling.

## 1.3. PEP carboxylase : a key enzyme in $C_4$ photosynthesis

### 1.3.1. Mechanism and function in higher plants

In recent years a great deal of information has been accumulated on many aspects of PEP carboxylase. In this section some of the main areas of research into the structure and function of this enzyme will be covered with particular reference to the photosynthetic form from $C_4$ plants.

PEP carboxylase (orthophosphate : oxaloacetate carboxylyase (phosphorylating), E.C. 4.1.1.31.) catalyses the highly exergonic and essentially irreversible reaction :



PEP OAA

The mechanism requires the involvement of a divalent metal ion, probably magnesium *in vivo* although other metal ions can also activate PEP carboxylase (O'Leary, 1982; Nguyen *et al.*, 1988). The detailed reaction mechanism has yet to be determined for the enzyme from higher plants (O'Leary, 1990) although it is believed to involve a carboxyphosphate intermediate (Gonzalez and Andreo, 1989; O'Leary, 1990).

Although it is believed to be ubiquitous in plants, algae and bacteria (Andreo *et al.*, 1987) the function and properties of the enzyme vary depending on its source. In $C_3$ plants and in non-photosynthetic tissue in $C_4$ and CAM plants the enzyme has been considered to have an anaplerotic role having up to eleven possible functions, with tissue type determining which function predominates (Latzko and Kelly, 1983). These functions include replenishment of tricarboxylic acid cycle intermediates, NADPH generation, pH maintenance, nitrogen assimilation and amino acid synthesis and recapture of respired $CO_2$. In guard cells PEP carboxylase seems to be involved in the regulation of the ion fluxes which control stomatal opening and closing (Willmer, 1983).

The photosynthetic form of the enzyme exists as a distinct isozyme which is formed *de novo* during greening of etiolated leaves whereas the non-photosynthetic form is present in etiolated as well as greened leaves (Hague and Sims, 1980). These two forms have been separated and shown to have different kinetic behaviours indicative of their separate metabolic roles (Ting and Osmond, 1973a). Ting and Osmond

(1973b) suggested that four different forms of PEP carboxylases might exist in higher plants based on such kinetic analysis, each associated with a different pathway : a $C_3$ form, a $C_4$ photosynthetic form, a CAM photosynthetic form and a non-green or root form. These types all vary in their $K_m$ values for PEP and magnesium or $V_{max}$. Goatly and Smith (1974) showed that PEP carboxylase from etiolated leaves of sugar cane had similar kinetic properties to the enzyme from $C_3$ plants.

PEP carboxylase plays a particularly important role, however, in atmospheric $CO_2$ fixation by $C_4$ and CAM plants. Further discussion on the enzyme will be confined to the photosynthetic form from these plants. Evidence from cell fractionation studies and immunocytochemical experiments have shown that this form of the enzyme in $C_4$ plants is located solely in the cytoplasm of the mesophyll cells (Schnarrenberger and Herbert, 1983), where it is an extremely abundant enzyme. It constitutes about 15% of the total soluble leaf protein in maize leaves (Uedan and Sugiyama, 1976).

### 1.3.2. Structure

Studies comparing PEP carboxylase protein by immunological methods or comparing mRNA transcripts have shown that there is some similarity between the $C_4$ and CAM photosynthetic enzymes and the $C_3$ and non-photosynthetic enzymes from $C_4$ and CAM plants (Muller *et al.*, 1980; Matsuoka and Hata, 1987; Miller *et al.*, 1987; Thomas *et al.*, 1987). This indicates the possible existence of conserved regions mainly in the C-terminal half of the protein from different sources. These regions may be involved in catalysis, in particular a 14 amino acid sequence might be involved in binding PEP (Ishijima *et al.*, 1985). The gene encoding the active photosynthetic form of PEP carboxylase from maize has been sequenced (Izui *et al.*, 1986; Yanagisawa *et al.*, 1988). The deduced amino acid sequence comprises 970 amino acids with a $M_r$ of 109 408. The gene has ten exons and the 5'-flanking region has been reported to possess sequences which are believed to be involved in the response of gene expression to light (Hudspeth and Grula, 1989; Matsuoka and Minami, 1989). Some information has been provided to suggest that there is a small gene family in maize though evidence on this point is conflicting (contrast Hudspeth *et al.*, 1986; Yanagisawa *et al.*, 1988; Matsuoka and Minami, 1989). Comparison of the deduced amino acid sequence of $C_4$ and CAM PEP carboxylases has shown that there is a high degree of similarity between them (about 75% identity) which is much higher than the similarity of the maize photosynthetic PEP carboxylase with the enzyme from *E. coli* and cyanobacteria

(Rickers *et al.*, 1989).

Much work has been directed towards the elucidation of the active site structure of PEP carboxylase. Certain amino acid residues have been implicated in catalysis and are involved in the binding of PEP at the active site (Andreo *et al.*, 1987). These include histidine residues (Iglesias and Andreo, 1983), cysteine residues (Manetas and Gavalas, 1982; Stiborova and Leblova, 1983b) and arginine and lysine residues (Andreo *et al.*, 1987) and it has been demonstrated that the enzyme can be inactivated by the oxidation of a vicinal dithiol (Iglesias and Andreo, 1984) which may play a role in the activity of the enzyme (Manetas and Gavalas, 1982; Karabourniotis *et al.*, 1983; Stiborova and Leblova, 1983b; Iglesias and Andreo, 1984). The active site structure has been extensively investigated using chemical modifiers and substrate analogues (reviewed by Gonzalez and Andreo, 1989) and a tentative model of this region has been proposed (Andreo *et al.*, 1987) although further characterisation of the structure is still needed. In the model, positively charged lysine and arginine residues interact with the carboxylate and phosphate moieties of PEP while imidazole groups could bind $Mg^{2+}$ and the phosphate moiety of the substrate. The binding site of bicarbonate is not yet known (Andreo *et al.*, 1987).

PEP carboxylase is an oligomeric protein, usually thought to exist as a tetramer ($M_r$ 400 000) of identical subunits with interaction of the subunits perhaps playing an important role in its kinetic and regulatory properties (O'Leary, 1982). This topic will be discussed in greater detail in Chapter 5.

### 1.3.3. Regulation

PEP carboxylase from the green leaves of $C_4$ plants is regulated allosterically. The main regulatory metabolites are glucose 6-phosphate (G6P) and L-malate (O'Leary, 1982). G6P, a precursor of starch and sucrose activates the enzyme by decreasing the $K_m$ for PEP (Coombs *et al.*, 1973; Ting and Osmond, 1973a; Huber and Edwards, 1975; Uedan and Sugiyama, 1976;.Mukerji, 1977; Stiborova and Leblova, 1985; Rodrigues-Sotres *et al.*, 1987). Malate, a secondary product of the carboxylation reaction acts as a feedback inhibitor of the enzyme (Huber and Edwards, 1975; Mares *et al.*, 1979; Mares and Leblova , 1980; Rodrigues-Sotres *et al.*, 1987) as does aspartate (a carboxylation product in some species). Malate inhibition can be decreased by G6P (Huber and Edwards, 1975). OAA can also inhibit PEP carboxylase, though only at concentrations that are too high to pertain *in vivo* (O'Leary, 1982). Glycine is a specific activator of PEP carboxylase from monocotyledonous $C_4$ plants, and seems to act by increasing the velocity of the reaction (Nishikido and Takanashi, 1973; Uedan and Sugiyama, 1976).

The pH also affects the activity of PEP carboxylase and significantly alters its sensitivity to G6P and malate. This is particularly true between pH 7 and pH 8, values which are probably physiologically relevent in the cytoplasm, with the enzyme being more sensitive to the effects of the metabolites at the lower pH (Gonzalez *et al.*, 1984) Indeed, malate may enhance its inhibitory effect on the enzyme by causing a decrease in pH on its accumulation in the cytoplasm (Andreo *et al.*, 1987). The possible significance of these allosteric properties in relation to light regulation of PEP carboxylase will be discussed in the following chapters.

The activity of the enzyme may be affected by other factors *in vivo*. The effects of NaCl on the kinetic properties of PEP carboxylase have been shown to differ amongst different species (Osmond and Greenway, 1972). PEP carboxylase from the leaves of $C_4$ plants is particularly sensitive to inhibition by salt when compared with the enzyme from the roots of $C_4$ plants or the roots or shoots of $C_3$ plants (Osmond and Greenway, 1972). However, the degree of inhibition varies between $C_4$ species with some being more tolerant to high concentrations of NaCl than others. NaCl may even activate the enzyme at moderate concentrations of salt, possibly by changing the tertiary and quaternary structure of the protein to a more active form (Shomer-Ilan et al., 1985). At superoptimal salt concentrations, hysteresis might occur causing inactivation of the enzyme (Shomer-Ilan et al., 1985). Manetas *et al.* (1986) suggested that PEP carboxylase from $C_4$ species which grow in saline soils might have a higher tolerance of salt than $C_4$ species which do not, because osmotic balance is maintained by the accumulation of specific organic solutes in the cytoplasm. Further studies using a range of different organic cosolutes have shown that these molecules might also protect the acivity of PEP carboxylase from cold inactivation (Angelopoulos and Gavalas, 1988) since PEP carboxylase has been found to be sensitive to low temperature inactivation (e.g. Uedan and Sugiyama, 1976). They may also protect the enzyme from inactivation caused by dilution, a problem that some workers have found when using dilute concentrations of PEP carboxylase in extracts and assays (Selinioti *et al.*, 1987).

Reversible phosphorylation is believed to control the activity of PEP carboxylase from CAM plants which is also subject to inhibition by malate (O'Leary, 1982). Nimmo and colleagues demonstrated that PEP carboxylase from *Bryophyllum fedtschenkoi* exists in a phosphorylated form during the night and this is ten times less sensitive to inhibition by malate than the day form of the enzyme which is

dephosphorylated (Nimmo *et al.*, 1984, 1986). This phosphorylation occurs on one or more serine residues per enzyme subunit (Nimmo *et al.*, 1986). Brulfert *et al.* (1986) found similar results for other species of CAM plants. Before the work for this thesis was started there was no evidence to suggest that PEP carboxylase from $C_4$ plants was controlled in such a manner.

## 1.4. Objectives

The objectives of this study were to investigate the regulation by light of the photosynthetic form of PEP carboxylase from the leaves of maize, a $C_4$ plant, and in particular to find out if phosphorylation/dephosphorylation of the enzyme might be responsible for its activation and inactivation in an analagous manner to PEP carboxylase from CAM plants. The fact that two anatomically different groups of plants each use PEP carboxylase to fix atmospheric $CO_2$ (see Section 1.1.) makes the comparison and contrast of the structure and function of this enzyme in these two systems a topic of considerable interest especially since the activation of enzymes from the $C_4$ pathway seems to occur in response illumination whereas the activity of the CAM pathway is regulated by an endogenous circadian rhythm independent of illumination (Warren and Wilkins, 1961).

Chapter 2

# MATERIALS AND METHODS

## 2.1. Materials

[$\gamma$-$^{32}$P] ATP (triethylammonium salt, approx. 5000 Ci/mmol) and carrier free [$^{32}$P] orthophosphate were from Amersham International, Bucks, U.K.

Antipain hydrochloride, benzamidine hydrochloride, blue dextran-agarose, Bromophenol Blue, bovine serum albumin (BSA), Coomassie Brilliant Blue G250, chymostatin, $M_r$ marker proteins for SDS/polyacrylamide-gel electrophoresis, Hepes, glucose 6-phosphate (monosodium salt), D-isoascorbic acid, leupeptin (hemisulphate salt), L-malate (disodium salt), Mes, phenylmethanesulphonyl fluoride (PMSF) and silver nitrate were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K.

Ferritin, Sephadex G-25M, FPLC Mono Q and Superose 6 pre-packed columns were from Pharmacia, Milton Keynes, Bucks., U.K.

Calf intestinal alkaline phosphatase, dithiothreitol (DTT), NADH (disodium salt), NADPH (tetrasodium salt), oxaloacetate (free acid), phosphoenolpyruvate (monosodium salt), ATP (disodium salt), Tris, enzymes were from Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K.

Hydroxylapatite (Bio-gel HTP) was from Bio-Rad Laboratories (England) Ltd., Bramley, Kent, U.K.

Freund's complete and incomplete adjuvants were from Miles Laboratories, East Mosley, Surrey, U.K.

Acrylamide monomer, ammonium persulphate, glycine, hydrogen peroxide, 2-mercaptoethanol, N',N' methylenebisacrylamide, sodium dodecyl sulphate (SDS), sorbitol and N,N,N',N'-tetramethylethylenediamine (TEMED) were 'AnalaR' grade materials from BDH Chemicals, Poole, Dorset, U.K.

Mammalian protein phosphatases 1 and 2A, okadaic acid, mammalian inhibitor 1, [$^{32}$P] phosphorylase a, calmodulin and trifluoperizine were gifts from Dr C. MacKintosh and Prof. P. Cohen of the Department of Biochemistry, University of Dundee, Dundee, Scotland.

All other reagents used were of the highest grade commercially available.

## 2.2. Plant material

Maize (*Zea mays* L.) varieties Bastille and Goal (Sinclair McGill plc, 13 Whitfield Drive, Heathfield Industrial Estate, Ayr, KA8 9RX) were grown from seed in SAI potting compost (ICI Agrochemicals, Woolmead House West, Bear Lane, Farnham, Surrey, U.K.) in 60 x 30 x 8 cm seed trays with seeds being spaced about 5 cm apart. They were then grown in a glasshouse with supplemental fluorescent lighting (12 h photoperiod) and a minimum temperature of about 10 °C for 3-5 weeks. At least 7 days and usually 2-3 weeks before being required for experimental purposes, plants were transferred to a controlled environment room. A 12 h photoperiod was maintained using 75/85 W Phillips warm white fluorescent lamps and 60 W tungsten lamps giving a quantum fluence rate of 250 $\mu$mol $m^{-2}s^{-1}$ at plant height. The temperature of the room was maintained at 27 °C during the photoperiod and at 18 °C during the dark period. Two such rooms were available with similar lighting and temperature regimes. In one room the photoperiod extended from 0700 h to 1900 h while in the other the photoperiod extended from 1900 h to 0700 h. This allowed darkened leaves to be harvested at any time in the 24 h cycle. Plants were watered every two to three days.

Sugar cane (*Saccharum officinarum*) plants were grown in SAI potting compost in 2 litre pots. Plants were propagated by division every two to three months and were grown under the same growth room conditions as described for maize.

Detached fully expanded leaves were either darkened or illuminated at 27 °C for 1 h at 1000 $\mu$mol $m^{-2}s^{-1}$ (unless otherwise stated) by tungsten halogen headlamps (12 V, 55 W, H3 bulbs). The light was passed through 15 cm of water to reduce heating effects.

Quantum fluence rates were measured using a quantum measuring system type SKP 215 (Skye Instruments Ltd., Unit 5, Ddole Industrial Estate, Llandrindod Wells, Powys, LD1 6DF, U.K.).

## 2.3. General biochemical methods

**2.3.1. Measurements of pH** were made with a Russell pH probe calibrated at room temperature.

**2.3.2. Conductivity** measurement was carried out at 4 °C using a Radiometer conductivity meter, type CDM 2e (Radiometer, Copenhagen, Denmark).

**2.3.3. Glassware and plastics** were washed in "Haemo-sol" solution (Alfred Cox (Surgical) Ltd., U.K.), rinsed with tap water and then distilled water and dried in an oven.

**2.3.4. Protein concentrations** were estimated using the method of Bradford (1976) which is based on the binding of Coomassie Brilliant Blue to proteins. This reagent was prepared by dissolving 100 mg Coomassie Brilliant Blue G250 in 50 ml of 95% (v/v) ethanol and 100 ml of 85% (w/v) orthophosphoric acid before being made up to 1 litre with distilled water and filtered. Sample (10 µl) was added to test-tubes containing 2.5 ml of the reagent. BSA was used as a standard (concentrations of BSA solutions were determined assuming that a 1 mg/ml solution has an $A_{280}$ of 0.67). Samples were vortexed and allowed to stand for 2 min before the absorbances were read at 595 nm. A standard curve of absorbance versus BSA concentration (0-25 µg) was plotted, from which the protein concentration in the unknown could be determined. Protein concentrations of the fractions eluted from columns were measured spectrophotometrically at 280 nm.

**2.3.5. Concentration of protein samples** was done using Centricon 30 microconcentrators (Amicon Ltd., Stonehouse, Glos., U.K.).

**2.3.6. Preparation of chromatographic media.**

Sephadex G-25 Medium and hydroxylapatite were swollen and poured according to the manufacturers' instructions.

All columns were poured at room temperature and then transferred to a 4 °C cold room. Columns were stored in 0.02% (w/v) sodium azide and equilibrated with starting buffer before use. Blue dextran-agarose columns were regenerated by washing with 8 M urea.

## 2.4. Enzyme Assays

**2.4.1. Instrumentation and micropipetting.**

Spectrophotometric assays were carried out in semi-micro quartz cuvettes or plastic cuvettes (1 cm path, 1 ml). The instrument used for kinetic experiments was a Pye Unicam SP8-500 UV/VIS spectrophotometer while a Cecil CE272 linear ultraviolet spectrophotometer series 2 was used to assay PEP carboxylase activity from columns.

Volumes of 5 µl-1 ml were dispensed using adjustable Finnpipettes (Labsystems Oy, Pulttitie 9-11, 00810 Helsinki 81, Finland) or Gilson pipetmen (Gilson Medical Electronics, 72 rue Gambetta, 95400 Villiers-le-Bel, France). Microsyringes (Scientific Glass Engineering Ply Ltd., North Melbourne, Australia 3051) were used to dispense 0.1-25 µl volumes.

### 2.4.2. Assays

One unit of enzyme activity is defined as the amount of enzyme required to catalyse the disappearance of 1 μmol of substrate or the formation of 1 μmol of product per minute.

**(i) PEP carboxylase.** The standard spectrophotometric assay mixture for the measurement of PEP carboxylase activity contained, in 1 ml, 50 mM-Tris/HCl, pH 7.8, 5 mM-$MgCl_2$, 2 mM-PEP, 0.2 mM-NADH, 10 mM-$NaHCO_3$, 5 units of MDH and the enzyme sample (approx. 2-20 munits unless otherwise stated).

For the routine determination of the malate sensitivity of the enzyme the assay mixture consisted of 50 mM-Hepes/KOH, pH 7.0, 10 mM-$MgCl_2$, 10 mM-$NaHCO_3$, 0.2 mM-NADH, 5 mM-glucose 6-phosphate, 0.5 mM-PEP, 5 units of MDH, malate (0-1.8 mM) and the enzyme sample (concentration as described in the text) in 1 ml as in Nimmo *et al.* (1987).

In both types of reaction mixture used to detect PEP carboxylase activity, the decrease in $A_{340}$ at 25 °C caused by oxidation of NADH by the coupling enzyme MDH was proportional to the amount of PEP carboxylase activity. Any departure from the standard assay conditions for measurement of PEP carboxylase activity are described in the text.

**(ii) Pyruvate, phosphate dikinase** was assayed by the method of Chapman and Hatch (1981). 1 ml reaction mixtures contained 50 mM-Hepes/KOH, pH 8.0, 5 mM-DTT, 8 mM-$MgSO_4$, 1 mM-glucose 6-phosphate, 10 mM-$NaHCO_3$, 5 mM-$(NH_4)_2SO_4$, 5 mM-pyruvate, 2.5 mM-$NaH_2PO_4$, 0.2 mM-NADH, 1 unit of MDH, about 0.2 units of PEP carboxylase and 10 μl of sample. The activity was followed by the change in absorbance at 340 nm at 25 °C as NADH was oxidised by OAA formed from PEP via PEP carboxylase.

**(iii) NADP-Malate dehydrogenase.** This method was based on that of Johnson and Hatch (1970). The decrease in absorbance at 340 nm was measured at 25 °C in reaction mixtures containing 25 mM-Tris/HCl, pH 8.0, 1 mM-EDTA, 0.5 mM-OAA, 0.2 mM-NADPH and the extract to a final volume of 1 ml.

Kinetic parameters were determined, where appropriate, by direct linear plots using the ENZPACK computer program (Williams, 1985).

## 2.5. Methods developed for the purification of enzymes

### 2.5.1. Purification buffers

Buffer A (extraction buffer) : 50 mM-Hepes/KOH, pH 7.3, containing 5mM-$MgCl_2$, 5 mM-DTT, 1 mM-EDTA, 10 µg of chymostatin/ml, 2% (w/v) insoluble polyvinyl-pyrrolidone (PVP) and 0.5% (w/v) isoascorbate.

Buffer B : 50 mM-Hepes/KOH, pH 7.1, containing 5 mM-$MgCl_2$, 5 mM-DTT, 1 mM-EDTA and 10 µg of chymostatin/ml.

Buffer C : 50 mM-Tris/HCl, pH7.5, containing 1 mM-DTT, 5% (v/v) glycerol.

Buffer D : 50 mM-Tris/HCl, pH 7.8, containing 1 mM-DTT, 20% (v/v) glycerol.

Buffers A and B were derived from buffers used by Hatch and Heldt (1984) for the purification of PEP carboxylase from maize leaves. Buffers C and D are buffers which were used by Jiao and Chollet (1989) during the partial purification of PEP carboxylase kinase.

Benzamidine and DTT were stored as 1 M solutions at -20 °C and added just before use. Chymostatin was made up fresh by dissolving it in a minimal volume of glacial acetic acid and diluting with water to a stock solution of 10 mg/ml and was added just before use. PMSF was dissolved in ethanol (20mg/ml) and added just before use. Isoascorbate was added to the extraction buffer just prior to use and the buffer pH re-adjusted to pH 7.3.

All purification steps were carried out at 4 °C except Mono Q chromatography which was done at room temperature. The pH of all buffers was adjusted at room temperature.

### 2.5.2. Methods used for the preparation of leaf extracts

Two methods were used to prepare leaf extracts. The standard extraction buffer (Buffer A) was used in both methods. Unless otherwise stated, 4 ml of extraction buffer was used per gram of leaf tissue. Extracts were made either by chopping the leaves up with scissors and homogenising them in a Waring blender for 30 s at low speed, or alternatively leaves were frozen in liquid nitrogen and then pulverised using a pestle with acid-washed sand and extraction buffer in a chilled mortar. The extract made by either method was filtered through two layers of muslin before use. No difference in the amount of extractable protein or PEP carboxylase activity was observed on comparison of the two extraction methods.

### 2.5.3. Purification of PEP carboxylase

**(i) Extraction.** Maize leaves (10 g) were taken from plants which had been maintained in darkness for at least 2 h in the growth rooms. The leaves were chopped up with scissors and then homogenised in 40 ml of extraction buffer (Buffer A) in a Waring blender (see Section 2.5.2.). The extract was filtered through two layers of muslin and centrifuged for 15 min at 10 000 g.

**(ii) Ammonium sulphate fractionation.** The supernatant was brought to 30% saturation with ammonium sulphate and centrifuged for 15 min at 15 000 g. The supernatant from this centrifugation was then taken to 50% ammonium sulphate saturation, recentrifuged and the supernatant discarded. The pellet from the 30-50% ammonium sulphate fraction, which contained the PEP carboxylase activity, was dissolved in 2 ml of Buffer B. This buffer was used in all subsequent purification steps.

**(iii) Sephadex G-25M chromatography.** The dissolved pellet from the 30-50% ammonium sulphate fraction was desalted into Buffer B on a 1 x 12 cm (10 ml) column of Sephadex G-25M. The flow rate was 4 ml/min and 0.5 ml fractions were collected.

**(iv) Hydroxylapatite chromatography.** Active fractions from the Sephadex column were pooled and loaded on to a 3.3 x 1.2 cm (10 ml) column of hydroxylapatite Bio-Gel HTP equilibrated in Buffer B. After washing the column in this buffer until the $A_{280}$ fell to zero, PEP carboxylase was eluted by running a 0-150 mM-potassium phosphate gradient in 200 ml of Buffer B. The flow rate was 2 ml/min and 2 min fractions were collected. Enzyme was eluted from 80-120 mM-potassium phosphate and fractions containing enzyme activity were pooled and desalted on a 2.2 x 45 cm (175 ml) Sephadex G-25M column into Buffer B as described above.

**(v) Mono Q chromatography.** The active fractions from the Sephadex column were pooled and chromatographed on a Mono Q column (0.5 x 5 cm) connected to a Pharmacia Fast Protein Liquid Chromatography (FPLC) system. A linear 25 ml gradient of 50-400 mM-KCl in the same buffer was used to elute the PEP carboxylase from the column. The flow rate was 1 ml/min and 0.5 min fractions were collected. Enzyme activity was eluted at 210-240 mM-KCl. Purified enzyme from the Mono Q column was mixed with an equal volume of glycerol and stored at -20 °C or dialysed into Buffer B plus 50 % glycerol overnight before storage at -20 °C.

### 2.5.4. Partial purification of PEP carboxylase kinase

**(i) Extraction.** 40 g of leaves were taken from plants which had been illuminated (at least 2 h into the light phase) in the growth cabinet (250 $\mu$mol $m^{-2}s^{-1}$). These leaves were chopped up and homogenised in 160 ml of extraction buffer in a Waring blender, filtered through two layers of muslin and centrifuged for 10 min at 10 000 g.

**(ii) Ammonium sulphate precipitation.** The supernatant was brought to 50% saturation with ammonium sulphate and centrifuged for 15 min at 15 000 g. The pellet was resuspended in a total volume of 4 ml of Buffer C. This buffer was used in all subsequent purification steps.

**(iii) Sephadex G-25M chromatography.** The dissolved pellet was desalted into Buffer C on a 1.7 x 22 cm (50 ml) Sephadex G-25M column. The flow rate was 4 ml/min and 1 min fractions were collected. The green, desalted fraction was clarified by centrifugation at 11 600 g for 5 min.

**(iv) Blue-dextran agarose chromatography.** The clarified supernatant was loaded on to a 2.7 x 5.0 cm (28 ml) column of blue-dextran agarose equilibrated in Buffer C. The flow rate was 0.2 ml/min and 5 min fractions were collected. The column was washed with Buffer C until the $A_{280}$ fell to zero. It was then washed in Buffer C plus 0.5 M-NaCl and the fractions collected from this wash which contained protein were pooled and dialysed overnight into Buffer C.

**(v) Mono Q chromatography.** The dialysed sample was loaded on to a FPLC Mono Q column (0.5 x 5 cm) equilibrated in Buffer C. A linear 10 ml gradient of 0-350 mM-NaCl was run in the same buffer in order to elute the kinase activity from the column. The flow rate was 1 ml/min and 1 min fractions were collected. These fractions were individually dialysed overnight into Buffer D before being assayed for kinase activity (by testing their ability to alter the malate sensitivity of purified PEP carboxylase on incubation with ATP and $MgCl_2$ as described in Section 2.7.6). Fractions containing kinase activity were pooled and concentrated using Centricon 30 microconcentrators to approx. 1 ml and stored at -20 °C.

## 2.6. Polyacrylamide gel electrophoresis techniques

### 2.6.1. SDS/polyacrylamide gel electrophoresis (discontinuous system)

This was carried out using 8% and 15% polyacryamide gels in the presence of 0.1% SDS essentially by the method of Laemmli (1970). The following stock solutions were used for the preparation of both types of gel :

| Stock solution | Separating gel | | Stacking gel |
|---|---|---|---|
| (all volumes in ml) | 8% | 15% | 3% |
| Acrylamide 30% (w/v) and Bisacrylamide 0.8% (w/v) | 8 | 15 | 3 |
| 1.5 M-Tris/HCl pH 8.8 | 7.5 | 7.5 | - |
| 0.5 M-Tris/HCl pH 6.8 | - | - | 3.75 |
| $H_2O$ | 14.05 | 7.05 | 9.45 |
| 10% (w/v) SDS | 0.3 | 0.3 | 0.15 |
| 10% (w/v) Ammonium persulphate | 0.15 | 0.15 | 0.15 |
| TEMED | 0.01 | 0.01 | 0.01 |

Glass plates which were separated with 1.5 mm thick spacers were clamped together and sealed with 1% (w/v) agarose. In the preparation of either type of gel, the acrylamide solution, Tris buffer and $H_2O$ were mixed together and degassed before the SDS, freshly prepared ammonium persulphate and TEMED were added. The gel solution was then poured immediately between the plates, carefully overlayed with a thin layer of water-saturated-butanol and allowed to set. After the gel had set (usually about 45 min) the water-saturated-butanol was removed and a comb used for forming the wells for loading the samples into was inserted. A 3 cm stacking gel was then poured on top of the separating gel and allowed to set (usually for 60-90 min). The wells were rinsed with $H_2O$ after polymerisation. The gel was clamped into the gel tank and the two reservoirs filled with electrophoresis buffer, which contained 3 g Tris, 14.4 g glycine and 0.1% SDS per litre, making sure to avoid air bubbles being trapped under the gel plates or in the sample wells.

Samples were denatured by adding the protein samples to at least an equal volume of "sample buffer" which comprised 50 mM-Tris/HCl pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol, 0.01% (w/v) Bromophenol Blue and 1% (v/v) 2-mercaptoethanol. The mixtures were immediately boiled for 2 min. The samples were loaded carefully into the wells and electrophresis was carried out at a constant current of 45 mA per gel until the tracking dye had reached the foot of the gel (usually about 3 h).

### 2.6.2. Non-denaturing polyacrylamide gel electrophoresis

This was carried out essentially by the method of Davis (1964). The following stock solutions were prepared and stored at 4 °C :

Solution A : 36.3 g Tris, 0.23 ml TEMED, 48 ml 1 M-HCl, $H_2O$ to 100 ml

Solution B : 28 g acrylamide, 0.74 g N,N'-methylenebisacrylamide (bis), $H_2O$ to 100 ml

Solution C : 30 g Tris, 144 g glycine, $H_2O$ to 1 litre

7% gels were prepared by mixing 6 ml of Solution A with 12 ml of Solution B and 29.7 ml $H_2O$, degassing and adding 0.3 ml of freshly made up 10% (w/v) ammonium persulphate. This solution was mixed thoroughly and poured into 0.5 x 10 cm gel tubes. Gels of 9 cm length were poured, overlayed with $H_2O$ and allowed to set.

The tank buffer was a 25-fold dilution of Solution C containing 0.1% (v/v) 2-mercaptoethanol. The gels were pre-electrophoresed for 30 min at 3 mA per tube at 4 °C. Tracking dye (10 µl) of 0.1% (w/v) Bromophenol Blue in 20% (v/v) glycerol was layered on to the top of each gel and electrophoresis was carried out until the dye had penetrated the gel by about 1 mm. Samples which had been diluted in 20% (v/v) glycerol were loaded on to the gels and electrophoresis was carried out at 3 mA per gel at 4 °C until the tracking dye had reached the foot of the gel. The dye front of each gel was marked with a piece of wire.

### 2.6.3. Staining of SDS/polyacrylamide gels and non denaturing polyacrylamide gels

Gels were routinely stained for protein in 0.1% Coomassie Brilliant Blue G250, 50% (v/v) methanol, 10% (v/v) acetic acid for 30 min at 45 °C and destained in several changes of 10% (v/v) methanol, 10% (v/v) acetic acid at 45 °C.

Silver staining, essentially using the method of Wray *et al.* (1981), was used as a more sensitive method of protein detection. Gels were soaked for 1 or 2 days in 50% methanol. The staining solution was prepared by adding solution A (0.8 g $AgNO_3$ in 4 ml $H_2O$) to solution B (1.4 ml 14.8 M-$NH_4OH$ and 21 ml 0.36% (w/v) NaOH) dropwise while stirring vigorously. This mixture was made up to 100 ml with distilled water. The gel was then shaken gently for 10 min in the staining solution and rinsed for 1 h with 6 changes of distilled water. To develop the stain the gel was immersed in a solution comprising 2.5 ml 1% (w/v) citric acid and 0.25 ml of 38% (v/v) formaldehyde in 500 ml distilled water.

Once the bands had appeared the staining was stopped by removing the gel to distilled water.

Staining of non-denaturing gels for PEP carboxylase activity was carried out using the method of Nimmo and Nimmo (1982). Gels were stained at 30 °C in 50 mM-Tris/HCl, pH 8.0, 5 mM-$MgCl_2$, 10 mM-$NaHCO_3$, 2 mM-PEP, 0.1% (v/v) 2-mercaptoethanol and 10 mM-$CaCl_2$. Once an activity stain of sufficient intensity had been obtained (seen as a white band of $CaPO_4$ precipitate) the gels were removed from the staining mixture and stored in 50 mM-glycine/KOH, pH 10, 5 mM-$CaCl_2$ and 0.02% (w/v) sodium azide at 4 °C.

### 2.6.4. Estimation of $M_r$ by SDS/polyacrylamide gel electrophoresis

$M_r$ estimation was carried out on destained gels before they were dried. This was done by calculating the relative electrophoretic mobility ($R_f$) for each protein. $R_f$ is defined as:

$$R_f = \frac{\text{distance migrated by protein}}{\text{distance migrated by tracking dye}}$$

The following set of standard $M_r$ proteins were used for calibration by this method : carbonic anhydrase (29 000), ovalbumin (45 000), bovine serum albumin (66 000), phosphorylase b (97 400 subunit $M_r$), ß-galactosidase (116 000 subunit $M_r$), myosin (205 000 subunit $M_r$).

### 2.6.5. Drying and autoradiography of polyacrylamide gels.

Slab gels were dried for easy storage or autoradiography. Destained gels were dried on to Whatman 3MM chromatography paper using a Biorad Laboratories Slab Gel Drier model 1125 connected to an Aquavac Junior multi-purpose vacuum unit (Uniscience Ltd., 12-14 Ann's Crescent, London SW18 2LS, U.K.). Gels which had been dried, were autoradiographed using Fuji RX X-ray film with an intensifying screen. Autoradiography was carried out at -80 °C before developing the film using a Kodak X-OMAT Processor Model ME-3. The period of exposure usually varied from 3 h to 14 days.

### 2.6.6. Gel slicing and counting.

The band of interest was excised from destained gels using a scalpel. In cases where the gel had been dried for autoradiography it was re-swollen in $H_20$ before excision. The gel segments were placed in scintillation insert vials, covered with 0.3 ml hydrogen peroxide (30% (w/v)) and incubated at 37 °C for at least 24 h. Ecoscint (2 ml) was then added to each vial and the samples counted in a Beckman LS8100 liquid scintillation counter.

