

EVIDENCE FOR ALLOSTERIC INHIBITION OF RIBULOSE-1,5-BISPHOSPHATE
CARBOXYLASE/OXYGENASE BY PHOSPHOMETABOLITES,

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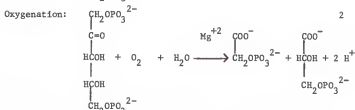
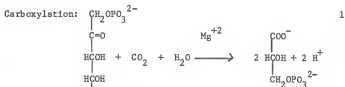
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Introduction

The most abundant protein on this earth is thought to be the enzyme, Ribulose-1,5-bisphosphate carboxylase/oxygenase¹ (EC. 4.1.1.39), a major constituent of all photosynthetic organisms (Kung, 1976). RuBPCase catalyzes the carboxylation and cleavage of ribulose-1,5-bisphosphate in the Calvin cycle to form two molecules of 3-phospho-D-glyceric acid which in turn are converted into sugars the organism needs for further growth (Quayle et al., 1954; Weisbach et al., 1954; Racker, 1955; Jacoby et al., 1956). RuBPCase is also known to have a competitive oxygenase activity which converts RuBP and molecular oxygen to one molecule of phosphoglycolate and one molecule of 3-phospho-D-glyceric acid (Bowes et al., 1971; Andrews et al., 1973; Ryan and Tolbert, 1975). Carboxylase activity is maximized under high NaHCO_3 and low oxygen concentrations while the converse is true for the

¹Abbreviations: Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCase); D-ribulose-1,5-bisphosphate (RuBP); 3-phospho-D-glyceric acid (3-PGA); phosphoglycolate (PG); inorganic phosphate (P_i); fructose-1,6-bisphosphate (F-1,6-BP); dithiothreitol (DTT); adenosine-5'-triphosphate (ATP); adenosine-5'diphosphate (ADP); ribose-5-phosphate (R-5-P); fructose-6-phosphate (F-6-P); 2-carboxy-hexitol-1,6-bisphosphate (CHBP); dihydroxyacetone phosphate (DHAP); 1,4-Bis [2-(5-phenyloxazoly)] benzene (POPOP); 2,5-diphenyloxzole (PPO); phenylmethylsulfonyl fluoride (PMSF); sodium dodecyl sulfate (SDS); polyacrylamide gel electrophoresis (PAGE); polyvinylpyrrolidone (PVP); 6-phosphogluconic acid (6-PGA); ultra violet (UV).

the oxygenase activity. Thus, most investigators agree that both reactions occur at the same active site (Badger and Andrews, 1974; Bahr and Jensen, 1974).



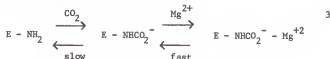
RuBPCase has been isolated from many sources including comfrey (Simpson et al., 1983). In most cases, RuBPCase of higher plant forms consists of eight large subunits ($\text{Mr}=53 \times 10^3$) and eight small subunits ($\text{Mr}=12$ to 14×10^3) (Baker et al., 1975 and 1977; McFadden and Purohit, 1978; Gray et al., 1980). In eukaryotes, the large subunit is encoded by the chloroplast DNA (Smille et al., 1967; Kawashima, 1970; Criddle et al., 1970; Chan and Wildman, 1972; Blair and Ellis, 1973) and synthesized with help of chloroplast ribosomes. The small subunit on the other hand is encoded by nuclear DNA (Kawashima and Wildman, 1972) and synthesized with the help of cytoplasmic ribosomes as a precursor protein of $\text{Mr}=20 \times 10^3$ (Dobberstein et al., 1977; Highfield and Ellis, 1979) which is processed to its mature size of $\text{Mr}=12-14 \times 10^3$ and crosses the chloroplastic membrane (Chua and Schmidt, 1978; Smith and Ellis, 1979).

Present evidence has indicated that the large subunit is the product of a single gene (Bedbrook et al., 1979), whereas the small subunit is encoded by a multigene family (Berry-Lowe et al.; Smith et al., 1983). This is thought to occur because the large subunit contains both the catalytic site and activation site of RuBPCase in contrast to the small subunit having few functional and structural constraints known. However, the function of the small subunit appears to be regulatory (Andrews and Abel, 1981; Andrews and Ballment, 1983).

In all higher plant forms studied thus far, the large and small subunits of RuBPCase have appeared to be heterogeneous. This heterogeneity has generally been shown with isoelectric focusing experiments of S-carboxymethylated subunits in 8 or 9 M urea solutions. The modified large subunits in tobacco and wheat gave three bands of unequal intensity on isoelectric focusing gels while the small subunit of the tobacco enzyme gave two bands of equal intensity (Gray et al., 1974; Chen et al., 1975). Simpson and Mueller (unpublished results) demonstrated similar types of heterogeneity for comfrey RuBPCase large and small subunits with isoelectric focusing in 9 M urea and SDS PAGE of the focused gels without carboxymethylation. At least four bands of unequal strength were seen for the large subunit while the small subunit showed two separate bands. The number of bands for both the large and small subunits of comfrey RuBPCase did not change when PSMF was present during isolation suggesting that heterogeneity in the subunits was not due to partial degradation by serine proteases.

Activation by CO_2 in the presence of a divalent metal ion is an absolute requirement for activity and occurs through the reaction of CO_2 with a lysine residue on the large subunit (Lys-201) to form a carbamate

(Lorimer et al., 1976; Lorimer and Mizioro, 1980). The rate-limiting step is the formation of the carbamate and it is stabilized by the rapid interaction with the divalent metal cofactor. The CO_2 molecule needed for activation is not the same CO_2 molecule that is fixed to RuBP during catalysis (Lorimer, 1979; Mizioro, 1979).



For a long time, there have been many questions raised over the exact control mechanisms of RuBPCase activation and catalysis in vivo. In 1981, Lorimer's group and Tolbert's group published papers addressing the question of how high enzyme activity could be attained in the plant at physiological concentrations of CO_2 and Mg^{+2} since in vitro studies showed the amount of CO_2 and Mg^{+2} required for full activation of RuBPCase was much higher than that present at physiological levels. Only 18-20% of the total enzyme present in the plant would be activated by CO_2 at physiological conditions (McCurry et al., 1981) in the absence of additional activation mechanisms. In this regard, it is well known that a number of phosphometabolites (notably 6-PGA, 3-PGA and F-1,6-BP) are activators of the carboxylase and oxygenase activities of RuBPCases (Buchanan and Schurmann, 1973; Chu and Bassham, 1973 and 1975; Lawlis et al., 1979; Hatch and Jensen, 1980) when preincubated with the enzyme and Mg^{+2} at suboptimal levels of CO_2 . Badger and Lorimer (1981) and McCurry et al. (1981) suggest that with spinach RuBPCase, certain of these sugar phosphates, when preincubated in vitro with RuBPCase under suboptimal conditions of activation (1 mM NaHCO_3), provide stabilization for the enzyme- CO_2 - Mg^{+2} activated complex. The same authors suggest

that activation results only through active site binding. It has recently been shown that positive effectors of activation shift the equilibrium of the activation reaction toward the carbamate formation by decreasing the rate of dissociation (Jordan et al., 1983). Other metabolites, however, are negative effectors of activation which produce an increased rate of carbamate dissociation (Jordan et al., 1983). Enhancement of CO_2 activation through active site binding thus can explain why the same effectors always inhibit activity if the effector is added simultaneously or immediately after the addition of RuBP to the CO_2 -activated enzyme. In other words, all the known effectors are also competitive inhibitors with respect to the substrate, RuBP (Badger and Lorimer, 1981).

The recent evidence suggesting an allosteric binding site by difference UV methods on comfrey RuBPCase and its apparent absence from lack of detection on the spinach enzyme for the same effectors (Bolden and Mueller, 1983; Bolden, 1983) has raised several important questions in regard to the nature of the effector activation and inhibition of higher plant RuBPCases. First, what is the function, if any, of the nonactive site binding and how does it correlate with the competitive inhibition reported previously? Secondly, is this site unique to comfrey RuBPCase or is the site spectroscopically silent in the spinach enzyme? Since analysis of the difference UV data indicated that filling of the active site enhanced affinity at the allosteric site substantially, the experiments designed to test the function of the second site would best be done when the active site is saturated. Therefore, it was decided initially to investigate the effect of various levels of several effectors on the carboxylase activity at saturating

concentrations of the substrate, ribulose-1,5-bisphosphate. This thesis describes the results of those studies along with some further characterization of the comfrey enzyme and refinements in techniques.

Experimental

Isolation and Purification of Ribulose-1,5-bisphosphate

Carboxylase/Oxygenase

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCase) was purified from comfrey according to Simpson et al. (1983). Fresh leaves were homogenized in TBMESP² buffer, pH 7.5, cellular debris removed, brought to approximately 40% saturation with ammonium sulfate, and spun 20 hours on a 0.2 to 0.8 M TBME-DTT^a sucrose gradient at 27,000 rpm in a swinging bucket preparative ultracentrifuge at 4°C. Leupeptin (10 µM) was added to the homogenization buffers and the sucrose solutions. Fractions from the sucrose gradients were collected on ice and the protein position was determined by A₂₈₀ using a Cary-14 spectrophotometer and concentration from $\epsilon(280)=1.7 \text{ ml mg}^{-1} \text{ cm}^{-1}$ for comfrey RuBPCase (Bolden, 1983). Once pooled, the protein was purged with N₂ and stored at -70°C in plastic containers (typically 2 ml in a 5 ml stoppered test tube).

² Buffers: TBMESP is 50 mM Tris-HCl, 50 mM NaHCO₃, 10 mM MgCl₂, 0.1 mM EDTA, 5 mM sodium dithionite, and 0.1 mM PMSF; TBME-DTT^a is 50 mM Tris-HCl, 50 mM NaHCO₃, 10 mM MgCl₂, 0.1 mM EDTA, 2 mM DTT; TME-DTT is 50 mM Tris-HCl, 10 mM MgCl₂, 1 mM EDTA, 2 mM DTT; TBME-DTT^b is 50 mM Tris-HCl, 10 mM MgCl₂, 1 mM EDTA, 18-20.6 mM NaHCO₃/1-2 mM NaH¹⁴CO₃, 2 mM DTT; TB'ME-DTT is 50 mM Tris-HCl, 1 mM NaH¹⁴CO₃, 10 mM MgCl₂, 1 mM EDTA, 2 mM DTT; T'ME-DTT is 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM EDTA, 2 mM DTT; TE is 50 mM Tris-HCl, 1 mM EDTA, 2 mM DTT.

Spinach RuBPCase was obtained from the Sigma Chemical Company (St. Louis, Mo.) as a partially purified product and further purified by the comfrey procedure starting with sucrose density centrifugation and stored in the same manner. Protein position was determined by A_{280} , and concentration from $\left((280) = 1.6 \text{ ml mg}^{-1} \text{ cm}^{-1} \right)$ for spinach RuBPCase.

Both spinach and comfrey RuBPCases were further purified for use on a 1.5×3.5 cm DEAE-cellulose column. After washing with the 2M NaCl to rid the DEAE-cellulose of sodium azide, it was equilibrated with TME-DTT buffer, pH 7.5. The RuBPCase from -70°C storage was heat-activated for 10 min at 45°C , cooled for 10 min, and the solution applied twice to the column (Simpson *et al.*, 1983). After washing out the sucrose with the column buffer, the enzyme was eluted with 0.15 M NaCl TME-DTT, pH 7.5. A Gilson microfractionator was used to collect 0.8 ml fractions. Protein concentration was determined by A_{280} as before.

Preparation and Purification of D-Ribulose-1,5-bisphosphate

RuBP, the substrate, was synthesized as a barium salt according to the method of Horecker *et al.* (1958) using R-5-P, phosphoriboisomerase, phosphoribokinase, and a slight excess of ATP. Both phosphoriboisomerase and phosphoribokinase were thoroughly tested to determine accurate activities at 25°C before use (Ashwell and Hickman, 1956; Horecker *et al.* 1958). The barium salt of RuBP obtained by isopropanol precipitation was dried and stored at -70°C until needed.

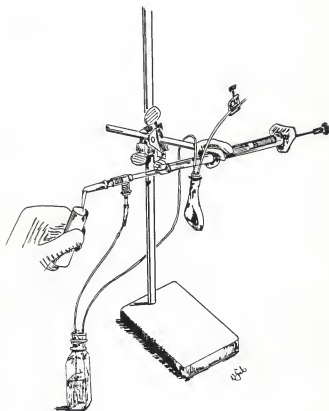
To further purify the barium salt, it was dissolved in 0.1 N HCl, applied to a Dowex-1 8x (400 mesh) column (2.5×43 cm) in the H^+ form. Following a 500 ml, 100 mM LiCl wash, it was eluted with a two liter 100 to 250 mM assumed linear LiCl gradient in 1 mM HCl (Kuehn and Hsu,

1978). Fifteen ml fractions were collected. Fractions were initially analyzed with an orcinol reagent (2 g orcinol, 0.2 g ferric chloride, 1 liter concentrated HCl, 1 ml of reagent to 0.5 ml of sample) to locate the Li^+ salt of RuBP, followed by analysis with enzymatic activity. Peak tubes identified as containing RuBP were pooled, lyophilized, and the excess LiCl extracted with a cold 8:1 acetone-methanol mixture in which both the methanol and acetone had been distilled to remove any H_2O .

Standard Enzyme Carboxylase Assay

The standard enzyme assay utilizing fully activated enzyme under saturating substrate conditions was similar to that of Simpson et al. (1983) except that the assay volume was reduced to 300 μl from 553. Standard assays were performed with each experiment to compare specific activities for all samples of enzyme. No effort was made to purge atmospheric O_2 from the enzyme when activation was at 20-22.6 mM NaHCO_3 (18-20.6 mM NaHCO_3 from a 123 mM NaHCO_3 stock, pH 7.5, and 1-2 mM $\text{NaH}^{14}\text{CO}_3$ from a 135 mM $\text{NaH}^{14}\text{CO}_3$ stock, pH 7.5). For studies done at 1 mM $\text{NaH}^{14}\text{CO}_3$, where atmospheric O_2 competes more effectively with CO_2 , both enzyme and buffer were purged with N_2 gas for at least 45 min before addition of any $\text{NaH}^{14}\text{CO}_3$, and maintained under N_2 throughout the experiment using the system diagramed in Fig. 1. The micropipettor system used worked by positive displacement. The micropipettor was attached to the sample reservoir which was essentially free from atmospheric O_2 . The sample reservoir, in turn, was attached to a balloon filled with N_2 gas. As the micropipettor delivered the sample into the reaction vial, which was then immediately sealed, N_2 from the

Figure 1. The positive displacement micropipettor system used for inhibition studies done with N_2 purging of RuBPCase.



balloon replaced the delivered volume so that no atmospheric O_2 could contaminate the rest of the sample pool. All vials used for studies at 1 mM $NaH^{14}CO_3$ were placed in a sealed glove bag and also purged with N_2 gas for 2 to 3 hr. The vials were sealed while still under N_2 and kept sealed until used.

CO_2 activation of the enzyme for all experiments was carried out for at least 45 min in a pool of TBME-DTT^b or TB'ME, pH 7.5. The standard enzyme concentration for all assays was 20 μ g. Reactions were initiated by adding 10 μ l of 39 mM RuBP (1.3 mM final concentration) to 290 μ l of activated enzyme-buffer mixture in a 5 ml plastic scintillation vial. After 90 s, the reaction was quenched with 0.2 ml of glacial acetic acid and the samples dried in a hood by placing the vials in an aluminum block on a hot plate. The temperature was maintained overnight at 75°C and finally for 2 hours the next morning at 90°C. After cooling, each sample was brought up in 0.3 ml of deionized H_2O and 4.5 ml of liquid scintillation cocktail (8 g PPO, 0.2 g POPOP, 2 liters toluene, and 1 liter of Triton X-100). The residual radioactivity was measured on a Beckman Liquid Scintillation Spectrometer using a ^{14}C isoset and a gain setting of 2.5 (Esser, 1982). Heated (background) and unheated (total $NaH^{14}CO_3$ counts available for incorporation) blanks without RuBP were done in duplicate with each pool of assays.

Calculation of specific activity was by the following method:
(CPM=counts of ^{14}C per minute)

Ratio of ^{14}C counts = $\frac{\text{CPM of sample} - \text{CPM of heated blank}}{\text{CPM of cold blank} - \text{CPM of heated blank}}$
incorporated

$\mu\text{moles of } ^{14}CO_2 = \frac{(\text{ratio of } ^{14}CO_2 \text{ fixed})(0.3 \text{ ml})(20 \text{ mM } NaHCO_3)}{\text{fixed/min-mg prot} \quad (0.020 \text{ mg enzyme})(1.5 \text{ minutes reaction time})}$

where 0.3 ml was the total reaction volume and 20 mM NaHCO_3 was the total amount of bicarbonate available.

