Inactivation of Spinach Ribulose-1,5-bisphosphate Carboxylase/Oxygenase by 1-Hydroxy-3-buten-2-one Phosphate†

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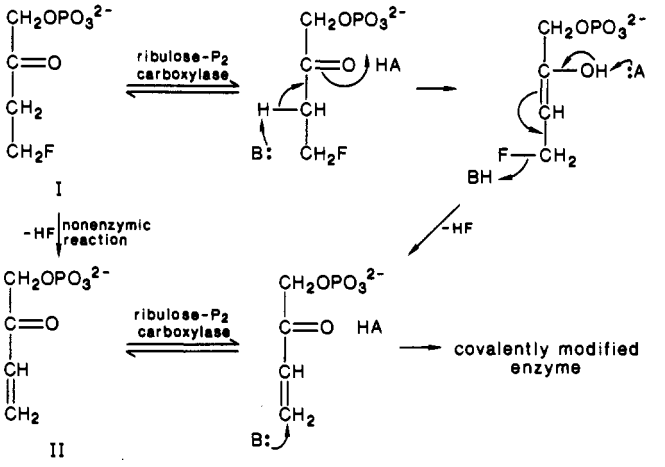
ABSTRACT: 1-Hydroxy-4-fluoro-2-butanone phosphate (I) was designed and synthesized as a potential suicide inhibitor of ribulose-1,5-bisphosphate carboxylase (ribulose-P_2 carboxylase). I is unstable at neutral pH and rapidly hydrolyzes to 1-hydroxy-3-buten-2-one phosphate (II) by elimination of HF (k_{obsd} = 2.51 × 10^{-2} min^{-1}, at pH 8.2). II reacts rapidly with sulfhydryl reagents. The rate of elimination of HF from I was not significantly affected by the presence of ribulose-P_2 carboxylase, when active sites were free or contained bound carboxypentitol bisphosphate. Both I and II appeared to be potent irreversible inhibitors of ribulose-P_2 carboxylase. Inactivation was pseudo first order and demonstrated rate saturation. The concentration of inhibitor yielding half the maximum rate of inactivation K_I was 1.48 ± 0.21 mM for I and 1.80 ± 0.34 mM for II. The rate constant observed for inactivation at infinite concentration k_2 was 0.97 ± 0.11 min^{-1} for I and 1.45 ± 0.20 min^{-1} for II. Fluoride did not contribute to the observed rates of inactivation by I and II. The data suggest that the observed inactivation by I arises from the spontaneous generation of II in solution, which consequently inactivates ribulose-P_2 carboxylase. There was no differential effect on the inactivation of carboxylase or oxygenase activity. 6-Phosphogluconate protected against inactivation, which with the demonstration of rate saturation suggests II combines with a residue(s) at the enzyme active site. II reacts with sulfhydryl groups on the enzyme as determined by quantitation of exposed sulfhydryl groups with 5,5′-dithiobis(2-nitrobenzoic acid). In the absence of II, 5.5 sulfhydryl groups per protomer were determined. Carboxypentitol bisphosphate protected about three of the exposed sulfhydryl groups modified by II. Thus, II reacts with sulfhydryl groups with evidence that these residues are in the active site region.

Ribulose-P_2 carboxylase[1] lies at a branch point in photosynthetic carbon metabolism and functions in both a catalytic and a regulatory capacity in the pathways of photosynthesis and photorespiration (Tolbert, 1983). In an attempt to understand how this bifunctional enzyme operates, it is important to identify functional groups at the active site that are responsible for catalytic and regulatory activity. By use of affinity labeling techniques, two lysine residues essential to catalysis have been located (Hartman et al., 1978). An additional lysine residue has been implicated in activation (Lorimer, 1981).

We have prepared two potential active site directed chemical probes and have examined their reactivity with ribulose-P_2 carboxylase. 4-Fluoro-1-hydroxy-2-butanone phosphate (I) was designed as a possible suicide inhibitor while 1-hydroxy-3-buten-2-one phosphate (II) was considered a potential affinity label. Their proposed interaction with the enzyme is outlined in Scheme I. Compound I contains a latently reactive β-fluoro ketone group that can be transferred by elimination of HF into a highly reactive α,β-unsaturated ketone. Since I is structurally analogous to the "top half" of the enzyme's natural substrate ribulose-P_2, it was thought that I might bind to the ribulose-P_2 binding site and be activated enzymatically

