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Rubisco: Characteristics of misfire during catalysis and the properties of the substrate binding sites

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RUBISCO: CHARACTERISTICS OF MISFIRE DURING CATALYSIS AND THE PROPERTIES OF THE SUBSTRATE BINDING SITES

by

Genhai Zhu

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Genhai Zhu

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director	Date 11/19/91

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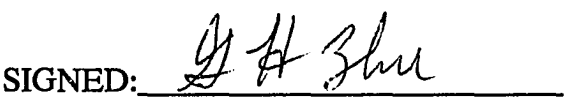
SIGNED: 

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ABSTRACT

The binding of carboxy-arabinitol bisphosphate (CABP), carboxy-arabinitol 1-phosphate (CA1P), carboxy-ribitol bisphosphate (CRBP), to carbamylated sites or xylulose bisphosphate (XuBP) and ribulose bisphosphate (RuBP) to decarbamylated sites on ribulose bisphosphate carboxylase/ oxygenase (rubisco) exhibits negative cooperativity. The binding of ligands to decarbamylated sites was highly pH-dependent between 7.5 and 8.5. Lower pH enhanced binding affinity. The binding of ligands to carbamylated sites was pH-independent. A binding model for negative cooperative interactions among catalytic sites is proposed based on the observations and the crystallographic structure of rubisco.

Fully activated, purified rubisco slowly loses its activity during *in vitro* catalysis after exposure to RuBP. This time-dependent kinetics is termed as "fallover". Two different fallover patterns were demonstrated during CO_2 fixation, one with little loss of activator CO_2 at pH 8.5 and the second with loss of activator CO_2 at pH 7.5. The two inhibitors being produced during fallover were isolated by high performance anion exchange chromatography (HPAE) and identified as XuBP and 3-ketoarabinitol 1,5-bisphosphate by pulsed amperometric detection (PAD) either directly or after reduction by $NaBH_4$. Because of the weak binding

affinity of XuBP to catalytic sites of rubisco at pH 8.5, there was little loss of activator CO_2 during fallover. 3-keto- arabinitol-P_2 which binds to carbamylated rubisco sites became the major inhibitor at that pH. However, at pH 7.5, the binding of XuBP to decarbamylated sites can cause a shift in equilibrium with loss of carbamylated sites even in the presence of excess CO_2 and Mg^{2+}, resulting in the loss of activator CO_2 during fallover.

XuBP was isolated and identified from celery leaves by HPAE-PAD.

A new method was developed for the determination of the substrate specificity of rubisco. It is based on the specific ^{14}C-labeling of 3-phosphoglycerate (PGA) from the carboxylase reaction and its dilution from the oxygenase reaction. Therefore, the ratio of carboxylation to oxygenation can be measured directly by determining the specific radioactivity of PGA produced from both reactions with HPAE-PAD separation of the total PGA and scintillation counting of ^{14}C-labeled PGA.

CHAPTER 1

LITERATURE REVIEW

The physiological process of photosynthetic CO_2 uptake by green plants, algae and photosynthetic bacteria is a manifestation of reactions catalyzed by ribulose 1,5-bisphosphate carboxylase/oxygenase (EC.4.1.1.39) (rubisco). This enzyme, purified from green leaves as a fraction I protein in 1947 (Wildman and Bonner, 1947) and identified as a carboxylase in 1956 (Weissbach et al., 1956), often accounts for up to 50% of the soluble proteins in a green leaf and is probably the most abundant protein in the world. It appears to be a grossly inefficient catalyst with a turnover number (k_{cat}) of 3.3 s^{-1} $site^{-1}$ (Woodrow and Berry, 1988). The catalytic sites of rubisco are unable to strictly distinguish between the substrate of photosynthesis, CO_2 and its product, O_2, resulting in both reactions of carboxylation and oxygenation of ribulose 1,5-bisphosphate (RuBP) being catalyzed by the same enzyme (Bowes et al., 1971; Andrews et al., 1973; Lorimer et al., 1973). Although rubisco is an abundant protein in leaves, the step catalyzed by this enzyme still appears as a rate-limiting factor in photosynthetic CO_2 assimilation (Perchorowicz et al., 1981). Because of these curious properties and its pivotal function in photosynthesis, the studies on rubisco have attracted a

lot of attention over last 25 years. The literature reviewed in this chapter will concern research primarily related to the projects in this dissertation rather than review all that is known about rubisco.

Activation of Rubisco. Purified rubisco requires preincubation with CO_2 and Mg^{2+} to achieve its catalytically competent form (Laing et al., 1975; Lorimer et al., 1976). The process of activation has been known to involve the slow, reversible reaction of a molecule of CO_2 with the ϵ-amino group of lysine (201 for L_8S_8 and 191 for L_2 rubiscos) located at the catalytic sites to form a carbamate (Lorimer et al., 1976; Lorimer and Miziorko, 1980; Lorimer, 1981). The carbamate CO_2, referred to as activator CO_2 or $^ACO_2$, is different from the substrate CO_2 (Miziorko, 1979; Lorimer, 1979). The negatively charged carbamate form is stabilized by the rapid addition of Mg^{2+} to form the active ternary complex (ECM). Thus, the process of activation is called carbamylation and proceeds by the following mechanism:

$$\underset{(E)}{\text{lys-NH}_3} \overset{\pm H^+}{\leftrightarrow} \text{lys-NH}_2 + {}^A\text{CO}_2 \overset{\pm H^+}{\leftrightarrow} \underset{(EC)}{\text{lys-NH-}^A\text{CO}_2} \overset{\pm Mg^{2+}}{\leftrightarrow} \underset{(ECM)}{\text{lys-NH-}^A\text{CO}_2\text{...Mg}^{2+}}$$

Inactive ——— Active

Since $^{A}CO_2$ only reacts with the uncharged form of lysine, an alkaline pH favors equilibrium toward the active form. The $K_{act}(CO_2)$, which is the concentration of CO_2 necessary for half maximal activation of the enzyme, measured in purified rubisco is about 25 to 30 μM CO_2 (reviewed by Ogren et al., 1986), about 3-fold higher than the physiological chloroplast CO_2 concentration (about 10 μM). This value of $K_{act}(CO_2)$, obviously, cannot explain *in vivo* activation of rubisco. Binding of effector ligands to the catalytic site can change the equilibrium to favor either the E or ECM form depending on which form is best stabilized by a given effector (Hatch and Jensen, 1980; Badger and Lorimer, 1981; McCurry et al., 1981; Jordan et al., 1983). Hatch and Jensen (1980) classified effectors into two groups. One is the positive effectors, such as NADPH and 6-phosphogluconate, enhancing carbamylation and reducing $K_{act}(CO_2)$ from 47 μM to 7.4 and 3.5 μM. The second consists of negative effectors, such as ribulose 5-phosphate, favoring the decarbamylated form. A chloroplast protein known as rubisco activase, discovered by Salvucci et al. (1985), is involved in *in vivo* activation of rubisco (Portis et al., 1986). The $K_{act}(CO_2)$ measured in *in vitro* activation of purified spinach rubisco with the rubisco activase system is about 4 μM (Portis et al., 1986) and may reflect the *in vivo* situation.

Catalytic Properties. Rubisco catalyzes a reaction where RuBP is either carboxylated or oxygenated by addition of a gaseous substrate CO_2 or O_2. There is ample evidence for the binding of RuBP to ECM, indicating the presence of the enzyme-RuBP complex (Lorimer et al., 1984), but no evidence to show the binding of the substrate CO_2 or O_2 to rubisco in the absence of RuBP (Pierce et al., 1986), suggesting this is an ordered process. Both reactions of carboxylation and oxygenation are initiated by abstraction of the C-3 proton of RuBP to form a carbanion, that will be stabilized on the enzyme as the ene-diol. CO_2 and O_2 will compete for the ene-diol of RuBP to form 2-carboxy-3-oxo-pentitol 1,5-bisphosphate and 2-hydroperoxy-3-oxo-pentitol 1,5-bisphosphate respectively. These intermediates, then, are cleaved between C-2 and C-3 by hydration and abstraction of a proton. As a result, the initial products formed during carboxylation are two 3-phosphoglyceric acid (PGA), and during oxygenation are one PGA and one 2-phosphoglycolate (Fig. 1.1). Since both carboxylation and oxygenation share the same catalytic site of rubisco and the ene-diol RuBP attacked by CO_2 in carboxylation is also susceptible to attack by O_2, Lorimer and Andrews (1973, 1978) suggest that oxygenation of RuBP is unavoidable by the mechanism.

Since CO_2 and O_2 are linear competitive inhibitors with respect to

each other (Badger and Andrews, 1974), the ratio of carboxylation (v_c) to oxygenation (v_o) can be derived as following equation (Laing et al., 1974):

$$\frac{v_c}{v_o} = \frac{V_c/K_c}{V_o/K_o} \cdot \frac{[CO_2]}{[O_2]}$$

Where V_c and V_o represent the maximum velocities for carboxylation and oxygenation. v_c and v_o are the observed velocities for carboxylation and oxygenation at the given conditions. K_c and K_o are the Michaelis constants for CO_2 and O_2 respectively. From the equation, it is known that the relative rates of carboxylation and oxygenation are proportional to the ratio of concentrations of gaseous substrates, and the proportionality constant V_cK_o/V_oK_c or s_{rel}, has been defined as the specificity factor. In a broad survey of rubiscos from different sources, it has been shown that enzymes from different species have intrinsically different values of the specificity factor (Jordan and Ogren, 1981, 1983; Andrews and Lorimer, 1985; Jordan and Chollet, 1985), and within species, the values are more constant. The changes in specificity factor are somewhat related to the evolutionary levels. For higher plant rubiscos, the values of the specificity factor are around 80 (Jordan and Ogren, 1981; Kent and Tomany, 1984). The large subunit dimer rubisco of *Rhodospirillum rubrum* has a value about 15 (Jordan and

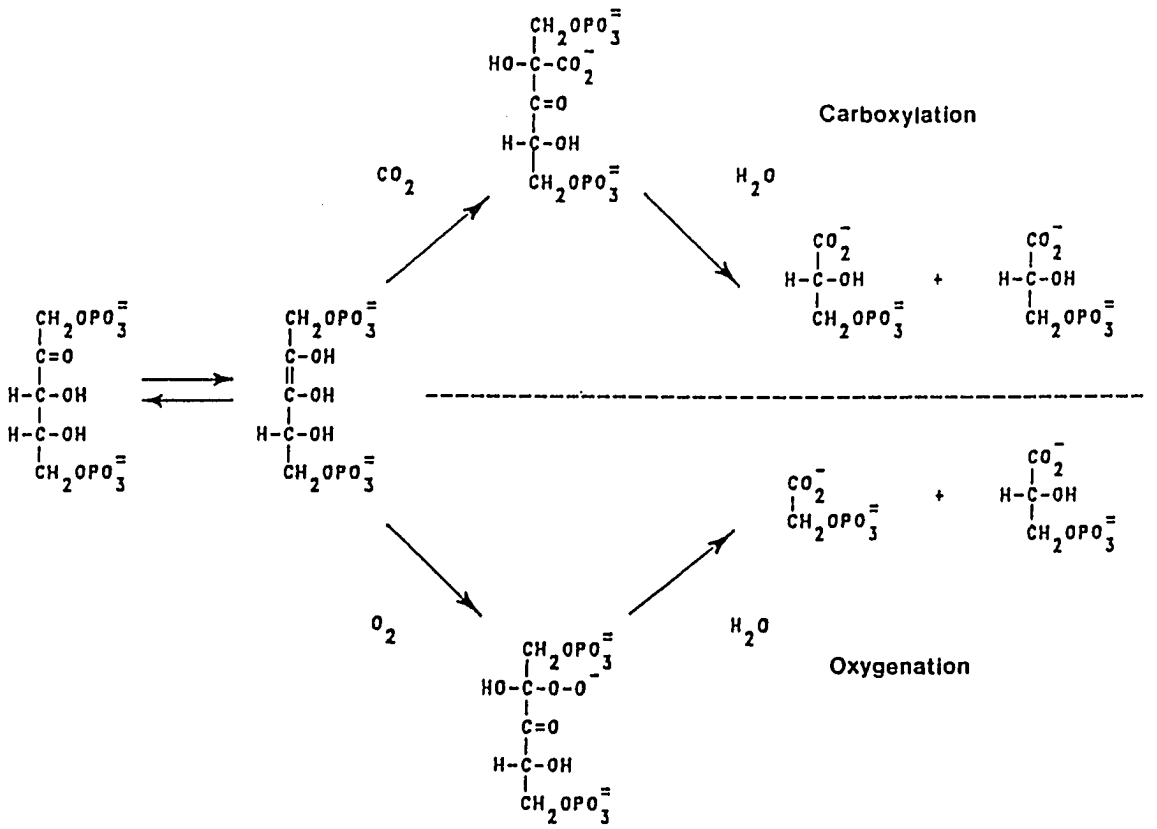


Figure 1.1 The chemical mechanism of the carboxylation and oxygenation of RuBP by rubisco

Ogren, 1981; Andrews and Lorimer, 1985). Cyanobacterial and green algal rubiscos have intermediate values around 50 to 70 (Jordan and Ogren, 1981, 1983; Andrews and Lorimer, 1985). The variation of specificity factor among species may reflect the environmental selection.

Tight Binding of Inhibitors. Although many of the Calvin cycle intermediates are able to bind to rubisco catalytic sites and influence its carbamylation state (Hatch and Jensen, 1980), these effectors do not form a tight binding complex with rubisco. Therefore, the binding of these effectors can be easily competed away upon addition of substrate RuBP. There are a few phosphorylated compounds found to bind to the rubisco catalytic sites and able to form a tight binding rubisco-inhibitor complex. The well-known tight binding inhibitors include: 2-carboxy-D-arabinitol 1,5-bisphophate (CABP), a synthesized transition state analog (Pierce et al., 1980); 2-carboxy-D-arabinitol 1-phosphate (CA1P) which is a natural rubisco inhibitor existing in some plant species (Gutteridge et al., 1986; Berry et al., 1987; Servaites, 1985; Servaites et al., 1986); D-xylulose 1,5-bisphosphate (XuBP), an epimerization product of RuBP, which was thought to be formed by de- and re-protonation during RuBP preparation and storage (Paech et al., 1978), but recently has been proved to be formed by rubisco during catalysis

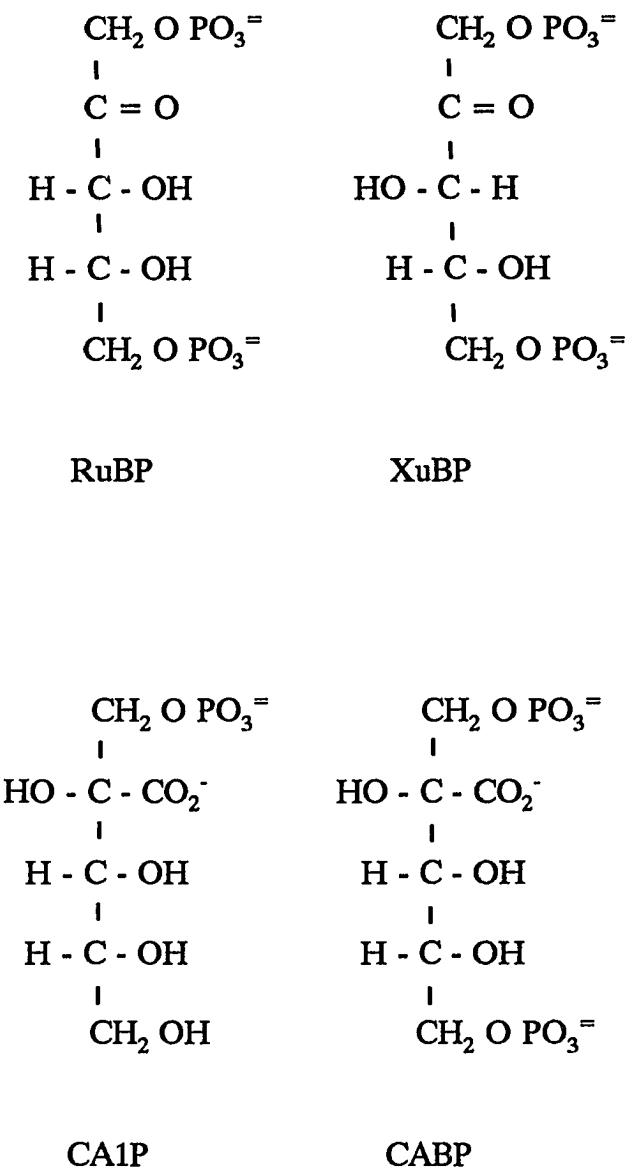


Figure 1.2 Structure of the tight binding inhibitors of rubisco

(Edmondson et al., 1990); RuBP, a substrate of rubisco, which also tightly binds to decarbamylated rubisco sites (Jordan and Chollet, 1983). To use these tight binding inhibitors as a tool to study the properties of the rubisco substrate binding sites and to analyze inhibitor binding kinetics are the major focus of this dissertation.

The mechanism for tight binding of inhibitor has been proposed as following process:

$$EI^{*} \underset{k'_4}{\overset{k'_3}{\leftrightarrow}} EI \underset{k'_2}{\overset{k'_1}{\leftrightarrow}} I + E \overset{CO_2}{\leftrightarrow} EC \overset{Mg}{\leftrightarrow} ECM + I \underset{k_2}{\overset{k_1}{\leftrightarrow}} ECMI \underset{k_4}{\overset{k_3}{\leftrightarrow}} ECMI^{*}$$

Where EI^{*} and $ECMI^{*}$ represent the tight binding complex of decarbamylated or carbamylated rubisco with inhibitor respectively. EI and ECMI are loose binding complexes. k is either the association or dissociation rate constant.

This equation indicates that the tight binding of inhibitors to rubisco sites undergoes two different phases. One is a rapid equilibrium between enzyme and inhibitors. The equilibrium can be achieved within 5 s. K_i usually reflects k_2/k_1. The second phase is a rate limiting step and requires conformational change of the protein. The overall dissociation constant ($K_d = k_2k_4/k_1k_3$) is mostly

Table 1.1 Binding Parameters[a] of Rubisco Inhibitors

	K_i	K_d	$t_{1/2}$
CABP	0.4 μM[b]	0.2 pM[c]	530 days[c]
CA1P	1.2 μM[d]	32 nM[e]	6.2 min[e]
RuBP[g]		21 nM[f]	3.93 hr.[f] 7.5 min[h]
XuBP	1.6 μM[d]	0.5 μM[d]	15.3 min

a. For spinach rubisco.

b. From Pierce et al., 1980.

c. From Schloss, 1988.

d. From Smrcka, Ph.D. dissertation, 1990.

e. From Berry et al., 1987

f. From Jordan and Chollet, 1983, measured at 2 °C.

g. Binds to decarbamylated rubisco.

h. From Cardon and Mott, 1989, measured at 22 °C.

influenced by the dissociation rate constant k_4, because k_4 usually is slower than the other rate constants. CABP has an overall K_d of about 0.2 pM with a half time for dissociation of about 530 days (Schloss, 1988), while RuBP (K_d = 21 nM)has a half time of about 3.93 hours (Jordan and Chollet, 1983) (Table 1.1)

Both carbamylated and decarbamylated sites of rubisco are able to form a tight binding complex depending on which kind of inhibitor is binding to it. CABP and CA1P have been known to bind to carbamylated rubisco, whereas RuBP can form a tight binding only with decarbamylated sites. Binding of CA1P and RuBP to rubisco clearly play a physiological role in regulation of rubisco activity *in vivo* (For CA1P, see a review paper by Servaites, 1990; Brooks and Portis, 1988; Cardon and Mott, 1989), and their binding can be relieved by rubisco activase in *in vitro* measurement (Robinson and Portis, 1988; Salvucci, 1989). However, binding of CABP, which does not exist *in vivo*, can not be released by rubisco activase.

Conformational Changes and Cooperative Binding. The most common form of rubisco, which occurs in all eukaryotes and the majority of prokaryotes, is composed of eight large subunits (L) with a molecular weight of 50 to 55 kDa each and eight small subunits (S) with a 12 to 18 kDa each (L_8S_8). The other

form of rubisco, so far found only in *Rhodospirillum rubrum*, is a dimer of large subunits (L_2) (Tabita and McFadden, 1974). In L_8S_8, eight large subunits are also organized as four dimers. The catalytic site is located at the interface of two L in a dimer.

There is sufficient evidence that the formation of the carbamylated ternary complex is accompanied by a change in the conformation of the enzyme. For example, using the group specific acylation reagent (trinitrobenzene, TNBS), Hartman et al. (1985) found only one major site reacting with TNBS in the carbamylated L_2 enzyme, but additional three sites were found to become accessible to TNBS in decarbamylated enzyme. In L_8S_8, the cross-linking of two large subunits of spinach rubisco is almost doubled upon carbamylation (Grebanier et al., 1978). Changes in the circular dichroism spectrum are also observed in response to carbamylation.

Binding of phosphorylated effectors to either carbamylated or decarbamylated rubisco sites can induce conformational transitions, which might be associated with cooperative interactions between the sites. Experiments with 6-phosphogluconate have shown that concentrations of this molecule equal to one eighth the total catalytic site concentration stabilized the carbamylated form at all eight catalytic sites (Jordan et al., 1983; Vater et al., 1983). Other research

indicating interactions between sites is that the tight binding of CABP is negatively cooperative (Johal et al., 1985; Zhu and Jensen, 1990). For example, Johal et al. were able to demonstrate that added CABP preferentially binds to the unbound rubisco in solution where native and partially CABP-bound rubisco were present. I report here (Zhu and Jensen 1990) that the first half of the sites on the rubisco holoenzyme bind CABP more than 10-fold faster than the second half of the sites, suggesting that cooperative binding occurs within a dimer. One site of the dimer bound with CABP affects binding to the second site on the same dimer. Kwok and Wildman (1974) found that the conformational change was completed after 4 substrate (RuBP) molecules bound to one rubisco molecule, although each enzyme provides 8 binding sites.

Many of the experiments in this dissertation have been designed to examine the properties of the cooperative binding among substrate binding sites. More results and discussions will be described in individual chapters.

"Fallover" of Rubisco Activity during *in vitro* Catalysis. A curious characteristic of higher plant rubisco is that when purified and fully activated enzyme is exposed to the substrate RuBP and CO_2, the reaction becomes nonlinear after 5 to 10 min of catalysis. This time-dependent, slow inactivation phenomenon has long been

observed and reported by a number of workers (Andrews and Hatch, 1971; Sicher et al., 1981; Mott and Berry, 1986; Robinson and Portis, 1989; Edmondson et al., 1990). The term "fallover" has been used to describe this phenomenon. Various explanations for the fallover of rubisco activity during *in vitro* catalysis have been made. The presence of slow, tight binding inhibitors (e.g. XuBP and other degradation products of RuBP) formed during preparation of RuBP and during storage could certainly account for such behavior (Paech et al., 1978). However, more recent experiments using either carefully prepared substrate devoid of these inhibitors or RuBP generated *in situ* from ribose 5-phosphate by the action of ribose-P isomerase and phosphoribulokinase still showed time-dependent loss of activity (Mott and Berry, 1986; Edmondson et al., 1990). Since the substrate RuBP binds to decarbamylated rubisco much tighter than to carbamylated or active sites, one can expect that binding of RuBP to inactive sites will shift the overall equilibrium toward the inactive form. However, experiments have shown that RuBP can stabilize the carbamylation state of rubisco rather than stimulating decarbamylation when CO_2 and Mg^{2+} in rubisco activation medium were highly diluted (Seftor et al., 1986; Edmondson et al., 1990).

The activity of rubisco following fallover cannot be fully recovered by removal of RuBP through gel filtration, suggesting that inhibitor(s) might be

formed and are tightly binding to the catalytic sites of rubisco during fallover (Robinson and Portis, 1989). An epimerization product, xylulose 1,5-bisphosphate (XuBP), has been identified from the fallover system and has been proved to be formed by carbamylated rubisco during catalysis (Edmondson et al., 1990; Zhu and Jensen, 1990). It is suggested that the formation of XuBP is due to misreprotonation on C-3 of enolized RuBP. Based on this mechanism, misreprotonation of the C-2 of enolized RuBP will also yield 3-keto arabinitol 1,5-bisphosphate and its epimer, 3-keto ribitol 1,5-bisphosphate (Edmondson et al., 1990). These misfire products account for only a very small fraction of the RuBP used in reaction mixture compared with the initial RuBP concentration and product PGA. These misfire products are labile and it is difficult to isolate and identify them. The major experiments of this dissertation were designed to identify and characterize these misfire products and their binding properties. Although the time-dependent loss of rubisco activity is observed in *in vitro* catalysis, it is possible that they could occur *in vivo*, because the loss of activity is initiated by exposure of rubisco to RuBP. After addition of a rubisco activase system to the *in vitro* carboxylation reaction mixture, the time-dependent loss of activity is eliminated (Robinson and Portis, 1989), indicating that the fallover of rubisco activity can be corrected by rubisco activase, possibly by the inhibitors

formed on catalytic sites being continuously removed by rubisco activase.

Regulation of Rubisco Activity *in vivo*. RuBP, the substrate of rubisco, is generated from the Calvin cycle by consuming ATP which, in turn, is regenerated by photosynthetic electron transport driven by light. One may expect that the activity of rubisco is regulated by control of RuBP supply under varying light conditions. However, experiments have shown that the pool size of RuBP is stable under low and high light, but that the activation state of rubisco is changed by varying light intensities (Bahr and Jensen, 1978; Perchorowicz et al., 1981). The rate of photosynthetic CO_2 assimilation is proportional only to the initial activity of rubisco, which represents the amount of rubisco that is catalytically functional at that particular physiological condition (Perchorowicz et al., 1981). Most plants exhibit this light-modulated rubisco activation/deactivation regulatory mechanism. The early explanation for this light regulatory mechanism is that the concentration of Mg^{2+} and pH in chloroplast stroma are influenced by light through photosynthetic electron transport and generation of a proton motive force across the membrane (Werdan and Heldt, 1972; Walker, 1973; Bahr and Jensen, 1974; Chu and Bassham, 1975; Laing et al., 1975; Andrews et al., 1975; Lorimer et al., 1976). The Mg^{2+} concentration and pH in the chloroplast stroma are lower

under low light than under high light. Changing the Mg^{2+} concentration and pH will cause changes in the carbamylation state of rubisco. However, experiments that have examined the light dependence of pH and Mg^{2+} fluctuations in chloroplasts have found that they are unresponsive to light intensities greater than 100 μE, while the major changes in rubisco carbamylation in response to light occur only at higher light intensities (Portis, 1981; Oja et al., 1986; Portis and Heldt, 1986). Recent observations have shown that the light activation of rubisco by rubisco activase in lysed chloroplasts is related to the operation of photosystem I which is not required for supplying the ATP needed by the rubisco activation reaction (Campbell and Ogren, 1990). Approaches through the rubisco activase system may provide a clue to understanding the light dependence of activation of rubisco, but the detailed mechanisms still remain unclear.

Binding of a sugar phosphate inhibitor, CA1P, which exists in some plants, to the catalytic sites of rubisco represents another mechanism regulating the activity of rubisco *in vivo* (reviewed by Servaites, 1990). The tight binding property of CA1P results from its similarity to the transition state intermediate of the carboxylase reaction (Fig. 1.2). The amounts of CA1P present in leaves vary with the level of light, giving CA1P the characteristics of a diurnal modulator of rubisco activity. Since this inhibitor binds to carbamylated sites of rubisco and

occupation by CA1P prevents catalytic sites of rubisco from binding the substrate, RuBP, changes in rubisco activity do not alter the carbamylation state. A specific phosphatase, which hydrolyzes CA1P in chloroplasts, has been found (Holbrook et al., 1989). The concentration of CA1P and the initial activity of rubisco in a leaf roughly follow the daily changes in light intensity (Kobza and Seemann, 1989), indicating the synthesis and degradation of CA1P are fast enough to function as a daily modulator of rubisco activity. Is the regulation of rubisco activity by binding of inhibitor a universal regulatory mechanism or only specific to some plants? Do other plants have other kinds of inhibitors like those observed during *in vitro* catalysis reaction (such as misfire products)? These questions will be discussed in individual chapters.

Objectives. Most experiments done in this dissertation involve examining the binding properties of rubisco for ligands and relating this to their *in vitro* catalytic behavior. Efforts will be made in this dissertation to connect this *in vitro* data to possible *in vivo* regulatory mechanisms for rubisco activity. For ligand binding, the emphasis has been put on the cooperative binding induced by the transition state analog CABP, natural inhibitor CA1P or the substrate of rubisco, RuBP. The pH effects, which may occur in the chloroplast with variation of light intensities, on

the binding affinity of rubisco for ligands, and on the fallover of rubisco activity during *in vitro* catalysis have been examined. The evidence obtained in this dissertation may assist in understanding some basic catalytic properties of rubisco and possible mechanisms for rubisco-rubisco activase interactions.

CHAPTER 2

MATERIALS AND METHODS

In this section, the basic methods that were used throughout this dissertation are outlined. These will be divided into two sections as follows: A) Protein Preparation and Assay, B) Sugar Phosphate Synthesis and Assay. Methods that were developed as part of the work for a given chapter are described in the Materials and Methods section in that chapter. Other specific methods are described in the figure legends.

A. Protein Preparation and Assay

1. Purification of Spinach Rubisco. This procedure was similar to that published by McCurry et al., 1982. Approximately 200 to 500 g of fresh spinach leaves were deribbed and homogenized 100 g at a time at 4° C using a Polytron (Brinkman) tissue homogenizer in 200 ml of buffer A [25 mM N,N-bis(2-hydroxyethyl)glycine (Bicine), pH 7.8), 10 mM 2-mercaptoethanol, 1 mM EDTA] plus 2% insoluble polyvinylpolypyrrolidone (PVP). The resulting homogenate was filtered through ten layers of cheese cloth and 2 layers of Miracloth (Calbiochem) and centrifuged

at 30,000 x g (ss34 rotor) for 30 min. The resulting supernatant was made 35% saturation at 0° C with $(NH_4)_2SO_4$ by slow addition of a 100% saturated $(NH_4)_2SO_4$ solution and stirred slowly at 4° C. The suspension was centrifuged at 10,000 x g for 15 min and the supernatant was decanted and made 55% saturated at 0° C in $(NH_4)_2SO_4$ by slow addition of solid ammonium sulfate. This suspension was stirred slowly for 30 min and centrifuged at 10,000 x g for 15 min. The pellet containing the precipitated rubisco was resuspended in buffer A and centrifuged at 30,000 x g to remove insoluble material.

The resulting supernatant was loaded on a 2.5 by 110 cm Sephacryl S-300 (Pharmacia) gel filtration column, equilibrated with buffer A, and eluted at a flow rate of 0.75 ml/min. Fractions were monitored for absorbance at 280 nm and peak fractions were assayed for RuBP dependent CO_2 fixation. Fractions containing peak activity of CO_2 fixation were examined for purity by SDS-PAGE as described in A.4. and fractions containing the most rubisco least protein contamination were pooled.

The pooled fractions were loaded onto a 3 x 30 cm DEAE-cellulose (Whatman-DE52) ion-exchange column, washed with 100 ml of buffer A and eluted with a 0.0 to 0.4 M $NaHCO_3$ gradient in buffer A. Protein elution was monitored at 280 nm, assayed for rubisco activity and checked for purity by

denaturing gel electrophoresis as described in A.4. Fractions determined to be at least 95% pure were pooled and the protein was precipitated by addition of solid $(NH_4)_2SO_4$ to 50% saturation at 0° C. The resulting suspension was slowly dripped into liquid N_2 and the resulting beads were stored at -70° C. To prepare rubisco for use, frozen beads were thawed at room temperature and centrifuged in an Eppendorf centrifuge for 2 min, the supernatant discarded and the pellets resuspended in buffer. This solution was incubated with 50 mM dithiothreitol (DTT) for at least 3 hours or overnight in refrigerator, and then dialyzed overnight at 4° C against the specific buffer to be used in a particular experiment to remove DTT and the remaining $(NH_4)_2SO_4$.

2. Rubisco Enzyme Assay. Rubisco was incubated in 500 μl assay solutions containing 100 mM Bicine-NaOH, pH 8.0, 10 mM $KH^{14}CO_3$ (1 μCi/μmol), 10 mM $MgCl_2$ and 3 mM DTT (optional). Reactions were usually initiated by addition of RuBP to 0.5 to 1 mM and stopped after 30 s by addition of 500 μl of 1 N HCl. Samples were dried to remove unfixed $^{14}CO_2$ and the acid stable radioactivity remaining was assayed by liquid scintillation counting.

3. CABP Binding. Samples containing rubisco were incubated for 15 to 30 min with 1 to 5 μM [^{14}C]CABP (10 to 20 μCi/μmol) which had been synthesized and purified from other isomers according to section B.2., in 25 mM Tris-Cl, pH 8.0,

10 mM $KHCO_3$, 10 mM $MgCl_2$ followed by a 1 to 5 h incubation with purified 50 to 250 μM unlabeled CABP unless otherwise indicated. Bound CABP was separated from unbound CABP by gel filtration on either a Pharmacia Superose 12 or a Bio-Rad Econo-Pac 10DG column. Since one molecule of CABP binds per active site, protomer amounts were calculated from the amount of [^{14}C]CABP bound. The amount of rubisco protein was measured by reading absorbance at 280 nm after column (A.6). Then the maximum sites available for CABP binding per holoenzyme was calculated.

4. Electrophoresis. Sodium dodecyl sulfate, polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method described by Laemmli, 1970. Holoenzyme was analyzed on 15% polyacrylamide gels. Stacking gels were 4% polyacrylamide. For rapid analysis, a Hoeffer minigel apparatus was used. The running gels were pre-cast at 0.75 mm in sets of eight and stored at 4° C. At the time of use gels were attached to the apparatus and 4% polyacrylamide stacking gels were cast on top of the gels with 10 to 15 wells per gel. Samples were prepared for electrophoresis by boiling the samples in 1% SDS, and ß-mercaptoethanol. Gels were stained with Coomassie Brilliant Blue R-250.

5. FPLC Gel Filtration. Analytical gel filtration was performed on a Pharmacia FPLC system using either a Superose 12 or Superose 6 Pharmacia gel filtration

column. The eluate was monitored at 277 nm using an LKB Uvicord flow through spectrophotometer.

6. Protein Assay. Rubisco concentrations were determined in two ways. The concentration of purified spinach rubisco was determined by measuring the absorbance at 280 nm and using the extinction coefficient of 0.61 mg/cm^2 from Paulsen and Lane, 1966. The concentration of rubisco from other sources was determined by [^{14}C]CABP binding (section B.2.) Other protein determinations were made by the dye-binding method of Bradford, (1976) using BSA as the standard.

B. Sugar Phosphate Preparation and Assay

1. RuBP Synthesis and Purification.

Synthesis of RuBP from ribose 5-P: RuBP was synthesized from ribose 5-P purchased from Sigma. Chloroplasts were isolated from spinach using the procedure of Jensen and Bassham, 1966 and lysed in 10 mM Bicine buffer (pH 7.5) that had been purged with N_2. These chloroplast lysates were used as a source for ribose 5-P (R5P) isomerase and phosphoribulokinase. Solutions containing 12 mM R5P, 1 mM ATP, 15 mM creatine phosphate and creatine phosphokinase were flushed with nitrogen for 30 min to remove CO_2 and O_2. The

chloroplast lysate was added and the components were allowed to react for 30 min. The reaction was stopped by cooling the reaction mixture to 0° C and the pH was adjusted to 6.5 by adding 1 M HCl. Sugar phosphates including RuBP were precipitated as their barium salts by the addition of a 5 fold excess of 1 M barium acetate and an equal volume of 95% ethanol. The precipitate was solubilized in 5% trichloroacetic acid (TCA) and centrifuged to remove precipitated proteins. The supernatant was passed over a column of activated charcoal to remove ATP and ADP. The pH was adjusted to 6.5 with CO_2 free KOH and the RuBP was reprecipitated by addition of excess barium acetate and 1/3 volume of 95% ethanol. The RuBP was collected by centrifugation and solubilized by adding excess Dowex AG 50W-X4(H^+) (Bio-Rad) and then filtered to remove the Dowex. The pH was adjusted to 6.5 with CO_2 free KOH and the solution was stored at -20° C.

Purification of RuBP: To further purify the RuBP it was loaded on a 2 by 60 cm Dowex AG 1-X8(Cl^-) (BioRad) ion-exchange column. The column was equilibrated with 3 mM HCl and the RuBP was eluted with a 2-L, 0 to 0.4 M linear gradient of LiCl in 3 mM HCl. The eluate was monitored at 206 nm for the carbonyl moiety of the RuBP. The major peak was collected, pooled and concentrated to about 30 ml and the RuBP was precipitated by the addition of a 3

fold excess of barium acetate and 1/3 volume of 95% ethanol. The precipitated RuBP was solubilized by the addition of excess Dowex AG 50W-X4(H^+). The solution was filtered to remove the Dowex and the pH was adjusted to 6.5 with CO_2 free KOH. The concentration of RuBP was determined by incubating RuBP with rubisco as described in section A.1, except the reaction was allowed to proceed for 30 min and the concentration of $KH^{14}CO_3$ was 30 mM. Dilute RuBP concentrations were used to prevent inhibition of activity by the substrate. The amount of CO_2 fixed under these conditions was assumed to be equivalent to the amount of RuBP present since the oxygenase function was inhibited by excess CO_2.