### 2.6.7. Peptide mapping by polyacrylamide gel electrophoresis

The method of Cleveland *et al.* (1977) was followed. The protein bands of interest were excised from Coomassie Blue-stained gels. The gel chips were rinsed in Cleveland buffer (0.125 M-Tris/HCl pH 6.8, 0.1% (w/v) SDS) for 30 min and then loaded into the slots of a 15% SDS/polyacrylamide gel (Section 2.6.1.). Each chip was overlayed with 10 µl of Cleveland buffer containing 20% glycerol and then 20 µl of protease in Cleveland buffer containing 10% glycerol and bromophenol blue. Electrophoresis was carried out until the dye front had reached the end of the stacking gel and the current was switched off for 30 min to allow proteolytic digestion to take place. The current was then switched back on until the tracking dye had reached the foot of the gel. The gel was silver stained for protein (see Section 2.6.3.).

## 2.7. Miscellaneous techniques

### 2.7.1. Gel-filtration of PEP carboxylase

This was carried out at room temperature on a Superose 6 column linked to a Pharmacia FPLC system. The column buffer contained 50 mM-Hepes/KOH, pH 7.1, 5 mM-$MgCl_2$, 5 mM-DTT and 1 mM-EDTA as well as the relevant additions shown in Table 5.2. In one set of experiments $MgCl_2$ was omitted from the column buffer. Enzyme activity (0.01-1 unit) was loaded on to the column in a volume of 100 µl. This represented 0.5-50 µg of purified PEP carboxylase or about 5-500 µg of crude extract protein. Where additions were made to the buffer, the enzyme was preincubated with the addition at room temperature for 30 min before it was loaded on the column. The flow rate was 0.4 ml/min and 0.5 min fractions were collected. The column was calibrated using the following $M_r$ markers : thyroglobulin (669 000), ferritin (440 000), aldolase (157 000), lactate dehydrogenase (144 000), hexokinase (100 000) and ovalbumin (45 000).

### 2.7.2. Preparation of antiserum to PEP carboxylase

Antisera were raised in New Zealand White rabbits. Pre-immune serum was first collected from each rabbit and then 0.5 mg of purified PEP carboxylase, emulsified in 50% Freund's complete adjuvant, was subcutaneously injected at many sites. 4 weeks later the rabbit was injected subcutaneously with 0.25 mg of purified PEP carboxylase emulsified in 50% Freund's incomplete adjuvant and a further 14 days later blood was removed, allowed to coagulate and antiserum was prepared. Subsequent bleeds

were taken at 14 day intervals over the next 6-8 weeks.

### 2.7.3. Immunoprecipitation of PEP carboxylase from crude extacts

The antiserum titre from each bleed was measured by incubating various volumes of the antiserum with 0.12 units of purified PEP carboxylase activity on ice for 30 min. The total volume in each incubation was kept constant by the addition of phosphate buffered saline (diluted ten-fold before use from a stock solution consisting of : 8.5 g NaCl, 1.28 g $Na_2HPO_4$, 1.56 g $NaH_2PO_4$ in 100 ml of $H_2O$, pH 7.6). After 30 min the mixture was centrifuged at 11 600 g for 2 min and the supernatant assayed for PEP carboxylase activity. The amount of antiserum used for immunoprecipitation experiments was that which was sufficient to precipitate greater than 95% of the PEP carboxylase activity in the incubation. The antiserum raised was shown to precipitate but not to inhibit enzyme activity by assaying the mixture after leaving on ice for 30 min, but before centrifugation, and finding that the amount of PEP carboxylase activity left in the supernatant at this stage was still 0.12 units.

### 2.7.4. *In vivo* labelling with [$^{32}$P] orthophosphate

This was done by the method of Nimmo *et al.* (1987). Detached leaf portions (0.4 g) were allowed to take up 40 μCi of carrier free [$^{32}$P] orthophosphate in 250 μl of water. They were then left resting in water for 24 to 48 h in the growth cabinet before any light treatment and extraction in liquid nitrogen as described in Section 2.5.2.

### 2.7.5. Analysis of phosphorylated amino acid residues

**(i) Preparation of protein.** The PEP carboxylase protein band was excised from a dried SDS/polyacrylamide gel and the backing paper scraped off carefully. The gel pieces were then cut up and swollen in a small volume of 0.05 M-$NH_4HCO_3$ before being homogenised and made 0.1% in SDS and 5% in 2-mercaptoethanol. This sample was boiled for 5 min and left shaking overnight at 37 °C on a rotary shaking water bath to elute the protein. Gel fragments were pelleted by centrifugation for 10 min at 11 600 g at room temperature and the supernatant was kept aside. The pelleted gel fragments were washed in half the volume of the same buffer and left for 2 h at 37 °C in the shaking water bath. These were re-pelleted and the supernatants from the two centrifugations were combined. 75 μg of BSA was added as a carrier and mixed well before leaving to precipitate on ice for 4 h in 20% trichloroacetic acid. The sample was centrifuged at 11 600 g for 15 min at 4 °C and the pellet washed with 95% (v/v) ethanol

at -10 °C and then with 95% (v/v) ethanol : ether (1:1) at -10 °C, centrifuging each time.

The following steps were carried out using the method of Cooper *et al.*, (1983):

**(ii) Hydrolysis.** The dried pellet was resuspended in 250 µl of 5.7 M-HCl and transferred to a nitric acid washed Corex glass test-tube which was vacuum sealed and left at 110 °C on a heating block for 2 h to allow hydrolysis. The test-tube was then removed from the heating block, broken open to release the vacuum and the sample left overnight to dry in a desiccator.

**(iii) Electrophoresis.** Separation of the phosphoamino acids was carried out using two-dimensional thin-layer electrophoresis. The desiccated sample was solubilised in 20 µl of pH 1.9 buffer : 88% formic acid, glacial acetic acid, $H_2O$ (50 : 156 : 1794) and spotted in 0.3-0.5 µl aliquots on to the origin of a 10 x 10 cm silica gel plate. The plate was dried with a stream of cold air after each application. Unlabelled standards, 0.5 µl of each, of disodium hydrogen phosphate ($P_i$), phosphoserine, phosphothreonine and phosphotyrosine were also spotted on to the origin and dried. The plate was then wetted in pH 1.9 buffer for electrophoresis in the first dimension, assembled into the electrophoresis apparatus and electrical contact made with the two buffer tanks using two pieces of Whatman 3MM chromatography paper as wicks. Electrophoresis was carried out at 500 V for 40 min. The plate was then dried and wetted in pH 3.5 buffer : pyridine, glacial acetic acid, $H_2O$ (10 : 100 : 1890) and electrophoresis in the second dimension was carried out at 500 V for 30 min in pH 3.5 buffer.

**(iv) Staining.** The dried silica plate was firstly stained for inorganic phosphate using 60% (w/v) perchloric acid, 1 M-HCl, 4% ammonium molybdate, acetone (5 : 10 : 25 : 60) and the position of the spot marked. The plate was then stained for the phosphoamino acids by spraying it with 0.25% ninhydrin in acetone and then drying it under a stream of cold air. These spots were also marked and the plate autoradiographed to locate the label from the sample.

### 2.7.6. *In vitro* phosphorylation of PEP carboxylase

**(i) Leaf extracts.** In experiments involving leaf extracts, incubations of 100 µl total volume were carried out at 25 °C. In this volume extract containing 0.06 units of PEP carboxylase activity was incubated in 50 mM-Hepes/KOH, pH 7.1, 5 mM-DTT, 1 mM-EDTA, 10 µg of chymostatin/ml, 10 mM-$MgCl_2$ and 10 µM or 0.5 mM-[$\gamma$-$^{32}$P]ATP (5 µCi). In order to facilitate the interpretation of results parallel assays were done, one including 0.06 units of purified PEP carboxylase (see Section 2.5.3.) and the other including the same volume of Buffer B plus 50% glycerol (see Section 2.5.1.). To

ensure that any phosphorylation which occurred was of PEP carboxylase and not PPDK, which has a similar $M_r$ to PEP carboxylase and is known to be reversibly phosphorylated (see Budde and Chollet, 1986), immunoprecipitation of PEP carboxylase from the mixture was carried out using the specific antiserum raised against purified enzyme (see Sections 2.7.2. and 2.7.3.). The resulting pellet, which contained the PEP carboxylase protein, was washed with 1 ml of 1.5 M-NaCl, 2 mM-EDTA, pH 7.0, resuspended in 90 µl of SDS sample buffer (see Section 2.6.1.) and boiled for 2 min. Samples of 30 µl were then analysed by SDS/polyacrylamide gel electrophoresis and autoradiography.

**(ii) Partially purified protein kinase samples.** Assays using partially purified protein kinase were carried out essentially by the method of Jiao and Chollet (1989). Partially purified kinase sample (17 µl) which had been thoroughly dialysed into Buffer D (see Section 2.5.1.) was incubated at 30 °C with 16.5 pmol of purified PEP carboxylase, 5 mM-$MgCl_2$ and 0.5 mM-[$\gamma$-$^{32}$P]ATP (1.25 µCi) in Buffer D in a total volume of 25 µl. The reaction was terminated by adding of 25 µl of SDS sample buffer and boiling for 2 min. Samples (40 µl) of this mixture were then analysed by SDS polyacrylamide gel electrophoresis and autoradiography.

Investigation of the effect of incubating purified PEP carboxylase with the partially purified kinase sample on the malate sensitivity of the enzyme was carried out using the same incubation conditions described for the partially purified kinase samples. However, 0.5 mM of unlabelled ATP was used instead of [$\gamma$-$^{32}$P]ATP and the malate sensitivity of the PEP carboxylase was assayed as in Section 2.4.2.(i).

In all cases the reactions were started by the addition of ATP. Variations from these standard procedures are stated in the relevent text.

### 2.7.7. Investigation of protein phosphatase activity in leaf extracts

The measurement of PP-1 and PP-2A activities in extracts from darkened and illuminated leaves was carried out by the method of Cohen *et al.* (1988a). Extracts were made as described in Section 2.5.2. and were then centrifuged for 2 min at 11 600 g. The supernatant (cytosolic fraction) was diluted twenty-fold for use and the pellet was washed twice with extraction buffer before being resuspended in the same volume of extraction buffer and diluted five-fold for assay. Samples (10 µl) were pre-incubated for 15 min at 30 °C with 10 µl of 50 mM-Tris/HCl, pH 7.0, containing 0.1 mM-EGTA, 0.1% (v/v) 2-mercaptoethanol, 1 mg/ml BSA, 0.03% Brij-35. These samples were then assayed for protein

phosphatase activity by incubating at 30 °C with 10 µl of $^{32}$P-labelled phosphorylase a (3mg/ml) from rabbit skeletal muscle. The phosphorylase a had been stored crystallized at 4 °C and was resuspended in 50 mM-Tris/HCl, pH7.0 containing 0.1 mM-EGTA, 0.1% (v/v) 2-mercaptoethanol, 15 mM-caffeine. Assays were terminated by the addition of 0.2 ml of 10% trichloroacetic acid, to precipitate the protein, and leaving on ice for 2 min before centrifuging for 2 min at 11 600 g. A 0.2 ml sample of the supernatant was removed, mixed with 1 ml of Ecoscint and $^{32}$P-radioactivity determined by scintillation counting.

In order to assay the relative activities of PP-1 and PP-2A, the effect of the phosphatase inhibitors okadaic acid and inhibitor 1 were examined. This was done by carrying out the basic phosphatase assay described, in the presence of :

(a) no inhibitor

(b) 0.2 µM-inhibitor 1

(c) 1 nM-okadaic acid

(d) 0.2 µM-inhibitor 1 and 1 nM-okadaic acid

Control assays were carried out by assaying in the absence of leaf extract and inhibitors. PP-1-like activity was measured as both the activity sensitive to 0.2 µM-inhibitor 1 and as the activity not inactivated by 1 nM-okadaic acid, and these two values were averaged. PP-2A-like activity shown was the average of the activity which was insensitive to inhibitor 1 and the activity sensitive to 1 nM-okadaic acid.

One unit of activity was that amount which catalysed the dephosphorylation of 1 µmol of substrate in 1 min.

### 2.7.8. ATP determination

The measurement of ATP concentrations was carried out as in Trautschold *et al.* (1983). The reduction of $NADP^+$ was assayed at 340 nm in 1 ml reaction mixtures containing 50 mM-Tris/HCl, pH 7.5, 20 mM-$NADP^+$, 0.1 M-$MgCl_2$, 0.5 M-glucose and 100 µl of leaf extract. The assay was started by adding 5 l of hexokinase/G6P dehydrogenase mixture (3mg/ml). When ATPase activity was meaured, extracts were made 0.5 mM in ATP and assays carried out at time intervals to measure the loss of ATP with time.

## 2.8. Statistics

Statistical analysis of data was carried out using Student's t-test on the Apple Macintosh "Statview" computer program (Apple Computer Inc., 20525 Mariani Avenue, Cupertino, CA 95014, U.S.A.).

Chapter 3

# THE ACTIVATION OF PEP CARBOXYLASE BY LIGHT

## 3.1. Introduction

When this work was started there was very little experimental evidence to support the hypothesis that PEP carboxylase in the leaves of $C_4$ plants is activated by light *in vivo*. However, the allosteric nature of the enzyme had been well established (see Section 1.3.3. and references therein), raising the possibility that diurnal changes in the kinetic properties could increase the activity of the enzyme *in vivo* on illumination. After an early observation that PEP carboxylase activity was two-fold greater in extracts prepared from illuminated leaves than in extracts prepared from darkened leaves (Slack, 1968), it was not until extraction and assay conditions were found which revealed light/dark differences in the activity of the enzyme in leaf extracts of several $C_4$ species (Karabourniotis *et al.*, 1983; Manetas *et al.*, 1983) that further progress on this issue was made possible. However the light activation of PEP carboxylase was not detected in extracts of illuminated and darkened leaves from several other $C_4$ species, including maize, although this could have been attributable to inappropriate extraction and/or assay buffers being used (Karabourniotis *et al.*, 1983). It seemed that if such activation did occur *in vivo* then it would probably be found in all $C_4$ plants rather than just some.

The purpose of this section of work was threefold :

1. To make a preliminary investigation of the kinetic properties of PEP carboxylase in an attempt to identify any differences in the behaviour of the enzyme in crude extracts prepared from darkened and from illuminated maize leaves.

2. To try to find out the nature of the photoreceptor(s) responsible for perceiving the light stimulus which causes PEP carboxylase activation. Higher plants are known to have three different light receptors : the pigments of the photochemical apparatus, phytochrome and a blue light receptor. Any or all of these could play a role in PEP carboxylase activation.

3. To investigate the possibility that post-translational modification of the $C_4$-type PEP carboxylase could be involved in its activation by light. Preliminary experiments carried out in Glasgow by Dr G.A. Nimmo, before the work for this thesis was started, showed that $^{32}P_i$ could be incorporated into maize

leaf PEP carboxylase. If leaves were labelled with $^{32}P_i$, extracted and samples run on SDS/polyacrylamide gels, autoradiography then showed $^{32}P_i$ incorporation into a protein band with a $M_r$ similar to that of PEP carboxylase. This result suggested the possibility that PEP carboxylase from $C_4$ plants could undergo a covalent phosphorylation similar to that seen previously with the enzyme from CAM plants (Nimmo *et al.*, 1986; Brulfert *et al.*, 1986). The aim of this section of work therefore was to study the phosphorylation state of PEP carboxylase in leaves which had been labelled with $^{32}P_i$ and then subjected to various periods of illumination and darkness, and to investigate whether any possible diurnal variation in phosphorylation state might correlate with changes in the kinetic behaviour of the enzyme.

## 3.2. Results

### 3.2.1. Changes in the kinetic properties of PEP carboxylase in response to light and dark

The kinetic properties of PEP carboxylase were investigated in leaves which had been darkened or highly illuminated. The extracts were prepared as rapidly as possible and desalted rapidly using a 1 x 12 cm (10 ml) column of Sephadex G-25M. Similar data to those described were also obtained using extracts that had been desalted. Extracts prepared by either of the methods described in Section 2.5.2. gave similar results. The pH dependence of PEP carboxylase, the $K_m$ of the enzyme for the substrate PEP and its sensitivity to the feedback inhibitor malate were examined.

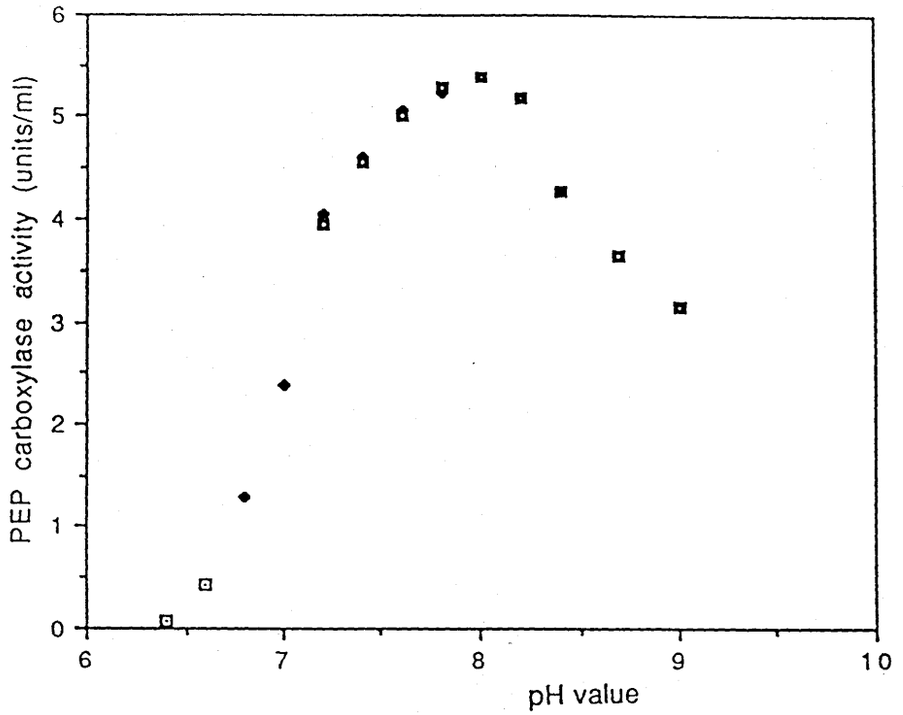
The activity of PEP carboxylase in extracts from illuminated and darkened leaves was compared at a range of pH values between pH 6.4 and pH 9.0 in the presence of 10 mM-$MgCl_2$ and 5 mM-PEP in the assay mixture. Enzyme activity in extracts from both types of leaves had similar pH profiles with maximal activity between pH 7.8 and pH 8.0 (Figure 3.1.). The specific activity of PEP carboxylase did not vary between extracts of illuminated and darkened leaves and there was no significant difference between the activity of the enzyme in illuminated and darkened extracts at any pH tested.

The values for the $K_m$ for PEP, $V_{max}$ and the apparent $K_i$ for malate of PEP carboxylase were tested at two different pH values under different assay conditions (Table 3.1.). Early kinetic studies were carried out at pH 7.8 using the assay conditions described in Section 2.4.2.(i) for this pH value. However, while the kinetic investigation at pH 7.8 was in progress, Huber and Sugiyama (1986) reported

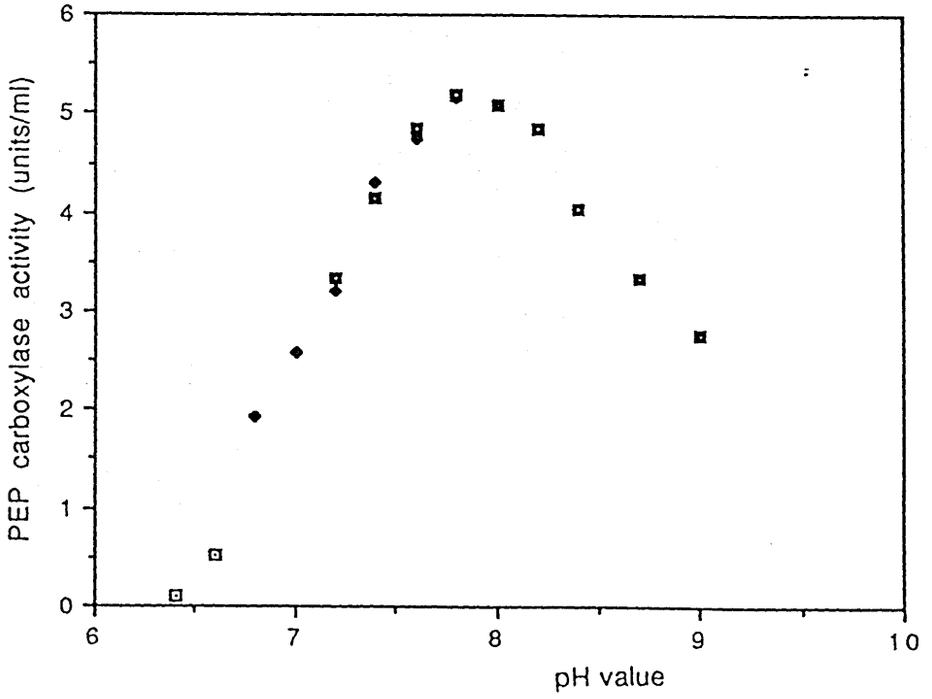
FIGURE 3.1. The effect of pH on the activity of PEP carboxylase in extracts prepared from (a) darkened and (b) illuminated leaves. The assays were carried out as described in section 2.4.2.(i). The buffers were 50 mM-Mes/KOH (pH 6.2-6.4), 50 mM-Hepes/KOH (pH 6.6-7.8), 50 mM-Tris/HCl (pH 7.2-9.0). Each assay contained 10 mM-$MgCl_2$, 5 mM-PEP, 10 mM-$NaHCO_3$, 0.2 mM-NADH, 5 units of MDH. Each point is the mean of three experiments. The spread of the values for the enzyme activity between different experiments was approx. ± 10-15%.

- ▫ Mes
- ◆ Hepes
- ▪ Tris

(a)


PEP carboxylase activity (units/ml)
0
1
2
3
4
5
6
6
7
8
9
10
pH value

(b)


PEP carboxylase activity (units/ml)
0
1
2
3
4
5
6
6
7
8
9
10
pH value

that PEP carboxylase in extracts prepared from illuminated maize leaves was less sensitive to inhibition by malate than enzyme in extracts prepared from darkened leaves, when assayed at pH 7.0 and in the presence of 0.5 mM-PEP and 5 mM-G6P. The present study was therefore widened to include an investigation of the kinetic properties of PEP carboxylase under similar conditions of assay to those developed by Huber and Sugiyama (1986). Studies on the effect of G6P, which was present in the pH 7.0 assay mixture showed that 5 mM-G6P stimulated PEP carboxylase activity 7-10-fold at this pH value. At pH 7.8, however, the enzyme activity was stimulated only 1.2-1.7-fold by 5 mM-G6P and did not produce any light/dark difference in the activity of the enzyme.

No statistical difference at the 0.1 level of confidence was observed between the $V_{max}$ values of PEP carboxylase in extracts prepared from illuminated or darkened leaves at either pH value (Table 3.1.). There was no statistical difference in the protein concentration of extracts prepared for either type of leaf sample.

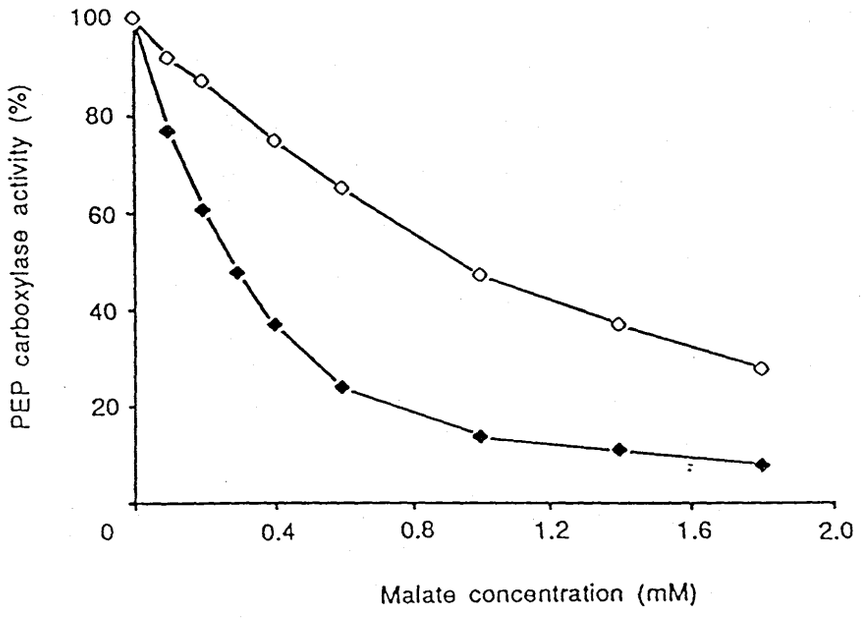
The $K_m$ for PEP did not differ significantly (at the 0.1 level of confidence) at pH 7.8. The affinity of PEP carboxylase for this substrate was greater at the lower pH value where there was a small statistically significant change in $K_m$ on illumination (significantly different at the 0.1 level but not at the 0.01 level of confidence). Under both assay conditions, but particularly at pH 7.8, higher than 10 mM concentrations of PEP led to a decrease in the enzyme activity, possibly due to PEP chelating $Mg^{2+}$.

Measurement of the apparent $K_i$ for malate at pH 7.8 showed that the enzyme in extracts from illuminated and darkened leaves had similar (not statistically different at the 0.1 level of confidence) high apparent $K_i$ values (Table 3.1.). However, the malate sensitivities of PEP carboxylase in the two types of leaf extract were found to be statistically different at pH 7.0 (statistically different at the 0.001 level of confidence). The apparent $K_i$ of the enzyme for malate was found to be 2-4-times higher in extracts prepared from illuminated leaves than in extracts prepared from leaves which had been kept in darkness. After illumination of leaves the enzyme in extracts was less sensitive to inhibition by malate, with an apparent $K_i$ value of 0.8-1.2 mM, than after darkening of leaves when the enzyme in extracts showed hyperbolic inhibition by malate and an apparent $K_i$ value of 0.2-0.5 mM (Figure 3.2.). The malate sensitivity at pH 7.0 and 5 mM-PEP was used as the kinetic criterion in all further studies of the

TABLE 3.1. **Comparison of the kinetic properties of PEP carboxylase measured in extracts prepared from darkened and illuminated leaves.** Assays were carried out using the conditions described in Section 2.4.2.(i) except that at pH 7.8 malate sensitivity was measured by including 0-30 mM-malate in the assay. In assay mixtures at both pH values, the PEP concentrations used to estimate the $K_m$ for PEP were 0.05-10 mM. Values given are expressed as means ± the S.D. for five experiments.

| Leaf treatment | Assay pH | $V_{max}$ (units/ml) | $K_m$ for PEP (mM) | $K_i$ for malate (mM) |
|---|---|---|---|---|
| Darkness | 7.8 | 4.65 ± 0.95 | 1.28 ± 0.26 | 18.25 ± 2.01 |
| Illumination | 7.8 | 4.42 ± 1.66 | 1.18 ± 0.15 | 19.40 ± 2.59 |
| Darkness | 7.0 | 3.24 ± 1.45 | 0.38 ± 0.19 | 0.32 ± 0.09 |
| Illumination | 7.0 | 4.35 ± 1.87 | 0.24 ± 0.17 | 0.98 ± 0.12 |

FIGURE 3.2. **Inhibition by malate of PEP carboxylase in extracts prepared from darkened and illuminated leaves.** Enzyme activity is expressed as a percentage of the activity measured in the absence of malate. Extracts were prepared from leaves maintained in darkness for 2 h at 27 °C (◆) or illuminated for 1 h at 27 °C (1000 μmol $m^{-2}s^{-1}$) (o).


100
80
60
40
20
0
0.4
0.8
1.2
1.6
2.0
PEP carboxylase activity (%)
Malate concentration (mM)

behaviour of PEP carboxylase in response to light.

The presence or absence of the thiol compounds, DTT (5 mM) or 2-mercaptoethanol (0.05% v/v), in either the extraction or assay buffers did not affect the extent of the difference in the malate sensitivity of PEP carboxylase in extracts from illuminated or darkened leaves.

The addition of lactate dehydrogenase (5 units) to some assays showed that the non-enzymic decarboxylation of OAA, which has been suggested to lead to an under- estimation of the enzyme activity (Walker *et al.*, 1986a), was not significant in assays at pH 7.0 or pH 7.8.

Figure 3.3. shows the effect of light intensity at 27 °C on the $K_i$ for malate of PEP carboxylase. When leaves were illuminated at light intensities below 200 μmol $m^{-2}s^{-1}$ there was little or no increase in the apparent $K_i$ for malate. Above about 600 μmol $m^{-2}s^{-1}$ the increase in the malate sensitivity was complete within 1 h and the extent of the increase was independent of the photon fluence rate.

The time taken for the increase in the $K_i$ for malate to reach completion was measured using leaves illuminated at 1 000 μmol $m^{-2}s^{-1}$ at 27 °C (Figure 3.4.(a)). The change in malate sensitivity was complete after about 1 h of illumination. Similarly, leaves which had been illuminated for 1 h and then transferred to darkness at 27 °C (Figure 3.4.(b)) also took about 1 h for the $K_i$ for malate to return to the value for leaves which had not been highly illuminated. These results are similar to those found for leaves from plants which had been grown, illuminated and extracted under slightly different conditions (Nimmo *et al.*, 1987).

Immunotitration curves for PEP carboxylase from illuminated and darkened leaves were identical, suggesting that the amount of PEP carboxylase protein does not change in the short term (1 h) response to light (not shown).

### 3.2.2. The nature of the photoreceptor(s) responsible for PEP carboxylase activation

Initial studies consisted of darkening leaves or illuminating them using a xenon arc lamp and then investigating the apparent $K_i$ for malate of PEP carboxylase in the crude leaf extracts. The xenon arc lamp enabled high light intensities to be attained and, by using blue and red light filters with sharp cut-offs at 505 nm, the light spectrum could be split precisely into the blue and red portions allowing leaves to be illuminated with white light or either blue light or red light (Figure 3.5). The filters decreased the maximum light intensity achievable to 600 μmol $m^{-2}s^{-1}$ which was sufficiently high to see a difference in malate sensitivity between illuminated and darkened leaves. Thus leaves were treated in four ways :

FIGURE 3.3. **The effect of light intensity on the apparent $K_i$ for malate of PEP carboxylase.** Leaves were illuminated for 1 h at 27 °C at the various light intensities (see Section 3.2.2.). Values shown are expressed as means ± S.D. for three experiments.

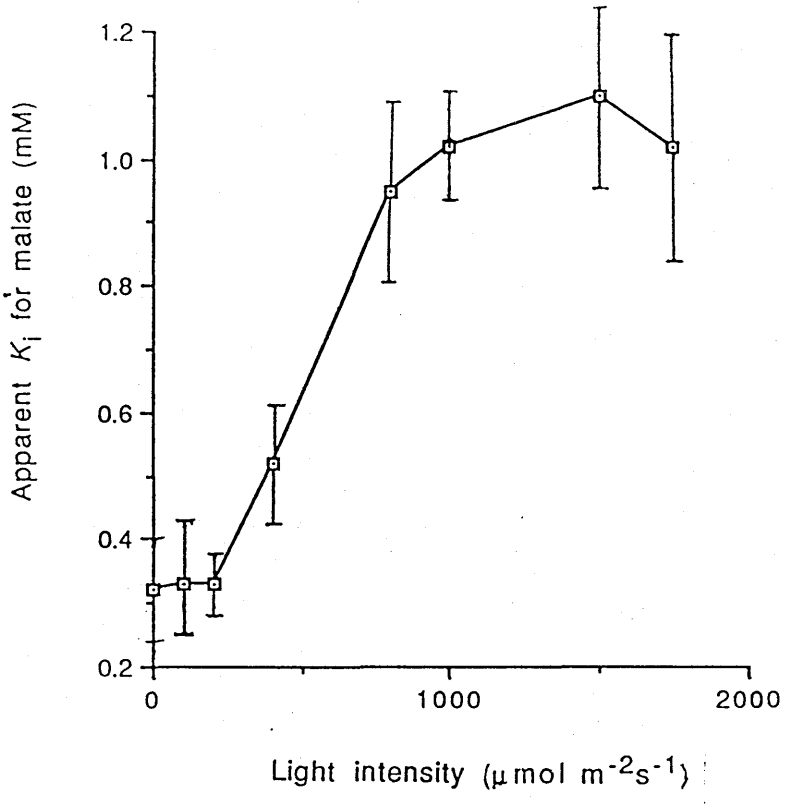
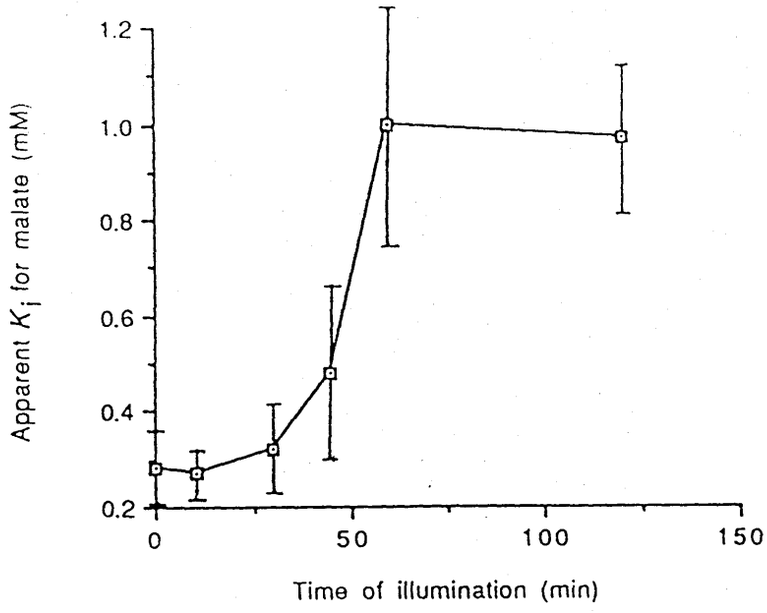

Apparent $K_i'$ for malate (mM)
1.2
1.0
0.8
0.6
0.4
0.2
0
1000
2000
Light intensity ($\mu$mol $m^{-2}s^{-1}$)

FIGURE 3.4. **Time courses of the changes in the malate sensitivity of PEP carboxylase in response to light and dark.** Leaves were illuminated as described in Section 3.2.2. before extraction and assay. (a) Leaves were kept in darkness for 10-12 h at 18 °C and then detached and transferred to light (1000 μmol $m^{-2}s^{-1}$) at 27 °C at zero time. (b) Detached leaves were illuminated (1000 μmol $m^{-2}s^{-1}$) for 1 h at 27 °C and then transferred to darkness at 27 °C at zero time. Values shown are expressed as means ± S.D. for three experiments.

