Plant Physiol. (1990) 93, 1376–1382
0032-0889/90/93/1376/07/$01.00/0

Received for publication December 29, 1989
Accepted March 6, 1990

A Kinetic Characterization of Slow Inactivation of Ribulosebisphosphate Carboxylase during Catalysis

Daryl L. Edmondson[1], Murray R. Badger, and T. John Andrews*

Research School of Biological Sciences, Australian National University, P. O. Box 475, Canberra ACT 2601, Australia

ABSTRACT

The catalytic activity of ribulosebisphosphate carboxylase (Rubisco) declined as soon as catalysis was initiated by exposure to its substrate, D-ribulose-1,5-bisphosphate (ribulose-P_2). The decline continued exponentially, with a half-time of approximately 7 minutes until, eventually, a steady state level of activity was reached which could be as low as 15% of the initial activity. The ratio of the steady state activity to the initial activity was lower at low CO_2 concentration and at low pH. The inhibitors 6-phosphogluconate and H_2O_2 alleviated the inactivation, increasing the final/initial rate ratio and the half-time. Varying ribulose-P_2 concentration in the range above that required to saturate catalysis did not affect the kinetics of inactivation. The affinities for CO_2 and ribulose-P_2 were unaffected by the inactivation. The decline in activity occurred with preparations of ribulose-P_2 which contained no detectable D-xylulose-1,5-bisphosphate and also with ribulose-P_2 which had been generated enzymatically immediately before use. Inclusion of an aldolase system for removing D-xylulose-1,5-bisphosphate also did not alter the inactivation process. The inactivated Rubisco did not recover after complete exhaustion of ribulose-P_2. We conclude that the inactivation is not caused by readily-reversible binding of ribulose-P_2 at a site different from the active site and that it is unlikely to be attributable to inhibitory contaminants in ribulose-P_2 preparations.

Rubisco[2] catalyzes the carboxylation and oxygenation of ribulose-P_2, thus initiating both of the mutually opposing plant processes of photosynthetic CO_2 fixation and photorespiration (3). When assayed *in vitro*, the activity of purified Rubisco from higher plants decreases with a half-time of several minutes after contact with its substrate, ribulose-P_2 (2, 5, 12, 15, 17, 22, 24, 26). This loss in activity, to which we have applied the term 'fallover,' is not due to substrate exhaustion or product accumulation (2) and no explanation for this unusual behavior has been established. The phenomenon is displayed to a much lesser degree, if at all, by cyanobacterial (1) and algal (26) Rubiscos.

Proposed explanations for fallover have fallen into three categories: (a) Rubisco becomes catalytically competent only after carbamylation, by CO_2, of the ϵ-amino group of lys-201 at the catalytic site, which enables the catalytically essential divalent metal ion to bind (reviewed by Andrews and Lorimer [3]). There have been many proposals that ribulose-P_2 binds more tightly to the uncarbamylated enzyme (E) than to the carbamylated, metal-complexed form (ECM), thus promoting decarbamylation with concomitant loss of activity (11, 12, 15). This plausible hypothesis has been tested, and found to be incorrect, in the work described in the second paper of this series (8). (b) Readily reversible binding of ribulose-P_2 to a regulatory site, different from the catalytic site, causing a reduction in activity of the fully carbamylated enzyme has been proposed by Yokota and Kitaoka (26) who suggested that fallover is a natural phenomenon, reflecting *in vivo* control of Rubisco activity by ribulose-P_2. (c) Slow, tight-binding inhibition by isomerization or degradation products which accumulate in ribulose-P_2 preparations during storage is another possible explanation for fallover (19).

It is unlikely that extensive inactivation of Rubisco could be tolerated in the chloroplast during active photosynthesis. Indeed, the activity of Rubisco after rapid extraction of brightly illuminated leaves appears to be close to the initial, uninhibited activity of the purified enzyme (6, 18, 23). Therefore, some mechanism for preventing or reversing fallover may exist *in vivo*. Sicher *et al.* (24) showed that, while the decline in activity did not reverse after exhaustion of ribulose-P_2 by purified Rubisco preparations, Rubisco activity in extracts of lysed chloroplasts quickly recovered in similar conditions. Recently, Robinson and Portis (22) showed that the Rubisco activase protein, in the presence of ATP, prevented and reversed fallover and suggested that binding of a phosphorylated compound was responsible for the inactivation.

Here we report a kinetic characterization of the fallover phenomenon *in vitro* under a range of conditions. In these studies, we have used ribulose-P_2 prepared under conditions designed to minimize inhibitory contaminants.

MATERIALS AND METHODS

Preparation of Rubisco

Rubisco was purified from spinach (*Spinacea oleracea* L.) leaves (often approximately 1 kg) by a method based on that of Lorimer *et al.* (14), involving ion-exchange chromatography on a 3.5 × 34 cm column of DEAE-Sephacel (Pharmacia), followed by gel filtration through a 3.5 × 87 cm column of Sephacryl S-400 (Pharmacia). However, the $(NH_4)_2SO_4$ frac-

[1] Present address: Centre for Molecular Biology and Biotechnology, University of Queensland, St Lucia QLD 4067, Australia.

