

Slow Inactivation of Ribulosebisphosphate Carboxylase during Catalysis Is Caused by Accumulation of a Slow, Tight-Binding Inhibitor at the Catalytic Site

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ABSTRACT

The slow inactivation which accompanies catalysis by higher-plant ribulose-P₂ carboxylase-oxygenase (Rubisco) *in vitro* was only partially reversed when the enzyme was gel filtered to remove small molecules. However, gel filtration or dialysis in the presence of high SO₄²⁻ concentrations induced full recovery. This suggests that the inactivation is caused by a tight-binding inhibitor whose effective affinity is reduced by competition with SO₄²⁻ ions, which are known to bind at the catalytic site. The involvement of an inhibitor was confirmed by observations that supernatants obtained after acid-precipitation of inactivated Rubisco were inhibitory when applied to fresh enzyme. The inhibitor bound slowly and tightly and showed strong negative cooperativity. The inhibitor was moderately unstable at pH 8.3, decaying with a half-life of several hours, but was more stable at pH 2. It was destroyed by phosphatase treatment but not by H₂O₂ or *o*-phenylenediamine, compounds which react with vicinal dicarbonyl groups. It did not contain a carbon atom derived from substrate CO₂. Possibilities concerning the identity, genesis, and physiological relevance of this inhibitor are discussed.

Rubisco² becomes slowly inactivated during catalysis after exposure to ribulose-P₂. This inactivation, or "fallover," continues until eventually a steady rate of catalysis is reached which is substantially less than the initial rate (6). The phenomenon is not a result of substrate depletion or product accumulation and it is worsened by lowering the pH or the CO₂ concentration and alleviated by inhibitors which bind at the active site (6). Earlier papers of this series indicated that it is unlikely that fallover is caused by readily reversible, inhibitory binding of ribulose-P₂ at a non-catalytic, regulatory site (6) and showed that fallover is not due to decarbamylation of the active-site ϵ -carbamyl-lysyl residue that is required for catalysis (7).

One possibility which remains is that fallover is caused by

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² Abbreviations: Rubisco, ribulose-P₂ carboxylase-oxygenase (EC 4.1.1.39); ribulose-P₂, D-ribulose-1,5-bisphosphate; P-glycerate, 3-phospho-D-glycerate; xylulose-P₂, D-xylulose-1,5-bisphosphate; ribose-P, D-ribose-5-phosphate; carboxyarabinitol-P, 2'-carboxy-D-arabinitol-1-phosphate; carboxyarabinitol-P₂, 2'-carboxy-D-arabinitol-1,5-bisphosphate.

progressive inhibition by a tight-binding inhibitor which is either present in all preparations of ribulose-P₂ or is produced during the course of the catalytic reaction. Robinson and Portis (19) interpreted the suppression of fallover by the Rubisco activase system in terms of the removal of such an inhibitor by the activase. The known tendency for ribulose-P₂ to epimerize to xylulose-P₂ via a 2,3-enediol intermediate, which is itself subject to β -elimination of the C-1 phosphoryl group to produce a variety of potentially inhibitory compounds, might be a means by which such an inhibitor is formed (12). Since the enediol compound is an intermediate in the catalytic reaction sequence of Rubisco (2), such reactions could occur at the catalytic site as well as in solution.

In this study, we show that a slow, tight-binding inhibitor is, indeed, responsible for fallover. The data are most consistent with the inhibitor being formed as a by-product during catalysis.

MATERIALS AND METHODS

Materials

Spinach (*Spinacia oleracea*) Rubisco, ribulose-P₂, [5-³H] carboxyarabinitol-P₂, and spinach phosphoribulokinase were prepared as described previously (6, 7). Bicine, bovine albumin, ATP, creatine phosphate, phosphocreatine kinase, and carbonic anhydrase, were obtained from Sigma Chemical Co., potato acid phosphatase from Calbiochem, and all other enzymes from Boehringer Mannheim. NaH¹⁴CO₃ came from Amersham. All other chemicals were of the highest purity commercially available.

Carboxylase Assays

Unless otherwise stated, the assay solution contained 100 mM Bicine-NaOH (pH 8.3), 20 mM MgCl₂, 10 mM NaH¹⁴CO₃ (50 Bq·nmol⁻¹), 1 mM ribulose-P₂, 0.1% (w/v) bovine albumin, and 50 μ g of carbonic anhydrase in a total volume of 0.5 mL. Assays were conducted at 25 °C and, generally, were initiated by addition of ribulose-P₂ after incubation of the otherwise complete mixture for at least 10 min. Catalysis was terminated by acidification after 1 min unless otherwise stated. Other details were as described previously (6). When assays were initiated with preactivated Rubisco instead of

ribulose-P₂, allowance was made for the increase in concentration and decrease in specific radioactivity of the H¹⁴CO₃ in the assay caused by H¹²CO₃ introduced with the enzyme.

Measurement of Catalytic Site Concentration

Aliquots of Rubisco preparations were added to a solution containing 100 mM Bicine-NaOH (pH 8.3), 20 mM MgCl₂, 100 mM NaHCO₃, 0.1% (w/v) bovine albumin, and 20 μM [5-³H]carboxyarabinitol-P₂ (350 Bq·nmol⁻¹). After overnight storage, protein-bound label was separated from unbound label by gel filtration as described previously (7).