(a)


1.2
1.0
0.8
0.6
0.4
0.2
Apparent $K_i$ for malate (mM)
0
50
100
150
Time of illumination (min)

(b)

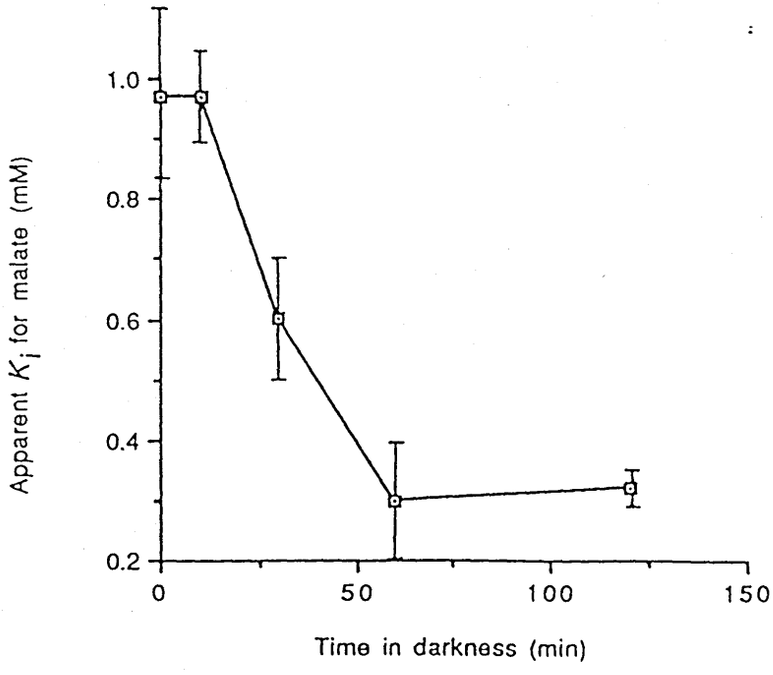
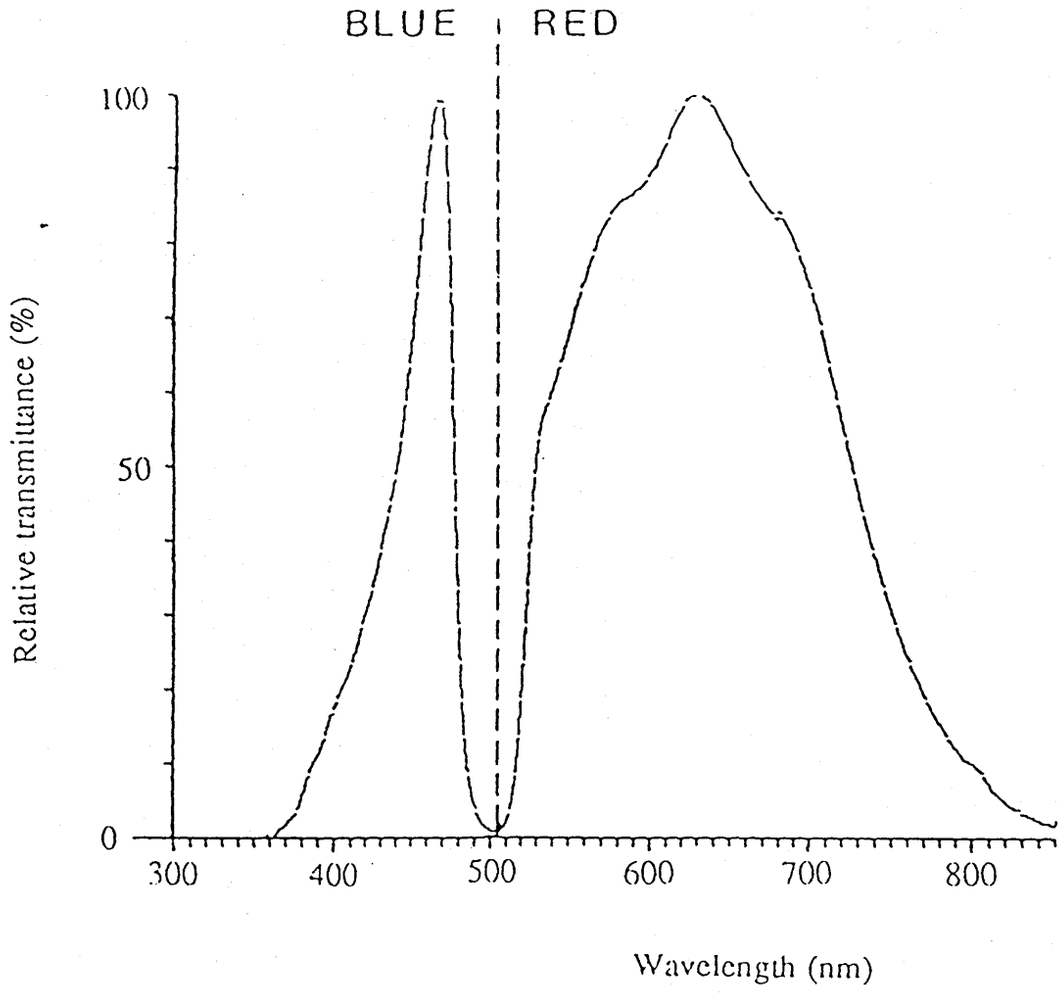

1.0
0.8
0.6
0.4
0.2
Apparent $K_i$ for malate (mM)
0
50
100
150
Time in darkness (min)

FIGURE 3.5. **Plot showing the transmittance of the blue and the red light filters described in Section 3.2.2.** The dashed vertical line shows the cut-off point between the filters. The blue filter did not permit light transmittance above 505 nm while the red filter did not allow transmittance of light below 505 nm (Figure courtesy of Dr G.I. Jenkins).


BLUE
RED
100
50
0
Relative transmittance (%)
300
400
500
600
700
800
Wavelength (nm)

darkened, illuminated with white light (full spectrum), illuminated with blue light (below 505 nm) or illuminated with red light (above 505 nm). After 1 h treatment the leaves were extracted and the malate sensitivity of PEP carboxylase measured.

The results in Table 3.2. show that the characteristic increase in the apparent $K_i$ for malate on illumination could be achieved by using either white, blue or red light. The $K_i$ value for leaves illuminated under any of the conditions was statistically different at the 0.005 probability level from the dark $K_i$ value. This suggests that the pigments of the photochemical apparatus are responsible for perceiving the light stimulus needed for PEP carboxylase activation and not the blue photoreceptor or phytochrome. However, this result does not exclude the involvement of the high irradiance response of phytochrome.

In an attempt to confirm the involvement of the photosynthetic pigments and photosynthetic electron transport in PEP carboxylase activation, the effect of DCMU was examined. DCMU is a specific inhibitor which blocks electron transport between the two photosystems (Izawa, 1982) (see Figure 1.1.). Detached leaves were floated on a 50 $\mu$M DCMU solution (aqueous + 0.25% v/v ethanol) for 90 min in darkness before 1 h further darkening or 1 h illumination at 1000 $\mu$mol $m^{-2}s^{-1}$. Parallel experiments were run using control leaves without DCMU added. After 90 min the DCMU had entered the leaves. This was shown using fluorescence analysis before the light treatment and extraction of the leaves. The fluorescence traces of DCMU treated leaves did not show the characteristic quenching of the fluorescence burst seen for traces from the control untreated leaves, instead the fluorescence burst remained largely unquenched. This is the expected effect of DCMU on fluorescence.

Table 3.3. shows the result of feeding leaves with DCMU before illuminating or darkening them. The expected increase in the apparent $K_i$ for malate on illumination which was seen for control leaves was not seen for leaves treated with DCMU prior to illumination. These leaves showed malate sensitivities which were similar to those found for dark control leaves. The activity of NADP-MDH was measured at the same time as the malate sensitivity of PEP carboxylase. NADP-MDH is an enzyme whose light activation is known to be blocked by DCMU (see Section 1.3.3.) and therefore acted as a further indication that DCMU had entered the leaf cells. NADP-MDH activity was inhibited in DCMU treated leaves in a similar manner to the decrease in the malate sensitivity of PEP carboxylase

TABLE 3.2. **The effect of blue and red light on the activation of PEP carboxylase.** Values are expressed as means ± S.D. for three experiments. The illumination with white, blue or red light was carried out at 600 μmol $m^{-2}s^{-1}$.

| Leaf treatment | Apparent $K_i$ for malate (mM) |
|---|---|
| Darkness | 0.40 ± 0.18 |
| Illumination with white light | 1.08 ± 0.32 |
| Illumination with blue light | 1.03 ± 0.23 |
| Illumination with red light | 0.98 ± 0.20 |

(Table 3.3.). In further experiments, a similar inhibition of the light activation of PEP carboxylase and NADP-MDH was also observed using methyl viologen, another inhibitor of the electron transport chain which shunts electrons from photosystem 1 to $O_2$ (Izawa, 1982). From these results it is apparent that the signal for the activation of PEP carboxylase must involve the pathway of photosynthetic electron transport.

The signal which activates PEP carboxylase on illumination does not seem to be transmitted from one part of the leaf to another. Detached leaves were illuminated on either the distal or the lower half of the leaf blade while the other half was kept in the dark. If the leaves were then cut in two, the two halves separately extracted and the apparent $K_i$ for malate measured, then the same light/dark difference in the malate sensitivity of the enzyme was seen as in leaves which had been cut in half before illuminating and darkening the separate halves. Clearly this does not rule out a more local transmission of the signal but it does show that shading of leaves may not allow activation of PEP carboxylase even when part of the plant is subjected to full sunlight and emphasises the need to ensure complete illumination of all areas of detached leaves for experimental purposes.

### 3.2.3. *In vivo* phosphorylation of PEP carboxylase

The experiments described in this section were carried out in collaboration with Dr G.A. Nimmo and Dr H.G. Nimmo.

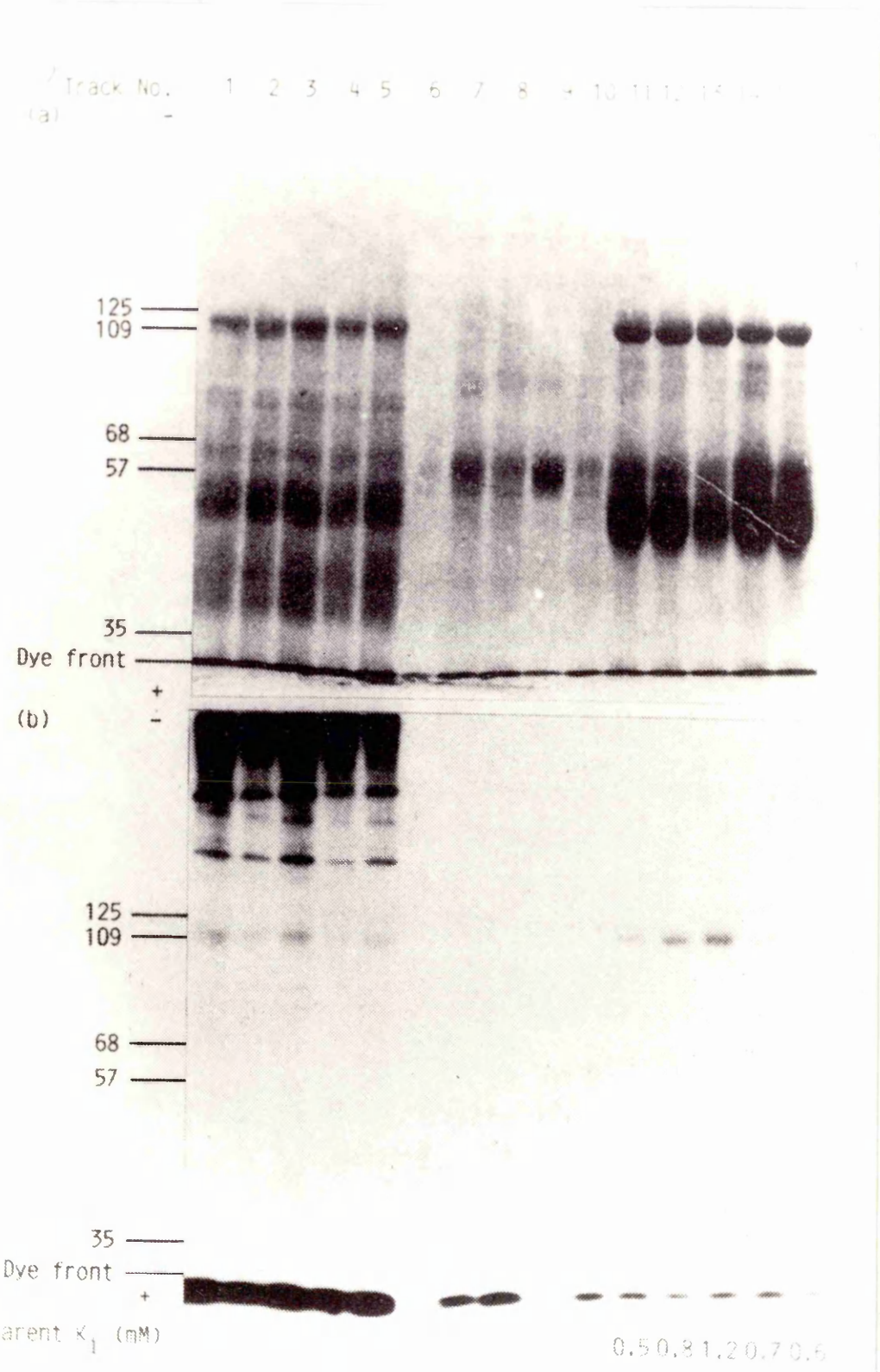
Leaves were labelled with $^{32}P_i$ for 24 h as described in Section 2.7.4. and then maintained in darkness at 15 °C for 10-12 h. They were then extracted : immediately, after 30 min illumination, after 60 min illumination, after 60 min illumination followed by 45 min darkness and after 60 min illumination followed by 90 min darkness, all at 27 °C (see Figure 3.6.). Samples containing 0.01 unit of PEP carboxylase activity from each of these extracts were immunoprecipitated as described in Section 2.7.3. except using 0.3 ml of antiserum raised against PEP carboxylase from *Bryophyllum fedtschenkoi*. The immunoprecipitated samples were then run on an SDS/polyacrylamide gel which was subsequently dried and autoradiographed. At the same time the apparent $K_i$ for malate for each extract was measured.

Similar amounts of PEP carboxylase protein of subunit $M_r$ 109 000 were immunoprecipitated from each extract as judged by the Coomassie Brilliant Blue stain (Figure 3.6.(a), tracks 11-15). The protein band was shown to be PEP carboxylase by using the Cleveland mapping procedure (see Section 2.6.7) to

TABLE 3.3. **The effect of DCMU on the activation by light of PEP carboxylase and NADP-malate dehydrogenase.** Values are expressed as means ± S.D. for the number of experiments shown in parentheses in the final column. Levels of significance are for a comparison of the apparent $K_i$ for malate value with the $K_i$ for malate value of the dark-DCMU control leaf sample : n.s., not significantly different at the 0.1 level; s., significantly different at the 0.001 level.

| Treatment of leaf | $K_i$ for malate of PEP carboxylase (mM) | Level of significance | Specific activity of PEP carboxylase (units/mg) | Specific activity of NADP-MDH dehydrogenase (units/mg) |
|---|---|---|---|---|
| Darkness -DCMU | 0.29 ± 0.04 | - | 1.14 ± 0.16 | 0.06 ± 0.03 (5) |
| Illumination -DCMU | 0.91 ± 0.11 | s | 1.26 ± 0.36 | 1.02 ± 0.37 (6) |
| Darkness +DCMU | 0.32 ± 0.13 | n.s. | 1.64 ± 0.74 | 0.06 ± 0.04 (5) |
| Illumination +DCMU | 0.39 ± 0.21 | n.s. | 1.38 ± 0.20 | 0.11 ± 0.04 (6) |

FIGURE 3.6. SDS/polyacrylamide gel electrophoresis of extracts from $^{32}P$-labelled leaves. Leaves were labelled with $^{32}P$ for 24 h as described in Section 2.7.4. and then maintained in darkness for 10-12 h. They were then extracted immediately (tracks 1,6,11), after 30 min illumination (tracks 2,7,12), after 60 min illumination (tracks 3,8,13), after 60 min illumination followed by 45 min darkness (tracks 4,9,14) and after 60 min illumination followed by 90 min darkness (tracks 5,10,15). Tracks 1-5, leaf extract containing 0.01 unit of PEP carboxylase activity. Tracks 11-15, immunoprecipitate containing 0.02 units of PEP carboxylase activity. Tracks 6-10, as tracks 11-15 but substituting pre-immune serum for antiserum. Illuminations were carried out at 800 $\mu$mol $m^{-2}s^{-1}$. (a) Coomassie Brilliant Blue-stained gel. (b) Autoradiograph. The numbers on the left indicate the $M_r$ values x $10^{-3}$ of the marker proteins (see section 2.6.4.) and the PEP carboxylase subunit (109 000). The apparent $K_i$ for malate was measured in each extract and is indicated beneath the appropriate immunoprecipitate.


Track No. 1 2 3 4 5 6 7 8 9 10 11 12 13 14
(a)
-
125
109
68
57
35
Dye front
+
(b)
-
125
109
68
57
35
Dye front
+
Apparent $K_I$ (mM)
0.5 0.8 1.2 0.7 0.5

compare the pattern of proteolytic fragments obtained from the $M_r$ 109 000 band which had been immunoprecipitated with the pattern obtained from purified PEP carboxylase from maize. No subunit $M_r$ 109 000 band was seen in control immunoprecipitates (Figure 3.6.(a), tracks 6-10).

The apparent $K_i$ for malate for PEP carboxylase from each of the leaf samples is also shown in Figure 3.6. The extracts with the lowest apparent $K_i$ for malate (0.5-0.6 mM) are those extracted immediately after 10 h darkness (Figure 3.6.(b), track 11) and after 60 min illumination followed by 90 min in the dark (Figure 3.6.(b), track 15). These samples also seemed to contain the least $^{32}P$. Further, the extract which had the highest apparent $K_i$ for malate (1.2 mM for the sample from leaves extracted after 60 min illumination) appeared to contain the most $^{32}P$ label.

The $^{32}P$ present did not constitute a covalent intermediate in the reaction mechanism of catalysis since pre-incubation of the $^{32}P$-labelled extracts with unlabelled substrates for 1 h at 25 °C before immunoprecipitating did not remove any $^{32}P$ from PEP carboxylase.

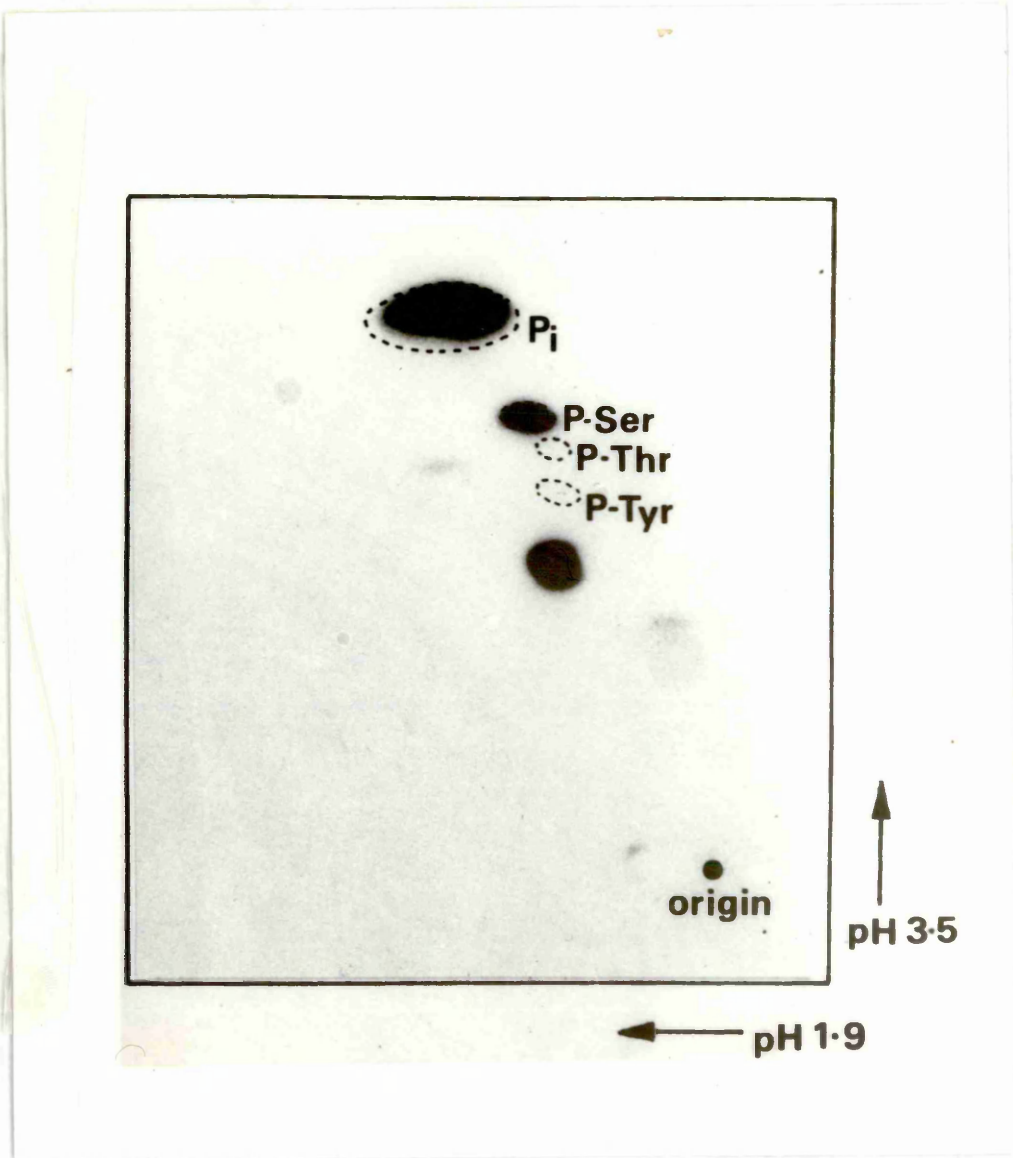
The $^{32}P$-labelled PEP carboxylase was analysed for phosphoamino acids as described in Section 2.7.5. Three radioactive spots were observed (Figure 3.7.). These corresponded to $^{32}P_i$, phosphoserine and an unknown. The unknown spot probably represents a phosphopeptide since a longer period of hydrolysis (4 h) removed the label from this spot and increased the amount of $^{32}P$ in $P_i$. It therefore seems that PEP carboxylase from maize is phosphorylated *in vivo* on one or more serine residues.

The results described are for leaves from plants which were grown in short (8 h) days and extracted in four volumes of 0.1 M-Tris/HCl, pH 7.0, 1 mM-EDTA, 10 mM-$MgCl_2$, 20% (w/v) sorbitol, 0.05% 2-mercaptoethanol and polyclar AT (analagous to PVP) to 10% of the leaf weight, as described in Nimmo *et al.* (1987), instead of the usual extraction buffer (Buffer A, Section 2.5.1.). However, very similar results were obtained using leaves from plants grown under 12 h days and extracted in Buffer A.

## 3.3 Discussion

The studies of the kinetic properties of leaves which have been darkened or highly illuminated show that, as previously found for other $C_4$ plants (Karabourniotis *et al.*, 1983, 1985; Manetas *et al.*, 1983), maize exhibits a light/dark difference in these properties under certain conditions of assay. Manetas *et al.* (1983) and Karabourniotis *et al.* (1983) reported that illumination of leaves from the $C_4$ plant *Salsola soda* resulted in an increase in the affinity of PEP carboxylase for PEP while Doncaster and Leegood (1987) found assay conditions where maize PEP carboxylase also showed a small decrease in the $K_m$ for

FIGURE 3.7. **Autoradiograph of a two-dimensional electrophoretogram of $^{32}P$-labelled PEP carboxylase which had been subjected to partial acid hydrolysis.** This was carried out as described in Section 2.7.5. The positions of the standard markers are indicated. The radioactive spot which is not marked is believed to be a phosphopeptide (see text).


Pi
P-Ser
P-Thr
P-Tyr
origin
pH 3·5
pH 1·9

PEP on illumination but no change in $V_{max}$. In the present study, however, under assay conditions at pH 7.8, no statistically significant difference was found in either the $V_{max}$, $K_m$ for PEP or the apparent $K_i$ for malate from darkened or illuminated leaves (Table 3.1.). Under the assay conditions used at pH 7.0, there was no statistically significant difference in the $V_{max}$ and only a slight statistical difference the $K_m$ for PEP. However, there was a much more significant, 2-4-fold, difference in the malate sensitivity observed between illuminated and darkened leaves at pH 7.0 (Table 3.1.). This diffence in kinetic behaviour could be seen only at suboptimal pH and at limiting concentrations of PEP (0.5 mM), as was found by Huber and Sugiyama (1986). Jiao and Chollet (1988, 1989) have used different conditions of assay (pH 7.3 and 2.5 mM-PEP) which also show a change in the $K_i$ for malate and $V_{max}$. When we tested this reaction mixture the difference in $V_{max}$ between illuminated and darkened leaves was not found although a similar decrease in $K_i$ for malate to that reported by Jiao and Chollet (1988) was seen.

Wu and Wedding (1987a) suggested that the observed decrease in the malate sensitivity of PEP carboxylase on illumination might be due to an increase in temperature. However, if leaves were both illuminated and darkened at 27 $^{o}$C then the light/dark difference is still seen (Figure 3.2.).

The fact that illumination with blue, red or white light can activate PEP carboxylase and that DCMU inhibits the light activation process implicates the photochemical apparatus in the light modulation process. Since this work was done, Samaras *et al.* (1988) have shown, by using a range of inhibitors of photosynthesis, that both photophosphorylation and electron flow at least to ferredoxin are needed for PEP carboxylase activation. Little is known as yet about the mechanism by which cytosolic enzymes are regulated by light (Anderson, 1986). Apart from $C_4$ PEP carboxylase (Karabourniotis *et al.*, 1983; Samaras *et al.*, 1988; this thesis) the light inactivation of pea leaf cytosolic G6P dehydrogenase is the only other cytosolic enzyme whose activity is known to be affected by DCMU (Anderson and Nehrlich, 1977). It is not, however, known whether these enzymes are regulated by the same mechanism. The complete activation spectrum for these enzymes or investigation using other inhibitors of the photosynthetic electron transport chain might further elucidate the mechanism.

The results presented in Section 3.2.3. suggest that the increase in the apparent $K_i$ for malate of PEP carboxylase, which occurs on illumination of maize leaves (Huber and Sugiyama, 1986; Nimmo *et*

*al.*, 1987; Doncaster and Leegood, 1987; Jiao and Chollet, 1988 and this chapter), is due to the phosphorylation of one or more serine residues on the enzyme. Guidici-Orticoni *et al.* (1988) have subsequently found a similar *in vivo* phosphorylation of serine residues of *Sorghum* leaf PEP carboxylase. This phosphorylation was also much greater in extracts made from leaves which had been illuminated than in extracts from darkened leaves although these authors did not attempt to correlate this phosphorylation with any possible kinetic change of the enzyme. The findings for $C_4$ PEP carboxylase are very similar to those for PEP carboxylase from CAM plants where it has also been proposed that phosphorylation of the enzyme renders it less sensitive to malate inhibition (Nimmo *et al.*,1984, 1986; Brulfert *et al.*, 1986). However, phosphorylation of the enzyme from $C_4$ plants occurs on illumination whereas the phosphorylation of PEP carboxylase from CAM plants is seen during darkness.

The time taken for this kinetic change to take place is relatively long (about 1 h) and is very similar to that obtained for *S. soda* (Karabourniotis *et al.*, 1983). However, the immunotitration experiments described here, together with the immunodiffusion experiments of Huber and Sugiyama (1986), suggest that *de novo* synthesis of PEP carboxylase is not involved in this effect. This might indicate that phosphorylation and the consequent decrease in malate sensitivity of the enzyme is a longer term response to light and that the faster changes in the concentrations of metabolites induced by illumination (Leegood and Furbank, 1984) act as a more rapid response to light.

Chapter 4

# THE PURIFICATION OF PEP CARBOXYLASE FROM MAIZE LEAVES

## 4.1. Introduction

In order to achieve many of the aims of this project, it was crucially important to develop an efficient and reliable method for the purification of maize leaf PEP carboxylase. The enzyme had been purified to apparent homogeneity from maize prior to this work and has subsequently been purified several more times by a variety of methods from maize (Uedan and Sugiyama, 1976; Mukerji, 1977; Mares *et al.*, 1979; O'Leary *et al.*, 1981; Iglesias and Andreo, 1983; Hatch and Heldt, 1984; Nimmo *et al.*, 1987; Jiao and Chollet, 1988; McNaughton *et al.*, 1989) and from sugar cane (Iglesias and Andreo, 1989). However, few if any of these studies have included a careful investigation into the kinetic properties of the purified enzyme in order to ensure that the enzyme that was purified had similar properties to those of the enzyme in crude extracts. The aim of this work, therefore, was to develop a procedure for the purification of PEP carboxylase which would enable an unaltered form of the enzyme to be obtained which maintained the kinetic properties of the enzyme observed in crude leaf extracts.

## 4.2. Results

The purification of PEP carboxylase from darkened maize leaves as described in Section 2.5.3. is summarized in Table 4.1. An SDS/polyacrylamide gel monitoring the purification at each step of the procedure is shown in Figure 4.1. The method used derived from initial attempts to purify the enzyme by the method of Hatch and Heldt (1984). This proved highly irreproducible however, and produced low recoveries of purified PEP carboxylase. In the purification scheme developed (see Section 2.5.3.), the 30-50% ammonium sulphate pellet contained nearly all the PEP carboxylase activity and, after desalting on Sephadex G-25M, chromatography on hydroxylapatite proved to be an excellent step in the protocol. This step has also been used successfully in the purification of PEP carboxylase from *B. fedschenkoi* (Nimmo *et al.*, 1986) As well as separating PEP carboxylase from many other proteins (Figure 4.2.) it also removed the contaminating pigments. A final Mono Q column (Figure 4.3.) yielded purified PEP carboxylase which was essentially homogeneous as judged by electrophoresis on SDS/polyacrylamide

TABLE 4.1. **Purification of PEP carboxylase from darkened maize leaves**

| Step | Volume (ml) | Total protein (mg) | Total enzyme activity (units) | Specific activity (units/mg) | Yield (%) | Purification (fold) | $K_i$ for malate (mM) |
|---|---|---|---|---|---|---|---|
| Homogenate | 40 | 92 | 189 | 2.0 | 100 | 1.0 | 0.30 |
| 10 000 g supernatant | 39 | 62 | 201 | 3.2 | 106 | 1.6 | 0.30 |
| Desalted 30-50% ammonium sulphate fraction | 4 | 44 | 174 | 4.0 | 92 | 2.0 | 0.35 |
| Desalted hydroxylapatite pool | 50 | 30 | 140 | 4.7 | 74 | 2.3 | 0.30 |
| Mono Q pool | 3 | 4.6 | 96 | 20.8 | 51 | 10.4 | 0.30 |

**FIGURE 4.1. Purification of PEP carboxylase from darkened maize leaves.** Purification was monitored by electrophoresis on an 8% (w/v) polyacrylamide-gel run in the presence of SDS and then stained with Coomassie Brilliant Blue. About 0.05 units of PEP carboxylase activity was loaded in each track. Track A, crude extract; track B, 10000 g supernatant; track C, desalted 30-50% ammonium sulphate fraction; track D, desalted hydroxylapatite pool, track E, dialysed Mono Q pool. The mobilities of the following $M_r$ marker proteins are indicated : 1, myosin; 2, ß-galactosidase; 3, phosphorylase b; 4, bovine serum albumin; 5, ovalbumin and 6, carbonic anhydrase.

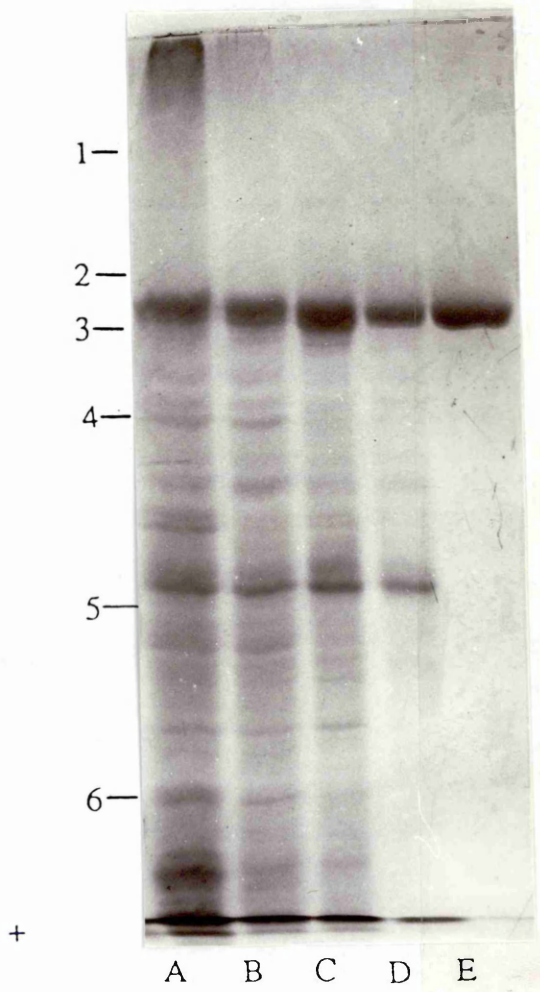

1
2
3
4
5
6
A
B
C
D
E

FIGURE 4.2. **Hydroxylapatite chromatography of PEP carboxylase.** Enzyme from the 30-50% ammonium sulphate fraction which had been desalted on a 1 x 12 cm (10 ml) column of G-25M Sephadex was loaded on to a 3.3 x 1.2 cm (10 ml) hydroxylapatite column equilibrated in Buffer B. The flow rate was 2 ml/min and 2 min fractions were collected. PEP carboxylase activity was eluted from the column by running a linear 200 ml gradient of 0-150 mM-potassium phosphate which is indicated by the diagonal straight line on the figure.