[2] Abbreviations: Rubisco, ribulose-P_2 carboxylase-oxygenase (EC 4.1.1.39); ribulose-P_2, D-ribulose-1,5-bisphosphate; P-glycerate, 3-phospho-D-glycerate; xylulose-P_2, D-xylulose-1,5-bisphosphate; ribose-P, D-ribose-5-phosphate; glycerol-P, glycerol-3-phosphate.

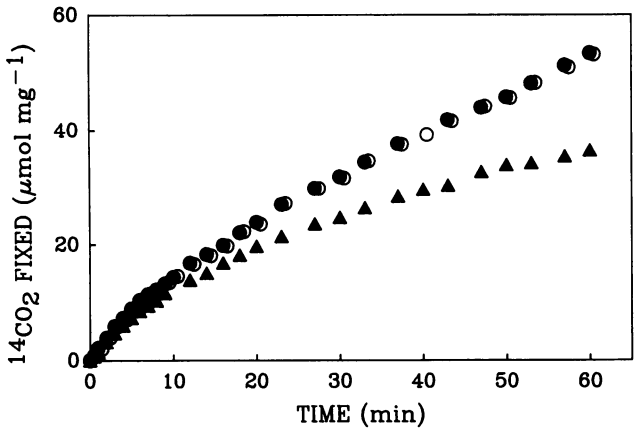

Figure 1. Effect of other proteins on the time course of Rubisco catalysis. Assays were carried out at 25°C, and contained (in a total volume of 6 mL) 100 mM Bicine-NaOH (pH 8.3), 20 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ (75 Bq·nmol^{-1}), 0.1 mg·mL^{-1} carbonic anhydrase, 4 μg·mL^{-1} Rubisco, 1 mM ribulose-P_2, and either 0.1% (w/v) gelatin (●), 0.1% (w/v) bovine albumin (○), or no further addition (▲). Rubisco was incubated in the assay mixture for 10 min before initiating catalysis by adding ribulose-P_2. Fifty μL aliquots were withdrawn at timed intervals, injected into 200 μL of 10% (v/v) formic acid, and dried on a hot plate. Acid-stable radioactivity was measured by scintillation spectrometry.

tionation of the original procedure was replaced by either of the following (a) The leaf extract was filtered using a Pellicon apparatus (Millipore Corporation) fitted with a 0.45 μm filter and then concentrated and diafiltered against the starting buffer for the DEAE-Sephacel column using the same device fitted with an ultrafiltration membrane with a 100,000 mol wt cut off. (b) The leaf extract was clarified by centrifugation and then subjected to the polyethyleneglycol precipitation procedure of Hall and Tolbert (9), followed by dissolution of the precipitate in the starting buffer for the DEAE-Sephacel column. In either case, pooled active fractions from the DEAE-Sephacel column were concentrated to 15 mL using a Minitan Ultrafiltration System (Millipore) fitted with a 30,000 mol wt cut off membrane, before being applied to the Sephacryl column which was equilibrated with 10 mM Na phosphate buffer (pH 7.6), containing 50 mM NaCl and 1 mM EDTA. Pooled active fractions from the latter column were made 20% (v/v) in glycerol and stored frozen at −80°C. When necessary, the stored enzyme was dialysed before use to remove phosphate.

Preparation of Ribulose-P Kinase

Spinach ribulose-P kinase was purified as a by-product during the preparation of Rubisco. It eluted from the DEAE-Sephacel column at slightly lower ionic strength than Rubisco. These fractions were pooled, concentrated, and chromatographed on the Sephacryl S-400 column as described for Rubisco. The pooled active Sephacryl fractions were then adjusted to pH 5 with acetic acid, stored for 2 h at 0°C to precipitate residual traces of Rubisco, and centrifuged. The supernatant was then neutralized to pH 7.6, made 20% (v/v) in glycerol and stored at −80°C.

Preparation of Ribulose-P_2

Ribulose-P_2 was prepared from ribose-P enzymatically, using ribose-P isomerase and the ribulose-P kinase prepared as described above, by the method of Horecker *et al.* (10) and purified by chromatography on Dowex 1-X8-Cl^-. At no stage was the product allowed to experience a pH above 7, and pH adjustment was always effected with dilute alkali and vigorous stirring. The product was stored at −80°C as the Na^+ salt in solution (20 mM) at pH 5. It contained less than 0.05% xylulose-P_2, assayed enzymatically as described by Sue and Knowles (25). Carboxylase activities achieved with this preparation were similar to those observed when ribulose-P_2 was generated enzymatically *in situ* from ribose-P and ATP, using ribose-P isomerase and ribulose-P kinase. By contrast, ribulose-P_2 obtained from the Sigma Chemical Co. (St. Louis, Mo) contained more than 1% xylulose-P_2 and supported lower carboxylase activities, particularly at longer assay times. At 20 min, the rate with Sigma ribulose-P_2 was only half of that observed with ribulose-P_2 prepared and stored as described above.

Other Materials

Bicine, Hepes, bovine albumin, ATP, creatine phosphate, NADH, phosphocreatine kinase, and carbonic anhydrase were obtained from Sigma Chemical Co., and all other enzymes from Boehringer Mannheim. $NaH^{14}CO_3$ came from Amersham.