2. CABP Synthesis and Purification. CABP was prepared by the method of Pierce et al., 1980. RuBP (100-200 μmoles) was incubated for 48 h at pH 8.5 with either KCN or $K^{14}CN$ such that the final ratio of KCN to RuBP was 1.1. The solution was treated with excess Dowex AG 50W-X4(H^+), filtered, and concentrated to dryness under vacuum overnight. The resulting lactones were dissolved in H_2O and adjusted to pH 5.5 with 1 M NaOH. This solution was loaded on a 2 by 60 cm Dowex AG 1-X8(Cl^-) equilibrated with 3 mM HCl and eluted with a linear, 2-L, 0.0-0.4 M LiCl gradient. Peak fractions were identified either by monitoring radioactivity or absorbance at 206 nm and fractions

containing CABP (Pierce et al., 1980) were pooled. When CABP elution was followed by monitoring absorbance at 206 nm, the absorbance of the starting solution was adjusted to that of the final solution using acetone. The pooled fractions were reduced in volume to 30 ml, the pH was adjusted to 8.5 using 2 M LiOH and CABP was precipitated by addition of 5 fold molar excess of 1 M barium acetate and an equal volume of 95% ethanol. After 1 h at -20° C, the precipitate was collected by centrifugation and washed twice with 95% ethanol. The precipitate was solubilized by addition of excess Dowex AG 50W-X4(H^+), filtered, adjusted to pH 6.5 and stored at -20° C until just before use. Prior to use, the pH of the solution was adjusted to 9.0 and incubated for 24 h to hydrolyze the lactones. CABP concentration was determined by phosphate analysis (B.5).

3. XuBP Synthesis and Purification. Xylulose 1,5- bisphosphate (XuBP) was synthesized by condensation of glycolaldehyde phosphate and dihydroxyacetone phosphate using rabbit muscle aldolase (EC 4.1.2.13). Glycolaldehyde phosphate was prepared by oxidative decarboxylation of α-glycerophosphate as described by Serianni et al., 1982. The products that resulted were then separated by ion-exchange chromatography on a 1.5 by 60 cm column of Dowex AG 1-X8(Cl^-). The column was eluted with a 0-0.4 M LiCl (in 3 mM HCl) gradient. The XuBP

was detected by the absorbance of the keto group at the 2 position at 206 nm. Fractions in the peak were pooled and the bisphosphates were precipitated by addition of an excess of barium acetate. The precipitate was resuspended by addition of Dowex AG 50W-X4(H^+) and the pH adjusted to 6.5 with NaOH.

The identity of the XuBP was confirmed by hydrolyzing the bisphosphate with alkaline phosphatase and comparing the retention time to that of standard xylulose on an HPLC system that separates sugars by anion-exchange chromatography (Carbopac, Dionex) at high pH and is coupled to pulsed amperometric detection (Dionex).

4. CA1P Synthesis and Purification. A method for the synthesis of CA1P from CABP was developed in this lab by limited alkaline phosphatase digestion of CABP (Smrcka and Jensen, 1988). [^{14}C]CABP was incubated with alkaline phosphatase in 50 mM glycine buffer at pH 9.3 containing 1 mM $ZnSO_4$ and 10 mM $MgCl_2$ for 15-20 min. The monophosphates were separated from unreacted CABP and enzyme by fractionation on a small silica anion-exchange precolumn (Sepralyte SAX, packed in a 1 ml syringe with bed volume of 0.5 ml). Monophosphates were selectively eluted with 4 ml of 0.05 N HCl, CABP was eluted with 4 ml of 1 N HCl. The monophosphates fractions were evaporated to dryness, dissolved in 1 ml water and adjusted pH to 9.0. After 12 h of incubation

at this pH no lactones were detected in the solution. The sample was loaded on a 1.5 by 55 cm Dowex AG 1-X8 (Cl^-) column and eluted with a 1 L linear gradient from 0 to 0.2 M LiCl in 3 mM HCl. Ten ml fractions were collected and assayed for radioactivity. The peak fractions were pooled, the total volume was reduced to approximately 10 ml with a rotating evaporator, the pH was adjusted to 8.2, and the CA1P was precipitated by addition of 1 M barium acetate. Sample were redissolved using Bio-Rad AG 50 (H^+) and analyzed by HPLC.

To insure that the synthetic CA1P was free of inorganic phosphate and CA5P the solution containing "pure" CA1P prepared as above was incubated with purified rubisco holoenzyme from tobacco at a ratio of CA1P to holoenzyme of 1.5 in 50 mM Tris-Cl, pH 8.0, 10 mM $KHCO_3$, 10 mM $MgCl_2$ and 0.2 mM EDTA. After 30 min the rubisco protein was precipitated by making the solution 20% (w/v) in polyethylene glycol 4000 (PEG) and 60 mM in $MgCl_2$ at 4° C and centrifugation at 15,000 x g for 15 min. In the incubation solution the only possible phosphate contaminants that could have been present were Pi, CA5P, and CABP (CABP was removed from monophosphates by both silica anion exchange column and Dowex AG 1-X8 (Cl^-) column) because of the way the synthetic CA1P was prepared and only CA1P binds tightly enough to the rubisco to remain associated with the rubisco during precipitation (Berry et al., 1987).

The precipitated protein was solubilized in the incubation solution and reprecipitated by the above procedure. This precipitate was dissolved in water and $HClO_4$ was added to 4%, to precipitate the rubisco and release the bound inhibitor. The $HClO_4$ was removed as $KClO_4$ by adjusting the pH to 8.5 with 0.1 N KOH at 4° C and centrifugation at 10,000 x g for 5 min. The CA1P was then precipitated by addition of a 10 fold molar excess of barium acetate and an equal volume of 95% ethanol. After storage at -20° C overnight the precipitated CA1P was harvested by centrifugation at 10,000 x g for 20 min. The Ba-CA1P salt was solubilized by addition of an excess of washed Dowex AG 50W-X4(H^+). The Dowex was removed by filtration and the pH was adjusted to 6.5 with 0.1 M NaOH and the solution was stored at -20° C. That this preparation was free of inorganic phosphate was determined by the phosphate assay (see B.5) without the 160° C heating step that hydrolyses organic phosphates. That the preparation was free of CA5P and CABP was shown by HPLC analysis (B.6). The concentration of the CA1P was determined by phosphate analysis (B.5).

5. Phosphate Analysis. Solutions containing organic phosphates and inorganic phosphate standards were incubated in 0.4 ml of 10 N H_2SO_4 at 160° C for at least 1 h to release the Pi from the organic phosphates. After cooling 10 μl of H_2O_2 was added and the solutions were heated for 20 min. After cooling, 2 ml of

water was added and the solutions refluxed at 100° C for 20 min to hydrolyze pyrophosphates. The solution was cooled to room temperature and 1.6 ml H_2O, 0.5 ml 6% ammonium molybdate and 0.5 ml 1% ascorbic acid were added. The color reaction was allowed to develop for 1.5 to 2.0 h at 42° C and the absorbances were read at 820 nm. Phosphate concentrations were determined by comparison with the standard inorganic phosphates. If determining the concentration of free Pi in organic phosphate preparations the 160° C heating, the H_2O_2 and the 100° C pyrophosphate hydrolysis steps were omitted.

6. Purity Assay of Sugar Phosphates by HPAE-PAD System. For enzyme kinetic assays and ligand binding assays, the sugar-P (RuBP, CABP, CA1P etc.) purified from Dowex AG 1-X8 (Cl^-) column was further assayed on a Dionex Carbopac column by HPAE-PAD system to ensure that the ligand was not contaminated by any other sugar-P and Pi. For details about HPAE-PAD analysis of sugar and sugar-P, see individual chapters (Chapter 5, 6 and 8).

CHAPTER 3

STATUS OF THE SUBSTRATE BINDING SITES OF RIBULOSE BISPHOSPHATE CARBOXYLASE AS DETERMINED WITH 2-C-CARBOXYARABINITOL 1,5-BISPHOSPHATE

ABSTRACT

The properties of the tight and specific binding of 2-C-carboxy-D-arabinitol 1,5-bisphosphate (CABP), which occurs only to reaction sites of ribulose 1,5-bisphosphate carboxylase (rubisco) that are activated by CO_2 and Mg^{2+}, were studied. With fully active purified spinach rubisco the rate of tight binding of [^{14}C]CABP fit a multiple exponential rate equation with half of the sites binding with a rate constant of 40 min^{-1} and the second half of the sites binding at 3.2 min^{-1}. This suggests that after CABP binds to one site of a dimer of rubisco large subunits, binding to the second site is considerably slower, indicating negative cooperativity as previously reported by Johal et al. (1985). The rate of CABP binding to partially activated rubisco was completed within 2 to 5 min, with slower binding to inactive sites as they formed the carbamate and bound Mg^{2+}. Addition

of [^{14}C]CABP with EDTA stopped binding of Mg^{2+} and allowed tight binding of the radiolabel only to sites that were CO_2/Mg^{2+}-activated at that moment. This approach estimated the amount of CO_2/Mg^{2+}-activated sites in the presence of inactive sites and carbamated sites lacking Mg^{2+}. The rate of CO_2 fixation was proportional to the CO_2/Mg^{2+}-activated sites. During light-dependent CO_2 fixation with isolated spinach chloroplasts, the amount of carbamation was proportional to rubisco activity either initially upon lysis of the plastids or following total activation with Mg^{2+} and CO_2. Lysis of chloroplasts in media with [^{14}C]CABP plus EDTA estimated those carbamated sites having Mg^{2+}. The loss of rubisco activation during illumination was partially due to the lack of Mg^{2+} to stabilize the carbamylated sites.

INTRODUCTION

Ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco) plays a major role in CO_2 assimilation during photosynthesis in plants. Its activity is regulated in light in concert with other metabolic processes so that the regeneration of RuBP equals its utilization by rubisco (Mott et al., 1984). CO_2 fixation is controlled by the amount of rubisco that is active, not by the total amount of rubisco protein (Perchorowicz et al., 1981). At the molecular level rubisco is activated by two essential effectors: CO_2 and Mg^{2+} (Lorimer et al., 1976). The activator CO_2 ($^{A}CO_2$) forms a carbamate which can be stabilized by Mg^{2+} at each of the eight active sites on rubisco. There appears to be good correlation between the net rate of photosynthetic CO_2 assimilation of a leaf and the "initial" activity of rubisco measured from an extract of the same leaf (Perchorowicz et al., 1981), although there are conditions where changes in net assimilation do not follow changes in rubisco activity.

The transition-state analog, 2-C-carboxy-D-arabinitol 1,5-bisphosphate (CABP), provides an excellent tool for determining the number of reaction sites on rubisco where the activator carbamate has been formed. It binds tightly and specifically to the active enzyme-CO_2-Mg^{2+} (ECM) sites of rubisco with a K_d of

10^{-11} M and does not exchange (Hall et al., 1981; Pierce et al., 1980). Although CABP does bind to both activated (ECM) sites and inactive (E, EC) sites, the binding is not nearly as tight to inactive sites and is exchanged with 20 to 100 fold excess of [^{12}C]CABP and removed upon gel filtration.

As carbamylation of the reaction sites is needed for rubisco activity, the initial activity should be proportional to the amount of bound CABP, which measures carbamylation. However, when an inhibitor of rubisco, such as CA1P, is present then the activity appears less than the amount of bound CABP because CABP, having a higher affinity, can displace CA1P from ECM sites (Butz and Sharkey, 1989).

I have used this tight and specific binding property of [^{14}C]CABP to study the kinetics of binding to spinach rubisco. The number of active sites of rubisco that were carbamylated have been compared to the enzyme activity using either purified rubisco or rubisco in lysed chloroplast extracts. Two forms of the carbamated enzyme, ECM and EC, were separately identified. The binding of CABP to purified rubisco suggested that after one CABP molecule is bound to a dimer of rubisco large subunits, binding of the second CABP is considerably less favorable.

MATERIALS AND METHODS

Materials. RuBP was synthesized as previously described (Bahr and Jensen, 1978). Both [2-^{14}C] and [2-^{12}C]CABP, were synthesized and purified by adaption of the procedures of Pierce et al (1980).

Methods. Rubisco from spinach (*Spinacia oleracea*) was purified according to McCurry et al (1982). The purified carboxylase was totally deactivated by dialysis for 24 to 36 h with 50 mM Bicine, pH 8 and 0.2 mM EDTA at room temperature while bubbling with N_2. It was reactivated by incubation in 50 mM HEPES, pH 8.0, with 20 mM $MgCl_2$ and 20 mM $KHCO_3$ for 30 min. Chloroplasts were isolated from 6 to 7 week old spinach plants (*var Viroflay*) as previously described (Stumpf and Jensen, 1982).

Two steps were used to ensure the tight and specific binding of [^{14}C]CABP to the active sites of rubisco and to eliminate non-specific binding of [^{14}C]CABP to other domains of the protein. Rubisco, either purified or in extracts of lysed chloroplasts, was incubated with 100 μM of the free acid form (not the lactone form) of [^{14}C]CABP with 1 mg/ml rubisco (having 14.5 μM binding sites) for 5 min at 24 to 25° C. The radiolabel was diluted with 20 to 100 fold excess of [^{12}C]CABP and further incubated for 1 to 5 h. The mixture was eluted on a

Pharmacia FPLC Superose 12 column with 50 mM Hepes, pH 8.0, 10 mM $MgCl_2$, 20 mM $KHCO_3$, 15 mM NaCl, and 0.5 ml fractions collected. The Rubisco-[^{14}C]CABP peak, identified by A_{277} and by the radioactivity, was cleanly separated from the unbound [^{14}C]CABP. The amount of rubisco protein in the pooled tubes containing the rubisco-[^{14}C]CABP complex was determined by absorbance at 280 nm (McCurry et al., 1982) and by rocket immunoelectrophoresis (Gustafson et al., 1987). Over 90 % of the protein in the pooled tubes fractionated from chloroplast extracts was rubisco.

The experimental kinetic data was analyzed using MEDAS (Multiple Exponential Decay Analysis Software), copyright by EMF Software, Knoxville, TN. Rate constants and coefficients were determined.

RESULTS

CABP is known to bind specifically and tightly to rubisco if the catalytic sites are carbamylated (Hall et al., 1981; Pierce et al., 1980). The kinetics of this binding has been investigated and its characteristics are reported here. A 20 to 50 fold excess of [^{12}C]CABP exchanged the non-specific bound [^{14}C]CABP on the rubisco protein. This also stopped the tight binding of [^{14}C]CABP which would continue as other inactive sites became carbamylated.

The protein-CABP complex was separated from unbound CABP by gel filtration through a Pharmacia Superose 12 column. Enzyme sites which were not carbamylated did not bind [^{14}C]CABP tightly and exchanged with [^{12}C]CABP. Routine separation of the rubisco-[^{14}C]CABP complex from unbound [^{14}C]CABP by gel filtration has not been widely used because of the long time required. The Pharmacia FPLC system using a Superose gel filtration column separated a sample every 35 min, pumping at 1 ml/min. It proved more reproducible than precipitation of the protein by PEG.

The maximal number of reaction sites that can bind CABP per rubisco molecule is eight, with one per large subunit (Hall et al., 1981; Wishnick et al., 1970). Although our binding numbers do approach eight, the maximum values

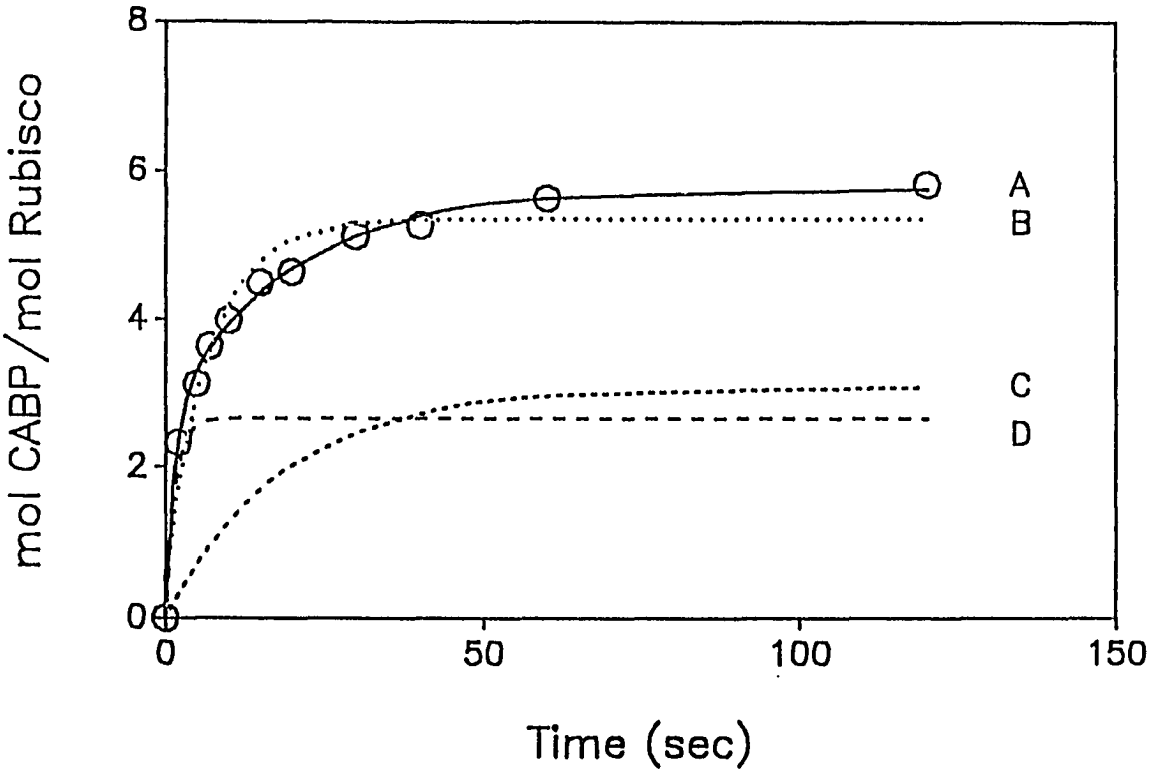


Figure 3.1. Rate of binding of [^{14}C]CABP to purified activated spinach rubisco. Rubisco (1 mg/ml) was fully activated 30 min with 20 mM $MgCl_2$ and $NaHCO_3$ in 50 mM Hepes, pH 8.0. [^{14}C]CABP (100 μM) was added to the enzyme to initiate the binding (zero time) and diluted with 5 mM [^{12}C]CABP at the times given. The squares indicate the measured amount of tightly bound [^{14}C]CABP. The solid line indicates the best fit generated by the Multiple Exponential Decay Analysis Software (MEDAS) where k_1 is 40.1 min^{-1} and k_2 is 3.18 min^{-1}.

Table 3.1. Rate Constants of CABP Binding to Rubisco and Their Coefficients

Solutions of purified spinach rubisco (1 mg/ml), either activated or deactivated, were incubated in 50 mM Hepes, pH 8.0, 20 mM each of $MgCl_2$ and $NaHCO_3$ and 100 μM [^{14}C]CABP. At various time points 5 mM [^{12}C]CABP was added and the tight binding of [^{14}C]CABP determined following gel filtration. The rate constants and coefficients were determined using MEDAS.

Condition of Sites	k_1[a]	k_2[a]	A_1[b]	A_2[b]
Fully Active Enzyme, 25° C	40.1	3.18	2.7	3.1
Fully Active Enzyme, 15° C	19.2	0.82	1.9	3.2
Inactive Enzyme, 25° C	9 to 16x10^{-3}	-	3.4	-
Inactive Enzyme, 2° C	1.6x10^{-3}	-	2.6	-

[a] min^{-1}

[b] mol CABP/mol Rubisco

(See Fig. 3.1)

observed were usually between 6.0 to 7.5 binding sites per rubisco molecule. These values agree with those of Hall etal who reported 6.6 [^{14}C]CABP bound per molecule with purified spinach rubisco (Hall et al., 1981).

Rate of CABP Binding. The rate of CABP binding to purified spinach rubisco was examined within seconds after adding [^{14}C]CABP. With fully active rubisco the rate of tight binding of [^{14}C]CABP was quite rapid with a half-time of about 2.5 sat 25° C (Fig. 3.1). Computer analysis of the data was best fit by two exponential rate constants. The faster rate constant was 40 min^{-1} and the slower was 3.2 min^{-1}. The solid line of Fig. 3.1 was generated using these exponents. Comparison to the observed data (squares) to the line indicates the completeness of the fit.

Fig. 3.2 represents the binding of [^{14}C]CABP to deactivated rubisco at 25° C. The rapid binding of [^{14}C]CABP during the first two minutes was due to sites that were already carbamylated, formed in equilibrium with air levels of CO_2. These sites (EC) then bound Mg^{2+} and [^{14}C]CABP. Binding of [^{14}C]CABP to the remainder of the inactive rubisco binding sites was considerably slower, with an apparent first order rate constant of 0.009 to 0.016 min^{-1} at 25° C (Table 3.1). After 24 h [^{14}C]CABP had bound to the same number of sites as found when rubisco was first fully Mg^{2+}/CO_2-activated before addition of CABP. The slow rate of [^{14}C]CABP binding to originally inactive rubisco sites was dependent on the

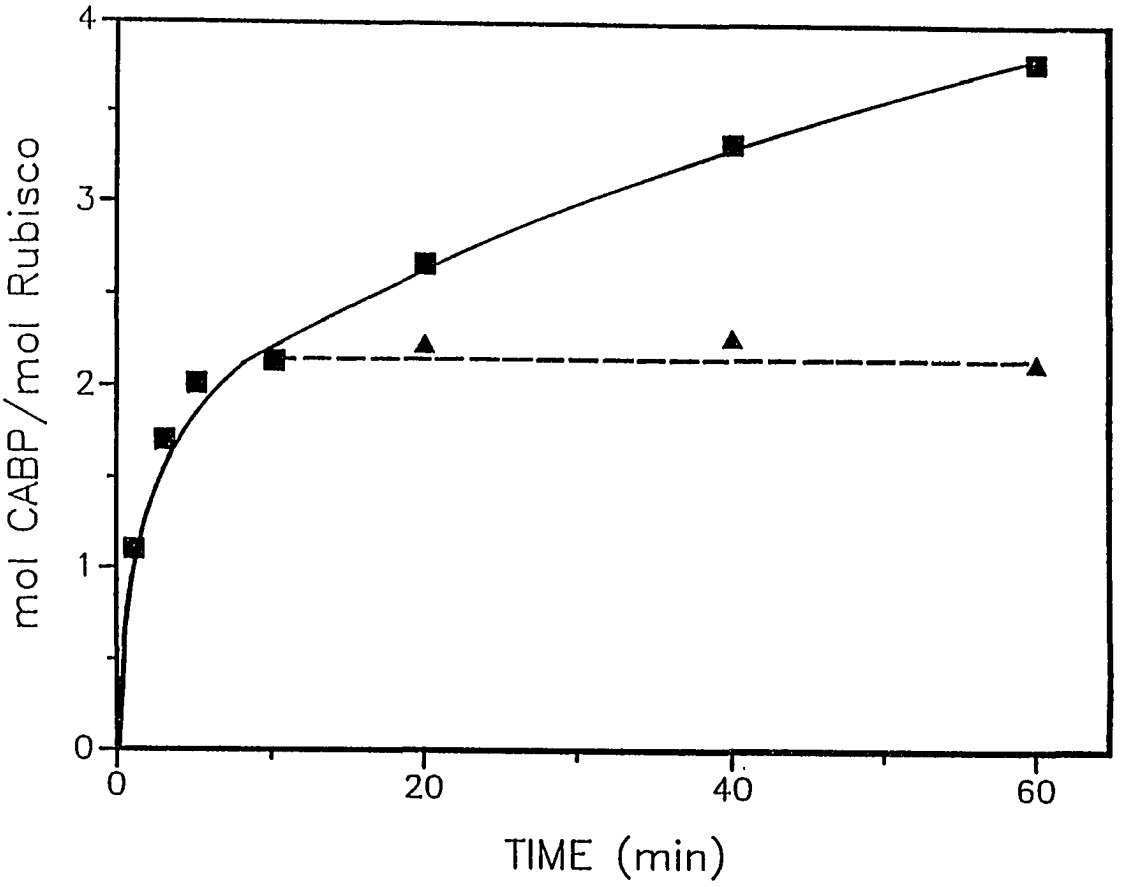


Figure 3.2. Rate of [^{14}C]CABP binding to deactivated spinach rubisco. Purified rubisco (1 mg/ml) from spinach was deactivated by dialysis in 50 mM Hepes, pH 8.0 and 0.2 mM EDTA. [^{14}C]CABP (100 μM) was added 1 min before addition of 10 mM each of $MgCl_2$ and $NaHCO_3$ (25° C) and the binding of label was terminated by removing samples into 5 mM [^{12}C]CABP. Binding of [^{14}C]CABP (■); binding of [^{14}C]CABP upon addition of 11 mM EDTA at 10 min (▲). The solid line represents the best fit as analyzed by MEDAS with a rate constant, k_1, of 16×10^{-3} min^{-1}.

rate of carbamylation of those sites rather than the rate of CABP binding. At 2°C the rate of binding of [^{14}C]CABP to inactive rubisco was reduced even further. This agrees with our reported observation that changes in activation are considerably slower at ice temperatures (Perchorowicz et al., 1981).

Comparison of Rubisco Initial Activity with Binding of CABP. The rate of CO_2 fixation with rubisco has been shown to depend on the "activation" of rubisco (Perchorowicz et al., 1981) and is proportional to the number of rubisco sites capable of CABP tight binding (Butz and Sharkey, 1989; Hall et al., 1981). We have observed similar relationships. With a given rubisco preparation, the CO_2 fixation activity will generally be directly proportional to the amount of [^{14}C]CABP bound after 5 min. However, this is not always the case. Rubisco protein, which had been kept more than a year in the freezer, had lost considerable activity which was restored following 24 h incubation in 50 mM dithiothreitol (DTT). Activity and CABP binding studies were conducted before and after DTT incubation (Table 3.2). The observed k_{cat} was little influenced by the bicarbonate concentration used in activation and the degree of carbamylation, but was greatly influenced by incubation with DTT. It is obvious that other factors in addition to the ability to tight bind CABP also influence the observed k_{cat}.

Separation of the EC and ECM Forms Using EDTA. The rapid conversion of EC

to ECM during [^{14}C]CABP binding was inhibited if EDTA was added to bind excess Mg^{2+} (Fig. 3.2). Note that when EDTA was added, no further binding of [^{14}C]CABP occurred.

The possibility that EDTA could remove Mg^{2+} from the ECM form resulting in reduced binding of [^{14}C]CABP was explored. In Fig. 3.3, 11 mM EDTA (1.1 times [Mg^{2+}]) was added up to 60 seconds before or after addition of [^{14}C]CABP. When added 10 s or more before [^{14}C]CABP, tight binding of the radiolabel was reduced. But if added with or after [^{14}C]CABP, EDTA did not affect the amount of [^{14}C]CABP bound. [^{14}C]CABP added with excess EDTA may be used to estimate the ECM form exclusive of the EC form, while [^{14}C]CABP binding in the presence of Mg^{2+} measures both the ECM and EC forms.

Measurement of Carbamylated Rubisco in Chloroplasts. Previously, it was reported that during prolonged CO_2 fixation in light with isolated spinach chloroplasts, the activity of Rubisco decreased (Seftor and Jensen, 1986; Stumpf and Jensen, 1982). Here I compare the activity of rubisco during 60 min CO_2 fixation with intact chloroplasts with the amount of carbamylation of the binding sites (Fig. 3.4). With 1.5 mM bicarbonate, light-dependent CO_2 fixation continued for 20 minutes, then declined to almost zero. The RuBP level was between 80 to 100 nmoles per mg Chl or about equivalent to the total binding

Table 3.2. Effect of DTT on Rubisco Activity and the Tight Binding of [^{14}C]CABP

	Before DTT Incubation		After DTT Incubation	
Activation[a]	1mM HCO_3^-	10mM HCO_3^-	1mM HCO_3^-	10mM HCO_3^-
Activity[b]	0.42	0.68	0.75	1.31
Bound [^{14}C]CABP[c]	2.16	4.12	3.01	5.44
k_{cat}[d]	1.7	1.5	2.3	2.2

[a] Purified spinach Rubisco (1 mg/ml) was activated in 50 mM Hepes, pH 8.0, 10 mM $MgCl_2$ and bicarbonate as given.

[b] μmole CO_2/mg Rubisco per min.

[c] mol CABP/mol Rubisco

[d] mol CO_2/mol CABP binding site per sec.

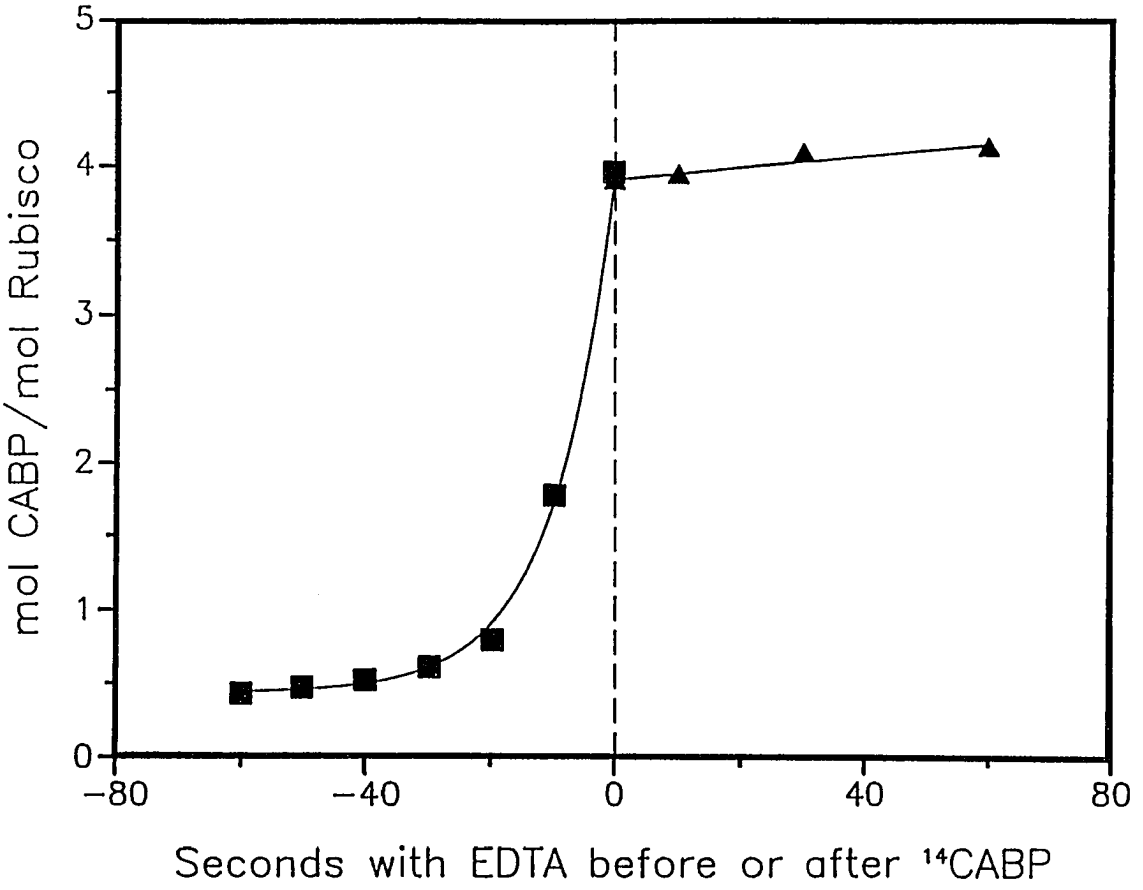


Figure 3.3. Effect of EDTA on the binding of [^{14}C]CABP. Purified spinach Rubisco (1 mg/ml) was partially activated with 5 mM $MgCl_2$ and 1.5 mM $NaHCO_3$. EDTA (5.5 mM) was added at times indicated. Binding of [^{14}C]CABP (50 μM) occurred for 5 min before [^{12}C]CABP (2.5 mM) was added. The slight increase in [^{14}C]CABP binding after zero was due to the slow increase in binding when inactive sites incubate with Mg^{2+}, CO_2 and [^{14}C]CABP

sites, yet CO_2 fixation had stopped by 60 min (data not shown, see Seftor and Jensen, 1986; Stumpf and Jensen, 1982).

The total activity and the total binding sites from the same chloroplast preparation were plotted equivalent to each other in Fig. 3.4, but using different axes. Again the initial activity and the number of initial CABP binding sites compared well to each other. At 60 min illumination, the initial CO_2 fixation activity of rubisco and the initial CABP binding sites had both decreased by half compared to the peak at 10 min.

The bottom curve of Fig. 3.4 represents the binding of [^{14}C]CABP in the presence of EDTA and estimates those sites which are ECM but not EC.

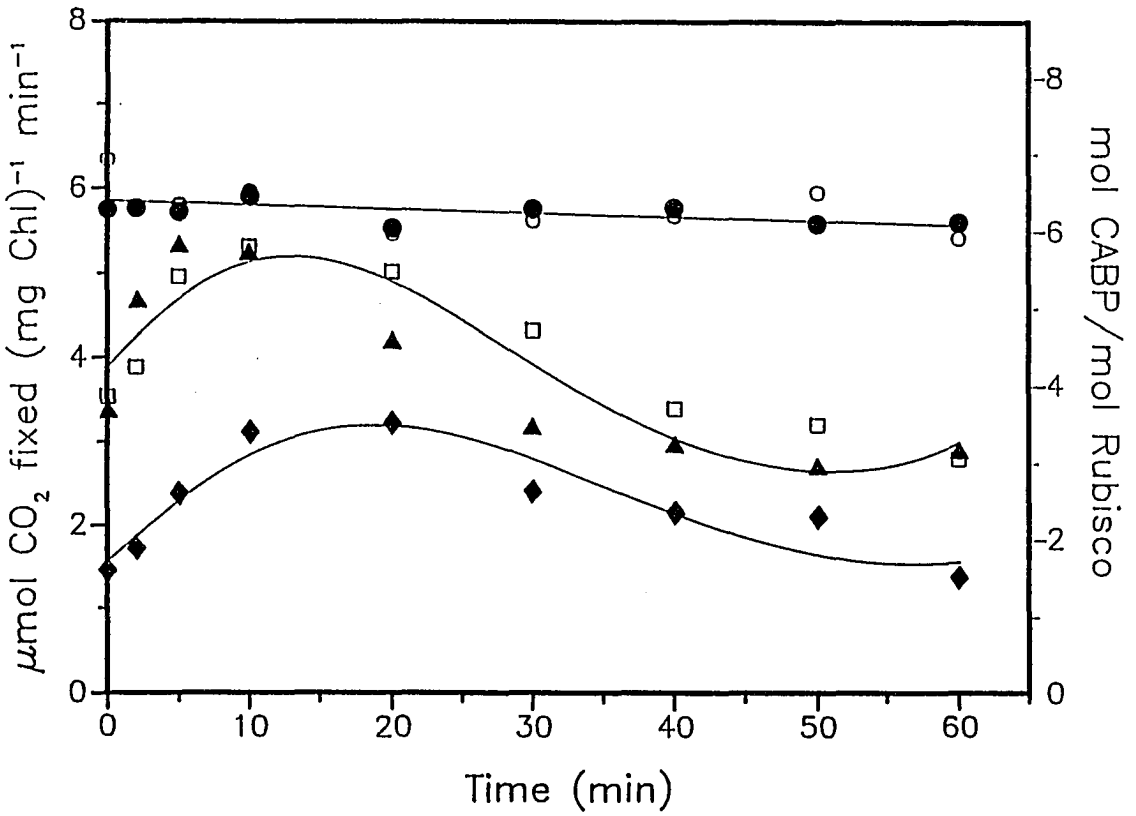


Figure 3.4. Comparison of rubisco total and initial activity and [^{14}C]CABP binding upon lysis of isolated spinach chloroplasts undergoing photosynthesis. Isolated chloroplasts having 30 μg Chl/ml, 2 ml were illuminated at 500 μE/m^2·s, 25° C with 1.5 mM $NaHCO_3$ (19). Samples were lysed and initial or total activities measured and [^{14}C]CABP binding determined under conditions of the initial or total activation. Total Rubisco activity (○); [^{14}C]CABP binding after total activation (●); initial Rubisco activity (□); [^{14}C]CABP binding of initial activation with excess Mg^{2+} (▲); [^{14}C]CABP binding of initial activation with excess EDTA (♦).

DISCUSSION

Conclusions to the Binding Kinetics. Analysis of the binding kinetics of CABP to ECM sites fits best an equation representing the sum of two first-order processes:

$$S_t = A_0 - (A_1e^{-k1t} + A_2e^{-k2t})$$

where S is the number of sites having bound [^{14}C]CABP per mol Rubisco at time t (min); A_0, the number of sites bound per mol rubisco at infinite time; and A_1 and A_2, the coefficients corresponding to the exponential rate constants, k_1 and k_2. The two rate constants differed by over ten fold, a difference easy to detect. The sum of the residuals (sum of the differences between the observed and calculated S_t values) as indicated by the close fit in Fig. 1 was less than 0.1. From the coefficients it appears that about half of the sites bind CABP at 40 min^{-1} with the second half binding at 3.2 min^{-1}.

Studies on the mechanism of binding of CABP indicate that it takes place in two steps (Pierce et al., 1980).