Measurement of Acid-Stable ¹⁴C Present in the Inhibitor Responsible for Fallover, following Carboxylation of Ribulose-P₂ with ¹⁴CO₂

Fallover proceeded in a 3 mL solution containing 100 mM Bicine-NaOH (pH 8.3), 20 mM MgCl₂, 10 mM NaH¹⁴CO₃ (60 Bq·nmol⁻¹), 0.1% (w/v) bovine albumin, 6 mM ribulose-P₂, and 0.3 mg Rubisco and was left for 1 h to completely consume the ribulose-P₂. Initial and final activities were assayed as described above. Then the mixture was gel filtered twice through a 0.9 × 25 cm column of Sephadex G-50 (fine), to remove the labeled P-glycerate and unreacted ¹⁴CO₂, and the catalytic activity was again assayed. A 2 mL aliquot of the pooled, high mol wt peak was acidified to release any bound ¹⁴C-labeled activator carbamate. The solution was dried, and the remaining radioactivity was determined by scintillation counting.

Other Methods

Rubisco concentrations (mg·mL⁻¹) were calculated by multiplying the A₂₈₀ of the solutions by 0.61 (13). Other details are given in the legends to the Figures and Tables.

RESULTS

Recovery of Activity after Fallover

When a solution of Rubisco which had undergone fallover was rapidly gel filtered to remove unbound small molecules, the inhibition was only partially reversed (Fig. 1). During fallover, the carboxylase activity had declined to approximately 25% of the initial activity and following separation of small molecules, the rate recovered to approximately 50% of the initial activity. This level did not change significantly during 60 min further storage at 25 °C. When ribulose-P₂ was omitted, full activity was preserved throughout the experiment (Fig. 1). Robinson and Portis (19) have also reported partial recovery of fallover after gel filtration.

The effects of a variety of further treatments on the activity of the gel-filtered, inhibited enzyme are summarized in Table I. Dilution by 50-fold did not relieve the inhibition any further. A second gel filtration under conditions identical to the first was equally ineffective but, when 200 mM (NH₄)₂SO₄ was included in the second column buffer, nearly complete restoration of activity occurred. Dialysis against a buffer containing 200 mM (NH₄)₂SO₄ completely restored the initial rate

while, in the absence of (NH₄)₂SO₄, it was no more effective than storage for the same period. However, storage for 24 h at room temperature itself gave partial relief of the inhibition, indicating that the inhibitory principle may be somewhat unstable. Incubation of the inhibited enzyme with alkaline phosphatase for 2 h only slightly relieved the inhibition and longer treatments led to greater inactivation (with the uninhibited control as well as with the inhibited enzyme) perhaps because of traces of protease in the phosphatase preparation.

Inhibitor Responsible for Fallover

Supernatants obtained after acid precipitation of Rubisco preparations after fallover were inhibitory when applied to fresh activated Rubisco, not previously exposed to ribulose-P₂ (Fig. 2). (In these experiments, catalysis was allowed to continue after fallover until the ribulose-P₂ was exhausted before acid precipitation.) When fresh Rubisco was preincubated with varying amounts of the acid-treated supernatant, the resulting reaction rate declined as the amount of supernatant increased (Fig. 2A). However, the reaction rate did not decline to zero, even with large amounts of supernatant. The extent of inhibition achieved in this way (measured as the ratio of maximum inhibition at the highest inhibitor level to

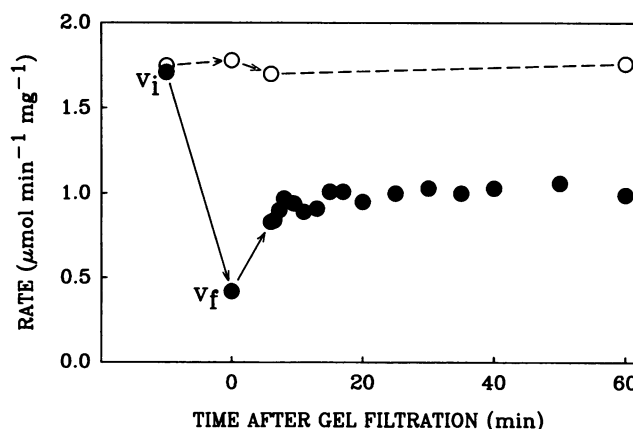


Figure 1. Partial recovery of carboxylation rate following gel filtration of Rubisco after fallover. Rubisco (0.1 mg·mL⁻¹) was activated in 100 mM Bicine-NaOH (pH 8.3), 20 mM MgCl₂, 10 mM NaHCO₃, 0.1% (w/v) bovine albumin, and 0.1 mg·mL⁻¹ carbonic anhydrase. After 10 min, aliquots were removed for assay and [³H]carboxyarabinitol-P₂ trapping to determine the initial rate (*v_i*) and the catalytic site concentration. Ribulose-P₂ was then added to 6 mM and the mixture was left in a tightly capped container at 25 °C for 1 h. Aliquots were removed for final rate assays (*v_f*), and at time zero, a 2 mL aliquot was filtered through a 0.9 × 25 cm Sephadex G50 (fine) column equilibrated with 100 mM Bicine-NaOH (pH 8.3), 10 mM NaHCO₃, 20 mM MgCl₂, and 0.1% (w/v) bovine albumin. Aliquots of the high mol wt fraction of the eluate were removed at intervals over the following hour for assay as described in "Materials and Methods" (●). Rubisco concentration in the eluate was determined by trapping with [³H]carboxyarabinitol-P₂ (see "Materials and Methods") and thus the 'recovered' rate was determined. Competitive effects of P-glycerate carried over into the assay were insignificant because of the large dilution involved. The control (○—○) contained no ribulose-P₂ initially.