Scheme I: Proposed Interaction of I and II with Ribulose-P_2 Carboxylase



according to the mechanism proposed by Calvin (1954). This would involve removal of the C-3 proton by a basic amino acid side chain. The resulting highly reactive moiety might then form a covalent adduct with a nucleophilic residue at the active

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[1] Abbreviations: Bicine, *N,N*-bis(2-hydroxyethyl)glycine; CPBP, carboxypentitol bisphosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; 6-P-gluconate, 6-phosphogluconate; ribulose-P_2, ribulose 1,5-bisphosphate; ribulose-P_2 carboxylase, ribulose-1,5-bisphosphate carboxylase; TNB, 5-thio-2-nitrobenzoate; I, 1-hydroxy-4-fluoro-2-butanone phosphate monosodium salt; II, 1-hydroxy-3-buten-2-one phosphate monosodium salt.

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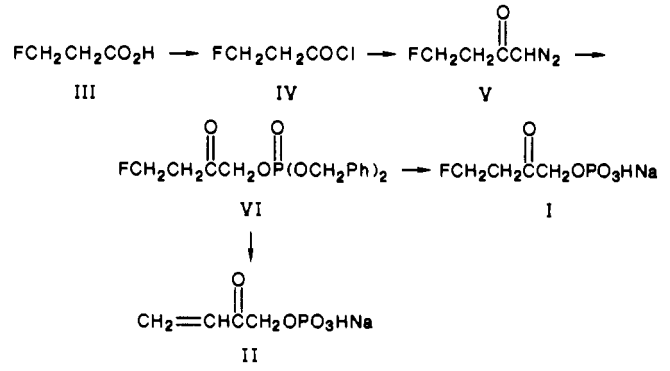
Scheme II



site. A competing reaction is the nonenzymatic elimination of HF to generate II, which may bind to the active site as an affinity label and lead to chemical modification of a reactive amino acid side chain. Either pathway could cause loss of enzyme activity if an essential residue were affected. The purpose of this study was to identify the mechanisms by which I and II interact with ribulose-P_2 carboxylase.

Experimental Procedures

Materials

Spinach ribulose-P_2 carboxylase/oxygenase was purified to homogeneity and stored at −80 °C (McCurry et al., 1981). The enzyme was treated with 50 mM dithiothreitol for several hours and then dialyzed against 25 mM Bicine–Na^+ (pH 8.2) prior to use. Ribulose-P_2 was prepared enzymatically from D-ribulose 5-phosphate and ATP (Horecker et al., 1958) and purified by chromatography on Dowex 1-Cl with a linear 0–0.5 M LiCl gradient prepared in 5 mM HCl. Carboxypentitol bisphosphate (an equimolar mixture of arabitol and ribitol isomers) was prepared according to Wishnick et al. (1970). 1-Hydroxy-4-fluoro-2-butanone phosphate monosodium salt (I) and 1-hydroxy-3-buten-2-one phosphate monosodium salt (II) were synthesized as described under Methods. $NaH^{14}CO_3$ was obtained from New England Nuclear, and other reagents were from Signa or Mallinckrodt in the highest grade available.

Methods

Synthesis of 4-Fluoro-1-hydroxy-2-butanone Phosphate (I) and 1-Hydroxy-3-buten-2-one Phosphate (II). See Scheme II for synthetic pathway.

(A) 3-Fluoropropionyl Chloride (IV). 3-Fluoropropionic acid (III) (Pattison et al., 1956) (8.74 g, 95 mmol) was added in small portions to oxalyl chloride (25 mL, 285 mmol) with stirring at room temperature. After 1.5 h, the reaction mixture was refluxed for 3 h and then stirred overnight at room temperature. Distillation at atmospheric pressure yielded 6.7 g (64%) of IV: bp 110–115 °C [lit. (Brocks et al., 1970) bp 34 °C at 25 mmHg; IR 1797 cm^{-1}.

(B) 4-Fluoro-1-diazo-2-butanone (V). 3-Fluoropropionyl chloride (IV) (6.7 g, 60.0 mmol) in 100 mL of ether was added dropwise with stirring to a cold, ether solution containing about 200 mmol of distilled CH_2N_2. After 1.5 h at 0 °C, ether and excess CH_2N_2 were removed with an aspirator. The residue was purified by bulb-to-bulb distillation at 0.5 mmHg and a bath temperature of 50 °C to give 6.0 g (86%) of a yellow liquid: NMR ($CDCl_3$) δ 5.43 (s, 1 H), 5.10 (t, 1 H), 4.33 (t, 1 H), 2.90 (t, 1 H), 2.48 (t, 1 H); IR 1640, 2116 cm^{-1}.