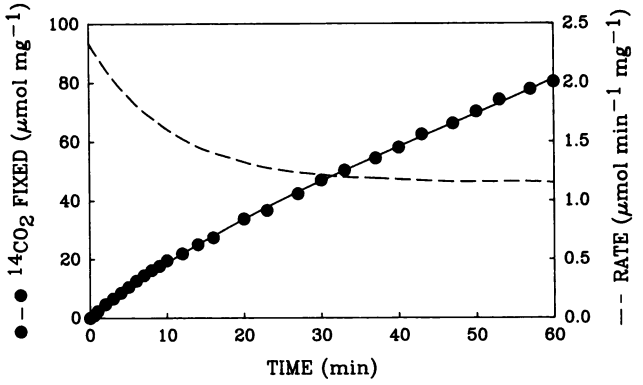

Figure 2. Slow inactivation of Rubisco during carboxylation of ribulose-P_2. The assay conditions were identical to those described in the legend to Figure 1, with 0.1% (w/v) gelatin. Data are shown plotted as product accumulated versus time (t) (●), and were fitted (solid line) to the equation:

$$\text{product} = v_f \cdot t + [(v_i - v_f)(1 - e^{-k_{obs} \cdot t})]/k_{obs} \cdot t$$

to estimate the initial rate (v_i), the final rate after infinite time (v_f), and the pseudo first-order rate constant (k_{obs}) for slow inactivation, and their standard errors. The equation was differentiated to give the rate at time t (v_t) (– – –):

$$v_t = v_f + (v_i - v_f)e^{-k_{obs} \cdot t}$$

Rubisco Assays

Carboxylase activity was measured by following the incorporation of $^{14}CO_2$ into acid-stable products. Details are given in the Figure legends. In some experiments, the spectrophotometric assay of Lilley and Walker (13), modified to include triosephosphate isomerase and glycerol-P dehydrogenase, was used. The two methods gave similar results, but the spectrophotometric assay had the disadvantage that there was an initial lag, lasting up to 2 min even at very high coupling enzyme concentrations, as coupling enzyme activities increased with the concentration of intermediates. Rubisco protein concentrations were estimated by multiplying the A_{280} of the solutions by 0.61 (20).

RESULTS

Kinetics of Slow Inactivation

The progress of the carboxylation reaction, containing fully carbamylated, Mg^{2+}-bound Rubisco (ECM), showed a gradual decline in rate for 30 min or more. Some, but not all, of this decline could be prevented by including 0.1% (w/v) of another protein, gelatin or bovine albumin being equally effective (Fig. 1). This preventable component may represent inactivation caused by binding to sites on the surfaces of the glass container. However, even when this form of inactivation was suppressed, the carboxylation rate still declined until, eventually, a steady rate was attained which was substantially less than the initial rate (Figs. 1 and 2). The final steady state activity depended on CO_2 concentration and pH (see later). The data fitted well to the equation given in the legend to Figure 2, which models a first-order decay in carboxylating activity until a final steady state activity is reached. Similar results were obtained when ribulose-P_2 was freshly generated enzymatically from ribose-P and ATP immediately before use, although the half-time of the decline increased a little (Table IB).

The slow decline in carboxylation rate was not due to protein denaturation (unless it was induced by ribulose-P_2), since the kinetics were not altered by varying the time for which Rubisco was incubated in the assay medium before catalysis was initiated by adding ribulose-P_2 (data not shown). Nor was the decline in rate due to substrate exhaustion, because no more than 15% of the ribulose-P_2 or CO_2 initially present was consumed during the reaction. Accumulation of the product, P-glycerate, to inhibitory levels may also be ruled out as the cause of this fallover, since the extent of the decline, under any given set of conditions, depended only on the time since initiation of catalysis, not on the extent of conversion of ribulose-P_2 to P-glycerate. If product build-up were causing the decline in rate, the decline should occur faster at higher enzyme concentrations and this was not observed (Table IA).

To determine if fallover was a result of ribulose-P_2 degradation under the assay conditions, perhaps by slow isomerization to xylulose-P_2 or other inhibitory compounds (19), a standard assay was compared to one in which the ribulose-P_2 had been left at pH 8.3, 25°C for 1 h. No difference in the kinetics of fallover was observed (data not shown), indicating that nonenzymatic isomerization of ribulose-P_2 is not rapid enough to explain the fallover phenomenon. Furthermore, inclusion of aldolase, glycerol-P dehydrogenase, and NADH (to remove any traces of xylulose-P_2) also had no effect on the kinetics of fallover (Table IC).