Table I. Recovery of Rubisco Activity after Fallover

Carboxylase assays during fallover and after gel filtration were carried out as described in the legend to Figure 1. The gel-filtered eluate was then treated as follows: A, The eluate was diluted 50-fold into a solution containing 100 mM Bicine-NaOH (pH 8.3), 10 mM $\text{NaH}^{14}\text{CO}_3$ (80 Bq·nmol⁻¹), 20 mM MgCl_2 , 0.1% (w/v) bovine albumin, and 0.1 mg·mL⁻¹ carbonic anhydrase. At intervals after dilution, 500 μL aliquots were removed and added to 0.5 μmol of ribulose-P₂ for carboxylase assays. The rate did not change with time over a 20 min period, and a mean value is shown. B, The eluate was gel filtered a second time, under conditions identical to the first gel filtration. C, The eluate was gel filtered a second time with the column buffer containing, in addition, 200 mM $(\text{NH}_4)_2\text{SO}_4$. D, The eluate was dialyzed against column buffer for 24 hr at room temperature. E, The eluate was dialyzed against column buffer containing 200 mM $(\text{NH}_4)_2\text{SO}_4$ for 24 h at room temperature, with a final change to $(\text{NH}_4)_2\text{SO}_4$ -free buffer to remove inhibitory SO_4^{2-} . F, The eluate was left for 24 h at room temperature. G, H, To 2 mL of gel filtered eluate was added 200 μL of 100 mM Bicine-NaOH buffer (pH 9), containing 1 mM MgCl_2 , 1 mM ZnSO_4 , and 2 units of alkaline phosphatase. After the stated intervals at 25°C, aliquots were taken to determine Rubisco activity. For treatments B and D to F, 50 μL aliquots were assayed in a total volume of 500 μL . For C, 25 μL aliquots were assayed in 3 mL to minimize competition by SO_4^{2-} . Assays after treatments G and H were the same as for C. For the assays before gel filtration, sufficient dilution of the fallover solution occurred to prevent competitive inhibition by P-glycerate produced during the fallover reaction. All assays were carried out in triplicate and the standard errors are shown. See "Materials and Methods" for other assay details. Controls, treated identically but without initial exposure to ribulose-P₂, retained >95% activity after 24 h, except in H, where the control decreased to 28%.

Stage	Carboxylation Activity $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ (percent of initial)
Before initial exposure to ribulose-P ₂	2.22 ± 0.03 (100)
After completion of fallover	0.53 ± 0.04 (24)
After gel filtration	1.19 ± 0.06 (54)
After further treatment of the gel-filtered eluate	
A. 50-fold dilution	1.23 ± 0.03 (55)
B. Second gel filtration	1.25 ± 0.07 (56)
C. Second gel filtration with 200 mM SO_4^{2-}	1.95 ± 0.07 (88)
D. 24 h dialysis	1.73 ± 0.04 (78)
E. 24 h dialysis with 200 mM SO_4^{2-}	2.29 ± 0.06 (103)
F. 24 h room temperature	1.75 ± 0.05 (79)
G. 2 h alkaline phosphatase	1.35 ± 0.06 (61)
H. 24 h alkaline phosphatase	0.51 ± 0.04 (23)

the rate in the absence of inhibitor) was similar to the lowest v/v_i ratios measured during fallover (6).

The concentration of the inhibitor in the acid-treated supernatant was estimated using similar assays, except that the supernatant volume was held constant while the Rubisco concentration was varied (Fig. 2B). The data were fitted to the equation:

$$v = k_{\text{cat}}\{E - I - K_D + [(E + I + K_D)^2 - 4E \cdot I]^{1/2}\} / 2 \quad (1)$$

where v is the measured turnover rate in the presence of inhibitor, k_{cat} is the rate measured in controls lacking inhibitor, and E is the total concentration of Rubisco active sites. In this way, the total inhibitor concentration, I , and the enzyme-inhibitor dissociation constant, K_D , were estimated. The equation, which is a suitably rearranged version of that used by Berry *et al.* (4), models a binding process where the total inhibitor concentration is such that binding significantly depletes the concentration of free inhibitor. The inhibitor concentration in the acid-treated supernatant was estimated to be $4.0 \pm 0.3 \mu\text{M}$, or 0.07% of the ribulose-P₂ initially present. Although the data appear to fit the equation well,

Figure 2A shows that the inhibition is not complete even at very high inhibitor concentrations. This contradicts the assumption implicit in the equation. Perhaps binding of the inhibitor to a catalytic site does not render it completely inactive or, more likely, the inhibitor completely inhibits any sites to which it binds but the binding itself is negatively cooperative. This anomaly is concealed in the graphical approach used in Figure 2B because it manifests itself only at high [inhibitor]:[site] ratios which occur near the origin. Because of this, only an apparent K_D , which neglects this negative cooperativity, could be estimated. The estimated value for Figure 2B was $0.22 \mu\text{M}$, and the mean apparent K_D for seven such experiments was 0.21 ± 0.05 (SE) μM .

Rate of Inhibitor Binding

The rate at which the inhibitor bound to Rubisco was determined by withdrawing aliquots at timed intervals after mixing activated Rubisco with the acid-treated supernatant and adding them to an assay solution with ribulose-P₂ for a 1 min rate determination. Inhibition was biphasic, with a relatively rapid phase in the first minute followed by a slower