Step 1: E-C-M + CABP <=> E-C-M-CABP

Step 2: E-C-M-CABP → E-C-M*-CABP

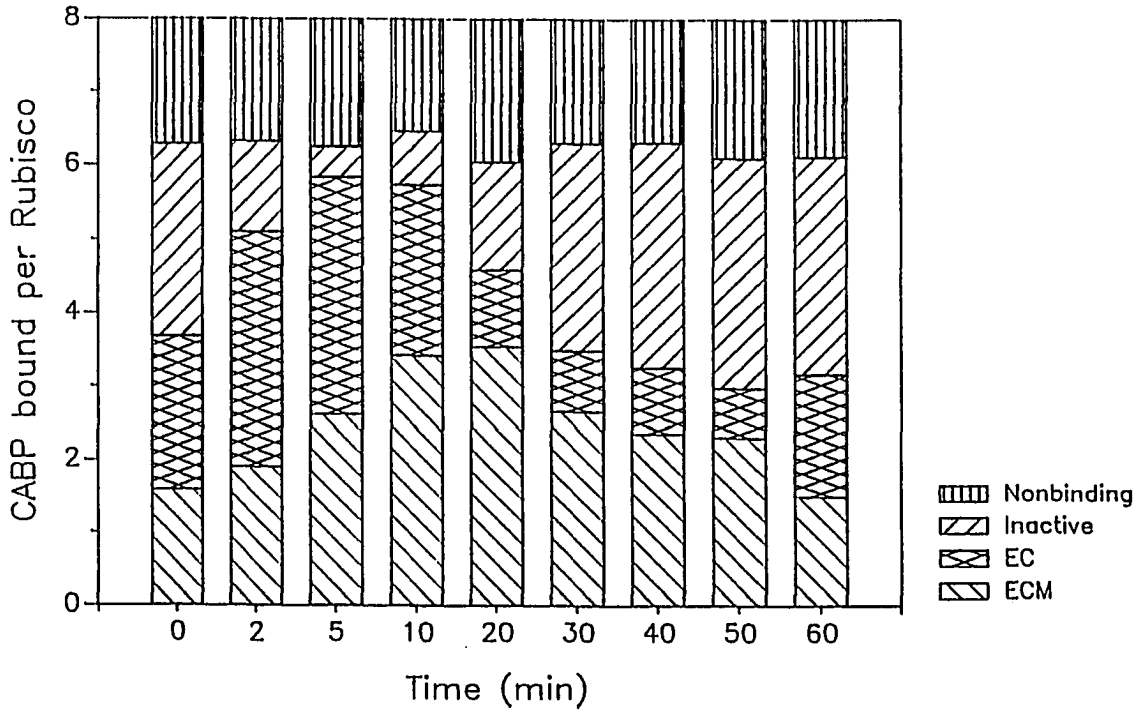


Figure 3.5. Graphic allocation of binding sites on partially activated Rubisco during photosynthesis with isolated spinach chloroplasts. From Figure 4 the active sites on Rubisco were put into four groups. From the top; sites not capable of binding CABP; inactive sites without bound $^{A}CO_2$, eg. E, E-RuBP; sites activated but without Mg^{2+}, e.g., EC; sites fully activated and involved in catalysis, e.g., ECM.

The first step is fast and in rapid equilibrium. Step 2 is slower, involving a conformational change, and is essentially nonreversible, being more than 10^{+5} in the forward direction (Pierce et al., 1980). It is this step that is measured.

Previous studies on the rate of Step 2 have obtained values of 2.4 and 2.2 min^{-1} (Pierce et al., 1980; Schloss, 1988), which is similar to our slower rate constant of 3.2 min^{-1}. These previous values were determined by measuring enzyme activity in the presence of limiting amounts of CABP.

Our kinetic experiments were done differently than those previously reported. We have used non-steady state kinetics in which the amount of CABP was saturating and stopped the reaction by dilution of the [^{14}C]CABP with [^{12}C]CABP. This has allowed us to observe the faster rate, which is mostly over when half of the sites are blocked.

Our observation that Step 2 of CABP binding involves two binding constants is consistent with the report of Johal et al (1985) that the tight binding of CABP is negatively cooperative; i.e. that it is much easier to bind CABP to unliganded rubisco than to rubisco having already bound CABP. Recent reports on the crystallographic structure of rubisco suggest that the active sites of rubisco are formed by a pocket at the opening of the alpha/beta barrel located in the C-terminal domain of one large subunit (L) and the N domain of the neighboring L

of a L_2 dimer (Andersson et al., 1989; Chapman et al., 1988). It is possible that with the six sites available for binding CABP, the binding of one CABP to each of three L_2 dimers causes the binding of the second CABP to be slower.

It is not clear why up to two sites, depending on the rubisco preparation, do not bind CABP tightly. Perhaps only six sites can form a tight binding complex with CABP with the last two having reduced affinity or blocked in some manner. It may be that they are "damaged" and could be repaired by treatment with thiol reagents like DTT. We have not investigated this further.

Relationship between Activity and CABP Binding. If Rubisco activity were simply controlled by the amount of carbamylated reaction sites, the k_{cat} (activity per carbamylated site) would remain constant. Other factors, such as the binding of an inhibitor of carboxylase, CA1P, could reduce the apparent k_{cat}, but this compound is apparently lacking in spinach (Seemann et al., 1985). Yet, the ability to tightly bind CABP is not always directly related to enzyme activity. We were able to increase the k_{cat} by incubation with DTT, a treatment previously reported to increase activity of Rubisco lost during storage (Hall et al., 1981). The modification of protein sulfhydryl groups has already been shown to be an important mechanism regulating *in vivo* enzyme activity in the chloroplast (Buchanan, 1980), but it is not known to what extent the protein of rubisco is

similarly influenced.

Nature of the Catalytic Sites on Rubisco in Chloroplasts. Based on studies with CABP the catalytic sites on rubisco can be assigned to one of four groups. 1. Sites not capable of binding CABP; 2. Sites without bound $^{A}CO_2$ which are free (E) or bound to other intermediates (E-RuBP, etc) but capable of binding CO_2 upon total activation of rubisco; 3. Sites binding $^{A}CO_2$ but not Mg^{2+} (EC); and 4. Sites binding both $^{A}CO_2$ and Mg^{2+} (ECM) and functional in catalysis.

These categories are depicted in Fig. 3.5, based on the data of Fig. 3.4. During the first five min of illumination of the isolated plastids there was a significant increase in the carbamylated EC form accompanying a decrease in the number of inactive E sites. ECM did increase but much slower that the EC form. This reflected a lack of available Mg^{2+} during illumination of the chloroplasts. The response is anomalous to isolated chloroplasts: do not see a build-up of the EC form when intact spinach leaves are illuminated.

By 20 min the rate of CO_2 fixation was severely reduced as the amount of activated sites (ECM and EC) declined. Apparently the rubisco activase (Portis et al., 1986) system could no longer maintain carbamylation of the reaction sites. Such changes varied between chloroplast preparations, partially as a function of the RuBP present. That the ECM form and the pool of RuBP did not decline to

zero when CO_2 fixation ceased may have been that RuBP was not available to those active sites, probably by it being bound to inactive sites and/or compartmentized in only some of the chloroplasts.

The binding of [^{14}C]CABP to partially or totally carbamylated reaction sites of rubisco is usually proportional to the partial or total activity of rubisco and indicates the carbamylation status of the reaction sites on the protein. By selecting the conditions for CABP binding, we have been able to indicate whether the sites are inactive, as E, or carbamylated as EC or ECM. Addition of [^{14}C]CABP with EDTA allows estimation of the ECM sites separate from the EC sites.

CHAPTER 4

NEGATIVE COOPERATIVE INTERACTIONS AMONG CATALYTIC SITES ON RIBULOSE BISPHOSPHATE CARBOXYLASE/OXYGENASE

ABSTRACT

The binding of ribulose 1,5-bisphosphate (RuBP) to decarbamylated sites and 2-C-carboxy-D-arabinitol 1-phosphate (CA1P) and 2-C-carboxy-D-ribitol 1,5-bisphosphate (CRBP) to carbamylated sites on ribulose 1,5-bisphosphate carboxylase/oxygenase displayed negative cooperativity. The Hill coefficients from equilibrium binding experiments are 0.7 to 0.8 for RuBP binding and 0.8 to 0.9 for CA1P and CRBP binding. The results also showed that the binding of ligand to decarbamylated sites was dependent on pH as previously reported by Mott and Berry, (1986). Lower pH enhanced binding affinity. However, no pH effect was observed for ligand binding to carbamylated sites. By using CABP-free rubisco and the rubisco with less than half of the sites bound with CABP, we measured two different K_m(RuBP) values (K_m^1 = 6.5 μM; K_m^2 = 13 μM). Rubisco with more than half of the sites bound with CABP had only one K_m(RuBP) = 12 μM

which corresponds to K_m^2. This suggests that the substrate binding affinity of the sites within the holoenzyme decreases as their neighboring sites bind RuBP or CABP. The evidence provided here suggests that communications between subunits within a large subunit dimer is significant. Binding of CABP to part of the rubisco sites has no effect on CO_2 dependent activation of the remaining unbound sites. Based on the observations here and the results from other workers, as well as the X-ray crystallographic structure of rubisco, a binding model which explains the behavior of rubisco binding ligand is proposed.

INTRODUCTION

Ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco) plays an important role in CO_2 fixation in photosynthesis. This enzyme is known to be composed of eight large and eight small subunits (L_8S_8) in higher plants, green algae and some photosynthetic bacteria. The eight large subunits are organized as four dimers and each dimer contains two substrate binding sites where carboxylation or oxygenation of ribulose bisphosphate (RuBP) occurs (Andersson et al., 1989).

In addition to the substrate RuBP, many other phosphorylated compounds can bind to the substrate binding sites on rubisco. The ligand, 2-carboxy-D-arabinitol bisphosphate (CABP), a transition state analog, binds tightly and specifically to carbamylated rubisco sites with a K_d less than 0.2 pM (Pierce et al., 1980; Schloss, 1988). The tight binding of CABP provides a useful tool for studying the properties of the rubisco substrate binding sites. Johal et al (1985) have demonstrated that the binding of CABP to higher plant rubisco was negatively cooperative by isoelectric focusing on non-denaturing gels to separate rubiscos with different sites bound with CABP. Belknap and Portis (1986) proved that the coordination of Mg^{2+} to activator CO_2 was negative cooperativity. The

results in Chapter 3 have shown that the rate of the binding of CABP to half of the sites was faster (with a binding rate constant of 40 per minute) than that of binding to the second half of the sites (3.2 per minute), suggesting that the binding of CABP to one site of a dimer affects binding affinity to the second site. This appears to be caused by transitions in protein conformation induced by the tight binding of CABP.

The interaction among catalytic sites represents communications between subunits. Theoretically, the structural properties of rubisco provides at least two different possibilities for communication between catalytic sites. One involves intra-dimer interactions within a dimer. The second involves inter-dimer interactions between dimers. These two kinds of interactions have a mixed effect on the apparent binding. As yet, there are no good methods by which these two kind of interactions can be clearly distinguished and analyzed in rubisco. In this chapter, these two different interactions will be discussed. Based on present and previous results, a binding model is also proposed.

The factors causing negative cooperative binding between sites may play an important role in regulation of rubisco activity *in vivo*. How the rest of the sites on rubisco behave when part of them are bound with ligand is not understood. In this chapter, an approach using partially CABP-bound rubiscos

studies the binding properties for the remaining unbound sites and shows negative interactions between sites. The results will be useful to understand the physiological implications of negative cooperativity.

MATERIALS AND METHODS

Materials Rubisco was purified from spinach according to McCurry et al., (1982). CABP and CRBP were synthesized from RuBP plus NaCN and purified by anion exchange chromatography (Pierce et al., 1980). CA1P was prepared by partially hydrolysis of CABP with alkaline phosphatase (Smrcka and Jensen, 1988). RuBP was synthesized and purified by methods described (Bahr and Jensen, 1978).

Binding of CABP to Rubisco in Different Molar Ratios Fully activated, purified spinach rubisco was incubated with [^{14}C]CABP in different molar ratios of ligand to holoenzyme for 30 min, then the rubisco was passed through a Pharmacia FPLC Superose 12 column to remove any unbound [^{14}C]CABP from the rubisco-[^{14}C]CABP complex. Rubisco peak fractions were collected. The amount of rubisco and [^{14}C]CABP binding to the protein were determined by absorbance at 280 nm (McCurry et al., 1982) and ^{14}C radioactivity respectively. The average number of sites which were bound with CABP per holoenzyme were then calculated from these data.

Activation of Rubisco at Air Levels of CO_2 and Different pH Unbound rubisco and rubisco with 3 sites bound with CABP were first decarbamylated by dialysis against 50 mM Hepes (pH 7.5) containing 0.2 mM EDTA overnight at 4 °C and

bubbled with N_2. The experimental procedures for activation at air levels of CO_2 and varying pH were similar to Lorimer et al. (1976).

Equilibrium Binding Rubisco was decarbamylated by dialyzing overnight against 25 mM Bicine, pH 8.0 and 0.2 mM EDTA at 4° C. The buffer for dialysis was bubbled with N_2 for 1 hour to remove CO_2 and was then sealed under N_2. Equilibrium binding was performed according to Na and Timasheff (1985) with some modifications. 60 mg of Bio-Gel P-100 was used for each tube in a total volume of 2 ml. After swelling the gel (remove air under vacuum) in 50 mM Bicine buffer containing 100 mM KCl for 20 hr, known amounts of RuBP and rubisco were added and mixed well, then the tubes were incubated at 25° C for 30 min. 0.5 ml of the liquid without gel was taken out for assay of RuBP by RuBP-dependent $[^{14}C]CO_2$ fixation under high CO_2 concentration (more than 300 μM). The buffer used for RuBP assay was free of both CO_2 and O_2 before addition of $^{14}CO_2$.

The internal volume (v_i) of the Bio-Gel P-100, the space available only to the low molecular weight materials, was determined separately using either blue dextran 2000 (Pharmacia) or rubisco according to Na and Timasheff (1985). The internal volumes determined were similar to each other (v_i = 0.597 ± 0.012 ml/60 mg dry gel, using blue dextran; v_i = 0.589 ± 0.009 ml/60 mg dry gel, using

deactivated rubisco; v_i = 0.599 ± 0.005 ml/60 mg dry gel, using activated rubisco), suggesting that the rubisco was totally excluded from the internal volume of the beads. The partition coefficient of the free ligand was estimated with [^{14}C]CABP or with [^{14}C]CA1P, and were close to unity (0.986). A control experiment where RuBP was incubated with or without deactivated rubisco showed that no RuBP was lost during the time required for equilibrium binding (data not shown).

The same method was used for determining the binding of CA1P and CRBP to carbamylated rubisco sites except 10 mM $MgCl_2$ and 10 mM $KHCO_3$ were included in the binding buffer and [^{14}C] labelled CA1P and CRBP were used.

Non-specific binding of ligand to rubisco was eliminated by 100 mM KCl in the binding solution. Presence of 100 to 150 mM KCl (or NaCl) in the elution solution was proved to remove non-specific binding to the protein during the separation of CABP-rubisco complex from free CABP by the Bio-Rad Econo Pac-10 gel filtration column (data not shown).

Data Analysis Negative cooperativity was analyzed either by Hill plots or Scatchard plots (Dahlquist, 1978).

RESULTS

Negative Cooperative Binding It is known that the eight sites on the rubisco holoenzyme do not behave independently. This was previously shown by the example where one of the eight sites on the holoenzyme was bound with 6-phosphogluconate, the activator CO_2 molecules on the other sites were stabilized (Jordan et al., 1983; Vater et al., 1983). This indicates cooperative interactions between sites within the holoenzyme.

Scatchard plots of equilibrium binding of RuBP to purified spinach rubisco show that the binding affinity to decarbamylated sites exhibits negative cooperativity (Fig. 4.1). The binding is also dependent on the pH and this will be discussed later. The first and second sites have the highest binding affinity and the last 3 to 4 sites have the lowest binding affinity (Table 4.1). Analysis by Hill plots further confirms this difference in binding. The Hill coefficients obtained at all three pH values were less than 1 (0.65 to 0.8), suggesting negative cooperative binding (Fig. 4.2 and Table 4.1).

The measurement of binding of RuBP to carbamylated sites on rubisco is difficult because of the turnover of the substrate. CA1P and CRBP are known to bind to carbamylated rubisco sites (Berry et al., 1987; Pierce et al., 1980). The

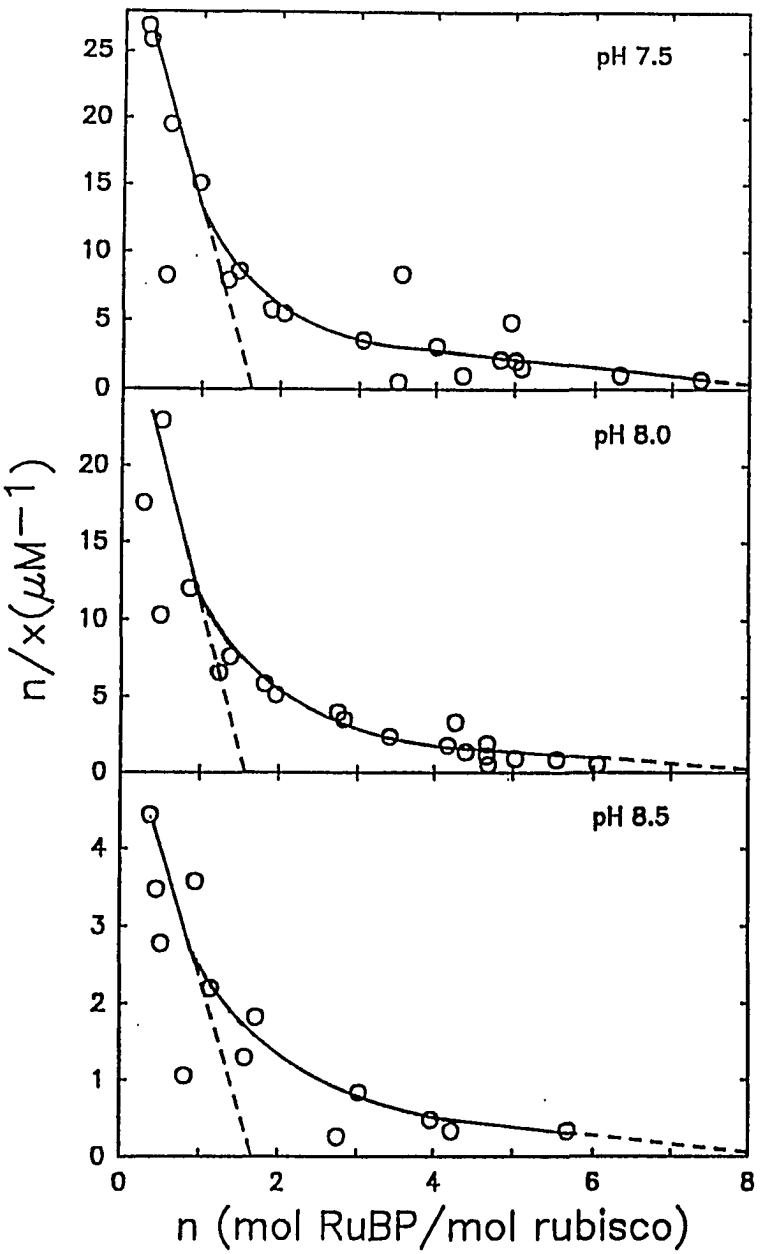


Figure 4.1. Scatchard plot for binding of RuBP to decarbamylated rubisco sites at various pH values. n = RuBP bound to rubisco (mol RuBP/mol rubisco); x = free RuBP (μM).

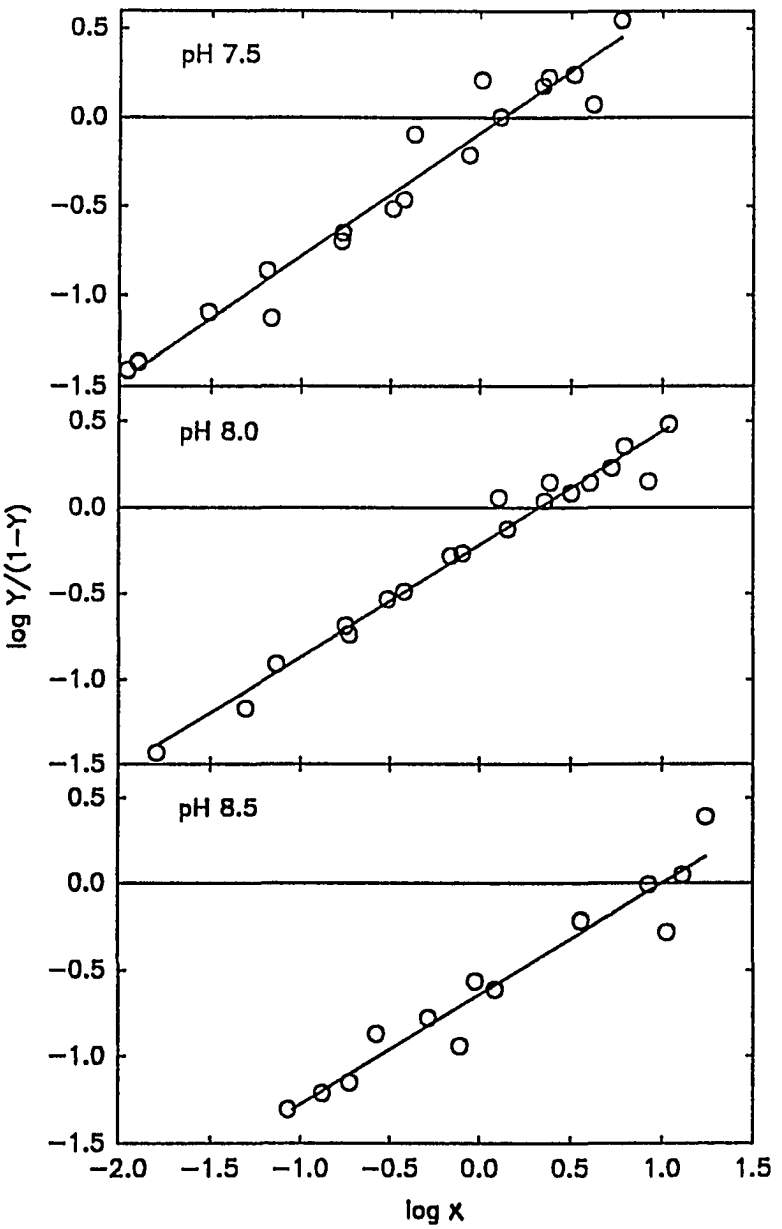


Figure 4.2. Hill plot for binding of RuBP to decarbamylated rubisco sites at various pH values.

Hill coefficients for binding of CA1P and CRBP to carbamylated sites was 0.8 to 0.9 (Fig. 4.3 and 4.4; Table 4.2), indicating that the binding of both ligands to carbamylated rubisco exhibits negative cooperativity.

Similar observations from the binding of 6-phosphogluconate to rubisco sites have been discussed by Andrews and Lorimer (1987). The K_d values reported (<1 μM) for the first site are considerably less than the bulk K_d (37 μM) measured by equilibrium binding experiments (Badger and Lorimer, 1983). My and previous results strongly suggest that binding of the first and second ligand (CA1P, CRBP and RuBP) to carbamylated and decarbamylated sites are considerably tighter than subsequent binding events.

Effect of pH on Ligand Binding The binding of RuBP to decarbamylated rubisco sites at three different pH values were presented in Fig. 4.1, 4.2 and Table 4.1. The K_d values were affected significantly by pH. Lower pH increased the binding affinity of rubisco for RuBP. This result agrees with the evidence of Mott and Berry (1986) and is in accordance with our previous observations concerning the binding of xylulose 1,5-bisphosphate to decarbamylated rubisco sites (Zhu and Jensen, 1991). This pH-dependence occurs only with decarbamylated rubisco sites. The binding of CA1P and CRBP to carbamylated rubiscosites was not affected by pH in the range of 7.5 to 8.5 (Table 4.2).

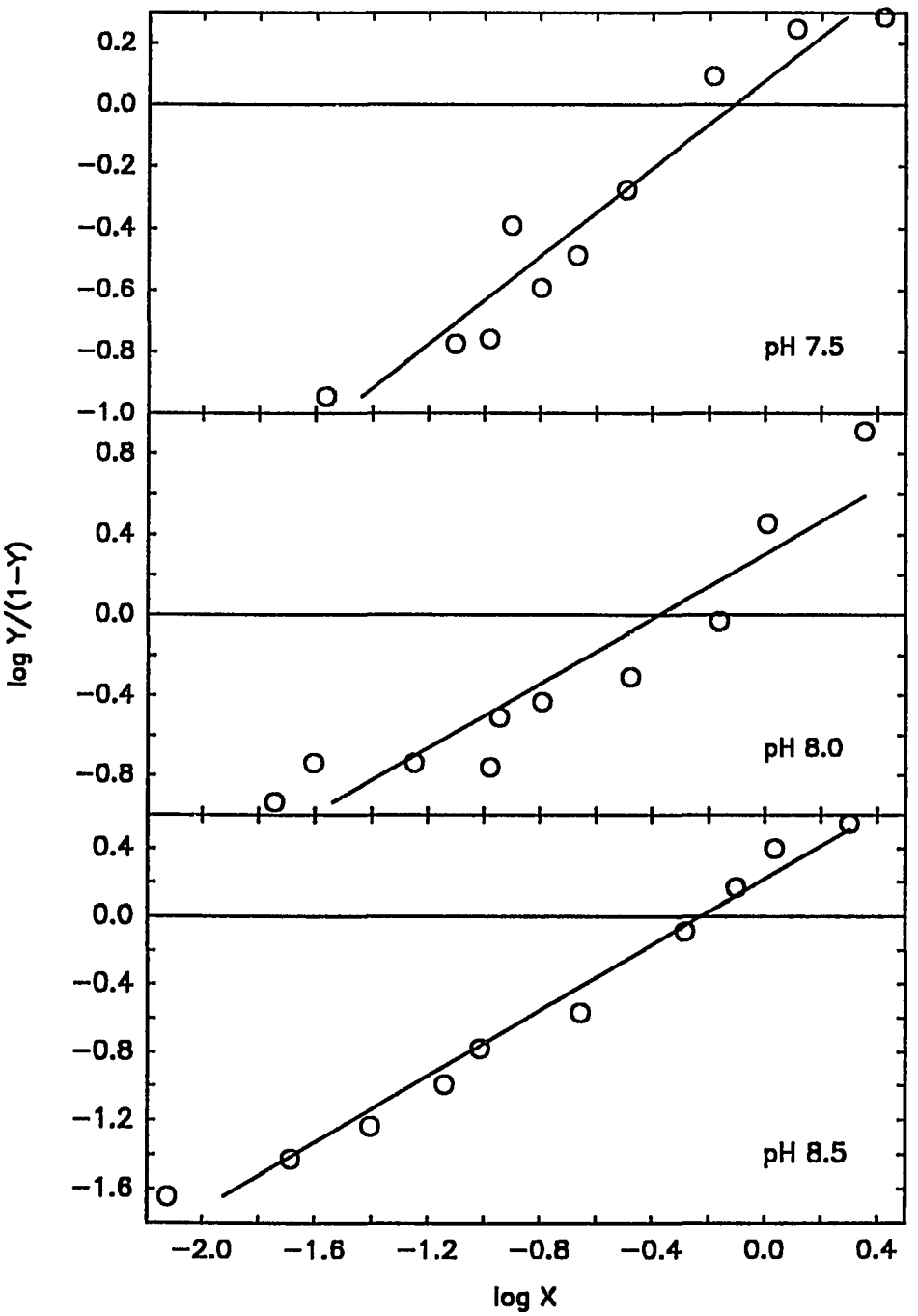


Figure 4.3. Hill plot for the binding of CA1P to carbamylated rubisco at various pH values.

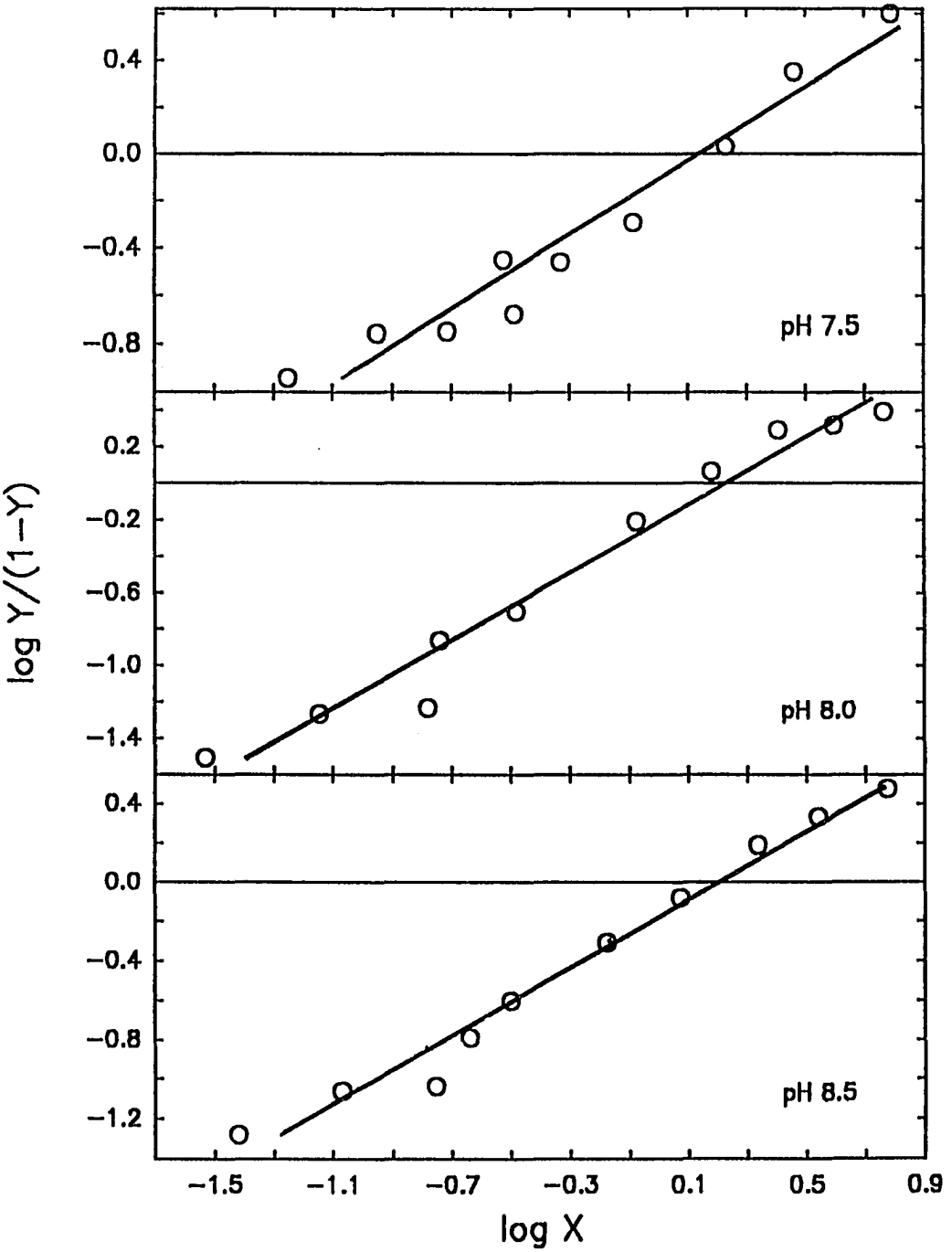


Figure 4.4. Hill plot for the binding of CRBP to carbamylated rubisco at various pH values.

Table 4.1. Binding Properties of Decarbamylated Rubisco Sites for RuBP

pH	Hill coefficient[a]	K_d(μM) overall[a]	beginning[b]	last[c]
8.5	0.65	8.86	0.62	9.85
8.0	0.79	2.27	0.09	2.70
7.5	0.69	1.37	0.07	1.74

[a]Values were estimated by Hill plots.

[b]K_d for the first 2 sites. Values were obtained from Scatchard plots.

[c]K_d for last 4 sites. Values were obtained from Scatchard plots.

Table 4.2. Binding Properties of Carbamylated Rubisco Sites for CA1P and CRBP

pH	Hill coefficient[a]		Overall[a] K_d (μM)	
	CA1P	CRBP	CA1P	CRBP
7.5	0.84	0.89	0.54	1.94
8.0	0.81	0.86	0.45	1.55
8.5	0.91	0.90	0.55	1.49

[a]Values calculated from Hill plots.

The lowering of the pH can stimulate the tight binding of CABP and CA1P to decarbamylated rubisco sites (Table 4.3). After passing through a Bio-Rad gel filtration column, a significant amount of CABP or CA1P were still bound to the decarbamylated rubisco at lower pH.

Effect of Ligand Binding on K_m(RuBP) Although recent evidence indicates that the binding of ligand, such as CABP, to some sites influences the binding affinity to the rest of the sites, there are few quantitative measurements to show this effect on substrate binding. Except for enzymes exhibiting simple Michaelis-Menten kinetics, the K_m is not the same as the substrate equilibrium dissociation constant, K_s. However, K_m can be considered as an apparent dissociation constant that may be treated as the overall dissociation constant of all enzyme-bound species in the reaction mechanism (Fersht, 1985). In this study, CABP-freerubisco and rubisco which had been bound with CABP in different molar ratios were used to measure the apparent K_m(RuBP). As can be seen from Fig. 4.5a, Lineweaver-Burk plots for rubisco unbound with CABP and rubisco with two sites bound with CABP show two linear segments, which extrapolate to two different K_m(RuBP) on the x-axis. The first apparent K_m is 6.5 μM and the other about 13 μM. The curves for rubisco with more than 4 sites bound with CABP fit best a single linear line (Fig. 4.5b) and a K_m of 12 μM, which is similar to 13 μM above. As RuBP

Table 4.3. The effect of pH on the relative binding of CABP and CA1P to decarbamylated rubisco sites. 80 μg (1.16 nmol sites) decarbamylated rubisco in 0.1 M buffer (MES for pH 6.5, Hepes for pH 7.0 and 7.5, Bicine for pH 8.0 and 8.5), 0.2 mM EDTA and 100 mM KCl was incubated first with 5 nmol CABP or 10 nmol CA1P in a volume of 0.6 ml for 1 h. Unbound ligand was separated by passing through a Bio-Rad P10 gel filtration column which was equilibrated with the same buffer as binding.

pH	number of sites bound per holoenzyme	
	CABP	CA1P
6.5	4.78	1.58
7.0	4.14	1.06
7.5	3.95	0.37
8.0	2.79	0.47
8.5	0.65	0.19

binds progressively to all of the carbamylated sites, two different substrate binding affinities can be seen, one with a high affinity for RuBP and the other with a lower affinity. If half or more of the sites are already bound with RuBP or CABP, the low affinity K_m is seen.

After consideration of RuBP-Mg^{2+} form which can be calculated with an association constant of 530 M^{-1} (Porits et al., 1986), I calculated $K_m(RuBP)^f$ values of 1.02 and 2.17 μM, which are dramatically lower than K_m(RuBP) based on total added RuBP.

No Effect of Ligand Binding on the Apparent pK_a of $K^{L8}201$ The difference of the binding affinity among eight sites on the holoenzyme is the result of negative cooperativity induced by the binding of ligand via conformational transitions in the protein. It is not known if partial binding of CABP to rubisco would cause rubisco conformational changes which may affect the local environment around some important amino acid residues, such as $K^{L8}201$, and influence the carbamylation of the remaining sites. To test this, the pH-dependence of carbamylation of the CABP-free rubisco and the rubisco having half of the sites bound with CABP per molecule were compared (Fig 4.6) following the procedures of Lorimer et al (1976). The results showed that both rubiscos had similar activation patterns as a function of pH, suggesting that binding of CABP to part of the sites on the

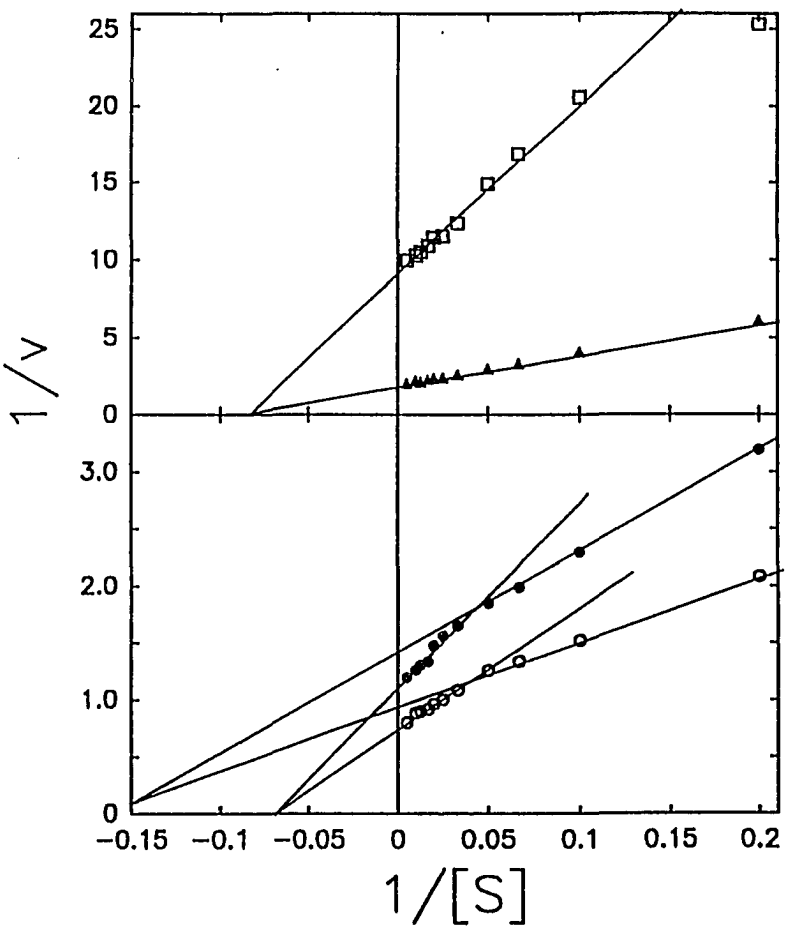


Figure 4.5. Km(RuBP) of native and partially CABP-bound rubisco. Total reaction volume is 1 ml containing 50 mM Bicine, pH 8.0, 10 mM $KH^{14}CO_3$ (2.5 μCi/μmol), 10 mM $MgCl_2$ and 2 - 20 μg rubisco (depending on the specific activity of the rubisco partially bound with CABP). RuBP concentration was between 5 to 200 μM. The reaction was started by addition of rubisco and lasted for 30 s. (○) Native rubisco; (●) Rubisco incubated with CABP in a ratio of 2:8 (CABP:sites); (▲) Rubisco with CABP in 4:8; (□) Rubisco with CABP in 5.5:8. The maximum binding sites per holoenzyme was 6.81.

v is μmol CO_2 mg^{-1} rubisco min^{-1}; S is total added RuBP (μM).

rubisco holoenzyme did not influence the pK_a for carbamylation of the ϵ-amino group of lysine 201 on the remaining sites.