Table I. *Kinetics of Slow Inactivation under Different Conditions*

Assays were carried out in 6 mL total volume at pH 8.3, and kinetic parameters were calculated, all as described in the legend to Figure 2. Where indicated in B, ribulose-P_2 was generated immediately before use in 1 mL of a CO_2- and O_2-free solution containing 100 mM Hepes-NaOH buffer (pH 7.6), 20 mM $MgCl_2$, 20 mM ribose 5-phosphate, 40 mM ATP, 5 mM dithiothreitol, 2 units of ribulose-P kinase and 20 units of ribose-P isomerase. After 45 min at 23°C, the enzymes were removed by filtration with an Amicon MPS-1 apparatus using a YMT membrane. One hundred and fifty μL of the filtrate was used to initiate the assay instead of ribulose-P_2. This gave a final concentration of 0.5 mM ribulose-P_2 and ATP and ADP were also carried over to final concentrations of 0.5 mM for each. In C, the "xylulose-P_2 trap" consisted of adding 20 units of aldolase, 20 units of glycerol-P dehydrogenase and 0.5 mM NADH. In D and E, the final rates shown (v_{60}) are the rates after 60 min of catalysis, as estimates of the extrapolated v_f were inaccurate when inactivation was slow.

	Conditions				Parameters			
	$[CO_2]$	[Ribulose-P_2]	[Rubiscol]	Other	v_i	v_f	v_f/v_i	t_{v_s}
	μM	mM	$\mu g \cdot mL^{-1}$		$\mu mol \cdot (min \cdot mg)^{-1}$	$\mu mol \cdot (min \cdot mg)^{-1}$		min
A.	70	1.0	0.68		1.45 ± 0.05	0.44 ± 0.01	0.30	5.1 ± 0.4
	70	1.0	1.9		1.58 ± 0.05	0.51 ± 0.01	0.32	4.5 ± 0.3
	70	1.0	5.2		1.60 ± 0.05	0.49 ± 0.01	0.31	6.9 ± 0.5
B.	70	0.5	1.3		1.87 ± 0.03	0.63 ± 0.01	0.34	8.7 ± 0.5
	70	0.5	1.3	Freshly generated ribulose-P_2	1.94 ± 0.03	0.61 ± 0.04	0.31	14 ± 1
C.	70	1.0	2.0		2.25 ± 0.04	0.71 ± 0.01	0.32	5.2 ± 0.3
	70	1.0	2.0	Xylulose-P_2 trap	2.32 ± 0.05	0.73 ± 0.01	0.31	5.7 ± 0.4
						v_{60}	v_{60}/v_i	
D.	7	1.0	32		0.29 ± 0.008	0.07 ± 0.001	0.24	5.2 ± 0.3
	7	1.0	32	2 mM H_2O_2	0.17 ± 0.003	0.09 ± 0.01	0.53	28 ± 6
E.	70	0.2	1.3		1.69 ± 0.04	0.48 ± 0.01	0.28	6.1 ± 0.4
	70	0.2	1.3	2 mM 6-phosphogluconate	0.29 ± 0.004	0.16 ± 0.007	0.55	15 ± 2

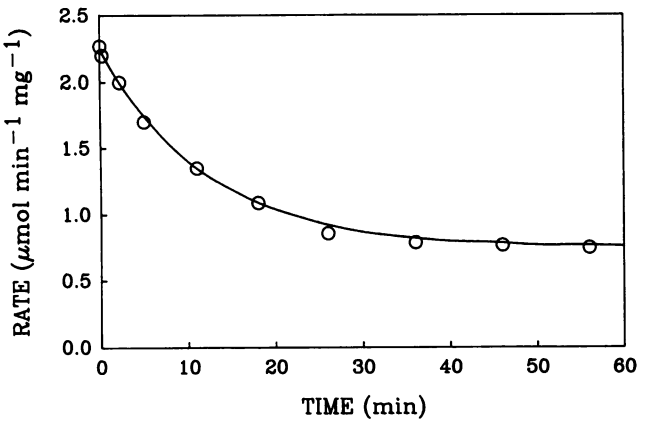

Figure 3. Effect of 50-fold dilution on slow inactivation. Carboxylation rate is shown during an assay identical to that described in the legend to Figure 2, but with a $^{14}CO_2$ specific radioactivity of 3 Bq·nmol^{-1}, and a Rubisco concentration of 40 μg mL^{-1} (——). Also shown (O) is the rate measured in 1 min assays, in which 20 μL aliquots (0.8 μg Rubisco) were removed from the assay mixture at given times and added to otherwise complete 1 mL assays which were identical to the original except that the $^{14}CO_2$ specific radioactivity was 150 Bq·nmol^{-1}. This gave sufficient $^{14}CO_2$ fixed in 1 min to be considerably higher than the background $^{14}CO_2$ fixed and carried over from the slow inactivation assay, which was subtracted.

Reversibility of Fallover

To determine if fallover could be reversed by dilution (which might be expected if the inactivation was due to loose and rapid-equilibrium binding of an inhibitor), aliquots of the fallover assay were removed at different times during a 1 h reaction period and the rate determined (in 1 min) after 50-fold dilution into a fresh assay mixture. As shown in Figure 3, the rate measured in the diluted samples paralleled that in the undiluted reaction, *i.e.* during the 1 min following dilution, inhibition was not relieved.

A further indication that fallover was not rapidly reversible was obtained when the assay conditions were arranged so that all of the ribulose-P_2 was consumed in approximately 2 h. A second addition of ribulose-P_2 to restore the initial concentration did not restore the initial carboxylation rate (Fig. 4).