PEP carboxylase activity —■—

$A_{280}$ ——

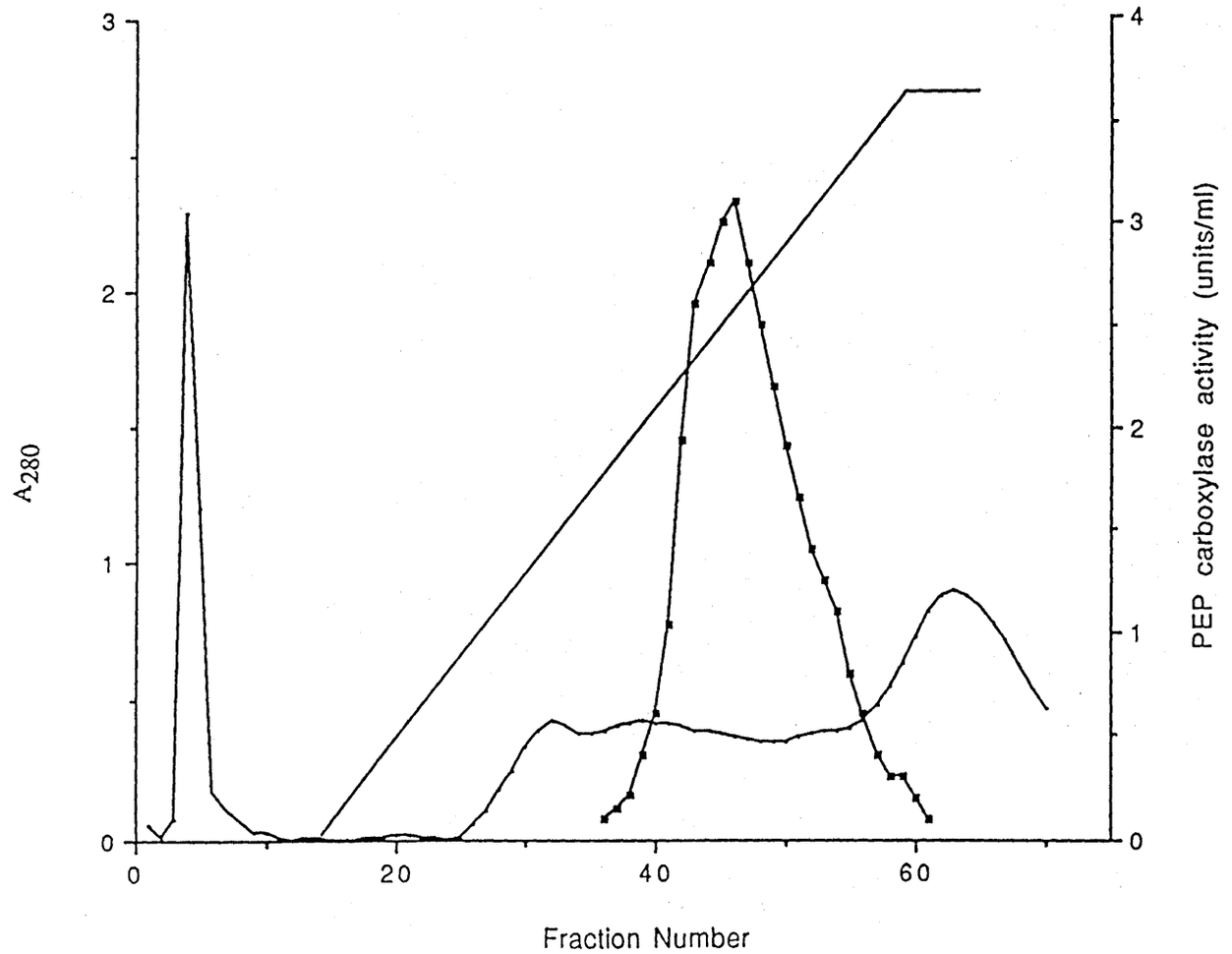
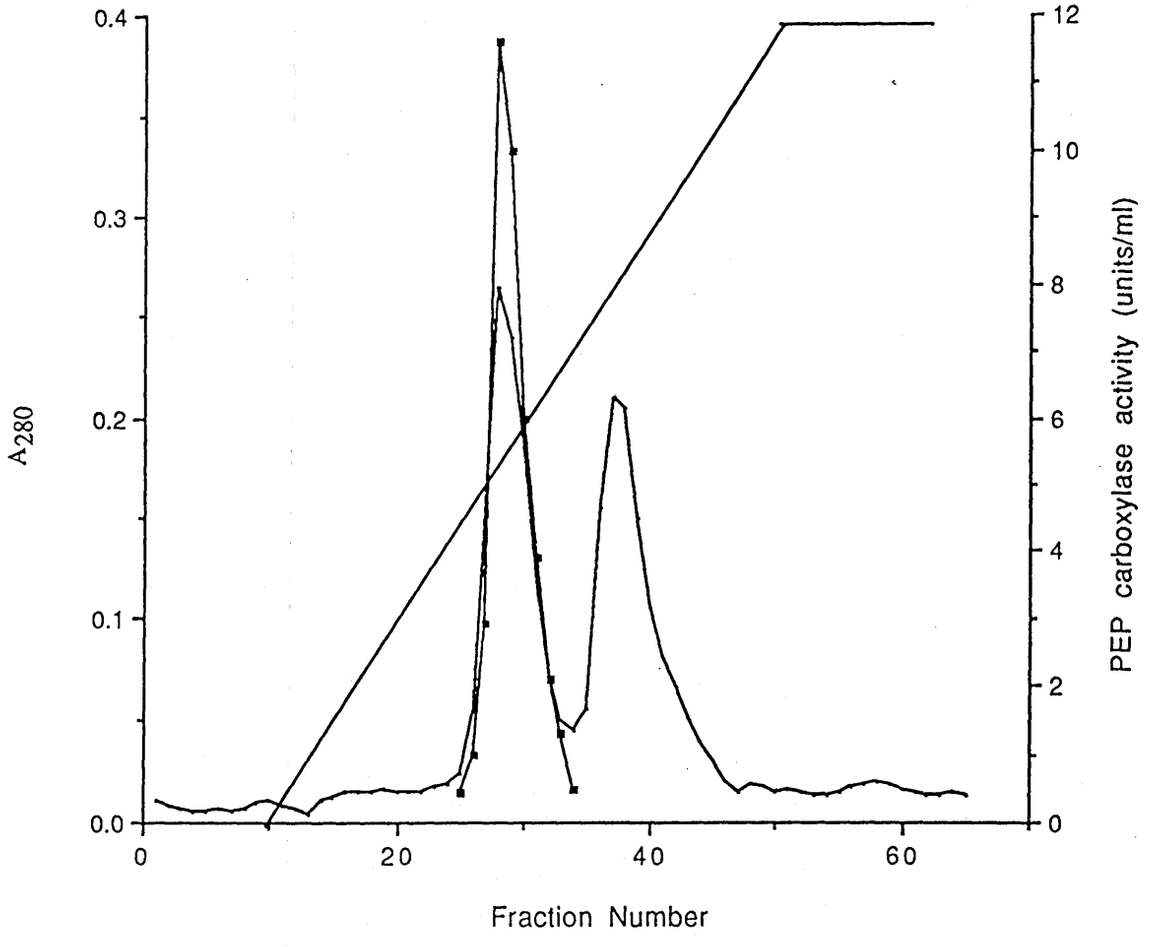

3
2
1
0
$A_{280}$
0
20
40
60
Fraction Number
4
3
2
1
0
PEP carboxylase activity (units/ml)

FIGURE 4.3. **Mono Q chromatography of PEP carboxylase.** Enzyme from the hydroxylapatite column was desalted on a 2.2 x 45 cm (175 ml) column of Sephadex G-25M, as described in Section 2.5.3.(iv), and then loaded on to a Mono Q column equilibrated in Buffer B. PEP carboxylase activity was eluted from the column with a linear 25 ml gradient of 50-400 mM-KCl in Buffer B which is indicted on the figure by a diagonal straight line. The flow rate was 1 ml/min and 0.5 min fractions were collected.

PEP carboxylase activity —■—

$A_{280}$ ——


0.4
0.3
0.2
0.1
0.0
$A_{280}$
0
20
40
60
Fraction Number
12
10
8
6
4
2
0
PEP carboxylase activity (units/ml)

gels (Figure 4.1.) (see Figure 5.2. for the homogeneity of the purified enzyme on non-denaturing gels). The enzyme retained the malate sensitivity of PEP carboxylase found in the crude extract (Table 4.1.) and maintained the $K_m$ for PEP of 1.35 ± 0.21 (mean ± S.D., n=4) when measured at pH 7.8 (compare with the value for crude extracts in Table 3.1.). The final specific activity varied from 17 to 22 units/mg of protein, and the apparent $K_i$ values for malate from 0.2 to 0.4 mM, amongst different enzyme preparations. PEP carboxylase purified by this method was free from detectable contamination by PPDK activity, another enzyme of the $C_4$ pathway which has a similar $M_r$ to PEP carboxylase and which has previously been reported to be a contaminant in purified PEP carboxylase (Budde and Chollet, 1986).

The presence of the protease inhibitor chymostatin throughout the enzyme preparation was found to be essential for the maintenance of the malate sensitivity of PEP carboxylase during the purification. Chymostatin is an inhibitor of chymotrypsin-like proteases and has been successfully used to prevent the proteolysis of ADPglucose pyrophosphorylase from maize endosperm (Plaxton and Preiss, 1987). Malate, which has previously been used to stabilise the $C_4$ and CAM enzymes (Winter, 1981; Wu and Wedding, 1985b; Huber *et al.*, 1986; Nimmo *et al.*, 1986; Kruger and Kluge, 1988; Jiao and Chollet, 1988), and other protease inhibitors such as benzamidine or PMSF in the buffers used in the preparation, all failed to prevent the enzyme from losing its sensitivity to malate. Omission of chymostatin from the buffers used in the purification protocol did not affect the final specific activity of the PEP carboxylase, but enzyme purified in this manner had a much reduced sensitivity to inhibition by malate with an apparent $K_i$ for malate of 1.8 mM or greater. Further, the enzyme prepared in the absence of chymostatin had a slightly greater mobility on SDS/polyacrylamide gels than enzyme prepared when chymostatin was present during the purification. The subunit $M_r$ of PEP carboxylase was about 109 000 for enzyme prepared in the presence of chymostatin (Figure 4.4.), while enzyme prepared in its absence had a subunit $M_r$ of about 105 000. The two species could be resolved by gel electrophoresis (Figure 4.5.) only if protein loading on the gel was low (0.25 μg). The difference in $M_r$ values was confirmed by immunoblotting (not illustrated). These results suggest that chymostatin protects the enzyme against the cleavage of a peptide bond close to one end of the PEP carboxylase subunit and thus prevents the loss of the enzyme's malate sensitivity. The malate sensitivity, $K_m$ for PEP and specific activity remained unaltered over several months provided that the purified enzyme had been dialysed

FIGURE 4.4. **Plot of mobility ($R_f$) against subunit $M_r$ of protein standards on SDS/polyacrylamide gel electrophoresis.** Analysis was carried out on 8% slab gels as described in Section 2.6.4. The relative mobility of purified PEP carboxylase is indicated by the arrow. The marker proteins are : 1, carbonic anhydrase; 2, ovalbumin; 3, bovine serum albumin; 4, phosphorylase b; 5, β-galactosidase; 6, myosin.

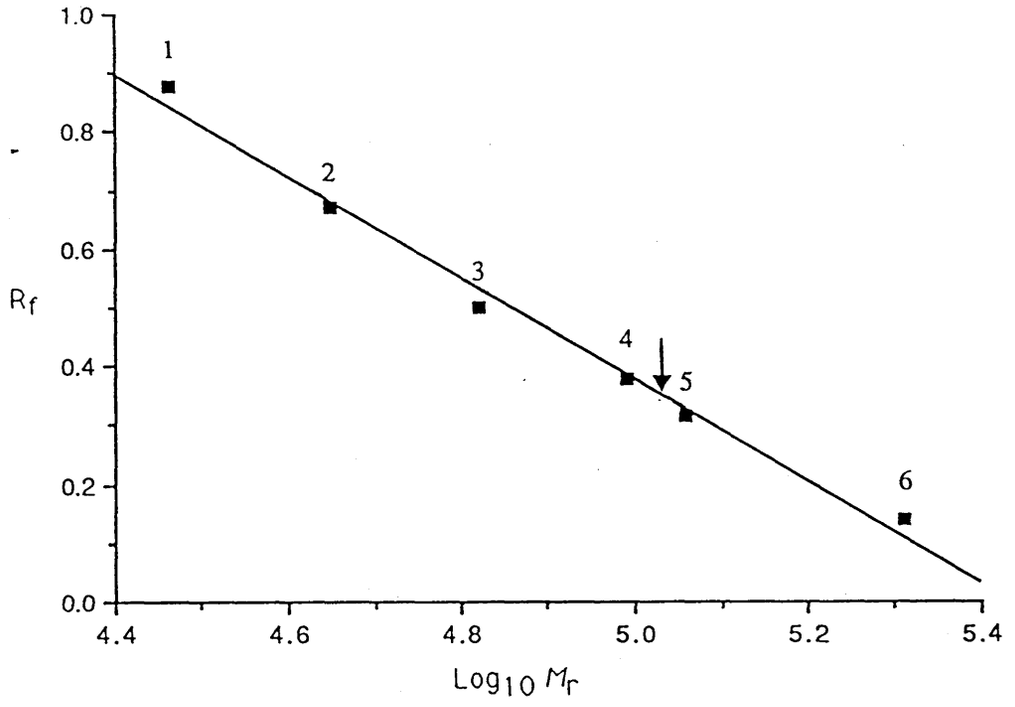
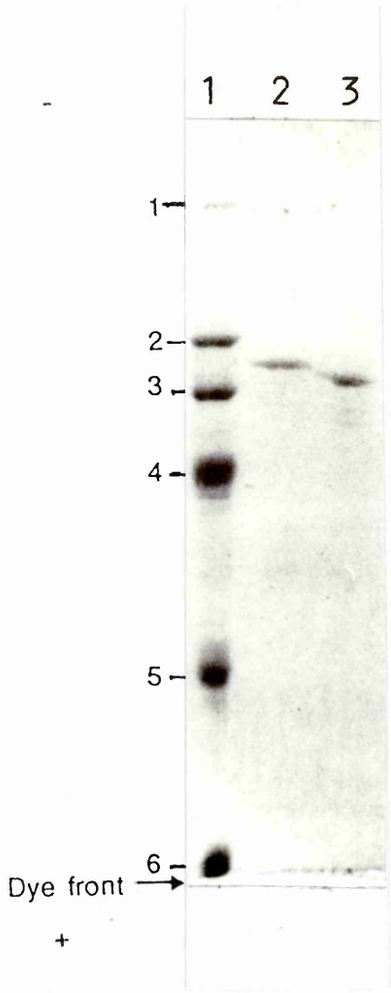

1.0
0.8
0.6
0.4
0.2
0.0
$R_f$
1
2
3
4
5
6
4.4
4.6
4.8
5.0
5.2
5.4
$Log_{10} M_r$

FIGURE 4.5. **SDS/polyacrylamide (8%, w/v)-gel electrophoresis of purified PEP carboxylase.** Track 1, $M_r$ marker proteins. As indicated by the arrows these are: 1, myosin; 2, ß-galactosidase; 3, phosphorylase b; 4, bovine serum albumin; 5, ovalbumin and 6, carbonic anhydrase. Track 2, PEP carboxylase purified in the presence of chymostatin (0.25 µg). Track 3, PEP carboxylase purified in the absence of chymostatin (0.25 µg).


1 2 3
1
2
3
4
5
6
Dye front
+

overnight into 50% (v/v) glycerol and stored at -20 °C.

## 4.3. Discussion

The method described in this thesis affords a rapid purification of PEP carboxylase from darkened maize leaves to apparent homogeneity. The final specific activity of 17 to 22 units/mg of protein is comparable with most other reported maximum specific activities of purified maize PEP carboxylase (Uedan and Sugiyama, 1976; Iglesias and Andreo, 1983; Hatch and Heldt, 1984). Although several other groups have isolated PEP carboxylase from maize, in some cases the enzyme has apparently been partially proteolysed during the purification (e.g. Hague and Sims, 1980; Harpster and Taylor, 1986) and in others there was little or no attempt to show that the purified enzyme had retained its *in vivo* regulatory properties. It was therefore necessary to assess whether the enzyme had been isolated in its native state by the present procedure. The inclusion of chymostatin in the buffers during purification was necessary to prevent proteolysis of the enzyme and to preserve its sensitivity to malate. The purified PEP carboxylase was indistinguishable from enzyme from crude extracts as judged by malate sensitivity (Table 4.1.), $K_m$ for PEP and SDS/polyacrylamide-gel electrophoresis and immunoblotting.

Chapter 5

# THE OLIGOMERIZATION STATE AND MALATE SENSITIVITY OF PEP CARBOXYLASE

## 5.1. Introduction

It is generally thought that active purified PEP carboxylase is a tetramer ($M_r$ ~400 000) of four identical subunits (Uedan and Sugiyama; 1976, O'Leary, 1982; Stiborova and Leblova, 1986). There have, however, been many reports that the enzyme can exist in different oligomeric forms (Jones *et al.*, 1978; Nott and Osmond, 1982; Wu and Wedding, 1985a, 1985b, 1987b; Stiborova and Leblova, 1986; Walker *et al.*, 1986b, 1986c; Wedding and Black, 1986, 1987; Wagner *et al.*, 1987; Budde and Chollet, 1986; Kruger and Kluge, 1988; Podesta and Andreo, 1989). These studies have been carried out on PEP carboxylase from a number of $C_4$ and CAM species using several different techniques such as gel-filtration (Jones *et al.*, 1978; Nott and Osmond, 1982; Wu and Wedding, 1985a, 1985b, 1987b; Stiborova and Leblova, 1986; Walker *et al.*, 1986b, 1986c; Wedding and Black, 1986, 1987; Wagner *et al.*, 1987), non-denaturing polyacrylamide-gel electrophoresis (Wu and Wedding, 1985b; Budde and Chollet, 1986) and ultracentrifugation (Kruger and Kluge, 1988). However, the question of whether changes in oligomerization state are involved in the regulation of PEP carboxylase activity by light remained unanswered. In the present study a systematic investigation was therefore made of conditions which might cause alterations in the aggregation state of purified maize-leaf PEP carboxylase. Conditions were identified where the purified enzyme behaves as a tetramer and as a dimer and subsequently the effects of illumination on the aggregation state and malate sensitivity of PEP carboxylase in extracts were studied.

## 5.2. Results

### 5.2.1. Analysis of the oligomerization state of PEP carboxylase by gel filtration

Table 5.1. summarizes the effects on the apparent $M_r$ of purified PEP carboxylase of changing the protein concentration under various conditions. At high protein loadings (10-50 µg of purified enzyme) PEP carboxylase activity was eluted from the Superose 6 column as a single, slightly asymmetrical peak

TABLE 5.1. **Apparent $M_r$ values for purified PEP carboxylase under various conditions.** $M_r$ values and recoveries obtained from Superose 6 gel-filtration are expressed as means ± S.D. for the number of experiments shown in parentheses in the final column. Levels of significance are a comparison of $M_r$ values with the $M_r$ value obtained when 50 μg of protein was loaded without any addition.

| Addition to column buffer | Protein loading (µg) | Apparent $M_r$ (x $10^{-3}$) | Level of significance+ | Recovery of activity from column (%) |
|---|---|---|---|---|
| None | 50 | 415 ± 40 | - | 93 ± 10 (8) |
| | 25 | 405 ± 35 | n.s. | 87 ± 13 (14) |
| | 10 | 410 ± 50 | n.s. | 78 ± 14 (22) |
| | 0.5 | 260 ± 50 | ** | 55 ± 16 (25) |
| 200 mM-NaCl | 25 | 380 ± 65 | n.s. | 98 ± 15 (8) |
| | 10 | 255 ± 60 | ** | 80 ± 15 (8) |
| | 0.5 | 200 ± 50 | ** | 46 ± 12 (8) |
| 5 mM-malate | 25 | 330 ± 40 | * | 101 ± 12 (8) |
| | 10 | 270 ± 35 | ** | 83 ± 16 (8) |
| | 0.5 | 270 ± 35 | ** | 74 ± 24 (8) |
| 5 mM-PEP | 25 | 405 ± 20 | n.s. | 110 ± 12 (5) |
| | 10 | 395 ± 15 | n.s. | 90 ± 5 (5) |
| | 0.5 | 365 ± 40 | n.s. | 74 ± 20 (8) |
| None, but buffer pH 8.0 | 25 | 420 ± 20 | n.s. | 29 ± 9 (4) |
| | 10 | 410 ± 30 | n.s. | 18 ± 7 (5) |
| | 0.5 | 260 ± 25 | ** | 10 ± 5 (4) |

+Not significantly different at the 0.1 level (n.s.), significantly different at the 0.01 level (*) and significantly different at the 0.001 level (**).

(Figure 5.1.) with an apparent $M_r$ which corresponds to a tetrameric aggregation state. At low protein loadings (0.5 µg of purified enzyme), enzyme activity was eluted from the column, again as a single slightly asymmetrical peak, with a lower apparent $M_r$ equivalent to that which might be expected for a dimer of PEP carboxylase (Figure 5.1.).

The inclusion of 200 mM-NaCl in the buffer appeared to promote the dissociation of PEP carboxylase (Table 5.1.). This is most clearly seen at a loading of 10 µg, where the apparent $M_r$ is significantly lower than that observed in the absence of NaCl. At a loading of 25 µg the enzyme behaved as a tetramer whereas at loadings of 10 µg and 0.5 µg the elution volume was close to that expected for a dimer. A similar effect was observed if 5 mM-malate was present (Table 5.1.). PEP carboxylase was eluted with a lower apparent $M_r$ than when malate was omitted from the buffers. This was the case for 25 µg loadings as well as for lower loadings although the $M_r$ was slightly larger than might have been expected for a dimer in the former case. The apparent $M_r$ values obtained in the presence of malate were significantly different from the apparent $M_r$ values in the absence of malate for 10 and 25 µg but not for 0.5 µg loadings of protein. The differences in the apparent $M_r$ values amongst the loadings in the presence of malate were not statistically significant, indicating that malate probably induces dissociation of PEP carboxylase to dimers over the entire concentration range of enzyme tested. The inclusion of 5 mM-PEP in the buffer resulted in the elution of PEP carboxylase at an apparent $M_r$ corresponding to the tetramer even at a loading of 0.5 µg (Table 5.1.). Increasing the pH of the column buffer from pH 7.1 to pH 8.0 did not affect the oligomeric state of PEP carboxylase although the recovery of enzyme activity from the column was much lower at pH 8.0 (Table 5.1.).

In the absence of magnesium from the buffer, PEP carboxylase was eluted from the column at about 13.2 ml, a much greater apparent $M_r$ than in its presence. The aggregation was so great that the $M_r$ could not be estimated accurately by Superose 6 chromatography. Further, there was no concentration-dependent dissociation of this aggregation state.

Extracts from illuminated or darkened leaves were subject to gel-filtration in the absence of additions to the buffer. There were no detectable differences in the behaviour of PEP carboxylase between the two extracts (Table 5.2.). In both cases the enzyme showed a dissociation to dimers occurring at low enzyme concentrations, similar to that found with the purified enzyme under the same conditions.

FIGURE 5.1. **Superose 6 gel-filtration of purified maize PEP carboxylase.** This experiment was carried out in the absence of effectors (see Section 2.7.1.). PEP carboxylase activity is indicated on the left-hand ordinate for a 25 μg loading (•), and for a 0.5 μg loading (o) on the right-hand ordinate. The arrows indicate the following $M_r$ marker proteins: 1, thyroglobulin; 2, ferritin; 3, aldolase; 4, lactate dehydrogenase; 5, hexokinase and 6, ovalbumin.

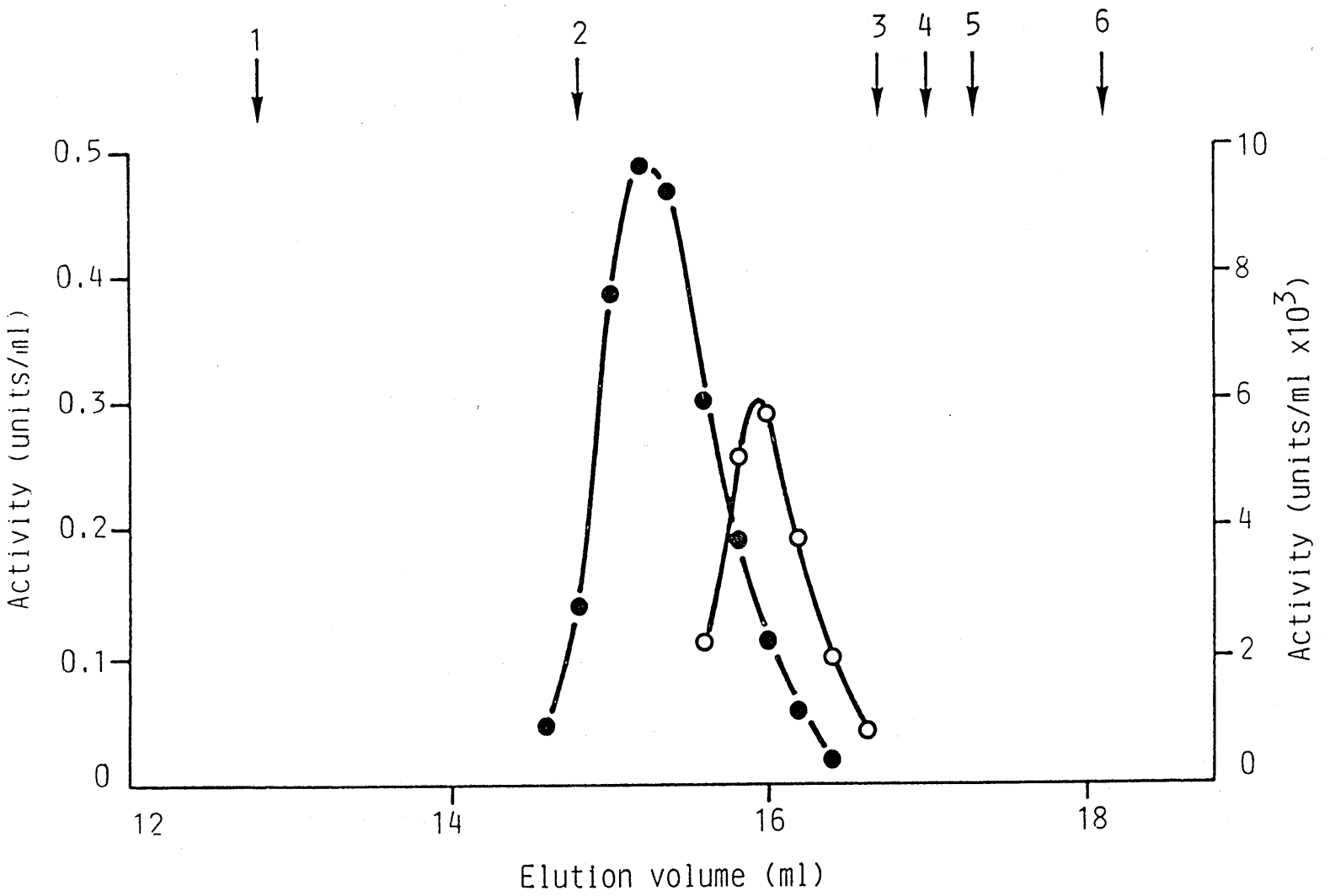

1
2
3
4
5
6
0.5
0.4
0.3
0.2
0.1
0
Activity (units/ml)
10
8
6
4
2
0
Activity (units/ml x10³)
12
14
16
18
Elution volume (ml)

TABLE 5.2. **Apparent $M_r$ values for PEP carboxylase in extracts prepared from darkened and illuminated leaves.** $M_r$ values and recoveries of activities from Superose 6 gel-filtration are expressed as means ± S.D. for 4 experiments.

| | Enzyme loading* (units) | Apparent $M_r$ (x $10^{-3}$) | Recovery of activity from column (%) |
|---|---|---|---|
| Darkened leaf extract | 0.2 | 420 ± 25 | 104 ± 18 |
| | 0.01 | 260 ± 35 | 46 ± 15 |
| Illuminated leaf extract | 0.2 | 410 ± 35 | 86 ± 16 |
| | 0.01 | 285 ± 50 | 40 ± 16 |

* Loadings were equivalent in enzyme activity to 10 μg (0.2 units) and 0.5 μg (0.01 units) of purified PEP carboxylase (see Table 5.1.).

### 5.2.2. Non-denaturing polyacrylamide gel electrophoresis

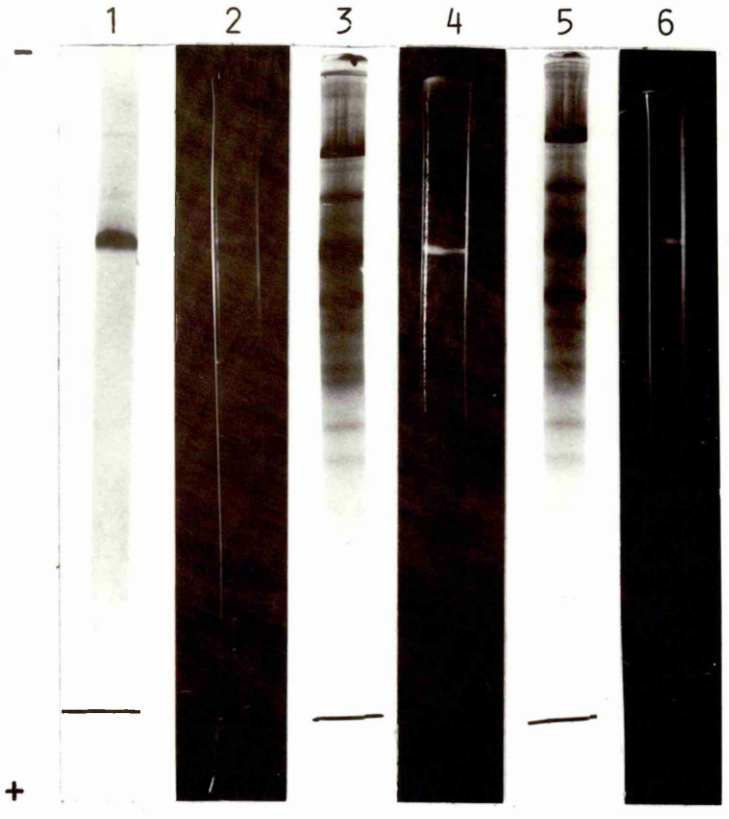
Analysis by non-denaturing polyacrylamide gel electrophoresis of purified PEP carboxylase showed a single protein band with the same mobility at either high (10 μg) or low (0.5 μg) loadings. This band was coincident with a single band of enzyme activity (Figure 5.2.). Crude extracts from both darkened and illuminated leaves gave a single band of PEP carboxylase activity with the same mobility when electrophoresed under the same conditions as the purified enzyme (Figure 5.2.). Only one band of activity was observed when darkened and illuminated crude extracts were electrophoresed together on one gel (not shown). These findings with the crude extracts are in contrast to the results of Budde and Chollet (1986), who showed the existence of dissociated forms of PEP carboxylase in leaf extracts from illuminated plants using a similar procedure.

### 5.2.3. Concentration-dependence of malate sensitivity of purified PEP carboxylase and of enzyme in extracts of illuminated and darkened leaves

These experiments were designed to investigate the effects of enzyme concentration and oligomerization state on the malate sensitivity of PEP carboxylase. The apparent $K_i$ for malate was measured by diluting enzyme samples to concentrations analagous to those at which it was eluted from the Superose 6 column as a tetramer (approx. 10 μg/ml of eluate) or a dimer (approx. 0.5 μg/ml of eluate). The enzyme was diluted 30 min before assay in order to mimic the time taken for the enzyme to pass down the column. It was then assayed at final concentrations in the cuvette (2 or 0.2 munits/ml respectively) which were equivalent to those used when assaying samples from a column loaded with 100 μl volumes containing 10 μg (tetramer) or 0.5 μg (dimer) of protein.

At both concentrations of PEP carboxylase, non-linear progress curves were seen which became more curved as the concentration of malate present in the assay was increased. In experiments involving purified PEP carboxylase, when the reaction was initiated by adding PEP a slight acceleration period in the rate occurred before a steady-state rate was reached, as would be expected for any coupled assay system. When the enzyme was added last, the rate slowed for the first few minutes before becoming linear. In experiments using crude extracts the rate accelerated during a short lag period before linearity was reached, irrespective of whether assays were initiated with PEP carboxylase or PEP. Desalted extracts showed the same kinetic behaviour as crude extracts. In all cases the final linear rate was measured; it was the same

FIGURE 5.2. **Non-denaturing polyacrylamide-gel electrophoresis of PEP carboxylase.** Gels 1 and 2, purified PEP carboxylase (2.5 μg). Gels 3 and 4, crude extract of darkened leaf (from 0.01 g of leaf). Gels 5 and 6, crude extract of illuminated leaf (from 0.01 g of leaf). Gels 1, 3 and 5 were stained for protein with Coomassie Brilliant Blue. Gels 2, 4 and 6 were stained for PEP carboxylase activity (see Section 2.6.3.). The position of the tracking dye was marked with wire.


1
2
3
4
5
6
−
+

irrespective of the order of addition of enzyme and PEP, and was proportional to enzyme concentration. The discrepancy in kinetic behaviour between purified PEP carboxylase and enzyme in crude extracts observed when assays were started by addition of enzyme is difficult to explain. It might imply that there is some difference between purified PEP carboxylase and the enzyme in crude extracts that is not manifested on polyacrylamide-gel electrophoresis or gel-filtration.

Dilution of purified PEP carboxylase to 10 or 0.5 µg/ml (2 or 0.2 munits/ml in the reaction mixture), concentrations at which it behaves as a tetramer or as a dimer respectively, had no effect on its sensitivity to malate (Table 5.3.). The malate sensitivity remained constant over an even wider range of concentrations of enzyme, up to 0.2 units/ml in the reaction mixture. At 10 µg/ml, PEP carboxylase activity was stable. At 0.5 µg/ml the specific activity of the enzyme declined slowly (a 50% decrease in 90-120 min) while the malate sensitivity was not affected. Crude extracts were diluted to give PEP carboxylase activities of 0.2 and 0.01 units/ml (2 and 0.2 munits/ml in the reaction mixture), concentrations at which the enzyme behaves as a tetramer or a dimer (Table 5.2.). This did not affect the malate sensitivity of the enzyme. The characteristic difference in sensitivity between extracts from darkened or illuminated leaves was observed at both concentrations (Table 5.3.). Similar results were obtained at higher enzyme concentrations (up to 0.2 units/ml in the reaction mixture). As found for the purified enzyme, the specific activity of the crude enzyme declined with time at the lower concentration. This instability of the dimer could explain the varied recovery of enzyme activity from gel-filtration, depending on the conditions used.