Initial Velocity Studies

To study the initial velocity of both spinach and comfrey RuBPCases in the absence or presence of certain phosphometabolites, 25 μl of 60 mM 3-PGA-TME, pH 7.5, was added to 265 μl of the fully activated enzyme-buffer assay mixture (22.6 mM NaHCO_3) 1-2 s after 1.3 mM RuBP was added. Reaction times varied from 3 to 180 s. Duplicate and triplicate assays were done for each reaction time. Quenching of the reactions was with 200 μl of glacial acetic acid. Drying and counting the samples were as before. Standard assays were done at the beginning and the end of the initial velocity studies for a measure of the specific activity of the enzyme used.

Inhibition of RuBPCase by Phosphometabolites

To study the inhibition of RuBPCase activity by several phosphometabolites, 25 μl of various concentrations of effectors in assay buffer were added to 265 μl of the activated enzyme-buffer assay mixture 1-2 s after initiation of the reaction by 10 μl of 39 mM RuBP. Activation of the enzyme for these inhibition studies was always at 22.6 mM NaHCO_3 so that when the 25 μl of effector were added to 265 μl of the activated enzyme-buffer mixture, the NaHCO_3 concentration in a total volume of 300 μl would remain at saturating 20 mM NaHCO_3 during the reaction. Stock phosphometabolite solutions were prepared in TME buffer readjusted to pH 7.5 after dissolution. The concentrations of the stock solutions were such that 25 μl of the solution would give the desired

concentration of effector in the final 300 μ l assay volume. Quenching after 90 s, drying and counting the samples were as before. Heated, unheated controls and samples without effectors were obtained for each pool of activated enzyme solutions. Standard assays at the beginning and end of the inhibition studies also were done to assess the stability of the enzyme solution.

Dependence of Effector Inhibition on the State of Activation at Saturating Levels of RuBP

Comfrey RuBPCase, the TME-DTT buffer, and vials were purged either directly or in a glove bag with N_2 for about 45 min before activation. Aliquots of the enzyme and buffer were activated at the various concentrations of $NaHCO_3$ used in the assays. Concentrations of $NaHCO_3$ for activation were made so that upon dilution by effector in the assay, the reaction would be carried out at the desired concentration. The concentrations of $NaHCO_3$ ranged from 1 mM $NaH^{14}CO_3$ to 22.6 mM $NaHCO_3$. Except for the 1 mM $NaH^{14}CO_3$, all enzyme pools contained a combination of 2 mM $NaH^{14}CO_3$ plus enough cold $NaHCO_3$ to make the desired total $NaHCO_3$ concentration. The positive displacement micropipettor discussed earlier was used for all additions of activated enzyme solutions into the reaction vials. Activated enzyme was assayed at each total $NaHCO_3$ concentration in the absence and presence of 2 mM and 5 mM 3-PGA. To initiate the reaction, 10 μ l of 39 mM RuBP were added to 265 μ l of the enzyme-buffer mixture followed by 25 μ l of the effector within 1-2 s. Reactions were stopped after 90 s with acetic acid. Drying and counting were as previously described.

Dependence of Effector Inhibition on Mg^{+2} Concentration

Comfrey RuBPCase (1.12 mg/ml) was eluted from a DEAE-cellulose column with 0.15 M NaCl-TME buffer with DTT, pH 7.5. For CO_2 activation, aliquots of enzyme (20 μ g/tube) were incubated either in a TE, 10 mM $MgCl_2$ buffer, or a TE, 20 mM $MgCl_2$ buffer either containing 1 mM $NaH^{14}CO_3$, or 20-22.6 mM $NaHCO_3$. Before 1 mM $NaH^{14}CO_3$ activation, the enzyme, buffers, and vials used were purged with N_2 for 45 min. A syringe was used to add the 1 mM $NaH^{14}CO_3$ activated enzyme to the reaction vials. After a 45 min incubation period with $NaHCO_3$, 10 μ l of 39 mM RuBP were added to 265 μ l of activated enzyme followed by 25 μ l of either 5 mM or 10 mM 3-PGA. After a 90 s reaction, 200 μ l of glacial acetic acid were added to quench the reaction. Heating and counting were as before. Concentration corrections had to be made for the Mg^{+2} (10 mM) in the substrate, effector, enzyme and bicarbonate solutions for the assays at 20 mM Mg^{+2} . The final concentrations of Mg^{+2} were 16.5 mM for fully activated enzyme and 18 mM for partially activated enzyme at 1 mM $NaH^{14}CO_3$.

Determination of K_I for 3-PGA for Comfrey RuBPCase

A K_I for 3-PGA was determined by measuring the initial rate of fully activated comfrey RuBPCase for 10 s in the presence of various concentrations of RuBP and 3-PGA. To initiate the reaction, 200 μ l of activated enzyme plus TME buffer were added to 50 μ l of RuBP and 50 μ l of pH adjusted 3-PGA solutions which had been thoroughly mixed. Concentrations of RuBP ranged from 5 to 25 μ M and concentrations of 3-PGA were from 0 to 0.75 mM. Dilutions were made of the highest

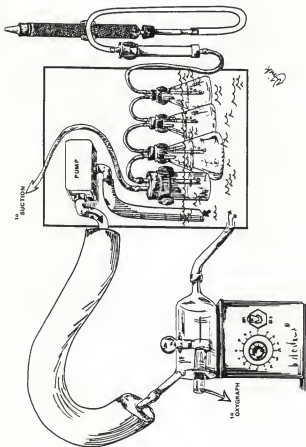
concentration of effector and substrate to get the lower concentrations needed. Standard assays were done throughout the time period of the entire experiment to determine the stability of the enzyme solution. To improve accuracy, 4 mM $\text{NaH}^{14}\text{CO}_3$ was used to increase the number of counts of $^{14}\text{CO}_2$ incorporated by RuBPCase during the short assay time.

Determination of Oxygenase Activity and pH Optimum

Spinach and comfrey RuBPCases were further purified on a DEAE-cellulose column eluted with a T'ME-DTT buffer, pH 8.2. The higher pH (8.2 vs 7.5) was selected after preliminary experiments had shown that the pH optimum was likely to be near that value. In this way the final pH of the assay was changed less upon addition of concentrated enzyme (1 mg/ml) to the buffer in the oxygen electrode chamber. The enzyme was then purged with CO_2 free air (Fig. 2) for 45 min before activation. Activation was initiated by adding an appropriate amount of a 20 mM NaHCO_3 T'ME solution, pH 8.2, to the enzyme pool to give 1 mM NaHCO_3 . TME buffers without DTT were adjusted to pH values between 7.9 and 9.3 and used for the assays. Before use, all buffers were purged with CO_2 free air for at least 30 min. Then, 1 mM NaHCO_3 was added to the buffers (2 mg solid NaHCO_3 /25 ml buffer) and the buffers equilibrated approximately 15 min before being used. Carboxylase assays also were performed on each enzyme pool.

Throughout the entire experiment, the temperature of the enzyme and buffers was kept constant at 25.0°C by the constant temperature water bath seen in Fig. 2. The water from the bath also circulated around the reaction chamber so that the temperature of the buffer and enzyme never changed between activation and reaction. For reaction, atmospheric O_2

Figure 2. The constant temperature water bath and Clark electrode system used in studying oxygenase activity of RuBPCase.

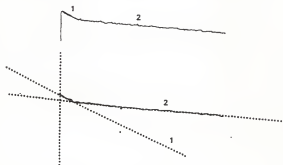


consumption was measured by a Clark electrode. Approximately 1560 μ l of the appropriate buffer and 80 μ l of activated enzyme were mixed in the chamber containing the electrode with a magnetic stirring bar and allowed to equilibrate for 4 min at a constant speed of mixing to establish a stable 100% line before initiating the reaction. During mixing, the chamber was completely filled to the capillary portion of the stopper and the top of the capillary sealed from the atmosphere with parafilm. In all assays, 120 μ g of RuBPCase were used.

To initiate the reaction, 7 μ l of 390 mM RuBP in CO_2 free T'ME without DTT were added to the enzyme-buffer mixture in the chamber with a Pipetman by removing the capillary stopper and immediately replacing it after the addition of RuBP. Oxygen consumption was followed on the 10 mV scale of an Omni-Scribe recorder for 4 min. Immediately following the reaction, the pH of the reaction mixture was taken. Between reactions, the mixing chamber was rinsed three times with T'ME buffer without DTT. This proved to be important to eliminate of any traces of enzyme, substrate, or DTT so that a reproducible stable baseline could be established before the next reaction.

For oxygenase studies at 20 mM NaHCO_3 enzyme activation, the buffers were purged as before but no NaHCO_3 was added. Activation of the enzyme at 20 mM NaHCO_3 was accomplished by adding the appropriate amount of a 400 mM NaHCO_3 T'ME solution, pH 8.2, to the enzyme aliquot. In addition, the enzyme concentration was adjusted so that the addition of 120 μ g gave a 1 mM NaHCO_3 final solution in the oxygen electrode chamber. Everything else followed the same procedure as the 1 mM NaHCO_3 activation studies. In most cases, carboxylase reactions were run along with the oxygenase reactions.

Oxygenase activity was calculated as follows: the rate of O_2 depletion of the solution with just the enzyme plus buffer, the 100% baseline (slope 1), was subtracted from the rate of O_2 depletion of the solution during the reaction of RuBPCase (slope 2) which equalled the rate of O_2 depletion of the solution by the RuBPCase reaction alone per min (decrease in the number of divisions per unit time from the 100% baseline). The time scale (abscissa) was 0.25 cm/min for slope 1 and 2 cm/min for slope 2.



slope 2 - slope 1 = rate of O_2 depletion/min

$$\frac{\text{nmoles } O_2}{\text{ml}} = \frac{(0.02831)(740 \text{ mm Hg})(0.21)}{(22,400 \text{ ml})(760 \text{ mm Hg/atm.})} = 258.42 \text{ nmoles } O_2/\text{ml}$$

where 0.02831 is α , the Bunsen coefficient for pure water at 25°C (Handbook of Chemistry and Physics, 33rd Edition, 1951 through 1952), 740 mm Hg is the estimated barometric pressure at the time of the experiment, 0.21 is the % of O_2 in the air, 22,400 ml is the volume of 1 mole of gas at Standard Temperature and Pressure (STP), and 247 is the total number of small divisions between 0 and 100% on the chart paper of the recorder.

$$\text{nmoles } O_2/\text{ml-div} = (258.42)(1/247) = 1.046$$

$$\begin{aligned}
 \text{Oxygenase activity} &= \frac{(\text{rate of } O_2 \text{ deplet.}/\text{min.})}{\text{ml-division}} \frac{(\text{nmoles } O_2)}{\text{volume}} \text{ (ml of reaction)} \\
 &= \frac{(\text{nmoles } O_2/\text{min}) (1 \text{ } \mu\text{mole}/1000 \text{ nmoles})}{\text{mg protein}} \\
 &= \text{ } \mu\text{moles } O_2 \text{ fixed/min-mg protein}
 \end{aligned}$$

Materials

Most metabolites studied were obtained as sodium salts in the purest forms available from the Sigma Chemical Company and used without further purification. Radioactive ^{14}C was purchased from ICN Chemical and Radioisotope Division as $\text{NaH}^{14}\text{CO}_3$ in 1 mCi quantities with a specific activity of approximately 8 mCi/mMole. When not being used, the isotope was stored at 4°C . The Dowex-1, Amberlite and DEAE-cellulose resins were purchased from the Sigma Chemical Company. After initial swelling of the resin in deionized water, the resin was cleaned by alternating washes with 0.5 M NaOH and 0.5 M HCl for several hours. Between each wash, the resin was brought to neutrality by exhaustive rinsing with deionized water and the fine particles were removed at each step throughout the entire procedure.

Results

Preparations of Comfrey and Spinach RuBPCase

Generally, the standard specific activity obtained for all comfrey RuBPCase preparations ranged from 0.60 to 1.50 $\mu\text{moles } ^{14}\text{CO}_2 \text{ fixed/min-mg}$ protein. Specific activities from spinach RuBPCase ranged from 0.90 to 1.50 $\mu\text{moles } ^{14}\text{CO}_2 \text{ fixed/min-mg}$ protein. When the specific activity dropped as low as 0.60 $\mu\text{moles } ^{14}\text{CO}_2 \text{ fixed/min-mg}$ protein for comfrey RuBPCase preparations, several chemicals and techniques used in the preparation were checked out.

One change that was tried to raise the activity was the addition of 10 μM leupeptin (Johal and Chollet, 1983) to all solutions used in the isolation procedure. This tripeptide contains an aldehyde functional group which reacts specifically with thiol proteases. The addition of leupeptin alone did not raise the specific activity to its former level (1.5 units) indicating that elevated amounts of thiol proteases in the leaves as the plants had aged was not a problem. Other purification techniques, such as heat activation and DEAE-cellulose column preparation, also were reinvestigated and found to give consistent results with that seen previously (Simpson *et al.*, 1983). The results are summarized below.

Heat Activation: Aliquots of the September 23, 1983 preparation of comfrey RuBPCase were heat-activated at 40°, 45°, and 50° C for 10 min and then cooled at room temperature for 10 min before being applied to three separate DEAE-cellulose columns. All three columns were poured from the same batch of DEAE-cellulose. Elution from the columns was stepwise using 0.25 M NaCl TME-DTT, pH 7.5 buffer. RuBPCase

was fully activated with NaHCO_3 while concentrated (1-2 mg/ml) instead first of diluting with buffer prior to activation (Table 1).

NaCl Gradient Elution: Aliquots of the same preparation of comfrey RuBPCase which had not been heat-activated were applied to two separate columns poured from the same batch of DEAE-cellulose. Elution from one of the columns was with 0.25 M NaCl in TME-DTT, pH 7.5 whereas elution from the other column was with a TME-DTT 0 to 0.20 M NaCl gradient, pH 7.5. As before, the enzyme was fully CO_2 -activated while concentrated instead of first being diluted with buffer (Table 2).

DEAE-Cellulose Preparation: Further aliquots of the same comfrey RuBPCase preparation without heat-activation were applied to a DEAE-cellulose column made from the media previously mentioned. Other aliquots of the same comfrey RuBPCase were heat-activated at 45°C for 10 min, cooled at room temperature for 10 min and applied to a DEAE-cellulose column made from a newly processed batch of media. Elution from both columns was stepwise with 0.25 M NaCl. NaHCO_3 activation before assay was performed as with the gradient elution study (Table 3).

Activity Revival with DTT: It had been reported (Hall *et al.*, 1981) that partially inactivated spinach RuBPCase could be reactivated by adding high concentrations of DTT (50 to 100 mM) to enzyme preparations which had been stored for long periods of time (apparently as an $(\text{NH}_4)_2\text{SO}_4$ precipitate at 4°C). The activity of the comfrey RuBPCase was not raised when 100 mM DTT and 200 mM DTT were added to separate aliquots of RuBPCase indicating that low activity had not resulted from oxidation of cysteines (Table 4).

NaHCO_3 Activation: Both spinach and comfrey RuBPCases from the October,

1983 preparations were heat-activated at 45°C and cooled for 10 min before being applied to separate DEAE-cellulose columns. Elution was stepwise with 0.25 M NaCl TME-DTT. Aliquots of both spinach and comfrey RuBPCases were activated with NaHCO_3 in both concentrated and diluted volumes (Table 5).

One item that did seem to correlate with the onset of low specific activity was the purchase of a new lot # of insoluble PVP, a free radical scavenger and polyphenol absorbent, from Sigma Chemical Co. Therefore, PVP from Chemalog and GAF Corporation were tested with different preparations of the comfrey enzyme. A more normal specific activity was obtained with the GAF product and it was used from then on since Sigma Chemical Co. no longer had any lot #99C-0315 insoluble PVP (Table 6).