Effect of Varying CO_2 Concentration

The extent of fallover at pH 8.3 varied inversely with the CO_2 concentration prevailing in the assay mixture (Fig. 5). Thus, the ratio v_f/v_i increased from 0.23 at 3 μM CO_2 to 0.67 at 300 μM CO_2. On the other hand, the pseudo-first-order rate constant for the attainment of the final steady state rate (k_{obs}) was relatively little affected by CO_2 concentration. k_{obs} remained constant at approximately 0.1 min^{-1} except at very low CO_2 levels (below 10 μM) where it increased (0.21 min^{-1} at 3 μM CO_2).

Effect of Varying pH

The extent of fallover at a constant CO_2 concentration (100 μM) was greater at more acid pH, v_f/v_i being 0.14 at pH 7.6 and increasing to 0.65 at pH 8.8 (Fig. 6). Although the initial rate dropped steeply above pH 8, the final rate was much less affected.

Effect of Varying Ribulose-P_2 Concentration

Varying ribulose-P_2 concentration in the range where it remained saturating for catalysis, *i.e.* above 0.2 mM, had no effect on the kinetics of fallover (Table I, compare A and E). In other experiments (not shown), ribulose-P_2 concentrations as high as 6 mM were used without altering the kinetics of fallover. At lower concentrations, *i.e.* below 0.2 mM, fallover becomes difficult to analyse because the decline in activity due to the substrate becoming progressively less saturating as it is consumed is superimposed on the basic fallover phenomenon.

Effects of Inhibitors

Both the rate and the extent of fallover were decreased by the inhibitors, H_2O_2 and 6-phosphogluconate (Table I, D and E). 2 mM H_2O_2 (7 μM CO_2, 1 mM ribulose-P_2, pH 8.3) caused the half-time to increase more than 5-fold. The final rates shown in Table I in these cases are those observed after 60 min of reaction, not the extrapolated estimates for infinite

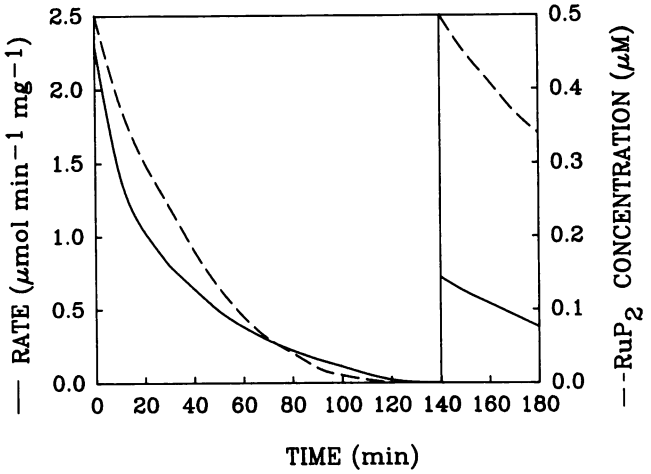

Figure 4. Effect of further addition of ribulose-P_2 after complete consumption of ribulose-P_2. The spectrophotometric assay was used. Assay solutions contained (in a total volume of 1 mL) 100 mM Bicine-NaOH (pH 8.3), 20 mM $MgCl_2$, 15 mM $NaHCO_3$, 1.25 mM ATP, 5 mM dithiothreitol, 12 mM creatine phosphate, 0.2 mM NADH, 60 units of P-glycerate kinase, 25 units of glyceraldehyde-3-phosphate dehydrogenase, 60 units of triosephosphate isomerase, 20 units of glycerol-P dehydrogenase, 15 units of phosphocreatine kinase, 0.1 mg of carbonic anhydrase, and 7 μg of Rubisco. The reaction was initiated after 10 min preincubation by the addition of ribulose-P_2 to give a final concentration of 0.5 mM. The coupling enzymes were desalted by ultrafiltration. Rubisco rate (——) was determined from the slope of a plot of A_{340} versus time, and ribulose-P_2 concentration (– – –) was determined from the extent of the change in A_{340}. Once the ribulose-P_2 was depleted, the mixture was left for a further 15 min, and then the initial ribulose-P_2 concentration was restored at the time indicated by the vertical line.

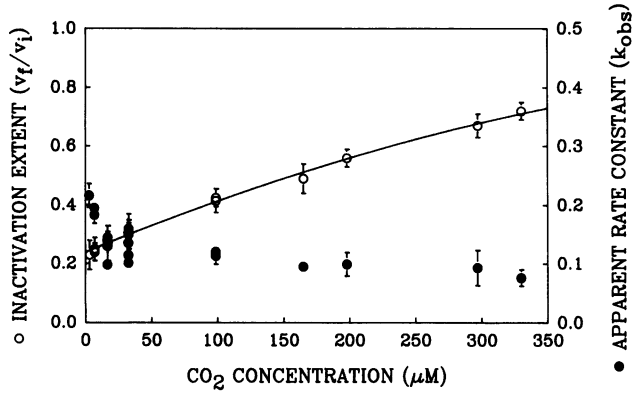

Figure 5. Effect of varying CO_2 concentration on slow inactivation. Assays for slow inactivation were carried out, and the kinetic parameters estimated, as described in the legend to Figure 2, at a range of CO_2 concentrations. The effect of CO_2 concentration on the extent (measured as v_f/v_i, O——O) and on the rate (k_{obs}, ●) of slow inactivation are shown. The bars show the standard errors.