(C) 4-Fluoro-1-hydroxy-2-butanone Dibenzyl Phosphate (VI). A solution of V (4.84 g, 41.7 mmol) in 100 mL of ether was added to a stirred suspension of dibenzyl phosphate (12.18 g, 43.8 mmol) in 400 mL of ether at room temperature. A few drops of boron trifluoride etherate were added, and the reaction mixture was stirred overnight at ice-bath temperature. Solvent was removed on a Roto Vap to give 17 g of a red-orange oil. This material was divided into three portions, and each was chromatographed through a C18 silica gel column (96 g, 2.3 × 50 cm, 6:4 ethanol–water). Fractions containing a single spot at R_f 0.37 on TLC C18 silica gel, 6:4 ethanol–water) were combined. Solvent was removed under vacuum to give 7.89 g (52%) of a colorless liquid: NMR ($CDCl_3$) δ 7.26 (s, 10 H), 5.03 (d, 4 H), 4.93 (t, 1 H), 4.40 (d, 2 H), 4.19 (t, 1 H), 2.85 (t, 1 H), 2.45 (t, 1 H).

(D) 4-Fluoro-1-hydroxy-2-butanone Phosphate Monosodium Salt (I). A solution of VI (1.0 g, 2.73 mmol) in 40 mL of ethyl acetate was combined with 150 mg of 5% Pd/C and stirred under a N_2 blanket. The mixture was cooled in an ice–salt bath and H_2 bubbled through for 1 h, at which time no VI could be detected by TLC (R_f 0.48, silica gel, ethyl acetate). The reaction mixture was filtered cold through a 0.22-μm Teflon filter into a flask blanketed with N_2. The clear, colorless solution was stirred under N_2 and cooled to dry ice–acetone temperature. A standardized solution of sodium ethoxide in ethanol (1.90 mL of 1.43 M, 2.73 mmol) was added dropwise over a period of 20 min. The reaction mixture was allowed to warm to room temperature. Solvent was removed under vacuum to give 0.56 g (99%) of a white solid: NMR (D_2O) δ 5.10 (t, 1 H), 4.57 (d, 2 H), 4.35 (t, 1 H), 3.20 (t, 1 H), 2.74 (t, 1 H); ^{19}F NMR (D_2O) δ 221 ($^2J_{HF}$ = 49.8 Hz, $^3J_{HF}$ = 30.0 Hz). Anal. Calcd for $C_4H_7O_5FNaP$: C, 23.09; H, 3.39; Na, 11.05. Found: C, 22.67; H, 3.71; Na, 10.9.

(E) 1-Hydroxy-3-buten-2-one Phosphate Monosodium Salt (II). A solution of VI (0.5 g, 1.36 mmol) in 10 mL of ethyl acetate was combined with 75 mg of Pd/C and stirred under a N_2 blanket. The mixture was cooled in an ice–salt bath, and H_2 was bubbled through for 50 min, at which time no VI could be detected by TLC (R_f 0.48, silica gel, ethyl acetate). The reaction mixture was filtered cold through a 0.22-μm Teflon filter into a flask blanketed with N_2. Ethanol (10 mL) was added, and the mixture was stirred under N_2 at 0 °C. A standardized solution of sodium ethoxide in ethanol (1.90 mL of 1.43 M, 2.73 mmol) was slowly added. After 45 min, the reaction mixture was allowed to warm to room temperature. Solvent was removed under vacuum to give 240 mg (77%) of white solid; no attempt was made to remove the 1 equiv of NaF that is cogenerated: NMR (D_2O) δ 6.35 (m, 2 H), 5.90 (m, 1 H), 4.7 (d, 2 H). The NMR spectrum also contained peaks due to I at the level of about 15%. Anal. Calcd for $C_4H_6O_5PNa_2F$ (II + NaF): C, 20.89; H, 2.63. Found: C, 20.31; H, 2.75. UV (pH 7.0, 10 mM phosphate buffer) showed λ_{max} at 215 nm, ϵ = 6150. The UV spectrum of I in pH 7.0 10 mM phosphate buffer changes over a period of 2 h to a spectrum that is stable for many hours: λ_{max} 215, ϵ 7560. If this spectrum is assumed to be due solely to the generation of II (see NMR experiment), then the purity of the above preparation of II can be estimated to be 81%. These spectra may be compared to that reported for II generated in situ: λ_{max} 215 nm, ϵ 6500 (Motiu-DeGrood et al., 1979).