RuBPCase Elution from DEAE-Cellulose

Elution of comfrey RuBPCase from a DEAE-cellulose column with an assumed 0 to 0.20 M NaCl in TME-DTT, pH 7.5 gradient showed that protein came off the column with approximately 0.13 M added NaCl (Fig. 3) based on A_{280} absorbance readings. Carboxylase assays on each A_{280} peak tube of the profile confirmed the presence of RuBPCase in those tubes. The difference in NaCl elution concentration with the gradient compared to the 0.25 M NaCl used in a stepwise manner by Simpson *et al.* (1983) may be attributable to the techniques used in running the column. In the gradient, as much as possible of excess buffer was removed from the column before the elution buffer was applied. Therefore, the NaCl applied was diluted less when it entered the column medium. It was considered important to minimize the NaCl concentration necessary to

TABLE 1
Effects of Heat-Activation on RuBPCase Activity

Activation Temperature	Specific Activity of Comfrey RuBPCase*	
	20 μ g protein	70 μ g protein
40°C	0.42	0.43
45°C	0.48	0.43
50°C	0.43	0.44

*Specific activity: μ moles $^{14}\text{CO}_2$ fixed/min-mg protein (9/23/83 preparation)

#Amounts of protein in assay

TABLE 2
Effects of Column Elution on RuBPCase Activity

Type of DEAE-Cellulose Column Elution	Specific Activity of Comfrey RuBPCase*	
	20 μ g protein	70 μ g protein
One-Step 0.25 M NaCl	0.83	0.97
0 to 0.2 M NaCl Gradient	0.70	0.80

*Specific activity: μ moles $^{14}\text{CO}_2$ fixed/min-mg protein (9/23/83 preparation)

#Amounts of protein used per assay

TABLE 3
Effects of DEAE-Cellulose Preparation on RuBPCase Activity

DEAE-Cellulose Preparation	Specific Activity of Comfrey RuBPCase [*]	
	20 μ g protein	70 μ g protein
Prepared 4/29/83	0.83	0.97
Prepared 9/28/83	0.72	0.80

*Specific activity: μ moles $^{14}\text{CO}_2$ fixed/min-mg protein (9/23/83 preparation)

#Amount of protein used per assay

TABLE 4
Effects of High Concentration of DTT on RuBPCase Activity

Specific Activity: Comfrey RuBPCase					Spinach RuBPCase		
DTT ^a mM	Non-heat act.	DTT ^b mM	Heat* act.	Reheat [#] act.	DTT ^c mM	Heat* act.	Reheat [#] act.
0	0.50	0	0.93	----	0	1.12	----
100	0.42	100	0.67	----	100	1.06	----
200	0.42	200	----	0.58	200	----	1.01

Specific activity: μ moles $^{14}\text{CO}_2$ fixed/min-mg protein (9/23/83 preparation)

a,b,c: Enzyme (1-2 mg/ml) activated with 22.6 mM NaHCO_3 for 45 min before assay.

*Heat activation (act.) was for 10 min at 45°C and cooling for 10 min at room temperature before addition of DTT.

#Process repeated after the addition of DTT for reheat-activation.

TABLE 5
Effects of the Type of NaHCO_3 Activation on RuBPCase Activity

Type of NaHCO_3 Activation	Specific Activity of Cofrey RuBPCase [#]		Specific Activity of Spinach RuBPCase [#]	
	20 μg	70 μg	20 μg	70 μg
Concentrated (1-2 mg/ml)	0.93	1.07	1.12	1.30
Diluted (20 μg /265 μl buffer)	0.75	----	1.20	----

Specific Activity: $\mu\text{moles } ^{14}\text{CO}_2 \text{ fixed/min-mg protein (10/83 preparation)}$

[#]Amount of protein used per assay

TABLE 6
Effects of Different Sources of Insoluble PVP on RuBPCase Activity

Time	Source of PVP	Lot #	Specific Activity
Spring 84	GAF Corporation	0803D	1.50
Fall 83	Chemalog	121869	----*
Summer 83	Sigma	92F-0395	0.70
Spring 83	Sigma	99C-0315	1.20

Specific activity: $\mu\text{moles } ^{14}\text{CO}_2 \text{ fixed/min-mg protein}$

* proved to be soluble

Figure 3. Elution profile of comfrey RuBPCase from a DEAE-cellulose column with a 0-0.20 M NaCl TME-DTT gradient. Comfrey RuBPCase (2.2 mg/ml) was applied to a 1.3 x 3.5 cm DEAE-cellulose column and eluted with an assumed linear 0-0.20 M gradient (...) produced by a conical gradient maker. Thirty-one fractions (0.63 ml per fraction) were collected and protein position (O) determined by A_{280} . A carboxylase activity (A) (20 μ g of fully activated enzyme per assay) was done on aliquots from each of the A_{280} peak tubes to confirm the presence of RuBPCase. The specific activity of the enzyme was approximately 0.60 μ moles $^{14}\text{CO}_2$ fixed/min-mg protein.

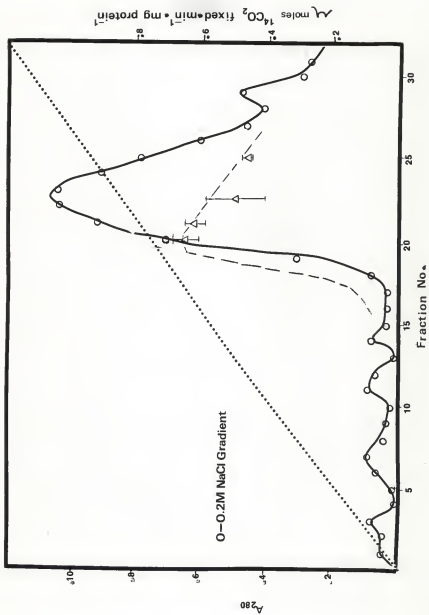
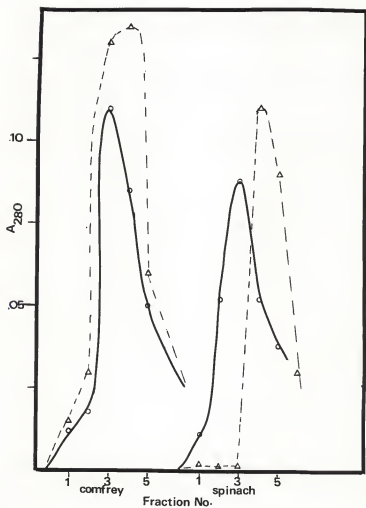


Figure 4. Elution profile of comfrey and spinach RuBPCases from DEAE-cellulose columns with one-step elutions using 0.25 or 0.15 M NaCl. Comfrey and spinach RuBPCases (2-3 mg/ml) were applied to 1.3 x 3.5 cm DEAE-cellulose columns and eluted with either 0.15 M NaCl (O) or 0.25 M NaCl (Δ) stepwise elutions. Fractions of 0.80 ml per tube were collected and protein position determined by A_{280} .

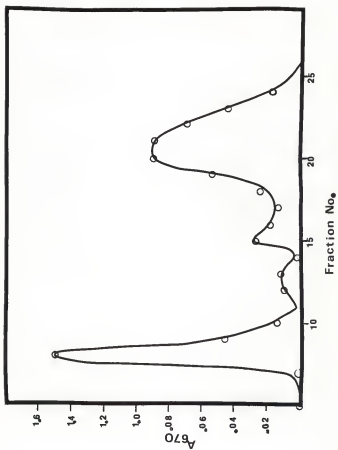


elute the enzyme since Cl^- had been shown to be a weak inhibitor of RuBPCase (Bonsall, 1981). Consequently, a concentration of 0.15 M NaCl was used routinely for stepwise elution of RuBPCase instead of 0.25 M NaCl. Both spinach and comfrey RuBPCases gave similar stepwise elution profiles with 0.15 M NaCl as they did with 0.25 M NaCl as can be seen in Fig. 4.

Synthesis of RuBP

Following synthesis and storage of RuBP as described in the experimental section, the Ba^{+2} form of RuBP was dissolved in a minimum of 0.1 M HCl, filtered, and applied to the Dowex-1 column. Fractions (15 ml) were collected for analysis from an initial 500 ml of a 100 mM LiCl, 1 mM HCl wash to make sure that the gradient could be started at 100 mM LiCl as had been surmised from published procedures. When the fractions were analyzed by the orcinol method, two or three peaks representing pentose-containing compounds were identified (Fig. 5). The first compound peaked at fraction #8 and was a light green color after reaction with the orcinol reagent. The second compound peaked around fraction #20 and also gave a light green colored reaction with the orcinol reagent. A possible third pentose-containing compound was located in fractions 23 to 25 distinct from the compound in fraction 20 because of its straw color. The compound in fractions 23 to 25, instead of having a maximum wavelength of 670 nm as the green colored compounds, showed a maximum absorbance at 540 nm. The most prominent compounds found were most likely R-5-P and ADP, the expected byproducts of RuBP synthesis which had not been completely removed by the Horecker procedures. The third compound remained unidentified.

Figure 5. Orcinol reaction profile of a 100 mM LiCl, 1 mM HCl wash of a Dowex-1 column after addition of RuBP for purification. Approximately 200 mg of $(\text{Ba}^+)_2\text{RuBP}$ dissolved in 0.1 N HCl were applied to a Dowex 1 column (400 mesh, 2.5 x 43 cms) which had been equilibrated with 1 mM HCl. Five hundred ml of 100 mM LiCl, 1 mM HCl was run through the column and 15 ml fractions collected. Each fraction was analyzed with an orcinol reagent for pentose-containing compounds. Positions of the pentose-containing compounds reacting with the orcinol reagent were then identified by their A_{670} (O).



A fourth compound giving a prominent band was eluted after addition of approximately 750 ml of the two liter 100 to 250 mM LiCl gradient. The orcinol test with this compound gave a dark green colored solution. From the results of the carboxylase assay (Fig. 6), this compound was identified as RuBP. Sixteen peak fractions (39-54) were pooled, adjusted to pH 6.5 with 1 N NaOH and yielded, after lypholization, 132 mg of $(Li^+)_4RuBP$ representing a recovery of approximately 66% assuming all the added material was $(Ba)_2RuBP$. The RuBPCase activity produced by this product was compared to other preparations of RuBP. One of the preparations was obtained by exchanging the Ba^{+2} form by passage over an Amberlite resin (Li^+ form) ion exchange column at room temperature using deionized water for elution but without analysis of any fractions. The other RuBP was purchased from Sigma Chemical Company as a tetrasodium salt. The carboxylase activities obtained from these products are compared in Table 7. The Dowex-1 column purified RuBP gave considerably higher activity than those from either Sigma or the batchwise ion exchange of RuBP. The Sigma RuBP was not representative but showed what can be obtained when commercial sources are used. Therefore, it was concluded that the Dowex-1 column purification gave a superior RuBP and consequently was used in all further studies.

Initial Velocity of Comfrey and Spinach RuBPCase Carboxylase Activities in the Presence or Absence of the Effector, 3-PCA

Time studies of comfrey RuBPCase carboxylase activity showed that the effector, 3-PCA, when added to the assay mixture 1-2 s after the addition of 1.3 mM RuBP, exerted its effect within 3 s since there was no lag time observable, but only an immediate shift to the lower

Figure 6. Elution of $(\text{Li}^+)_4\text{RuBP}$ from a Dowex-1 column with a 100-250 mM LiCl , 1 mM HCl gradient. $(\text{Li}^+)_4\text{RuBP}$ was eluted from the Dowex 1 column with an assumed linear 2 liter 100 to 250 mM LiCl , 1 mM gradient. Fifteen ml fractions were collected and analyzed with an orcinol reagent for pentose-containing compounds. Positions of the pentose-containing compounds reacting with the orcinol reagent were determined by A_{670} (O). The presence of $(\text{Li}^+)_4\text{RuBP}$ was confirmed by assay with RuBPCase. 100 μl of each of the A_{670} peak tubes were added to 200 μl of fully activated comfrey RuBPCase for the carboxylase assay (A).

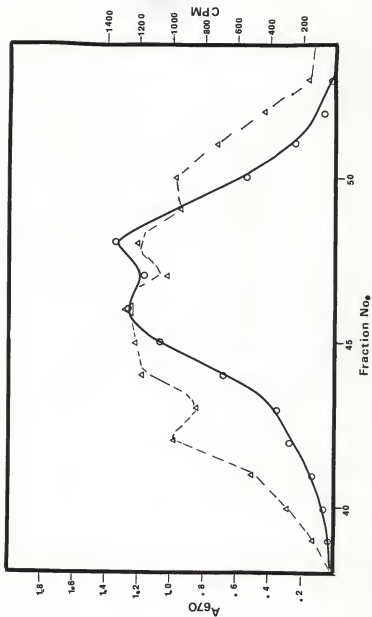


TABLE 7
 Comfrey RuBPCase Activity with Different Sources of RuBP

Source of RuBP	Specific Activity of Comfrey RuBPCase
Dowex-1 Column	1.40
Amberlite Column	0.83
Sigma	0.02
Specific activity: $\mu\text{moles } ^{14}\text{CO}_2 \text{ fixed/min-mg protein.}$	

activity. In general, the activity in the presence of 5 mM 3-PGA was lowered approximately 30-40%. In the absence or presence of effector, activity for fully activated comfrey RuBPCase increased linearly with time for about 50 s with a noticeable dropoff by 90 s (Fig. 7). The steady state rate after 90 s of reaction persisted for the duration of the experiment (3 min).

Similar studies with spinach RuBPCase also showed that the effector, 3-PGA, when added within 1-2 s after the addition of 1.3 mM RuBP, exerted its effect immediately (Fig. 8). As had been observed with comfrey RuBPCase, the activity decreased approximately 30-40% in the presence of 5 mM 3-PGA. For spinach RuBPCase, the initial velocity was not maintained quite as long since there was a noticeable decrease in the rate after 40 s.

Inhibition of Comfrey RuBPCase by 3-PGA at Saturating Levels of RuBP

RuBPCase, a multisubunit enzyme, starts the Calvin cycle and carries out an essentially irreversible reaction. Therefore, it is likely to be highly regulated by some of the products of the Calvin cycle. Since two molecules of 3-PGA are produced in the carboxylation and cleavage reaction of RuBPCase, 3-PGA represents a potential regulator of RuBPCase activity in vivo through a type of feedback inhibition. Stitt et al. (1980) observed that within the first 4 min of illumination of spinach palisade protoplasts, the concentration of 3-PGA in the chloroplast compartment shot up to a maximum level after 2 min of illumination and then steadily decreased with further illumination to an apparent steady state rate. During the same time period, the concentration of P_i decreased several-fold. Furthermore,

Figure 7. Initial velocity of comfrey RuBPCase carboxylase activity in the presence or absence of the effector, 3-PGA. Initial rates in the absence of 3-PGA, Δ ; rates in the presence of 5 mM 3-PGA added to the fully activated enzyme assay mixture within 1-2 s after 1.3 mM RuBP, \bullet . Specific activity was 0.70 μ moles $^{14}\text{CO}_2$ fixed/min-mg protein.

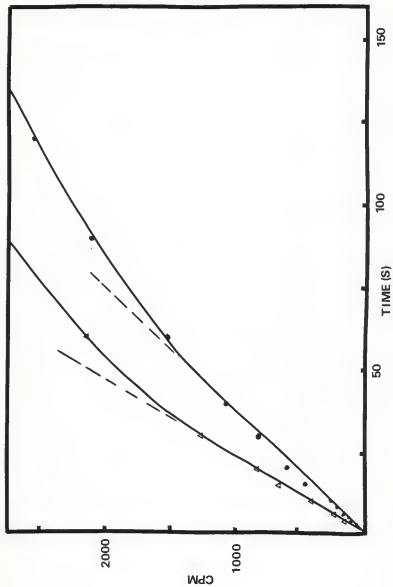
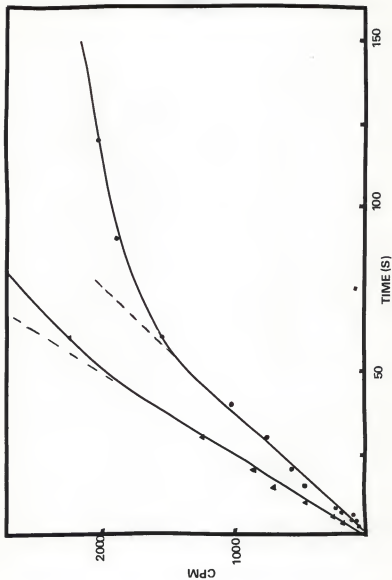


Figure 8. Initial velocity of spinach RuBPCase carboxylase activity in the presence or absence of the effector, 3-PGA. Velocity in the absence of 3-PGA, ▲; velocity in the presence of 5 mM 3-PGA added to the fully activated enzyme assay mixture 1-2 s after 1.3 mM RuBP, ●. Specific activity was 1.20 $\mu\text{moles } ^{14}\text{CO}_2 \text{ fixed/min-mg protein}$.



Bolden (1983) showed evidence that 3-PGA had one of the highest affinities for the allosteric site on comfrey RuBPCase of all the phosphometabolites investigated and therefore represented a prime prospect for a negative effector. 3-PGA inhibition in vitro was then studied to try and further characterize possible 3-PGA regulation of RuBPCase.