time, because the latter became inaccurate when the half time was very long. These rates will be a little higher than the true equilibrium v_f. Nevertheless, v_{60}/v_i was 2-fold greater when 2 mM H_2O_2 was present. Fallover was similarly relieved by 6-phosphogluconate. With 2 mM 6-phosphogluconate (70 μM CO_2, 0.2 mM ribulose-P_2, pH 8.3), a 2.5-fold increase in half-time and a 2-fold greater v_{60}/v_i ratio were observed. H_2O_2 or 6-phosphogluconate were effective only at concentrations sufficient to cause substantial inhibition of the initial rate. For example, the 6-phosphogluconate concentration required to double v_{60}/v_i caused 80% inhibition of v_i. H_2O_2 was less inhibitory as far as the initial rate was concerned, achieving a doubling of v_{60}/v_i at a concentration which inhibited v_i by only 40%.

Catalytic Properties of Rubisco After Fallover

Kinetic characteristics of Rubisco which had undergone fallover were compared with those of the enzyme with no prior exposure to ribulose-P_2. Neither the K_m(ribulose-P_2) nor the $K_m(CO_2)$ was significantly affected, the effect being restricted to V_{max} in each case (Table II).

DISCUSSION

Kinetics

Fallover is characterized by an apparently first-order decline in Rubisco's activity, without alteration in its substrate affinities (Table II), which occurs on exposure to ribulose-P_2 with a half-time of approximately 7 min (Fig. 2). It does not continue to complete inhibition but eventually stabilizes at a level of activity which is dependent on CO_2 concentration (Fig. 5), pH (Fig. 6), and the presence of inhibitors (Table I). Explanations of the phenomenon must take all of these salient features into account.

There have been reports of inhibition with similar characteristics, but more severe in the sense that it occurred faster and proceeded to a greater extent (*e.g.* 17, 26). We attribute this more severe inhibition to the super-imposition of other inhibitory processes, such as the tendency for Rubisco to be inactivated at low concentration by adhesion to surfaces (Fig. 1) and the use of commercial ribulose-P_2 preparations which may be contaminated with epimerization and degradation products (19) on the underlying fallover process. Suppression of these other kinds of inhibition reveals a fallover phenomenon which is particularly reproducible under defined conditions in its rate (k_{obs}) and extent (v_f/v_i) (Table I).

We confirm previous observations (2) that fallover is not a reflection of substrate exhaustion or product accumulation (Table IA). Regardless of the [ribulose-P_2]/[Rubisco] ratio, the inhibition depended only on the time of exposure to ribulose-P_2 (*i.e.* the half-time of fallover was unaffected (Table IB)). Indeed, it appears that the extent of fallover depends only on the number of catalytic turnovers which a particular active site has experienced. The reduction in severity of fallover with increase in pH (Fig. 6) also confirms previous observations (17).

The suppression of the extent of fallover by high CO_2 concentration (Fig. 5) has not been previously reported. It is important to note that the suppressive effect of CO_2 concentration does not parallel the affinity for CO_2 in the carboxylation reaction but continues far beyond concentrations required to saturate catalysis. This is another salient feature which must be accommodated by any explanation of the fallover phenomenon.

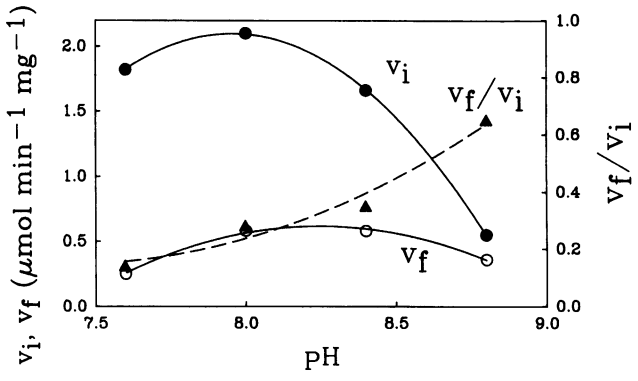

Figure 6. Effect of pH on slow inactivation. The initial rate (v_i, ●——●), the final rate (v_f, O——O), and the extent (v_f/v_i, ▲——▲) of slow inactivation are shown. Data were obtained using the spectrophotometric assay as described in the legend to Figure 4, at varying pH. Hepes buffer was used instead of Bicine when the pH was below 8. The $NaHCO_3$ concentration was varied so that the CO_2 concentration remained constant at 100 μM regardless of the pH. (A pK′ value of 6.12 for the hydration of CO_2 was assumed.) The Rubisco concentration was 0.5 μg mL^{-1}. After 10 min preincubation, catalysis was initiated by addition of 1 mM ribulose-P_2. Because this assay method has an inherent lag as intermediate substrate pools within the coupling system build up, v_i was determined after 2.5 min and thus is consistently slightly lower than that measured with the $^{14}CO_2$ method. The ratio v_f/v_i is, therefore, correspondingly higher. No significant variation in the rate of inactivation (k_{obs}) was observed across this range of pH.