The K_{act} for CO_2 was also not affected by partial binding of CABP (Fig 4.7). The response of activation of CABP-free rubisco and the rubisco with half of the sites bound with CABP was determined at various CO_2 concentrations at pH 8.3. K_{act} for CO_2 was calculated using the method of Wilkinson (1961) and the value of 17 μM (CABP-free rubisco) and 18 μM (rubisco with half of the sites bound with CABP) are similar to each other. These results appear to agree with the observations of Belknap and Portis (1986) who found no cooperative effect with respect to CO_2 during carbamylation of all sites.

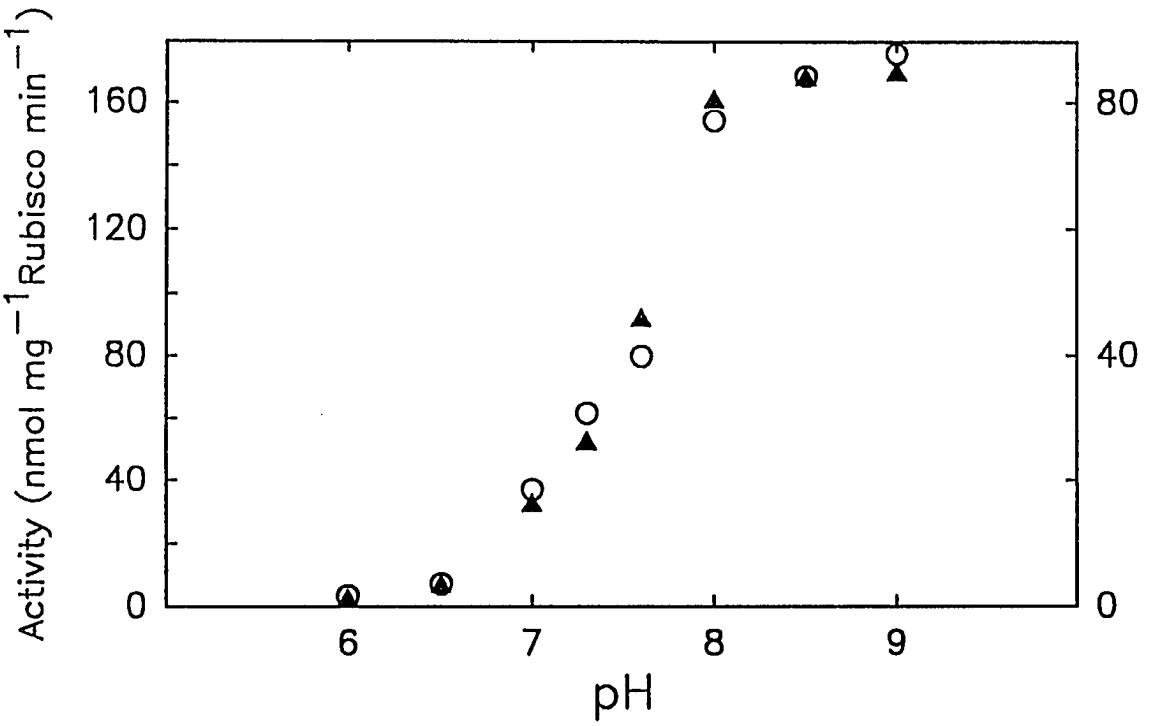


Figure 4.6. pH-Dependent carbamylation of native and partially CABP-bound rubisco. 0.7 mg/ml natural rubisco (▲) and the rubisco with 3 sites bound CABP (O) were deactivated by dialysis overnight against 50 mM Hepes, pH 7.5, 0.2 mM EDTA and bubbled with N_2. Deactivated rubisco was incubated in 0.1 M buffer (Mops for pH 6.0, 6.5 and 7.0; Hepes for pH 7.3 and 7.6; Tris for pH 8.0, 8.5 and 9.0), 10 mM $MgCl_2$, 0.5 mM DTT, 3 μg of carbonic anhydrase and air level CO_2 for 30 min. All activities were assayed at pH 8.0 with 0.1 M Hepes, 10 mM $MgCl_2$, 1 mM $KH^{14}CO_3$ (1 μCi/μmole), 25° C.

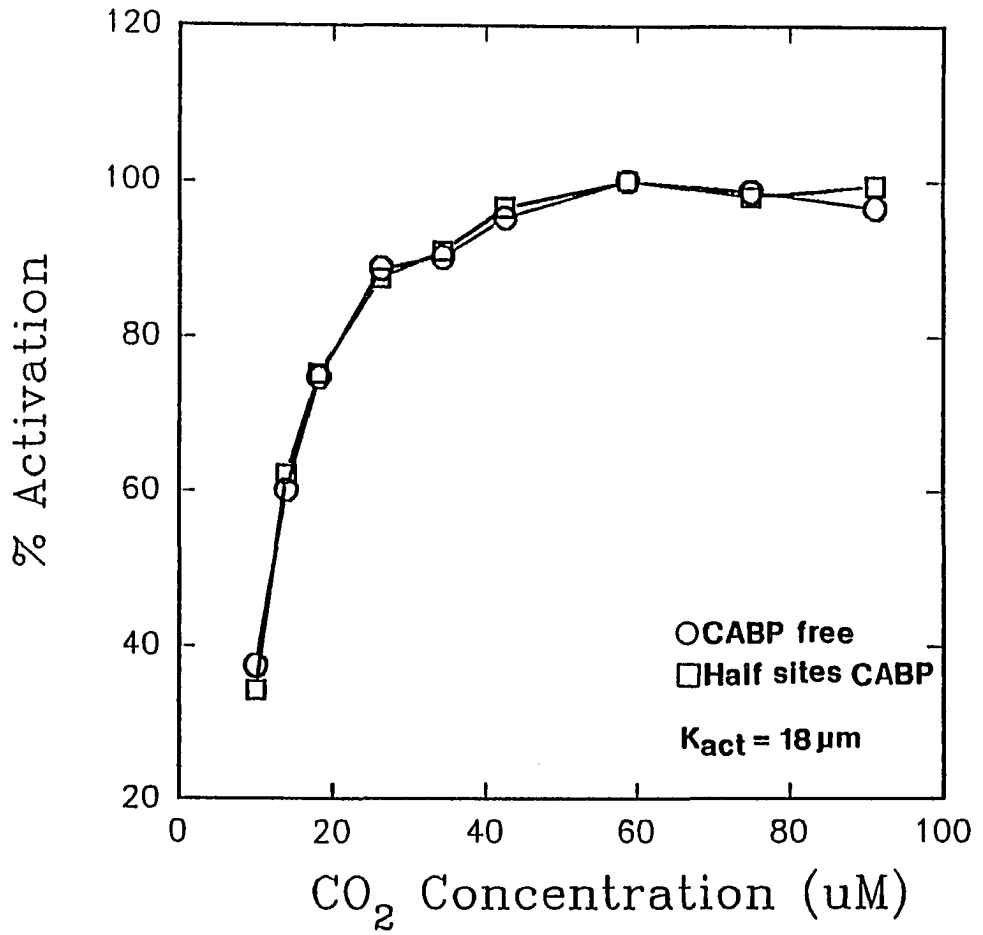


Figure 4.7. $K_{act}(CO_2)$ is not influenced by binding of CABP. CABP-free rubisco (O) and the rubisco with half sites bound with CABP (□) were incubated at pH 8.3 in 50 mM Bicine, 20 mM $MgCl_2$, 1 mM DTT and various concentrations of $KH^{14}CO_3$. CO_2 fixation was assayed in the same solution except for 5 mM $KH^{14}CO_3$ and 1 mM RuBP used.

DISCUSSION

Mechanisms for Negative Cooperative Binding. The crystallographic structure of rubisco reveals that the catalytic site is located at the intra-dimer interface between the C-terminal domain of one large subunit and the N-terminal domain of the second large subunit. The two large subunits in a L_2 dimer have the highest interactions in the L_8S_8 molecule, with 25% of the surface of the two subunits buried in the L_2 dimer interface with 136 residues from each subunit involved in this interaction (Knight et al., 1990). This close relationship between two large subunits in an L_2 dimer provides a major potential for the communication between the two catalytic sites.

Binding of ligand to one site in an L_2 dimer appears to cause protein conformational changes which transfers to the second site on the same dimer, resulting in a decrease in binding affinity of the second site. Kwok and Wildman (1974) found that the binding of RuBP to inactive rubisco (with 0.1 mM EDTA) showed cooperativity in that the conformational change measured with UV-difference spectroscopy was completed after 4 substrate molecules per holoenzyme were bound. There are two possible explanations for this observation. One is four ligands equally bind to four dimers, each dimer with one;

The second is four ligands bind to two dimers, each dimer with two and the other two dimers remaining unoccupied. Negative cooperative binding properties support the first explanation. If the second explanation were true, the interaction for the two binding sites on the same dimer would most likely be positive cooperative. However, so far there is on evidence for positive cooperativity between binding sites. This suggested that there were major interactions during binding of RuBP to decarbamylated rubisco within a dimer. Our previous results from the binding of CABP to carbamylated rubisco sites also suggest that the cooperativity occurred within the dimer (Chapter 3). The observations of Fig. 4.5 indicates that the binding of RuBP to rubisco having less than half of the sites bound with CABP induced conformational changes in the protein which exhibited at least two different K_m values. However, the K_m(RuBP) did not change when more than half of the sites were bound with CABP. This results consistent with the logic that the interactions between the two catalytic sites within a dimer are more prominent than those between dimers.

Inter-dimer interactions of the catalytic sites within the holoenzyme is also well documented (Jordan et al., 1983; Vater et al., 1983; Johal et al., 1985; Belknap and Portis, 1985). This kind of interaction occurs both by dimer-dimer contact and by mutual contact to a small subunit. Eight percent of the solvent-

accessible surface of the large subunits is involved in direct dimer to dimer contact with a neighboring dimer, with 28 charged side-chains at the dimer-dimer interface (Knight et al., 1990).

The other long-distance communication between L_2-L_2 dimers is via interactions with small subunits. From the crystallographic structure, the large subunit in one L_2 dimer has much more interaction with the nearest small subunit than with the neighboring L_2 dimer. Each large subunit interacts with 3 small subunits. There is evidence for long-distance communication between catalytic sites by way of a small subunit. The catalytic properties exhibited by large subunits was significantly affected by isomorphic replacement studies of amino acids in the small subunit (Voordouw et al., 1987).

A model is proposed based on the interactions given by the crystallographic structure of rubisco and the binding kinetic data (Fig. 4.8). This model is supported by the following observations:

Belknap and Portis (1985) demonstrated that the catalytic sites on rubisco were not heterogeneous and that negative cooperativity was due to true allosteric interactions. This fact and the symmetric property of rubisco structure support that the sites on rubisco are all equal before binding of ligand.

After the first ligand binds to rubisco, the most affected site would be

its neighboring site on the same dimer. This assumption is supported by the evidence of Kwok and Wildman (1974), Zhu and Jensen (1990) and the results in Fig 4.5. All these observations suggest that the strongest interaction between catalytic sites occurs within a dimer.

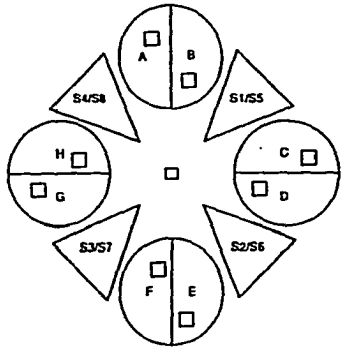
Stabilization of most activator CO_2 by binding of 6-phosphogluconate to only one eighth of the sites (demonstrated by Jordan et al., 1983 and Vater et al., 1983) indicates that the interaction also occurs between dimers. The crystallographic structure reveals that a dimer has direct contact with its neighboring dimers, but not the opposite one. Thus, the highest probability for the second ligand binding would occur on the opposite dimer. The sites on the remaining two unoccupied dimers would be equally influenced by their neighboring dimers.

After four sites occupied, the remaining four sites would exhibit similar binding affinities. This 2:2:4 model is especially supported by the Scatchard plots (Fig 4.1) where the points for first 2 sites are virtually on the same linear line and the last 4 site on the another linear line. The previous results discussed above and the data shown in Fig 4.5 well demonstrate that after 4 sites are occupied, the remaining 4 sites exhibit the same binding affinity on the Lineweaver-Burk plot. Also, after half of the sites on rubisco were bound with RuBP, Kwok and

Figure 4.8. A proposed model of the binding mechanism for rubisco. The diagram of L_8S_8 rubisco structure is taken from Knight et al. (1990). The squares represent substrate binding sites. The triangles, circles and diamonds represent sites after conformational changes induced by neighboring sites upon binding a ligand. Open symbols represent unoccupied sites and the filled symbols represent occupied sites.

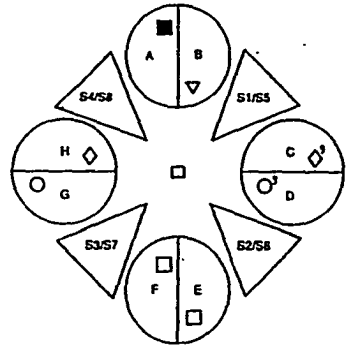
Before binding:

$K_A = K_B = K_C = K_D = K_E = K_F = K_G = K_H$



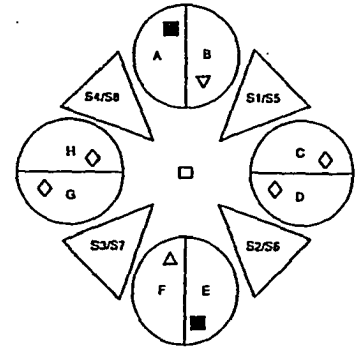
After binding to A:

$\underset{\sim}{K_A} = K_E = K_F > K_G = K_D > K_H = K_C > K_B$



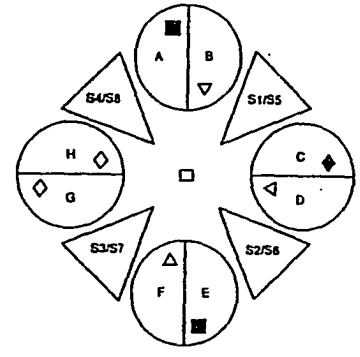
After binding to A, E:

$\underset{\sim}{K_A} = \underset{\sim}{K_E} > K_C = K_D = K_G = K_H > K_B = K_F$



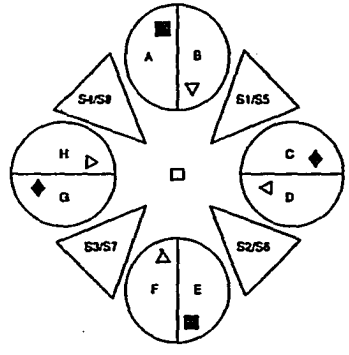
After binding to A, E, C:

$\underset{\sim}{K_A} = \underset{\sim}{K_E} > \underset{\sim}{K_C} = K_G = K_H > K_B = K_D = K_F$



After binding to A, E, C, G:

$\underset{\sim}{K_A} = \underset{\sim}{K_E} > \underset{\sim}{K_C} = \underset{\sim}{K_G} > K_B = K_D = K_F = K_H$



$E + 2I \rightleftharpoons EI_2 \qquad K_{1\text{-}2} = [EI_2]/[E][I]$

$EI_2 + 2I \rightleftharpoons EI_4 \qquad K_{3\text{-}4} = [EI_4]/[EI_2][I]$

$EI_4 + 4I \rightleftharpoons EI_8 \qquad K_{5\text{-}8} = [EI_8]/[EI_4][I]$

$K_{1\text{-}2} > K_{3\text{-}4} > K_{5\text{-}8}$

Wildman (1974) were unable to detect further conformational change for remaining sites bound with ligand. This suggests that the interaction among remaining four sites are not significant.

pH Effect on Ligand Binding The binding of CABP to catalytic sites involves a number of amino acid residues plus the co-ordination of Mg^{2+} in the carbamylated site (Andersson et al., 1989). The phosphate groups form mainly H-bonds with the NH-groups of nearby peptide residues and with the charged amino groups of Arg, Lys and His (Fig. 4.9). Our results clearly show that the binding of ligand to carbamylated sites is independent of pH between 7.5 to 8.5 (Table 4.2), while the binding to decarbamylated sites is highly pH dependent. The binding affinities of RuBP (Fig. 1 and 2, Table I) or XuBP (Zhu and Jensen, 1991) to decarbamylated sites are similar in magnitude, suggesting that it is the orientation of the bisphosphate groups which provides the major binding strength rather than the arrangement of the sugar hydroxyl groups and this binding is pH sensitive. The opposite appears true during binding of ligands to carbamylated sites. CABP and CA1P both bind quite tightly, while CABP and CRBP exhibit large differences in binding affinities. Ligand binding strength depends more on coordination of Mg^{2+} to the 2-carboxyl group and the C-2 and C-4 hydroxyl groups than on phosphate binding. Mg^{2+} coordination appears not of be greatly influenced by pH between

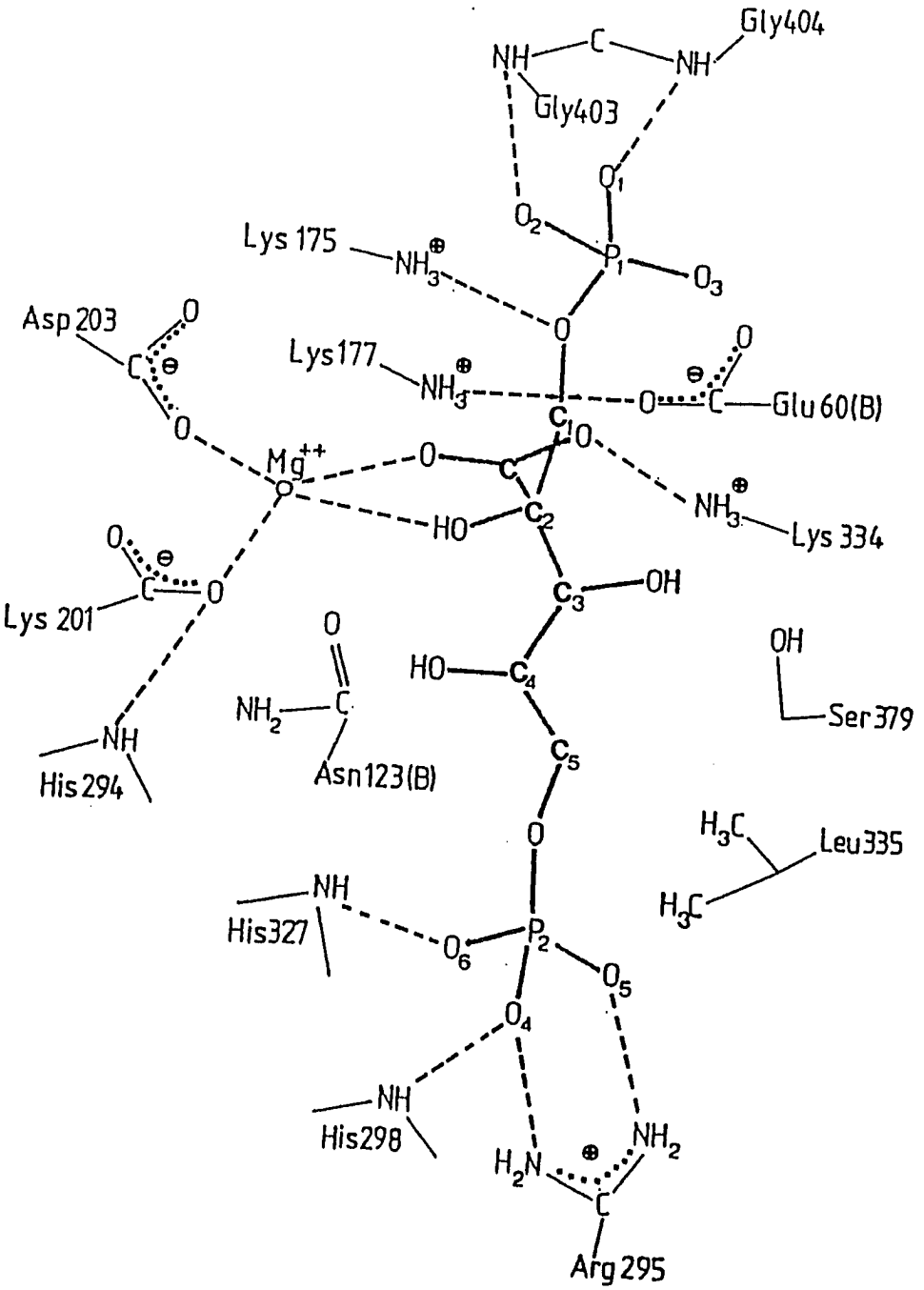


Figure 4.9. Active site of spinach rubisco, showing interactions between protein side chains, Mg^{2+} and a bound transition state analog, CABP. (Taken from Andersson et al., 1989)

7.5 and 8.5 while the binding of bisphosphates is. The differences between carbarmylated sites in binding ligand most likely explains why pH has little effect on carbamylated sites.

Physiological Implications. The study on negative cooperative interaction among catalytic sites on rubisco provides insights into the interactions between rubisco and rubisco activase. A roughly quantitative estimation reveals that the molar ratio of rubisco to rubisco activase is about 9:1 or a ratio of catalytic sites toactivase of 72:1 (Robinson et al., 1988). Each rubisco activase would have to react with several rubisco holoenzyme proteins rather than each individual catalytic site. The properties of negative cooperativity among catalytic sites offers potentially a way for effective interactions. The possible mechanism for the rubisco-rubisco activase interactions will be discussed in the final discussion.

CHAPTER 5

XYLULOSE 1,5-BISPHOSPHATE SYNTHESIZED BY RIBULOSE BISPHOSPHATE CARBOXYLASE DURING CATALYSIS BINDS TO DECARBAMYLATED ENZYME

ABSTRACT

Xylulose 1,5-bisphosphate (XuBP) has been separated and identified from compounds binding to rubisco during *in vitro* catalysis following HPAE-PAD separation and detection by pulsed amperometry (Dionex HPAE-PAD). The results indicate that the XuBP could be synthesized from RuBP by carbamylated rubisco during catalysis, but it is not formed by decarbamylated rubisco. Further investigation showed that the amount of XuBP formed by rubisco was pH-dependent. Low pH favored formation of XuBP. When rubisco catalyzed CO_2 fixation at a pH lower than 8.0, significant amounts of XuBP were formed.

Like RuBP, XuBP was found to bind to inactive rubisco. Upon incubation of fully activated rubisco with 5 μM XuBP, the effect of XuBP on the inhibition of rubisco activity started with loss of activator CO_2. This observation suggests that

the decarbamylation of rubisco occurs first before XuBP binds to enzyme sites, by causing a shift in the equilibrium to the inactive form even in the presence of excess CO_2 and Mg^{2+}. Since XuBP is also present *in vivo* (see Chapter 7), regulation of rubisco catalysis in plants may occur by decarbamylation of the catalytic sites which is stabilized by XuBP or RuBP binding.

Binding of XuBP to decarbamylated rubisco sites was found to be highly pH-dependent. At pH 7.0 and 7.5, apparent K_d is 0.03 μM, at pH 8.0 and 8.5, K_d increases up to 0.35 and 2.0 μM respectively. This difference was caused by increasing association rate constant (k_{on}) and decreasing dissociation rate constant (k_{off}). Binding of CA1P to carbamylated rubisco sites was not significantly influenced by pH.

INTRODUCTION

The enzyme, ribulose 1,5-bisphosphate carboxylase/ oxygenase (rubisco) has to be carbamylated by addition of an activator CO_2 followed by binding of Mg^{2+} to become catalytically active. Rubisco activity in plants is regulated by changing its carbamylation state dependent on light and/or binding an inhibitor such as CA1P, a potent rubisco inhibitor (Berry et al., 1987; Kobza and Seemann, 1988; Perchorowicz et al., 1981; Seemann et al., 1985). A second chloroplast protein, rubisco activase, is also involved (Portis et al., 1986).

Rubisco can be spontaneously activated *in vitro* by incubation with CO_2 and Mg^{2+} at alkaline pH (Laing et al., 1975; Lorimer and Miziorko, 1980). *In vitro* upon addition of RuBP the catalytic rate decreases with time in a first order manner to a final steady state rate as activated rubisco fixes CO_2. The time-dependent kinetics of rubisco have been described (Badger et al., 1980, Edmondson, 1990; Mott and Berry, 1986; Sicher et al., 1981). The term "fallover" describes this die-off phenomenon (Edmondson et al., 1990). Almost at the same time, Edmondson et al (1990) and our results demonstrated that the products of RuBP isomerization, such as XuBP, could inhibit rubisco activity during catalysis. The inhibitors formed by rubisco per se during catalysis explain the fallover

phenomenon. They further proposed that the loss of rubisco activity during catalysis occurs without loss of carbamate CO_2. This is due to the tight binding of an unknown inhibitor, other than XuBP, to carbamylated sites.

In this study I have used a pulsed amperometric detector following HPAE-PAD separation to separate and identify the compounds bound to rubisco during catalysis. The observations of Edmondson et al (1990) were corroborated, but I found that their conclusions were only part of the story. The formation of XuBP is pH-dependent and XuBP is bound slowly but tightly to inactive sites of rubisco. Lower pH increases the rate of synthesis as well as the binding affinity of rubisco for XuBP. The decarbamylation of rubisco during fallover does occur under low pH, but does not under high pH (see next chapter).

From celery leaf extracts I have separated and identified XuBP (see Chapter 7), indicating that XuBP could also be formed *in vivo*. From these and other results (Brooks and Portis, 1988; Cardon and Mott, 1989; McCurry and Tolbert, 1977), it appears that the regulation of rubisco catalysis does occur by decarbamylation induced by RuBP or XuBP binding to inactive rubisco both *in vitro* and *in vivo* if pH of the chloroplast stroma drops lower than pH 8.0.

MATERIALS AND METHODS

RuBP was synthesized and purified as previously described (Bahr and Jensen, 1978) and shown to be free of other sugar phosphates, including XuBP by HPAE-PAD. CABP was synthesized and purified as described (Pierce et al., 1980). Rubisco from spinach leaves was purified (McCurry et al., 1982). XuBP was synthesized by Alan Smrcka by aldolase-catalyzed condensation of glycolaldehyde phosphate and dihydroxyacetone phosphate (Byrne and Lardy, 1954; Paech et al., 1978). Xylulose was obtained from Sigma.

Rubisco was activated by incubation with 10 mM $KHCO_3$ and 10 mM $MgCl_2$ in 50 mM Hepes, pH 8.0 for 15 to 30 min. Activity of rubisco was measured in 50 mM Hepes, pH 8.0, 10 mM $NaH[^{14}C]CO_3$ (1 Ci/mol), 10 mM $MgCl_2$ and 0.5 mM RuBP. After 30 sec. 1 ml of 1 N HCl was added and the unfixed $[^{14}C]CO_2$ released upon heating at 80° C.

Rubisco-Bound Compounds. After 30 to 60 min CO_2 fixation with RuBP during which fallover occurred, the rubisco reaction solution was eluted through a Bio-Rad Econo-Pac^R 10DG gel filtration column to separate rubisco and protein-bound compounds from unbound substrates and product. The rubisco protein fractions were combined and $HClO_4$ added to 5%. Precipitated protein was

removed, the supernatant was neutralized to pH 6.5-7.0 with 4 N KOH and the $KClO_4$ precipitate removed. The supernatant was put either directly on the HPAE-PAD or passed through a silica ion-exchange column (Sepralyte SAX, 1 ml syringe with a 0.6 ml bed volume), washed with 1 ml H_2O and the sugar monophosphates and PGA eluted with 5 ml of 0.05 N HCl. The bisphosphates were eluted next with 5 ml of 0.15 N HCl (Smrcka and Jensen, 1988). The fractions were evaporated almost to dryness under vacuum in a Savant SpeedVac concentrator to remove HCl and redissolved in a small volume of water. Large amounts of PGA in the samples on the HPAE-PAD interfered with separation of the bisphosphates.

Separation and Identification of XuBP by HPAE-PAD. The HPAE-PAD system used a Dionex CarboPac™ PA1 or an Ion PAC column, a Dionex pulsed amperometric detector (Model PAD-2) with a pump and solvent delivery system (Spectra Physics SP8700) at 0.8 ml/min. For sugar phosphate separation, the column was equilibrated with 50 mM NaOH and 400 mM K acetate. Five min after injecting the sample, a gradient of K acetate from 400 to 500 mM was run. In this system, RuBP and XuBP were well separated (Fig. 5.1). For sugar separation, the column was equilibrated and the sample eluted with 100 mM NaOH. Ribulose and xylulose were well separated under these conditions (Fig.

5.3).

Trapping of Activator CO_2 with CABP. Rubisco was first activated by incubation with 10 mM [^{14}C]$KHCO_3$ (10 Ci/mol) and 10 mM $MgCl_2$ in 50 mM Hepes, pH 8.0 for 15 min. After addition of XuBP or RuBP, 0.2 ml was taken and added to 50 μM [^{12}C]CABP plus 0.5 M [^{12}C]$KHCO_3$ to dilute the radiolabel. The rubisco-CABP complex was separated by passing it through a 10DG gel filtration column. The amount of rubisco after the column was estimated by absorbance at 280 nm and multiplied by 0.61. The $^{A}CO_2$ trapped was determined by counting the rubisco-CABP fractions.

RESULTS

XuBP Can Be Formed during Catalysis by Carbamylated Rubisco. Purified spinach rubisco was activated in 10 mM $KHCO_3$ and 10 mM $MgCl_2$. Freshly prepared RuBP, which was shown to be XuBP free on the HPAE-PAD system, was added to start CO_2 fixation. After 30 min reaction, inhibitor was prepared from this reaction mixture according to a method described in Materials and Methods section and was assayed on HPAE-PAD system to separate sugar phosphates. An XuBP peak was identified in this inhibitor preparation (Fig. 5.1a). With decarbamylated rubisco, CO_2 fixation did not occur and also no XuBP was formed (Fig. 5.1b).

pH and Formation of XuBP during Fallover. When purified, activated spinach rubisco catalyzed carboxylation of RuBP at pH 6.6, 7.0, 7.7 and 8.2 for 30 min, different amounts of XuBP were formed depending on the pH (Fig. 5.2). The pH values indicated were measured at the end of the reaction. The addition of 5 mM RuBP(pH 6.0) plus the formation of PGA by rubisco did reduce the initial pH about 0.3 units. A lower pH favored XuBP formation during fallover. The relative area of XuBP peaks read from the integrator (SP 4290) were 7.7, 5.3, 3.0, 1 for pH 6.6, 7.0, 7.7 and 8.2 respectively.

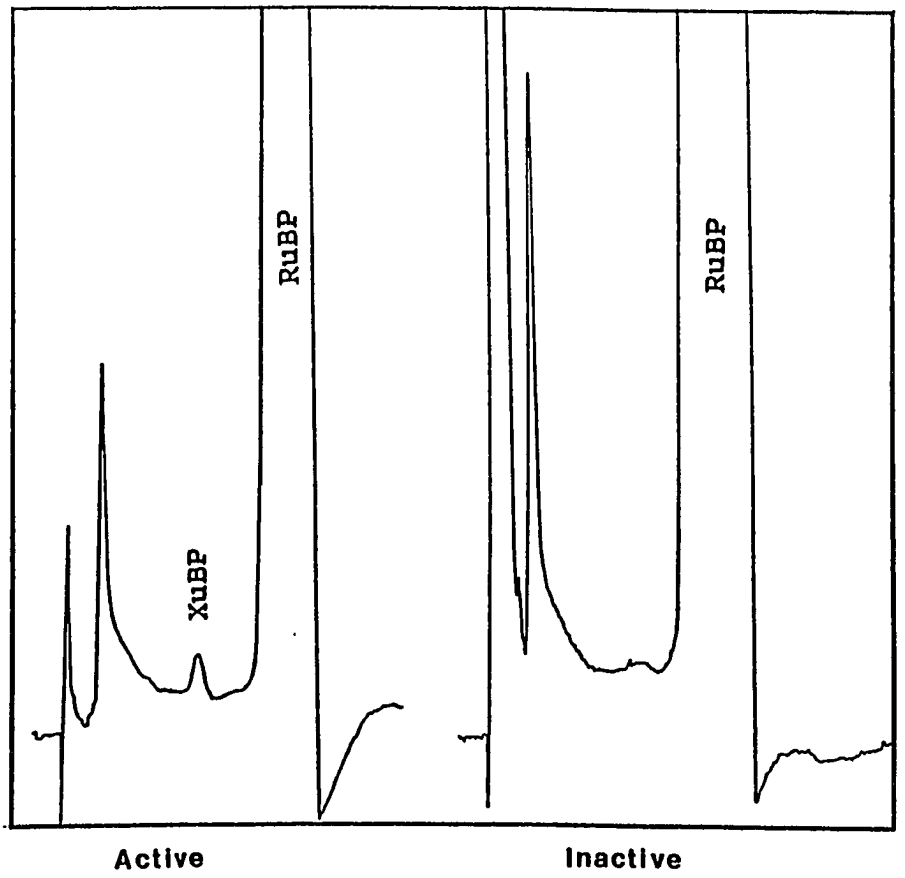


Figure 5.1. XuBP can be formed by carbamylated rubisco, but not decarbamylated rubisco from RuBP during *in vitro* catalysis. Two assay conditions were identical except that 10 mM $KHCO_3$ and 10 mM $MgCl_2$ were omitted in b. The detailed assay conditions were described in Fig. 5.2.

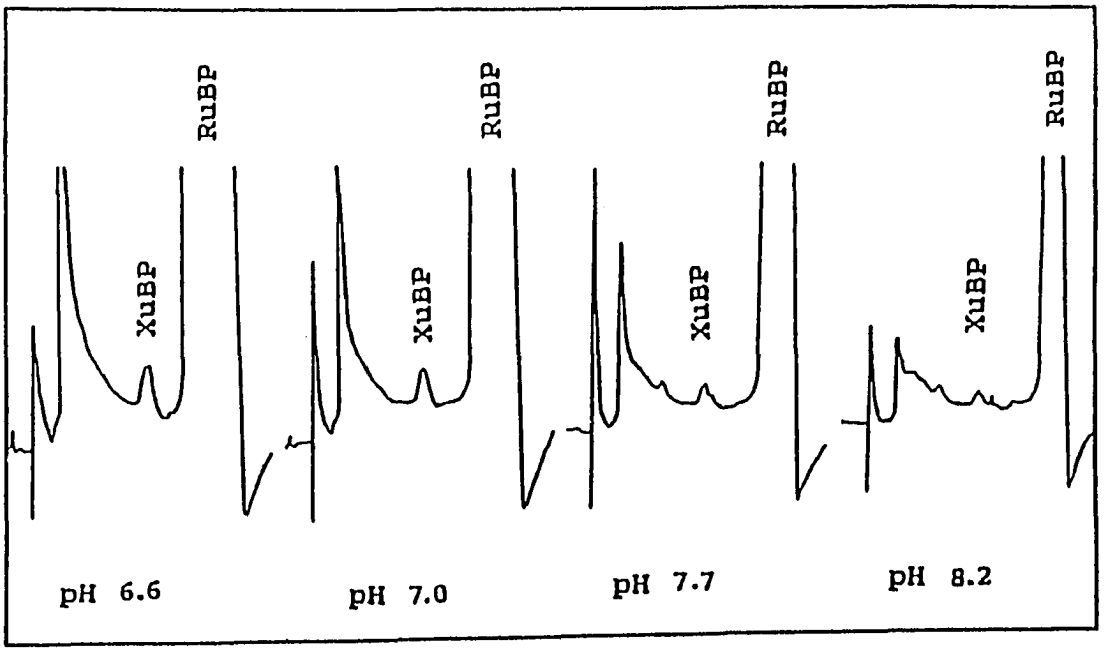


Figure 5.2. HPAE-PAD analysis of the bisphosphate fraction indicating more XuBP was formed at lower pH by carbamylated rubisco during *in vitro* catalysis. Rubisco used in this experiment was 3 mg/ml and activated first by incubation of enzyme with 10 mM $KHCO_3$ and 10 mM $MgCl_2$ in 50 mM Hepes. The reaction was started by addition of 5 mM RuBP and went for 30 min. The final pH of the reaction solutions is given. Perchloric acid to 5 % was added directly to the reaction solution after 30 min. PGA was first separated from the bisphosphates by a silica ion exchange column before the sample was added to the HPAE-PAD.