To test this possibility, the effect of various concentrations of 3-PGA on the activity of comfrey RuBPCase was investigated at saturating levels (1.3 mM) of RuBP in a standard assay. 3-PGA concentrations of 0.1 to 20 mM showed more than just competitive inhibition with RuBP. This is illustrated in Fig. 9 where activities are plotted as a % of the control vs 3-PGA concentration.

$$\% \text{ control} = \frac{\text{CPM of sample w/effector} - \text{CPM of heated blank}}{\text{CPM of control w/out effector} - \text{CPM of heated blank}} \times 100$$

The dashed lines in the graphs indicate competitive inhibition patterns predicted from the relative inhibition, i , as defined (Segel, 1975) by equation 4:

$$i = \frac{(I)}{(I) + K_I \left[1 + (S)/K_M \right]} \quad 4$$

where K_I is the competitive inhibition constant for the effector in question, K_M is the Michaelis-Menten constant for RuBP, S and I are the concentrations of RuBP and effector used in the assays, respectively. The percent relative activity was obtained by subtracting 100% from 100%. A K_M of 10 μM and a K_D of 990 μM were used in calculations.

Between 1 and 5 mM 3-PGA, an 8 to 10-fold increase in inhibition over that predicted on a competitive basis alone was observed. At higher concentrations of 3-PGA, this difference dropped to a 3-fold

increase and seemed to parallel the competitive curve above approximately 10 mM effector. Also, the shape of the experimental curve remained the same whether the enzyme in 10 mM Mg^{+2} was fully activated with 22.6 mM $NaHCO_3$ or partially activated with 1 mM $NaH^{14}CO_3$. To determine if there was an effect from changes in ionic strength in vitro upon addition of the inhibitors, NaCl was used in place of the effector at concentrations that were equivalent to the ionic strength produced by each 3-PGA solution. Comparable ionic strength NaCl solutions not only failed to inhibit the enzyme, but they actually appeared slightly activating at lower concentrations (Fig. 9).

Inhibition of Different Preparations of Comfrey RuBPCase Activity by 3-PGA at Saturating Levels of RuBP

Essentially the same inhibitory effect was seen by 3-PGA between different comfrey RuBPCases prepared in June of 1983 and January of 1984 (Fig. 10). The same results between the two different preparations also reduced the possibility of the inhibitory effect being an artefact of the length of storage at $-70^{\circ}C$ since the one had been stored six months longer than the other.

Inhibition of Spinach RuBPCase Activity by 3-PGA at Saturating Levels of RuBP

It was important to see if 3-PGA affected spinach RuBPCase activity as it had comfrey RuBPCase since it was not known whether the enzyme from spinach contained an allosteric site. Therefore, studies similar to those using 3-PGA with comfrey RuBPCase were done with the spinach

Figure 9. Inhibition of comfrey RuBPCase activity by 3-PGA at saturating levels of RuBP. \circ , 3-PGA inhibition at 20 mM NaHCO_3 ; \blacktriangle , 3-PGA inhibition at 1 mM $\text{NaH}^{14}\text{CO}_3$; \square , NaCl activation at 20 mM NaHCO_3 . The dashed line shows how simple competitive inhibition with respect to 1.3 mM RuBP would vary with 3-PGA concentrations based on Equation 4 using $K_M=10 \mu\text{M}$ and $K_D=990 \mu\text{M}$ while the solid line indicates the observed trends with 3-PGA and NaCl. The error bars are 1 standard deviation from the mean based on two to eight determinations for each effector concentration using enzyme at 20 mM NaHCO_3 with a specific activity of $0.80 \mu\text{moles } ^{14}\text{CO}_2 \text{ fixed/min-mg protein}$ and enzyme at 1 mM $\text{NaH}^{14}\text{CO}_3$ with a specific activity of $0.10 \mu\text{moles } ^{14}\text{CO}_2 \text{ fixed/min-mg protein}$. The counts per minute (CPM) of ^{14}C of the control (100% value) at 20 mM NaHCO_3 was 1450 while the CPM of the control at 1 mM $\text{NaH}^{14}\text{CO}_3$ was 2498.

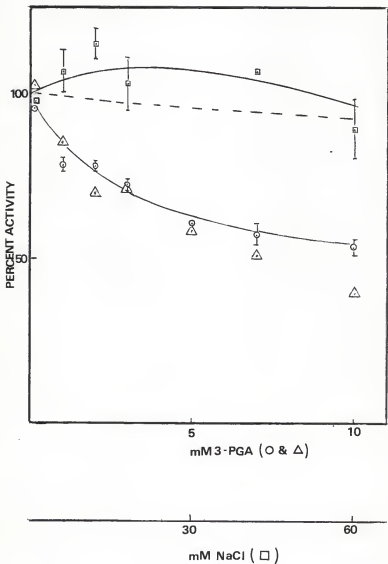
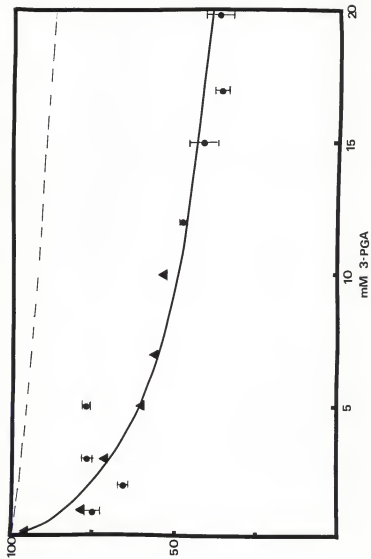


Figure 10. Inhibition of different preparations of confrey RuBPCase activity by 3-PGA at saturating levels of RuBP. ●, 3-PGA inhibition of RuBPCase (specific activity=1.30 μ moles $^{14}\text{CO}_2$ fixed/min-mg protein, CPM of control=2235);▲, 3-PGA average inhibition of RuBPCase by duplicate determination (specific activity=0.80 μ moles $^{14}\text{CO}_2$ fixed/min-mg protein, CPM of control=1450). Both enzyme preparations were activated at 20 mM NaHCO_3 . The dashed line is the same as in Figure 9. The solid line indicates observed trends with 3-PGA with different preparations of confrey RuBPCase. The error bars represent extremes of duplicate determinations.



PER CENT ACTIVITY

enzyme. A K_M of 20 μM (Jensen and Bahr, 1977) and a K_I of 840 μM (Badger and Lorimer, 1981) were used to predict competitive inhibition.

Spinach RuBPCase when activated and assayed at 10 and 20 mM NaHCO_3 in the presence of 3-PGA gave essentially the same curves. Similar to the effect seen with comfrey RuBPCase, extra inhibition was observed beyond competitive levels (Fig. 11). At low concentrations of 3-PGA, a 3-fold increase in inhibition of spinach RuBPCase activity at 10 and 20 mM NaHCO_3 (10 mM Mg^{+2}) was observed over and above that predicted for competitive inhibition alone, whereas at higher concentrations, 3-PGA showed little extra inhibition. Quantitatively, 3-PGA with spinach RuBPCase at 10 and 20 mM NaHCO_3 seemed to give only about 40% of the inhibitory effect that 3-PGA gave with comfrey RuBPCase. However, with 1 mM $\text{NaH}^{14}\text{CO}_3$ activation of spinach RuBPCase, 3-PGA inhibition was greater at concentrations above 5 mM 3-PGA than seen at 10 and 20 mM NaHCO_3 activation and more like the inhibition seen with comfrey RuBPCase at either 1 or 20 mM NaHCO_3 .

Inhibition of Different Preparations of Spinach RuBPCase Activity by 3-PGA at Saturating Levels of RuBP

As had been observed with the comfrey preparations (Fig. 10), there were no detectable differences in inhibition patterns of 3-PGA for spinach RuBPCase purified from different commercial lot numbers (Fig. 12).

Inhibition of Comfrey and Spinach RuBPCases by P_i at Saturating Levels of RuBP.

In the dark, RuBPCase activity essentially shuts down bringing the

Figure 11. Inhibition of spinach RuBPCase activity by 3-PGA at saturating levels of RuBP. ●, 3-PGA inhibition at 20 mM NaHCO_3 ; ▲, average 3-PGA inhibition of duplicate determinations at 10 mM NaHCO_3 ; □, 3-PGA inhibition at 1 mM $\text{NaH}^{14}\text{CO}_3$. The dashed line shows how simple competitive inhibition with respect to 1.3 mM RuBP would vary with 3-PGA concentration based on Equation 4 using a $K_M=20 \mu\text{M}$ and a $K_I=840 \mu\text{M}$ while the solid line indicates the observed trends with 3-PGA at 1, 10, and 20 mM NaHCO_3 . The error bars represent extremes of duplicate determinations. Specific activity was 1.20 $\mu\text{moles } ^{14}\text{CO}_2 \text{ fixed/min-mg protein}$ for the enzyme at 10 and 20 mM NaHCO_3 (CPM of control=3240 at 20 mM NaHCO_3 and 5135 at 10 mM NaHCO_3) whereas specific activity for the enzyme at 1 mM $\text{NaH}^{14}\text{CO}_3$ was 0.14 $\mu\text{moles } ^{14}\text{CO}_2 \text{ fixed/min-mg protein}$ (CPM of control=10321).

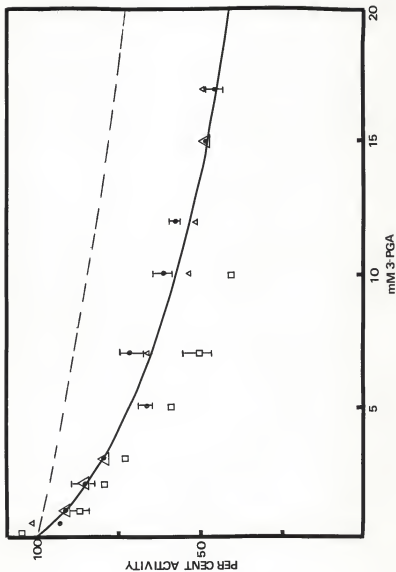
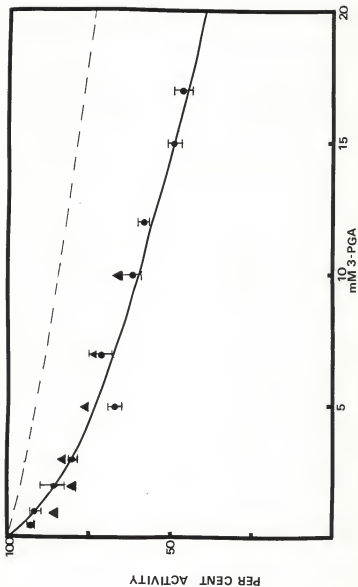


Figure 12. Inhibition of different preparations of spinach RuBPCase activity by 3-PGA at saturating levels of RuBP. ●, 3-PGA inhibition of RuBPCase (specific activity=1.20 μ moles $^{14}\text{CO}_2$ fixed/min-mg protein, CPM of control=816); ▲, 3-PGA average inhibition of a different RuBPCase (specific activity=1.10 μ moles $^{14}\text{CO}_2$ fixed/min-mg protein, CPM of control=3240). Both enzyme preparations were activated and assayed at 20 mM NaHCO_3 . The dashed line is the same as in Figure 11. The solid line indicates the observed trends with 3-PGA with different preparations of spinach RuBPCase. Error bars show extremes of duplicate determinations.



Calvin cycle to a halt. P_i , a compound found to be at very high levels (320 \pm 59 nmols/mg chlorophyll) in the chloroplast compartment (Stitt *et al.*, 1980) in the dark and at the onset of plant protoplast illumination, could represent another potential regulator of RuBPCase activity. In the dark when the P_i level is high and the concentrations of cofactors for the Calvin cycle reactions are low, RuBPCase activity, the first reaction in the Calvin cycle, is essentially nonmeasurable. But, upon illumination of the plant protoplasts, P_i levels rapidly drop several fold (Stitt *et al.*, 1980). It is reasonable that P_i concentrations also are decreasing in the chloroplast since P_i is being used to make ATP and Calvin cycle cofactors. Therefore, P_i might act as a regulator of RuBPCase activity between the light and dark transitions. Consequently, experiments were performed in the presence of P_i as they had been with 3-PGA to see if P_i might possibly effect RuBPCase activity *in vitro*.

When concentrations of P_i between 1 and 20 mM were added to comfrey RuBPCase activated either at 1 mM $\text{NaH}^{14}\text{CO}_3$ or 22.6 mM NaHCO_3 , inhibition curves shaped similarly to those of Fig. 9 were obtained (Fig. 13). Quantitatively, the amount of extra inhibition appeared to be less than that for the same concentration of 3-PGA over the entire range of effector concentrations but still considerably more than that predicted by simple competitive inhibition.

With spinach RuBPCase, P_i showed double the inhibition predicted on a strictly competitive basis compared to a 3-fold increase at low concentrations of 3-PGA with spinach RuBPCase (Fig. 14). Furthermore, the inhibition continued to increase over the whole range of 1 to 20 mM P_i and was slightly greater at 1 mM than at 20 mM NaHCO_3 .

Figure 13. Inhibition of comfrey RuBPCase activity by P_i at saturating levels of RuBP. ●, P_i inhibition at 20 mM NaHCO_3 ; ▲, P_i average inhibition of duplicate determinations at 1 mM $\text{NaH}^{14}\text{CO}_3$. The dashed line shows how simple competitive inhibition with respect to 1.3 mM RuBP would vary with P_i concentrations based on Equation 4 using a $K_M=10$ μM and a $K_D=750$ μM whereas the solid line shows the observed trends with P_i at 20 and 1 mM NaHCO_3 . Error bars indicate extremes of duplicate determinations. Specific activity was 0.60 $\mu\text{moles } ^{14}\text{CO}_2 \text{ fixed/min-mg protein}$ for the enzyme at 20 mM NaHCO_3 (CPM of control=1450) and 0.10 $\mu\text{moles } ^{14}\text{CO}_2 \text{ fixed/min-mg protein}$ at 1 mM $\text{NaH}^{14}\text{CO}_3$ (CPM of control=2498).

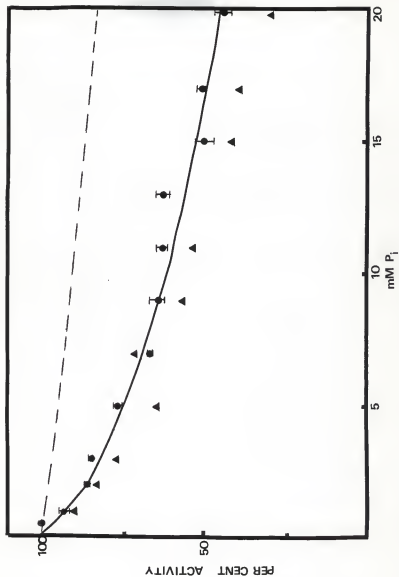
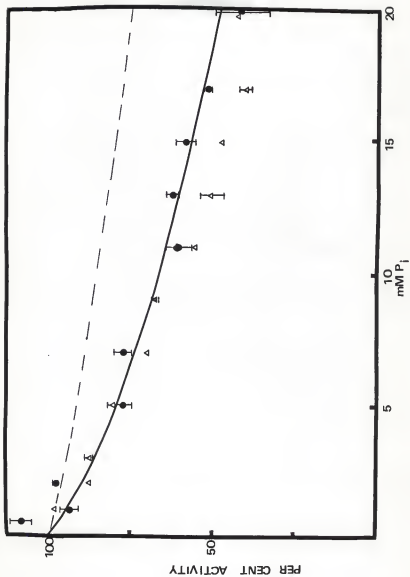


Figure 14. Inhibition of spinach RuBPCase by P_i at saturating levels of RuBP. Δ , Average P_i inhibition of duplicate determinations at 1 mM $\text{NaH}^{14}\text{CO}_3$; \bullet , P_i inhibition at 20 mM NaHCO_3 . The dashed line shows how simple competitive inhibition with respect to 1.3 mM RuBP would vary with P_i concentration based on Equation 4 using a $K_M=20 \mu\text{M}$ and a $K_I=900 \mu\text{M}$ while the solid line indicates the observed trends with P_i at 1 and 20 mM NaHCO_3 . Error bars show extremes of duplicate determinations. Specific activity was 1.20 $\mu\text{moles } ^{14}\text{CO}_2$ fixed/min-mg protein (CPM of control=816) for the enzyme at 20 mM NaHCO_3 and 0.15 $\mu\text{moles } ^{14}\text{CO}_2$ fixed/min-mg protein (CPM of control=10321) at 1 mM $\text{NaH}^{14}\text{CO}_3$.



concentrations somewhat as seen for 3-PGA (Fig. 11). The measured degree of inhibition at 1 and 20 mM NaHCO_3 for comfrey and spinach RuBPCases at the same P_i concentrations were very similar as they had been for 3-PGA at 1 mM $\text{NaH}^{14}\text{CO}_3$.