Table II. *Kinetic Characteristics of Rubisco After Fallover*

Fallover was allowed to proceed in a solution containing 100 mM Bicine-NaOH (pH 8.3), 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 5 mM ribulose-P_2, 75 $\mu g \cdot ml^{-1}$ of preactivated Rubisco, 0.1% (w/v) bovine albumin and 0.1 $mg \cdot mL^{-1}$ carbonic anhydrase. The solution was left in a tightly-stoppered container for 12 h to ensure that all of the ribulose-P_2 was consumed before 25 μL aliquots were added to initiate $K_m(CO_2)$ or K_m(ribulose-P_2) assays containing all other components. The control was treated in a similar manner but with 10 mM P-glycerate substituted for ribulose-P_2. $K_m(CO_2)$ was determined using a CO_2 concentration range of 1.6-100 μM (100 $Bq \cdot nmol^{-1}$) and a constant ribulose-P_2 concentration of 0.5 mM. All solutions, except the Rubisco, were made CO_2-free by bubbling with N_2 overnight, and pH adjustment was made with CO_2-free NaOH. K_m(ribulose-P_2) was determined using a range of ribulose-P_2 concentrations of 5 to 160 μM and a constant $NaHCO_3$ concentration of 10 mM. In addition, all assay solutions contained 100 mM Bicine-NaOH (pH 8.3), 20 mM $MgCl_2$, 0.1% (w/v) bovine albumin and 0.1 $mg \cdot mL^{-1}$ bovine carbonic anhydrase in a total volume of 3 mL. Catalysis was terminated by acidification after 1 min at 25°C and fixed ^{14}C measured by scintillation counting. Never more than 15% of the CO_2 or ribulose-P_2 initially present was consumed during the reaction. In calculating the concentration and specific radioactivity of CO_2, allowance was made for the CO_2 carried over with Rubisco. K_m and V_{max} were determined (±SE) by fitting the data to the Michaelis-Menten equation.

	After Fallover	Control
CO_2 as variable substrate		
K_m (CO_2), μM	11.9 ± 0.5	10.1 ± 0.4
V_{max}, $\mu mol \cdot min^{-1} \cdot mg^{-1}$	1.57 ± 0.02	2.92 ± 0.04
Ribulose-P_2 as variable substrate		
K_m (ribulose-P_2), μM	24.4 ± 0.4	20.1 ± 0.8
V_{max}, $\mu mol \cdot min^{-1} \cdot mg^{-1}$	1.23 ± 0.01	2.01 ± 0.03

Inhibitory Contaminants in Ribulose-P_2 Preparation

It could be argued that our efforts to eliminate contaminants from the ribulose-P_2 preparations used in this study have been only partially successful and that fallover would not occur with totally pure ribulose-P_2. We have addressed this possibility in two ways. First, we have shown that the level of one of the most likely contaminants, xylulose-P_2, was below the level of detection (see "Materials and Methods") and further, that inclusion of an enzymatic scavenging system for removal of xylulose-P_2 did not alter the rate or extent of fallover (Table I). Second, fallover was still observed with ribulose-P_2 that was freshly generated immediately before use (Table I). Furthermore, since prolonged exposure of ribulose-P_2 to the assay conditions prior to addition of Rubisco did not worsen the inhibition, it is unlikely that slow nonenzymatic degradation of ribulose-P_2 during the course of the assay was responsible for producing an inhibitor. Therefore, if fallover is caused by a contaminant, it is not likely to be xylulose-P_2 and it must be either present in or produced by the enzymatic system used to generate ribulose-P_2 from ribose-P. Of course the possibility that the inhibitor is produced by Rubisco itself still remains.

Reversibility

Fifty-fold dilution did not reverse the inhibition at all (Fig. 3). Therefore, it must not be caused by the simple, freely reversible binding of an inhibitor. Nor did the inhibition reverse after complete exhaustion of ribulose-P_2 (Fig. 4), which might be expected if it was caused by readily reversible binding of ribulose-P_2 at a noncatalytic regulatory site as has been suggested by Yokota and Kitaoka (26). While this conclusion differs from that of Yokota and Kitaoka, our data are consistent with those of Sicher *et al.* (24) for purified Rubisco, and this matter of reversibility is addressed further in the third paper of this series (7).

Effects of Inhibitors

Fallover is alleviated by the presence of compounds which inhibit catalysis, such as 6-phosphogluconate and H_2O_2 (Table I) (4, 5). This may be a result of competition between a tight-binding inhibitor responsible for fallover and other, more freely reversible, inhibitors which bind at the catalytic site. The situation with H_2O_2, which competes with the gaseous substrate rather than ribulose-P_2 (4), is complicated by the possibility of reaction of H_2O_2 with the carbonyl group of ribulose-P_2, either nonenzymatically or at the catalytic site, to produce 2′-peroxypentitol-1,5-bisphosphate which would be expected to be a strong competitive inhibitor with respect to ribulose-P_2 (3). It is possible that the alleviating effect of high CO_2 concentrations (Fig. 5) is also explicable in terms of competition by HCO_3^- ions, which are known to bind at the catalytic site (16, 21). If HCO_3^- ions alleviate fallover, then the reduced extent of fallover at high pH (Fig. 6) may also be partially or completely explained in these terms because the experiment described in Figure 6 was conducted at a constant CO_2 concentration and, therefore, the HCO_3^- concentration increased with pH. Other possible explanations for the effects of CO_2 concentration and pH on fallover will be discussed in a later paper in this series (7).