Binding of XuBP to Rubisco Causes Loss of Activator CO_2. The inhibition of rubisco activity by XuBP is a slow process, the maximum inhibition of rubisco activity was reached only after incubation of enzyme with XuBP for 20 to 30 min (McCurry and Tolbert, 1977). This delay would be consistent with the catalytic site first losing its Mg^{+2} and $^{A}CO_2$. To test this XuBP was added to carbamylated rubisco, which had been activated with $[^{14}C]CO_2$ and Mg^{+2} . Samples were treated with $[^{12}C]$CABP to trap the radiolabel and the rubisco-$[^{14}C]CO_2$ Mg^{+2}-$[^{12}C]$CABP complex was separated from the unbound $[^{14}C]CO_2$ by gel filtration. The binding of XuBP induced loss of the $^{A}CO_2$ linearly with inhibition of rubisco activity (Fig. 5.3). While XuBP binding to carbamylated sites is competitive with RuBP (McCurry and Tolbert, 1977), it binds tightly to inactive, decarbamylated sites and not to carbamylated sites on rubisco.

XuBP Inhibition of Rubisco Activity Increases at Lower pH. The binding of XuBP to decarbamylated rubisco sites was examined at various pH values. Activated rubisco was incubated 30 min with up to 2 μM XuBP at various pH values followed by CO_2 fixation assay at that pH. As shown in Fig. 5.4 at pH 8.5, the maximum inhibition by XuBP was less than 40% in spite of saturating amounts of inhibitor (2 μM). Inhibition was 70 % at pH 8. Inhibition was similar at pH 7.5

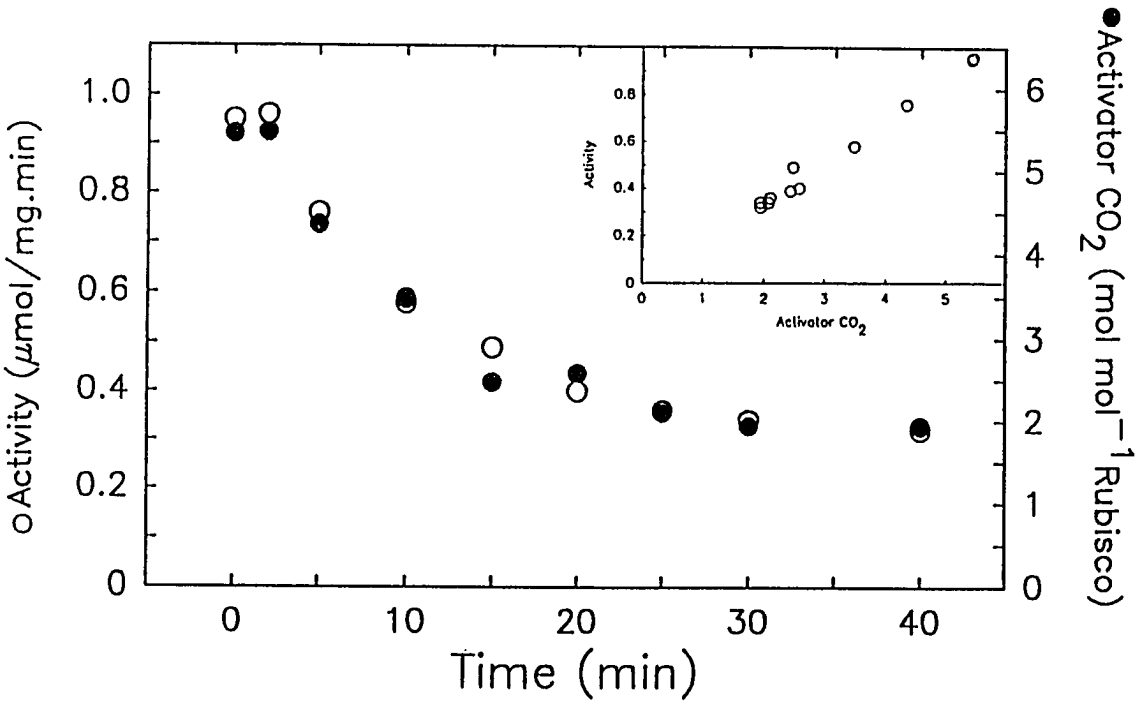


Figure 5.3. Loss of $^{A}CO_2$ and activity of rubisco during incubation with XuBP. Rubisco (70 μg/ml) was incubated with 5 μM XuBP in 50 mM Hepes, pH 7.8, 10 mM $MgCl_2$ and 10 mM $NaHCO_3$. Samples (0.2 ml) were taken and either enzyme activity or the amount of $^{A}CO_2$ determined. (○) enzyme activity; (●) amount of activator CO_2.

and 7.0 with about 90% of the rubisco activity inhibited at about 0.8 μM XuBP. These results indicate that the binding affinity for XuBP increased at lower pH (Table 5.1).

Using similar methods to titrate rubisco activity with CA1P, there was little change with pH. Only at pH 8.5 could a slight difference be observed (Fig. 5.4). CA1P is known to bind tightly to carbamylated rubisco (Seemann et al., 1985). This was also the case in our hands.

Not only did XuBP binding to rubisco increase but XuBP bound faster to rubisco at pH 7.5 than at pH 8.0 and 8.5. The effect of pH on the rate of XuBP binding was studied (Fig. 5.5). By using Multiple Exponential Decay Analysis Software (MEDAS) (EMF Software; Knoxville, TN), the data was best fitted with a first order decay equation containing a single exponential term, and the k_{on}'s were estimated (Table 5.1). The $t_{1/2}$ was 13.9, 3.5 and 1.6 min for pH 8.5, 8.0 and 7.5 respectively.

The Rates of Rubisco-XuBP Dissociation Differ with pH. To determine the k_{off}, we incubated deactivated rubisco with 20 μM XuBP for 30 min. After incubation, a 200-fold dilution was made with simultaneous addition of 10 mM $KHCO_3$ and 10 mM $MgCl_2$. The activation was measured at different time points after dilution (Fig. 5.6). A control experiment without XuBP was conducted at the same time.

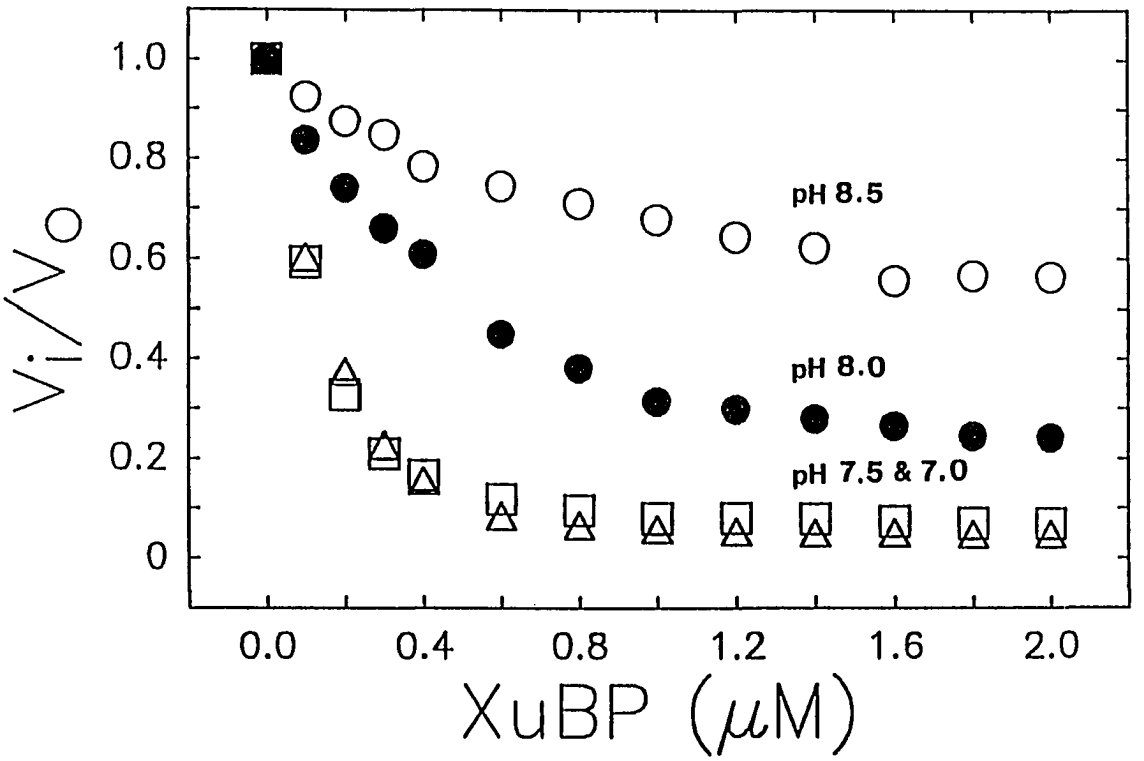


Figure 5.4. Inhibition of rubisco activity after incubation with XuBP. Activated rubisco (0.34 μM of enzyme sites) was incubated with various concentrations of XuBP for 30 min in 50 mM Hepes (for pH 7.0 and 7.5) or Tris (for pH 8.0 and 8.5), then CO_2 fixation activities were measured for 30 s at the same pH by addition of 0.5 mM RuBP. V_o is the activity in the absence of added XuBP and V_i is the activity after incubation with XuBP. (○) pH 8.5, (●) pH 8.0, (△) pH 7.5 and (□) pH 7.0.

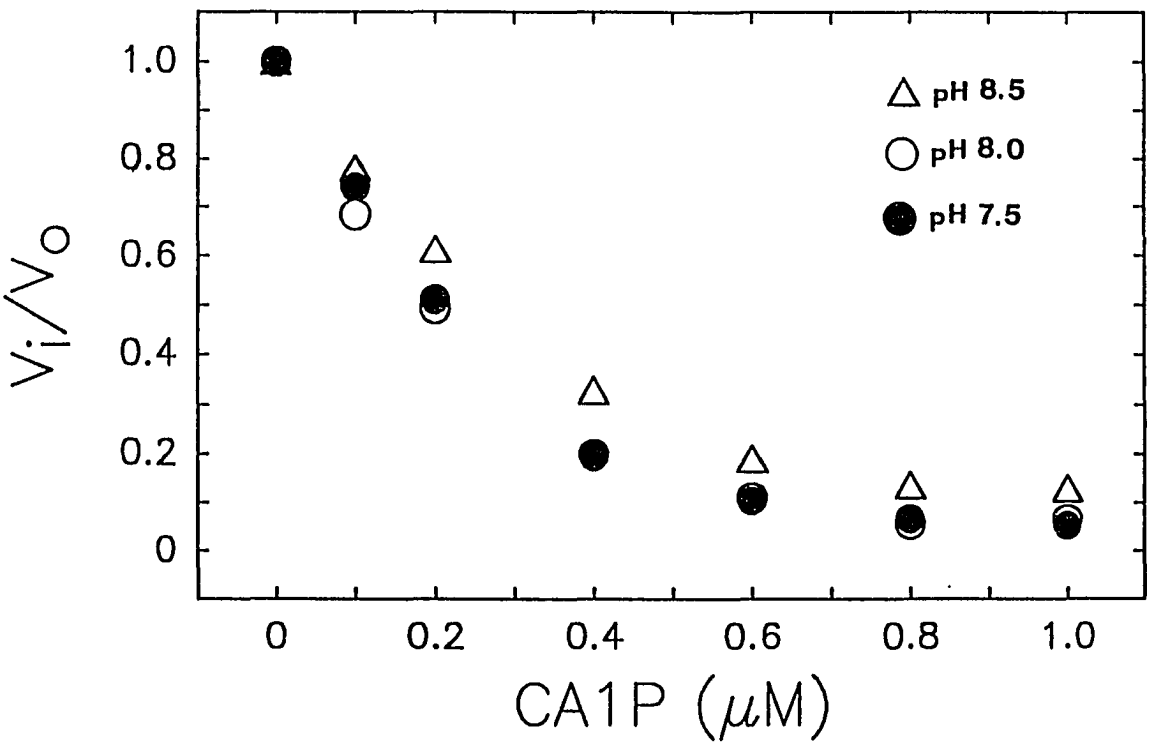


Figure 5.5. Inhibition of rubisco activity after incubation with CA1P. The method used the same conditions as Fig. 3. (△) pH 8.5, (●) pH 8.0 and (○) pH 7.5.

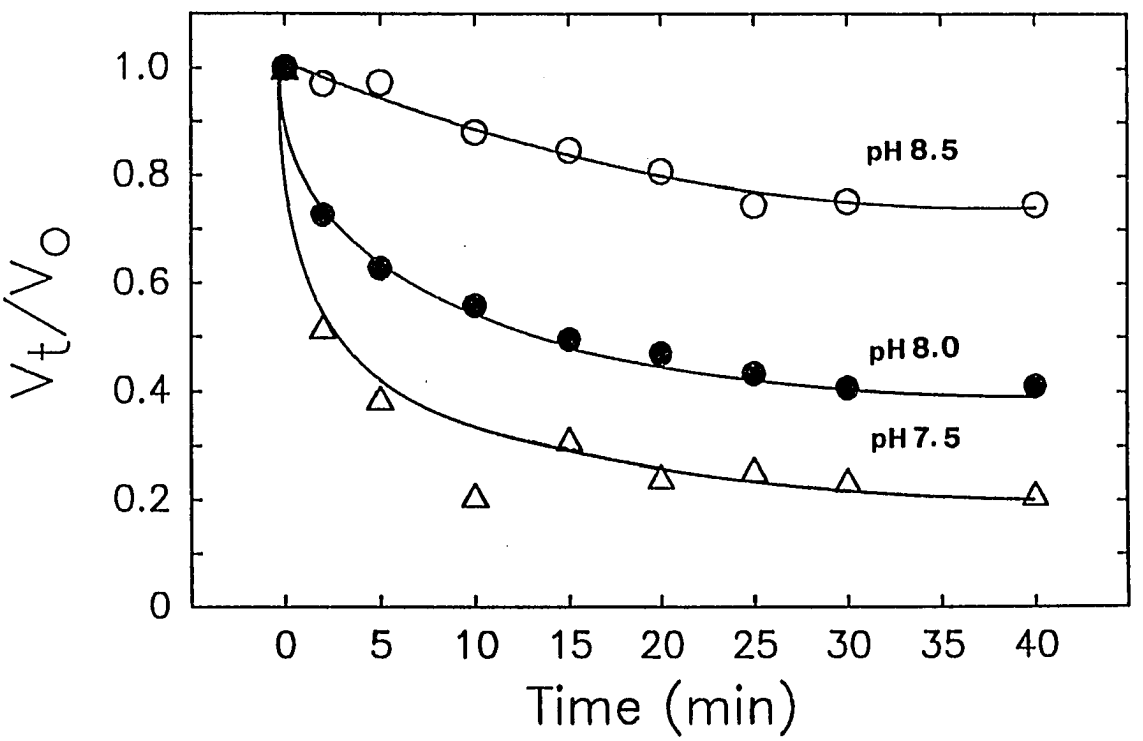


Figure 5.6. Dependence of the rate of XuBP binding to rubisco on pH. Rubisco was 70 μg/ml. The concentration of XuBP was 5 μM. Rubisco was first activated in 50 mM Hepes (pH 7.0 and 7.5) or Tris (pH 8.0 and 8.5) buffer, 10 mM $KHCO_3$ and 10 mM $MgCl_2$. The data was best fitted with a first order decay equation and the k_{on}'s at different pH's were estimated (see Table 5.1).

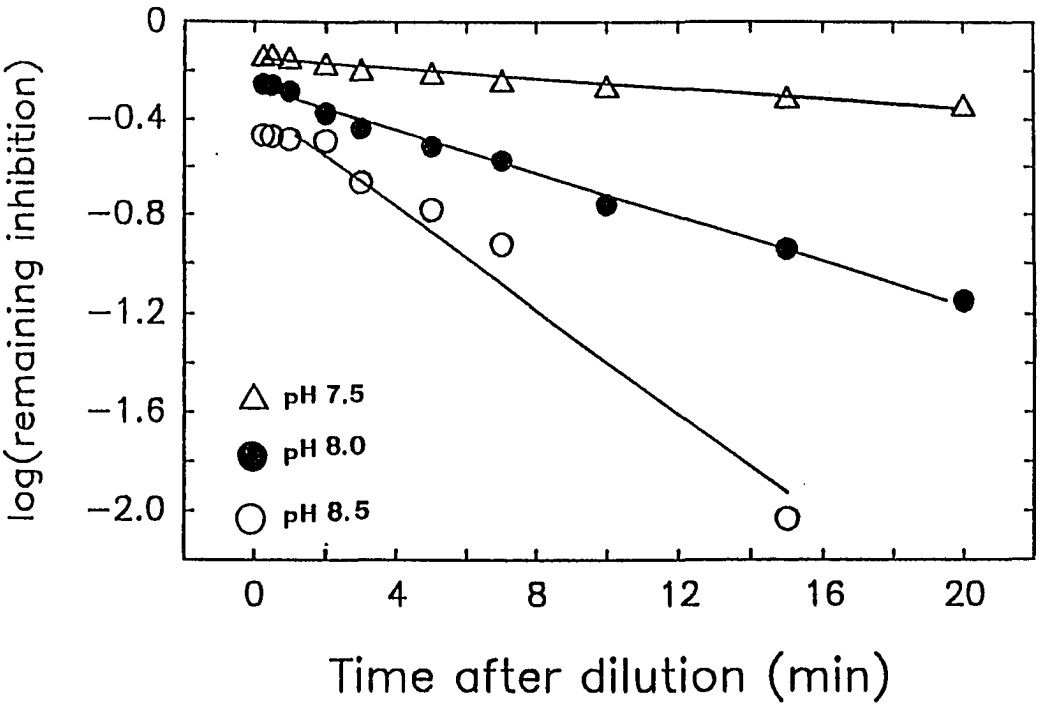


Figure 5.7. The rate of dissociation of XuBP from Rubisco. 1 mg/ml rubisco which had been deactivated by dialysis overnight in 50 mM Hepes, pH 8.0, 0.2 mM EDTA free of added CO_2 was used. XuBP (20 μM) was added to the deactivated rubisco and incubated for 30 min in the given pH at 24 ° C. after incubation, 200x dilution was made to the same buffer but containing 10 mM $KHCO_3$ and 10 mM $MgCl_2$. Rubisco activities were measured at given time points after dilution. The remaining inhibition was the percent difference of activities between control and XuBP incubated rubisco at the same time point and the same pH. The slope of the lines is the dissociation rate constant and was determined by linear regression.

The data was expressed as the ln(remaining inhibition) and plotted versus time, the slope was the k_{off}. From the data in Fig. 5.6, the k_{off}'s at different pH's were calculated (Table 5.1). The $t_{1/2}$ was 65.6 min for pH 7.5, 15.3 min for pH 8.0 and 6.6 min for pH 8.5. The changes of k_{off} and k_{on} at different pH's are quite consistent with the results from the titration experiment (Table 5.1).

DISCUSSION

According to the mechanism of RuBP carboxylation proposed by Calvin (1956), the first step catalyzed by rubisco is deprotonation of C-3 of RuBP to generate a 2,3-enediol which has a nucleophilic center. This first step, ie. enolization, is the same reaction as the conversion of ribulose 5-P to xylulose 5-P catalyzed by ribulose 5-phosphate-3-epimerase. The difference is that ribulose 5-P 3-epimerase puts the proton back on the same carbon (C-3) with inversion of configuration, whereas rubisco adds a CO_2 molecule to form the 6-carbon intermediate, 2-carboxy 3-ketoarabinitol 1,5-P_2. It is not surprising that XuBP can be formed by rubisco during catalysis because enolization of RuBP can occur quite readily without carboxylation (Gutteridge et al., 1984; Pierce et al., 1986).

Paech et al (1978) proposed that treatment of RuBP at pH 11 without rubisco resulted in formation of XuBP by deprotonation and reprotonation of C-3 of RuBP. When we adjusted purified RuBP to pH 9.0 and let it sit at room temperature for 6, 12 and 24 hours or incubated it at 50 °C for 30 min, RuBP was partially degraded as determined by HPAE-PAD. However, we were unable to detect XuBP as a degradation product. Whereas with rubisco and RuBP, the formation of XuBP occurred much more vigorously at lower pH such as 7.0.

Table 5.1. Effect of pH on the Binding Affinity of Rubisco for XuBP and CA1P. K_d's from titration (Fig. 5.3 and 5.4) were calculated according to Berry et al (1987)

pH	k_{on} (s^{-1})	k_{off} (s^{-1})	K_d (μM)	
XuBP			k_{off}/k_{on}	Titration
8.5	8.32×10^{-4}	1.75×10^{-3}	2.103	1.95
8.0	3.34×10^{-3}	7.55×10^{-4}	0.226	0.43
7.7	7.46×10^{-3}	1.76×10^{-4}	0.024	0.03
CA1P				
8.5				0.100
8.0				0.035
7.5				0.029

Conditions such as low pH and/or CO_2, which are still favorable for RuBP binding and enediol formation but less favorable for carboxylation, increase the likelihood of reprotonation of the enediol with formation of XuBP.

XuBP as an inhibitor of rubisco activity has been known for many years. McCurry and Tolbert (1977) observed that a 20 to 30 min preincubation was needed for maximum inhibition. They also noted that inhibition by XuBP was competitive with RuBP but appeared noncompetitive after 20-min preincubation. This could be explained by the slower time needed for decarbamylation of rubisco before XuBP tight binding.

Mott and Berry (1986) reported that the binding of RuBP to Rubisco was pH-dependent. As well the binding affinity of rubisco for XuBP is greatly affected by pH. Lower pH increased the binding affinity for XuBP. The pH affects both the binding rate constant (k_{on}) and the dissociation rate constant (k_{off}) with a difference of two orders of magnitude between pH 7.5 and 8.5. The effect of pH on the tight binding affinity of rubisco for ligands seems to only be with decarbamylated rubisco sites, as CA1P tight binding to carbamylated sites was little influenced by pH. The difference is that decarbamylated sites have no $^{A}CO_2$ bound to lysine ($K^{L8}201$), implying that this lysine influences inhibitor binding (Jordan and Chollet, 1983).

XuBP can be formed only by carbamylated sites on rubisco, while inhibition of rubisco activity by XuBP involves decarbamylated sites. In a solution with rubisco, CO_2, Mg^{+2} and XuBP, the following equilibrium will be established:

$$EX^{\bullet} \underset{k_4}{\overset{k_3}{\leftrightarrow}} EX \underset{k_2}{\overset{k_1}{\leftrightarrow}} E + X \leftrightarrow E + CO_2 \underset{k_{-c}}{\overset{k_c}{\leftrightarrow}} EC + Mg^{2+} \underset{k_{-M}}{\overset{k_M}{\leftrightarrow}} ECM$$

Where **E** represents a decarbamylated site on rubisco, **X** is XuBP, **C** is CO_2, **M** is Mg^{2+}. K_d, the dissociation constant for XuBP, is k_2k_4/k_1k_3. $K_c = k_c/k_{-c}$ and $K_{Mg} = k_M/k_{-M}$ are the association constants for CO_2 and Mg^{2+} respectively. The K_d values reported here represent the apparent K_d and not the true values; they represent the overall binding affinity which also includes decarbamylation. With computer assistance, we have calculated the overall dissociation constants (K_d) for XuBP in the system above at various pH values from the data of Fig. 5.3 (Berry et al., 1987). The K_d's were estimated and shown in Table 5.1. From the k_{on} and k_{off} data as k_{off}/k_{on} also equals K_d and the values are quite similar to the K_d's from the titration experiment (Table 5.1), indicating that the increased binding of XuBP at lower pH involves changing both the binding and dissociation rates.

At equilibrium the concentration of tight binding $EX^{\bullet}$ will be:

$$[EX^{*}] = \frac{[X]\,[ECM]}{K_D\,K_c\,K_{Mg}\,[C]\,[M]}$$

The K_d for XuBP in the activation system is between 2 μM to 30 nM, depending on pH values. The equilibrium will be shifted toward decarbamylation and more tight binding inhibitor, EX^{*}, at lower pH than at higher pH even in presence of high CO_2 and Mg^{2+}.

In plants, stromal pH of chloroplasts changes with the change in light intensity. The pH of illuminated isolated chloroplasts was estimated to be approximately 8.2 (Heldt et al., 1973). In low light or dark, the stromal pH could be expected to be lower than 8. Kobza and Seemann (1988) have noted that the enzyme extracted from leaves of *Spinacea* adapted to low light could not be restored to full activity upon incubation with saturating concentrations of CO_2 and Mg^{2+} at ice temperatures. In contrast, enzyme extracted from leaves in the dark or at saturating PPFD could achieve full activity under these conditions. They suggested that either RuBP or an inhibitor that binds only to the decarbamylated rubisco had bound to the enzyme at low PPFD. It could be expected from observations of the present study that XuBP may be formed in low light-adapted chloroplasts where the pH is low and plenty of RuBP is present. Confirmation of this awaits further study.

CHAPTER 6

FALLOVER OF RUBISCO ACTIVITY: DECARBAMYLATION OF CATALYTIC SITES DEPENDS ON pH

ABSTRACT

Loss of ribulose bisphosphate carboxylase (rubisco) activity during CO_2 fixation, called fallover, occurred with or without loss of activator CO_2 from catalytic sites depending on pH. At pH 7.5, but not pH 8.5, the fraction of rubisco sites that were carbamylated decreased during fallover. Inhibitors which formed during fallover were identified following $NaBH_4$ reduction and separation of the products by high-performance anion-exchange chromatography and pulsed amperometric detection. They were xylulose 1,5-bisphosphate (XuBP) and 3-ketoarabinitol 1,5-bisphosphate. During fallover at pH 8.5, 3-ketoarabinitol-P_2 was the only inhibitor binding to rubisco and this binding was at carbamylated sites, although both inhibitors were made. At pH 7.5, both inhibitors were bound to catalytic sites of rubisco with XuBP bound tightly to decarbamylated sites while 3-ketoarabinitol P_2 bound to carbamylated sites. The pH during fallover also

influenced the ratio of 3-ketoarabinitol-P_2 to XuBP formed. When fallover occurred at pH 7.5, both the formation of XuBP and its binding affinity to decarbamylated rubisco sites were increased compared to pH 8.5. The total amount of inhibitors formed during fallover was influenced by the concentration of CO_2. 3-ketoribitol-P_2 was not found at either pH.

INTRODUCTION

Fully-activated, purified rubisco slowly loses its activity during CO_2 fixation after exposure to RuBP, a process known as fallover. Edmondson et al (1990) have shown that fallover of rubisco activity at pH 8.3 can occur without decarbamylation of the catalytic sites on the protein. They suggested that two inhibitors were involved, xylulose 1,5-bisphosphate (XuBP), which was positively identified, and 3-ketoarabinitol 1,5-bisphosphate, whose identity was tentative based on the appearance of arabinitol with xylitol following hydrolysis of the phosphates and $NaBH_4$ reduction of the sugars (Edmondson et al., 1990). For positive proof of 3-ketoarabinitol, their chromatogram should only have had arabinitol as $NaBH_4$ reduction of 3-ketoarabinitol gives only arabinitol.

The synthesis of XuBP from RuBP by rubisco had been reported before (Paech et al., 1978). As we have shown, XuBP, like RuBP, binds tightly only to rubisco sites that are decarbamylated (Zhu and Jensen, 1990). Indeed, its presence stabilizes rubisco sites in the decarbamylated state. The dilemma is: If the fallover does not involve a loss of $^{A}CO_2$ at rubisco catalytic sites, what role, if any, does XuBP play in the fallover?

In this chapter, I demonstrate using HPAE-PAD that both inhibitors, XuBP

and 3-ketoarabinitol-P_2, are formed during fallover of rubisco activity. I further show that at pH 8.5, rubisco activity was mainly inhibited by 3-ketoarabinitol-P_2 binding and stabilizing the carbamylated sites. At pH 7.5, the loss of enzyme activity favored XuBP binding to decarbamylated sites of rubisco. The ratio of XuBP to 3-ketoarabinitol-P_2 is also determined.

MATERIALS AND METHODS

Materials. RuBP and CABP were synthesized and purified as described in Chapter 2. XuBP was made by an aldolase-catalyzed condensation of glycolaldehyde phosphate and dihydroxyacetone phosphate according to (Byrne and Lardy, 1954, Paech et al., 1978). Rubisco was purified from spinach leaves.

CO_2 Fixation with Rubisco to Produce Inhibitors. Rubisco (2-4 mg in 3 ml) was activated by incubation with 10 mM $KHCO_3$ and 10 mM $MgCl_2$ in 100 mM Bicine (pH 8.5) or 100 mM Hepes (pH 7.5) for 30 minutes. The reaction was started by addition of 5-8 mM RuBP.

Formation and Detection of Inhibitors. After 1-2 hours catalysis, $HClO_4$ to 3 % was added to the 3 ml rubisco reaction mixture, the precipitated protein removed and the supernatant neutralized to pH 5 with 4 N KOH. After removal of the $KClO_4$ precipitate, this solution was used for inhibition of rubisco activity or was reduced with $NaBH_4$ for analysis of the polyol bisphosphates. Other procedures, such as the trapping of the $^{A}CO_2$ with CABP were the same as described in Chapter 5.

The polyol bisphosphates were separated and identified by HPAE-PAD. The HPAE-PAD system used in this study was the same as described in Chapter 5

with some minor modifications. The NaOH concentration in the eluant (1 ml/min) was decreased to 1 mM plus 450 mM Na acetate. Post-column addition of 750 mM NaOH at 0.25 ml/min gave 150 mM NaOH at the detector. After the extracts containing the inhibitors were reacted with $NaBH_4$ (2-3 mg/3 ml), most of the PGA was selectively removed over a silica SAX ion-exchange column (Smrcka and Jensen, 1988) before HPAE separation. Xylitol-P_2, arabinitol-P_2,and ribitol-P_2 were made by reducing purified RuBP or XuBP with $NaBH_4$ and identified by HPAE-PAD.

Total inhibitors formed during catalysis were estimated by titrating a known amount of rubisco protein at pH 8.0 with inhibitor solution, and allowing the inhibitory process to go to completion for 30 min (Edmondson et al., 1990). The change in rubisco activity was assayed. The amount of enzyme catalytic sites capable of tight binding was determined with each rubisco preparation using [^{14}C]CABP (Chapter 3) and was usually about 6 CABP binding sites per mole of spinach rubisco. XuBP was determined enzymatically using aldolase, glycerol-P dehydrogenase and NADH (Sue and Knowles, 1982).

RESULTS

Fallover of Rubisco Activity Occurs with or without Loss of $^{A}CO_2$. Two different fallover patterns involving loss of activity were observed; one at pH 8.5 having little loss of $^{A}CO_2$ and the second at pH 7.5 with a loss of $^{A}CO_2$ (Fig. 6.1). These results corroborate the observations that fallover with rubisco does not involve decarbamylation at pH 8.3 (Edmondson et al., 1990), but it extends them further. At pH 7.5, about 40% of the $^{A}CO_2$ was lost during 60 min of catalysis, which clearly indicates that carbamylation of rubisco can change during catalysis. We find that decarbamylation of rubisco increases the lower that the reaction pH is from 8.0.

Loss of $^{A}CO_2$ During Fallover Depends on Which Inhibitor Binds to the Catalytic Sites. To investigate differences in fallover products, neutralized extracts containing the inhibitors produced at pH 7.5 or 8.5 were added back to solutions at pH 8.0 having fresh, fully-carbamylated spinach rubisco. After 30 min incubation, the carbamylation state as mol $^{A}CO_2$ bound per mol rubisco was measured. The inhibitor concentration was adjusted so that 50 μl of an extract gave 50% inhibition of CO_2 fixation (Fig. 6.2A). The pH 7.5 extract caused a significant change in $^{A}CO_2$ bound to rubisco (Fig. 6.2B). These observations

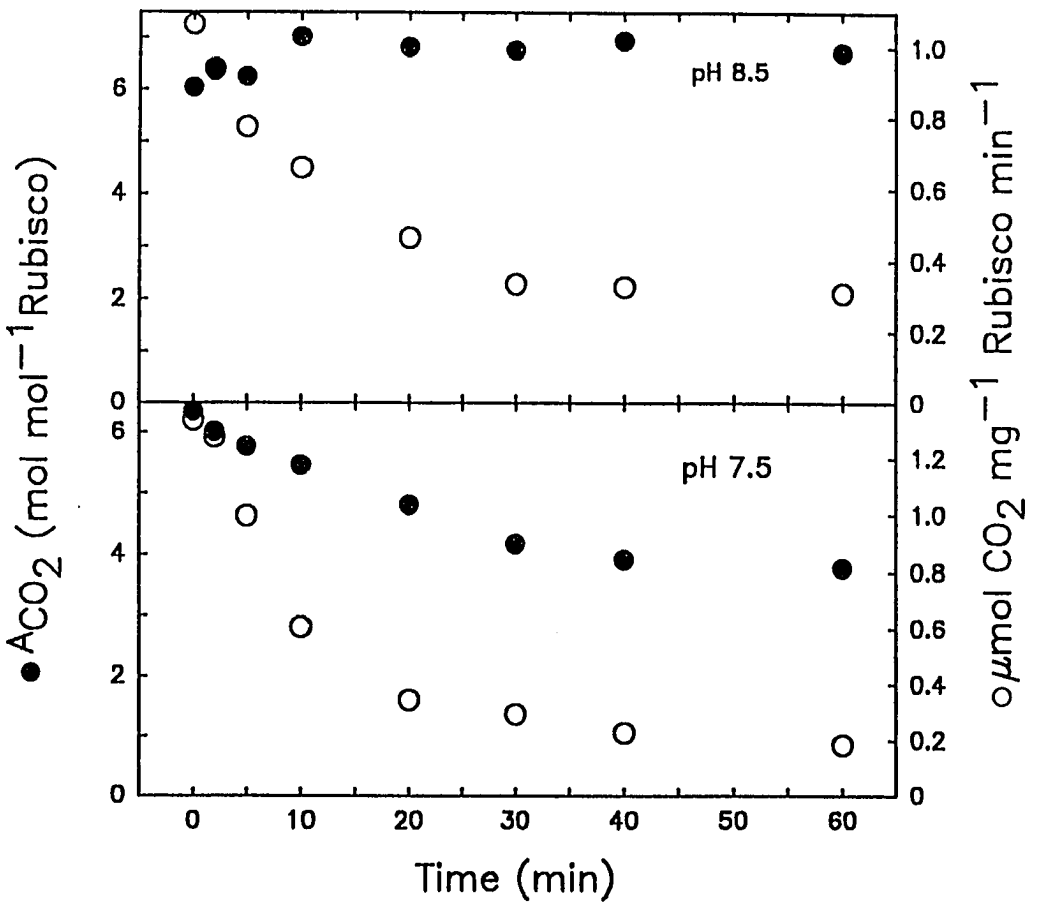
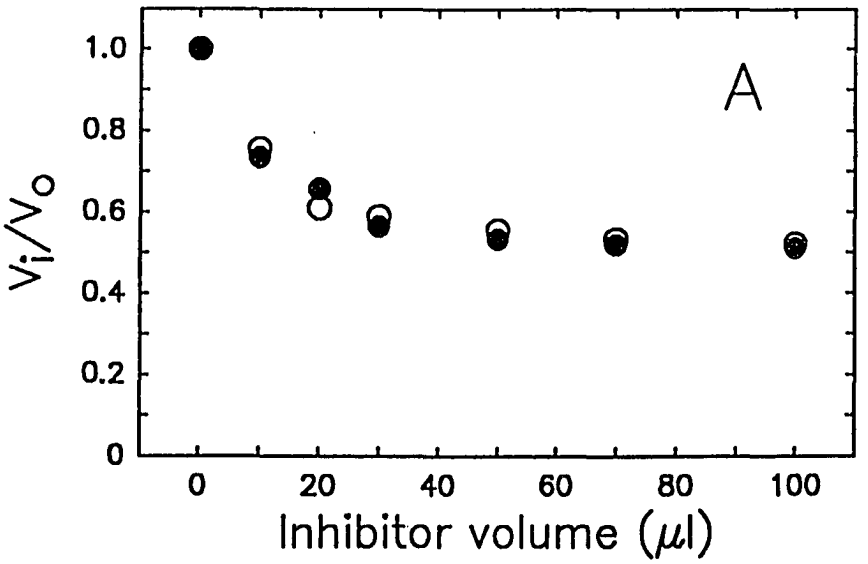
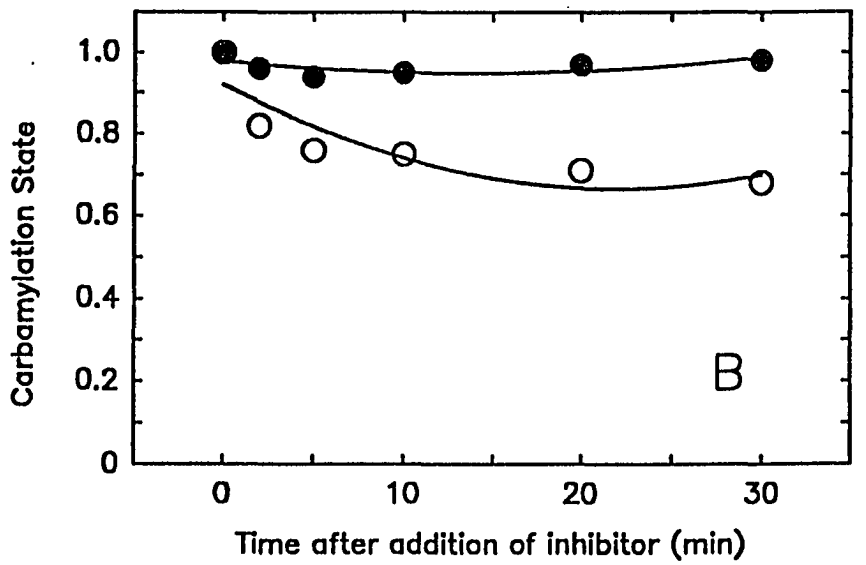


Figure 6.1. Effect of pH during catalysis on the activity (○) and carbamylation state (●) of purified rubisco during fallover. Rubisco (0.1 mg/ml) was first activated in 100 mM Hepes, pH 7.5 or 100 mM Bicine, pH 8.5 containing 10 mM $KH^{14}CO_3$ (10 Ci/mol) and 10 mM $MgCl_2$ for 30 min. The fallover reaction was started by addition of 4 mM RuBP. At times given, 10 μl of the reaction was acidified with 1 N HCl, dried at 80° C and the amount of CO_2 fixed determined by liquid scintillation counting. The $[^{14}C]^{A}CO_2$ was trapped by $[^{12}C]$CABP.

