Fallower of Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase Activity

Decarbamylation of Catalytic Sites Depends on pH

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ABSTRACT

Loss of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity during CO₂ fixation, called fallover, occurred with or without loss of activator CO₂ from catalytic sites depending on pH. At pH 7.5, but not at pH 8.5, the fraction of Rubisco sites that were carboxylated decreased during fallover. Inhibitors which formed during fallover were identified following NaBH₄ 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-P₂ was the only inhibitor binding to Rubisco and this binding was at carboxylated 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, whereas 3-ketoarabinitol-P₂ bound to carboxylated sites. The pH during fallover also influenced the ratio of 3-ketoarabinitol-P₂ 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 with those at pH 8.5. 3-Ketoribitol-P₂ was not found at either pH.

FULLY ACTIVATED, PURIFIED RUBISCO SLOWLY LOSES ITS ACTIVITY DURING CO₂ FIXATION AFTER EXPOSURE TO RuBP,² A PROCESS KNOWN AS FALLOVER. EDMONDSON ET AL. (4–6) 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, 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 NaBH₄ REDUCTION OF THE SUGARS (3). FOR POSITIVE PROOF OF 3-KETOARABINITOL, THEIR CHROMATOGRAM SHOULD ONLY HAVE HAD ARABINITOL AS NaBH₄ REDUCTION OF 3-KETOARABINITOL GIVES ARABINITOL.

THE SYNTHESIS OF XuBP FROM RuBP BY RUBISCO HAD BEEN REPORTED BEFORE (9). AS WE HAVE SHOWN, XuBP, LIKE RuBP, BINDS TIGHTLY ONLY TO RUBISCO SITES THAT ARE CARBOXYLATED (16). INDEED, ITS PRESENCE STABILIZES RUBISCO SITES IN THE DECARBAMYLATED STATE. THE DILEMMA IS: IF THE FALLOVER DOES NOT INVOLVE A LOSS OF ^14CO₂ AT RUBISCO CATALYTIC SITES, WHAT ROLE, IF ANY, DOES XuBP PLAY IN THE FALLOVER?

IN THIS PAPER, WE DEMONSTRATE USING HPAE-PAD THAT BOTH INHIBITORS, XuBP AND 3-KETOARABINITOL-P₂, ARE FORMED DURING FALLOVER OF RUBISCO ACTIVITY. WE FURTHER SHOW THAT, AT pH 8.5, RUBISCO ACTIVITY WAS MAINLY INHIBITED BY 3-KETOARABINITOL-P₂ BINDING AND STABILIZING THE CARBOXYLATED 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-P₂ IS ALSO DETERMINED.

MATERIALS AND METHODS

Materials

RuBP and CABP were synthesized and purified as previously described (1, 10, 15). XuBP was made by an aldolase-catalyzed condensation of glycolaldehyde phosphate and dihydroxyacetone phosphate according to Byrne and Lardy (2) and Paech et al. (9). Rubisco was purified from spinach (Spinacia oleracea) leaves (8).

CO₂ Fixation with Rubisco to Produce Inhibitors

Rubisco (2–4 mg in 3 mL) was activated by incubation with 10 mM KHCO₃ and 10 mM MgCl₂ in 100 mM Bicine (pH 8.5) or 100 mM Hepes (pH 7.5) for 30 min. The reaction was started by addition of 5 to 8 mM RuBP.

Formation and Detection of Inhibitors

After 1 to 2 h catalysis, HClO₄ 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 KClO₄ precipitate, this solution was used for inhibition of Rubisco activity or was reduced with NaBH₄ for analysis of the polyal bisphosphates. Other procedures, such as the trapping of the ^14CO₂ with CABP were previously described (15, 16).

The polyal bisphosphates were separated and identified by HPAE-PAD. The HPAE-PAD system used in this study was the same as previously described (16) with some minor mod-

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¹ This research was supported partially by the Arizona Agricultural Experiment Station.

² Abbreviations: RuBP, ribulose 1,5-bisphosphate; ^14CO₂, activator CO₂; CABP, 2-C-carboxy-arabinitol 1,5-bisphosphate; HPAE-PAD, high performance anion-exchange chromatography with pulsed amperometric detection; PGA, 3-phosphoglycerate; XuBP, xylulose 1,5-bisphosphate.
ifications. 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 extracting 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 (16) 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 (6). The change in Rubisco activity was assayed. The amount of enzyme catalytic sites capable of tight binding was determined with each Rubisco preparation using $[^1^3C]$CABP (15) and was usually about six CABP binding sites per mole of spinach Rubisco. XuBP was determined enzymatically using aldolase, glycerol-P dehydrogenase, and NADH (14).

RESULTS

Fallover of Rubisco Activity Occurs with or without Loss of $^1^3$CO$_2$

Two different fallover patterns involving loss of activity were observed; one at pH 8.5 having little loss of $^1^3$CO$_2$ and the second at pH 7.5 with a loss of $^1^3$CO$_2$ (Fig. 1). These results corroborate the observations that fallover with Rubisco does not involve decarbamylation at pH 8.3 (5), but it extends them further. At pH 7.5, about 40% of the $^1^3$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 $^1^3$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 $^1^3$CO$_2$ bound per mol Rubisco was measured. The inhibitor concentration was adjusted so that 50 $\mu$L of an extract gave 50% inhibition of CO$_2$ fixation (not shown). The pH 7.5 extract caused a significant change in $^1^3$CO$_2$ bound to Rubisco (Fig. 2). These observations 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.