## 5.3. Discussion

Using gel-filtration, a concentration-dependent dissociation of tetramers to a less stable form with a lower apparent $M_r$ was detected. Dissociation of the enzyme was not detected using non-denaturing polyacrylamide gel-electrophoresis, possibly because of the higher protein concentration used with this technique. Similar effects were observed with purified PEP carboxylase and with enzyme in extracts of darkened and illuminated leaves. The dissociated enzyme form observed on gel-filtration is most probably a dimer, even though the elution volume is slightly lower than would be expected. It is unlikely that cross-linking experiments could resolve this point because the enzyme loses activity rapidly under the slighly alkaline conditions (pH 8.0) normally used for such experiments. Jones *et al.* (1978) found a

TABLE 5.3. **Malate sensitivity of PEP carboxylase at high and low concentrations of the enzyme in the reaction mixture.** Values are expressed as means ± S.D. for 4 experiments.

| | PEP carboxylase concentration in the reaction mixture* (munits/ml) | Apparent $K_i$ for malate (mM) |
|---|---|---|
| Purified enzyme | 2.0 | 0.26 ± 0.04 |
| | 0.2 | 0.21 ± 0.06 |
| Enzyme in an extract from a darkened leaf | 2.0 | 0.32 ± 0.04 |
| | 0.2 | 0.32 ± 0.04 |
| Enzyme in an extract from an illuminated leaf | 2.0 | 0.95 ± 0.16 |
| | 0.2 | 0.97 ± 0.18 |

*Purified PEP carboxylase was diluted to 10 or 0.5 μg/ml and then assayed as described in the text. Crude extracts were diluted to 0.2 or 0.01 units/ml and then assayed. The concentrations were chosen to be equivalent to the concentrations at which the enzyme was eluted from the Superose 6 column loaded with 10 μg or 0.5 μg protein respectively.

similar concentration-dependent dissociation of PEP carboxylase purified from the CAM plant *Bryophyllum fedtschenkoi*. The maize dimer appears to be unstable whereas the dimer of *Bryophyllum* PEP carboxylase was stable but had only about 50% of the activity of the tetramer. Nevertheless, it appears that PEP carboxylases from $C_4$ and CAM plants undergo similar concentration-dependent changes in oligomerization state. The results of the kinetic experiments on purified maize PEP carboxylase show that the tetrameric and dimeric forms of PEP carboxylase are equally sensitive to inhibition by malate (Table 5.3.). Wu and Wedding (1987b) found that gel filtration of partially purified PEP carboxylase from the CAM plant *Crassula argentea* yielded a mixture of dimers and tetramers with the tetramer being much less sensitive to inhibition by malate than the dimer. This does not seem to be the case for purified maize PEP carboxylase.

The apparent $M_r$ of PEP carboxylase was also affected by the composition of the buffer used for gel-filtration. The presence of 200 mM-NaCl promoted the dissociation of the enzyme into dimers at concentrations above those at which dilution alone caused this effect (Table 5.1.). A fast dissociation into dimers induced by high ionic strength was also observed by Wagner *et al.* (1987) using HPLC gel filtration and similar conditions to those employed in this investigation. In the presence of malate the enzyme exhibited a lower apparent $M_r$ than the tetramer, which was only slightly affected by changes in the protein concentration. These results could indicate that in the presence of malate the dissociation does not go to completion. Alternatively, the binding of malate to dimers may induce a conformational change which results in increased asymmetry and a higher apparent $M_r$. Huber *et al.* (1986) found that in the presence of 2 mM-malate during gel-filtration, partially purified PEP carboxylase from illuminated and darkened maize leaves had slightly different elution profiles which both corresponded to tetrameric conformations of the enzyme. If malate was omitted from the buffers, no such subtle difference was seen. The enzyme concentrations that they used were much higher than those used in this study. Presumably the enzyme does not dissociate to dimers at such high concentrations even in the presence of malate. The presence of 5 mM-PEP in the buffers maintained the tetrameric state of the enzyme on dilution, with no dissociation to dimers being observed at low enzyme concentrations (Table 5.1.). Similar results have been observed by others (Wu and Wedding, 1985a; Wagner *et al.*, 1987; Andreo *et al.*, 1987). Wu and Wedding (1985a) reported that the PEP carboxylase from *C. argentea* dissociated from tetramers to dimers in the absence of a divalent metal ion, whereas our results indicate that magnesium causes

aggregation of the maize enzyme. Wu and Wedding (1985a) suggested that magnesium might play some role in the light regulation of the CAM enzyme. However studies on the purified enzyme in the presence of single effectors are of limited relevance in this context, since it is probably the relative concentrations of several effectors in the cytosol that is of importance.

In conclusion, the identification of conditions which affect the oligomerization state of maize PEP carboxylase has allowed the comparison of the association/dissociation behaviour of PEP carboxylase from illuminated and darkened extracts. Both forms of the enzyme dissociated at low enzyme concentrations, as did the purified enzyme. This dissociation did not affect the characteristic difference in apparent $K_i$ for malate between the enzyme from illuminated and darkened leaves. These results therefore suggest that changes in the oligomerization state of PEP carboxylase of the type reported here are not directly involved in its regulation by light.

Chapter 6

# STUDIES ON THE PHOSPHORYLATION AND DEPHOSPHORYLATION OF PEP CARBOXYLASE *IN VITRO*

## 6.1. Introduction

Krebs (1973) suggested that there are several criteria that must be fulfilled in order to demonstrate that a cyclic AMP-induced effect is mediated by phosphorylation of a protein. These criteria were updated by Nimmo and Cohen (1977) but they can be modified to establish the physiological relevence of any protein phosphorylation occurring in response to a given stimulus. Four main criteria must be met :

1. The rate of phosphorylation of the protein substrate, in its native state, should be adequate to account for the speed at which the alteration in the function of the protein occurs *in vivo*.

2. The function of the protein should be shown to undergo a reversible alteration *in vitro* by phosphorylation and dephosphorylation, catalysed by a protein kinase and a protein phosphatase.

3. A reversible change in the function of the protein should occur *in vivo* in response to the stimulus.

4. Phosphorylation of the protein should occur *in vivo* in response to the stimulus at the same site(s) phosphorylated by the protein kinase *in vitro*.

Having established that maize leaf PEP carboxylase can be phosphorylated *in vivo* and that this phosphorylation seems to be accompanied by a change in the malate sensitivity of the enzyme (see Chapter 3), further investigation of the phosphorylation/dephosphorylation process was carried out using a reconstituted system. Such an *in vitro* system of assay was used in an attempt to meet the above criteria for PEP carboxylase. In particular, the objectives were (i) to try to establish whether there is a direct correlation between the malate sensitivity and the phosphorylation state of the enzyme, (ii) to determine the stoichiometry of phosphorylation and (iii) to investigate the light dependence of the phosphorylation. Also, such a system would allow the study of the putative protein kinase and protein phosphatase responsible for carrying out the phosphorylation and dephosphorylation reactions.

An additional approach to the study of the protein phosphatases in maize leaves was also used. MacKintosh and Cohen (1989) reported that seeds of *Brassica napus* contain high levels of protein phosphatase activity which show properties remarkably similar to those of mammalian PP-1 and PP-2A

type protein phosphatases. We therefore decided to investigate the protein phosphatase activity in maize leaves using a similar approach to that employed by MacKintosh and Cohen (1989) in the hope that this might provide some information about the putative protein phosphatase involved in dephosphorylating PEP carboxylase *in vivo*.

## 6.2. Results

### 6.2.1. *In vitro* phosphorylation of PEP carboxylase using leaf extracts

The experiments described in this section were carried out using the assay method outlined in Section 2.7.6.(i). After incubation with [$\gamma$-$^{32}$P]ATP and $MgCl_2$, PEP carboxylase was immunoprecipitated from the reaction mixture to ensure that any phosphorylation was due to labelling of PEP carboxylase and not of PPDK (see Section 4.2.) or other proteins. PEP carboxylase purified from darkened leaves was used as a substrate for the kinase since it is presumably in the dephosphorylated form.

An important initial finding was that when extracts were desalted through Sephadex G-25M using Buffer B (as described in Section 2.5.3.(iii)), a much greater level of phosphorylation was attained (Figure 6.1.). Desalted samples were therfore used in all future experiments involving crude leaf extracts. Figure 6.1. also shows that greater phosphorylation was achieved when purified PEP carboxylase was added to the incubation mixture.

The phosphorylated band was shown to be PEP carboxylase by comparing, by Cleveland mapping (as described in Section 2.6.7.), the pattern of proteolytic fragments which was obtained for the immunoprecipitated enzyme with the pattern obtained for purified PEP carboxylase (not shown).

A distinct difference was observed in the ability of extracts of illuminated and darkened leaves to phosphorylate PEP carboxylase. Extracts of leaves which had been illuminated seemed to contain much more PEP carboxylase kinase than extracts from darkened leaves (Figure 6.2.). Extracts from leaves taken directly from the growth room during the light phase (after 10-12 h at 250 $\mu$mol $m^{-2}s^{-1}$ at 27 $^{o}$C) also contained PEP carboxylase kinase, though the amount of activity was less than in extracts prepared from leaves which had been illuminated at 1000 $\mu$mol $m^{-2}s^{-1}$ (Figure 6.3.). When leaves were illuminated for various lengths of time at 27 $^{o}$C and 1000 $\mu$mol $m^{-2}s^{-1}$ before extraction and assay, it was seen that phosphorylation increased with time of illumination up to about 1 h before reaching a maximum (Figure 6.4.).

FIGURE 6.1. **The effect of desalting on the *in vitro* phosphorylation of purified PEP carboxylase.** Tracks from an autoradiograph of an 8% SDS/polyacrylamide gel. Incubations were carried out as described in Section 2.7.6.(i) for 30 min before the PEP carboxylase was immunoprecipitated from the reaction mixture and the resuspended sample electrophoresed. (a) Undesalted extract prepared from illuminated leaves. Track 1, exogenous purified PEP carboxylase added; track 2, no exogenous purified PEP carboxylase added. (b) The same leaf extract desalted before incubation. Track 1, exogenous purified PEP carboxylase added; track 2, no exogenous purified PEP carboxylase added. The arrow indicates the position of PEP carboxylase on the gel.

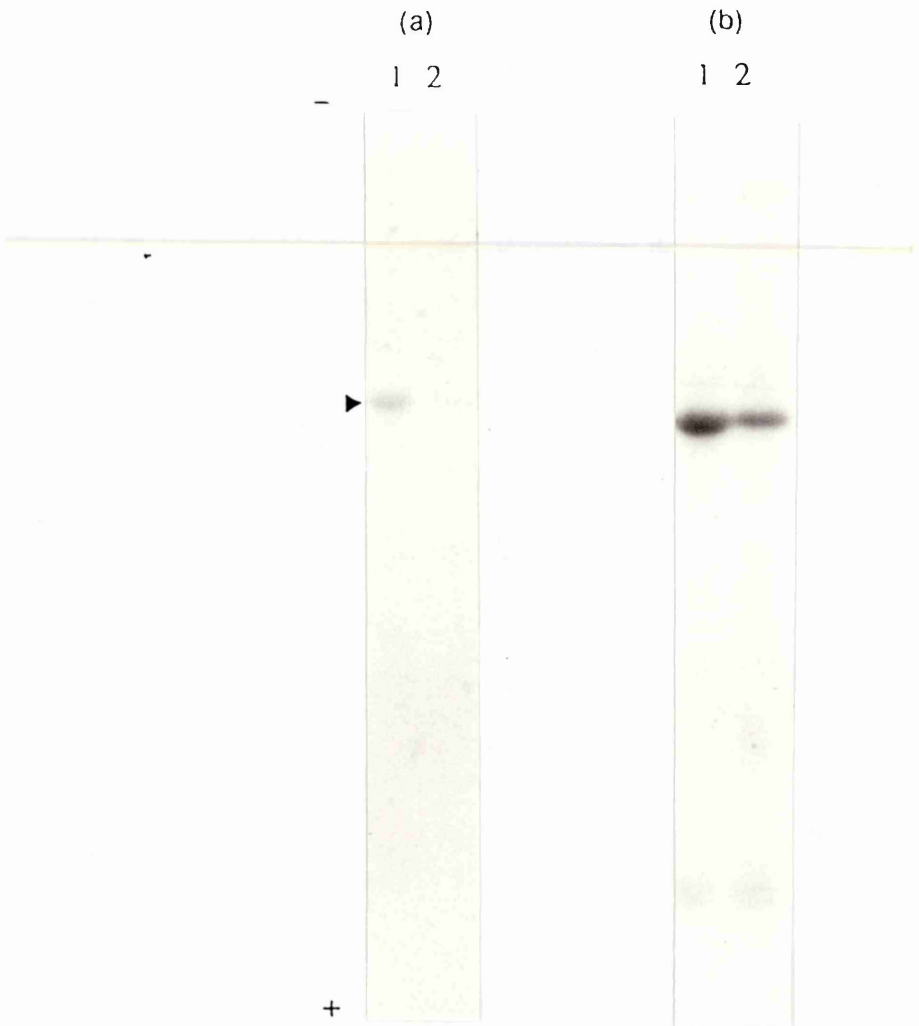

(a)
1 2
(b)
1 2
−
+

FIGURE 6.2. **Comparison of the *in vitro* phosphorylation of PEP carboxylase by extracts from darkened or illuminated leaves.** Leaves were either (a) darkened or (b) illuminated for 60 min before extraction and kinase incubations carried out as described in Section 2.7.6.(i). Tracks 1 and 4, 5 min incubation; tracks 2 and 5, 15 min incubation; tracks 3 and 6, 30 min incubation. Tracks 1-3, exogenous purified PEP carboxylase added; tracks 4-6, no exogenous purified PEP carboxylase added. (i) Coomassie Brilliant Blue stained gel; (ii) autoradiograph. The arrow indicates the mobility of PEP carboxylase on the gel.

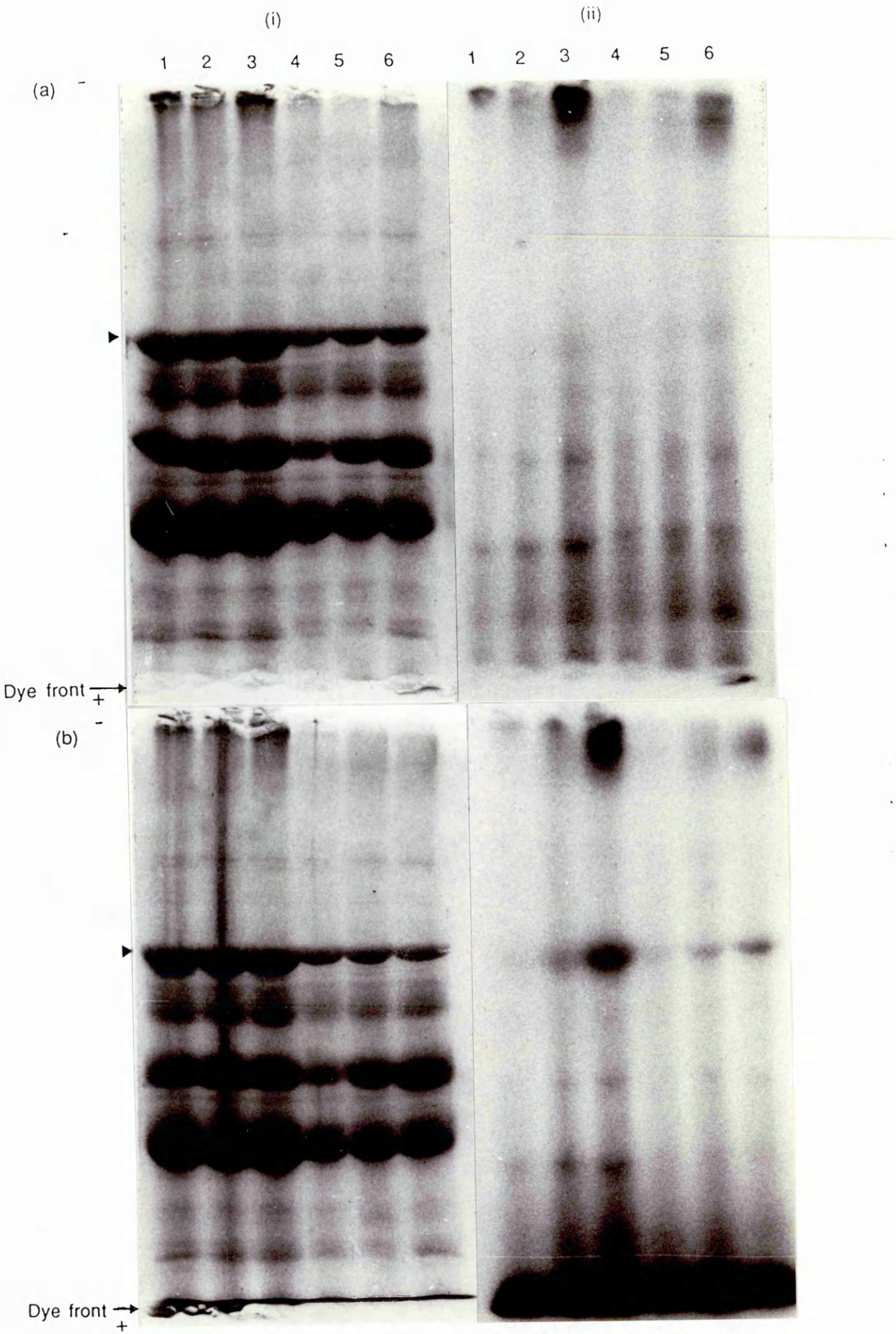

(i)
(ii)
1 2 3 4 5 6
1 2 3 4 5 6
(a)
Dye front
+
-
(b)
Dye front
+

FIGURE 6.3. **Comparison of the *in vitro* phosphorylation of PEP carboxylase by extracts from leaves illuminated at 250 μmol $m^{-2}s^{-1}$ and 1000 μmol $m^{-2}s^{-1}$.** Leaves were illuminated for 60 min and 30 min kinase incubations carried out as described in Section 2.7.6.(i). Tracks 1 and 2, extract from leaf illuminated at 1000 μmol $m^{-2}s^{-1}$; tracks 3 and 4, extract from leaves illuminated at 250 μmol $m^{-2}s^{-1}$. Tracks 1 and 3, no exogenous purified PEP carboxylase added; tracks 2 and 4, exogenous purified PEP carboxylase added. (a) Coomassie Brilliant Blue stained gel, (b) autoradiograph. The arrow indicates the mobility of PEP carboxylase on the gel.

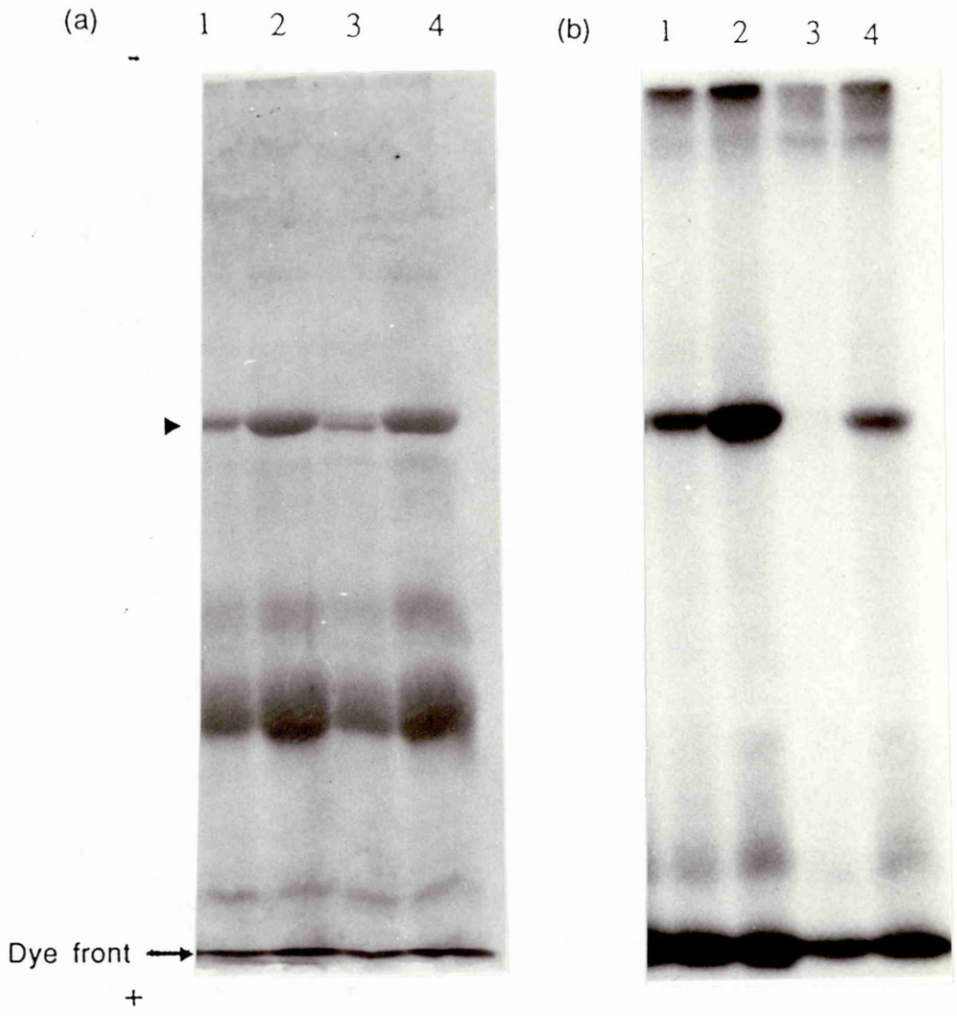
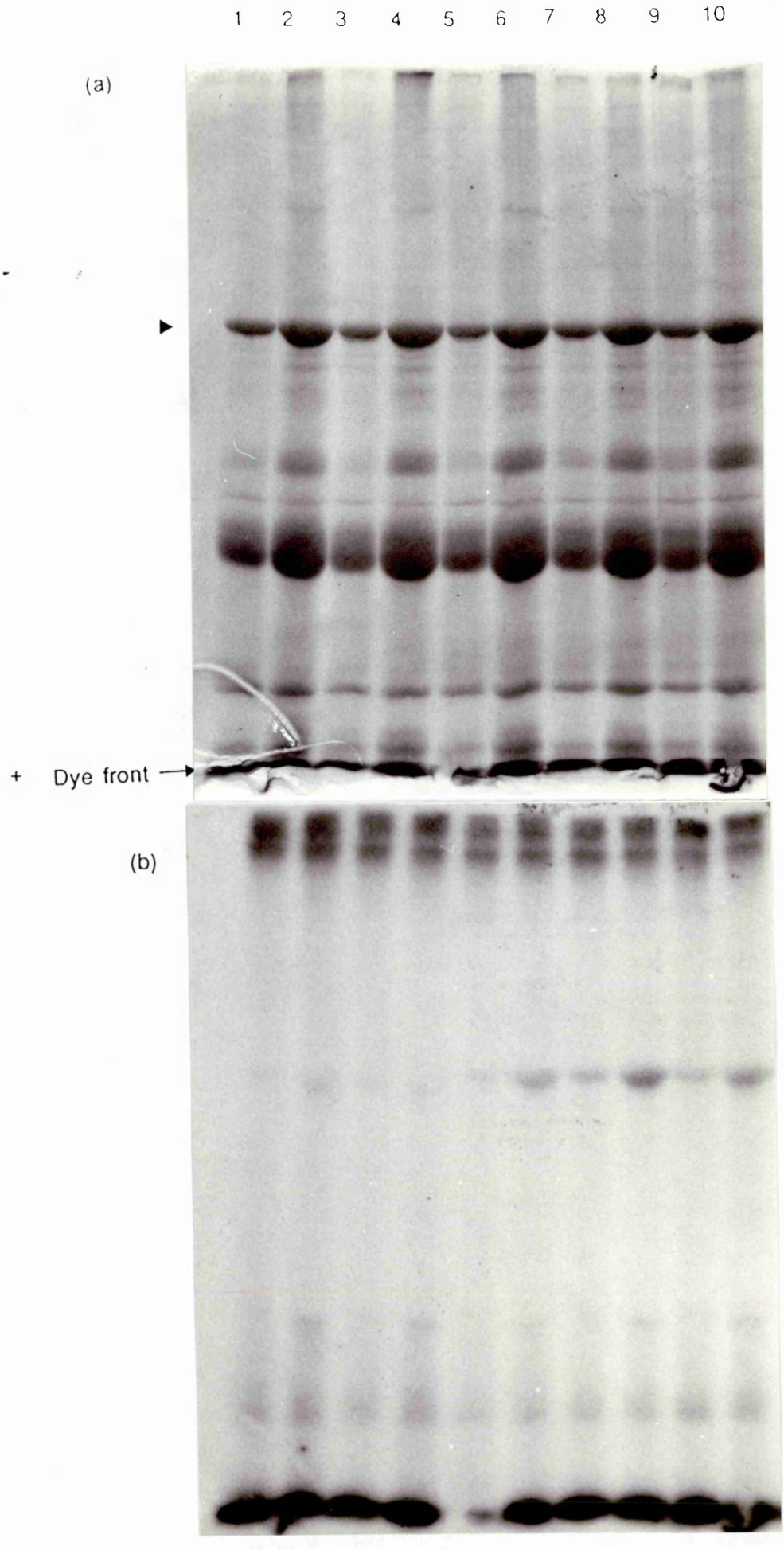

(a)
1
2
3
4
(b)
1
2
3
4
Dye front
-
+

FIGURE 6.4. **The effect of illumination on the *in vitro* phosphorylation of PEP carboxylase.** Leaves were either placed in darkness or illuminated at 1000 μmol $m^{-2}s^{-1}$ for various times before extracts were prepared from them and 30 min kinase incubations carried out as described in Section 2.7.6.(i). (a) Coomassie Brillinat Blue stained gel. (b) Autoradiograph. Tracks 1,3,5,7,9, no exogenous PEP carboxylase added; tracks 2,4,6,8,10, exogenous PEP carboxylase added. Tracks 1 and 2, darkened leaf; tracks 3 and 4, leaf illuminated for 5 min; tracks 5 and 6, leaf illuminated for 20 min; tracks 7 and 8, leaf illuminated for 60 min; tracks, 9 and 10, leaf illuminated for 120 min. The arrow indicates the position of PEP carboxylase on the gel.


1 2 3 4 5 6 7 8 9 10
(a)
+ Dye front
(b)

The presence of chymostatin in the *in vitro* assay using desalted leaf extracts prevented the proteolysis of the PEP carboxylase present. This was very important since proteolysed enzyme could not be phosphorylated (Figure 6.5.). Indeed purified PEP carboxylase which had lost only a tiny fragment due to proteolysis (e.g. Figure 4.5., track 3) could not be phosphorylated to any detectable degree (not shown).

In an attempt to find out whether the kinase activity in crude extracts could alter the apparent $K_i$ for malate of purified dephosphorylated PEP carboxylase as well as being able to phosphorylate it, desalted leaf extracts were incubated with purified PEP carboxylase, ATP and $MgCl_2$. Chymostatin was also included in the reaction mixture in order to try to prevent the proteolysis of PEP carboxylase. No change in the apparent $K_i$ for malate was seen when incubations were carried out using extracts prepared from either darkened or illuminated leaves. However, the results from this experiment were difficult to interpret since incubation of purified PEP carboxylase with extracts of illuminated leaves, which contained the kinase activity, also contained PEP carboxylase which had already been converted to the less malate sensitive form. This led to apparent $K_i$ values which were high to start with and which could therefore have masked any small change in the apparent $K_i$ value.

Assays were run in order to check whether ATPase activity in crude extracts was destroying the added ATP before it was able to be used to phosphorylate PEP carboxylase. The ATPase activity was found to be not significant in desalted extracts. After 2 h, four times the duration of most kinase incubations, greater than 85% of the ATP was found still to be present.

### 6.2.2. *In vitro* studies using extracts prepared from sugar cane leaves

PEP carboxylase in extracts of sugar cane leaves could be immunoprecipitated by the antiserum raised against the purified maize enzyme. The PEP carboxylase from sugar cane was distinguishable from the purified maize enzyme because it had a slightly greater mobility on SDS/polyacrylamide gels (Figure 6.6.). Desalted extracts from illuminated sugar cane leaves were also able to phosphorylate purified maize PEP carboxylase but did not phosphorylate the PEP carboxylase from the sugar cane extracts. The PEP carboxylase in such extracts of illuminated sugar cane leaves also showed a higher apparent $K_i$ for malate than in desalted extracts of darkened leaves. The assay conditions used for the sugar cane enzyme were the same as those which were used for maize and the two plants showed a similar effect of illumination

FIGURE 6.5. **The effect of chymostatin on the proteolysis and *in vitro* phosphorylation of PEP carboxylase.** Tracks 1 and 2, extract made and incubations carried out in the presence of chymostatin; tracks 3 and 4 extract made and assays carried out in the absence of chymostatin. Tracks 1 and 3, no purified PEP carboxylase added, tracks 2 and 4, purfied PEP carboxylase added. Leaves were illuminated at 1 000 $\mu molm^{-2}s^{-1}$ for 1 h before extraction. Incubations (30 min) were carried out as described in Section 2.7.6.(i) and immunoprecipitation of PEP carboxylase protein carried out as described in Section 2.7.3. prior to electrophoresis. (a) Coomassie Brilliant Blue stained gel, (b) autoradiograph. The arrow marks the position of PEP carboxylase on the gel.

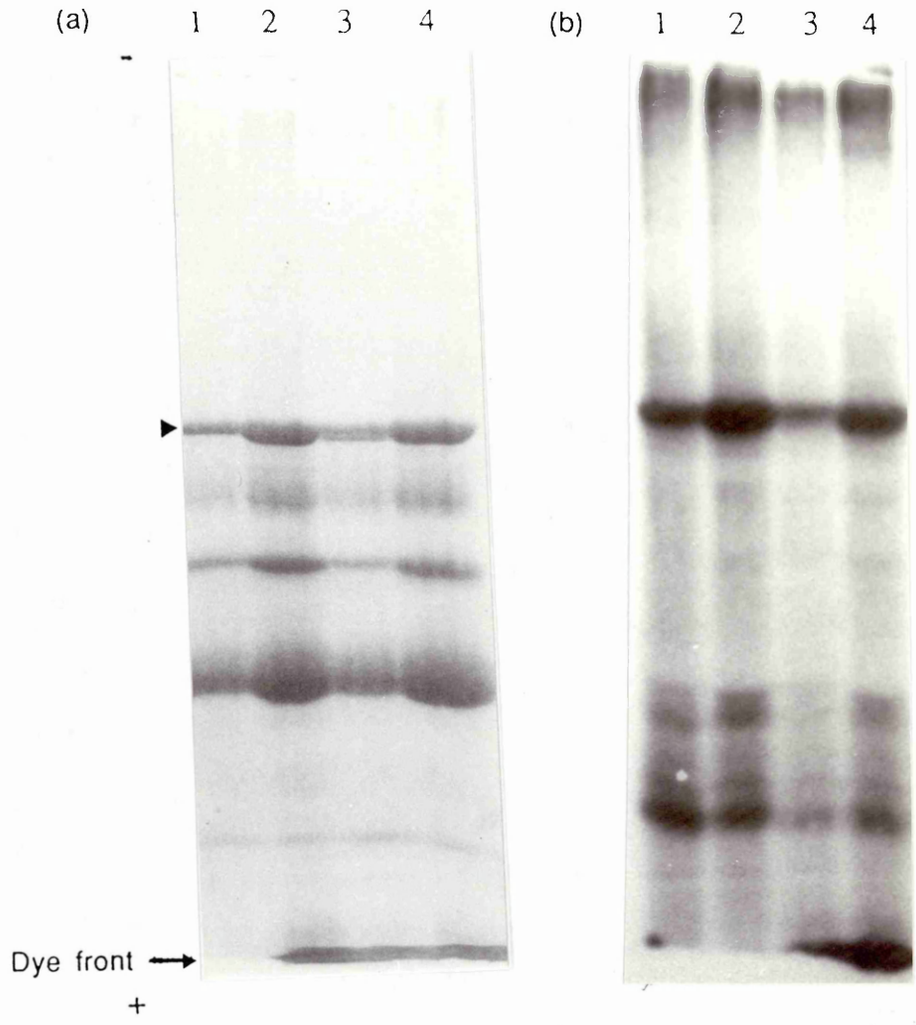
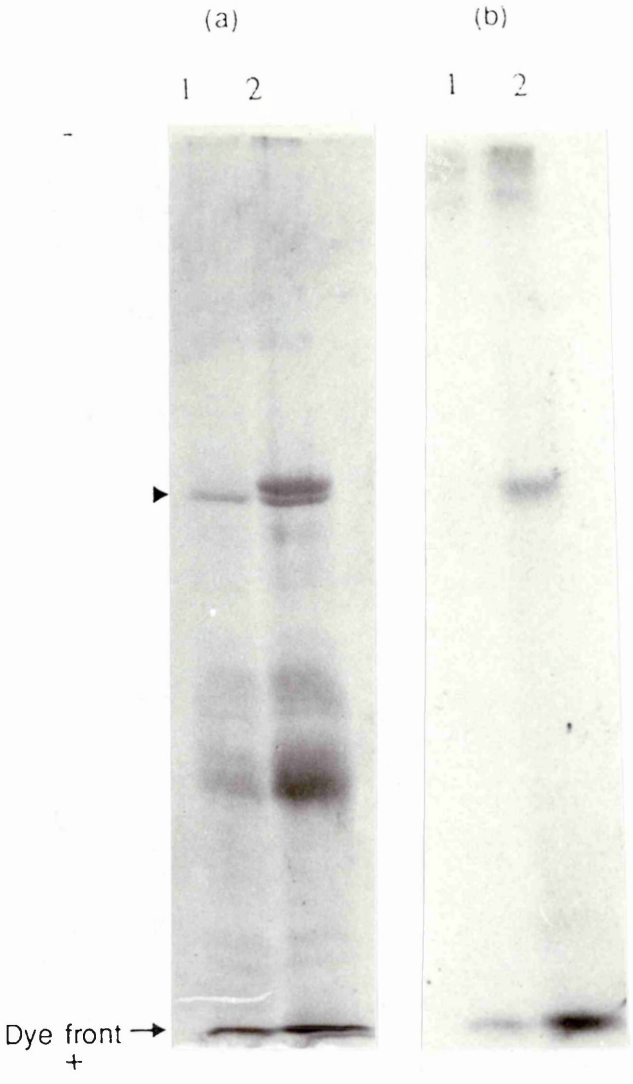

(a)
1
2
3
4
(b)
1
2
3
4
Dye front
+

FIGURE 6.6. ***In vitro*** **phosphorylation of purified PEP carboxylase from maize leaves by an extract prepared from illuminated sugar cane leaves.** Incubations were carried out as described in Section 2.7.6.(i) and immunoprecipitation of PEP carboxylase protein carried out as described in Section 2.7.3. prior to electrophoresis. (a) Coomassie Brilliant Blue stained gel. (b) autoradiograph. Track 1, no exogenous purified maize PEP carboxylase added; track 2, exogenous purified maize PEP carboxylase added. The arrow indicates the mobility on the gel of PEP carboxylase from sugar cane leaves. In track 2 the protein band with the greater mobility is PEP carboxylase from sugar cane leaves while the band of protein which has migrated slightly less far down the gel is PEP carboxylase purified from maize leaves.