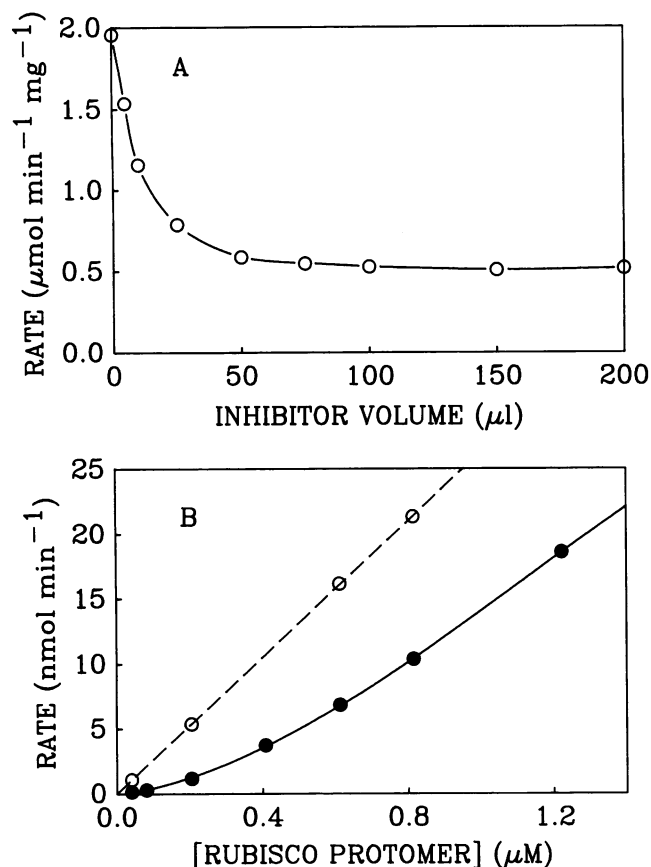


Figure 2. Inhibition of Rubisco by the inhibitor responsible for fallover. Fallover was carried out as described in the legend to Figure 1, except that the Rubisco concentration was $1 \text{ mg} \cdot \text{mL}^{-1}$, and the reaction was allowed to continue until all of the ribulose- P_2 was consumed. The solution was then acidified to pH 2 with 70% perchloric acid and left on ice for 30 min before centrifugation. The supernatant was adjusted to pH 5.0 by slow addition of 1 M KOH, left for a further 30 min on ice and again centrifuged. The supernatant contained the inhibitor. Inhibition by this 'acid-treated inhibitor' was measured in two ways: A, Varying inhibitor concentration. Eight mixtures containing 100 mM Bicine-NaOH (pH 8.3), 2 μg Rubisco, 20 mM MgCl_2 , 10 mM NaHCO_3 , and 0.1% (w/v) bovine albumin, were left for 10 min and then the stated volumes of acid-treated inhibitor were added to give final volumes of 300 μL . After 60 min at room temperature, 200 μL aliquots were taken for carboxylase assays as described in "Materials and Methods," except that the total assay volume was 1.5 ml. B, Varying Rubisco concentration. Eight mixtures, each containing 100 mM Bicine-NaOH (pH 8.3), 20 mM MgCl_2 , 10 mM NaHCO_3 , 0.1% (w/v) bovine albumin, and the stated amounts of Rubisco, were left to activate for 10 min before the addition of 50 μL of acid-treated inhibitor, bringing the total volume to 300 μL . After 60 min, 200 μL aliquots were assayed (\bullet). The solid line was obtained by fitting the data to Equation 1, using a k_{cat} value derived from a control containing no inhibitor (\circ).

decline in activity. The inhibition did not proceed to completion, even at long times (Fig. 3). This lack of complete inhibition is expected in view of the data shown in Figure 2A and also because the concentrations of both inhibitor and Rubisco sites were not much larger than the K_D .

Stability of the Inhibitor

To determine the stability of the inhibitor after release from Rubisco, portions of the acid-treated supernatant were stored at room temperature, at either pH 2 or pH 8.3, for up to 24 h before the inhibitor was reassayed by the method shown in Figure 2A. In this method, the inhibitor concentration is indicated by the initial slope of the inhibition curve. The results (Fig. 4) showed that the inhibitor was considerably more stable at pH 2 than at pH 8.3.

A further investigation of the stability of the inhibitor in various conditions was conducted using the method for quantitation of the inhibitor shown in Figure 2B (Table II). Neither 20 mM H_2O_2 nor 100 mM *o*-phenylenediamine significantly reduced the concentration of inhibitor, or altered the apparent K_D of its binding, during 1 h incubation at 25 $^\circ\text{C}$ at pH 7 or at pH 8.5. In contrast, the inhibitor was rendered completely inactive by overnight incubation with acid phosphatase at pH 5, a pH at which the inhibitor was relatively stable as demonstrated in the control incubation lacking acid phosphatase.

Conditions Leading to Inhibitor Formation

Inhibitor formation was not dependent on the time taken for complete consumption of ribulose- P_2 , or on the ratio between the ribulose- P_2 and catalytic site concentrations, because the inhibitor was still formed when sufficient Rubisco was used to completely consume 6 mM ribulose- P_2 in <5 min (instead of the usual approximately 60 min) (Table IIIA). Apparently, the amount of inhibitor produced depends simply on the number of catalytic turnovers completed. The inhibitor was also produced when ribose-P, in conjunction with ribose-P isomerase and ribulose-P kinase, was used instead of ribu-

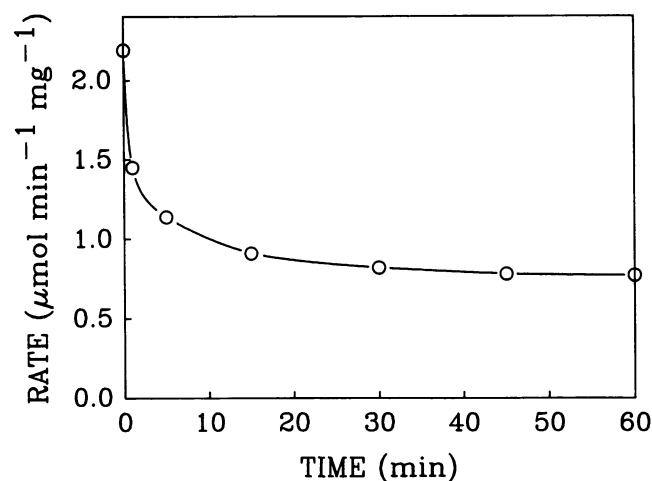


Figure 3. Rate of inhibitor binding to Rubisco. A 500 μL aliquot of acid-treated inhibitor (produced as described in the legend to Fig. 2) was added to 300 μL containing (final concentrations) 80 $\mu\text{g} \cdot \text{mL}^{-1}$ Rubisco, 15 mM NaHCO_3 , 20 mM MgCl_2 , 0.1% (w/v) bovine albumin, and 100 mM Bicine-NaOH (pH 8.3), which had been left for 10 min for the Rubisco to activate before adding the inhibitor. At the stated times, 75 μL aliquots were withdrawn and assayed, in a total volume of 1.5 mL, as described in "Materials and Methods."