Figure 6.2. Changes in carbamylation [^{14}C]$^{A}CO_2$) during incubation of rubisco in inhibitor preparations. The inhibitor preparations were made following CO_2 fixation with rubisco at either pH 7.5 or 8.5, for 1 h in order to use up the RuBP and leave the inhibitors behind. $HClO_4$ was added to 3 %, the precipitated protein removed, supernatant neutralized with KOH and the $KClO_4$ removed by centrifugation. The two preparations were diluted so that 50 μl of each equally inhibited CO_2 fixation 50 % with fresh rubisco at pH 8.0 after 30 min incubation (A). The inhibitor preparations (50 μl) from either pH were added back to fresh, activated rubisco (10 μg) in 100 mM Bicine, pH 8.0, 10 mM $KH^{14}CO_3$ (10 Ci/mol), 10 mM $MgCl_2$ in 100 μl and the carbamylation states of rubisco were measured at the times given by adding [^{12}C]CABP to trap the [^{14}C]$^{A}CO_2$ (B). At pH 8.0 the inhibitor preparation from pH 7.5 (○) caused about 30 % decarbamylation of rubisco while that from pH 8.5 (●) was decarbamylated about 8 %.


1.0
0.8
0.6
0.4
0.2
0
V_i/V_o
A
0
20
40
60
80
100
Inhibitor volume (μl)


1.0
0.8
0.6
0.4
0.2
0
Carbamylation State
B
0
10
20
30
Time after addition of inhibitor (min)

suggest that at least two different kinds of inhibitors were formed during fallover depending on pH; one binding to decarbamylated rubisco sites and the other to carbamylated sites.

After fractionation of inhibitor preparations from fallover solutions by a silica anion-exchange column to monophosphates and bisphosphates, the inhibition of rubisco activity was observed only by addition of bisphosphates fraction,indicating that the inhibitors produced during fallover were sugar bisphosphates rather than monophosphates (Fig. 6.3).

Identification of Inhibitors Formed during Fallover of CO_2 Fixation. XuBP has been directly separated and identified by HPAE-PAD. The loss of rubisco activity with loss of carbamylation at pH 7.5 (Fig. 6.1) suggests that, at the lower pH, formation of XuBP and binding to decarbamylated sites had occurred.

Before analysis of the unknown inhibitors, purified RuBP and XuBP were reduced and their product profiles determined by HPAE-PAD (Fig. 6.4). As expected, reduction of RuBP gave two peaks, which corresponded to ribitol 1,5-P_2 and arabinitol 1,5-P_2, while reduction of XuBP gave xylitol 1,5-P_2 and arabinitol 1,5-P_2. The identity of the sugar alcohol portion of the bisphosphates was determined following hydrolysis of the phosphates and comparison of the HPAE-PAD behavior to known polyols, xylitol, arabinitol, and ribitol.

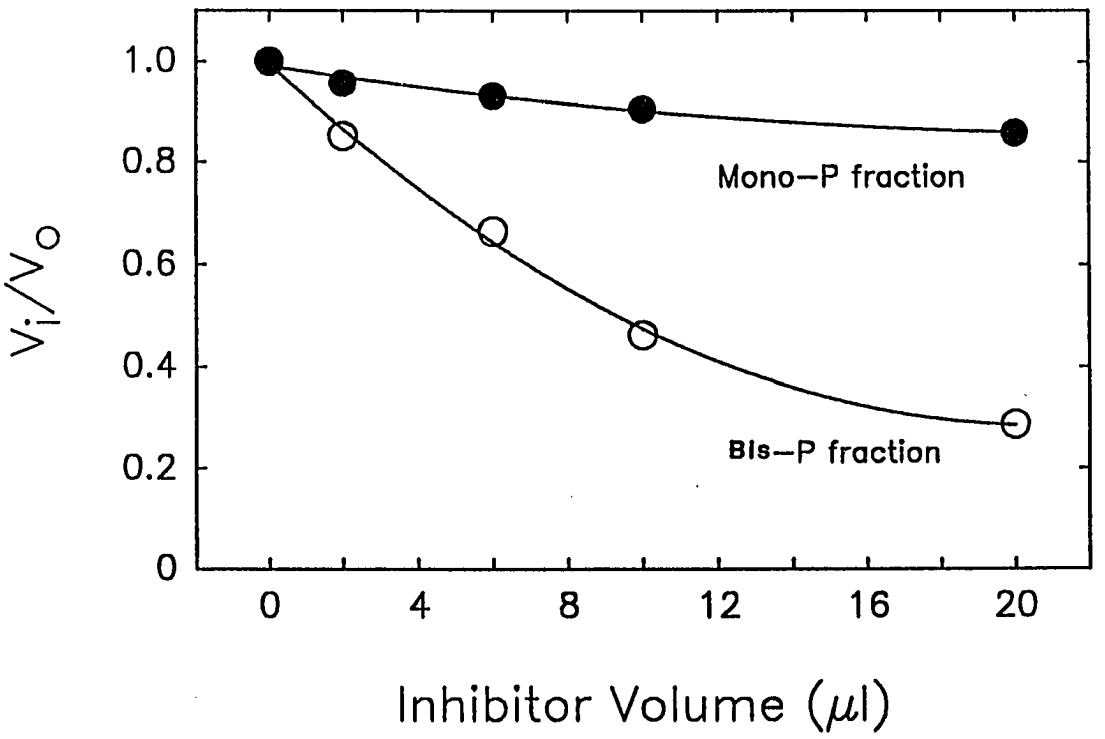
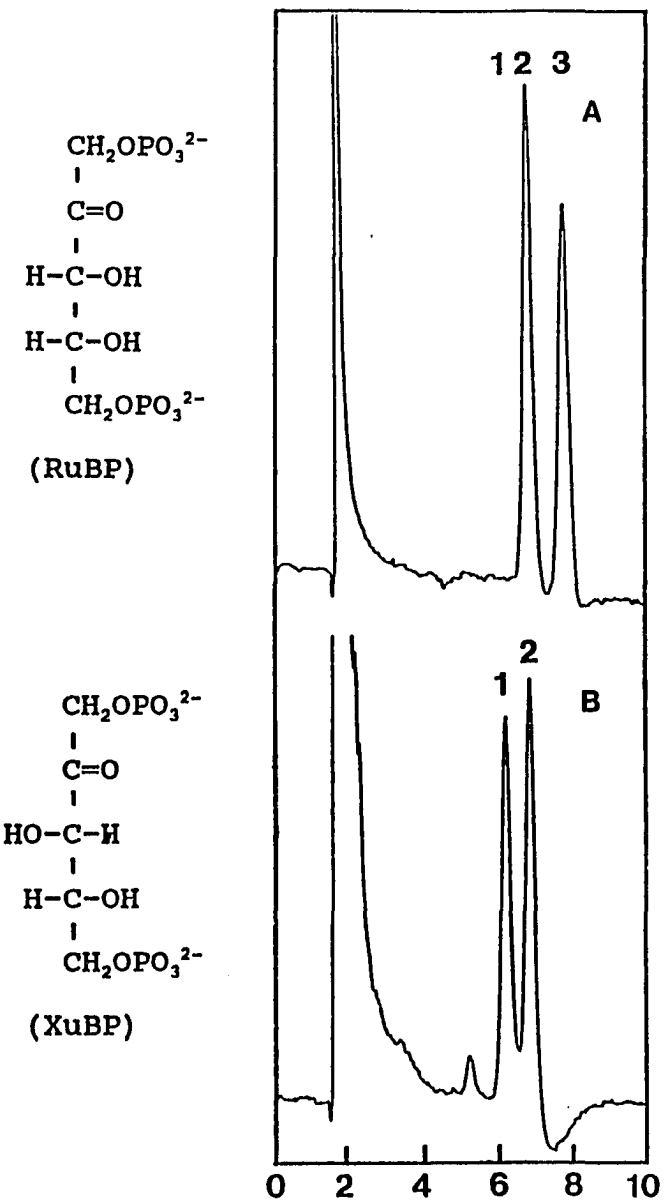


Figure 6.3. Inhibitory effects of mono- and bis-phosphate fractions from the fallover solution.

Figure 6.4. HPAE separation of the products of $NaBH_4$ reduction of purified RuBP (Curve A) and XuBP (Curve B). RuBP or XuBP (6 μmol) was reduced with 2-3 mg $NaBH_4$ at pH 6.5 for 2 min. Barium acetate (30 μl, 1 M) and one volume of ethanol were added and the precipitate collected. The Ba salts of the polyol bisphosphates were solubilized upon adding 200 μl of Dowex 50W, H^+ form. In order of appearance, the peaks in A were arabinitol-P_2 and ribitol-P_2: the peaks in B were xylitol-P_2 and arabinitol-P_2. Each peak represents the response of about 2 nmol of the corresponding polyol bisphosphate. In A, the ratio of the peak areas, arabinitol-P_2/ribitol-P_2, was 1.16. In B, the ratio of the peak areas, arabinitol-P_2/xylitol-P_2, was 1.20. The small peak to the left of xylitol-P_2 in B corresponds to PGA.



$CH_2OPO_3^{2-}$
C=O
H-C-OH
H-C-OH
$CH_2OPO_3^{2-}$
(RuBP)

$CH_2OPO_3^{2-}$
C=O
HO-C-H
H-C-OH
$CH_2OPO_3^{2-}$
(XuBP)

1
$CH_2OPO_3^{2-}$
H-C-OH
HO-C-H
H-C-OH
$CH_2OPO_3^{2-}$
(xylitol-P_2)

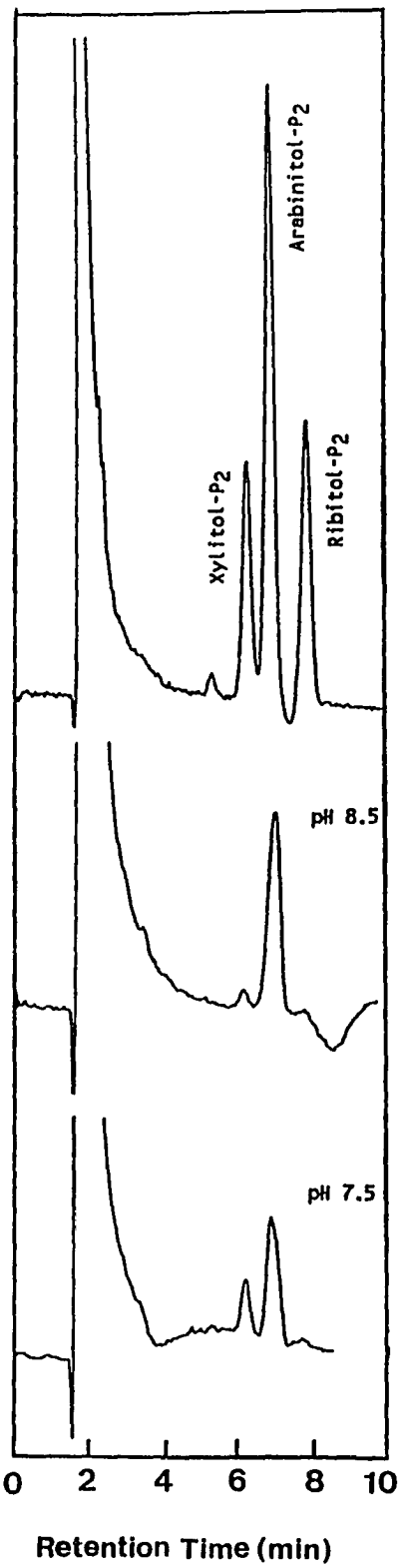
2
$CH_2OPO_3^{2-}$
HO-C-H
H-C-OH
H-C-OH
$CH_2OPO_3^{2-}$
(arabinitol-P_2)

3
$CH_2OPO_3^{2-}$
H-C-OH
H-C-OH
H-C-OH
$CH_2OPO_3^{2-}$
(ribitol-P_2)

The inhibitor(s) formed at either pH 8.5 or pH 7.5 and which bound to carbamylated rubisco sites have been separated and identified. After catalysis for 1.5 h at pH 8.5 or pH 7.5, rubisco was separated from unbound compounds by gel filtration and the protein bound compounds then removed from the rubisco fraction by addition of concentrated $HClO_4$. The neutralized products were stabilized by reduction with $NaBH_4$ to form the polyol bisphosphates. From the pH 8.5 sample only one major peak corresponding to arabinitol-P_2 and a tiny peak corresponding to xylitol-P_2 were detected following HPAE separation (Fig. 6.5). As the reduced product from 3-ketoarabinitol-P_2 is symmetrical, only arabinitol-P_2 would be expected. From the ratio of the xylitol-P_2 to arabinitol-P_2 areas of reduced XuBP from Fig 6.4, the pH 8.5 sample had 7 % XuBP and 93 % 3-ketoarabinitol-P_2. 3-Ketoarabinitol-P_2 was the major inhibitor formed at pH 8.5 and binding to rubisco sites at that pH. Since the carbamylation state of rubisco at pH 8.5 remained nearly unchanged, 3-ketoarabinitol-P_2 must bind tightest to carbamylated sites.

From the reduced products of the inhibitors formed at pH 7.5 both xylitol-P_2 and arabinitol-P_2 were detected (Fig. 6.5). About 40 % of the inhibitors bound to rubisco following incubation at pH 7.5 was XuBP, the rest being 3-ketoarabinitol-P_2.

Figure 6.5. HPAE-PAD separation and detection of the polyol bisphosphates formed upon $NaBH_4$ reduction of the inhibitors originally bound to rubisco. After about 1.5 h catalysis at either pH 8.5 or 7.5, rubisco (8 mg) was separated from unbound compounds by passing through an Econo-Pac^R 10 DG gel filtration column and $HClO_4$ to 3 % added to the protein fraction. After neutralization with KOH and centrifugation to remove the $KClO_4$, 5-10 mg $NaBH_4$ was added to the supernatant. The solution (0.5 ml) was loaded on a silica SAX column and washed with 1 ml H_2O. Any PGA plus monophosphates were eluted by 3 ml 0.05 N HCl. The bisphosphates were eluted by another 5 ml 0.15 N HCl. The eluate was concentrated to near dryness under vacuum at room temperature, H_2O added to a known volume and 50 μl was put on the HPAE-PAD. The top curve indicates the position of retention of xylitol-P_2, arabinitol-P_2 and ribitol-P_2, made by mixing the reduction products of XuBP and RuBP from Figure 3. The middle and bottom curves indicate the position of the $NaBH_4$-reduced inhibitors made and bound to rubisco during fallover at pH 8.5 or 7.5 respectively.


Arabinitol-P2
Xylitol-P2
Ribitol-P2
pH 8.5
pH 7.5
0
2
4
6
8
10
Retention Time (min)

Total Amounts of XuBP and 3-Ketoarabinitol-P_2. Although Fig. 6.5 shows a chromatogram of the inhibitors made and bound to rubisco, much of the XuBP formed during fallover remained unbound to the protein. The total amount of inhibitors produced during fallover at either pH 8.5 or pH 7.5 were assayed by titrating fresh, fully-activated rubisco with various amounts of neutralized inhibitor. After allowing complete inhibition over 30 min (Edmondson et al., 1990) the resulting activity was measured. The amount of inhibition was linear with the volume of inhibitor solution when inhibition of enzyme activity was less than 25 %. With greater amounts of the inhibitor solution the reaction rate only slowly declined to zero. After consuming 16.6 μmol RuBP, 12.2 and 11.1 nmol of inhibitors were formed at pH 8.5 and 7.5 respectively (Table 6.1). The RuBP turnover rate to inhibitor formed was similar at the two pH values. About 30 % of the inhibitors formed at pH 8.5 was XuBP, but most of the XuBP did not bind to rubisco (Fig. 6.5). At pH 7.5, 74 % of the inhibitors was XuBP.

When rubisco catalyzed carboxylation and oxygenation reactions at the same pH (8.0), but different CO_2 concentrations, different amounts of inhibitors were formed. As shown in Table 6.2, lower CO_2 concentration in the reaction mixture enhanced formation of inhibitors. However, the ratio of XuBP to 3-keto arabinitol-P_2 showed no big change between two CO_2 concentrations (Table 6.2).

Table 6.1 Formation of Inhibitors by Rubisco during Catalysis.

Rubisco (2 mg) was activated in 3 ml of 100 mM Tris-HCl, pH 8.5 or 100 mM Hepes, pH 7.5, each containing 10 mM $KHCO_3$ and 10 mM $MgCl_2$, for 30 min, then 0.5 ml of 33.2 mM RuBP was added (16.60 μmol). The reaction lasted for 1.5 h until all of the RuBP was consumed. The protein was removed with $HClO_4$ (see METHODS) and the supernatant used for analysis of inhibitors.

pH	Total[a] Inhibitors (nmol)	XuBP[b] (nmol)	Turnover[c] per Inhibitor	XuBP per Total Inhibitors (%)
8.5	12.2	3.61	1360	29.6
7.5	11.1	7.46	1495	73.9

[a]Total inhibitors were estimated by titration of rubisco activity at pH 8.0 and extrapolation of the initial inhibition to zero activity. [b]XuBP was determined enzymatically. The reaction mixture contained: 100 mM Hepes, pH 7.5, 9 units of glycerol-P dehydrogenase, 75 μM NADH and 37 units of aldolase in 0.5 ml. [c]The ratio of RuBP consumed to inhibitors formed.

Table 6.2. Effect of CO_2 Concentration on the Formation of Inhibitors. 2 mg rubisco in 2 mL of 100 mM Bicine, pH 8.0, 10 mM $MgCl_2$. CO_2 concentrations are indicated in the table. The other methods are the same as in table 6.1.

CO_2 (μM)	Total Inhibitor (nmol)	RuBP (μmol)	XuBP (nmol)	Turnover per inhibitor	XuBP per total inhibitor (%)
12	17.2	9.96	5.74	578	33.4
130	7.3	9.96	2.97	1370	40.7

DISCUSSION

Two different fallover patterns have been demonstrated during catalysis, one with little loss of $^{A}CO_2$ at pH 8.5 and the second with loss of $^{A}CO_2$ at pH 7.5. Two inhibitors have been identified as being produced during fallover. They are XuBP and 3-ketoarabinitol-P_2. Because of the weak binding affinity of XuBP to decarbamylated catalytic sites of rubisco at pH 8.5, there is little loss of $^{A}CO_2$ during fallover. XuBP does not bind tightly to carbamylated sites (Chapter 5) and thus the loss in rubisco activity at pH 8.5 was mostly due to the binding of 3-ketoarabinitol-P_2 to carbamylated sites.

According to the scheme proposed by Edmondson et al (1990) for the formation of potential inhibitors from RuBP by rubisco, once the enediolate of RuBP is formed, both C-2 and C-3 could be reprotonated. As a result, three possible isomerization products of RuBP might be formed, i.e. XuBP, 3-ketoarabinitol-P_2 and 3-ketoribitol-P_2. XuBP, formed by reprotonation at C-3, was confirmed to be formed by rubisco during catalysis (Edmondson et al., 1990, Chapter 5). Following breakdown of the XuBP with aldolase and glycerol-P dehydrogenase, Edmondson et al (1990) suggested that a second inhibitor was present which might be 3-ketoarabinitol-P_2. Since xylulose produces both

arabinitol and xylitol after $NaBH_4$ reduction, they were not able to confirm how much 3-ketoarabinitol-P_2 was present when significant amounts of XuBP were in their inhibitor solutions.

An important question arises: why was there no 3-ketoribitol-P_2 formed? According to the scheme, reprotonation of C-2 will give both 3-ketoarabinitol-P_2 and 3-ketoribitol-P_2. It is known that the C-2 of RuBP provides 2 faces, si and re, available for attack by proton. The resulting products would be 3-ketoarabinitol-P_2 and 3-ketoribitol-P_2. After reduction of these by $NaBH_4$, only arabinitol-P_2 would come from the first and ribitol-P_2 and arabinitol-P_2 from the second. The presence of arabinitol-P_2 and not ribitol-P_2 is consistent with only 3-ketoarabinitol-P_2 being formed. This agrees with the observations of Lorimer et al (1984) who found that when RuBP was bound to the catalytic sites of rubisco, a selective attack of $NaBH_4$ on the si face was observed, resulted in formation of arabinitol-P_2. This means that the protonation of C-2 of RuBP, when bound to catalytic sites is oriented by the enzyme and explains why only 3-ketoarabinitol-P_2 and XuBP are the only "misfire" products formed on active sites of rubisco during catalysis.

It should be noted from Fig. 6.1 that decarbamylation of rubisco at pH 7.5 only accounts for about half of the loss of activity. There were still half of the

sites that were carbamylated but their activities were inhibited. The observation of Fig. 6.5 (pH 7.5) shows that these sites were, most likely, occupied by 3-ketoarabinitol-P_2. From Fig. 6.4 where $NaBH_4$ reduction of XuBP at 25° C gives a ratio of xylitol-P_2 to arabinitol-P_2 of 1:1.2, the ratio of XuBP to 3-ketoarabinitol-P_2 was about 0.41:0.59 and close to the concluded ratio of inhibitors of Fig 6.1 (pH 7.5). Indeed, Edmondson et al (1990) have shown that decarbamylation of rubisco sites was faster at pH 7.3 than at pH 8.3 either in the presence or absence of RuBP. They noted that the rate of decarbamylation in the presence of RuBP could not fully account for the rate of fallover. We explain this by presence of both inhibitors which bind to decarbamylated and carbamylated rubisco sites, but both cause fallover.

In plants, an enzymatic system for relieving inhibition of rubisco, the rubisco activase, has been demonstrated (Portis et al., 1986). Rubisco activase can remove rubisco inhibitors from both active and inactive sites (Portis et al., 1986; Robinson and Portis, 1988). We have isolated and identified XuBP from celery leaves (Chapter 7), which implies that the mechanism for the formation of inhibitors *in vitro* catalysis may also exist *in vivo*. Robinson and Portis (1988) have proved that the fallover of rubisco activity can be overcame by addition of an activase system to the rubisco *in vitro*. Most likely, the activase also relieves

inhibition of rubisco catalytic sites *in vivo* by the misfire products, XuBP and 3-ketoarabinitol-P_2.

CHAPTER 7

REGULATION OF RUBISCO ACTIVITY IN CELERY LEAVES

ABSTRACT

The activation state of rubisco in celery leaves has been investigated. The results show that the initial activity measured immediately after grinding leaves at ice temperature was higher than total activity assayed after incubation of leaf extract with 10 mM $KHCO_3$ and 10 mM $MgCl_2$ at 25° C in high light adapted leaves, resulting in over 100% activation. This is the result of loss of total rubisco activity during incubation. However, when CABP was used to determine initial and total binding sites, the ratio of initial sites to total sites was always less than 1, suggesting that loss of rubisco total activity during incubation is not due to loss of substrate binding sites. After de-proteination, the celery leaf extract still inhibited rubisco activity, indicating that some compounds which bind to rubisco catalytic sites might exist in these leaves. After an anion exchange column prepurification, HPAE-PAD separation and identification, a potent rubisco inhibitor, D-xylulose 1,5-bisphosphate (XuBP), was found in celery leaves. Since XuBP and 3-

ketoarabinitol 1,5-bisphosphate have been found to be formed by rubisco from RuBP during *in vitro* CO_2 fixation and bind to rubisco substrate binding sites, the existence of XuBP in celery leaves suggests that XuBP and probably also 3-ketoarabinitol-P_2 could be formed as misfire products during in vivo catalysis. As the initial activity of rubisco in high light-adapted leaves was high, the misfire products formed *in vivo* were mostly likely removed from rubisco catalytic sites by rubisco activase.

INTRODUCTION

Plants which produce sugar alcohols (polyols) exhibit high rates of photosynthesis. The photosynthetic rates in apple, a sorbitol synthesizer, are generally very high (Avery 1977, Larcher 1969) and substantially higher than other trees (Larcher 1969) and other C_3 plants. Celery, a mannitol translocating species, has rates of photosynthesis not only higher than most C_3 plants, but as high as some C_4 plants such as corn and sugar cane. The biomass of celery is also among the highest reported for crop plants and even close to the maximum reported biomass yield of sugar cane (Larcher et al., 1985).

The rate of photosynthetic CO_2 assimilation by plant leaves is a direct result of the activity of rubisco. It has been proven that the photosynthetic rate is proportional only to the rubisco sites which are active, not to the total catalytic sites present in chloroplasts. In other words the rubisco activation state controls the photosynthetic rate (Perchorowicz and Jensen, 1983). Being curious about the photosynthetic behavior of polyol plants, we investigated the rubisco activity and its activation state in celery leaves. Although the results did not explain why polyol plants exhibit higher photosynthetic rates than other C_3 plants, there are some interesting observations. The most interesting result is that xylulose 1,5-

bisphosphate (XuBP) was separated and identified by the HPAE-PAD system from celery leaves. XuBP and 3-ketoarabinitol 1,5-bisphosphate have been found to be formed by rubisco from RuBP during *in vitro* CO_2 fixation (see Chapter 6 and 7). Both the formation of XuBP and the binding affinity of rubisco sites for XuBP are pH dependent. The presence of XuBP in celery leaves suggests that the regulation of rubisco activity responding to the variation of light intensities might be manipulated by binding an inhibitor (misfire products) to catalytic sites on rubisco in these plants which do not produce CA1P. Thus, the regulation of rubisco activity *in vivo* by binding of inhibitor(s) could be a universal mechanism in all plants.

MATERIALS AND METHODS

Celery plants (*Apium graveolens* L.) were grown for 7 - 9 weeks in a greenhouse and watered by half strength of Hoaglands solution.

Rubisco Activity Assay in Leaf Extracts. The leaf samples, which were immediately frozen in liquid nitrogen after removal from the plant, were ground in a chilled mortar to fine powder in liquid nitrogen. Rubisco protein was extracted by grinding in 100 mM Bicine buffer, pH 8.0, containing 5 mM $MgCl_2$, 5 mM DDT, 1% BSA and 1% PVP (insoluble). Rubisco initial activity was assayed by putting 50 μl of the ice-cold leaf extract into an assay solution of 450 μl containing 50 mM Hepes (pH 8.0), 10 mM $MgCl_2$, 10 mM $KH^{14}CO_3$ and 0.6 mM RuBP at 25° C. The total activity was assayed after incubation of the leaf extract in 10 mM $MgCl_2$ and 10 mM $KHCO_3$ at 25° C for 5 min. Rubisco substrate binding sites were determined by [^{14}C]CABP binding (see Chapter 2).

Separation of XuBP from Celery Leaves. The celery leaves, following adaption of the plants to dark or low light, were ground in 5% $HClO_4$ and the homogenate was filtered through three layers of Miracloth. The filtrate was centrifuged at 10,000 x g, 15 min. The supernatant was neutralized to pH 7 with 4 N KOH, $KClO_4$ removed and the supernatant passed through a cation exchange column,

H^+ form (AG 50W-X4, 60 ml syringe with about 50 ml bed volume). The column eluate was adjusted to pH 7, excess 1 M barium acetate was added and the initial precipitate, which was mostly nucleotides and UV-absorbing material, was discarded. An equal volume of 95% ethanol was added to the supernatant. The second precipitate was collected containing the bisphosphate fraction and was dissolved by adding AG 50W-X4(H^+). The filtrate was purified on a 55 x 1.5 cm column of AG 1-X8 (Cl^-) and eluted with a gradient of 0 to 0.4 M of LiCl (1 ml/min). The fractions that caused inhibition of rubisco activity were collected and concentrated, the barium precipitate formed and redissolved with AG 50W-X4(H^+). The product from this step was applied to the HPAE-PAD for separation and identification.

HPAE-PAD Separation. The system used was same as described in Chapter 5. The XuBP sample from celery leaves was identified by comparing the retention times of both the sugar phosphate and the sugar with standard XuBP and xylulose. The sample was hydrolyzed by incubation with *E. coli* alkaline phosphatase to hydrolyze organic phosphate.

RESULTS

Initial/Total Activities. When the celery leaves were adapted in high light (>600 $\mu E.m^{-1}s^{-1}$), the initial activity of rubisco in the leaf extract was higher than the total activity. The total activity assayed after incubation of leaf homogenate with 10 mM $KHCO_3$ and 10 mM $MgCl_2$ was lower than initial activity and kept going down with incubation, resulting in the percentage of activation over 100% (Table 7.1). The inhibition of rubisco activity during incubation could not be relieved by addition of BSA, phenylmethyl-sulfonyl fluoride (a protease inhibitor), PVP, or EDTA. However, the total number of CABP binding sites was still higher than the initial binding sites and only changed slightly during a 20 min incubation (Table 7.1). This observation indicates that the loss of rubisco activity during incubation is not due to loss of substrate binding sites. However, the substrate binding sites might be occupied by other compounds causing the loss of rubisco activity during incubation, but such binding can be replaced by CABP.

Inhibition of Rubisco Activity by De-proteinated Leaf Extract. Celery leaves were ground in 3% perchloric acid. Soluble proteins were precipitated along with cell debris by centrifugation. The supernatant, which was neutralized to pH 6 to 7, was added back to the purified, freshly activated spinach rubisco. The resulting

Table 7.1. Initial/Total Rubisco Activity and CABP Binding Sites Measured in Celery Leaf Extract

	Incubation time (min)				
	0	5	10	15	20
Activity (μmol mg^{-1}chl min^{-1})	15.08	12.67	10.56	9.73	8.81
Activation (%)		119.0	142.8	155.0	171.2
CABP binding sites (nmole mg^{-1} chl)	80.9	92.14	95.14	96.31	89.77
Activation (%)		87.8	85.0	84.0	90.1

rubisco activity was assayed after incubation of enzyme with supernatant for 15 - 30 min, and is shown in Fig. 7.1. With an increase in volume of celery leaf preparation, rubisco activity decreased, indicating that there are some compounds which can inhibit rubisco activity in this preparation. However, it is well known that celery leaf produces mannitol-P which might also inhibit rubisco activity in an appropriate concentration in *in vitro* assay. *in vivo*, mannitol-P is located in the cytoplasm (Loescher, 1987), so there would be no physiological significance if this inhibition were caused by mannitol-P. In order to identify the inhibitors, further separation and purification of sugar-P was conducted.

Separation of Rubisco Inhibitor from Celery Leaves by Anion Exchange Column. After de-proteination of celery leaf extract and barium precipitation of sugar phosphates, the inhibitors were first separated on an anion exchange column. Since the inhibitor accounts for only a very small portion of total sugar phosphates in celery leaf extract, one cannot expect that the inhibitor should form a nicely separated peak. The fractions were assayed for rubisco inhibition. The fractions which caused inhibition of rubisco activityare marked with a bar in Fig. 7.2. The elution volume for this inhibitor was just before RuBP which comes out at around 0.2 M LiCl.

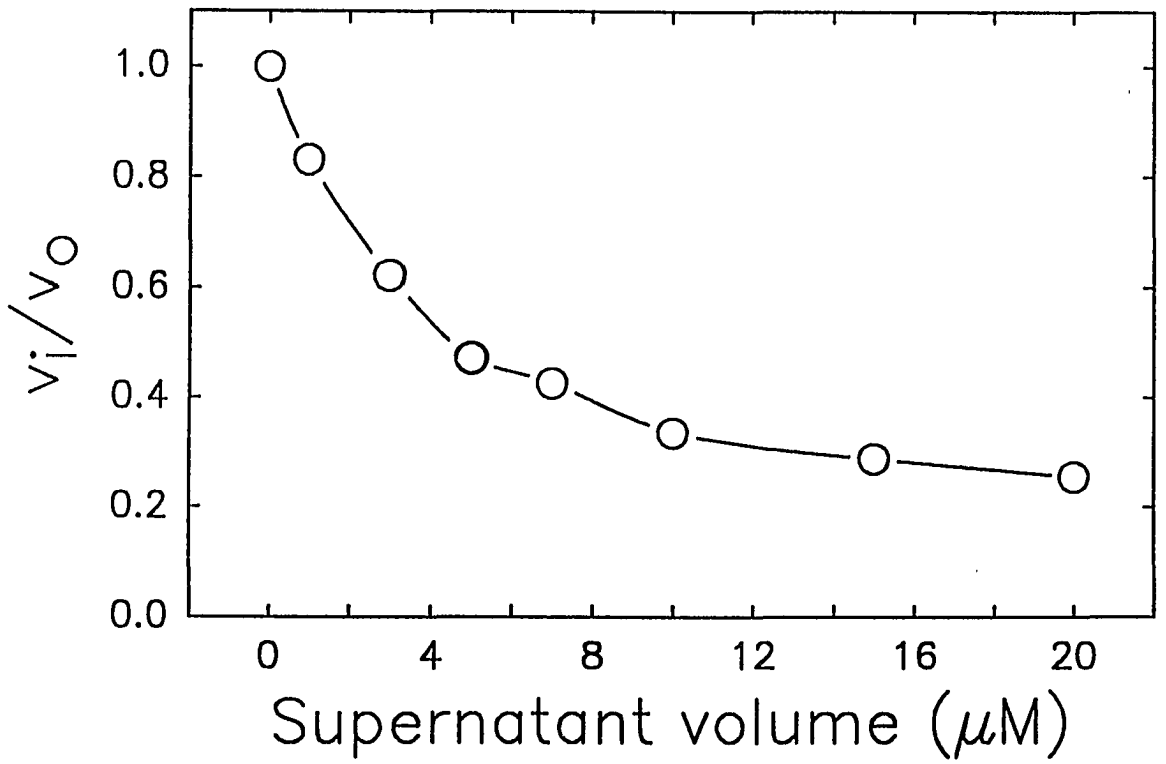


Figure 7.1. Inhibition of rubisco activity with celery leaf preparation. Approximately 5 g of celery leaves were ground in 10 ml 5% $HClO_4$, homogenate was centrifuged at 10,000 rpm for 20 min. The supernatant was neutralized to pH 6.5 with 4 N KOH and $KClO_4$ was removed by spin. The resulting solution was passed through a Dowex AG 50W-X4 (H^+) column (25 ml syringe) and neutralized again with 4 N KOH. This solution was used for inhibition assay. 15 μg spinach rubisco was used for each assay.

Separation and Identification of Inhibitor by HPAE-PAD. After the inhibitor fractions were pooled and concentrated, the sugar phosphates were precipitated with barium acetate and ethanol. The HPAE-PAD system was used to identify the sugar bisphosphates from various leaf extracts. XuBP was identified from the extracts. These leaves were first adapted to low light for 1 h before freezing the leaves in liquid N_2. The results shown in Fig. 7.3 indicate that the leaf sample peaks were identical with both standards of XuBP and xylulose. This is the first report of XuBP being found in leaves.