Ribulose-P_2 Carboxylase/Oxygenase Assays. Enzyme was activated with 10 mM $NaHCO_3$, 20 mM $MgCl_2$, 100 mM Bicine–Na^+ (pH 8.2), and 0.1 mM EDTA for 30 min at 30 °C prior to assay. Ribulose-P_2 carboxylase activity was determined by a radiometric assay (Pierce et al., 1981) consisting of 100 mM Bicine–Na^+ (pH 8.2), 10 mM $NaH^{14}CO_3$ (0.2 Ci/mol), 20 mM $MgCl_2$, 0.1 mM EDTA, and 0.5 mM ribu-

lose-P_2 in 0.5 mL. Assays were initiated by the addition of activated enzyme (40 μg of protein) and terminated with 200 μL of 2 M HCl. Reaction vials were dried at 80 °C, and CO_2 fixation was determined from acid-stable material remaining.

Ribulose-P_2 oxygenase activity was measured polarographically with an oxygen electrode (*14*). The electrode was calibrated with air-saturated water at 30 °C. Assays (1 mL) consisted of 100 mM Bicine–Na^+ (pH 8.2), 20 mM $MgCl_2$, 0.1 mM EDTA, and 0.5 mM ribulose-P_2 and were initiated by the addition of activated enzyme. Dithiothreitol was omitted from all reaction mixtures since sulfhydryl reducing agents reacted rapidly with compound II.

Solutions of Compounds I and II. Compound I was dissolved in distilled H_2O, maintained on ice, and prepared fresh daily. Compound II contained 1 equiv of NaF, generated during the production of the enone salt, and also contained 10% of compound I. Therefore, compound II was prepared in 25 mM Bicine–Na^+ (pH 8.2) and allowed to stand on ice for 1–2 h before use. This procedure caused elimination of HF from any residual I with the formation of II.

Determination of Sulfhydryl Groups. The number of exposed enzyme sulfhydryl groups was determined with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Ellman, 1959). A 10 times excess of DTNB (prepared in distilled H_2O and adjusted to pH 6.0 with Na_2CO_3) over enzyme sulfhydryl groups was added to the enzyme. The solutions were maintained at 30 °C, and the absorbance at 412 nm was monitored until the reaction had gone to completion. The number of sulfhydryl groups was calculated from the release of TNB with an extinction coefficient of $\epsilon = 14\,000\ M^{-1}\ cm^{-1}$ at A_{412nm}. Reagent blanks were prepared to correct for hydrolysis of DTNB at alkaline pH.

Results

NMR Studies on Reaction of I and II. A pD 7.5, 0.1 M phosphate buffer solution was prepared in D_2O from Na_2HPO_4 (preexchanged with D_2O) and DCl. A 0.07 M solution of I in this buffer [containing 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt as reference] was prepared, and NMR spectra were taken at various times. Within 30 min (probe temperature 40 °C), the spectrum showed that I had been converted completely to II. Over the next 18.5 h (at room temperature), no further change was observed by monitoring the vinyl protons of II relative to the internal standard. Ethanol (5 μL) was added, and a decrease of about 50% in the vinyl protons of II was observed over the next 49 h. Mercaptoethanol (5 μL) was added, and a spectrum, taken immediately, showed complete loss of vinyl protons. This experiment demonstrates that compound I is relatively unstable at neutral pH and is converted into compound II. Compound II, however, is relatively stable at neutral pH even in the presence of ethanol. Since 2-mercaptoethanol was observed to react rapidly with II, sulfhydryl reducing agents were found to be a convenient means of quenching reactions containing this compound.

pH–Rate Profile for Elimination of HF from I. The rate of elimination of HF from I was monitored by measuring the appearance of II at 215 nm on a Zeiss spectrophotometer thermostated at 25 °C (Figure 1). At pH values above 5.0, reactions were followed to completion, and pseudo-first-order rate constants (k_{obsd}) were calculated from semilog plots. At pH 4.0 and 5.0, calculated A_∞ values were used to compute k_{obsd}.