Virtually all the data with 3-PGA and P_i indicated two types of inhibition which probably resulted from binding at different sites. One way to test for two-site binding is a Dixon plot. The data used for these plots, however are normally obtained at less than saturating concentrations of substrate. Even though our data were obtained at high levels of RuBP where competitive inhibition was correspondingly small, Dixon plots of the inhibition of comfrey and spinach RuBPCases by 3-PGA and P_i are shown in Figs. 15-18. The Dixon plot for inhibition of fully activated (22.6 mM NaHCO_3) comfrey RuBPCase by 3-PGA at saturating levels of RuBP showed possible concave curvature at low concentrations of 3-PGA indicating two-site binding (Fig. 15). However at the higher concentrations (2 to 20 mM) of 3-PGA (data not shown), the Dixon plot appeared linear. Linear plots could also be seen with the Dixon plot of spinach RuBPCase and 3-PGA (Fig. 16) from 1 to 20 mM 3-PGA. However, extensive inhibition studies at low levels of 3-PGA (0-3 mM) were not performed for spinach RuBPCase.

With P_i , which has a 2-fold lower affinity for the allosteric site than 3-PGA (Bolden, 1983), second site binding to partially activated comfrey and spinach RuBPCases was observed since the Dixon plots (Figs. 17 and 18) curved upward more gently, but over a much larger concentration range than that for 3-PGA (Fig. 15). The wider range of curvature suggested that higher concentrations of P_i were required to exert the same inhibition as 3-PGA in agreement with the higher

Figure 15. Dixon plot of 3-PGA inhibition of comfrey RuBPCase. ○, reciprocal velocity ($1/V$) of fully activated RuBPCase in the presence of 0-3 mM 3-PGA and with velocity expressed as $\mu\text{moles } ^{14}\text{CO}_2 \text{ fixed/min}$. The solid line represents the observed trend of reciprocal velocity in the presence of 0 to 3 mM 3-PGA. Error bars show extremes of duplicate determinations. Specific activity of the enzyme was 0.60 $\mu\text{moles } ^{14}\text{CO}_2 \text{ fixed/min-mg protein}$ when activated and assayed at 20 and 22.6 mM NaHCO_3 , respectively.

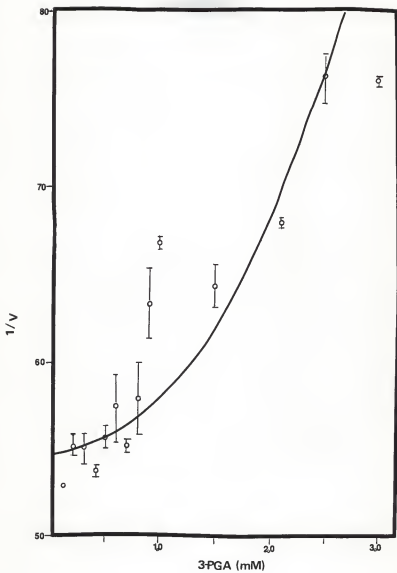
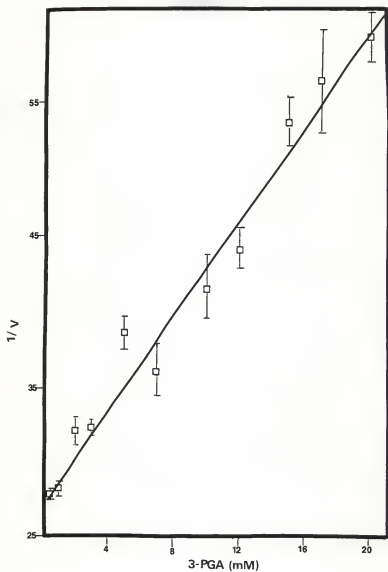


Figure 16. Dixon plot of 3-PGA inhibition of spinach RuBPCase. The 20 mM NaHCO_3 data of Fig. 11 (\square) replotted as in Fig. 15.



allosteric site K_D for P_1 .

To see if this second site binding was cooperative, kinetic Hill plots $\ln(100\% \text{ activity}/\% \text{ activity})$ vs $\ln(\text{effector})_T$ of 3-PGA and P_1 inhibition data of comfrey and spinach RuBPCases were prepared. Percent activity would be related to the fraction of sites not bound with effector (the activity), while $(100\% \text{ activity})$ would be related to the fraction of sites bound by the effector (inhibition). The reciprocal of the amount of sites bound over free was taken so that the sigmoidal curve would not be opposite of what normally is seen with a Hill plot. The Hill plot of 3-PGA inhibition of comfrey RuBPCase at low concentrations of 3-PGA (Fig. 19) indicated some site-site interaction by giving a sigmoidal curve compared to the linear line given by theoretical competitive inhibition. A Hill coefficient for 3-PGA inhibition of comfrey RuBPCase of 1.7 can be estimated from the region of maximum slope as indicated by the dotted line in Fig. 19. A Hill coefficient of 1.7 for a 16 total subunit, or 8 large subunit, enzyme would indicate only weak or moderate interaction. A better idea as to the degree of the cooperativity might be gained if the data were fit to a particular model by a computer program, but at this time, such a program was unavailable.

The cooperativity seen with 3-PGA inhibition also could be another reason why the Dixon plot of 3-PGA inhibition appeared linear at high concentrations of 3-PGA for comfrey RuBPCase since the cooperative inhibitory effect was completed by approximately 5 mM 3-PGA (Fig. 16).

Data for the Hill plot of 3-PGA inhibition with spinach RuBPCase probably were not in an appropriate range of 3-PGA concentrations to see the full sigmoidal curve as also indicated by the linear Dixon plot

Figure 17. Dixon plot of P_i inhibition of comfrey RuBPCase. The 1 mM $\text{NaH}^{14}\text{CO}_3$ data of Fig. 13 (Δ) replotted as in Fig. 15.

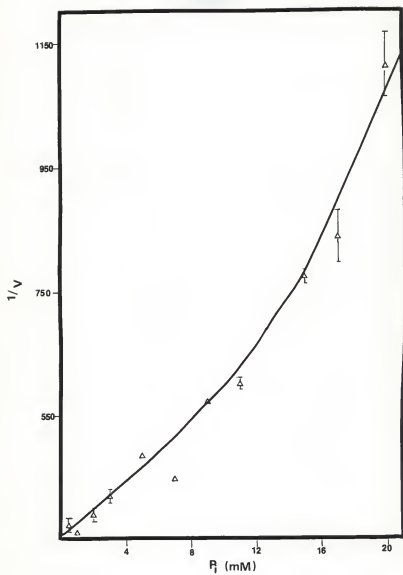


Figure 18. Dixon plot of P_i inhibition of spinach RuBPCase. The 1 mM $\text{NaH}^{14}\text{CO}_3$ data of Fig. 14 (O) replotted as in Fig. 15.

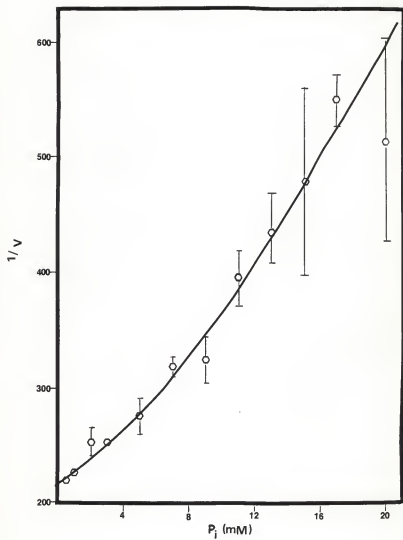
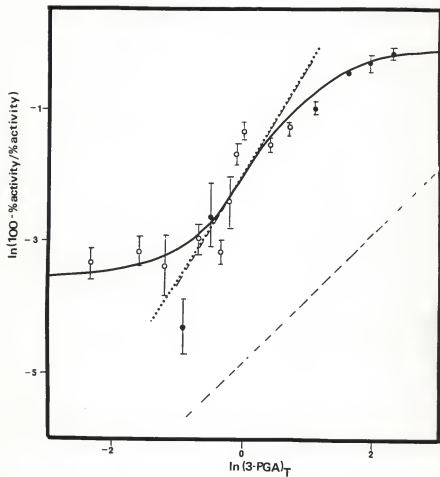


Figure 19. Hill Plot: $\ln(100-\%activity/\%activity)$ vs $\ln(3-PGA)_T$ for comfrey RuBPCase. \bigcirc , $\ln(100-\%activity/\%activity)$ for fully activated comfrey RuBPCase with specific activity of 0.60 $\mu\text{moles } ^{14}\text{CO}_2$ fixed/min-mg protein in the presence of 0-3 mM 3-PGA; \bullet , $\ln(100-\%activity/\%activity)$ for another sample of fully activated comfrey RuBPCase with the same specific activity. The solid line indicates the trend of inhibition as a function of 3-PGA concentration. The dotted line (...) through the sigmoidal curve indicates the area of maximum slope from which a Hill coefficient of 1.7 was calculated. The dashed line (---) indicates the theoretical changes in $\ln(100-\%activity/\%activity)$ with 3-PGA concentration as calculated from Equation 4 using $K_M=10 \mu\text{M}$ and $K_D=990 \mu\text{M}$. Error bars show extremes of duplicate determinations.



(Fig. 20). However, this Hill plot showed some curvature again indicating more of a sigmoidal than rectilinear type of kinetics. Hill plots of P_i inhibition of comfrey and spinach RuBPCases also curved indicating at least some cooperativity with P_i inhibition of both comfrey and spinach RuBPCases (Figs. 21 and 22). A clear maximum slope was not apparent in any of the spinach RuBPCase data, nor with comfrey RuBPCase and P_i , so Hill coefficients were not obtained from those data.

Determination of a Competitive K_i for 3-PGA for Comfrey RuBPCase

An attempt was made to determine a competitive K_i for 3-PGA using classical kinetic procedures at low RuBP concentrations, but with fully activated enzyme. Since it was proposed that certain metabolites like 3-PGA not only competed at the active site with RuBP, but also bound at the allosteric site and thereby exerted more inhibition on RuBPCase activity, the concentrations of 3-PGA were kept low in these experiments to minimize any second site effects. This appeared possible since a competitive K_i (3-PGA) for spinach RuBPCase of 840 μ M had been reported (Badger and Lorimer, 1981) whereas the K_D for allosteric site binding of 3-PGA to comfrey RuBPCase was 2.9 mM (Bolden and Mueller, 1983). In addition, the value of 840 mM suggested that 3-PGA was a weak competitive inhibitor of spinach carboxylase activity (Badger and Lorimer, 1981) which necessitated moderately low levels of RuBP for the experiment. This, in turn, should have helped to lower the affinity of the allosteric site which appeared to be greater when the active site was saturated. Unfortunately, these conditions resulted in low CPM's for the zero inhibition concentrations and even lower in the presence of 3-PGA. Consequently, the scatter in the data was larger than normally

Figure 20. Hill plot: $\ln(100 - \% \text{activity} / \% \text{activity})$ vs $\ln(3\text{-PGA})_T$ for spinach RuBPCase. \square , $\ln(100 - \% \text{activity} / \% \text{activity})$ for fully activated spinach RuBPCase with specific activity of 1.10 $\mu\text{moles } ^{14}\text{CO}_2 \text{ fixed/min-mg protein}$ in the presence of 3-PGA concentrations from 0-20 mM. The solid line indicates the observed trends of cooperativity with 3-PGA inhibition of spinach RuBPCase. Error bars show extremes of duplicate determinations.

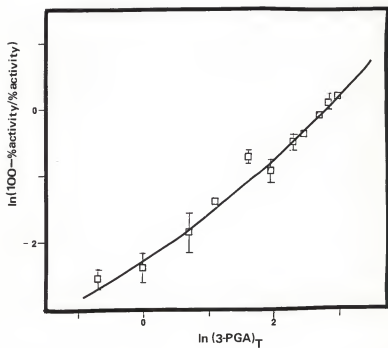


Figure 21. Hill plot: $\ln(100-\%activity/\%activity)$ vs $\ln(P_i)_T$ for comfrey RuBPCase. Δ , $\ln(100-\%activity/\%activity)$ for partially activated (1 mM $NaH^{14}CO_3$) comfrey RuBPCase with specific activity of 0.10 μ moles $^{14}CO_2$ fixed/min-mg protein in the presence of 0-20 mM P_i . The solid line indicates the observed trends of cooperativity with P_i inhibition of comfrey RuBPCase. Error bars show extremes of duplicate determinations.

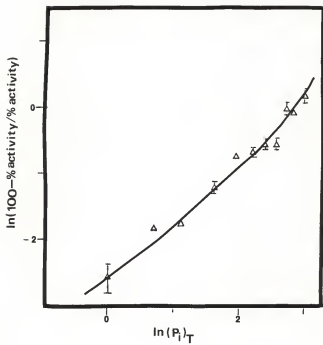
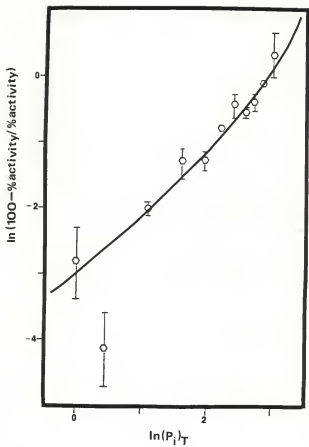


Figure 22. Hill plot: $\ln(100 - \% \text{activity} / \% \text{activity})$ vs $\ln(P_i)_T$ for spinach RuBPCase. \bigcirc , $\ln(100 - \% \text{activity} / \% \text{activity})$ for partially activated ($1 \text{ mM NaH}^{14}\text{CO}_3$) spinach RuBPCase with specific activity of $0.14 \text{ } \mu\text{moles } ^{14}\text{CO}_2 \text{ fixed/min-mg protein}$ in the presence of $0\text{--}20 \text{ mM } P_i$. The solid line indicates the observed trends of cooperativity with P_i inhibition of spinach RuBPCase. Error bars show extremes of duplicate determinations.



would be desirable. The data from several trials were analyzed with the aid of a computer program designed to statistically compare different kinetic models which was available in Dr. Larry Davis' laboratory (Table 8-11).

From the computer analysis, it could be concluded that 3-PGA was not an uncompetitive inhibitor of RuBPCase (Table 9-11). The overall error (reported as sigma) from the computer fit for uncompetitive inhibition was as much as two times more than that of either competitive or noncompetitive models. However, competitive and noncompetitive fits had essentially the same error indicating no statistically significant difference between the two types of inhibition between groups 1 vs 2. This was equally true for the combined groups after normalization of 1 to 2. Competitive inhibition fits gave K_I 's (K_{1S} calculated from the replots of the slopes) ranging from 690 +/- 275 to 883 +/- 514 which were the same within calculated errors. The value of 734 +/- 137 was considered the most representative competitive K_I because of the greater precision in the Group 1 set of data. The value also was within experimental error of the 840 μ M value reported for spinach RuBPCase (Badger and Lorimer, 1981).

A noncompetitive fit, on the other hand, gave reasonable K_I values but the raw data were not of sufficient precision that K_{II} (K_{1i} calculated from the replots of the intercepts) values could be determined accurately (Table 10; Fig. 23). Overall, the classical kinetic data may be most compatible with a mixed competitive and noncompetitive inhibition as the data presented earlier at saturating levels of RuBP had suggested.

TABLE 8
Apparent K_M 's (RuBP) for Comfrey RuBPCase from Initial Velocities in the
Absence or Presence of 3-PGA

Concentration 3-PGA	0	0.20	0.40	0.60	0.75
Group 1	22.9 +/-5	----	25.9 +/-10.4	----	44.2 +/-18.7
Group 2	5.9 +/-4.3	8.0 +/-5.2	5.1 +/-2.9	----	8.0 +/-4.8
Group 3	7.4 +/-2	13.4 +/-11.3	----	12.5 +/-7.7	----
Group 1 normalized to 2	22.9 +/-5	----	23.4 +/-8.6	----	44.2 +/-18.8
Group 1 normalized to 2 plus 2	11.2 +/-4.7	8.0 +/-5	10.2 +/-4.5	----	15.0 +/-8
Group 1 + 3 normalized to 2 plus 2	10.3 +/-2.8	10.3 +/-5	13.4 +/-4.4	12.5 +/-7.7	8.8 +/-5.5

Group 2 was the group chosen to normalize the other groups to because it had the highest activity.

K_M 's were calculated from a hyperbolic computer fit of the data.

The computer program was by Cleland, W. W. (1979) Methods in Enzymology 63, 103-137.

Group 1 was done with 0, 0.4, 0.75 mM 3-PGA; group 2 was done with 0, 0.2, 0.4, 0.75 mM 3-PGA; group 3 was done with 0, 0.2, 0.6 mM 3-PGA.

TABLE 9
Competitive Inhibition Kinetic Model

Experiment	K_M μM	V_{MAX} nmol/s/min	K_I μM	Sigma $\times 10^6$
1	20 +/-4	16 +/-2	734 +/-137	4.65
1 normalized to 2	20 +/-4	20 +/-2	713 +/-130	5.98
2	5.1 +/-1.7	14.2 +/-1.5	883 +/-514	13.6
1 normalized to 2 + combined	8.2 +/-2.5	13.8 +/-1.9	690 +/-275	16.3

The computer program was by Cleland, W. W. (1979) Methods in Enzymology 63, 103-137.