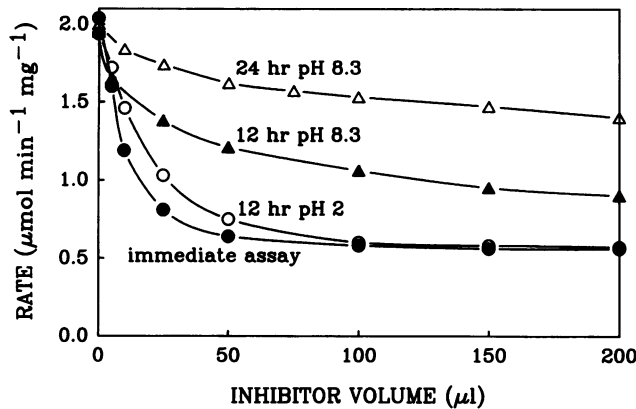


Figure 4. Stability of the inhibitor after release from Rubisco. The inhibitor, prepared as described in the legend to Figure 2, was assayed by the method shown in Figure 2A before storage (●), and after storage for 12 h at pH 2 (○), 12 h at pH 8.3 (▲), and 24 h at pH 8.3 (△), all at room temperature. The inhibitor concentration is reflected by the initial slopes of the lines at low inhibitor volumes.

lose-P₂ (Table IIIB). In this case, the ribulose-P kinase:Rubisco activity ratio was set low to minimise the steady state pool size of ribulose-P₂ during the synthesis. Therefore, it is unlikely that the inhibitor is derived by nonenzymatic decay of ribulose-P₂.

Substrate CO₂ Is Not Incorporated into the Inhibitor

A fallover reaction to produce the inhibitor was conducted with ¹⁴CO₂ present as described in "Materials and Methods." The initial carboxylase activity of 1.85 μmol·min⁻¹·mg⁻¹ decayed to a final activity of 0.47 μmol·min⁻¹·mg⁻¹. Then the solution was gel filtered twice to remove all labelled P-glycerate and unreacted ¹⁴CO₂, after which the activity was 0.96 μmol·min⁻¹·mg⁻¹. Therefore, 48% of the catalytic sites had inhibitor bound at this stage. An aliquot, containing 1.1 nmol of inhibitor, was then acidified to discharge any ¹⁴C-labeled carbamate attached to the enzyme. The acid-stable radioactivity measured in this aliquot was not significantly above background (<0.03 nmol), indicating that the inhibitor did not contain any incorporated ¹⁴CO₂.

DISCUSSION

Fallover Is Caused by an Inhibitor

We conclude that Rubisco loses activity during catalysis because its catalytic sites become progressively sequestered by an inhibitor. This is attested by observations that a substance could be released by acidification from Rubisco after fallover and used to inhibit fresh Rubisco (Figs. 2 and 3).

The partial recovery of activity following gel filtration of Rubisco after fallover, and the lack of further recovery in the filtrate during subsequent incubation (Fig. 1), or extensive dilution, or a second gel filtration under the same conditions (Table I), may be explained in two ways. The inhibitor might be composed of two compounds with differing binding affinities (only one being loose enough to be lost during gel

filtration) or, if only a single compound, its binding could be negatively cooperative (*i.e.* the affinity declines as its occupancy of Rubisco's eight active sites increases.) The shape of the binding curve in Figure 2A, showing lack of complete inhibition even at high inhibitor concentrations, supports the latter possibility.

Complete removal of the inhibitor and full recovery of activity was achieved by exchange in a high concentration of SO₄²⁻, an anion known to compete with ribulose-P₂ at the active site (3). Alleviation of fallover by other compounds which bind at the catalytic site, such as 6-phosphogluconate or the ribulose-P₂/H₂O₂ adduct (6) might be attributable to a similar mechanism.

Kinetic Characteristics of the Inhibitor

The material isolated from the enzyme after fallover shows slow, tight-binding, and negatively cooperative inhibition. The slowness (Fig. 3) implies that the inhibition is not a simple, rapidly reversible phenomenon but may be biphasic, involving isomerization of an initial, loose and rapidly reversible enzyme-inhibitor complex to a much tighter complex. Slow, biphasic inhibition has been reported for other tight-binding inhibitors of Rubisco (10, 15, 20, 22). The negative cooperativity (Fig. 2A) means that the *K_D* estimated by the approach shown in Figure 2B is only an apparent value because Equation 1 does not take cooperativity into account (*i.e.* it is an average value which neglects the progressive loosening of the binding as site occupancy increases). In its affinity, negative cooperativity, and slowness of binding, the fallover inhibitor resembles other tight-binding inhibitors of Rubisco, such as carboxyarabinitol-P (4, 22), xylitol-1,5-bisphosphate (20), and xylulose-P₂ (10).