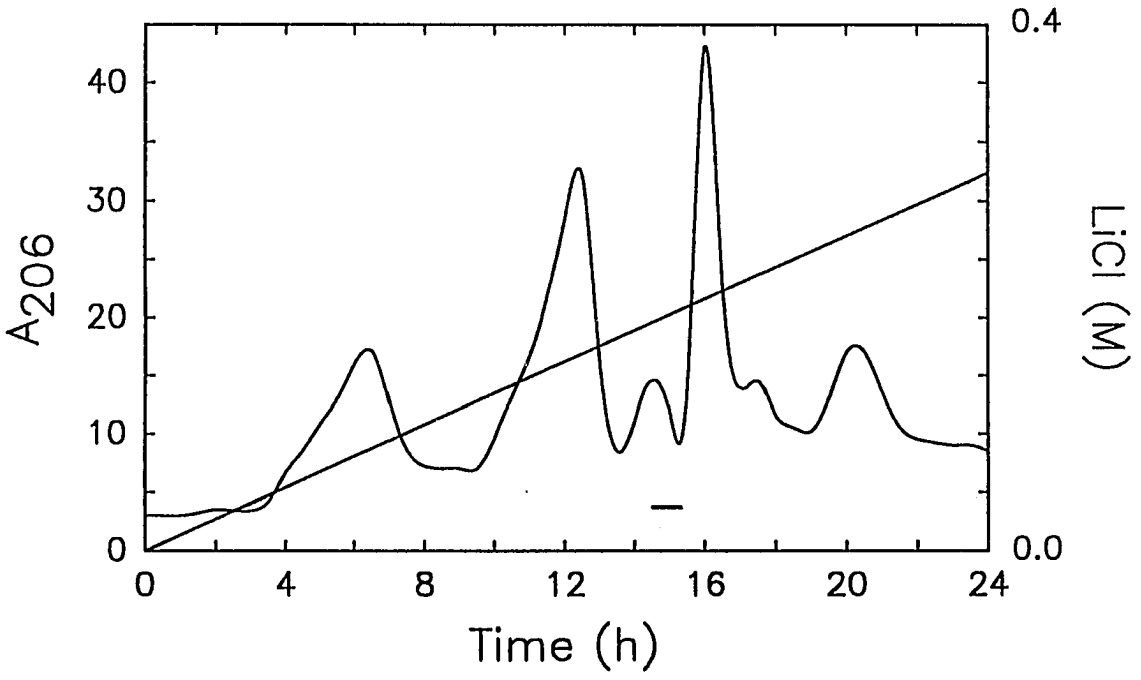
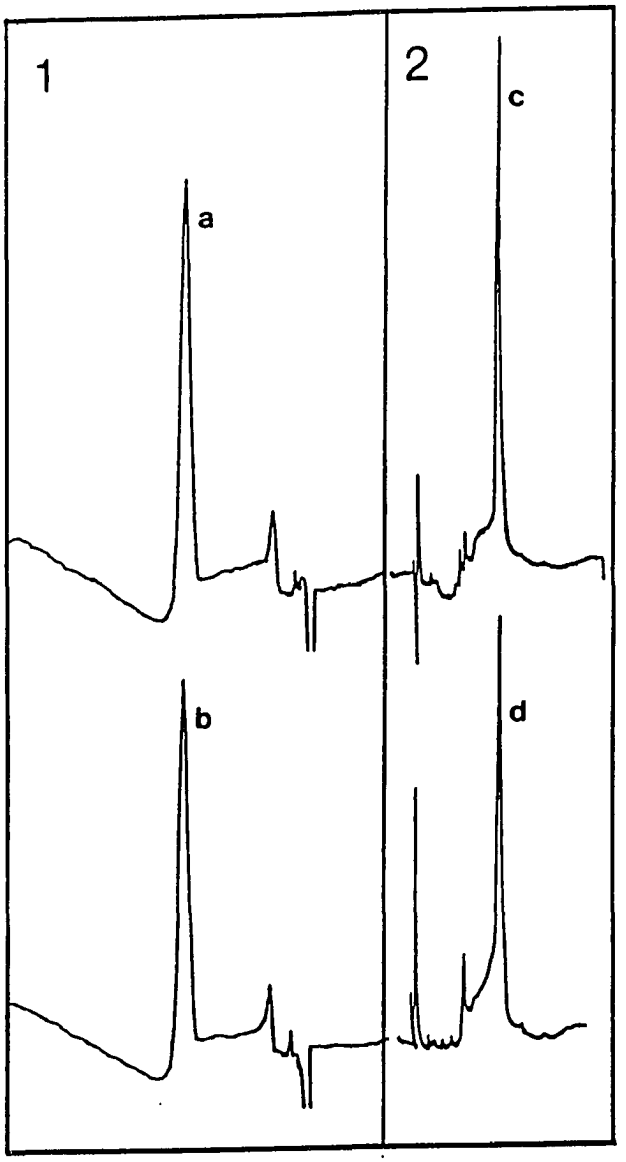


Figure 7.2. Elution profile of celery leaf extract on a Dowex AG 1-X8 (Cl^-) anion exchange column (1.5 x 60 cm) detected by absorbance at 206 nm. Sugar phosphates were eluted with a linear gradient of 0 - 0.4 M LiCl in 3 mM HCl. Flow rate was 1 ml per min.

Figure 7.3. The HPAE-PAD profiles of known XuBP and xylulose with samples purified from celery leaves. 1. Sugar phosphates. (a). XuBP standard, retention time = 14.3 min. (b). XuBP purified from celery leaves. 2. Sugars. (c). Xylulose standard, retention time = 6.3 min. (d). Xylulose from celery leaves after removal of the phosphates from XuBP by incubation with alkaline phosphatase. Phosphates were removed by incubation of 60 μl extract plus 25 mM ammonium bicarbonate with about 0.3 IU *E. coli* alkaline phosphatase at pH 9 for 60 min at 37° C. Protein was precipitated by perchloric acid and neutralized by KOH.


1
2
a
b
c
d

DISCUSSION

It has been found that rubisco activity is modulated in plants by at least two mechanisms: one is carbamylation/ decarbamylation equilibrium induced by varying light intensity (Perchorowicz et al., 1981) which is a function of the pH values and Mg^{2+} concentration in chloroplast (Heldt et al., 1973). Under low light, the stromal acidification promoted by excessively high phosphoglycerate concentrations would inhibit rubisco activity by driving the equilibrium of the CO_2/Mg^{2+}-induced carbamylation reaction back toward decarbamylation state which can be further stabilized by binding of RuBP (Perchorowicz et al., 1981; Mott and Berry, 1986; Brooks and Portis, 1988). This mechanism seems to provide a direct means of regulating rubisco activity according to the demand for phosphoglycerate. The second is to bind an inhibitor, CA1P, to the catalytic sites (Servaites et al., 1985; Kobza and Seemann, 1988; Seemann et al., 1985; Berry et al., 1987). Since this inhibitor is synthesized in low light or dark conditions and is broken down under high light, it acts as a light modulator of rubisco activity (reviewed by Servaites, 1989). However, this inhibitor exists only in some plant species (Servaites et al., 1986).

From *in vitro* experiments, Edmondson et al (1990) and our results

(described in Chapter 5 and 6) have shown that XuBP and 3-ketoarabinitol-P_2 can be made from purified RuBP by rubisco during CO_2 fixation. These two inhibitors are responsible for the fallover of rubisco activity during catalysis. Separation of XuBP from celery leaves indicates that XuBP, as one of the misfire products, also can be made by rubisco during *in vivo* catalysis. Since rubisco activase is able to remove these inhibitors from catalytic sites, rubisco activity in chloroplasts seems not to be inhibited by misfire products under high light.

As measured from high light adapted celery leaves, rubisco initial activity is higher than total activity, suggesting that misfire products possibly made during catalysis must be accumulated in a compartment or in a situation having little access to rubisco, or perhaps XuBP cannot bind to rubisco sites because of the function of rubisco activase. During incubation of the leaf homogenate with high concentration of HCO_3^- and Mg^{2+}, these free inhibitors then slowly bind back to rubisco and inhibit rubisco activity and causing the total activity to appear lower than the initial activity.

If XuBP is formed *in vivo* by the same mechanism as *in vitro*, i.e. XuBP is formed as misfire product, 3-ketoarabinitol-P_2, another misfire product, should also exist in celery leaves. Since 3-ketoarabinitol-P_2 is more labile than XuBP, especially in alkaline conditions (Edmondson et al., 1990), it does not rule out the

presence of this compound *in vivo*, although we have not detected it directly on the HPAE-PAD system. Like CA1P, 3-ketoarabinitol-P_2 binds to carbamylated rubisco sites with a K_d about 0.1 μM at pH 8.0. This compound will inhibit rubisco activity, but keep enzyme catalytic sites carbamylated upon its binding to the enzyme sites.

Unlike CA1P, the formation of misfire products is from catalysis. One could expect that more inhibitors might be made and accumulated in the chloroplast under high light. However, since the binding affinity of XuBP is highly pH-dependent and the activity of rubisco activase is higher under high light, XuBP can bind to rubisco sites only at low light or dark conditions. This may make it possible for XuBP to act as a modulator of rubisco activity.

CHAPTER 8

DETERMINATION OF THE CO_2/O_2 SPECIFICITY FACTOR OF RUBISCO BY SPECIFIC RADIOACTIVITY OF PGA

ABSTRACT

A new method was developed for measurement of the CO_2/O_2 specificity factor of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco). The specific 14C-labeling of 3-phosphoglycerate (PGA) from the rubisco carboxylase reaction and its dilution by the rubisco oxygenase reaction was monitored by directly measuring the specific radioactivity of PGA produced in both reactions. PGA was separated from other compounds by high-performance anion-exchange chromatography with detection of the total PGA by pulsed amperometry and scintillation counting of the ^{14}C-labeled PGA.

The calculation of the simultaneous carboxylase and oxygenase activities was simplified. It uses the peak area integrated from the amount of PGA as determined by pulsed amperometry, and the radioactive counts of the ^{14}C-PGA in the same peak.

The method was used to determine the specificity factor of RuBP carboxylase/oxygenase from the higher plant spinach, the green algae *Chlamydomonas reinhardtii*, cyanobacterium *Synechococcus* and the photosynthetic bacterium *Rhodospirillum rubrum*. The values obtained were comparable with the published values measured by different techniques.

INTRODUCTION

The bifunctional enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco) catalyzes both carboxylation and oxygenation of RuBP. The dual functions of this enzyme result in two opposing metabolic pathways, photosynthetic carbon assimilation and photorespiration, operating simultaneously. The kinetic parameters of rubisco determine the partitioning of RuBP between carboxylation and oxygenation at given concentrations of CO_2 and O_2. The specificity factor is defined as V_cK_o/V_oK_c, where V_c and V_o are the maximal velocities for the carboxylase and oxygenase reaction, and the K_c and K_o are the Michaelis constants for CO_2 and O_2, respectively (Laing et al., 1974; Jordan and Ogren, 1983). This describes the ultimate relationship between photosynthesis and photorespiration at any given concentrations of CO_2 and O_2.

The specificity factor of rubisco varies with evolution of the enzyme. The facultative anaerobic photosynthetic bacterium *Rhodospirillum rubrum* has the lowest value of specificity factor compared with cyanobacteria, green algae and higher plants (Jordan and Ogren, 1981, 1983; Kent and Tomany, 1984; Parry et al., 1989). The possibility of changing the ratio of carboxylase to oxygenase in order to increase plant productivity has stimulated interest in determining this

ratio in the form of the specificity factor. Several different methods have been developed to determine the specificity factor. The method developed by Jordan and Ogren (1981) was to use ^{14}C-CO_2 to label PGA from carboxylation and 1-^{3}H-RuBP to label phosphoglycolate, the product of oxygenation. Kent and Tomany (1984) used ^{14}C-CO_2 to label PGA from carboxylation and to use 5-^{3}H-RuBP to label PGA from both carboxylation and oxygenation. The third method (Parry et al., 1989) provides an alternative technique which measured the oxygen uptake during the total consumption of RuBP in the presence of various concentrations of the substrate, O_2 and CO_2.

In this chapter, a new, relatively simple and inexpensive method is described for determining the specificity factor by directly determining the specific radiolabeling of the product, PGA. With recent developments for anion separation by HPAE and detection by pulsed amperometry (PAD), a procedure was developed to measure the specific radioactivity of the formed [^{14}C]PGA.

The method was applied to determine the specificity factor of rubisco isolated from spinach, *Chlamydomonas reinhardtii*, *Synechococcus* and *Rhodospirillum rubrum*.

PRINCIPLE

The method is based on measuring changes in the specific radioactivity of PGA formed when both $[^{14}C]CO_2$ and O_2 are used as gaseous substrates compared to $[^{14}C]CO_2$ alone as the substrate. The RuBP carboxylase reaction produces equimolar amounts of $[^{14}C]$-labeled and unlabeled PGA, whereas oxygenase yields only unlabeled PGA. Therefore, the rates of carboxylation and oxygenation can be determined simultaneously by directly quantifying both the total PGA and $[^{14}C]$PGA. The dilution of specific radioactivity of $[^{14}C]$PGA (the ratio of $[^{14}C]$PGA to total PGA) reflects oxygenase activity.

The specificity factor, V_cK_o/V_oK_c, is related to the observed carboxylase and oxygenase rates and aqueous concentrations of CO_2 and O_2 by the following equation (Laing et al., 1974):

$$\frac{V_cK_o}{V_oK_c} = \frac{v_c/v_o}{[CO_2]/[O_2]} \quad (1)$$

The rate for the carboxylase reaction, v_c, is proportional to the incorporation of $[^{14}C]$ into PGA per reaction time (RT), whereas the reaction rate for the oxygenase reaction, v_o, in the presence of $[^{14}C]CO_2$, equals the amount of

total PGA produced less twice the [^{14}C]PGA per RT. The subscripts, $_{c}$ and $_{c,o}$, indicate the reaction conditions: $_{c,}$ carboxylation in the presence of [^{14}C]CO_2 and N_2 and no O_2; $_{c,o,}$ carboxylation and oxygenation in the presence of [^{14}C]CO_2 and O_2. The ratio of the rate of carboxylation and oxygenation, v_c/v_o can be represented by the following equation:

$$\frac{v_c}{v_o} = \frac{[^{14}C]PGA_{c,o}/RT}{(PGA_{c,o} - 2([^{14}C]PGA))/RT} \quad (2)$$

When both terms are multiplied by $RT/PGA_{c,o}$, equation 2 can be rewritten as

$$\frac{v_c}{v_o} = \frac{[^{14}C]PGA_{c,o}/PGA_{c,o}}{(PGA_{c,o} - 2([^{14}C]PGA))/PGA_{c,o}} \quad (3)$$

or

$$\frac{v_c}{v_o} = \frac{[^{14}C]PGA_{c,o}/PGA_{c,o}}{2\{(PGA_{c,o}/2PGA_{c,o}) - ([^{14}C]PGA_{c,o}/PGA_{c,o})\}} \quad (4)$$

The specific radioactivity of PGA formed by carboxylation in the absence of O_2 is [^{14}C]PGA_c/PGA_c, but PGA_c is equal to twice [^{14}C]PGA, giving the specific

activity as $[^{14}C]PGA_c/2[^{14}C]PGA_c$ or 1/2 of the specific activity of the $[^{14}C]CO_2$ used. As both the $_{c,o}$ and $_c$ reactions used the same $[^{14}C]CO_2$ having the same specific activity, $[^{14}C]PGA_c/PGA_c$ can be substituted for $PGA_{c,o}/2PGA_{c,o}$ in the denominator of Eq. 4, giving

$$\frac{v_c}{v_o} = \frac{[^{14}C]PGA_{c,o}/PGA_{c,o}}{2\{([^{14}C]PGA_c/PGA_c) - ([^{14}C]PGA_{c,o}/PGA_{c,o})\}} \quad (5)$$

The amount of $[^{14}C]PGA$ in the PGA peak is proportional to the radioactive counts (DPM), and the total amount of PGA is proportional to the peak area of PGA. Upon inserting these into Eq. 5, v_c/v_o can be calculated using the experimental data.

$$\frac{v_c}{v_o} = \frac{DPM_{c,o}/\text{peak area}_{c,o}}{2\{DPM_c/\text{peak area}_c - DPM_{c,o}/\text{peak area}_{c,o}\}} \quad (6)$$

The specificity factor, V_cK_o/V_oK_c, in turn can be calculated by Eq. 1.

MATERIALS AND METHODS

Preparation of Rubisco from *Chlamydomonas reinhardtii*. The cells were grown photoautotrophically (air level CO_2) and harvested in the late logarithmic growth phase. The cell pellets were resuspended in 50 mM bicine buffer, pH 7.8, containing 0.2 mM EDTA and 10 mM mercaptoethanol, and shortly sonicated to almost complete breakage. The sonicates were centrifuged (12,000 g, 20 min) and the supernatants subjected to ammonium sulfate precipitation. The precipitates of the 35 - 55% saturation fraction were dissolved in the same buffer mentioned above and dialyzed against 5 mM Tris-Cl, pH 8.0, 10 mM $MgCl_2$, 5 mM $NaHCO_3$ and 10 mM mercaptoethanol overnight at 4° C. The dialysates were clarified by centrifugation (39,000 g, 10 min) and further desalted by chromatography on a Sephadex G-50 column, equilibrated with 5 mM Tris-Cl buffer, pH 8.0. The first protein fractions were collected and further purified by perfusion chromatography. Approximately 10 mg protein was loaded on a Poros Q prepacked column, M particle size (PerSeptive Biosystems, Cambridge, MA) (bed volume: 1 ml). Rubisco was eluted with a binary salt gradient from 5 mM Tris-Cl, pH 8.0, to 1 M KCl in the same buffer. The first peak of the elution profile contained the rubisco activity. The enzyme preparations were stored in presence of 20% glycerol at -75°

C.

Spinach rubisco was prepared according to McCurry et al. (1982). The procedures have been described in Chapter 2.

The enzyme from *Rhodospirillum rubrum* was a gift from Dr. F. C. Hartman (Oak Ridge, Natl. Lab.).

Assay Conditions for the Carboxylase/Oxygenase Reactions. Reactions were performed in a total volume of 1 mL in 1.5-ml screw cap septum reaction vials (Pierce, Rockford. IL) in a 25° C bath. For simultaneous measurement of the carboxylase and oxygenase reactions the mixture consisted of 80 mM Tris-Cl, pH 8.2, 10 mM $MgCl_2$, 25 units of carbonic anhydrase, 0.66 mM RuBP and enzyme in the range of 20 to 50 μg protein. The level of $[CO_2]/[O_2]$ in the routine assay was equivalent to 40 μM CO_2 as calculated from pK' of the CO_2/HCO_3^- equilibrium measured by Yokota and Kitaoka (1985) versus 1131 μM O_2 (100% O_2 at air pressure in Tucson and the temperature employed) according to the solubility of O_2 given by Lange's Handbook of Chemistry (edition 13, Dean, J.A., ed., 1985). For the carboxylation only reactions, the assay conditions were identical except that the vials contained 20 mM $KHCO_3$ so that the $[CO_2]$ was 160 μM. The vials were flushed with O_2 (carboxylase and oxygenase) or with N_2 (carboxylase only), both saturated with water vapor, for 5 min before adding the CO_2-free solutions

which were bubbled by either N_2 or O_2 for at least 30 min. The reaction mixtures were allowed to sit under a layer of the appropriate gas for 15 min to activate the rubisco prior to initiation of the reaction by the addition of RuBP by a syringe injection. The reactions were terminated by addition of 14 μL of 70% $HClO_4$ to precipitate the enzyme proteins. The acidified reaction mixture was centrifuged at 12,000g for 2 min and the supernatants were adjusted to pH 6.0 - 7.0 with about 30 μl of 4 N KOH.

Pre-HPLC Purification of PGA. It is extremely difficult to separate PGA on the HPAE in the presence of 500 fold or more amounts of RuBP. Also, high amounts of Cl^- and Tris complicate detection. Therefore, the protein-free, neutralized supernatants were first separated by absorbing the PGA and residual RuBP on a silica anion exchange column (Sepralyte SAX, 1 mL syringe with a 0.6 mL bed volume). The newly packed column was washed with 2 ml methanol followed by 5 ml H_2O. The bound PGA was first washed with 1 mL of H_2O after loading and eluted with 3 mL of 0.05 N HCl. The 0.5 ml fractions were collected and the radioactive peak fractions were pooled and concentrated to dryness in a speedvac concentrator. The residue was dissolved in a small volume of H_2O (200 - 400 μl) and 20 μl was applied to the HPAE-PAD system.

HPAE-PAD Separation of PGA. The system used for separation and quantitation of PGA is composed of a Dionex Carbopac column, a Dionex pulsed amperometric detector (Model PAD-2) which was heated to 50° C, a pump and solvent delivery system (Spectra Physics SP8700). The peak area was read from an integrator (SP4290). The mobile phase contained 450 mM sodium acetate and 50 mM NaHO. Flow rate was 0.8 ml/min.

RESULTS

DEVELOPMENT OF THE METHOD

Test of HPAE-PAD System for Separation and Quantitation of PGA. The CarboPac PA1 column provides the necessary selectivity for the determination of PGA in the presence of phosphate and RuBP. After prepurification of a reaction mixture by running through a silica ion-exchange column, the PGA was fairly separated from most of buffer ions, salts and unused RuBP. A nice PGA peak shape, which is necessary for accurately integrating the peak area, may not be achieved in the presence of buffer ions and Cl^- in the sample. A typical separation of PGA from RuBP is shown in Fig. 8.1a and compared with the prepurified sample of PGA (Fig. 8.1b). It can be seen that the separation of PGA (retention time = 8.24 min) from RuBP (retention time = 14.96 min) is distinct enough for the integration of the peaks. Phosphoglycolate could not be detected due to the lack of an oxidizable hydroxyl-group.

The quantitative analysis of PGA detection is presented in Fig. 8.2. In the range of 0 to 25 nmoles a linear correlation (R = 0.998) could be observed between the integrated peak area values and the amounts of PGA applied to the column. The sensitivity of the detection system was sufficient to guarantee a

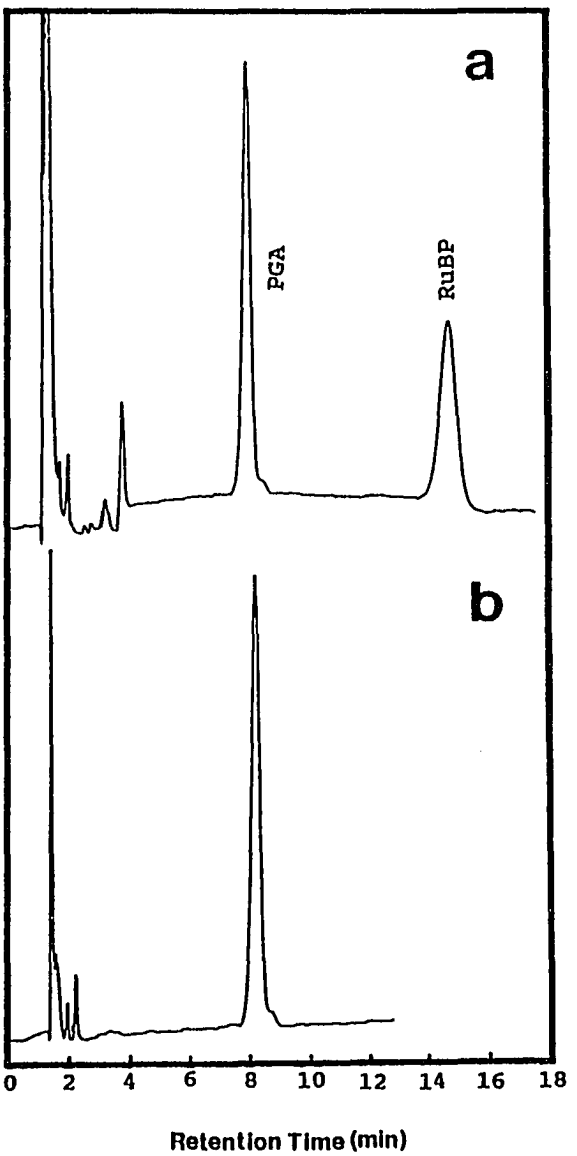


Figure 8.1 Separation of PGA from RuBP in HPAE-PAD system. a. a 100 μl mixture of 20 nmole PGA and 6 nmole RuBP was injected. b. 20 nmole PGA from rubisco reaction mixture was loaded. The retention time for PGA was 8.24 min; for RuBP was 14.96 min.

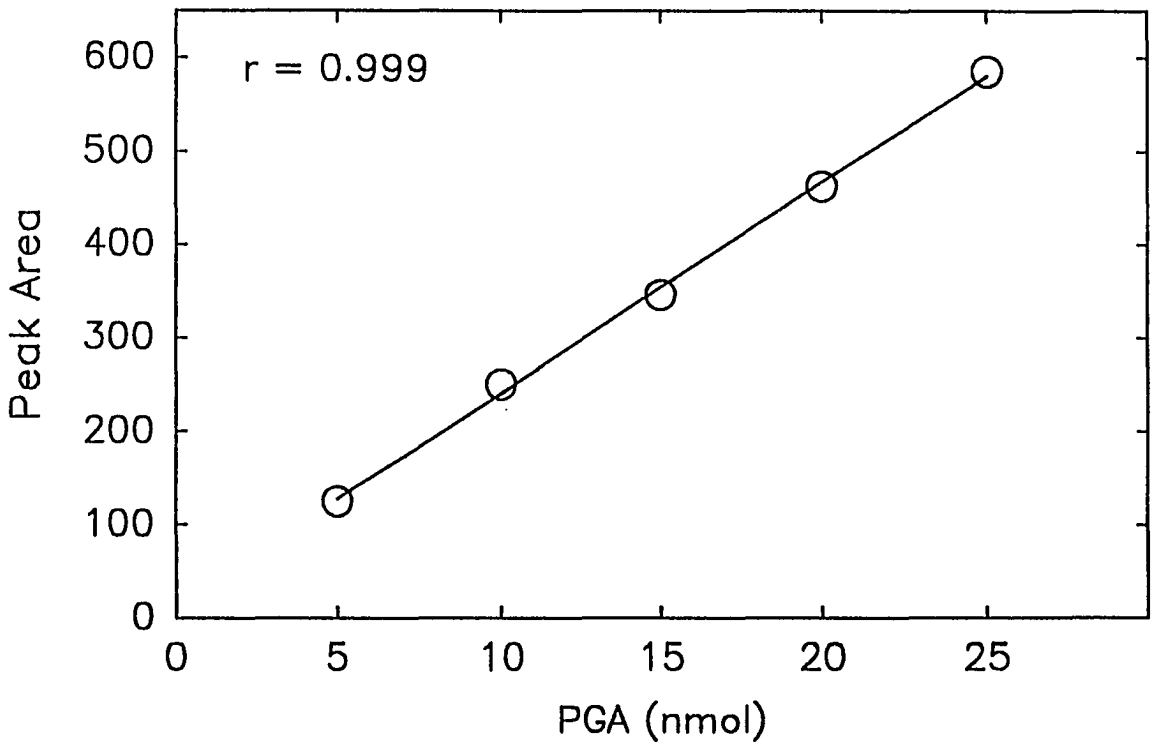


Figure 8.2 Pulsed amperometric detection following HPAE separation of PGA shows a linear relationship between peak area and amount of PGA loaded over the range shown.

quantitative evaluation of the PGA content of the assay samples.

The Ratio v_c/v_o Is Proportional to the Ratio of $[CO_2]/[O_2]$. From equation 1, one can expect that the measured ratio of v_c/v_o is directly proportional to the $[CO_2]/[O_2]$. The plot of v_c/v_o versus [CO2]/[O2] in Fig. 8.3 was used to determine the constant VcKo/VoKc from measuring the slope. A value of 93 ± 4 was obtained for the purified enzyme from spinach. The correlation coefficient was equivalent to 0.997 for this series of experiments. Those experiments were carried out at one specific RuBP concentration (0.66 mM). It was shown previously that the V_c/V_o ratio was found to be independent of the RuBP concentration in the reaction mixture (Badger and Collatz, 1977, Laing et al., 1974; Jordan and Ogren, 1981).

APPLICATION OF THE METHOD

The specificity factor was determined as described above for the enzyme isolated from spinach leaves, *Chlamydomonas reinhardtii*, *Synechococcus* and *Rhodospirillum* cells. The enzyme from spinach, *Synechococcus* and *Rhodospirillum rubrum* have been purified to homogeneity whereas the enzyme isolated from *Chlamydomonas reinhardtii* cells were almost 90% pure as judged by the measured specific activities for RuBP carboxylase and by SDS-gel

electrophoresis of the protein samples on polyacrylamide gels.

The values of the specificity factors determined with this method were shown in Table 8.1. A highest value of 93 ± 4 was obtained for the enzyme isolated from spinach. The medium values of 66 ± 1 and 45 were for the enzymes isolated from wild-type *Chlamydomonas reinhardtii* cells and from *Synechoccocus*. The lowest value of 13 was from the enzyme of *Rhodospirillum rubrum* were measured. These values were compared with those obtained using other procedures in Table 8.1. The different values obtained by various techniques from different laboratories mostly are due to using different parameters for estimating amounts of CO_2 and O_2 dissolved in solution as well as other correction factors. These differences will be discussed in the discussion section.

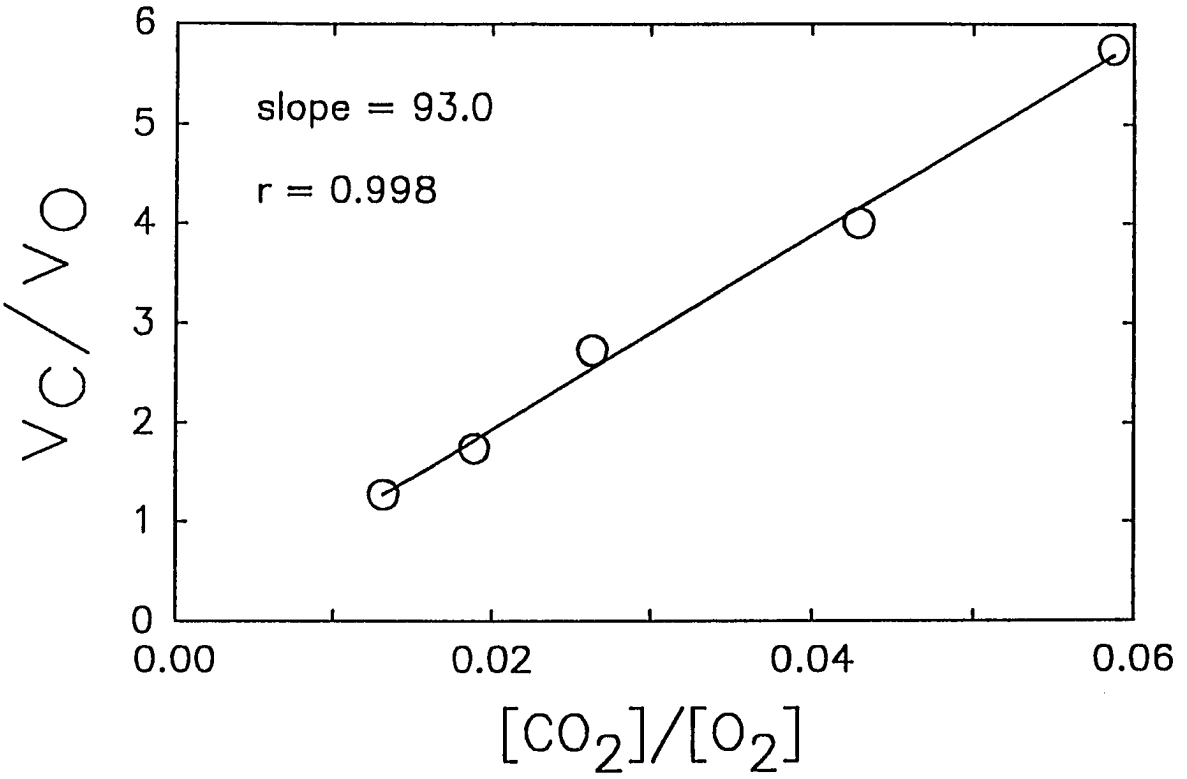


Figure 8.3 v_c/v_o measured at varying $[CO_2]/[O_2]$ shows a linear relationship. The slope of the linear curve is equal V_cK_o/V_oK_c (see Eq. 6).

Table 8.1. The Substrate Specificity Factor Derived by Different Procedures.

The values for spinach, *Chlamydomonas reinhardtii*, *Synechococcus* and *Rhodospirillum rubrum* were derived from Eq. 6. All experiments were done at 25° C.

Applied Method	Enzyme source				
	Higher plant	C.r	R.r	Syn.	Ref.
1-^{3}H-RuBP, $^{14}CO_2$	82±5 (soybean)	61±5	15±1		1
5-^{3}H-RuBP, $^{14}CO_2$	74 (spinach)		8		2
O_2 consumption	89±4 (tobacco)		9±1		3
^{14}C-PGA	93±4 (spinach)	64±1	13	49	4

C. r is *Chlamydomonas reinhardtii.*

R. r is *Rhodospirillum rubrum.*

Syn. is *Synechococcus*

1. From Jordan and Ogren, 1981

2. From Kent and Tomany, 1984

3. From Parry et al., 1989

4. From this method

DISCUSSION

Traditional methods of liquid chromatography have not been capable of a selective and efficient analysis of PGA. Anion exchange chromatography with pulsed amperometric detection takes advantage of the oxidizable hydroxyl group of PGA which can be monitored at a level down to a few nmole. This method combined with the radioisotope labeling techniques was applied for the accurate measurements of the specific radioactivity of [^{14}C]-labeled PGA in the reaction mixtures of rubisco, and therefore, also for the calculation of the specificity factor of this enzyme. Detection by pulsed amperometry was originally developed for the qualitative and quantitative analysis of carbohydrates in biological fluids. The pulsed amperometric detector is sensitive enough to detect sugar phosphates at picomole levels.

This procedure provides a simple, direct method for determining the CO_2/O_2 specificity factor for rubisco, based on changes in the specific activity of the PGA formed. All PGA produced must be made during the reaction. If PGA is a significant contaminant of the RuBP or found in the rubisco preparation, then false results will occur. This is usually not a problem and can be determined by control assays at time zero after adding the RuBP.

This procedure eliminates the expensive and complex synthesis of [^{3}H]RuBP needed for the double-label measurements with [^{14}C]CO_2 and the difficulties in counting ^{3}H and ^{14}C together. It is not necessary to know the specific activity of the [^{14}C]CO_2 as long as DPM is proportional to the amount of radioactivity. The carboxylation and carboxylation with oxygenation reactions must use the same [^{14}C]CO_2. The amount of PGA does not have to be standardized to calculate the specificity factor as long as the area of the PGA is linearly dependent on the amount of PGA. All proportionality constants are eliminated because of the ratio, v_c/v_o. If the area of the PGA peak is standardized with a known amount of PGA, then the $_c$ reaction could be used to determine the specific activity of the [^{14}C]CO_2.

Reaction times with rubisco can be short so that there is little loss in the CO_2 or O_2 levels during the assay. Quantitative recovery of the PGA is not necessary, only that the same PGA sample giving a peak on the HPAE-PAD be collected for assay of the [^{14}C]. The HPAE-PAD only needs about 10 - 25 nmoles of PGA to give a very adequate peak. Separation of [^{14}C]PGA takes only minutes, indeed two samples can be loaded two min apart on the column (Fig. 8.4). The sample appears highly reproducible between injections. The differences in the absolute values of the specificity factors reported by the various laboratories

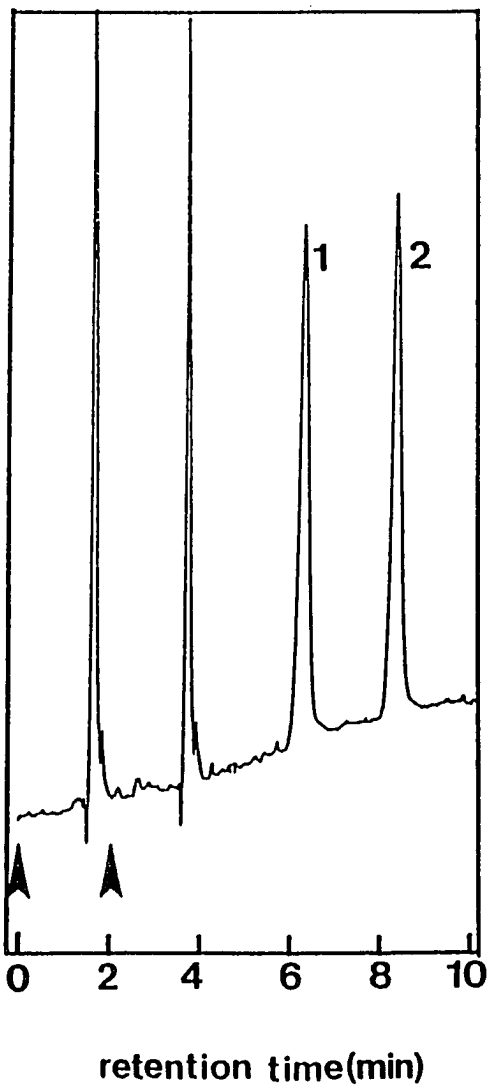


Figure 8.4 Samples from carboxylase (1) and carboxylase with oxygenase (2) loaded two min apart improves the accuracy of the measurement. Two arrows show two injections.

are largely dependent on the ionization and solubility constants used to establish the CO_2 and O_2 concentrations. The solubility coefficients for the dissolved O_2 in equilibrium with 100% O_2 at atmospheric pressure were often not corrected for ionic strength and the solute composition of the reaction mixtures. As well, where aqueous CO_2 concentrations were calculated from the added bicarbonate, the pk_1' for H_2CO_3 may not have been corrected for ionic strength and temperature. These corrections are arbitrarily based on the effects of NaCl or KCl on the solubility constants or ionization constants in water without consideration of the other organic components of the reaction mixture. Furthermore, the partition of CO_2 between the air space in the reaction vials and the solution, and the loss of bicarbonate by flushing with O_2 usually are ignored. This could cause about 2 - 10% variations especially when using a low concentration of bicarbonate and a large head space.