Possible Causes of Fallover

These studies consolidate and extend observations of the fallover phenomenon and they eliminate from consideration some of the proposals about its cause (see introduction). Since the inhibition was not relieved upon complete exhaustion of ribulose-P_2, it cannot be due to readily-reversible binding of ribulose-P_2 at a noncatalytic, regulatory site. Furthermore, fallover seems unlikely to be caused by inhibition by products of nonenzymatic isomerization or decay of ribulose-P_2. Other possibilities are addressed in the subsequent papers in this series.

ACKNOWLEDGMENTS

We thank S. von Caemmerer for helpful discussions and for reading the manuscript.

LITERATURE CITED

1. **Andrews TJ, Ballment B** (1984) Active site carbamate formation and reaction-intermediate-analog binding by ribulose bisphosphate carboxylase-oxygenase in the absence of its small subunits. Proc Natl Acad Sci USA **81**: 3660–3664
2. **Andrews TJ, Hatch MD** (1969) Properties and mechanism of action of pyruvate, phosphate dikinase from leaves. Biochem J **114**: 117–125

3. **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
4. **Badger MR, Andrews TJ, Canvin DT, Lormier GH** (1980) Interaction of hydrogen peroxide with ribulose bisphosphate carboxylase-oxygenase. J Biol Chem **255:** 7870–7875
5. **Bassham JA, Krohne S, Lendzian K** (1978) *In vivo* control mechanism of the carboxylation reaction. *In* HW Siegelman, G. Hind, eds, Photosynthetic Carbon Assimilation. Plenum Press, New York, pp 77–93.
6. **Butz ND, Sharkey TD** (1989) Activity ratios of ribulose-1,5-bisphosphate carboxylase accurately reflect carbamylation ratios. Plant Physiol **89:** 735–739
7. **Edmondson DL, Badger MR, Andrews TJ** (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
8. **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
9. **Hall NP, Tolbert NE** (1978) A rapid procedure for the isolation of ribulose bisphosphate carboxylase/oxygenase from spinach leaves. FEBS Lett **96:** 167–169
10. **Horecker BL, Hurwitz J, Weissbach A** (1958) Ribulose diphosphate. Biochem Prep **6:** 83–90
11. **Jordan DB, Chollet R** (1983) Inhibition of ribulose bisphosphate carboxylase by substrate ribulose 1,5-bisphosphate. J Biol Chem **258:** 13752–13758
12. **Laing WA, Christeller JT** (1976) A model for the kinetics of activation and catalysis of ribulose 1,5-bisphosphate carboxylase. Biochem J **159:** 563–570
13. **Lilley RM, Walker DA** (1974) An improved spectrophotometric assay for ribulosebisphosphate carboxylase. Biochem Biophys Acta **358:** 226–229
14. **Lorimer GH, Badger MR, Andrews TJ** (1976) The activation of ribulose-1,5-bisphosphate carboxylase by carbon dioxide and magnesium ions. Equilibria, kinetics, a suggested mechanism and physiological implications. Biochemistry **15:** 529–536
15. **McCurry SD, Pierce J, Tolbert NE, Orme-Johnson WH** (1981) On the mechanism of effector-mediated activation of ribulose bisphosphate carboxylase/oxygenase. J Biol Chem **256:** 6623–6628
16. **Miziorko HM, Mildvan AS** (1974) Electron paramagnetic resonance, ^{1}H, and ^{13}C nuclear magnetic resonance studies of the interaction of manganese and bicarbonate with ribulose 1,5-bisphosphate carboxylase. J Biol Chem **249:** 2743–2750
17. **Mott KA, Berry JA** (1986) Effects of pH on activity and activation of ribulose 1,5-bisphosphate carboxylase at air level CO_2. Plant Physiol **82:** 77–82
18. **Mächler F, Nösberger J** (1980) Regulation of ribulose bisphosphate carboxylase activity in intact wheat leaves by light, CO_2, and temperature. J Exp Bot **31:** 1485–1491
19. **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
20. **Paulsen JM, Lane MD** (1966) Spinach ribulose diphosphate carboxylase. I. Purification and properties of the enzyme. Biochemistry **5:** 2350–2357
21. **Pierce J, Lorimer GH. Reddy GS** (1986) Kinetic mechanism of ribulosebisphosphate carboxylase: evidence for an ordered, sequential reaction. Biochemistry **25:** 1636–1644
22. **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
23. **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 CO_2 in *Phaseolus vulgaris* L. Planta **174:** 407–416
24. **Sicher RC, Hatch AL, Stumpf DK, Jensen RG** (1981) Ribulose 1,5-bisphosphate and activation of the carboxylase in the chloroplast. Plant Physiol **68:** 252–255
25. **Sue JM, Knowles JR** (1982) Ribulose-1,5-bisphosphate carboxylase: Primary deuterium kinetic isotope effect using [3-^{2}H] ribulose 1,5-bisphosphate. Biochemistry **21:** 5410–5414
26. **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