The apparent *K_D* estimated for the inhibitor (0.21 μM) should be treated as a maximum estimate. The inhibitor preparations contained P-glycerate, a known competitive inhibitor of Rubisco (3), as well as residual perchlorate anions, which might also be inhibitory. These species would compete with the inhibitor, at least during the inhibitor's initial, reversible-binding phase, and it is conceivable that this might loosen the overall binding affinity to some extent.

Stability of the Inhibitor

The inhibitor was moderately unstable at pH 8.3 at room temperature, decaying with a half-time of several hours, but was more stable in acid conditions (Fig. 4). This instability may have allowed the very slow further recovery of activity of the gel filtered enzyme after fallover (Fig. 1; Table I, D and F).

Inhibitor Is a Phosphorylated Compound

Inhibitory potency was destroyed by acid phosphatase (Table II), indicating that the inhibitor must be a phosphate ester. However, alkaline phosphatase was not effective in promoting further recovery of activity of Rubisco when gel filtered after fallover (Table I). Apparently, alkaline phosphatase must not have access to the inhibitor while it is bound at Rubisco's active site and the rate of release of the inhibitor

Table II. Properties of the Inhibitor Responsible for Fallover

Preparations of Rubisco after fallover were acidified to release the inhibitor as described in the legend to Figure 2. The acid-treated supernatants were then further treated as follows: A, 1 mL aliquots were adjusted to pH 7 or 8.5 and H₂O₂ was added to 20 mM. After 1 h at 25°C, the H₂O₂ was discharged by adding 4000 units of catalase. B, Aliquots were adjusted to pH 7 or 8.5, *o*-phenylenediamine was added to 100 mM and incubated for 1 h at 25°C. C, Aliquots were incubated overnight at pH 5 and room temperature with 50 units of potato acid phosphatase. This mixture was then adjusted to pH 1 to denature the phosphatase and centrifuged before readjustment to pH 8.3. The concentration of inhibitor remaining after the various treatments was determined as shown in Figure 2B. The inhibitor concentration (\pm SE) was estimated by fitting the data to Equation 1. The estimates of apparent K_D were not significantly different, ranging from 0.16 to 0.40 μ M for all determinations. Blanks, omitting inhibitor, showed that k_{cat} was unaffected by the concentration of H₂O₂/catalase (A) or *o*-phenylenediamine (B) carried over with the treated inhibitor.

Treatment	Control	Inhibitor Concentration	
		Treatment	Control
μ M			
A. 1 h, 20 mM H ₂ O ₂			
pH 7	No H ₂ O ₂	5.2 \pm 0.1	5.3 \pm 0.2
pH 8.5	No H ₂ O ₂	3.5 \pm 0.2	3.5 \pm 0.2
B. 1 h, 100 mM <i>o</i> -phenylenediamine			
pH 7	No <i>o</i> -phenylenediamine	3.7 \pm 0.2	5.3 \pm 0.2
pH 8.5	No <i>o</i> -phenylenediamine	4.4 \pm 0.2	3.5 \pm 0.2
C. 24 h, acid phosphatase			
pH 5	No phosphatase	0	2.7 \pm 0.3

must be very slow under the conditions prevailing in the gel filtrate. This observation contrasts with the reversal of fallover by alkaline phosphatase demonstrated by Robinson and Portis (19). However, Robinson and Portis did not gel filter before applying the phosphatase and, therefore, a high concentration (approaching 8 mM) of P-glycerate, produced by carboxylation of ribulose-P₂ during fallover, would have been present. Since P-glycerate also competes for binding to the active site ($K_i = 0.8$ mM [3]), it would have loosened the inhibitor's binding in a similar manner to SO₄²⁻ ions (Table I) and allowed the phosphatase greater access.

Inhibitor Appears Not to Be an α -Dicarbonyl Compound

A potential candidate for the inhibitor is 1-deoxy-2,3-pentodiulose-5-phosphate. It might be produced by β -elimination of the C-1 phosphoryl group of the enediol intermediate, either at the catalytic site or after release of the enediol from the enzyme. The dicarbonyl compound is known to be the predominant fate of the enediol intermediate in solution (12, 14). Its binding to Rubisco might be strengthened by interaction of the α -dicarbonyl moiety with a guanidino group of an arginine residue (16). However, neither *o*-phenylenediamine nor H₂O₂, compounds known to react with vicinal dicarbonyl groups (8, 12), caused significant reduction in the inhibitor's potency or altered the apparent K_D of its binding (Table II). While it is just conceivable that the *o*-phenylenediamine-dicarbonyl adduct could also be a tight-binding inhibitor, H₂O₂ would convert the dicarbonyl compound to acetate and P-glycerate, neither of which are tight-binding inhibitors. Therefore, it seems unlikely that this α -dicarbonyl compound could be the inhibitor.

Inhibitor Is Likely to Be a Catalytic By-product

The inhibitor was still produced when ribulose-P₂ was replaced by an enzymatic system for generating it from ribose-P under conditions where ribulose-P₂ did not accumulate to significant levels during the course of its consumption (Table IIIB). Nor did rapid consumption of ribulose-P₂ by a large amount of Rubisco reduce the amount of inhibitor (Table IIIA). These observations are not consistent with the inhibitor being a contaminant in the starting ribulose-P₂ or a derivative of ribulose-P₂ formed nonenzymatically during the course of fallover. However, there is still the remote possibility that the inhibitor was present in the ribose-P used, or produced from it during the synthesis (and not removed by chromatographic purification of ribulose-P₂). Except for this reservation, the weight of the evidence is in favor of the inhibitor being produced by a side reaction of Rubisco's catalytic mechanism. If so, the catalytic intermediate responsible cannot be the six-carbon, carboxylated intermediate, 2'-carboxy-3-keto-D-arabinitol-1,5-bisphosphate (2), because the inhibitor does not contain a carbon atom derived from substrate CO₂. A more likely precursor for the inhibitor is the enediol intermediate formed by abstraction of the C-3 proton from ribulose-P₂ by an enzymatic base. If this enediol was reprotonated incorrectly, potentially inhibitory pentulose bisphosphate isomers would be produced. The product of stereochemically incorrect protonation at C-3 (*i.e.* protonation from the side of the plane of the double bond opposite to that from which protonation would recreate the substrate, ribulose-P₂) would be xylulose-P₂, which has inhibitory characteristics identical to those shown in Figures 2 and 3 (10), while misprotonation at C-2, rather than at C-3, would yield either 3-keto-D-arabinitol-1,5-bisphosphate or 3-keto-D-ribitol-1,5-bisphosphate, or both.