The following buffers were used to maintain pH: carbonate (pH 11.0 and 10.5), borate (pH 10.0 and 9.0), phosphate (pH 8.0, 7.0, and 6.0), and acetate (pH 5.0 and 4.0). Rates were measured at two different buffer concentrations, 10 and 2 mM

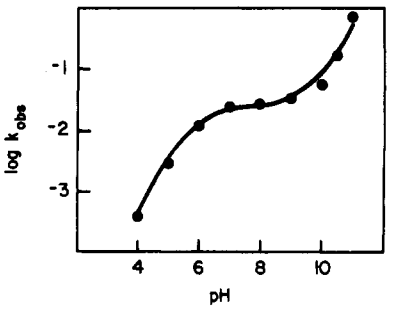


FIGURE 1: pH–rate profile for the conversion of I to II. Initial [I] = 2.42×10^{-4} M.

at each pH (Figure 1). Buffer catalysis of the rate of elimination of HF was not observed. The solid line in Figure 1 represents the best fit of the data to the expression in eq 1,

$$k_{obsd} = (k_1 + k_2K_w/[H^+])[K_a/(K_a + [H^+])] \quad (1)$$

where k_1 is a first-order rate constant for reaction of doubly ionized I, k_2 is a second-order rate constant for attack of hydroxide on double ionized I, K_w is the ionization product of water, and K_a is the second ionization constant for I. The rate law is given by eq 2; the pK_a was calculated to be 5.85.

$$v = (2.44 \times 10^{-2}\ min^{-1})[I^{2-}] + (4.91 \times 10^2\ M^{-1}\ min^{-1})[OH^-][I^{2-}] \quad (2)$$

Effect of Ribulose-P_2 Carboxylase on Rate of Elimination of HF from I. The inactivation of ribulose-P_2 carboxylase by the suicide mechanism proposed in Scheme I indicates that for each inactivation one HF is released. Such a mechanism would result in an enzyme-catalyzed elimination of HF in addition to the spontaneous elimination rate observed at a given pH in buffer. Since the proposed scheme involves an active site mechanism, the effect of enzyme on the rate of HF elimination was determined in the presence of ribulose-P_2 carboxylase with functional active sites and in the presence of enzyme where the active sites were blocked by bound CPBP.

The elimination of HF from I was measured with a fluoride ion electrode connected to a pH meter and chart recorder. The electrode was standardized by diluting a 0.1 M NaF commercial standard solution into 0.1 M Bicine–Na^+ (pH 8.2) containing 10 mM $NaHCO_3$, 20 mM $MgCl_2$, and 0.2 mM EDTA, to give NaF concentrations of 1 μM to 10 mM. A linear relationship between millivolts and log [F^-] was obtained for all but the lowest F^- concentration. All solutions were prepared in polypropylene labware.

The rate of HF elimination was determined in the presence and absence of ribulose-P_2 carboxylase (2.25 mg/mL; equivalent to an active site concentration of 33 μM based on eight catalytic sites per 550 000 molecular weight) under two sets of conditions: (a) concentration of I approximately equal to the enzyme active sites (35 μM) and (b) [I] equal to 5 mM, a large excess over the active site concentration. Under condition a, reactions were initiated by the addition of I (freshly prepared in 1 mM HCl) to 10 mL of 0.1 M Bicine–Na^+ (pH 8.2) containing 10 mM $NaHCO_2$, 20 mM $MgCl_2$, 0.2 mM EDTA, and 2.25 mg/mL ribulose-P_2 carboxylase (when present) to give a final concentration of 35 μM I. Under condition b, reactions were initiated by the addition of a weighed quantity of I (to give a final concentration of 5 mM) to solutions of (i) buffer alone, (ii) buffer and ribulose-P_2 carboxylase, and (iii) buffer and enzyme treated with CPBP (309 μM). The final pH of these reactions was 7.9.

Kinetic runs were followed to completion and pseudo-first-order rate constants (k_{obsd}) were calculated from semilog

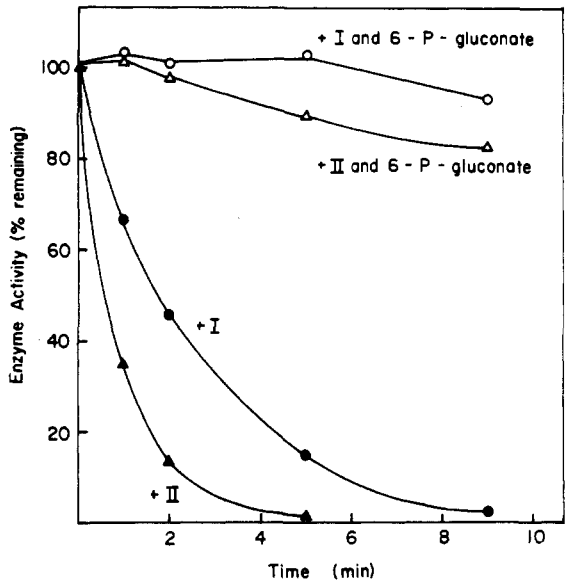


FIGURE 2: Time course of inhibition of ribulose-P_2 carboxylase activity by I and II in the presence and absence of 6-P-gluconate. Activated enzyme (2 mg/mL) was incubated with and without 5 mM 6-P-gluconate for 30 min at 30 °C and was then treated with 1 mM I or II. Samples were taken over the time course and assayed in the absence of either I or II. The initial specific activity was 1.0 μmol of CO_2 fixed min^{-1} (mg of protein)$^{-1}$.

plots. Under condition a, HF is released 8% faster in the presence of enzyme while under condition b a 5% increase is observed. These differences are considered too small to be significant. Enzyme activity was determined after the runs, with 67% of the initial activity remaining after treatment with 35 μM I and no activity remaining after treatment with 5 mM I.