Group 1 was done with 0, 0.4, 0.75 mM 3-PGA; group 2 was done with 0, 0.2, 0.4, 0.75 mM 3-PGA.

Group 2 was the group chosen to normalize the other group to because it had the highest activity.

TABLE 10
Noncompetitive Inhibition Kinetic Model

Experiment	K_M μM	V_{MAX} nmoles/min	K_I μM	K_{II} mM	Sigma $\times 10^4$
1	22 +/-6	17 +/-3	855 +/-359	4 +/-10	4.78
1 normalized to 2	22 +/-6	22 +/-4	882 +/-370	3 +/-5	6.11
2	6.1 +/-2.7	15.2 +/-2.5	1779 +/-3085	4 +/-7	13.9
1 normalized to 2 + combined	9 +/-4	15.3 +/-3.1	1227 +/-1274	2.4 +/-3.4	16.4

The computer program was by Cleland, W. W. (1979) Methods in Enzymology 63, 103-137.

Group 1 was done with 0, 0.4, 0.75 mM 3-PGA; group 2 was done with 0, 0.2, 0.4, 0.75 mM 3-PGA.

Group 2 was the group chosen to normalize the other group to because it had the highest activity.

TABLE 11
Uncompetitive Inhibition Kinetic Model

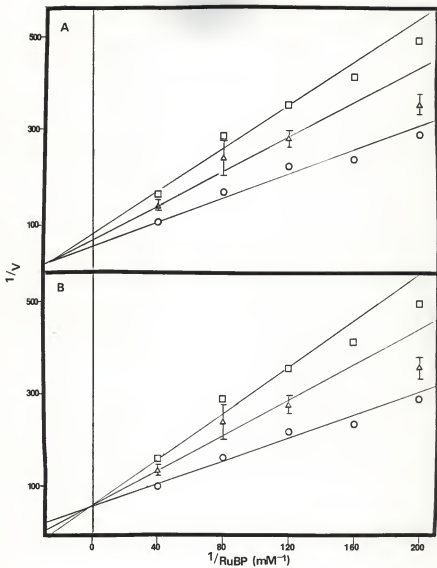
Experiment	K_M μM	V_{MAX} nmoles/min	K_{II} mM	Sigma $\times 10^4$
1	41 +/-24	21 +/-11	419 +/-253	10.3
1 normalized to 2	39 +/-21	27 +/-13	418 +/-237	13.0
2	7.5 +/-2.2	16.1 +/-2.2	1728 +/-882	13.7
1 normalized to 2 + combined	13.6 +/-5.3	15.9 +/- 4.2	891 +/-447	21.7

Group 1 was done with 0, 0.4, 0.75 mM 3-PGA; group 2 was done with 0, 0.2, 0.4, 0.75 mM 3-PGA.

The computer program was by Clelsnd, W. W. (1979) Methods in Enzymology 63, 103-137.

Group 2 was the group chosen to normalize the other group to because it had the highest activity.

Figure 23. Lineweaver-Burk plot of 3-PGA inhibition of comfrey RuBPCase. A: Noncompetitive fit \bigcirc , reciprocal velocities ($1/V$) of fully activated comfrey RuBPCase over a range of 5 to 25 μM RuBP in the absence of 3-PGA where velocity was expressed in $\mu\text{moles } ^{14}\text{CO}_2 \text{ fixed/min}$; \blacktriangle , 0.4 mM 3-PGA; \square , 0.75 mM 3-PGA. The solid lines indicate the calculated reciprocal velocities based on noncompetitive inhibition. Error bars show extremes of duplicate determinations while single data points show average reciprocal velocities from two or three determinations. Specific activity of the enzyme used was 0.90 $\mu\text{moles } ^{14}\text{CO}_2 \text{ fixed/min-mg protein}$. B: Same as A but for 3-PGA as a competitive inhibitor.



Other Potential Effectors of RuBPCase Activity

F-1,6-BP, another Calvin cycle metabolite, can be found further away from the direct action of RuBPCase in the chloroplast. F-1,6-BP is the precursor to the other product made in the Calvin cycle, F-6-P, which is transported out of the chloroplasts for gluconeogenesis or starch synthesis. Therefore, F-1,6-BP was studied as another potential regulator of RuBPCase activity to see if regulation by the precursor of the end product of the cycle could affect the initial reaction.

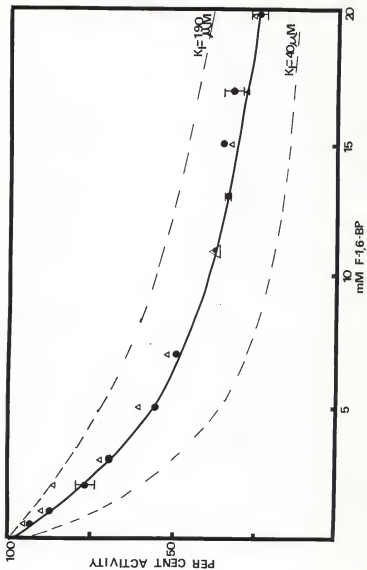
R-5-P, the precursor to the enzyme's substrate, RuBP, probably would not be expected to be an inhibitor of RuBPCase activity because a high concentration of R-5-P should lead to more RuBP production and that would not seem to be the time to shut down the Calvin cycle. If that were the case, R-5-P should not show much extra inhibition over that predicted from competition with RuBP.

As seen with either 1 mM or 20 mM NaHCO_3 in vitro, experimental values of inhibition of spinach RuBPCase in the presence of F-1,6-BP between 1 and 20 mM, fell directly in the middle of competitive inhibition as predicted by the K_I values of Chu and Bassham (1975) (190 μM) and Badger and Lorimer (1981) (40 μM). Therefore, it was concluded that F-1,6-BP probably showed little, if any, extra inhibition for spinach RuBPCase (Fig. 24) and would, therefore, probably not be an in vivo regulator of RuBPCase activity.

A K_I value for F-1,6-BP is not available at this time for comfrey RuBPCase, but the inhibition pattern both at 1 and 20 mM NaHCO_3 concentrations was virtually identical to that of the spinach enzyme and, therefore, probably is not a viable in vivo regulator (Fig. 25).

In 20 mM NaHCO_3 using two different enzyme preparations, R-5-P

Figure 24. Inhibition of spinach RuBPCase by F-1,6-BP at saturating levels of RuBP. ●, F-1,6-BP inhibition at 20 mM NaHCO₃; ▲, F-1,6-BP average inhibition by duplicate determinations at 1 mM NaH¹⁴CO₃. The dashed lines show how simple competitive inhibition with respect to 1.3 mM RuBP would vary with F-1,6-BP concentrations based on Equation 4 using a K_M=20 μM and K_I's=40 and 190 μM while the solid line indicates the observed trends with F-1,6-BP at 1 and 20 mM NaHCO₃. The error bars represent extremes of duplicate determinations. Specific activity was 1.10 μmoles ¹⁴CO₂ fixed/min-mg protein (CPM of control=2215) for the enzyme at 20 mM NaHCO₃ and 0.24 μmoles ¹⁴CO₂ fixed/min-mg protein (CPM of control=4093) at 1 mM NaH¹⁴CO₃.



showed inhibition not very different from the competitive values for comfrey RuBPCase (Fig. 26).

Dependence of Effector Inhibition on State of Activation at Saturating Levels of RuBP

The percent of inhibition of RuBPCase carboxylase activity by 2 mM and 5 mM 3-PGA was measured for the comfrey enzyme at various states of NaHCO_3 activation to determine more completely if the extra inhibition seen by 3-PGA and P_i was dependent on the enzyme's state of activation since in most cases, no differences had been observed between 1 and 20 mM activations and assays (Figs. 9-14). The concentrations of NaHCO_3 chosen for activation and assay ranged from 1 mM to 20 mM. The assay procedure was the same as that followed in the phosphometabolite inhibition studies using the positive displacement micropipettor. Based on the standard deviation of four data points at most NaHCO_3 concentrations, the percent inhibition by 3-PGA did not seem to depend on the state of activation of RuBPCase (Fig. 27). Essentially the same percentage of inhibition was observed with 2 mM and 5 mM 3-PGA no matter what concentration of NaHCO_3 was used for activation and assay. The one exception may be at lower effector concentrations and at the lowest bicarbonate concentrations where the average of four determinations appeared activating rather than inhibiting. Overall, this data confirmed the observations of Figs. 9, 13, 14, 24 and 25 where the same inhibition curves at 1 mM and 20 mM NaHCO_3 were observed for 3-PGA, P_i , and F-1,6-BP. This leaves the result with spinach and 3-PGA (Fig. 11) in question despite its apparent reproducibility.

Figure 25. Inhibition of comfrey RuBPCase by F-1,6-BP at saturating levels of RuBP. ●, F-1,6-BP inhibition at 20 mM NaHCO₃; Δ, F-1,6-BP average inhibition from duplicate determinations at 1 mM NaH¹⁴CO₃. The solid line indicates the observed trends with F-1,6-BP at 1 and 20 mM NaHCO₃. The error bars are 1 standard deviation from the mean based on three to four determinations for each effector concentration using enzyme with a specific activity of 0.80 μmoles ¹⁴CO₂ fixed/min-mg protein (CPM of control=1814) for the enzyme at 20 mM NaHCO₃ and 0.20 μmoles ¹⁴CO₂ fixed/min-mg protein (CPM of control=2824) at 1 mM NaH¹⁴CO₃.

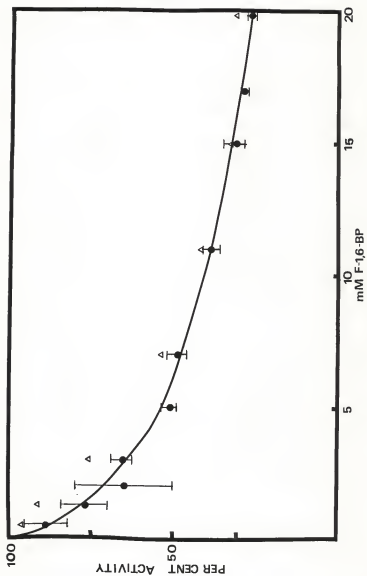


Figure 26. Inhibition of comfrey RuBPCase by R-5-P at saturating levels of RuBP. \blacktriangle , R-5-P inhibition at 20 mM NaHCO_3 (specific activity=1.30 $\mu\text{moles } ^{14}\text{CO}_2$ fixed/min-mg protein, CPM of control=2235); \bullet , R-5-P inhibition at 20 mM NaHCO_3 (specific activity=0.80 $\mu\text{moles } ^{14}\text{CO}_2$ fixed/min-mg protein, CPM of control=1814). The dashed line shows how simple competitive inhibition with respect to 1.3 mM RuBP would vary with R-5-P concentrations based on Equation 4 using a $K_M=10 \mu\text{M}$ and a $K_D=2.9 \text{ mM}$. The solid line indicates the observed trends of R-5-P inhibition. The error bars represent extremes of duplicate determinations.

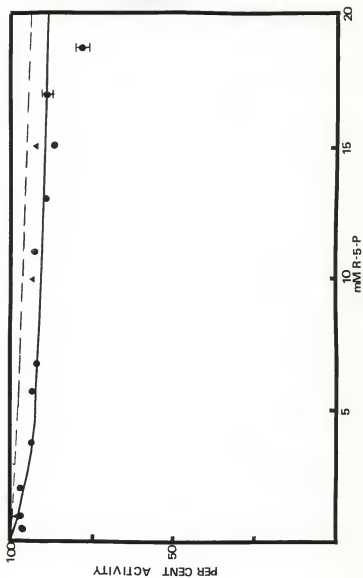
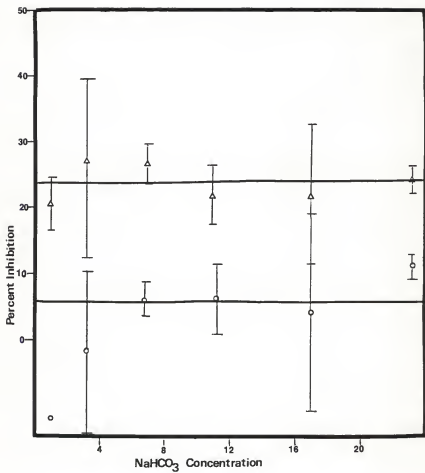


Figure 27. Dependence of allosteric inhibition on the state of activation of comfrey RuBPCase. \circ , 2 mM 3-PGA % inhibition over a range of activation states from 1-22.6 mM NaHCO_3 ; Δ , 5 mM 3-PGA % inhibition over the same range of activation states. Before NaHCO_3 activation, all samples of RuBPCase (1.5-2 mg/ml) were purged for at least 45 min. The positive displacement micropipettor used to deliver the appropriate amount of activated enzyme to the vial for reaction was sealed under N_2 . The 100% controls were assayed at each NaHCO_3 concentration in the absence of 3-PGA. The solid lines represent observed inhibition trends with 2 and 5 mM 3-PGA. The error bars are 1 standard deviation from the mean based on three to four determinations for each NaHCO_3 concentration using enzyme with an overall specific activity of 0.88 $\mu\text{moles } ^{14}\text{CO}_2 \text{ fixed/min-mg protein}$ when activated and assayed at 20 mM NaHCO_3 .



Dependence of Effector Inhibition on Mg^{+2} Concentration of Comfrey

RuBPCase

To see if effector inhibition was dependent on Mg^{+2} concentration, comfrey RuBPCase was assayed at 1 and 20 mM $NaHCO_3$ with either 10 or 16-18 mM Mg^{+2} in the presence of 5 and 10 mM 3-PGA. No differences in activity without effector, or in inhibition with 5 or 10 mM 3-PGA could be observed between either activation state indicating that it was not the Mg^{+2} *3-PGA complex doing the inhibiting of RuBPCase activity (Table 12). This also ruled out the paralleling effect of the experimental and theoretical competitive curves at high 3-PGA concentrations (greater than 10 mM) being caused by a limited amount of free Mg^{+2} (10 mM) in solution. Overall, the data suggested that 3-PGA or the 3-PGA* Mg^{+2} complex was causing the observed inhibition of RuBPCase activity.

Oxygenase Activity and pH Optimum

Oxygenase activity was measured for both spinach and comfrey RuBPCases. The enzymes were eluted from the DEAE-cellulose column in 10 mM Tris-HCl instead of 50 mM Tris-HCl so that when mixed with the TME buffer at a different pH, the pH of the mixture would be closer to that of the added buffer than that of the original enzyme solution. In that way, better control of the final pH was possible and a pH optimum could be found for the oxygenase activity more easily. The enzyme and buffer were exhaustively purged before activation with known exact amounts of $NaHCO_3$ present. This was also part of the reason for completely filling the mixing chamber with enzyme plus buffer and why the mixing chamber was sealed from the atmosphere during equilibration and reaction.

TABLE 12
Effect of Mg^{+2} Concentration on 3-PGA Inhibition of Comfrey RuBPCase

Assay conditions	Specific activity ^a	% Inhibition ^b	
		5 mM 3-PGA	10 mM 3-PGA
20 mM $NaHCO_3$ /10 mM Mg^{+2}	1.16	----	----
1 mM $NaH^{14}CO_3$ /10 mM Mg^{+2}	0.06	33.9	70.1
20 mM $NaHCO_3$ /20 mM Mg^{+2}	1.04	----	----
1 mM $NaH^{14}CO_3$ /20 mM Mg^{+2}	0.09	32.9	70.9
22.6 mM $NaHCO_3$ /20 mM Mg^{+2}	1.25	38.0	66.6

a: Specific activity: μ moles $^{14}CO_2$ fixed/min-mg protein.

b: Percent inhibition figured from a control without effector at each $NaHCO_3$ and Mg^{+2} concentration.

Keeping the mixing chamber filled and sealed also helped maintain a constant level of O_2 before the reaction. Since CO_2 activation is an absolute requirement for oxygenase activity, but inhibitory during assay, keeping $NaHCO_3$ only at 1 mM helped maximize oxygenase activity with a minimum amount of competitive carboxylation.

For comfrey RuBPCase oxygenase activity, the pH optimum seemed to be between pH 8.40 and pH 8.80 (Fig. 28). By assaying different preparations of the enzyme, it was observed that when the amount of $^{14}CO_2$ fixed for the carboxylase activity was low, the amount of O_2 fixed from the atmosphere also decreased a corresponding amount. For enzyme isolated in September of 1982 (carboxylase activity=1.10 μ moles $^{14}CO_2$ fixed/min-mg protein), the amount of O_2 fixed at optimum pH was 0.062 μ moles O_2 /min-mg protein. But for enzyme isolated in June of 1983 (carboxylase activity=0.60 μ moles $^{14}CO_2$ fixed/min-mg protein), the amount of O_2 fixed at optimum pH was only 0.031 μ moles O_2 /min-mg protein.