Table III. Effect of Rubisco:Ribulose-P₂ Ratio and Ribulose-P₂ Concentration on Inhibitor Formation

A, Fallover was allowed to proceed at a Rubisco concentration (10 mg · mL⁻¹) high enough to completely consume the ribulose-P₂ (6 mM) in <5 min. Other conditions were identical to those described in the legend to Figure 1. The mixture was left a further 20 min before acidifying to release the inhibitor as described in the legend to Figure 2. B, Alternatively, fallover was carried out in a reaction in which ribulose-P₂ was replaced by an enzymatic system for generating it from ribose-P. In this experiment, the steady state concentration of ribulose-P₂ was kept low throughout by using a large excess of Rubisco compared to the capacity of the generating system. The assay mixture contained, in a final volume of 1 mL, 20 mM MgCl₂, 1 mM NaHCO₃, 0.1 mM ATP, 7 mM phosphocreatine, 6 mM ribose-P, and 5 mM DTT. The mixture was adjusted to pH 7.5, and the following were added: 10 units of phosphocreatine kinase, 10 units of ribose-P isomerase, 0.1 mg of carbonic anhydrase, 0.3 units of phosphoribulokinase, and 20 mg (approximately 4 units at this CO₂ level) of Rubisco. In both experiments, reaction progress was monitored by the pH change (P-glycerate production releases protons), and the pH was continuously adjusted by addition of 24 mM Na₂CO₃. This also served to replenish the CO₂ and the volume of carbonate added provided another measure (besides there being no further pH change) of reaction completion. The inhibitor was then released by acidification as before. In order to remove all of the Rubisco in these experiments, it was necessary to carry out the perchloric acid/KOH cycle twice. The inhibitor concentration in the acid-treated supernatant was determined as described for Figure 2B and Table II.

Conditions	Inhibitor Concentration
	μM
A. High Rubisco:ribulose-P ₂ ratio (rapid consumption of ribulose-P ₂)	3.5 ± 0.3
B. Ribulose-P ₂ replaced by generating system (low steady-state concn. of ribulose-P ₂)	2.5 ± 0.2

Formation of the inhibitor by misprotonation of the enediol intermediate would be consistent with the progressive suppression of fallover by increasing CO₂ concentration in the range above that required to saturate catalysis (6). Since CO₂ reacts with the enediol intermediate, the fraction of Rubisco complexed by the enediol intermediate during steady state catalysis would decrease as the CO₂ concentration increased. Furthermore, this decrease might continue as the CO₂ concentration increased beyond the catalytically saturating level. While, in this region, catalysis is limited by other steps of the catalytic cycle, the reaction of CO₂ with the enediol would continue to be stimulated by increasing CO₂ concentration. Relief of fallover at high pH (6) might simply reflect scarcity of protons involved in the misprotonation reaction. Algal and cyanobacterial Rubiscos might be less susceptible to fallover (1, 23, 24) if their equilibria associated with the enediol intermediate were adjusted so that the fraction of the enzyme in the enediol form during steady state catalysis at a given CO₂ concentration was smaller than for the higher-plant enzyme. However, such a strategy would tend to reduce catalytic effectiveness and lower $k_{\text{cat}}/K_m(\text{CO}_2)$ ratios are, indeed, observed for algal and cyanobacterial Rubiscos (2, 23).

Of course, as mentioned in the earlier report (6), the effects of CO₂ concentration and pH on fallover might also be attributable to competition between the inhibitor and HCO₃⁻ ions.

Amount of Inhibitor Produced

If the inhibitor is a catalytic by-product, then the amounts of it detected in the acid supernatants indicate that the side reaction must occur approximately once for every 1500 carboxylation turnovers under the conditions used for producing the inhibitor (see legend to Fig. 2). Presumably, the side reaction would be more favored at lower CO₂ concentration or lower pH.

Physiological Significance of Fallover

Since the activity of freshly extracted Rubisco is often near the maximum rate predicted from the maximal specific activity of the purified enzyme before fallover (5, 11, 21), it seems that fallover inhibition is not tolerated *in vivo* during vigorous photosynthesis. How is it prevented? The recent observations of Robinson and Portis (19) that Rubisco activase, in the presence of ATP, both prevents fallover and reverses it after it has occurred may provide the necessary mechanism for prevention of fallover. Furthermore, the modulation of the activity of Rubisco activase by light via the stromal ATP/ADP ratio (18) could well mean that the degree to which fallover was suppressed was also modulated by light. This would provide a means for regulating Rubisco's activity according to the prevailing light intensity (*i.e.* in effect, adjusting the rate at which Rubisco produces P-glycerate to the rate at which P-glycerate is consumed using the products of the light reactions). This mechanism is entirely analogous to the regulation of Rubisco's activity by the nocturnal Rubisco inhibitor, carboxyarabinitol-P (9), which is also released from Rubisco's active site by Rubisco activase (17). However, many plant species (*e.g.* spinach [9]) lack the carboxyarabinitol-P regulatory system and inhibitory pentulose bisphosphates produced by the fallover mechanism could well provide a more general means for regulating Rubisco's activity. Indeed, Kobza and Seemann (9) have shown that a tight-binding inhibitor other than carboxyarabinitol-P causes a reduction in the level of total Rubisco activity, after full activation, in extracts of spinach leaves exposed to low light levels. They speculated that this inhibitor was ribulose-P₂, binding to decarbamylated Rubisco, but their data are equally consistent with it being the fallover inhibitor(s), binding to the fully carbamylated enzyme. In any event, the identification of the fallover inhibitor(s) and elucidation of the mechanisms by which they are detoxified and recycled to photosynthetic metabolism will be interesting areas for future research.