Effect of Compounds I and II on Ribulose-P_2 Carboxylase Activity. Compounds I and II were tested on ribulose-P_2 carboxylase in the presence and absence of 5 mM 6-P-gluconate, a competitive inhibitor with respect to ribulose-P_2. The effect on activity over a time course is shown in Figure 2. In the absence of 6-P-gluconate, inactivation was rapid with a half-life of inactivation $t_{1/2}$(inact) of 1.8 min for I and 0.8 min for II. The rate of inactivation was much reduced in the presence of 6-P-gluconate, with $t_{1/2}$(inact) equal to 47 min for I and 30 min for II, suggesting inactivation occurred by I and II binding at the active site.

Effect of Fluoride on Ribulose-P_2 Carboxylase Activity. Since Fluoride ion is a contaminant in II and is also produced during hydrolysis of I, an experiment was conducted to measure its effect on enzyme activity. The effect of fluoride was examined under two conditions: (1) preincubation of enzyme with a range (0–5 mM) of NaF concentrations followed by dilution (1:25) of an aliquot into the standard assay and (2) effect of fluoride in the assay. The presence of up to 5 mM fluoride in the preincubation with activated enzyme had no effect on activity when diluted into the assay, but when similar concentrations were present in the assay, 60% inhibition was observed. Since the concentration of either I or II did not exceed 1 mM in this study and enzyme was assayed by dilution into the assay, it can be concluded that the level of fluoride encountered in these experiments was insufficient to contribute to the inhibition of enzyme activity observed with either I or II.

Examination of Reversibility of Inhibition by II. Ribulose-P_2 carboxylase inhibited with 1 mM II was treated by (a) dialysis or (b) precipitation with 50% saturated ammonium sulfate followed by dialysis, to remove excess inhibitor. The enzyme was then assayed for the recovery of activity. The results presented in Table I demonstrate that inhibition was not reversible by either of the above treatments. Incubation with an excess of sulfhydryl reagent (10 mM 2-mercaptoethanol) in addition to the above was also ineffective in displacing compound II. Incubation of the enzyme with 1 mM I also resulted in an irreversibly inhibited complex. However, interpretation of this result is difficult due to in situ generation of II from I during incubation.

Table I: Irreversibility of Inhibition by Compound II (1-Hydroxy-3-buten-2-one Phosphate)[a]

	sp act. [μmol of CO_2 fixed min^{-1} (mg of protein)$^{-1}$]			
	initial	dialysis (24 h)	dialysis + SH reagent	precipitation [50% $(NH_4)_2SO_4$]
control, activated enzyme	1.5	1.1	1.2	1.3
activated enzyme plus 1 mM II	0	0.02	0.06	0.01

[a] Activated ribulose-P_2 carboxylase (2 mg/mL) was incubated in the presence and absence of 1 mM II for 30 min at 30 °C. Enzyme activity was determined before and after treatment (column 1). Excess II was removed by (i) dialysis against 500 volumes of 25 mM Bicine–Na^+ (pH 8.2) containing 0.5 mM EDTA for 24 h, (ii) dialysis followed by incubation with a sulfhydryl reagent (10 mM 2-mercaptoethanol) for 8 h at 25 °C, or (iii) precipitation with 50% saturated $(NH_4)_2SO_4$, followed by washing and dialysis of the precipitated material. Enzyme activity and protein concentration were redetermined after each treatment.

Effect of Inhibitor Concentration on Rate of Inhibition of Ribulose-P_2 Carboxylase by I and II. Although I appeared to be inhibitory to ribulose-P_2 carboxylase activity, an important consideration was the fact that significant amounts of II were generated from I during the time course of an experiment. The rate constant (k_{obsd}) for the elimination of HF from I was shown to be 2.51×10^{-2} min^{-1} at pH 8.2 with a corresponding half-life of 28 min. Therefore, in order to test the effect of I on activity, it was necessary to determine inhibition kinetics over a rapid time course, minimizing conversion to II and thereby ensuring a large excess of I over II.