CHAPTER 9

FINAL DISCUSSION

Rubisco's central role in photosynthesis and photorespiration places it in a position of regulation. It has been known that the regulatory mechanisms for rubisco activity in plants involve activation/deactivation (Perchorowicz et al., 1981) and the binding of inhibitors to catalytic sites (Vu et al., 1983, 1984; Servaites et al., 1984). Based on current knowledge, it seems that rubisco activity in some plants (such as spinach) is controlled only by activation/deactivation mechanisms, whereas in *Phaseolus vulgaris* it is almost completely regulated by the amount of CA1P (Kobza and Seemann, 1988, 1989). However, most species probably lie somewhere in between. The evidence obtained by Brooks and Portis (1988) and Cardon and Mott (1989) showed that even in spinach, rubisco activation/ deactivation was involved in the binding of RuBP to decarbamylated sites. Rubisco activase has both functions of increasing rubisco activation state by reducing its $K_{act}(CO_2)$ and releasing inhibitors from rubisco catalytic sites (Portis et al., 1986; Robinson and Portis, 1988). Apparently, these two mechanisms are related by rubisco activase to regulate rubisco activity in plants. What is the

general mechanism for rubisco-rubisco activase interactions?

Physiologically, rubisco activase is needed to keep the catalytic sites on rubisco from being blocked due to the binding of inhibitors, such as CA1P and 3-ketoarabinitol-P_2, to carbamylated sites or RuBP and XuBP to decarbamylated sites (Kobza and Seemann, 1988, 1989; Brooks and Portis, 1988; Cardon and Mott, 1989; Robinson and Portis, 1988). Once free of an inhibitor, the catalytic site can be carbamylated, bind Mg^{2+} and RuBP and go on to carboxylation/ oxygenation. Activase could influence the binding of inhibitors by utilizing the cooperativity between sites. Activase could directly recognize the protein topological change induced by binding of inhibitors.

The results from this dissertation and from other workers (Hatch and Jensen, 1980; Jordan et al., 1983; McCurry et al., 1981) have shown that the binding of ligands to catalytic sites can influence rubisco activation state by preferentially binding to carbamylated or decarbamylated sites. Although rubisco protein undergoes a conformational change involving two tight binding situations (either the carbamylated and decarbamylated form), the conformation for tight binding to carbamylated form is different from the conformation for decarbamylated binding. As we have shown, at low pH (lower than 8), rubisco has a conformation which prefers binding to decarbamylated sites (Chapter 4).

Since rubisco activase releases inhibitors from catalytic sites, but does not metabolize them (Robinson and Portis, 1988), the mechanism for this function must involve a reduction in the binding affinity of rubisco for ligand by changing protein conformation. Also, because rubisco activase appears to reduce the $K_{act}(CO_2)$ only in the presence of RuBP (ER form) (Portis et al., 1986), rubisco activase must specifically recognize conformations different from the conformation achieved by normal catalytic-binding (ECMR). Rubisco activase turns the ER conformation back to the form which favors catalytic binding. Under such a situation, RuBP can only bind to and stabilize the carbamylated form. It would pull the equilibrium toward further carbamylation and, as a result, $K_{act}(CO_2)$ is reduced.

Since rubisco activase is able to restore activity to CA1P-inhibited rubisco, rubisco activase must function to reduce the tightness of the binding of CA1P to carbamylated rubisco. The extent of reversal of CA1P-inhibited rubisco activity by rubisco activase is greatly enhanced by the presence of RuBP. Because the binding of CA1P has a lower K_d than RuBP to carbamylated sites, after binding of CA1P to carbamylated rubisco, it might undergo a conformational change which is greater than that induced by catalytic binding. The function of rubisco activase for releasing CA1P could be that rubisco activase corrects the extreme conformation

induced by binding of CA1P, thus reducing the binding affinity of CA1P equal to or less than the binding affinity for RuBP, so that RuBP is able to compete with CA1P and restores rubisco activity.

Based on the above, I propose that the general mechanism for rubisco-rubisco activase interaction could be that rubisco activase changes and holds rubisco conformation in a form which favors only catalytic binding and reduces the affinity of any non-catalytic binding (except CABP which is extremely tight binding) equal to or less than that of catalytic binding so that RuBP can compete. Any other apparent effects from rubisco activase, such as reduced $K_{act}(CO_2)$ and release of inhibitors from catalytic sites, are the results of the equilibrium binding kinetics with negative cooperative interactions among catalytic sites of rubisco.

Johal et al. (1985) have demonstrated that the surface properties of the rubisco molecule are changed as it binds CABP. A 16% decrease in apparent molecular weight was seen upon complete binding of CABP. A significant change in pI was also observed after binding of ligand. This indicates that the ligand induces a conformational reorganization which extends to the surface of the protein molecule. Such changes in surface property provide a signal for rubisco activase to recognize rubisco involved in non-catalytic binding and corrects it by protein-protein interactions.

Compared with most other enzymes, rubisco is an inefficient catalyst with a poor substrate specificity. It catalyzes oxygenation of RuBP, resulting in loss of fixed carbon. Furthermore, this enzyme produces inhibitors from RuBP by misfiring. From the reaction mechanism, we know that both oxygenation and misfiring are related to the enolization of RuBP during the carboxylase reaction. O_2, in competition with CO_2, attacks the enediol resulting in the oxygenase reaction. Similarly, reprotonation of the enediol can result in the production of inhibitors. Are these two events related to each other in some way?

The diversity of evolution has produced various rubiscos with CO_2/O_2 specificity from the lowest value of 13 to the highest near 100. Just as with misfiring, the oxygenation of RuBP has little useful biological purpose, but many disadvantages. Of interest, rubisco with a low specificity factor also exhibits low carboxylase activity (such as *Rhodospirillum rubrum* rubisco) at air levels of CO_2. If the rate of enolization of RuBP remains constant, low carboxylase activity would provide a high probability for misfire with increased presence of enediol. This has been proved from the data in Chapter 6 (Table 6.2). Lower CO_2 increases the formation of misfire products compared with total substrate turnover. The indirect evidence suggests that the steady state level of the enediol on the spinach enzyme is much lower than on the *Rhodospirillum rubrum* enzyme (reviewed by

Andrews and Lorimer, 1987). Does a high level of enediol also increase the opportunity for attack by O_2 and promote oxygenation? The data from mutant rubiscos (Chen and Spreitzer, 1988) showed that reduced CO_2/O_2 specificity was always accompanied by a greatly reduced carboxylase activity. The recovery of reduced specificity was also accompanied by an increased carboxylase activity (Chen and Spreitzer, 1990). Different results from rubisco small subunit mutations showed that reduced carboxylase activity did not influence CO_2/O_2 specificity (Voordouw et al., 1987). However, we do not know if these mutations change the level of enediol. Based on current evidence, we can neither confirm nor rule out the possible relationship between the level of enediol and the rate of oxygenation. However, clarification of this relationship will benefit our understanding of the mechanism for oxygenation and choosing strategies for improving this pivotal enzyme.

To prove this possible relationship between the level of enediol and oxygenation is to chose a technique for measuring the stead state level of enediol. In addition to the ^3H exchange method, the measurement of the misfire products provides an indirect way to estimate the level of enediol because the formation of misfire products is directly related to the level of available enediol. If the rate of oxygenation is controlled by the steady state level of enediol, any rubisco with

reduced specificity would increase the formation of misfire products, and any rubisco with reduced carboxylase activity but unchanged specificity would have no change in the formation of misfire products.

REFERENCES

Andersson, I., Knight, S., Schneider, G., Lindqvist, Y., Lundqvist, T., Branden, C. I. and Lorimer G. H. (1989) Crystal structure of the active site of ribulose-bisphosphate carboxylase. Nature 337, 229-234

Andrews, T. J. and Lorimer, G. H. (1985) Catalytic properties of a hybrid between cyanobacterial large subunits and higher plant small subunits of ribulose bisphosphate carboxylase/oxygenase. J. Biol. Chem. 260, 4632-4636.

Andrews, T. J. and Lorimer, G. H. (1987) Rubisco: Structure, mechanisms, and prospects for improvement. in "the Biochemistry of plants", Vol. 10, M. D. Hatch and N. K. Boardman, eds., pp. 131-218. Academic Press, San Diego.

Atkins, C. A. and Canvin, D. T. (1971) Analysis of ^{14}C-labeled acidic photosynthetic products by ion-exchange chromatography, Photosynthetica 5, 341-351.

Avery, D. J. (1977) Maximum photosynthetic rate - a case study in apple. New Phytol. 78, 55-63.

Badger, M. R. (1987) The CO_2 concentrating mechanism in aquatic photoautotrophs, in: "The Biochemistry of Plants", Vol. 10, M. D. Hatch and N. K. Boardman, eds., pp. 219-274. Academic Press, San Diego.

Badger, M. R. and Andrews, T. J. (1974) Effects of CO_2, O_2 and temperature on a high-affinity form of ribulose diphosphate carboxylase-oxygenase from spinach. Biochem. Biophys. Res. Commun. 60, 204-210.

Badger, M. R., Andrews, J. T., Canvin, D. T. and Lorimer, G. H. (1980) Interaction of hydrogen peroxide with ribulose bisphosphate carboxylase oxygenase. J. Biol. Chem. 255, 7870-7875

Badger, M. R. and Collatz, G. J. (1977) Studies on the kinetic mechanism ribulose-1,5-bisphosphate carboxylase and oxygenase reactions, with particular reference to the effect of temperature on kinetic parameters. Carnegie Inst. Wash. Year Book 76, 355-361

Badger, M. R. and Lorimer, G. H. (1981) Interaction of sugar phosphates with the catalytic site of ribulose 1,5-bisphosphate carboxylase. Biochem. 20, 2219-2225.

Bahr, J. T. and Jensen, R. G. (1978) Activation of ribulose bisphosphate carboxylase in intact chloroplasts by CO_2 and light. Arch. Biochem. Biophys. 185, 39-44

Berry, J. A., Lorimer, G. H., Pierce, J., Seemann, J. R., Meek, J. and Freas, S. (1987) Isolation, identification, and synthesis of 2-carboxyarabinitol 1-phosphate, a diurnal regulator of ribulose-bisphosphate carboxylase activity. Proc. Natl. Acad. Sci. USA 84, 734-738

Bowes, G., Ogren, W. L. and Hageman, R. H. (1971) Phosphoglycolate production catalyzed by ribulose diphosphate carboxylase. Biochem. Biophys. Res. Commun. 45, 716-722.

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

Brooks, A. and Portis, A. R., Jr (1988) Protein-bound ribulose bisphosphate correlates with deactivation of ribulose bisphosphate carboxylase in leaves. Plant Physiol. 87, 244-249

Bryan, P., Pantoliano, M. W., Quill, S. G., Hsiao, H-Y. and Poulos, T. (1986) Site-directed mutagenesis and the role of the oxyanion hole in subtilisim. Proc. Natl. Acad. USA 83, 3743-3745

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

Butz, N. D. and Sharkey, T. D. (1989) Activity ratios of ribulose-1,5-bisphosphate carboxylase accurately reflect carbamylation ratios. Plant Physiol. 89, 735-739

Byrne, W. L. and Lardy, H. A. (1954) Pentose phosphates formed by muscle aldolase. Biochim. Biophys. Acta. 14: 495-501

Calvin, M. (1956) The photosynthetic carbon cycle. J. Chem. Soc. 1895-1915

Cambpell, W. J. and Ogren, W. L. (1990) Electron transport through photosystem I stimulates light activation of ribulose bisphosphate carboxylase/oxygenase (rubisco) by rubisco activase. Plant Physiol. 94, 479-484.

Cardon, Z. G. and Mott, K. A. (1989) Evidence that ribulose 1,5-bisphosphate (RuBP) binds to inactive sites of RuBP carboxylase *in vivo* and an estimate of the rate constant for dissociation. Plant Physiol. 89, 1253-1257

Chapman, M. S., Suh, S. W., Curmi, P. M. G., Cascio, D., Smith, W. W. and Eisenberg, D. S. (1988) Tertiary structure of plant RuBusCo: Domains and their contacts. Science 241, 71-74

Chen, Z., Chastain, C. J., Al-Abed, S. R., Chollet, R. and Spreitzer, R. J. (1988) Reduced CO_2/O_2 specificity of ribulose-bisphosphate carboxylase/oxygenase in a temperature-sensitive chloroplast mutant of *Chlamydomonas*. Proc. Natl. Acad. Sci. USA 85, 4696-4699

Chen, Z., Green, D., Westhoff, C. and Spreitzer, R. J. (1990) Nuclear mutation restores the reduced CO_2/O_2 specificity of ribulosebisphosphate carboxylase/oxygenase in a temperature-conditional chloroplast mutant of *Chlamydomonas reinhardtii*. Arch. Biochem. Biophys. 263, 60-67

Chen, Z. and Spreitzer, R. J. (1991) Proteolysis and transition-state-analogue binding of mutant forms of ribulose-1,5-bisphosphate carboxylase/oxygenase from *Chlamydomonas reinhardtii*. Planta 183, 597-603

Christeller, J. T. (1982) Effects of divalent cations on the activity of ribulosediphosphate carboxylase: Interactions with pH and with D_2O as solvent. Arch. Biochem. Biophys. 217, 485-490.

Christeller, J. T. and Laing, W. A. (1978) A kinetic study of ribulose bisphosphate carboxylase from the photosynthetic bacterium *Rhodospirillum rubrum*. Biochem. J. 173, 467-473.

Dahlquist, F. W. (1978) The meaning of Scatchard and Hill plots. Methods Enzymol. 48, 270-299

Dean, J. A. (1985) ed, Lange's Handbook of Chemistry, Ed 13, McGraw-Hill Book Co., New York, Sec. 10, pp 3

Edmondson, D. L., Badger, M. R. and Andrews, T. J. (1990) A kinetic characterization of slow inactivation of ribulose bisphosphate carboxylase during catalysis. Plant Physiol. 93, 1376-1382

Edmondson, D. L., Badger, M. R. and Andrews, T. J. (1990) Slow inactivation of ribulosebisphosphate carboxylase during catalysis is not due to decarbamylation of the catalytic site. Plant Physiol. 93, 1383-1389

Edmondson, D. L., Badger, M. R. and Andrews, T. J. (1990) Slow inactivation of ribulosebisphosphate carboxylase during catalysis is caused by accumulation of a slow, tight-binding inhibitor at the catalytic site. Plant Physiol. 93, 1390-1397

Edmondson, D. L., Kane, H. J. and Andrews, T. J. (1990) Substrate isomerization inhibits ribulosebisphosphate carboxylase-oxygenase during catalysis. FEBS Lett. 260, 62-66

Fersht, A. (1985) "Enzyme Structure and Mechanism", 2nd ed. Freeman, New York.

Fox, T. C., Kennedy, R. A. and Loescher, W. H. (1986) Developmental changes in photosynthetic gas exchange in the polyol-synthesizing species, *Apium graveolens* L. (celery). Plant Physiol. 82, 307-311

Giersch, C. (1979) Quantitative high-performance liquid chromatographic analysis of ^{14}C-labelled photosynthetic intermediates in isolated intact chloroplasts, J. Chromatogr. 172, 153-161.

Grebanier, A. E., Champagne, D. and Roy, H. (1978) Effects of Mg^{2+} and substrates on the conformation of ribulose 1,5-bisphosphate carboxylase, Biochemistry 17, 5150-5155.

Gustafson, S. W., Raynes, D. A. and Jensen, R. G. (1987) Photosynthesis and activity of ribulose bisphosphate carboxylase of wheat and maize seedlings during and following exposure of O_2-low, CO_2-free N_2. Plant Physiol. 83, 170-176

Gutteridge, S., Parry, M. A. J., Burton, S., Keys, A. J., Mudd, A., Feeney, J., Servaites, J. C. and Pierce, J. (1986) A nocturnal inhibitor of carboxylation in leaves, Nature 324, 274-276.

Gutteridge, S., Parry, M. A. J., Schmidt, C. N. G. and Feeney, J. (1984) An investigation of ribulosebisphosphate carboxylase activity by high resolution ^1H NMR. FEBS Lett. 170, 355-359

Hall, N. P., McCurry, S. D. and Tolbert, N. E. (1981) Storage and maintaining activity of ribulose bisphosphate carboxylase/oxygenase. Plant Physiol. 67, 1220-1223

Hall, N. P., Pierce, J. and Tolbert, N. E. (1981) Formation of a carboxyarabinitol bisphosphate complex with ribulose bisphosphate carboxylase/oxygenase and theoretical specific activity of the enzyme. Arch. Biochem. Biophys. 212, 115-119

Hatch, A. L. and Jensen, R. G. (1980) Regulation of ribulose 1,5-bisphosphate carboxylase from tobacco: Changes in pH response and affinity for CO_2 and Mg^{2+} induced by chloroplast intermediates. Arch. Biochem. Biophys. 205, 587-594.

Heldt, H. W., Werdan, K., Milovancev, M. and Geller, G. (1973) Alkalization of the chloroplast stroma caused by light-dependent proton flux into the thylakoid space. Biochem. Biophys. Acta. 314, 224-241

Henderson, P. J. F. (1972) A linear equation that describes the steady-state kinetics of enzymes and subcelluar particles interacting with tightly bound inhibitors, Biochem. J. 127, 321-333.

Holbrook, G. P., Bowes, G. and Salvucci, M. E. (1989) Degradation of 2-carboxyarabinitol 1-phosphate by a specific chloroplast phosphatase. Plant Physiol. 90, 673-678.

Jensen, R. G. and Bahr, J. T. (1977) Ribulose 1,5-bisphosphate carboxylase-oxygenase, Ann. Rev. Plant Physiol. 28, 379-400.

Jensen, R. G. and Bassham, J. A. (1966) Photosynthesis by isolated chloroplasts, Proc. Natl. Acad. Sci. USA 56, 1095-1101.

Johal, S., Partridge, B. E. and Chollet, R. (1985) Structural characterization and the determination of negative cooperativity in the tight binding of 2-carboxyarabinitol bisphosphate to higher plant ribulose bisphosphate carboxylase. J. Biol. Chem. 260, 9894-9904

Jordan, D. B. and Ogren, W. L. (1981) Species variation in the specificity of ribulose bisphosphate carboxylase/oxygenase. Nature (London) 291, 513-515.

Jordan, D. B. and Ogren, W. L. (1981) A sensitivity assay procedure for simultaneous determination of ribulose 1,5-bisphosphate carboxylase and oxygenase activities. Plant Physiol. 67, 237-245

Jordan, D. B. and Ogren, W. L. (1983) Species variation in kinetic properties of ribulose 1,5-bisphosphate carboxylase/oxygenase. Arch. Biochem. Biophys. 227, 425-433.

Jordan, D. B. and Ogren, W. L. (1984) The CO_2/O_2 specificity of ribulose 1,5-bisphosphate carboxylase/oxygenase. Planta 161, 308-313

Jordan, D. B., Chollet, R. and Ogren, W. L. (1983) Binding of phosphorylated effectors by active and inactive forms of ribulose 1,5-bisphosphate carboxylase. Biochemistry 22, 3410-3418.

Jordan, D. B. and Chollet, R. (1985) Subunit dissociation and reconstitution of ribulose-1,5-bisphosphate carboxylase from *Chromatium vinosum*. Arch. Biochem. Biophys. 236, 487-496.

Jordan, D. B. and Chollet, R. (1983) Inhibition of ribulose bisphosphate carboxylase by substrate ribulose 1,5-bisphosphate. J. Biol. Chem. 25, 13752-13758

Kent, S. S. and Tomany, M. J. (1984) Kinetic variance of ribulose-1,5-bisphosphate carboxylase/oxygenase isolated from diverse taxonomic sources. Plant Physiol. 75, 645-650

Knight, S., Andersson, I. and Brandèn, C-I. (1990) Crystallographic analysis of ribulose 1,5-bisphosphate carboxylase from spinach at 2.4 Å resolution, subunit interactions and active site. J. Mol. Biol. 215, 113-160.

Kobza, J. and Seemann, J. R. (1988) Mechanisms for light-dependent regulation of ribulose-1,5-bisphosphate carboxylase activity and photosynthesis in intact leaves. Proc. Natl. Acad. Sci. USA 85, 3815-3819

Kwok, S. Y. and Wildman, S. G. (1974) Studies on a conformational change of tobacco ribulose diphosphate carboxylase induced by D-ribulose-1,5-bisphosphate. Arch. Biochem. Biophys. 161, 354-359.

Laing, W. A., Ogren, W. L. and Hageman, R. H. (1974) Regulation of soybean net photosynthetic CO_2 fixation by the interaction of CO_2, O_2, and ribulose 1,5-diphosphate carboxylase. Plant Physiol. 54, 678-685.

Laing, W. A., ogren, W. L. and Hageman, R. H. (1975) Bicarbonate stabilization of ribulose 1,5-diphosphate carboxylase. Biochemistry 14, 2269-2275.

Larcher, W. (1969) The effect of environment and physiological variables on the carbon dioxide exchange of trees. Photosynthetica 3, 167-198.

Leatherbarrow, R. J., Fersht, A. R. and Winter, G. (1985) Transition-state stabilization in the mechanism of tyrosyl-tRNA synthetase revealed by protein engineering. Proc. Natl. Acad. Sci. USA 82, 7840-7844

Lilley, R. McC., Fitzgerald, M. P., Rienits, K. G. and Walker, D. A. (1975) Criteria of intactness and the photosynthetic activity of spinach chloroplast preparations, New Phytol. 75, 1-10.

Loescher, W. H. (1987) Physiology and metabolism of sugar alcohols in higher plants. Physiol. Plantarum 70, 553-557

Lorimer, G. H. and Andrews, T. J. (1973) Plant photorespiration --- an inevitable consequence of the existence of atmospheric oxygen. Nature (London) 243, 359-360.

Lorimer, G. H., Andrews, T. J. and Tolbert, N. E. (1973) Ribulose diphosphate oxygenase. II. Further proof of reaction products and mechanism. Biochemistry 12, 11-18.

Lorimer, G. H., Badger, M. R. and Andrews, T. J. (1976) The activation of

ribulose 1,5-bisphosphate carboxylase by carbon dioxide and magnesium ions. Equilibria, kinetics, a suggested mechanism, and phyological implications. Biochemistry 15, 529-536.

Lorimer, G. H. (1979) Evidence for the existence of discrete activator and substrate sites for CO_2 on ribulose 1,5-bisphosphate carboxylase. J. Biol. Chem. 254, 5599-5601.

Lorimer, G. H. and Miziorko, H. M. (1980) Carbamate formation on the ϵ-amino group of a lysyl residue as the basis for the activation of ribulosebisphosphate carboxylase by CO_2 and Mg^{2+}. Biochemistry 19, 5321-5328.

Lorimer, G. H. (1981) Ribulosebisphosphate carboxylase: Amino acid sequence of a peptide bearing the activator carbon dioxide. Biochemistry 20, 1236-1240.

Lorimer, G. H. and Pierce, J. (1989) Carbonyl sulfide: An alternate substrate for but not an activator of ribulose-1,5-bisphosphate carboxylase, J. Biol. Chem. 264, 2764-2772.

Lorimer, G. H., Pierce, J., Gutteridge, S. and Schloss, J. V. (1984) Some mechanistic aspects of ribulose bisphosphate carboxylase. Adv. Photosyn. Res. Vol. 3, pp. 725-734.

Lorimer, G. H., Gutteridge, S. and Reddy, G. S. (1989) The orientation of substrate and reaction intermediates in the active site of ribulose-1,5-bisphosphate carboxylase. J. Biol. Chem. 264, 9873-9879.

Lundqvist, T. and Schneider, G. (1989) Crystal structure of the complex of ribulose-1,5-bisphosphate carboxylase and a transition state analogue, 2-carboxy-D-arabinitol 1,5-bisphosphate, J. Biol. Chem. 264, 7078-7083.

McCurry, S. D., Gee, R. and Tolbert, N. E. (1982) Ribulose-1,5- bisphosphate carboxylase/oxygenase from spinach, tomato or tobacco leaves. Methods Enzymol. 90, 515-521

McCurry, S. D. and Tolbert, N. E. (1977) Inhibition of ribulose-1,5-bisphosphate carboxylase/oxygenase by xylulose 1,5-bisphosphate. J. Biol. Chem. 252, 8344-8346

McCurry, S. D., Pierce, J., Tolbert, N. E. and Orme-Johnson, W. H. (1981) On the mechanism of effector-mediated activation of ribulose bisphosphate carboxylase/oxygenase. J. Biol. Chem. 256, 6623-6628.

Miziorko, H. M. (1979) Ribulose 1,5-bisphosphate carboxylase: Evidence in support of the existence of distinct activator and CO_2 substrate sites. J. Biol. Chem. 254, 270-272.

Miziorko, H. M. and Sealy, R. C. (1984) Electron resonance studies of ribulose bisphosphate carboxylase: Identification of activation cation ligands, Biochemistry 23, 479-485.

Mott, K. A. and Berry, J. A. (1986) Effect of pH on activity and activation of ribulose 1,5-bisphosphate carboxylase at air level CO_2. Plant physiol. 82, 77-82

Mott, K.A., Jensen, R. G., O'Leary, J. W. and Berry, J. A. (1984) Photosynthesis and ribulose 1,5-bisphosphate concentrations in intact leaves of *Xanthium strumarium* L. Plant Physiol. 76, 968-971

Ogren, W. L. (1984) Photorespiration: Pathways, regulation, and modification, Ann. Rev. Plant Physiol. 35, 415-442.

Ogren, W. L., Salvucci, M. E. and Portis, A. R., Jr. (1986) The regulation of rubisco activity. Phil. Trans. R. Soc. Lond. B313, 337-346.

Oja, V., Laisk, A. and Heber, U. (1986) Light-induced alkalization of the chloroplast stroma in vivo as estimated from the CO_2 capacity of intact sunflower leaves, Biochim. Biophys. Acta 849, 355-365.

Paech, C., Pierce, J., McCurry, S. D. and Tolbert, N. E. (1978) Inhibition of ribulose-1,5-bisphosphate carboxylase/oxygenase by ribulose-1,5-bisphosphate epimerization and degradation products. Biochem. Biophys. Res. Commun. 83, 1084-1092

Parry, M. A. J., Keys, A. J. and Gutteridge, S. (1989) Variation in the specificity factor of C_3 higher plant rubiscos determined by the total consumption of ribulose-P_2. J. Exper. Bot. 40, 317-320

Pauling, L. (1946) Molecular architecture and biological reactions. Chem. Eng. News 24, 1375-1377

Paulsen, J. M. and Lane, D. M. (1966) Spinach ribulose diphosphate carboxylase. I. Purification and properties of the enzyme, Biochemistry 5, 2350-2357.

Perchorowicz, J. T., Raynes, D. A. and Jensen, R. G. (1981) Light limitation of photosynthesis and activation of ribulose bisphosphate carboxylase in wheat seedlings. Proc. Natl. Acad. Sci. USA 78, 2985-2989.

Perchorowicz, J. T., Raynes, D. A. and Jensen, R. G. (1982) Measurement and preservation of the *in vivo* activation of ribulose 1,5-bisphosphate carboxylase in leaf extracts, Plant Physiol. 69, 1165-1168

Pierce, J., Andrews, T. J. and Lorimer, G. H. (1986a) Reaction intermediate partitioning by ribulose-bisphosphate carboxylases with differing substrate specificities, J. Biol. Chem. 261, 10248-10256.

Pierce, J., Lorimer, G. H. and Reddy, G. S. (1986) Kinetic mechanism of ribulosebisphosphate carboxylase: evidence for an ordered, sequential reaction. Biochem. 25, 1636-1644

Pierce, J. and Reddy, G. S. (1986) The sites for catalysis and activation of ribulosebisphosphate carboxylase share a common domain, Arch. Biochem. Biophys. 245, 483-493.

Pierce, J., Tolbert, N. E. and Barker, R. (1980) Interaction of ribulosebisphosphate carboxylase/oxygenase with transition-state analogues. Biochem. 19, 934-942

Portis, A. R. and Heldt, H. W. (1976) Light-dependent changes of the Mg^{2+} concentration in the stroma in relation to the Mg^{2+} dependency of CO_2 fixation in intact chloroplasts, Biochim. Biophys. Acta 449, 434-444.

Portis, A. R. (1981) Evidence of a low stromal Mg^{2+} concentration in intact chloroplasts in the dark, Plant Physiol. 67, 985-989.

Portis, A. R., Jr., Salvucci, M. E. and Ogren, W. L. (1986) Activation of

ribulosebisphosphate carboxylase/oxygenase at physiological CO_2 and ribulosebisphosphate concentrations by rubisco activase. Plant Physiol. 82, 967-971.

Portis, A. R., Jr. (1990) Rubisco activase. Biochem. Biophys. Acta. 1015, 15-28

Ranty, B., Lundqvist, T., Schneider, G., Madden, M., Howard, R. and Lorimer, G. (1990) Truncation of ribulose-1,5-bisphophate carboxylase/oxygenase (rubisco) from *Rhodospirillum rubrum* affects the holoenzyme assembly and activity. EMBO J. 9, 1365-1373.

Robinson, S. P. and Portis, A. R., Jr. (1988) Release of the nocturnal inhibitor, carboxyarabinitol-1-phosphate, from ribulose bisphosphate carboxylase/oxygenase by rubisco activase. FEBS Lett. 233, 413-416

Robinson, S. P. and Portis, A. R., Jr. (1989) Ribulose-1,5-bisphosphate carboxylase/oxygenase activase protein prevents the *in vitro* decline in activity of ribulose-1,5-bisphosphate carboxylase/oxygenase. Plant Physiol. 90, 968-971

Salvucci, M. E. and Holbrook, G. P. (1989) Purification and properties of 2-carboxy-D-arabinitol 1-phosphatase, Plant Physiol. 90, 679-685.

Salvucci, M. E., Holbrook, G. P., Anderson, J. C. and Bowes, G. (1988) NADPH-dependent metabolism of the ribulose bisphosphate carboxylase/oxygenase inhibitor 2-carboxyarabinitol 1-phosphate by a chloroplast protein, FEBS Lett. 231, 197-201.

Salvucci, M. E., Portis, A. R., Jr. and Ogren, W. L. (1985) A soluble chloroplast protein catalyzes ribulosebisphosphate carboxylase/oxygenase activation *in vivo*. Photosyn. Res. 7, 193-201.

Salvucci, M. E., Portis, A. R., Jr. and Ogren, W. L. (1986) Light and CO_2 response of ribulose-1,5-bisphosphate carboxylase/oxygenase in *Arabidopsis* leaves, Plant Physiol. 80, 655-659.

Salvucci, M. E., Wereneke, J. M., Ogren, W. L. and Portis, A. R., Jr. (1987) Purification and species distribution of rubisco activase, Plant Physiol. 84, 930-936.

Saver, B. G. and Knowles, J. R. (1982) Ribulose-1,5-bisphosphate carboxylase: Enzyme catalyzed appearance of solvent tritium at carbon 3 of ribulose 1,5-bisphosphate reisolated after partial reaction, Biochemistry 21, 5398-5403.

Schloss, J. V. (1988) Comparative affinities of the epimeric reaction intermediate analogs 2- and 4-carboxy-D-arabinitol 1,5-bisphosphate for spinach ribulose 1,5-bisphosphate carboxylase. J. Biol. Chem. 263, 4145-4150

Schloss, J. V. and Lorimer, G. H. (1982) The stereochemical course of ribulosebisphosphate carboxylase, J. Biol. Chem. 257, 4691-4694.

Seemann, J. R., Berry, J. A., Freas, S. M. and Krump, M. A. (1985) Regulation of ribulose bisphosphate carboxylase activity *in vivo* by a light-modulated inhibitor of catalysis. Proc. Natl. Acad. Sci. USA 12, 8024-8028

Seftor, R. E. B., Bahr, J. T. and Jensen, R. G. (1986) Measurement of the enzyme-CO_2-Mg^{2+} form of spinach ribulose 1,5-bisphosphate carboxylase/oxygenase. Plant Physiol. 80, 599-600

Servaites, J. C. (1985) Binding of a phosphorylated inhibitor to ribulose bisphosphate carboxylase/oxygenase during the night, Plant Physiol. 78, 839-843.

Sicher, R. C., Hatch, A. L., Stumpf, D. K. and Jensen, R. G. (1981) Ribulose-1,5-bisphosphate and activation of the carboxylase in the chloroplast. Plant Physiol. 68, 252-255

Smrcka, A. V. and Jensen, R. G. (1988) HPLC separation and indirect ultraviolet detection of phosphorylated sugars. Plant physiol. 86, 615-618

Somerville, C. R., Portis, A. R. and Ogren, W. L. (1982) A mutant of *Arabidopsis thaliana* which lacks activation of RuBP carboxylase *in vivo*, Plant Physiol. 70, 381-387.

Spreitzer, R. J., Jordan, D. B. and Ogren, W. L. (1982) Biochemical and genetic analysis of an RuBP carboxylase/oxygenase-deficient mutant and revertants of *Chlamydomonas reinhardtii*. FEBS. Lett. 148, 117-121

Spreitzer, R. J., Goldschmidt-Clermont, M., Rahire, M. and Rochaix, J-D. (1985)

Nonsense mutations in the *Chlamydomonas* chloroplast gene that codes for the large subunit of ribulosebisphosphate carboxylase/oxygenase. Proc. Natl. Acad. Sci. USA 82, 5460-5464

Spreitzer, R. J., Al-Abed, S. R. and Huether, M. J. (1988) Temperature-sensitive, photosynthesis-deficient mutants of *Chlamydomonas reinhardtii*. Plant Physiol. 86, 773-777

Streusand, V. J. and Portis, A. R., Jr. (1987) Rubisco activase mediates ATP-dependent activation of ribulose bisphosphate carboxylase, Plant Physiol. 85, 152-154.

Stumpf, D. K. and Jensen, R. G. (1982) Photosynthetic CO_2 fixation at air levels of CO_2 by isolated spinach chloroplasts. Plant Physiol. 69, 1263-1267

Sue, J. M. and Knowles, J. R. (1982) Ribulose-1,5-bisphosphate carboxylase: Primary deuterium kinetic isotope effect using [3-^{2}H] ribulose 1,5-bisphosphate. Biochem. 21, 5410-5414

Umbreit, W. W., Burris, R. H. and Stauffer, J. F. (1972) In Manometric and Biochemical Techniques. Burgess Publishing Co. Minn, MN pp 3-7, 267.

Voordouw, G., De Vries, P. A., Van Den Berg, W. A. M., De Clerck, E. P. J. (1987) Site-directed mutagenesis of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase from *Anacystis nidulans*. Eur. J. Biochem. 163, 591-598.

Vu, J. C. V., Allen, J. R., Jr. and Bowes, G. (1984) Dark/light modulation of ribulose bisphosphate carboxylase activity in plants from different photosynthetic categories, Plant Physiol. 76, 843-845.

Walker, D. A. (1973) Photosynthetic induction phenomena and the light activation of ribulose diphosphate carboxylase. New Phytol. 72, 209-235

Weissbach, A., Horecker, B. L. and Hurwitz, J. (1956) The enzymic fixation of phosphoglyceric acid from ribulose diphosphate and carbon dioxide. J. Bio. Chem. 218, 795-810.

Werdan, K. and Heldt, H. W. (1972) Accumulation of bicarbonate in intact

chloroplasts following a pH gradient. Biochim. Biophys. Acta 283, 430-441

Werdan, K., Heldt, H. W. and Milovancev, M. (1975) The role of pH in the regulation of carbon fixation in the chloroplast stroma. Studies on CO_2 fixation in the light and dark. Biochem. Biophys. Acta. 369, 276-292

Wildman, S. G. and Bonner, J. (1947) The proteins of green leaves. I. Isolation, enzymic properties, and auxin content of spinach cytoplasmic protein. Arch. Biochem. 14, 381-413.

Wilkinson, G. N. (1961) Statistical estimations in enzyme kinetics, Biochem. J. 80, 324-332.

Wishnick, M., Lane, M. D. and Scrutton, M. C. (1970) The interaction of metal ions with ribulose 1,5-diphosphate carboxylase from spinach. J. Biol. Chem. 245, 4939-4947

Wolfenden, R. (1969) Transition state analogues for enzyme catalysis, Nature 223, 704-705.

Woodrow, I. E. and Berry, J. A. (1988) Enzymatic regulation of photosynthetic CO_2 fixation in C_3 plants. Ann. Rev. Plant Physiol. Plant Mol. Biol. 39, 533-594.

Yeoh, H. -H., Badger, M. R. and Watson. L. (1981) Variations in kinetic properties of ribulose-1,5-bisphosphate carboxylase among plants, Plant Physiol. 67, 1151-1155.

Yokota, A. and Kitaoka, S. (1985) Correct pK values for dissociation constant of carbonic acid lowers the reported Km values of ribulose-1,5-bisphosphate carboxylase to half. Presentation of a nomograph and an equation for determining the pK values, Biochem. Biophys. Res. Comm. 131, 1075-1079.

Zhu, G. and Jensen, R. G. (1990) Status of the substrate binding sites of ribulose bisphosphate carboxylase as determined with 2-C-carboxyarabinitol 1,5-bisphosphate. Plant Physiol. 93, 244-249.

Zhu, G., Moon, J. W. and Jensen, R. G. (1990) Synthesis of xylulose bisphosphate by rubisco. Plant Physiol. 93, s-121.