Identification of Inhibitors

Formed during fallover of CO$_2$ fixation, XuBP has been directly separated and identified by HPAE-PAD (16). The loss of Rubisco activity with loss of carbamylation at pH 7.5 (Fig. 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. 3). As expected, reduction of RuBP gave two peaks, which corresponded to ribitol 1,5-P$_2$ and arabinitol 1,5-P$_2$, whereas 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 polysols, xylitol, arabinitol, and ribitol (16) (data not shown).

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$_2$ was detected following HPAE separation (Fig 4). As the reduced product from 3-ketoarabinitol-P$_2$ was 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 Figure 3, 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. Inasmuch as 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

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Effect of pH during catalysis on the activity (O) and carbamylation state (D) 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$^1^3$CO$_3$ (1 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 $\mu$L of the reaction were acidified with 1 N HCl and dried at 80°C, and the amount of CO$_2$ fixed was determined by liquid scintillation counting. The $[^1^3C]$CO$_2$ was trapped by $[^1^2C]$CABP (16).
fixation 50%

fresh, activated Rubisco (10 μL added to

The inhibitor preparations (50 μL) were made with Rubisco at either pH 7.5 or 8.5, for 1 h to use up the RuBP and leave the inhibitors behind. HClO₄ was added to 3%, the precipitated protein removed, supernatant neutralized with KOH and the KClO₄ removed by centrifugation. The two preparations were diluted so that 50 μL of each equally inhibited CO₂ fixation 50% with fresh Rubisco at pH 8.0 after 30 min incubation. 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 KH₂¹³CO₃ (10 Ci/mol), and 10 mM MgCl₂ in 100 μL, and the carbamylation state of Rubisco was measured at the times given by adding [¹⁴C]CABP to trap the [¹⁴C]CO₂ (16). At pH 8.0 the inhibitor preparation from pH 7.5 (O) caused about 30% decarbamylation of Rubisco, whereas that from pH 8.5 (●) was decarbamylation about 8%.

pH 7.5 both xylitol-P₂ and arabinitol-P₂ were detected (Fig. 4). About 40% of the inhibitors bound to Rubisco following incubation at pH 7.5 was XuBP, the rest being 3-ketoarabinitol-P₂.

**Total Amounts of XuBP and 3-Ketoarabinitol-P₂**

Although Figure 4 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 (6) 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 (data not shown) (6). 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 I). 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. 4). At pH 7.5, 74% of the inhibitors was XuBP.

**DISCUSSION**

Two different fallover patterns have been demonstrated during catalysis, one with little loss of ¹⁴CO₂ at pH 8.5 and the second with loss of ¹⁴CO₂ at pH 7.5. Two inhibitors have been identified as being produced during fallover. They are XuBP and 3-ketoarabinitol-P₂. Because of the weak binding affinity of XuBP to decarbamylated catalytic sites of Rubisco at pH 8.5, there is little loss of ¹⁴CO₂ during fallover. XuBP does not bind tightly to carbamylated sites (16) and thus the loss in Rubisco activity at pH 8.5 was mostly due to the binding of 3-ketoarabinitol-P₂ to carbamylated sites.

According to the scheme proposed by Edmondson et al. (3) 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 isomer-
FALLOVER OF RUBISCO ACTIVITY

Figure 4. HPAE-PAD separation and detection of the polyol bisphosphates formed upon NaBH₄ 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 10 DG gel filtration column and HClO₄ to 3% added to the protein fraction. After neutralization with KOH and centrifugation to remove the KClO₄, 5 to 10 mg NaBH₄ were added to the supernatant. The solution (0.5 mL) was loaded on a silica SAX column and washed with 1 mL H₂O. 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, H₂O was added to a known volume, and 50 μL were put on the HPAE-PAD. The top curve indicates the position of retention of xylitol-P₂, arabinitol-P₂, and ribitol-P₂, made by mixing the reduction products of XuBP and RuBP from Figure 3. The middle and bottom curves indicate the position of the NaBH₄-reduced inhibitors made and bound to Rubisco during fallover at pH 8.5 or 7.5, respectively.

Table I. 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 100 mM KHCO₃ and 10 mM MgCl₂, for 30 min, then 0.5 mL of 33.2 mg 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 HClO₄ (see *Materials and Methods*) and the supernatant used for analysis of inhibitors.

<table>
<thead>
<tr>
<th>pH</th>
<th>Total* Inhibitors</th>
<th>XuBP*</th>
<th>Turnover† per Inhibitor</th>
<th>XuBP per Total Inhibitors</th>
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* Total inhibitors were estimated by titration of Rubisco activity at pH 8.0 and extrapolation of the initial inhibition to zero activity (6). † XuBP was determined enzymatically. The reaction mixture contained: 100 mM Hepes (pH 7.5), 9 units of glyceral-P dehydrogenase, 75 μM NADH, and 37 units of aldolase in 0.5 mL. ‡ The ratio of RuBP consumed to inhibitors formed during the 1.5 h.
at pH 7.3 than at pH 8.3 either in the presence or absence of RuBP. As they noted that the rate of decarbamylation in the presence of RuBP could not fully account for the rate of fallower, we explain this by presence of both inhibitors which bind to decarbamylated and carbamylated Rubisco sites, but both cause fallower.

In plants, an enzymatic system for relieving inhibition of Rubisco, the Rubisco activase, has been demonstrated (11). Rubisco activase can remove Rubisco inhibitors from both active and inactive sites (11, 12). We have isolated and identified XuBP from celery leaves (16), which implies that the mechanism for the formation of inhibitors in vitro catalysis may also exist in vivo. Robinson and Portis (13) have proved that the fallower 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 misfired products, XuBP and 3-ketoarabinitol-P₂.

LITERATURE CITED