For spinach RuBPCase oxygenase activity at 1 mM $NaHCO_3$ activation concentration, the pH optimum was broad but occurred between pH 8.4 and pH 8.9. At 20 mM $NaHCO_3$ activation, the pH optimum for the oxygenase activity seemed to shift slightly to a higher range between pH 8.5 and pH 9.1 (Fig. 29). Even with the slight shift to a higher pH optimum, the amount of O_2 fixed at pH optimum in 20 mM activation was essentially the same amount of O_2 fixed at 1 mM activation. This spinach oxygenase activity of 0.60 μ moles O_2 fixed/min-mg protein (carboxylase activity=1.0 μ moles $^{14}CO_2$ fixed/min-mg protein) was also the same as the comfrey oxygenase activity of 0.062 μ moles O_2 fixed/min-mg protein

Figure 28. pH optimum for comfrey RuBPCase oxygenase activity at 1 mM NaHCO_3 activation. \circ , oxygenase activity of RuBPCase with a carboxylase specific activity of 0.60 $\mu\text{moles } ^{14}\text{CO}_2$ fixed/min-mg protein; \bigcirc , oxygenase activity of RuBPCase with a carboxylase specific activity of 1.10 $\mu\text{moles } ^{14}\text{CO}_2$ fixed/min-mg protein; Δ , oxygenase activity of RuBPCase with a carboxylase specific activity of 1.07 $\mu\text{moles } ^{14}\text{CO}_2$ fixed/min-mg protein. Before oxygenase assay, all RuBPCase samples were purged with CO_2 free air and then activated with 1 mM NaHCO_3 . The final NaHCO_3 concentration was 1 mM after dilution of the activated enzyme for oxygenase assay. The solid lines indicate the observed trend of oxygenase activity from pH 7.8 to 9.4 for several different samples of RuBPCase. The error bars represent extremes of duplicate determinations.

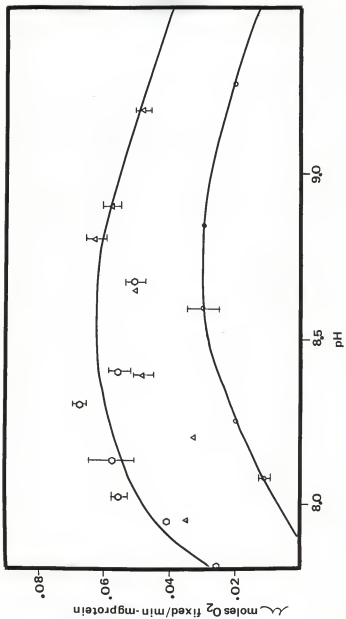
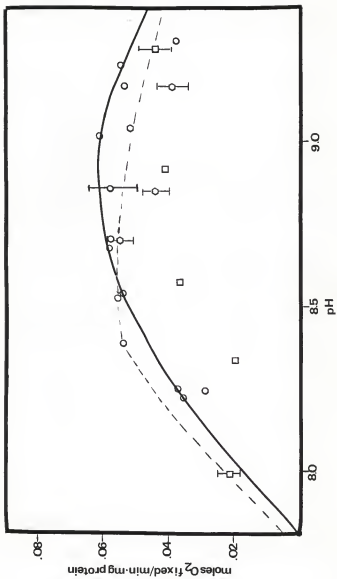


Figure 29. pH optimum for spinach RuBPCase oxygenase activity at 1 and 20 mM NaHCO_3 activation. \circ , oxygenase activity at 1 mM NaHCO_3 activation of RuBPCase with a carboxylase specific activity of 1.00 $\mu\text{moles } ^{14}\text{CO}_2$ fixed/min-mg protein; \square , oxygenase activity at 1 mM NaHCO_3 activation for a different sample of RuBPCase with a carboxylase specific activity of 1.00 $\mu\text{moles } ^{14}\text{CO}_2$ fixed/min-mg protein; \bigcirc , oxygenase activity after 20 mM NaHCO_3 activation of RuBPCase with a carboxylase specific activity of 1.00 $\mu\text{moles } ^{14}\text{CO}_2$ fixed/min-mg protein. Final NaHCO_3 concentrations at assay were 1 mM for 1 mM NaHCO_3 activation and 1.1 mM for 20 mM activation. Each single point is from a single determination. The solid line indicates the observed trend of oxygenase activity after 20 mM NaHCO_3 activation from pH 8.0 to 9.3, while the dashed line indicates the observed trend of oxygenase activity from two RuBPCase samples for 1 mM NaHCO_3 activation over the same range of pH. The error bars represent extremes of duplicate determinations.



(carboxylase activity=1.10 μ moles $^{14}\text{CO}_2$ fixed/min-mg protein) at a similar optimum pH.

Discussion

Refinement of Purification Techniques

The RuBPCase isolation and further purification procedures developed by Simpson *et al.* (1983) proved to give consistent results in the past so little was changed except for the final DEAE-cellulose column chromatography step and the inclusion of 10 μ M leupeptin in the isolation. The DEAE procedural change involved stepwise elution with TME, pH 7.5 buffer made 0.15 M in NaCl instead of the 0.25 M NaCl previously used. The elution profiles were nearly identical (Fig. 4) for the two salt concentrations and the lower concentration was selected because Cl^- had been shown to be a weak inhibitor of RuBPCase activity (Bonsall, 1981). Leupeptin was included routinely because Johal and Chollet (1983) found a modest change in the ratio of the intensities of the large subunit bands on isoelectric focusing gels in the presence of this thiol protease inhibitor. There is, however, some question as to whether leupeptin interacts with the reducing agent (dithionite and DTT) used with most higher plant RuBPCase purifications. This point needs to be checked since such reactions could inactivate the protease inhibitor.

A major departure from earlier procedures and assays involved the purification method for the substrate, RuBP. RuBP purification using a Dowex-1 column provided superior activity of RuBPCase over the stepwise Amberlite ion exchange resin method previously used (Bolden, 1983). Otherwise, the activation and assay of RuBPCase were done with essentially the same procedures established by Simpson *et al.* (1983) except for the reduction of the assay volume from 553 to 300 μ l so that

smaller proportions of reagents were required. The previous ratio of RuBP to RuBPCase was retained under the two sets of conditions (0.38 μ moles of RuBP and 20 μ g of RuBPCase) since the measured activities had been found to be somewhat sensitive to this ratio (Simpson et al., 1983).

At the onset of the work for this thesis, the specific activity of the comfrey RuBPCase preparations dropped dramatically and suddenly. Instead of the normal 1.2 to 1.5 units, specific activities of only 0.6 to 0.8 were obtained with several preparations in the summer and fall of 1983. There are a host of possible steps where activity can be lost. However, the purification of the commercial spinach enzyme, which begins with the sucrose density gradient step, still yielded good activities. That suggested that the problem was not in the assay or later stages of purification, but rather in an early step of the scheme, perhaps even the leaves themselves. Another item that stood out was that a new lot number of insoluble PVP had been used when the low activity first appeared. While awaiting the arrival of PVP from a new vendor (the first of which proved to be soluble) several early steps in the procedure were reinvestigated and some work toward the thesis initiated. In summary, none of the steps checked proved to alter the specific activity significantly until a new insoluble PVP was tried (Tables 1-6). No really good explanation comes to mind for why one PVP works while another does not, but the data strongly suggest that the first new batch of PVP was the culprit. Once normal specific activities were regained, most of the earlier inhibition studies were repeated. It was found that the percent inhibition of comfrey RuBPCase activity, as measured from a control sample without effector, was the same whether the specific

activity was 0.6 or 1.5 units (Fig. 10). This suggests that the low specific activity resulted from a mixture of active (or activatable) and inactive (or inactivatable) enzyme molecules and that the earlier inhibition data were valid.

Oxygenase activity and pH optimum

RuBPCase isolated from comfrey was found to have an oxygenase activity plus a carboxylase activity just like that for all other sources of the enzyme so far investigated (Andrews et al., 1973; Bowes and Ogren, 1971). Though the pH optimum for spinach and comfrey carboxylases appeared to differ somewhat (up to 0.5 pH units), the pH optimum between the oxygenases from the two sources seemed more similar and to fall between pH 8.4-pH 8.9. In agreement with more recent work (Andrews et al., 1973; Bahr and Jensen, 1974) the range is the same as that for the pH optimum for carboxylase activities and corroborates the common active site hypothesis for the two activities (Badger and Andrews, 1974; Bowes and Ogren, 1971). It also was observed that the oxygenase activities of both spinach and comfrey RuBPCases were dependent on the carboxylase activities. If the carboxylase activity was down, the oxygenase activity was also lower. This strongly suggests that at least the same activation process is required for both activities.

An oxygenase activity for spinach of approximately 0.06 $\mu\text{moles O}_2$ fixed/min-mg protein at both 1 and 20 mM NaHCO_3 activation was obtained compared to 0.20 $\mu\text{moles O}_2$ fixed/min-mg protein for the spinach enzyme activated at 10 mM NaHCO_3 reported by Badger et al. (1976). Apparently during the 4 min incubation of the activated enzyme and buffer in the electrode chamber to establish a rate to correct for the consumption of

O₂ by DTT, the enzyme activated at 20 mM NaHCO₃ had time to deactivate. This was possible because the enzyme activated at 20 mM bicarbonate was diluted into a buffer containing no NaHCO₃ to give a final 1 mM NaHCO₃ concentration like that used with the enzyme activated at 1 mM NaHCO₃. Since the two oxygenase activities were about the same for these two activation states, the fully activated enzyme must have reverted to the same state of activation as at 1 mM NaHCO₃. Therefore, the factor of 3-4 between these results and those of Badger et al. (1976) probably reflects a lower state of activation. That factor is somewhat smaller than the eight-fold increase in carboxylase activity between the same two states of activation. The oxygenase activity for the comfrey enzyme was also approximately 0.06 μ moles/min-mg protein for 1 mM NaHCO₃ activation and assay.

In regard to the results of Badger et al. (1976), the specific oxygenase activity reported for spinach was not corrected for a probable DTT reaction with O₂. The authors passed the enzyme through a G-25 Sephadex column apparently containing 1 mM DTT to strip the enzyme of CO₂ and Mg⁺² before oxygenase reaction was initiated. Since the enzyme was stripped of Mg⁺² after CO₂ activation, it is questionable how any oxygenase activity was observed in the first place over the 2 min reaction time since stripping Mg⁺² from comfrey RuBPCase almost totally deactivates the enzyme within 20 s (Esser, 1982). But taking their procedures as reported, the "oxygenase" activity measured probably was more of an oxygenase plus DTT reaction with O₂ assuming the enzyme from the Sephadex column was diluted comparably to that reported here. If the DTT rate had not been substrated in the oxygenase activities stated in this report, a 30% greater "oxygenase" activity would have been

obtained.

Therefore, an unknown amount of the overall activity reported by Badger et al. (1976) could be from DTT reaction with O_2 instead of oxygenase activity depending on the amount of enzyme added to start the reaction. One possible reason for this apparent oversight could be how the experiment was performed. Instead of letting the enzyme equilibrate in the electrode chamber before initiating the reaction with RuBP, Badger and coworkers initiated the oxygenase reactions by adding activated enzyme to buffer containing RuBP probably to keep a higher state of CO_2 activation during the initial time of the reactions. Therefore, the oxygenase activity they observed might be higher because of the higher state of activation, if for some reason stripping the Mg^{+2} did not cause as quick a loss of activation in their hands. They also reported a carboxylase specific activity of fully activated enzyme of 1.60 to 1.80 $\mu\text{moles } ^{14}CO_2 \text{ fixed/min-mg protein}$ compared to a carboxylase specific activity of 1.00 $\mu\text{moles } ^{14}CO_2 \text{ fixed/min-mg protein}$ for the fully activated enzyme used for the standard carboxylase assays here. Since the amount of carboxylase activity seems to correlate with the amount of oxygenase activity observed, the difference in carboxylase activities between these studies can also account for some of the difference between the oxygenase activities.

Determination of K_I for 3-PGA and K_M for RuBP for Comfrey RuBPCase

To determine if any of several Calvin cycle intermediates showed extra inhibition over and above that predicted from simple competition with RuBP for the active site, values of K_I for competitive inhibition were needed. Badger and Lorimer (1981) reported K_I values at 25°C for a

number of metabolites using spinach RuBPCase. Since our work was at the same temperature, these values could be used directly with that enzyme. The same type of information, however, was not available for comfrey RuBPCase. What was available were K_D values for active site binding of most of the intermediates as determined by difference UV spectroscopy and membrane partition experiments (Bolden, 1983). It had been noted that the K_D obtained for 3-PGA (Bolden and Mueller, 1983), along with those for several other metabolites, agreed reasonably well with the K_I values for spinach RuBPCase. What was not known, however, was whether the K_I 's were comparable for both enzymes. Therefore, attempts were made to determine K_I for 3-PGA using the comfrey enzyme. Unfortunately, the low inhibitor and substrate concentrations, deemed necessary to create a predominantly competitive state, gave less accurate data than normally would be desirable. This may have been an attributing factor to the inability to distinguish between competitive and noncompetitive kinetics using these procedures.

K_I for competitive inhibition of 3-PGA with comfrey RuBPCase was $734 \mu\text{M} \pm 137$ (Table 9) compared to a K_D of 990 ± 40 or 690 ± 50 (Bolden and Mueller, 1983; Bolden, 1983) and a K_I for spinach RuBPCase of $840 \mu\text{M}$ (Badger and Lorimer, 1981). Thus, it was concluded that the K_D 's determined for active site binding of phosphometabolites probably were reasonably close to the K_I 's and, therefore, could be used to predict competitive inhibition curves at least within the degree of accuracy required to detect extra inhibition. Tables 13 and 14 list K_I 's and K_D 's used to calculate theoretical competitive curves for spinach and comfrey RuBPCases in the presence of various effectors. From this same set of data, a K_M (RuBP) for comfrey RuBPCase was

TABLE 13
K_D's for Comfrey RuBPCase

Effector	K _D (μM)
3-PGA	990 ^a +/- 40 690 +/- 50
P _i	750 ^b
F-1,6-BP	-----
R-5-P	2900 ^b +/- 100

a. Bolden and Mueller (1983)

b. Bolden (1983)

TABLE 14
K_I's for Spinach RuBPCase

Effector	K _I (μM)
3-PGA	840 ^c
P _i	900 ^c
F-1,6-BP	400 ^c 190 ^d
R-5-P	-----

c. Badger and Lorimer (1981)

d. Chu and Bassham (1975)

determined. A K_M of 10 μM (average was 13 \pm 3.1, Table 8) was used for all calculations instead of the K_M of 30 μM reported previously (Simpson *et al.*, 1983). The K_M (RuBP) for spinach RuBPCase was taken as 20 μM (Jensen and Bahr, 1977).

However, based on the computer analysis of the inhibition data, it was not possible to distinguish between pure competitive inhibition and noncompetitive inhibition. The overall error (sigma) for a noncompetitive fit of the inhibition data was essentially the same as the error for the competitive fit. Active site K_I for the noncompetitive fit (855 \pm 359)(Table 10) was also essentially the same for the competitive fit (734 \pm 137)(Table 9). However, the K_{II} was not well determined since its error was larger than its value (4 \pm 10). Nevertheless, the value obtained was not far from the 2.9 mM K_D for allosteric binding of 3-PGA to the comfrey enzyme. Uncompetitive inhibition could be ruled out because of significantly higher sigma values for the same data sets. Overall, it appears that the classical enzyme inhibition experiments with 3-PGA and comfrey RuBPCase indicate a mixed mode of inhibition. This is also what was observed at saturating levels of RuBP with 3-PGA and P_1 .

Initial Velocity Studies of RuBPCases in the Presence or Absence of 3-PGA

To examine the potential inhibition of RuBPCase carboxylase activity by phosphometabolites, it was necessary to establish how fast maximum inhibition was reached. Initially, it was shown that phosphometabolites, such as 3-PGA, exert their inhibition immediately after mixing with RuBPCase and saturating concentrations of RuBP (1.3

mM) for both spinach and comfrey (Figs. 7 and 8) RuBPCases. The data showed that within 3 s after adding substrate and effector, in that order, to an activated enzyme solution, a new initial rate was established which persisted for about the same length of time as it had in the absence of effector. Consequently, the amount of inhibition observed was not dependent on the time after mixing using the current assay conditions. Furthermore, the behavior in the absence of effector was comparable to that observed earlier with comfrey RuBPCase (Simpson *et al.*, 1983; Esser, 1982) and with the spinach enzyme (Lorimer *et al.*, 1976; Whitman *et al.*, 1979).