(a)
(b)
1
2
1
2
Dye front
+

although the absolute values of $K_i$ were different. PEP carboxylase in extracts of darkened sugar cane leaves had an apparent $K_i$ of 1.33 ± 0.51 mM (mean ± S.D., n=3) while extracts of illuminated leaves had an apparent $K_i$ of 3.02 ± 0.70 mM (mean ± S.D., n=3). This compared to values for maize of about 0.3 mM for PEP carboxylase in extracts of darkened leaves and about 1.0 mM for the enzyme in extracts of illuminated leaves (see Section 3.2.1.).

### 6.2.3. Measurement of protein phosphatase activity in crude extracts of illuminated and darkened leaves

The activity of protein phosphatases in crude leaf extracts was investigated by measuring their activity towards phosphorylase a (which had been purified from rabbit muscle and $^{32}$P-labelled by phosphorylase kinase) as described in Section 2.7.7. Two of the four major mammalian protein phosphatases have significant activity towards phosphorylase a (Cohen *et al.*, 1988a). These are PP-1 and PP-2A, whose activities can be distinguished and quantified by the inclusionin the assay mixture of either inhibitor 1 or inhibitor 2, small heat and acid stable mammmalian proteins which inhibit PP-1 activity specifically (Cohen *et al.*, 1988b), or the tumour promoter okadaic acid which specifically inhibits PP-2A activity at 1 nM (Cohen, 1989, MacKintosh and Cohen, 1989). Using this method of assay, the activities of PP-1 and PP-2A were measured in the cytosolic and particulate fractions of leaf extracts.

Illuminated and darkened leaf portions (0.5 g) were extracted in liquid nitrogen (see Section 2.5.1.) into 2 ml of Buffer A. These samples were then centrifuged for 5 min at 11 600 g. The supernatant from this step was taken as the cytosolic fraction and used for assay. The pellet was washed twice, resuspended in 2 ml of Buffer A and then used for assay. The wash supernatants showed little protein phosphatase activity (less than 5% of the activity in the supernatant from the initial extract used for assay).

The amount of protein phosphatase activity in extracts prepared from darkened leaves was not statistically different from the amout of activity extracted from illuminated leaves (Table 6.1.). About 20-25% of the total protein phosphatase activity in both types of extract was located in the pellet, while about 25-30% of the total PP-1 activity and 12-19% of the total PP-2A activity was present in this fraction. In extracts from both illuminated and darkened leaves, more PP-1 activity than PP-2A

TABLE 6.1. **Specific activities of protein phosphatases PP-1 and PP-2A in the cytosol and resuspended pellet from extracts of darkened and illuminated maize leaves.** Cytosolic fractions were diluted 20-fold and pellets diluted 5-fold for measurements of phosphorylase a phosphatase activity. Assays were carried out as described in Section 2.7.7. Values are given ± S.D. for three experiments.

| Fraction | Protein concentration (mg/ml) | Total phosphatase activity (munits/mg) | Phosphatase activity due to PP-1 (munits/mg) | Phosphatase activity due to PP-2A (munits/mg) |
|---|---|---|---|---|
| Extract from darkened leaf | | | | |
| Cytosol | 2.23 ± 0.61 | 0.77 ± 0.16 | 0.44 ± 0.16 | 0.33 ± 0.17 |
| Pellet | 0.98 ± 0.21 | 0.56 ± 0.02 | 0.39 ± 0.03 | 0.17 ± 0.01 |
| Extract from illuminated leaf | | | | |
| Cytosol | 2.23 ± 0.57 | 1.02 ± 0.09 | 0.58 ± 0.14 | 0.44 ± 0.05 |
| Pellet | 0.95 ± 0.15 | 0.60 ± 0.11 | 0.45 ± 0.18 | 0.15 ± 0.04 |

activity was present in the cytosolic fraction (55-57% PP-1) and in the pellet (70-75% PP-1).

### 6.2.4. Partial purification and characterization of a soluble protein kinase capable of phosphorylating PEP carboxylase

The purification was carried out following the procedure described in Section 2.5.4. and using leaves which had been illuminated before extraction. In some experiments, where purification was only carried out as far as the chromatography on blue dextran-agarose, 1 g portions of excised leaves were illuminated at 1000 $\mu$mol $m^{-2}s^{-1}$ at 27 $^{o}C$ for 1 h, powdered with sand and PVP in liquid nitrogen and then stored until use under liquid nitrogen. No discernable difference was detected between the amount of kinase activity found in leaf samples frozen for several weeks under liquid nitrogen after illumination and that found in leaves which were extracted immediately after illumination. However, for scaled-up preparations, because of the difficulties of illuminating large quantities of leaf material in this way, leaves were taken directly from the growth cabinet 10-12 h into the light phase and homogenised in a Waring blender.

In initial experiments using ammonium sulphate fractionation, kinase activity was detected in both 0-30% and 30-50% fractions. Thus, a 0-50% ammonium sulphate step was used to ensure precipitation of all the kinase activity. After desalting, blue dextran-agarose chromatography (Figure 6.7.) permitted the separation of the kinase activity from the PEP carboxylase activity. Kinase activity bound to the column in Buffer C (see Section 2.5.1.) and was step eluted using Buffer C incuding 0.5 M-NaCl whereas PEP carboxylase did not bind to the column in Buffer C and was therefore removed before elution with 0.5 M-NaCl. No PEP carboxylase activity could be measured or PEP carboxylase protein seen on SDS/polyacrylamide gels of the 0.5 M-NaCl eluted fraction . Following overnight dialysis against Buffer C, Mono Q chromatography was carried out and kinase activity was eluted from the column by running a 0-350 mM-NaCl gradient (Figure 6.8.). Two peaks of kinase activity were usually resolved. A smaller initial activity peak was eluted by about 100 mM-NaCl while a second peak containing much more kinase activity was eluted by 200-300 mM-NaCl (Figure 6.9.). However, in one experiment 25 g of leaves which had been illuminated at 1000 $\mu$mol $m^{-2}s^{-1}$ together with 15 g of leaves taken directly from the illuminated growth cabinet, were used to carry out the purification. In this case much more kinase activity was obtained, particularly in the initial activity peak, than when 40 g of leaves taken

FIGURE 6.7. **Blue dextran-agarose chromatography of PEP carboxylase kinase.** Enzyme from the 0-50% ammonium sulphate fraction which had been desalted on a 1 x12 cm (10 ml) column of G-25 M Sephadex was loaded on to a 2.7 x 5.0 cm (28 ml) column of blue dextran-agarose equilibrated in Buffer C. Kinase activity was eluted from the column by washing with Buffer C containing 0.5 M-NaCl. The arrow indicates the point when the buffer change was carried out. The flow rate was 0.2 ml/min and 5 min fractions were collected. Fractions with $A_{280}$ greater than 0.2 were pooled

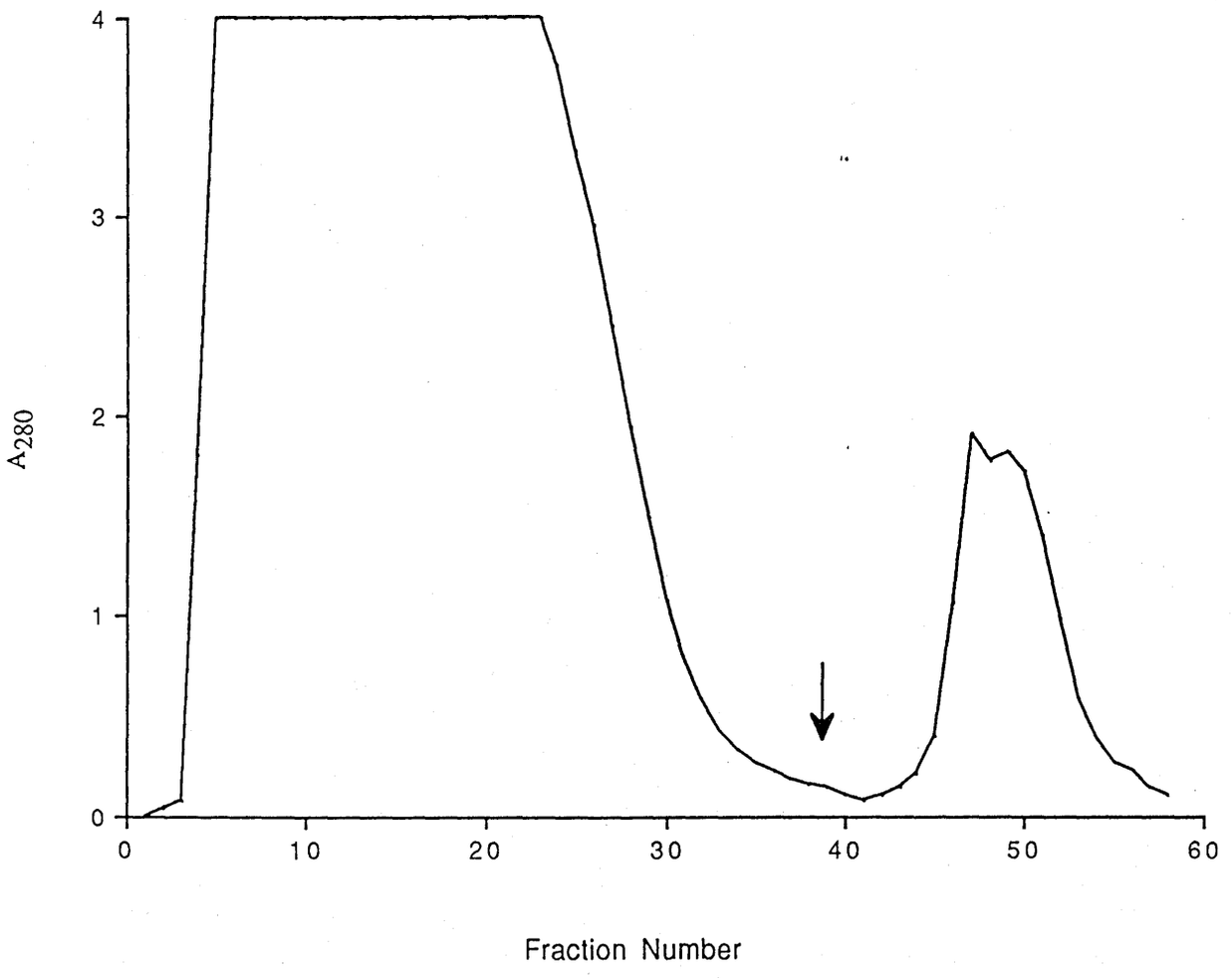

4
3
2
1
0
$A_{280}$
0
10
20
30
40
50
60
Fraction Number

FIGURE 6.8. **Mono Q chromatography of PEP carboxylase kinase.** The pooled kinase activity, which had been dialysed into Buffer C, was loaded on to a Mono Q column equilibrated in the same buffer. Activity was then eluted from the column with a linear gradient of 0-350 mM-NaCl as indicated on the figure by a diagonal straight line. The flow rate was 1 ml/min and 1 min fractions were collected.

The numbered arrows indicate the elution positons of the two peaks of kinase activity shown in Figure 6.9.

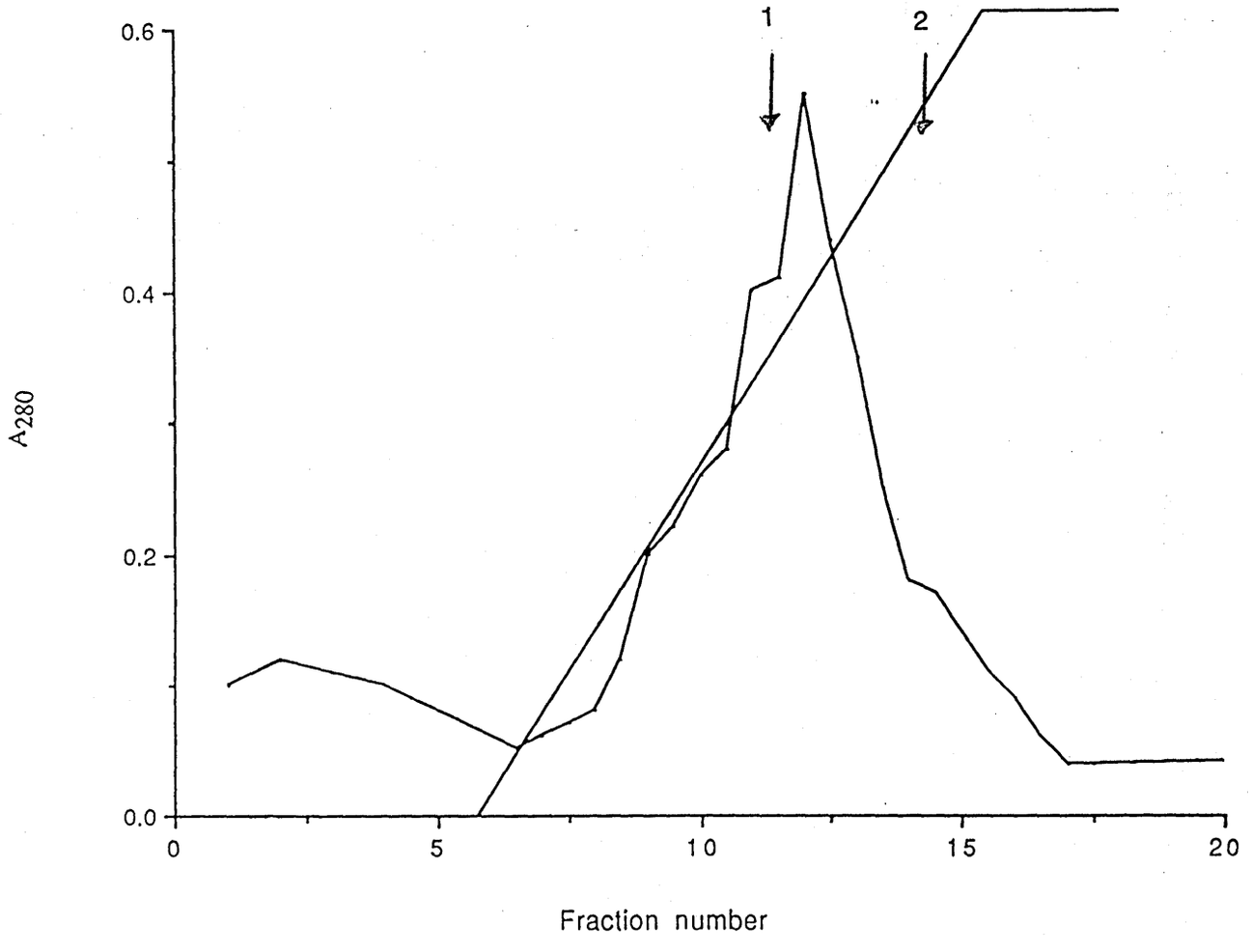
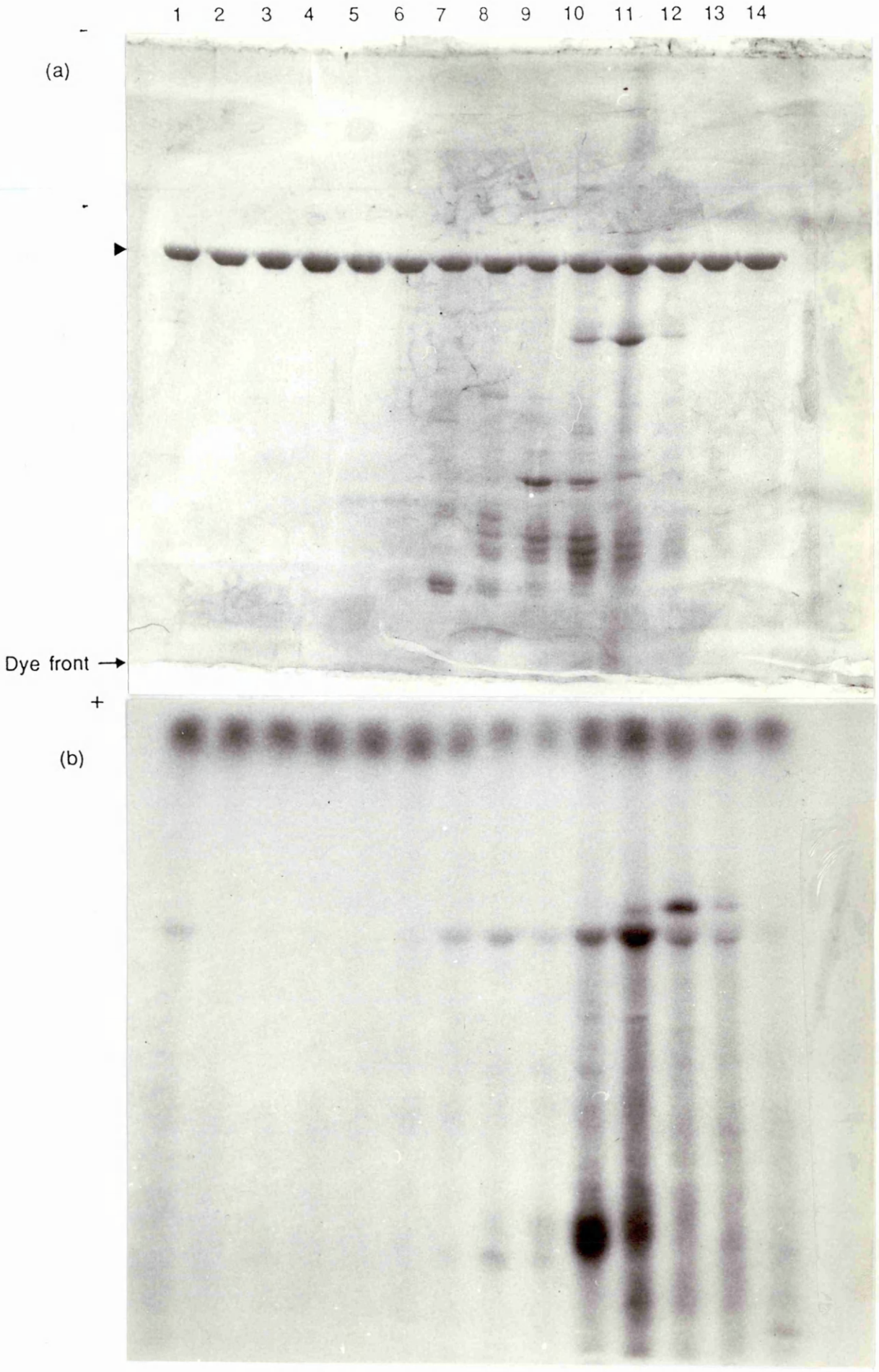

1
2
0.6
0.4
0.2
0.0
A280
0
5
10
15
20
Fraction number

FIGURE 6.9. **SDS/Polyacrylamide gel (8%) and autoradiograph of the *in vitro* kinase assays of individual fractions collected from the Mono Q column.** Incubations were carried out as described in Section 2.7.6.(ii). In all assays exogenous PEP carboxylase was added. Assays were from the MonoQ column run shown in Figure 6.8. and the blue dextran-agarose column run shown in Figure 6.7. Track 1, sample from the blue-dextran agarose column pool before loading on Mono Q; track 2, fraction 3 from Mono Q column; tracks 3-14, fractions 6-17 from Mono Q column respectively. (a) Coomassie Brilliant Blue stained gel, (b) autoradiograph. The arrow indicates the mobility of PEP carboxylase on the gel.


1 2 3 4 5 6 7 8 9 10 11 12 13 14
(a)
Dye front
+
(b)

directly from the illuminated growth cabinet were used (Figure 6.10.). This raises the possibility that two kinases or two forms of a single kinase exist in maize leaves which are capable of phosphorylating PEP carboxylase. After dialysis against Buffer D (see Section 2.5.1.) fractions containing kinase activity were pooled and concentrated. The kinase activity remained stable for several weeks when stored as samples at -20 °C.

This partially purified kinase fraction still contained many other contaminating polypeptides which were especially visible on SDS/polyacrylamide gels of kinase samples which had been concentrated before use (e.g. Figure 6.11.). The purification-fold and recovery of the kinase were not estimated because of the problems of quantifying assays which would have been difficult. As discussed by Jiao and Chollet (1989), the measurement of the kinase activity in crude extracts is unreliable, particularly due to the likelihood of endogenous protein phosphatase activity existing in the crude extracts.

`Using kinase samples which had been purified only as far as the blue-dextran agarose step, phosphorylation of PEP carboxylase by the kinase was shown to be $Mg^{2+}$ and ATP dependent. $Mg^{2+}$ was shown to be necessary for kinase activity since the inclusion of 10 mM-EDTA reduced the level of phosphorylation of PEP carboxylase by about 90% (96% and 85% in two separate experiments) when compared to control values. Randomization of the $^{32}P$ label using myokinase (adenylate kinase) effectively abolished the phosphorylation of PEP carboxylase by the kinase. The exact percentage inhibition was not measured, however, and was judged only by the visualisation of autoradiographs. It therefore seems that this is an ATP-dependent and not an ADP- or an AMP-dependent phosphorylation. Other phosphate donors (e.g. GTP) were not tested.

All further experiments in this section were carried out using kinase which had been purified as far as Mono Q chromatography. The stoichiometry of PEP carboxylase phosphorylation was investigated by carrying out time courses of phosphorylation using [$\gamma$-$^{32}P$]ATP. Figure 6.11. shows one such time course where the phosphorylation was complete after 2 h. The time at which the phosphorylation reached completion was strongly dependent on the amount of kinase activity present, i.e. the more concentrated the kinase sample used, the shorter the time taken to reach complete phosphorylation.

The maximum stoichiometry achieved was 0.76 moles of $P_i$ incorporated per mole of PEP carboxylase subunit (from scintillation counting fragments from the gel shown in Figure 6.11.). The

FIGURE 6.10. **SDS/Polyacrylamide gel (8%) and autoradiograph of the *in vitro* kinase assays of individual fractions collected from the Mono Q column of a kinase preparation from leaves illuminated which had been highly illuminated before extraction.** Incubations were carried out as described in Section 2.7.6.(ii). See text for details of illumination. In all assays exogenous PEP carboxylase was added. Tracks 1-12, fractions from Mono Q column NaCl gradient. (a) Coomassie Brilliant Blue stained gel, (b) autoradiograph. The arrow indicates the mobility of PEP carboxylase on the gel.

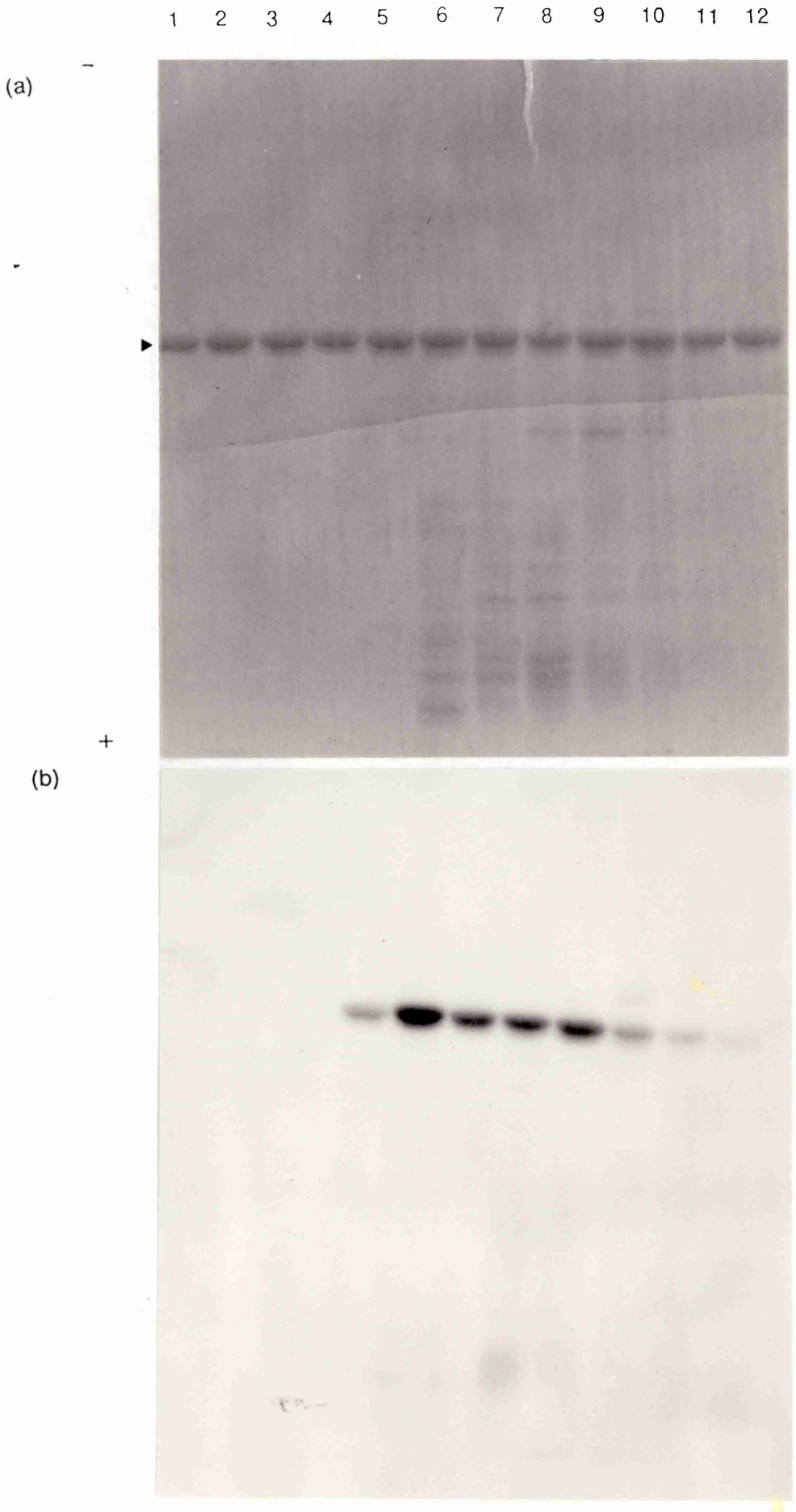
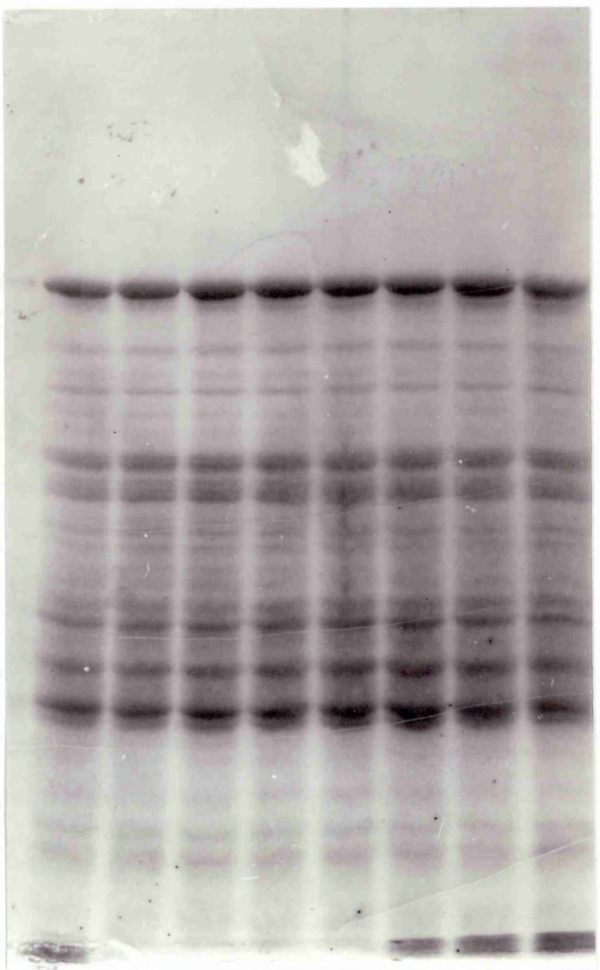

1 2 3 4 5 6 7 8 9 10 11 12
(a)
−
+
(b)

FIGURE 6.11. **Time course of phosphorylation of purified PEP carboxylase on incubation with the partially purified kinase.** Incubations were carried out as described in Section 2.7.6.(ii) and immunoprecipitation of PEP carboxylase protein carried out as described in Section 2.7.3. prior to electrophoresis. (a) Coomassie Brilliant Blue stained gel, (b) autoradiograph. Track 1, sample taken at time 0; track 2, sample taken after 5 min incubation; track 3, sample taken after 15 min incubation; track 4, sample taken after 30 min incubation; track 5, sample taken after 60 min incubation; track 6, sample taken after 90 min incubation; track 7, sample taken after 120 min incubation; track 8, sample taken after 240 min incubation. The arrow indicates the mobility of PEP carboxylase on the gel.

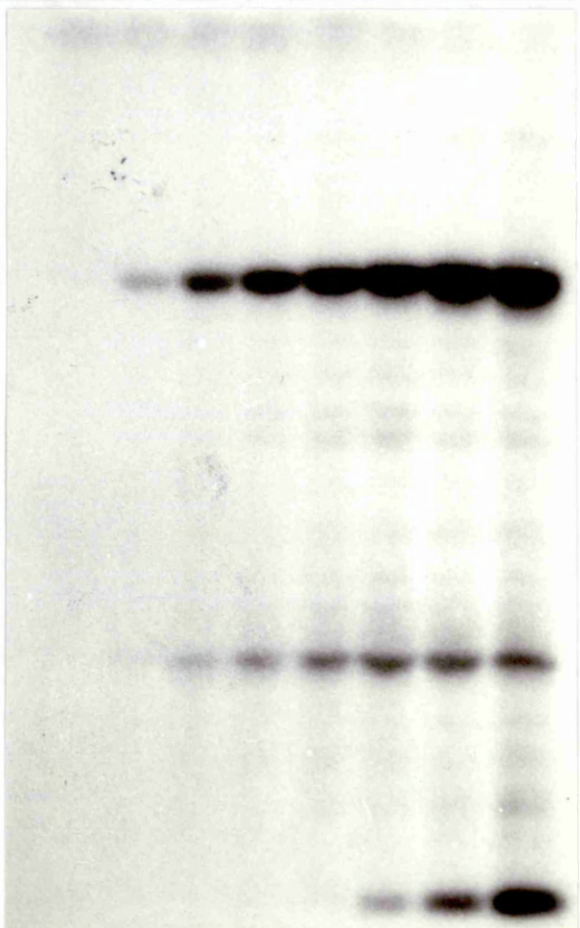
1 2 3 4 5 6 7 8

(a)



\+ Dye front →

(b)



difficulty in the measurement of the stoichiometry is that proteolytically damaged PEP carboxylase cannot be phosphorylated (Figure 6.6.). Careful examination of most time courses of phosphorylation (e.g. Figure 6.12.) showed that some proteolysis of PEP carboxylase occurred during incubation with the kinase even in the presence of protease inhibitors. In time course experiments where the protease inhibitors were omitted, the proteolysis was much more evident (Figure 6.13). Scintillation counting of the gel slices of the PEP carboxylase band also revealed that $^{32}P$ incorporation peaked and then fell again (Figure 6.14.) This proteolysis was slowed but not completely prevented by using a mixture of protease inhibitors consisting of 10 μg of chymostatin/ml, 10 μg of antipain/ml, 10 μg of leupeptin/ml, 1.2 mM-PMSF and 1 mM-benzamidine.

The increase in the phosphorylation state of PEP carboxylase was mirrored by an increase in the apparent $K_i$ for malate of the enzyme (Figure 6.14.). The time taken for this apparent $K_i$ change to reach completion was similar to the time taken for complete phosphorylation to occur. This $K_i$ change was dependent on the inclusion of ATP. When ATP was omitted from the incubation (see Figure 6.14.), or the non-hydrolysable ATP analogue adenylyl-imidodiphosphate (AMP-PNP) was added instead (data not shown), a similar apparent $K_i$ change to that seen in the presence of ATP did not occur. However, a small increase in the apparent $K_i$ for malate of PEP carboxylase was still seen in the absence of ATP (Figure 6.14.), or when AMP-PNP was used instead. This change was relatively small in comparison to that when ATP was present and a slow increase in the velocity of the reaction with time was seen in both the presence and absence of ATP. The slow increase in the apparent $K_i$ for malate seen in the absence of ATP, and the time-dependent increase in the velocity of the reaction appeared to coincide with the proteolysis and dephosphorylation of PEP carboxylase evident in $^{32}P$ labelling experiments carried out over the same time course. In some experiments this $K_i$ increase, which was presumably due to the proteolysis of PEP carboxylase during incubation, varied between different kinase preparations. In the experimental results shown in Figure 6.14., the proteolysis and the increase in the apparent $K_i$ for malate in the absence of ATP were relatively slight over the time course carried out. In other experiments both the proteolysis and the apparent $K_i$ increase in the absence of ATP were much greater. Indeed, the apparent $K_i$ for malate in the absence of ATP for the experiment shown in Figure 6.13. after 2 h was 0.60 mM compared to 1.20 mM in the presence of ATP (from an initial apparent $K_i$ for malate 0.25 mM). This change in the apparent $K_i$ , observed in the absence of ATP, was also substantially

FIGURE 6.12. **Time course of phosphorylation of PEP carboxylase showing proteolysis of purified PEP carboxylase on incubation with the partially purified kinase fraction in the presence of protease inhibitors.** Incubations were carried out as described in Section 2.7.6.(ii) and immunoprecipitation of PEP carboxylase protein carried out as described in Section 2.7.3. prior to electrophoresis. (a) Coomassie Brilliant Blue stained gel, (b) autoradiograph. Track 1, sample taken at time 0; track 2, sample taken after 5 min incubation; track 3, sample taken after 15 min incubation; track 4, sample taken after 30 min incubation; track 5, sample taken after 60 min incubation; track 6, sample taken after 90 min incubation; track 7, sample taken after 120 min incubation; track 8, sample taken after 240 min incubation. The arrow indicates the mobility of PEP carboxylase on the gel.