The effect of the concentration of I and II on the rate of inhibition of ribulose-P_2 carboxylase was determined under pseudo-first-order conditions (excess inhibitor). Pseudo-first-order rate constants for inhibition (k_{obsd}) were calculated from semilog lots. Inactivation rates appeared to be linear for each concentration of I, with no evidence for a lag or accelerated rate of inhibition over an extended time course, either of which could be associated with inhibition by II generated spontaneously in solution at pH 8.2. By use of the k_{obsd} value of 2.51×10^{-2} min^{-1} for the conversion of I to II at pH 8.2, it can be predicted that after 1 min, in the presence of enzyme (7.3 μM active sites) and 0.0625 mM I, 1.6 μM II has been formed and with 0.5 mM I 12.5 μM II has been formed. At 1 min, the concentration of I represents a 39 times excess over the concentration of II. After 12 min, a concentration of 0.0625 mM I generates 16.3 μM II, and 0.5 mM I generates 130 μM II. At 12 min, II is present in excess of the enzyme active site concentration, and only a 3 times excess of I over II exists. These considerations suggest that since II is a potent inhibitor of ribulose-P_2 carboxylase activity, the observed rate of inhibition in the presence of I could, in fact, be due to the spontaneous elimination of HF from I, forming sufficient II to demonstrate pseudo-first-order inactivation kinetics.

The reaction of an irreversible inhibitor with an enzyme may proceed initially by a collision between enzyme and inhibitor

Table II: Rate Constants for Inhibition of Ribulose-P_2 Carboxylase and Oxygenase Activities by 1 mM I and 1 mM II

	k_{obsd} (s^{-1})	$t_{1/2}$ (s)
compound I (1 mM)		
carboxylase activity	3.1×10^{-3}	224
oxygenase activity	3.0×10^{-3}	231
compound II (1 mM)		
carboxylase activity	5.2×10^{-3}	133
oxygenase activity	4.8×10^{-3}	144

to form a noncovalent complex (E.I), which then reacts to form a covalent bond (Dixon & Webb, 1979), eq 3.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_2} E{-}I \quad (3)$$

If it is assumed that the rate of conversion of E·I to E–I is very small in comparison to its breakdown ($k_2 \ll k_{-1}$), then the first part of eq 3 remains in equilibrium such that

$$k_{obsd} = k_2/(1 + K_I/[I]) \quad (4)$$

where k_2 is the rate constant observed for inactivation at infinite inhibitor concentration and K_I is the concentration of inhibitor yielding half the maximum rate of inhibition (Meloche, 1967). As noted above, K_I is equivalent to the dissociation constant of the E·I complex when k_2 is very small. The constants k_2 and K_I can be determined from a double-reciprocal plot of k_{obsd} against inhibitor concentration.

The inhibition data obtained for I and II when replotted in this manner demonstrated rate saturation, providing evidence that inhibition by I and II involves the initial formation of a noncovalent complex. The parameters k_2 and K_I were calculated for I and II: $k_2(I) = 0.97 \pm 0.11$ min^{-1}; $K_I(I) = 1.48 \pm 0.21$ mM; $k_2(II) = 1.45 \pm 0.20$ min^{-1}; $K_I(II) = 1.80 \pm 0.34$ mM. The parameters are of the same order for I and II, suggesting either that both compounds have similar inhibition kinetics or that solutions of I rapidly generate II such that the observed inhibition results from II in a pseudo-first-order manner. Since I and II are structurally similar to ribulose-P_2, it may be implied that they bind at the enzyme active site.

Effect of I and II on Ratio of Ribulose-P_2 Carboxylase to Oxygenase Activity. The possibility of a differential effect of I or II on carboxylase or oxygenase activity was examined by measuring the rate of loss of each activity (enzyme concentration 2 mg/mL) in the presence of 1 mM I or II. Samples were taken at timed intervals over a 3-min time course. The inhibition reaction was quenched in 2 mM DTT prior to assay of carboxylase and oxygenase activities. The observed rate of constants (k_{obsd}) calculated from semilog plots indicated that carboxylase and oxygenase activities were lost simultaneously in the presence of I or II (Table II). After the 3-min time course, the predicted concentration of II generated in solutions of I is 73 mM, a 2.5 times excess over the enzyme active site concentration.