Possible Explanations of Extra Inhibition Beyond Competitive by Phosphometabolites

When 3-PGA and P_i were added to spinach and comfrey RuBPCase assay mixtures at physiologically significant concentrations (low mM range), the inhibition produced was considerably more than that predicted from simple competition with RuBP for the active site whereas F-1,6-BP and R-5-P showed little, if any, extra inhibition. At low concentrations of 3-PGA (0.1 to 5 mM), the amount of inhibition was as much as 10 times the competitive value in the presence of saturating RuBP (1.3 mM) (Fig. 9) for comfrey RuBPCase. But, at 3-PGA concentrations above 5 mM inhibition reverted to a strictly competitive nature as evidenced by the absence of any further extra inhibition (Fig. 9). Similar trends were also observed for spinach RuBPCase but not to as great an extent (Fig. 10). In contrast, the amount of extra inhibition created by P_i was not as large as that with 3-PGA with enzyme from either source (Figs. 13 and 14). In addition, higher concentrations of P_i were required before its

inhibition reverted to a strictly competitive pattern. Dixon plots of the same total observed inhibition were concave upward for 3-PGA with comfrey RuBPCase only in the low mM range, compared to the same plots for P_i which curved upward more gently with either enzyme over the whole 0.5 to 20 mM span. Such concave upward Dixon plots were indicative of inhibition from two or more sites. The corresponding Hill plots were nonlinear in all cases with 3-PGA and P_i and distinctly sigmoidal for 3-PGA with comfrey RuBPCase where the data extended to lower concentrations. A sigmoidal Hill plot was indicative of site-site interactions among the inhibitory sites. The Hill coefficient estimated for 3-PGA from the low concentration data, however, indicated only a moderate degree of cooperativity for a hexadecameric enzyme. In contrast, the Hill plots for F-1,6-BP and R-5-P inhibition (Figs. 24-26) were essentially linear (plots not shown).

Competitive inhibition at subsaturating RuBP (approximately 50 μ M) has been reported for several phosphometabolites (Badger and Lorimer, 1981; McCurry et al., 1981) unlike the substantial extra inhibition seen by the phosphometabolites 3-PGA and P_i in the presence of saturating RuBP. There are several possible explanations for this observation. First, the extra inhibition seen particularly with 3-PGA could be from product inhibition through a deadend complex. However, spinach RuBPCase activity has been observed to follow a steady rate for at least 30 min (McCurry et al., 1981) as did comfrey RuBPCase activity for at least 3 min (Fig. 7). If 3-PGA, the product of the carboxylation reaction, formed irreversible inhibition with RuBPCase, the activity would be expected to have declined steadily over such a long time span. Also, since P_i , which is not a direct product of the carboxylase reaction,

shows extra inhibition with RuBPCase in the presence of saturating RuBP, the possibility of product inhibition by a deadend complex is substantially reduced. Secondly, this extra inhibition was not an ionic strength effect as seen in Fig. 9 with equivalent amounts of NaCl replacing 3-PGA. Therefore, it appeared unlikely that ionic strength effects alone were responsible for the extra inhibition seen with 3-PGA or P_i .

Another possibility was that K_I and/or K_M values used to predict the competitive curves were incorrect. However, even if the K_I value used for 3-PGA was as small as 400 μ M, extra inhibition would still have been observed. Similarly, a K_M (RuBP) of 50 μ M instead of 10 or 20 μ M would still have shown extra inhibition with 3-PGA. A combination of the two; that is, a K_I of half the 840 or 740 μ M used and a 2.5 to 5 times larger K_M , however, would be required to eliminate the extra inhibition. It seems unlikely that either was in error by that amount, especially since 20 μ M K_M (RuBP) for spinach RuBPCase has been reported from several laboratories and the K_I (3-PGA) for comfrey (740 \pm 130 μ M) agreed moderately well with 840 μ M reported for the spinach enzyme. In other words, although a degree of uncertainty existed in the K_I and K_M determinations, the extra inhibition would not have been expected to be a total consequence of these errors.

Extra inhibition by 3-PGA and P_i for spinach and comfrey RuBPCases also seemed to be independent of the state of activation. When activated and assayed at both 1 mM and 20 mM NaHCO_3 , usually the same amount of extra inhibition in the presence of 3-PGA and P_i was observed (Figs. 9-12). The only major exception seen was with 3-PGA and spinach RuBPCase at 1 mM $\text{NaH}^{14}\text{CO}_3$ activation. Extra inhibition of 3-PGA at 1 mM

$\text{NaH}^{14}\text{CO}_3$ was approximately 10% more than that observed either at 10 mM or 20 mM NaHCO_3 and appeared to be beyond the precision of the data. The reason for this difference remains unclear at this time. In general, it can be concluded from the data that no matter what the state of enzyme activation would be in the actual plant chloroplast, competitive inhibition or extra inhibition would be possible whenever the concentration of 3-PGA or P_i were in the low mM range.

Neither was the extra inhibition due to competition with the second substrate, CO_2 , during catalysis since again the extent of inhibition was the same at 20 mM NaHCO_3 as it was at 1 mM $\text{NaH}^{14}\text{CO}_3$. This was in agreement with the observations from other laboratories that phosphometabolite inhibition had been shown to be uncompetitive with the catalytic CO_2 (Paulsen and Lane, 1966; Laing and Christeller, 1980).

A more viable explanation of the extra inhibition appears to be that 3-PGA and P_i exert their extra inhibition through the recently discovered allosteric site in comfrey RuBPCase. As inferred from the difference UV data (Bolden and Mueller, 1983), the active site needed to be saturated before any significant binding at physiological concentrations of effector would have occurred at this second site. Based on the multiple, noninteracting site model used for effector binding in the absence of an active site inhibitor, the dissociation constants in the presence of saturating levels of inhibitor indicated at least a 5 to 20-fold increase in the affinity of the allosteric site when the active site was filled. There are no previous reports in the literature of inhibition studies at saturating RuBP concentrations. Therefore, if RuBP behaved like CHBP, the inhibitor used in the difference UV studies, the allosteric site should have had a greater

affinity for these studies at saturating RuBP than earlier reports using the subsaturating substrate levels employed in classical inhibition studies. The Dixon plots (Figs. 15 through 18) corroborated this possibility by indicating two site inhibition for 3-PGA and P_1 inhibition of spinach and comfrey RuBPCases.

The observation of similar extra inhibition by 3-PGA and P_1 for both spinach and comfrey RuBPCases suggests that the allosteric site exists in the spinach enzyme but is spectroscopically silent when bound. The Trp or Tyr environment that is perturbed when the allosteric site of comfrey RuBPCase is bound is not altered enough in the spinach enzyme to cause a significant absorbance change, or perhaps one of these aromatic amino acids is missing. There are other apparent differences between the allosteric sites in spinach and comfrey RuBPCases. Notably, the amount of extra inhibition is not as great with spinach RuBPCase at 10 and 20 mM NaHCO_3 as for comfrey RuBPCase at 20 mM NaHCO_3 .

Both enzymes, however, are affected by 3-PGA more in the lower than at higher concentrations. It seems that after a certain point (approximately 5-7 mM 3-PGA) the allosteric effect is saturated and above that point only competitive inhibition is observed but at a new lower overall level. This results in the theoretical competitive and experimental curves paralleling each other above about 10 mM 3-PGA for both spinach and comfrey RuBPCases. These same inhibitory effects were seen with various preparations of spinach and comfrey RuBPCases verifying that the overall allosteric effect was not an artefact of one preparation or of storage of the preparations at -70°C over a long period of time (Figs. 10 and 12).

There is some evidence in the literature for multiple site

inhibition by 3-PGA and P_i . Laing and Christeller (1980) used concentrations of 3-PGA large enough to possibly bind to an allosteric site (up to 20 mM 3-PGA) even at partially saturating levels of RuBP in their effector inhibition studies with soybean RuBPCase. Inhibition beyond competitive was clearly seen in the Dixon plots for 3-PGA and P_i which curved (concave) considerably. This observation was merely reported but not really discussed or apparently investigated further. Bhagwat (1981) also saw possible two site binding for P_i when he looked at P_i inhibition with the spinach enzyme. In his studies, he preincubated RuBPCase with 0.5 to 20 mM P_i , which activated the enzyme, and then assayed in the presence of the same P_i concentrations. He also assayed CO_2 activated enzyme in the presence of similar P_i concentrations which had not been preincubated with P_i (control). In both sets of assays, he observed inhibition beyond 6.5 mM P_i . With the control assay, inhibition was seen with as little as 0.5 mM P_i . To characterize this inhibition, a Dixon plot was made from the control assays. Instead of being linear, the plot definitely curved upward signifying two or more site binding. It appeared, therefore, that the presence of an inhibitory allosteric site could account for some of the observation seen in the literature as well.

A requirement for the allosteric site to be a type of feedback inhibition site would be site specificity. This is demonstrated to a certain extent by the current data. Some of the effectors such as 3-PGA and P_i seem to bind to the allosteric site and cause extra inhibition, while others such as F-1,6-BP and R-5-P seem to bind significantly only at the active site. These differences in inhibition suggest that only certain effectors exert an effect through the allosteric site. In

agreement with the selectivity reported by Bolden (1983), R-5-P and F-1,6-BP, both ring structures, would not be expected to bind the allosteric site effectively, whereas small linear molecules such as 3-PGA seem to be more suitable.

Based on the above literature observations and from the data presented here, it is a strong possibility that 3-PGA and P_i exert their extra inhibitory effects through a second site especially when the active site is filled. The overall importance could be that 3-PGA is acting as a feedback inhibitor in vivo. When the RuBP concentration rises and a large quantity of reduced CO_2 is fixed, the concentration of 3-PGA rises rapidly in the stroma of the chloroplast. The higher levels of 3-PGA may bind to the allosteric site decreasing RuBPCase activity until the concentration of 3-PGA can be stabilized by either breakdown of 3-PGA to DHAP or by translocation of 3-PGA to the cytosol by the P_i translocator (Lorimer et al., 1978). When the 3-PGA concentration has decreased to a minimum, 3-PGA would dissociate from the allosteric site and carboxylation would be enhanced. Thus, instead of an all-or-none control such as activation by CO_2 and Mg^{+2} , 3-PGA seems more likely to be part of a fine-tuning mechanism to keep metabolites at the appropriate steady state concentrations in vivo.

As to the role of P_i as an inhibitor, it is hard to pinpoint because it participates in a host of reactions. It is known that P_i is required for light activation of RuBPCase activity in the chloroplast (Lorimer et al., 1978). P_i also might be acting directly on the RuBPCase enzyme through the allosteric site during the dark cycle where its concentration is several fold higher than after several min of photosynthesis.

Summary

1. RuBPCase can be eluted from a DEAE-cellulose column with 0.15 M NaCl instead of 0.25 M NaCl. This probably is advantageous for RuBPCase since Cl^- can be a weak inhibitor.
2. Elution of RuBP from a Dowex-1 column to give $(\text{Li}^+)_4\text{RuBP}$ produced a superior product compared to that obtained in the past.
3. Comfrey RuBPCase has oxygenase activity with a pH optimum of 8.4 to 8.8.
4. Oxygenase and carboxylase activities are very closely correlated since when the carboxylase activity is low, the oxygenase activity is too, suggesting the same site of activation and/or activity for both enzyme functions.
5. All effectors, when added immediately after saturating amounts of RuBP exert their effects on the activity of both spinach and comfrey RuBPCases within 3 s.
6. Because the inhibition produced by 3-PGA and P_i was considerably more than that predicted from simple competition with saturating RuBP, they may be exerting their extra inhibition through the recently discovered allosteric site on comfrey RuBPCase. In that case, 3-PGA may act as a type of feedback inhibitor of RuBPCase activity.
7. Since similar amounts of extra inhibition beyond competitive were observed with 3-PGA and P_i with spinach RuBPCase, spinach RuBPCase most likely has an allosteric site as well through which the extra inhibition is exerted.

8. The same inhibitory allosteric effects were seen with various preparations of spinach and comfrey RuBPCases indicating the observed effects are not an artefact of any one preparation or procedure.
9. Curvature of Dixon and Hill plots of 3-PGA and P_i inhibition data also suggest two inhibition constants which can result in two site binding and some cooperativity between sites on both comfrey and spinach RuBPCases by these effectors.
10. 3-PGA, the product of the carboxylation reaction, exerts more of an inhibitory allosteric effect on RuBPCase activity than P_i . This is compatible with the specificity that had been observed for the allosteric site in that 3-PGA had over twice the affinity of P_i .
11. The amount of inhibition of the comfrey enzyme does not show any substantial dependence on NaHCO_3 concentration or ionic strength except possibly for the very lowest effector concentrations studied. There is, however, still some question in this regard for spinach RuBPCase.
12. F-1,6-BP and R-5-P, both ring structures, apparently do not bind the allosteric site in either spinach or comfrey RuBPCases as evidenced by their lack of any substantial extra inhibition. This is also compatible with the specificity of the allosteric site as studied by difference UV methods in that it showed almost no affinity for glucose-6-phosphate and only weak binding of R-5-P.

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EVIDENCE FOR ALLOSTERIC INHIBITION OF RIBULOSE-1,5-BISPHOSPHATE
CARBOXYLASE/OXYGENASE BY PHOSPHOMETABOLITES

by

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Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCase, E.C. 4.1.1.39), which is thought to be the world's most abundant protein, catalyzes the carboxylation and cleavage of ribulose-1,5-bisphosphate (RuBP) in the Calvin cycle to form two molecules of 3-phospho-D-glyceric acid (3-PGA). RuBPCase has a competitive oxygenase activity which converts RuBP and molecular oxygen to one molecule of phosphoglycolate (PG) and one molecule of 3-PGA. Higher plant RuBPCases consist of 8 large subunits encoded by chloroplastic DNA and 8 small partially heterogeneous subunits encoded by nuclear DNA. Activation by CO_2 in the presence of a divalent metal ion to form a carbamate with a lysine residue on the large subunit is an absolute requirement for activity. The most recent evidence suggests that activation is enhanced by preincubation with certain phosphometabolites and that the effect occurs via active site binding. Consequently, if added with or after the substrate, RuBP, these metabolites are competitive inhibitors. Recent evidence from difference UV studies from this laboratory has shown substantial nonactive site binding of the same phosphometabolites for comfrey RuBPCase when the active site was saturated. These studies have been extended to determine the function of this allosteric site. This was done by comparing the enzymatic activity at saturating levels of RuBP (1.3 mM) in the presence or absence of phosphometabolites. When 3-PGA was added 2-3 s after RuBP to the partially or fully CO_2 activated comfrey enzyme in a 50 mM Tris-HCl, 10 mM MgCl_2 , 1 mM EDTA, 2 mM dithiothreitol, pH 7.5 buffer containing 1 or 20 mM NaHCO_3 , the carboxylase activities were reduced markedly compared to those expected from competitive inhibition. Between 1 and 5 mM 3-PGA, the extra inhibition amounted to an 8 to 10-fold increase. At higher

concentrations of 3-PGA, the amount of extra inhibition declined to a 3-fold increase and ultimately seemed to parallel the predicted competitive curve between 10 and 20 mM effector. Addition of equivalent ionic strength amounts of NaCl slightly activated RuBPCase indicating the extra inhibition observed was not a generalized ionic strength effect. Furthermore, increasing Mg^{+2} concentration to 20 mM Mg^{+2} and varying $NaHCO_3$ concentrations during activation and assay, in general, did not alter the amount of inhibition observed at any concentration of 3-PGA. Thus, the extra inhibition was not a result of limiting amounts of Mg^{+2} nor apparently did it depend on the state of activation of the enzyme or on the levels of CO_2 during assay. Inorganic phosphate (P_i) also showed extra inhibition but to a smaller extent than found with 3-PGA. The effect, however, was observable over the whole 0.5-20 mM range investigated. Purified spinach RuBPCase gave analogous results with the same two effectors. Dixon plots of the inhibition data were concave up with P_i for both sources of the enzyme and distinctly concave up with 3-PGA for the comfrey RuBPCase when the data were extended down to 0.1 mM effector. Hill plots of the same data were all nonlinear and sigmoidal for 3-PGA when extended to low concentrations. A Hill coefficient of 1.7 was obtained for the inhibitory effects of 3-PGA with the comfrey enzyme. Fructose-1,6-bisphosphate (F-1,6-BP) and ribose-5-phosphate (R-5-P), on the other hand, did not show any substantial extra inhibition with either enzyme. That was in agreement with the known reduced affinity of R-5-P for the allosteric site on comfrey RuBPCase. Therefore, it appears likely that 3-PGA and P_i exerted their inhibitory effects through a selective second site, the allosteric site. Overall, 3-PGA may be acting as a type of feedback

inhibitor to control the activity of RuBPCase during the carboxylation reaction while P_1 could possibly be involved as an inhibitor to control RuBPCase activity in the dark.