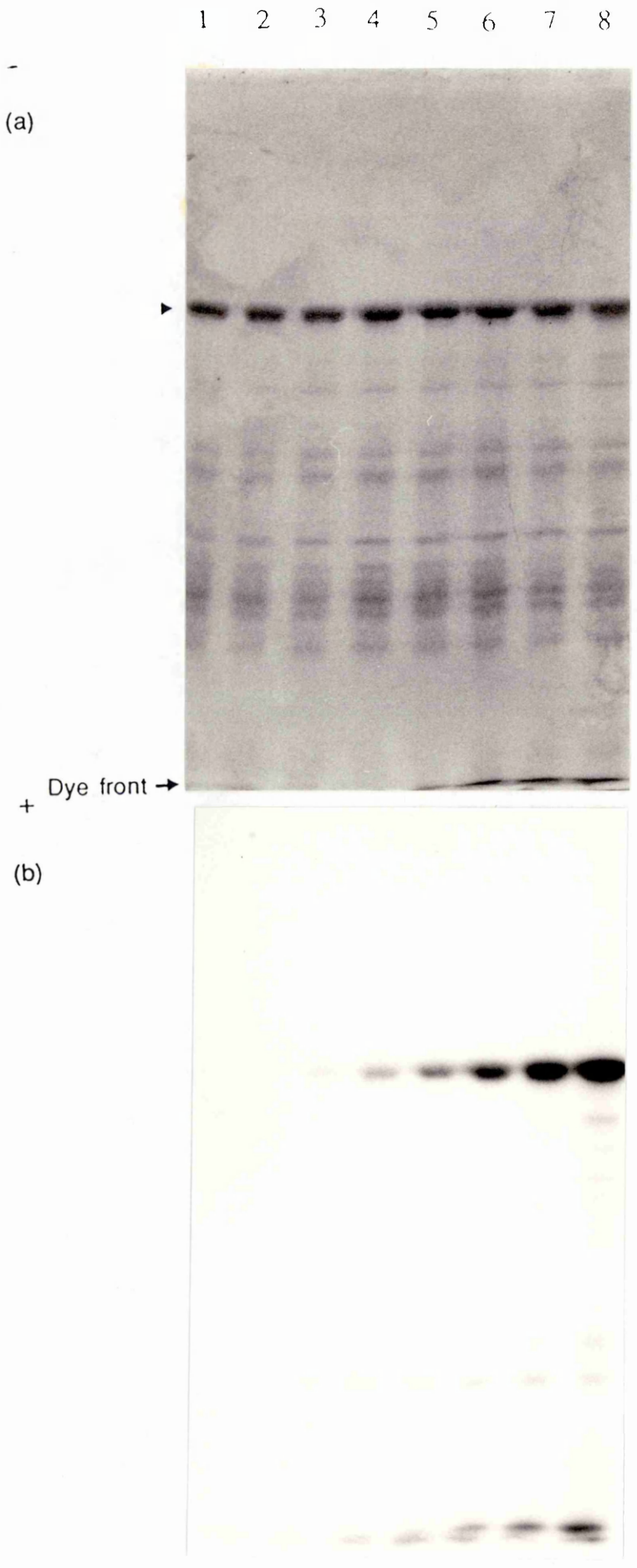

1 2 3 4 5 6 7 8
(a)
Dye front
+
(b)

FIGURE 6.13. **Time course of phosphorylation of PEP carboxylase showing proteolysis of purified PEP carboxylase on incubation with the partially purified kinase fraction in the absence of protease inhibitors.** Incubations were carried out as described in Section 2.7.6.(ii) and immunoprecipitation of PEP carboxylase protein carried out as described in Section 2.7.3. prior to electrophoresis. (a) Coomassie Brilliant Blue stained gel, (b) autoradiograph. Track 1, sample taken at time 0; track 2, sample taken after 5 min incubation; track 3, sample taken after 15 min incubation; track 4, sample taken after 30 min incubation; track 5, sample taken after 60 min incubation; track 6, sample taken after 90 min incubation; track 7, sample taken after 120 min incubation; track 8, sample taken after 240 min incubation; track 9 sample taken after 480 min incubation. The arrow indicates the mobility of PEP carboxylase on the gel.

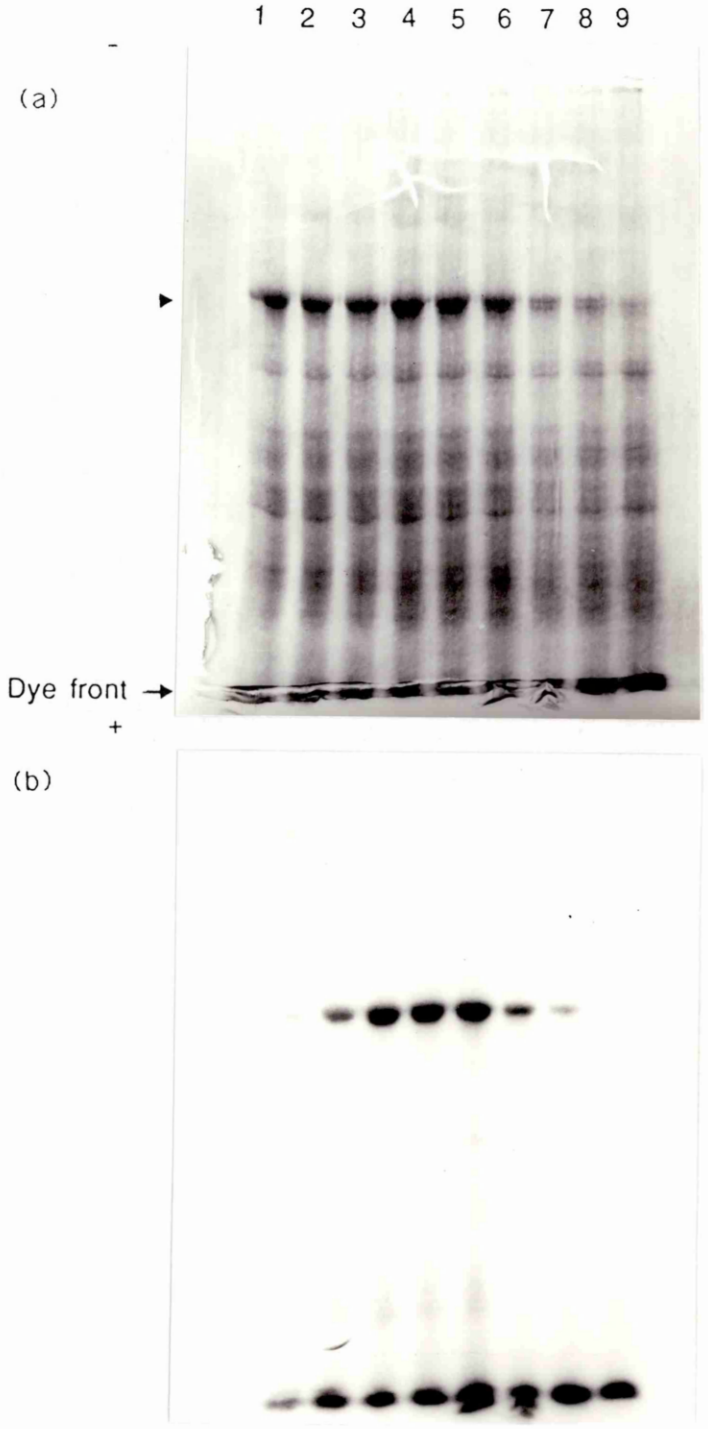
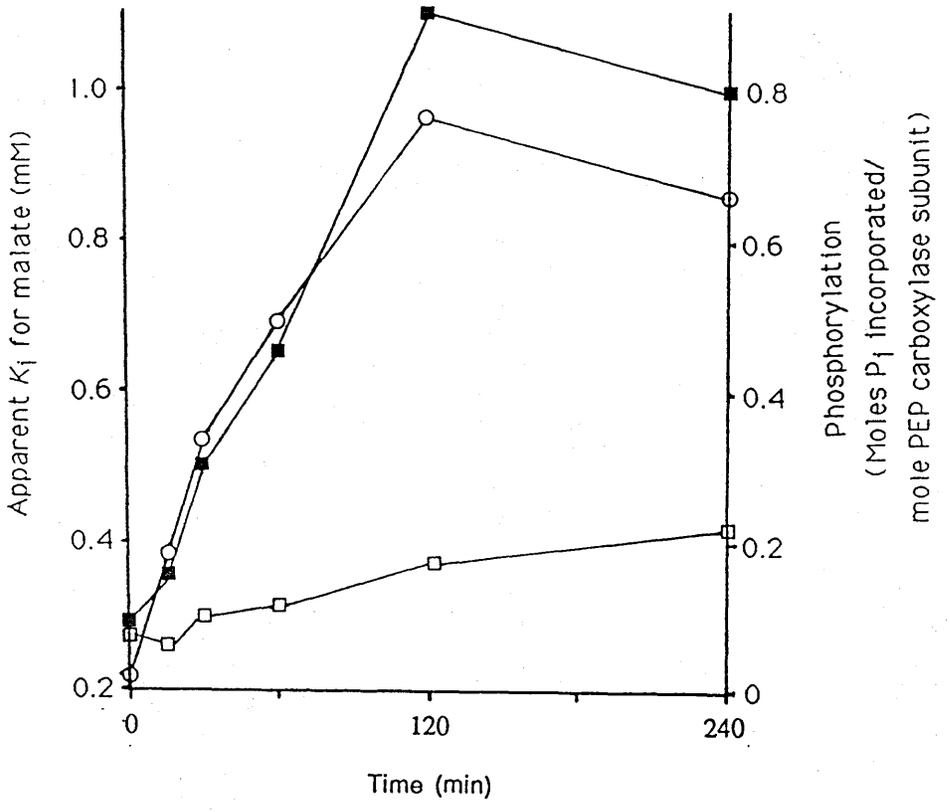

1 2 3 4 5 6 7 8 9
(a)
Dye front
+
(b)

FIGURE 6.14. **Graph showing the time course of phosphorylation and change in the apparent $K_i$ for malate of purified PEP carboxylase incubated with partially purified kinase.** Incubations were carried out as described in Section 2.7.6.(ii) The apparent $K_i$ for malate was measured for samples in the presence and in the absence of ATP for parallel assays to those used for the measurement of the stoichiometry of phosphorylation (gel slices from the gel shown in Figure 6.11.).

Apparent $K_i$ for malate in the presence of ATP —■—

Apparent $K_i$ for malate in the absence of ATP —□—

Phosphorylation (moles $P_i$ incorporated/mole PEP carboxylase subunit) —○—


Apparent $K_i$ for malate (mM)
1.0
0.8
0.6
0.4
0.2
0.8
0.6
0.4
0.2
0
0
120
240
Time (min)
Phosphorylation
(Moles $P_i$ incorporated/
mole PEP carboxylase subunit)

slowed by the inclusion of the same mixture of proteases used to try to stop the proteolysis seen in $^{32}P$ labelling experiments.

One of the objectives behind the purification of the kinase was to study the action of possible effectors of PEP carboxylase activity on the phosphorylation of the enzyme. The effects of a range of possible metabolites on the phosphorylation is summarized in Table 6.2. The results from this table show that $Ca^{2+}$, EGTA, calmodulin and the calmodulin antagonist trifluoperizine do not have any statistically significant effect on the phosphorylation of PEP carboxylase. $Ca^{2+}$/EGTA buffers which gave a range of free $Ca^{2+}$ concentrations ranging from 0-10 mM were also tested and showed that there was no significant effect at any concentration of free $Ca^{2+}$. Fluoride, $P_i$ and okadaic acid were used as possible inhibitors of any phosphatase activity present in the kinase sample. Neither fluoride nor okadaic acid showed any statistically significant effect on the level of phosphorylation, while the addition of $P_i$ gave a small but significant inhibition of phosphorylation. Various metabolite effectors were tested for their effects on the phosphorylation of PEP carboxylase. PEP, fructose 2,6-bisphosphate, glucose 6-phosphate and glycerol 3-phosphate did not have any statistically significant effect on the level of phosphorylation. However, 5 mM-malate showed a statistically significant (at the 0.001 level) inhibition of phosphorylation of over 40%. Using D-malate instead of L-malate (used in all other experiments in this thesis) produced a similar inhibition of phosphorylation (34% and 39% inhibition in two separate experiments).

The kinase was also used to test the effects of mammalian PP-1 and PP-2A on the phosphorylation of PEP carboxylase. PEP carboxylase was phosphorylated using the kinase fraction and then either PP-1 or PP-2A was added. It was found that purified mammalian PP-2A could dephosphorylate PEP carboxylase while purified mammalian PP-1, or PP-2A added in the presence of 10 nM okadaic acid could not (Figure 6.15.). This dephosphorylation by PP-2A was enhanced slightly by the addition of 7.5 mM-EDTA to the incubation, presumably because EDTA chelated the $Mg^{2+}$ needed for kinase activity and thus prevented the kinase from acting in opposition to the protein phosphatase. Further, addition of purified PP-2A also increased the sensitivity of PEP carboxylase to malate. However, the apparent $K_i$ did not return to the starting value for the purified enzyme before its phosphorylation by the kinase (Table 6.3.). This was probably because some proteolysis of PEP carboxylase occurred during the

FIGURE 6.15. **The effect of purified mammalian PP-1 and PP-2A on the phosphorylation of purified PEP carboxylase.** Autoradiograph of an 8% SDS/polyacrylamide gel. Kinase assays were carried out as described in Section 2.7.6.(ii). (a) Coomassie Brilliant Blue stained gel, (b) autoradiograph. Track 1, 60 min incubation; track 2, 120 min incubation; track 3, 60 min incubation plus 60 min in the presence of PP-1 (200 mU/ml final concentration); track 4, 60 min incubation plus 30 min in the presence of PP-2A (200 mU/ml final concentration); track 5, 60 min incubation plus 60 min in the presence of PP-2A (200 mU/ml final concentration); track 6, 60 min incubation plus 30 min in the presence of PP-2A (200 mU/ml final concentration) and 7.5 mM-EDTA; track 7, 60 min incubation plus 60 min in the presence of PP-2A (200 mU/ml final concentration) and 7.5 mM-EDTA; track 8, 60 min incubation plus 30 min in the presence of PP-2A (200 mU/ml final concentration) and 10 nM-okadaic acid; track 9, 60 min incubation plus 60 min in the presence of PP-2A (200 mU/ml final concentration) and 10 nM-okadaic acid.

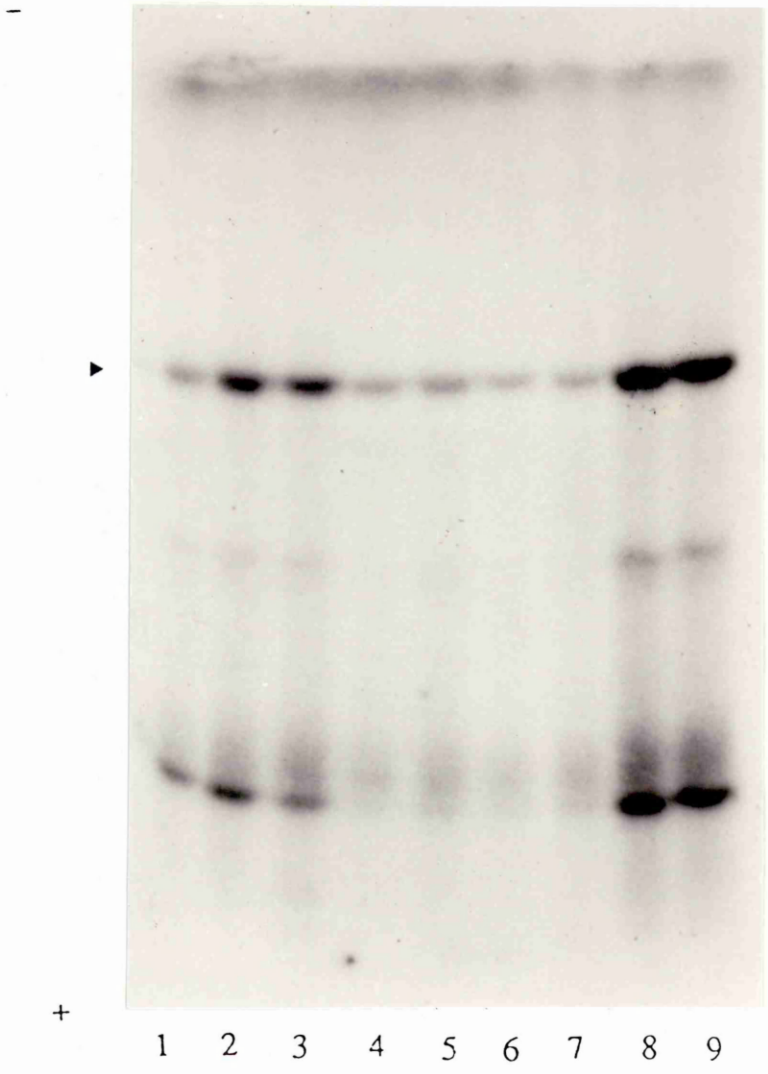

-
+
1 2 3 4 5 6 7 8 9

TABLE 6.2. **The effect of various compounds on the *in vitro* phosphorylation of maize leaf PEP carboxylase.** Values given are for a comparison of the percentage phosphorylation with the values for the standard (control) assay (see Section 2.7.6.(ii)) and are shown ± S.D. for three separate experiments.

| Addition to/omission from the standard in vitro assay | Relative phosphorylation (%) |
|---|---|
| None (control) | 100 |
| No kinase | 3.3 ± 2.9 |
| No PEP carboxylase | 8.7 ± 5.6 |
| $CaCl_2$ (1 mM) | 83.3 ± 7.7 |
| EGTA (1 mM) | 106.3 ± 8.1 |
| Trifluoperizine (1 mM) | 95.3 ± 22.4 |
| Calmodulin (0.2 μg/ml final concentration) | 90.3 ± 19.4 |
| NaF (10 mM) | 116.6 ± 48.2 |
| $NaH_2PO_4$ (10 mM) | 67.7 ± 12.0 |
| Okadaic acid (100 nM) | 112.0 ± 13.0 |
| Fructose 2,6-bisphosphate (20 μM) | 105.3 ± 5.5 |
| PEP (5 mM) | 93.0 ± 21.8 |
| L-Malate (5 mM) | 58.6 ± 8.6 |
| Glucose 6-phosphate (5 mM) | 81.3 ± 24.9 |
| Glycerol 3-phosphate (5 mM) | 95.6 ±10.2 |

TABLE 6.3. **The effect of PP-2A on the apparent $K_i$ for malate of PEP carboxylase.** *In vitro* kinase incubations were carried out for 60 min as described in Section 2.7.6.(ii). before any additions were made to the mixture. 200 mU of PP-2A/ml and 10 nM okadaic acid were used in the relevant incubations.

| | Apparent $K_i$ for malate (mM) | | |
|---|---|---|---|
| | (a) | (b) | (c) |
| Time (min) | Control | PP-2A added after 60 min | PP-2A and okadaic acid added after 60 min |
| 0 | 0.30 | | |
| 15 | 0.50 | | |
| 30 | 0.75 | | |
| 60 | 1.10 | | |
| 90 | 1.30 | 0.60 | 1.50 |
| 120 | 1.75 | 0.80 | 2.05 |

manipulations which increased the apparent $K_i$ for malate. PP-1 had no effect on the apparent $K_i$ for malate compared to control values (not shown), nor did PP-2A added in the presence of 10 nM okadaic acid (Table 6.3.).

Nimmo *et al.* (1986) showed that PEP carboxylase from the CAM plant *B. fedtschenkoi* could be dephosphorylated by calf intestinal alkaline phosphatase and that this led to a lowering of the apparent $K_i$ of the enzyme for malate. However, similar experiments designed to test the possible effects of calf intestinal alkaline phosphatase on the enzyme in extracts from maize leaves did not succeed. Under several different incubation conditions tested, addition of alkaline phosphatase to PEP carboxylase rapidly and completely abolished the malate sensitivity of the enzyme. However, using one of the conditions tested in this work, Jiao and Chollet (1988) were able to show the dephosphorylation and increased malate sensitivity of PEP carboxylase on incubation with alkaline phosphatase.

## 6.3. Discussion

It is apparent from the work described in this section, and from the results of Jiao and Chollet (1989), that purified PEP carboxylase from maize leaves can be phosphorylated *in vitro* by a soluble protein kinase also located in the leaves of maize. The kinase is much more active in extracts prepared from illuminated leaves than in extracts prepared from darkened leaves. This indicates that the phosphorylation of PEP carboxylase is dependent on light, as suggested by the *in vivo* phosphorylation studies described in Chapter 3.

Desalting extracts before carrying out assays for kinase activity increased the level of phosphorylation observed (Figure 6.1.). Perhaps the most likely explanation for this finding is that desalting removes a small molecule which can reduce the activity of the kinase. The results in Table 6.2 suggest that malate could be responsible for this effect. Alternatively, the desalting process could adversely effect the activity of the protein phosphatase. The effect of malate does not, however, appear to be stereospecific since both D- and L-malate affect the phosphorylation state of the enzyme to a similar degree. The level of phosphorylation in extracts seems to increase with the time of illumination up to about 1 h (Figure 6.4.), a similar duration to the time taken for the apparent $K_i$ change to be completed *in vivo* (see Figure 3.6.). For times up to 1 h, the apparent $K_i$ for malate of PEP carboxylase increased as the phosphorylation state of the enzyme increased. This stongly implies that the phosphorylation is

responsible for the reduced sensitivity to malate of PEP carboxylase on illumination of maize leaves. Further, since leaves which have been illuminated to 1 000 μmol $m^{-2}s^{-1}$ contain more kinase activity than leaves which have only been illuminated to 250 μmol $m^{-2}s^{-1}$, the activity of PEP carboxylase might vary with light intensity in a similar manner to NADP-MDH and PPDK from the $C_4$ pathway and the light regulated enzymes of the PCR cycle.

The maximum stoichiometry of phosphorylation was 0.76 moles of $P_i$ per mole of PEP carboxylase subunit, i.e. less than one $P_i$ group per subunit of PEP carboxylase. While this might be the maximum level of phosphorylation of the enzyme in these conditions, proteolysis of PEP carboxylase caused by a proteinase present in the kinase fraction seemed to prevent a higher phosphorylation level (possibly nearer to 1 mole of $P_i$ per mole of PEP carboxylase subunit) being attained. This proteolysis seems to be distinct from the proteolysis observed during the purification of PEP carboxylase in the absence of chymostatin (see Chapter 4) since it could not be prevented by the addition of chymostatin and led to a more complete destruction of the PEP carboxylase protein (as judged by SDS/polyacrylamide gel electrophoresis) and its activity. The *in vitro* level of kinase activity in extracts prepared from darkened leaves, although not accurately determined, seemed to be negligiable in comparison with the level of activity in extracts prepared from illuminated leaves (Figures 6.2. and 6.4.). However, this difference in the ability of extracts prepared from illuminated and darkened leaves to phosphorylate PEP carboxylase seemed to be much greater than the 50% increase in the level of phosphorylation found by Jiao and Chollet (1988) using extracts prepared from illuminated leaves compared to extracts prepared from darkened leaves. These authors found a maximum molar $^{32}P$ incorporation value of only about 0.25 moles of $P_i$ per mole of PEP carboxylase subunit (i.e. one per tetramer) using PEP carboxylase purified from darkened maize leaves (Jiao and Chollet, 1989) and suggested that substantial phosphorylation of PEP carboxylase existing in dark leaves could account for this. In the present study (see Figure 3.6.), the PEP carboxylase from darkened leaves was not phosphorylated or only phosphorylated to a slight extent. Thus the higher $P_i$ incorporation into PEP carboxylase obtained in our hands might have been due to a lower level of phosphorylation of the purified enzyme used as a substrate. Alternatively it may have been due to the occurrence of proteolysis of PEP carboxylase in the experiments of Jiao and Chollet (1989).

The experiments using extracts prepared from sugar cane leaves show that the phosphorylation of PEP carboxylase could also occur in this $C_4$ plant. However, the experiments described only show that the enzyme purified from maize leaves can be phosphorylated by some component present in extracts prepared from illuminated sugar cane leaves. Further studies are therefore needed in order to ascertain whether the PEP carboxylase from sugar cane can itself be phosphorylated. Echevarria *et al.* (1988) demonstrated the $Ca^{2+}$-calmodulin-dependent *in vitro* phosphorylation of *Sorghum* leaf PEP carboxylase although no data were presented on the correlation between the phosphorylation state and the kinetic state of the enzyme. It therefore seems likely that phosphorylation of PEP carboxylase in response to light may occur in all $C_4$ plants.

Studies on the activity of protein phosphatases in crude leaf extracts and experiments using purified mammalian protein phosphatases in the *in vitro* kinase assay mixture have provided good evidence that protein phosphatases present in maize leaves are very similar to protein phosphatases present in mammalian tissues. The properties of the maize leaf protein phosphatases are similar to mammalian PP-1 and PP-2A in that they can dephosphorylate phosphorylase a and are sensitive to similar concentrations of inhibitor 1 and okadaic acid. No attempt was made to investigate the possible existence in maize leaves of PP-2B- and PP-2C-like activities, although in this laboratory R.J. Bentley (unpublished data) has found some PP-2C-like activity. Purified PP-2A from rabbit muscle can dephosphorylate maize PEP carboxylase and render it more sensitive to malate inhibition. These facts suggest the possibility that a protein phosphatase in maize leaves which is similar to mammalian PP-2A might be responsible for the dephosphorylation of PEP carboxylase *in vivo*. No difference in the activity of these phosphatases was observed between extracts prepared from illuminated and darkened leaves (as judged by the dephosphorylation of phosphorylase a), suggesting that the phosphatase activity might not be light regulated. However, the kinase activity clearly is affected by light and this could explain the regulation of PEP carboxylase by light.

# Chapter 7

## GENERAL DISCUSSION

It is now established that the activity of the photosynthetic form of PEP carboxylase from maize leaves is regulated by light (this thesis; Huber and Sugiyama, 1986; Doncaster and Leegood, 1987; Jiao and Chollet, 1988, 1989). The activities of PPDK and MDH are also regulated by light (e.g. Edwards *et al.*, 1985), and so the $C_4$ pathway appears to be controlled at several points. This means that three enzymes which catalyse consecutive reactions of the $C_4$ pathway (see Figure 1.2.) are each regulated by light via a different mechanism. MDH is regulated by a redox mechanism (Section 1.2.2.); PPDK is regulated by a reversible ADP-dependent phosphorylation (Section 1.2.2) and PEP carboxylase has now been shown to be regulated by a reversible ATP-dependent phosphorylation (this study; Jiao and Chollet, 1989). This may give the system the flexibility to respond to several different signals. PEP carboxylase from CAM plants is also regulated by an ATP-dependent reversible phosphorylation (Nimmo *et al.*, 1986: Brulfert *et al.*, 1986; Kluge *et al.*, 1988), and so comparison with this system this provides a way of examining the mechanisms by which this is regulatory process is carried out in two different types of plants.

Samaras *et al.* (1988) pointed out that the time required for full activation of PEP carboxylase (30-60 min from Figure 3.6.), is relatively long in comparison with the time (2-10 min) needed for the activation of MDH and PPDK. These authors also found that DL-glyceraldehyde, which suppresses PCR cycle activity by inhibiting phosphoribulokinase, prevents PEP carboxylase activation. This finding could suggest a link between PEP carboxylase activation in mesophyll cells and $CO_2$ assimilation in the bundle sheath and could explain the slower activation of PEP carboxylase compared with the other light activated enzymes of the $C_4$ pathway which might not require the signal to be passed from one cell to another (Samaras *et al.*, 1988). Selinioti *et al.* (1985) showed that 3-PGA, a metabolite of the PCR cycle (see Section 1.1.1.), could activate PEP carboxylase from a range of $C_4$ species when assays were carried out at low pH and low PEP concentrations, conditions believed to pertain *in vivo* and under which the enzyme is more sensitive to metabolic effectors (Gonzalez *et al.*, 1984). This metabolite is believed to act as a transport metabolite between the bundle sheath and

mesophyll cells (Hatch and Osmond, 1976). It therefore seems that this observation could also lend weight to the idea that an integrated mechanism exists for the regulation of the PCR cycle and the $C_4$ pathway.

Since this work was started, progress has also been made in understanding the structure and reaction mechanism of PEP carboxylase. Sequence data are now available for the enzyme from $C_4$ plants (Izui *et al.*, 1986; Yanagisawa *et al.*, 1988) and from CAM plants (Rickers *et al.*, 1989). It should now be possible to locate the amino acid residues involved in the catalysis and substrate binding and regulatory sites by analysis of peptides and site directed mutagenesis. Using techniques such as circular dichroism and X-ray diffraction it could then be possible to relate the structure to the kinetic properties of the enzyme (Andreo *et al.*, 1987).

Further study of the protein kinase involved in the phosphorylation of PEP carboxylase should provide valuable information on its mechanism of action and on the light regulation of PEP carboxylase. This line of investigation could also provide a useful insight into the possible role of reversible phosphorylation in the regulation by light of the activities of other plant enzymes, a process which is little understood at present.

In order for progress to be achieved on the isolation and characterisation of the kinase, improvements need to be made to the basic purification procedure described in Section 2.5.4. Primarily this would involve scaling up the purification by using larger quantities of leaf material to allow more kinase to be purified. Since this study has shown that more kinase activity is extracted from maize leaves which have been highly illuminated (1000 $\mu$mol $m^{-2}s^{-1}$) than from leaves illuminated to only about 250 $\mu$mol $m^{-2}s^{-1}$, the use of a system allowing illumination of large quantities of leaves to 1000 $\mu$mol $m^{-2}s^{-1}$ would be advantageous. This should facilitate the further purification of the enzyme, perhaps to homogeneity. Once pure, amino acid sequence analysis and cloning and sequencing of the gene encoding the protein would allow comparison of the kinase which phosphorylates PEP carboxylase with other protein kinases from plants and also with the better characterised protein kinases in animals. This in turn could lead to information into the possible mechanism of action being obtained. In this context, Terada *et al.* (1990) have recently shown that maize leaf PEP carboxylase can be phosphorylated *in vitro* with a cyclic-AMP dependent protein

kinase from bovine heart. This phosphorylation was accompanied by a change in the kinetic properties of the enzyme which was very similar to that reported for the light activation. The phosphorylated amino acid residue was identified as serine 15 near the N-terminal end of the molecule (Terada *et al.*, 1990), the same residue which is phosphorylated by the maize leaf kinase *in vitro* (R. Chŏllet unpublished data). It is perhaps significant that the phosphorylation site occurs so close to one end of the molecule since, if the same residue is the site phosphorylated by the PEP carboxylase kinase *in vivo*, this could explain why proteolysis of only a small fragment of PEP carboxylase led to a loss in the ability of the molecule to undergo phosphorylation (see Section 6.2.1.).

The use of a more highly purified kinase sample might prevent the evident proteolysis of the purified PEP carboxylase during *in vitro* incubation as was found using the partially purified kinase sample in this study which clearly contains many contaminating polypeptides. This could allow a better calculation of the stoichiometry of phosphorylation and of the change in malate sensitivity observed on phosphorylation. Also, confirmation of the amino acid which is phosphorylated *in vitro* on the PEP carboxylase molecule is needed in order to check that this phosphorylation is a serine phosphorylation as found by *in vivo* phosphorylation studies (Figure 3.7.). This would help confirm that the kinase which has been partially purified is the same kinase that can phosphorylate PEP carboxylase *in vivo*. A serine phosphorylation was found by Budde and Chollet (1986) using crude extracts to phosphorylate PEP carboxylase *in vitro* but it is necessary to confirm that this is also the case for the partially purified kinase used in this work.

One possible method of investigating the kinase action might be to test its action on mammalian glycogen synthase. Glycogen synthase catalyses the "rate limiting" step in glycogen synthesis and is phosphorylated at multiple sites by a number of different protein kinases *in vitro* and *in vivo* (Cohen, 1988). The different specific phosphorylation sites are known for these protein kinases and can be determined by $^{32}$P-labelling glycogen synthase using the kinase of interest and then digesting the enzyme with trypsin or CNBr before separating the peptides by HPLC and locating the $^{32}$P label. It might be possible to use the kinase from maize leaves which phosphorylates PEP carboxylase to try to phosphorylate mammalian glycogen synthase. Location of any $^{32}$P label as described might then reveal any site(s) phosphorylated *in vitro* by the maize leaf kinase. If this were found to be the case

and the site(s) phosphorylated was found to be one of the known phosphorylation sites for one of the mammalian protein kinases known to act on glycogen synthase, then this could mean that the kinase from maize leaves bears some resemblance to the mammalian protein kinase and give some information as to the mode of action of the maize kinase.

The use of mammalian substrates and the specific protein phosphatase inhibitors okadaic acid and mammalian inhibitors 1 and 2 provides a powerful method for examining protein phosphatases from leaf tissue. It should prove possible to monitor the purification of the protein phosphatase involved in the dephosphorylation of PEP carboxylase using such a system to measure its activity. Indeed, progress is being made in this laboratory using this method to investigate the phosphatase responsible for the dephosphorylation of PEP carboxylase from *B fedtschenkoi* by Carter *et al.* (1990).