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LITERATURE CITED

1. **Andrews TJ, Ballment B** (1984) Active site carbamate formation and reaction-intermediate-analog binding by ribulose biphosphate carboxylase-oxygenase in the absence of its small subunits. *Proc Natl Acad Sci USA* **81**: 3660–3664
2. **Andrews TJ, Lorimer GH** (1987) Rubisco: Structure, mechanisms, and prospects for improvement. *In* MD Hatch, NK Boardman, eds, *The Biochemistry of Plants: A Comprehensive Treatise*, Vol 10, Photosynthesis. Academic Press, New York, pp 131–218
3. **Badger MR, Lorimer GH** (1981) Interaction of sugar phosphates with the catalytic site of ribulose-1,5-bisphosphate carboxylase. *Biochemistry* **20**: 2219–2225
4. **Berry JA, Lorimer GH, Pierce J, Seemann JR, Meek J, 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
5. **Butz ND, Sharkey TD** (1989) Activity ratios of ribulose-1,5-bisphosphate carboxylase accurately reflect carbamylation ratios. *Plant Physiol* **89**: 735–739
6. **Edmondson DL, Badger MR, Andrews TJ** (1990) Kinetic characterisation of slow inactivation of ribulosebisphosphate carboxylase during catalysis. *Plant Physiol* **93**: 1376–1382
7. **Edmondson DL, Badger MR, Andrews TJ** (1990) Slow inactivation of ribulosebisphosphate carboxylase during catalysis is not due to decarbamylation of the catalytic site. *Plant Physiol* **93**: 1383–1389
8. **Frankvoort W** (1978) The reaction between diacetyl and hydrogen peroxide: its mechanism and kinetic constants. *Thermochim Acta* **25**: 35–49
9. **Kobza J, Seemann JR** (1989) Regulation of ribulose-1,5-bisphosphate carboxylase activity in response to diurnal changes in irradiance. *Plant Physiol* **89**: 918–924
10. **McCurry SD, Tolbert NE** (1977) Inhibition of ribulose-1,5-bisphosphate carboxylase/oxygenase by xylulose 1,5-bisphosphate. *J Biol Chem* **252**: 8344–8346
11. **Mächler F, Nösberger J** (1980) Regulation of ribulose biphosphate carboxylase activity in intact wheat leaves by light, CO₂, and temperature. *J Exp Bot* **31**: 1485–1491
12. **Paech C, Pierce J, McCurry SD, Tolbert NE** (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
13. **Paulsen JM, Lane MD** (1966) Spinach ribulose diphosphate carboxylase. I. Purification and properties of the enzyme. *Biochemistry* **5**: 2350–2357
14. **Pierce J, Andrews TJ, Lorimer GH** (1986) Reaction intermediate partitioning by ribulose-bisphosphate carboxylases with differing substrate specificities. *J Biol Chem* **261**: 10248–10256
15. **Pierce J, Tolbert NE, Barker R** (1980) Interaction of ribulose-bisphosphate carboxylase/oxygenase with transition-state analogues. *Biochemistry* **19**: 934–942
16. **Riordan JF** (1973) Functional arginyl residues in carboxypeptidase A. Modification with butanedione. *Biochemistry* **12**: 3915–3923
17. **Robinson SP, Portis AR Jr** (1988) Release of the nocturnal inhibitor, carboxyarabinitol-1-phosphate, from ribulose bisphosphate carboxylase/oxygenase by rubisco activase. *FEBS Lett* **233**: 413–416
18. **Robinson SP, Portis AR Jr** (1988) Involvement of stromal ATP in the light activation of ribulose-1,5-bisphosphate carboxylase-oxygenase in intact isolated chloroplasts. *Plant Physiol* **86**: 293–298
19. **Robinson SP, Portis AR 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
20. **Ryan FJ, Barker R, Tolbert NE** (1975) Inhibition of ribulose diphosphate carboxylase/oxygenase by xylitol 1,5-diphosphate. *Biochem Biophys Res Commun* **65**: 39–46
21. **Sage RF, Sharkey TD, Seemann JR** (1988) The *in vivo* response of the ribulose-1,5-bisphosphate carboxylase activation state and the pool sizes of photosynthetic metabolites to elevated CO₂ in *Phaseolus vulgaris* L. *Planta* **174**: 407–416
22. **Seemann JR, Berry JA, Freas S, Krump MA** (1985) Regulation of ribulose bisphosphate carboxylase activity *in vivo* by a light-modulated inhibitor of catalysis. *Proc Natl Acad Sci USA* **82**: 8024–8028
23. **Yokota A, Harada A, Kitaoka S** (1989) Characterization of ribulose 1,5-bisphosphate carboxylase/oxygenase from *Euglena gracilis* Z. *J Biochem* **105**: 400–405
24. **Yokota A, Kitaoka S** (1989) Linearity and functioning forms in the carboxylase reaction of spinach ribulose 1,5-bisphosphate carboxylase/oxygenase. *Plant Cell Physiol* **30**: 183–191