Perhaps the most intriguing aspect of the regulation of PEP carboxylase remaining to be addressed concerns the elucidation of the mechanism of signal transduction of the light stimulus. The fact that DCMU prevents the activation of PEP carboxylase by light suggests that the stimulus is perceived by the photosynthetic pigments in the chloroplast and transmitted via the photosynthetic electron transport chain (Section 3.2.2.). At the other end of the chain of events, the activity of PEP carboxylase is altered by phosphorylation by a protein kinase in response to illumination. Samaras and Chollet (1990) have shown that illumination of maize mesophyll cell protoplasts or *Digitaria* mesophyll cells does not cause activation of PEP carboxylase. Taken with the effect of glyceraldehyde quoted above, this suggests that the "message" comes from the bundle sheath cell chloroplasts (Samaras, Y. and Chollet, R. unpublished data). Exactly how the signal could be transmitted and what further components may be involved in this signal transduction process from the photosynthetic pigments in the bundle sheath cells to PEP carboxylase in the cytoplasm of the mesophyll cells remain to be investigated. The method of the actual activation of the kinase itself as a result of exposure to light also needs to be investigated. If it is the kinase which is activated on illumination, and not the activity of the protein phosphatase which is suppressed as seems to be the case, then this activation could occur either by protein synthesis or by covalent modification. The idea that the kinase is activated by protein synthesis might be tested by using inhibitors of this process although these could have other effects on the metabolism of the plant. Covalent modification of the enzyme is an attractive proposition since this could form part of a cascade system as found in other systems (see

Section 1.2.1). This could be tested once further purification and characterisation of the protein kinase has been carried out.

Thus, there are still unresolved questions concerning the regulation by light of PEP carboxylase activity in $C_4$ plants. However, this work has made a contribution to the understanding of this process.

Chapter 8

# REFERENCES


Anderson, L.E. (1986) Light/dark modulation of enzyme activity in plants. Adv. Bot. Res. **12**, 3-46

Anderson, L.E. (1979) Interaction between photochemistry and activity of enzymes. In : Encyclopedia of Plant Physiol., New series, **6**, 271-281, Gibbs, M. and Latzko, E., ed., Springer-Verlag, Berlin

Anderson, L.E. and Nehrlich, S.C. (1977) Photosynthetic electron-transport system controls cytoplasmic glucose-6-phosphate dehydrogenase activity in pea leaves. FEBS Lett. **76**, 64-66

Andreo, C.S., Gonzalez, D.H. and Iglesias, A.A. (1987) Higher plant phosphoenolpyruvate carboxylase. Structure and regulation. FEBS Lett. **213**, 1-8

Angelopoulos, K. and Gavalas, N.A. (1988) Reversible cold inactivation of $C_4$-phosphoenolpyruvate carboxylase : factors affecting reactivation and stability. J. Plant Physiol. **132**, 714-719

Angelopoulos, K., Stamatakis, K., Manetas, Y. and Gavalas, N.A. (1988) Artifacts in the assay of maize leaf phosphoenolpyruvate carboxylase activity due to its instability. Photosynth. Res. **18**, 317-325

Bennett, J. (1984) Regulation of photosynthesis by reversible phosphorylation. In: Enzyme regulation by reversible phosphorylation - further advances. Cohen, P. ed., Mol. Asp. Cell. Reg. **3**, 227-246, Elsevier Biomedical Press, Amsterdam

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye binding. Anal. Biochem. **72**, 248-254

Brulfert, J., Vidal, J., Le Marechal, P., Gadal, P., Querioz, O., Kluge, M. and Kruger, I. (1986) Phosphorylation-dephosphorylation process as a probable mechanism for the diurnal regulatory changes of phosphoenolpyruvate carboxylase in CAM plants. Biochem. Biophys. Res. Commun. **136**, 151-159

Buchanan, B.B. (1980) Role of light in the regulation of chloroplast enzymes. Annu. Rev. Plant Physiol. **31**, 341-374

Budde, R.J.A. and Chollet, R. (1988) Regulation of enzyme activity in plants by reversible phosphorylation. Physiol. Plant. **72**, 435-439

Budde, R.J.A. and Chollet, R. (1986) *In vitro* phosphorylation of maize leaf phosphoenolpyruvate carboxylase. Plant Physiol. **82**, 1107-1114

Canvin, D.T. (1979) Photorespiration : comparison between $C_3$ and $C_4$ plants. In : Encyclopedia of Plant Physiol., New series, **6**, 368-398, Gibbs, M. and Latzko, E., ed., Springer-Verlag, Berlin

Carter, P.J., Nimmo, H.G., Fewson, C.A. and Wilkins, M.B. (1990) *Bryophyllum fedtscenkoi* protein phosphatase type 2A can dephosphorylate phosphoenolpyruvate carboxylase. FEBS Lett. **263**, 233-236

Chapman, K.S.R. and Hatch, M.D. (1981) Regulation of $C_4$ photosynthesis : mechanism of activation and inactivation of extracted pyruvate, inorganic phosphate dikinase in relation to dark/light regulation. Arch. Biochem. Biophys. **210**, 82-89

Chollet, R., Budde, R.J.A., Jiao, J-A. and Roeske, C.A. (1989) Light/dark-regulation of $C_4$ photosynthetic enzymes by reversible phosphorylation. Physiol Plant. **76** suppl., 35

Cleveland, D.W., Fischer, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) Peptide mapping by limited proteolysis in sodium dodecyl sulphate and analysis by gel electrophoresis. J. Biol. Chem. **252**, 1102-1106

Cohen, P. (1988) The regulation of phosphorylase kinase activity by calmodulin and troponin. Mol. Asp. Cell Reg., **5**, 123-193

Cohen, P. (1985) The coordinated control of metabolic pathways by broad specificty protein kinases and phosphatases. Curr. Top. Cell. Reg. **27**, 23-37

Cohen, P., Alemany, S., Hemmings, B.A., Resink, T.J., Stralfors, P. and Tung, H.Y.L. (1988a) Protein phosphatase-1 and protein phosphatase-2A from rabbit skeletal muscle. Meths in Enzymol. **159**, 390-409

Cohen, P., Foulkes, J.G., Holmes, C.F.B., Nimmo, G.A. and Tonks, N.K. (1988b) Protein phosphatase inhibitor-1 and inhibitor-2 from rabbit skeletal muscle. Meths in Enzymol. **159**, 427-437

Coombs, J., Baldry, C.W. and Buckes, C. (1973) The C-4 pathway in *Pennisetum purpureum* 1. The allosteric nature of PEP carboxylase. Planta (Berl.) **110**, 95-107

Coombs, J., Maw, S.L. and Baldry, C.W. (1975) Metabolic regulation in $C_4$ photosynthesis : the inorganic carbon substrate for PEP carboxylase. Plant Sci. Lett. **4**, 97-102

Cooper, J.A., Sefton, B.M. and Hunter, A. (1983) Detection and quantification of phosphotyrosine in proteins. Meths. in Enzymol. **99**, 387-402

Davies, D.D. (1979) The central role of phosphoenolpyruvate in plant metabolism. Annu. Rev. Plant Physiol. **30**, 131-158

Davis, B.J. (1964) Ann. N.Y. Acad. Sci. **121**, 404-427

Doncaster, H.D. and Leegood, R.C. (1987) Regulation of phosphoenolpyruvate carboxylase in maize leaves. Plant Physiol. **84**, 82-87

Echevarria, C., Vidal, J., Le Marechal, P., Brulfert, J., Ranjeva, R. and Gadal, P. (1988) The phosphorylation of *Sorghum* leaf phosphoenolpyruvate carboxylase is a $Ca^{++}$-calmodulin dependent process. Biochem. Biophys. Res. Commun. **155**, 835-840

Edwards, G.E. and Huber, S.C. (1981) The $C_4$ pathway. In : The Biochemistry of Plants. A comprehensive treatise, **8**, 238-281, Hatch, M.D. and Boardman, N.K., ed., Academic Press, New York

Edwards, G.E., Nakamoto, H., Burnell, J.N. and Hatch, M.D. (1985) Pyruvate, phosphate dikinase and NADP-malate dehydrogenase in $C_4$ photosynthesis - properties and mechanism of light regulation. Annu. Rev. Plant Physiol. **36**, 255-286

Edwards, G.E. and Walker, D.A. (1983) $C_3$, $C_4$ : mechanisms, and cellular and environmental regulation, of photosynthesis. Blackwell Scientific Publications, Oxford

Goatly, M. B. and Smith, H. (1974) Differential properties of phosphoenolpyruvate carboxylase from etiolated and green sugar cane. Planta **117**, 67-73

Gonzalez, D.H., Iglesias, A.A. and Andreo, C.S. (1984) On the regulation of phosphoenolpyruvate carboxylase activity from maize leaves by L-malate. Effect of pH. J. Plant Physiol. **116**, 425-434

Gonzalez, D.H. and Andreo, C.S. (1989) The use of substrate analogues to study the active site structure and mechanism of PEP carboxylase. Trends in Biochem. Sci. **14**, 24-27

Guidici-Orticoni, M-T., Vidal, J., Marechal, P., Thomas, M., Gadal, P. and Remy R. (1988) *In vivo* phosphorylation of *sorghum* leaf phosphoenolpyruvate carboxylase. Biochimie **70**, 769-772

Guitton, C. and Mache, R. (1987) Phosphorylation *in vitro* of the large subunit of the ribulose-1,5-bis phosphate carboxylase and of the glyceraldehyde-3-phosphate dehydrogenase. Eur. J. Biochem. **166**, 249-254

Hague, D.R. and Sims, T.L. (1980) Evidence for light-stimulated synthesis of phosphoenolpyruvate carboxylase in leaves of maize. Plant Physiol. **66**, 505-509

Harpster, M.H. and Taylor, W.C. (1986) Maize phosphoenolpyruvate carboxylase : cloning and characterization of mRNAs encoding isozymic forms. J. Biol. Chem. **261**, 6132-6136

Hatch, M.D.(1977) $C_4$ pathway photosynthesis : mechanism and physiological function. Trends in Biochem. Sci. **2**, 199-202

Hatch, M.D. and Heldt, H.W. (1984) Synthesis, storage and stability of [4-$^{14}$C] oxaloacetic acid. Anal. Biochem. **145**, 393-395

Hatch, M.D. and Osmond, C.B. (1976) Compartmentation and transport in $C_4$ photosynthesis. In : Encyclopedia of Plant Physiol., New series, **3**, 144-184, Stocking, C.R. and Huber, U., ed., Springer-Verlag, Berlin

Heldt, H.W. (1979) Light-dependent changes of stromal $H^+$ and $Mg^{2+}$ concentrations controlling $CO_2$ fixation. In : Encyclopedia of Plant Physiol., New series, **6**, 202-207, Gibbs, M. and Latzko, E., ed., Springer-Verlag, Berlin

Huber, S.C. and Edwards, G.E. (1975) Inhibition of phosphoenolpyruvate carboxylase from $C_4$ plants by malate and aspartate. Can. J. Bot. **53**, 1925-1933

Huber, J.L.A., Huber, S.C. and Neilsen, T.H. (1989) Protein phosphorylation as a mechanism for the regulation of spinach leaf sucrose phosphate synthase. Arch. Biochem. Biophys. **270**, 681-689

Huber, S.C. and Sugiyama, T. (1986) Changes in sensitivity to effectors of maize leaf phosphoenolpyruvate carboxylase during light/dark transitions. Plant Physiol. **81**, 674-677

Huber, S.C., Sugiyama, T. and Akazawa, T. (1986) Light modulation of maize leaf phosphoenolpyruvate carboxylase. Plant Physiol. **82**, 550-554

Hudspeth, R.L., Glackin, C.A., Bonner, J. and Grula, J.W. (1986) Genomic and cDNA clones for maize phosphoenolpyruvate carboxylase and pyruvate, orthophosphate dikinase : expression of different gene-family members in leaves and roots. Proc. Nat. Acad. Sci. USA. **83**, 2884-2888

Hudspeth, R.L. and Grula, J.W. (1989) Structure and expression of the maize gene encoding the phosphoenolpyruvate carboxylase isozyme involved in $C_4$ photosynthesis. Plant Mol. Biol. **12**, 579-589

Iglesias, A.A. and Andreo, C.S. (1989) Purification of NADP-malic enzyme and phosphoenolpyruvate carboxylase from sugar cane leaves. Plant Cell Physiol. **30**, 399-405

Iglesias, A.A. and Andreo, C.S. (1984) On the molecular mechanism of maize phosphoenolpyruvate carboxylase activation by thiol compounds. Plant Physiol. **75**, 983-987

Iglesias, A.A. and Andreo, C.S. (1983) The presence of essential histidine residues in phosphoenolpyruvate carboxylase from maize leaves. Biochim. et Biophys. Acta, **749**, 9-17

Ishijima, S., Katagiri, F., Kodaki, T., Izui, K., Katsuki, H., Nishikawa, K., Nakishima, H. and Ooi, T. (1985) Comparison of amino acid sequences between phosphoenolpyruvate carboxylases from *Escherichia coli* (allosteric) and *Anacystis nidulans* (non-allosteric) : identification of conserved and variable regions. Biochem. Biophs. Res. Commun. 133, 436-441

Izawa, S. (1982) Inhibitors of electron transport. In : Encyclopedia of Plant Physiol., New series, **5**, 266-282, Trebst, A. and Avron, M., ed., Springer-Verlag, Berlin

Izui, K., Ishijima, S., Yamaguchi, Y., Katagiri, F., Murata, T., Shigesada, K., Sugiyama T. and Katsuki, H. (1986) Cloning and sequencing analysis of cDNA encoding active phosphoenolpyruvate carboxylase of the $C_4$-pathway from maize. Nuc. Ac. Res. **14**, 1615-1628

Jenkins, G.I. (1988) Photoregulation of gene expression in plants. Photochem. Photobiol. **48**, 821-832

Jiao, J.-A. and Chollet, R. (1989) Regulatory seryl-phosphorylation of $C_4$ phosphoenolpyruvate carboxylase by a soluble protein kinase from maize leaves. Arch. Biochem. Biophys. **269**, 526-535

Jiao, J.-A. and Chollet, R. (1988) Light/dark regulation of maize leaf phosphoenolpyruvate carboxylase by *in vivo* phosphorylation. Arch. Biochem. Biophys. **261**, 409-417

Jiao, J.-A. and Shi, J.-N. (1987) Studies on the symmetry of quaternary structure of phosphoenolpyruvate carboxylase from sorghum leaves. Sci. Sin. **30**, 1060-1069

Johnson, H.S. and Hatch, M.D. (1970) Properties and regulation of leaf nicotinamide-adenine dinucleotide phosphate-malate dehydrogenase and 'malic' enzyme in plants with the $C_4$-dicarboxylic acid pathway of photosynthesis. Biochem. J. **119**, 273-280

Jones, R., Wilkins, M.B., Coggins, J.R., Fewson C.A. and Malcolm, A.D.B. (1978) Phosphoenolpyruvate carboxylase from the Crassulacean plant *Bryophyllum fedtschenkoi* Hamet *et* Perrier. Purification, molecular and kinetic properties. Biochem. J . **175**, 391-406

Karabourniotis, G., Manetas Y. and Gavalas, N.A. (1985) Detecting photoactivation of phosphoenolpyruvate carboxylase in $C_4$ plants. An effect of pH. Plant Physiol. **77**, 300-302

Karabourniotis, G., Manetas Y. and Gavalas, N.A. (1983) Photoregulation of phosphoenolpyruvate carboxylase in *Salsola soda* L. and other $C_4$ plants. Plant Physiol. **73**, 735-739

Kluge, M. (1979) The flow of carbon in crassulacean metabolism. In : Encyclopedia of Plant Physiol., New series, **6**, 113-125, Gibbs, M. and Latzko, E., ed., Springer-Verlag, Berlin

Kluge, M., Maier, P., Brulfert, J., Faist, K. and Wollny, E. (1988) Regulation of phosphoenolpyruvate carboxylase in Crassulacean acid metabolism : *in vitro* phosphorylation of the enzyme. J. Plant Physiol. **133**, 252-256

Kluge, M. and Ting, I.P. (1978) Crassulacean acid metabolism. Analysis of an ecological adaptation. Springer-Verlag, Berlin

Krebs, E.G. (1973) The mechanism of hormonal regulation by cyclic AMP. In : Endocrinology, Proceedings of the 4th International Congress. 17-29, Excerpta Medica, Amsterdam

Kruger, I. and Kluge, M. (1988) Diurnal changes in the regulatory properties of phosphoenolpyruvate carboxylase in CAM plants : are alterations in the quaternary structure involved? Botanica Acta **101**, 32-35

Ku, M.S.B. and Edwards, G.E. (1978) Oxygen inhibition of photosynthesis. III. Temperature dependence of quantum yield and its relation to $O_2/CO_2$ solubility ratio. Planta **140**, 1-6

Kurkdjian, A. and Guern, J. (1989) Intracellular pH : measurement and importance in cell activity. Annu. Rev. Plant Physiol. and Plant Mol. Biol. **40**, 271-303

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227, 680-685

Latzko, E. and Kelly, G.J. (1983) The many-faceted function of phosphoenolpyruvate carboxylase in $C_3$ plants. Physiol. Veg. **21**, 805-815

Leegood, R.C., Walker, D.A. and Foyer, C.H. (1985) Regulation of the Benson-Calvin cycle. In : Photosynthetic mechanisms and the environment. 191-258, Barber, J. and Baker, N., ed., Elsevier Biomedical Publications, Amsterdam

Leegood, R.C. and Furbank, R.T. (1984) Carbon metabolism and gas exchange in leaves of *Zea mays* L. Changes in $CO_2$ fixation, chlorophyll a fluorescence and metabolite levels during photosynthetic induction. Planta **162**, 450-456

Lorimer, G.H. and Andrews, T.J. (1981) The $C_2$ chemo- and photorespiratory carbon oxidation cycle. In : The Biochemistry of Plants. A comprehensive treatise, **8**, 329-374, Hatch, M.D. and Boardman, N.K., ed., Academic Press, New York

MacKintosh, C. and Cohen, P. (1989) Identification of high levels of type 1 and type 2A protein phosphatases in higher plants. Biochem. J. **262**, 335-339

McNaughton, G.A.L., Fewson, C.A., Wilkins M.B. and Nimmo, H.G. (1989) Purification, oligomerization state and malate sensitivity of maize leaf phosphoenolpyruvate carboxylase. Biochem. J. **261**, 349-355

Manetas, Y. and Gavalas, N.A. (1982) Evidence for essential sulfhydryl group(s) in photosynthetic phosphoenolpyruvate carboxylase : protection by substrate, metal-substrate and glucose-6-phosphate against p-chloromercuribenzoate inhibition. Photosynthetica **16**, 59-66

Manetas, Y., Karabouniotis, G. and Gavalas, N.A. (1983) Post-translational regulation of $C_4$ and CAM phosphoenolpyruvate carboxylase. Physiol. Veg. **21**, 911-917

Manetas, Y., Petropoulou, Y. and Karabourniotis, G. (1986) Compatible solutes and their effects on phosphoenolpyruvate carboxylase of $C_4$-halophytes. Plant, Cell, Environ. **9**, 145-151

Mares, J., Barthova, J. and Leblova, S. (1979) Purification and properties of phosphoenolpyruvate carboxylase from green leaves of maize. Collect Czech. Chem. Commun. **44**, 1835-1840

Mares, J. and Leblova, S. (1980) Phosphoenolpyruvate carboxylase from leaves of maize, sorghum and millet. Photosynthetica **14**, 25-31

Martiny-Baron, G. and Scherer F.E. (1989) Phospholipid-stimulated protein kinase in plants. J. Biol. Chem. **264**, 18052-18059

Matsuoka, M. and Hata, M. (1987) Comparative studies of phosphoenolpyruvate carboxylase from $C_3$ and $C_4$ plants. Plant Physiol. **85**, 947-951

Matsuoka, M. and Minami, E. (1989) Complete structure of the gene for phosphoenolpyruvate carboxylase from maize. Eur. J. Biochem. **181**, 593-598

Miller, S.S., Boylan K.L.M. and Vance, C.P. (1987) Alfalfa Root nodule carbon dioxide fixation III. Immunological studies of nodule phosphoenolpyruvate carboxylase. Plant Physiol. **84**, 501-508

Mukerji, S.K. (1977) Corn leaf phosphoenolpyruvate carboxylases, purification and properties of two isoenzymes. Arch. Biochem. Biophys. **182**, 343-351

Muller, D., Kluge, M. and Groschel-Stewart, U. (1980) Immunological studies on phosphoenolpyruvate carboxylase in *Sedum* species with crassulacean acid metabolism or $C_3$ photosynthesis. Plant, Cell, Environ. **3**, 249-253

Muto, S. and Shimogawara, K. (1985) Calcium- and phospholipid-dependent phosphorylation of ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit by a chloroplast envelope-bound protein kinase *in situ*. FEBS Lett. **193**, 88-92

Nguyen, T.T., Ngam-Ek, A., Jenkins, J. and Grover, S.D. (1988) Metal ion interactions with phosphoenolpyruvate carboxylase from *Crassula argentea* and *Zea mays*. Plant Physiol. **86**, 104-107

Nimmo, G.A., McNaughton, G.A.L., Fewson, C.A., Wilkins M.B. and Nimmo, H.G. (1987) Changes in the kinetic properties and phosphorylation state of phosphoenolpyruvate carboxylase in *Zea mays* leaves in response to light and dark. FEBS Lett. **213**, 18-22

Nimmo, G.A. and Nimmo, H.G. (1982) A general method for the localization of enzymes that produce phosphate, pyrophosphate, or $CO_2$ after polyacrylamide gel electrophoresis. Anal. Biochem. **121**, 17-22

Nimmo, G.A., Nimmo, H.G., Fewson C.A. and Wilkins, M.B. (1984) Diurnal changes in the properties of phosphoenolpyruvate carboxylase in *Bryophyllum* leaves : a possible covalent modification. FEBS Lett. **178**, 199-203

Nimmo, G.A., Nimmo, H.G., Hamilton, I.D., Fewson C.A. and Wilkins, M.B. (1986) Purification of the phosphorylated night form and dephosphorylated day form of phosphoenolpyruvate carboxylase from *Bryophyllum fedtschenkoi*. Biochem. J. **239**, 213-220

Nimmo, H.G. and Cohen, P. (1977) Hormonal control of protein phosphorylation. In : Advances in Cyclic Nucleotide Research **8**, 146-266, ed. P. Greengard and G.A. Robinson, Raven Press, New York

Nishikido, T. and Takanashi, H. (1973) Glycine activation of phosphoenolpyruvate carboxylase from monocotyledonous $C_4$ plants. Biochem. Biophys. Res. Commun. **53**, 126-133

Nott, D.L. and Osmond, C.B. (1982) Purification and properties of phosphoenolpyruvate carboxylase from plants with crassulacean acid metabolism. Aust. J. Plant Physiol. **9**, 409-422

O'Leary, M.H. (1990) Catalytic strategies in enzymatic carboxylation and decarboxylation. In : The Enzymes, **20**, in press

O'Leary, M.H. (1982) Phosphoenolpyruvate carboxylase : an enzymologists view. Ann. Rev. Plant Physiol. **33**, 297-315

O'Leary, M.H., Rife. J.E. and Slater, J.D. (1981) Kinetic and isotope effect studies of maize phosphoenolpyruvate carboxylase. Biochemistry **20**, 7308-7314

Osmond, C.B. and Greenway, H. (1972) Salt responses of carboxylation enzymes from species differing in salt tolerance. Plant Physiol. **49**, 260-263

Osmond, C.B. and Holtum, J.A.M. (1981) Crassulacean acid metabolism. In : The Biochemistry of Plants. A comprehensive treatise, **8**, 283-329, Hatch, M.D. and Boardman, N.K., ed., Academic Press, New York

Osmond, C.B., Winter, K. and Ziegler, H. (1982) Functional significance of different pathways of $CO_2$ fixation in photosynthesis. In : Encyclopedia of Plant Physiol., New series, **12B**, 453-479, Lange, O.J., Nobel, J.S., Osmond, C.B. and Ziegler, H., ed., Springer-Verlag, Berlin

Plaxton, W.C. and Preiss, J. (1987) Purification and properties of non-proteolytic degraded ADPglucose pyrophosphorylase from maize endosperm. Plant Physiol. **83**, 105-112

Podesta, F.E. and Andreo, C.S. (1989) Maize leaf phosphoenolpyruvate carboxylase. Oligomeric state and activity in the presence of glycerol. Plant Physiol. **90**, 427-433

Portis, A.R.Jr., Salvucci, M.E. and Ogren, W.L. (1986) Activation of ribulose bisphosphate carboxylase/oxygenase at physiological $CO_2$ and ribulose bisphosphate concentrations by rubisco activase. Plant Physiol. **82**, 967-971

Randall, D.D., Williams, M. and Rapp, B.J. (1981) Phosphorylation- dephosphorylation of the pyruvate dehydrogenase complex from pea leaf mitochondria. Arch. Biochem. Biophys. **207**, 437-444

Ranjeva, R. and Boudet, A.M. (1987) Phosphorylation of proteins in plants : regulatory effects and potential involvement in stimulus/response coupling. Annu. Rev. Plant Physiol. **38**, 73-93

Ray, T.B. and Black, C.C. (1979) The $C_4$ pathwayand its regulation. In : Encyclopedia of Plant Physiol., New series, **6**, 77-101, Gibbs, M. and Latzko, E., ed., Springer-Verlag, Berlin

Rickers, J., Cushman, J.C., Michalowski, C.B., Schmitt, J.M. and Bohnert, H.J. (1989) Expression of the CAM-form of phospho(enol)pyruvate carboxylase and nucleotide sequence of a full length cDNA from *Mesembryanthemum crystallinum*. Mol. Gen. Genet. **215**, 447-454

Robinson, S.P. and Walker, D.A. (1981) Photosynthetic carbon reduction cycle. In : The Biochemistry of Plants. A comprehensive treatise, **8**, 194-237, Hatch, M.D. and Boardman, N.K., ed., Academic Press, New York

Rodrigues-Sotres, R., Lopez-Pozos R. and Munoz-Clares, R.A. (1987) Further studies of the short-term regulation of maize leaf phosphoenolpyruvate carboxylase by light. J. Plant Physiol. **129**, 191-199

Russell, D.W., Knight, J.S. and Wilson, T.M. (1985) Pea seedling HMG-CoA reductases : regulation of activity *in vitro* by phosphorylation and $Ca^{2+}$, and posttranslational control *in vivo* by phytochrome and isoprenoid hormones. Curr. Top. Plant Biochem. Physiol. **4**, 191-206

Samaras, Y., Manetas, Y. and Gavalas, N.A. (1988) Effects of temperature and photosynthetic inhibitors on light activation of $C_4$-phosphoenolpyruvate carboxylase. Photosynth. Res. **16**, 233-242

Schacter, E., Chock, P.B. and Stadtman, E.R. (1984) Regulation through phosphorylation/dephosphorylation cascade systems. J. Biol. Chem. **259**, 12252-12259

Schnarrenberger, C and Herbert, M. (1983) Intracellular compartmentation of phosphoenolpyruvate carboxylase in green plant leaves. Physiol. Veg. **21**, 1047-1054

Selinioti, E., Karabourniotis, G., Manetas, Y. and Gavalas, N.A. (1985) Modulation of phosphoenolpyruvate carboxylase by 3-phosphoglycerate : probable physiological significance for $C_4$-photosynthesis. J. Plant Physiol. **121**, 353-360

Selinioti, E., Manetas Y. and Gavalas, N.A. (1986) Cooperative effects of light and temperature on the activity of phosphoenolpyruvate carboxylase from *Amaranthus paniculatus* L. Plant Physiol. **82**, 518-522

Selinioti, E., Nikolopoulos D. and Manetas, Y. (1987) Organic cosolutes as stabilisers of phosphoenolpyruvate carboxylase in storage : an interpretation of their action. Aust. J. Plant Physiol. **14**, 203-210

Shomer-Ilan, A., Moualem-Beno, D. and Waisel, Y. (1985) Effects of NaCl on the properties of phosphoenolpyruvate carboxylase from *Suaeda monica* and *Chloris gayana*. Physiol. Plant. **65**, 72-78

Slack, C.R. (1968) The photoactivation of a phosphopyruvate synthase in leaves of *Amaranthus palmeri*. Biochem. Biophys. Res. Commun. **30**, 483-488

Stadtman, E.R. (1970) Mechanisms of enzyme regulation in metabolism. In : The Enzymes, **1**, 398-459, Boyer, P.B. ed., Academic Press, New York

Stiborova, M. and Leblova, S. (1986) Structure of phosphoenolpyruvate carboxylase from maize leaves. FEBS Lett. **205**, 32-34

Stiborova, M. and Leblova, S. (1985) Activation of maize (*Zea mays* L.) phosphoenolpyruvate carboxylase by glucose-6-phosphate and glycine : effects of pH and $Mg^{2+}$. Photosynthetica **19**, 177-182

Stiborova, M. and Leblova, S. (1983a) Isolation and partial characterisation of two phosphoenolpyruvate carboxylases from maize (*Zea mays* L.). Photosynthetica **17**, 379-385

Stiborova, M. and Leblova, S. (1983b) The role of cystein SH groups in the phosphoenolpyruvate carboxylase molecule of maize. Physiol. Veg. **21**, 935-942

Stitt, M. and Heldt, H.W. (1985) Generation and maintenance of concentration gradients between the mesophyll and bundle sheath in maize ieaves. Biochim. et Biophys. Acta **808**, 400-414

Terada, K., Kai, T., Okuno, S., Fujisawa, H. and Izui, K. (1990) Maize leaf phosphoenolpyruvate carboxylase : phosphorylation of $ser^{15}$ with a mammalian cyclic AMP-dependent protein kinase diminishes the sensitivity to inhibition by malate. FEBS Lett. **259**, 241-244

Ting, I.P. (1985) Crassulacean acid metabolism. Annu. Rev. plant Physiol. **36**, 595-622

Ting, I.P. and Osmond, C.B. (1973a) Photosynthetic phosphoenolpyruvate carboxylases. Characteristics of allozymes from leaves of $C_3$ and $C_4$ plants. Plant Physiol. **51**, 439-447

Ting, I.P. and Osmond, C.B. (1973b) Multiple forms of plant phosphoenolpyruvate carboxylase associated with different metabolic pathways. Plant Physiol. **51**, 448-453

Thomas, M., Cretin, C., Keryer, E., Vidal, J. and Gadal, P. (1987) Photoregulation process of *sorghum* leaf phosphoenolpyruvate carboxylase : study with monoclonal antibodies. Biochem. Biophys. Res. Commun. **143**, 170-177

Tolbert, N.E. (1980) Photorespiration. In : The Biochemistry of Plants. A comprehensive treatise. **2**, 487-523, Davies, D.D. ed., Academic Press, New York

Trautschold, I., Lamprecht, W. and Schweitzer, G. (1983) UV-method with hexokinase and glucose-6-phosphate dehydrogenase for adenosine 5'-triphosphate determination. In : Methods of Enzymatic Analysis (3rd edition), 346-357, Bergemeyer, H.U. ed., VCH publishers

Uedan, K. and Sugiyama, T. (1976) Purification and characterisation of phosphoenolpyruvate carboxylase from maize leaves. Plant Physiol. **57**, 906-910

Usuda, H., Ku, M.S.B. and Edwards, G.E. (1984) Activation of NADP-malate dehydrogenase, pyruvate, $P_i$ dikinase and fructose-1,6-bisphosphatase in relation to photosynthetic rate in maize. Plant Physiol. **76**, 238-243

Wagner, R., Gonzalez, D.H., Podesta F.E. and Andreo, C.S. (1987) Changes in the quaternary structure of phosphoenolpyruvate carboxylase induced by ionic strength affect its catalytic activity. Eur. J. Biochem. **164**, 661-666

Walker, G.H. and Huber, S.C. (1987) Spinach leaf 6-phosphofructo-2-kinase. Isolation of a new enzyme form that undergoes ATP-dependent modification. FEBS Lett. **213**, 375-380

Walker, G.H., Ku, M.S.B. and Edwards, G.E. (1986a) Activity of maize leaf phosphoenolpyruvate carboxylase in relation to tautomerization and nonenzymatic decarboxylation of oxaloacetate. Arch. Biochem. Biophys. **248**, 489-501

Walker, G.H., Ku, M.S.B. and Edwards, G.E. (1986b) Catalytic activity of maize leaf phosphoenolpyruvate carboxylase in relation to oligomerisation. Plant Physiol. **80**, 848-855

Walker, G.H., Ku, M.S.B. and Edwards, G.E. (1986c) Purification of phosphoenolpyruvate carboxylase and characterization of changes in oligomerization using HPLC. J. Liq. Chromatogr. **9**, 861-874

Warren, D.M. and Wilkins, M.B. (1961) An endogenous rhythm in the rate of dark-fixation of carbon dioxide in leaves of *Bryophyllum fedtscenkoi.* Nature (London), **191**, 686-688

Wedding, R.T. and Black, M.K. (1987) Oligomerisation and the sensitivity of phosphoenolpyruvate carboxylase to inactivation by proteinases. Plant Physiol. **84**, 979-981

Wedding, R.T. and Black, M.K. (1986) Malate inhibition of phosphoenolpyruvate carboxylase from *Crassula.* Plant Physiol. **82**, 985-990

Williams, P.A. (1985) Enzpack, Elsevier-BIOSOFT, Cambridge

Willmer, C.M. (1983) phosphoenolpyruvate carboxylase activity and stomata operation. Physiol. Veg. **21**, 943-953

Winter, K. (1981) Change in properties of phosphoenolpyruvate carboxylase from the crassulacean acid metabolism plant *Mesembryanthemum crystallinum* after isolation. Aust. J. Plant Physiol. **8**, 115-119

Wray, W., Boulakis, T., Wray, V.P. and Hancock, R. (1981) Silver staining proteins in polyacrylamide gels. Anal. Biochem. **118**, 197-203

Wu, M.-X. and Wedding, R.T. (1987) Temperature effects on phosphoenolpyruvate carboxylase from a CAM and a $C_4$ plant. A comparative study. Plant Physiol. **85**, 497-501

Wu, M.-X. and Wedding, R.T. (1987) Regulation of phosphoenolpyruvate carboxylase from *Crassula argentea.* Further evidence on the dimer-tetramer interconversion. Plant Physiol. **84**, 1080-1083

Wu, M.-X. and Wedding, R.T. (1985a) Regulation of phosphoenolpyruvate carboxylase from *Crassula* by interconversion of oligomeric forms. Arch. Biochem. Biophys. **240**, 655-662

Wu, M.-X. and Wedding, R.T. (1985b) Diurnal regulation of phosphoenolpyruvate carboxylase from *Crassula.* Plant Physiol . **77**, 667-675

Yanagisawa, S., Izui, K., Yamaguchi,Y., Shigesada, K. and Katsui, H. (1988) Further analysis of cDNA clones for maize phosphoenolpyruvate carboxylase involved in $C_4$ photosynthesis. Nucleotide sequence of entire open reading frame and evidence for polyadenylation of mRNA at multiple sites *in vivo* . FEBS Lett. **229**, 107-110

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