Determination of Number of Exposed Sulfhydryl Groups on Ribulose-P_2 Carboxylase after Treatment with II. NMR studies demonstrated that sulfhydryl reacted rapidly with II, and therefore, it was proposed that II would react with sulfhydryl groups on the enzyme. Quantitation of the exposed sulfhydryl groups with DTNB demonstrated that fewer sulfhydryl groups were available to DTNB after treatment of the enzyme with II. The number of exposed sulfhydryl groups per protomer (L + S subunits) was determined to be 5.5 in the absence of II and 1.2 after treatment with II.

The rate of loss of exposed sulfhydryl groups was determined for enzyme incubated in the presence and absence of CPBP prior to treatment with 0.5 mM II (Figure 3). In the absence of CPBP, a decrease in the number of exposed sulfhydryl groups was observed with a similar loss of enzyme activity following the addition of II. In the presence of CPBP, the number of exposed sulfhydryl groups remained more or less unchanged from the number at zero time. Comparison of the zero-time values for the two conditions (±CPBP) indicated CPBP protected about three of the exposed sulfhydryl groups detectable by DTNB. The data suggest that II may selectively modify residues in a region protected by CPBP, that is, the active site, since when the active site is protected by CPBP the incorporation of II cannot be detected by the loss of exposed sulfhydryl groups.

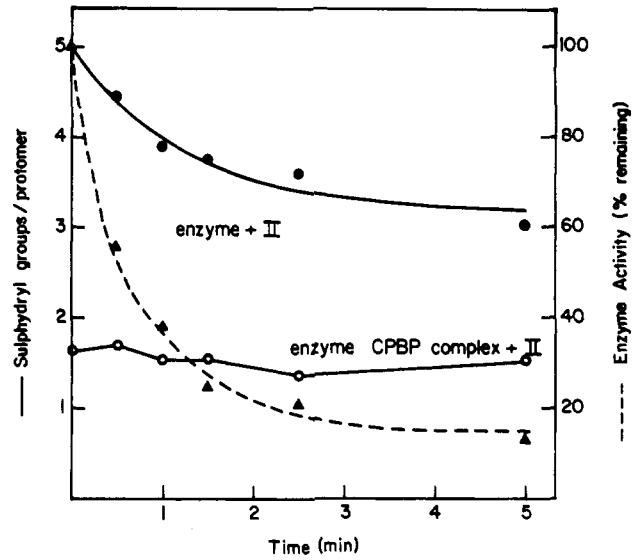


FIGURE 3: Time course following the loss of accessible sulfhydryl groups on ribulose-P_2 carboxylase [preincubated with (open circles) and without (closed circles) CPBP] following treatment with 0.5 mM II. Activated enzyme (0.5 mg/mL) was incubated with and without 0.3 mM CPBP for 1 h at 30 °C. The enzyme solutions were then made 0.5 mM with respect to II, and following the addition of II, samples (0.85 mL) were taken at the times indicated and quenched in 1 mM dithiothreitol. The sample was assayed for enzyme activity, and the remainder was precipitated to remove excess II. The precipitate was resuspended to the original volume in 0.1 M Bicine–Na^+ (pH 8.2) and 0.1 mM EDTA. The solutions were then treated with DTNB to quantitate exposed sulfhydryl groups.

Discussion

Inactivation of ribulose-P_2 carboxylase/oxygenase was observed in the presence of either of the two synthesized compounds 1-hydroxy-4-fluoro-2-butanone phosphate (I) or 1-hydroxy-3-buten-2-one phosphate (II). The reactivity studies showed that I is unstable at neutral pH and eliminates HF to form II. Under experimental conditions involving ribulose-P_2 carboxylase (pH 8.2), the rate constant for the elimination of HF from I was 2.51×10^{-2} min^{-1}, equivalent to a half-life of 28 min. Therefore, inherent in testing compound I on enzyme activity was the complication due to the generation of II in situ. The concentration of fluoride generated during elimination and that coproduced in the synthesis of II did not contribute to the observed rates of inactivation by I and II.

The observed rate of elimination of HF from I, as detected by a fluoride electrode, was not affected significantly by the presence of ribulose-P_2 carboxylase. The rate of HF elimination also remained unchanged when the enzyme active sites were blocked by bound CPBP. The production of inactive enzyme via a suicide mechanism (see Scheme I) requires that for each modified enzyme formed, one HF be released. This would cause an increased rate of fluoride formation, which would be observable under the condition where approximately

equimolar amounts (30–35 μM) of enzyme active sites and I are initially present. Since an increased rate of fluoride release was not observed, it is concluded that a suicide pathway does not operate. The fluoride electrode experiments suggest that the most probable explanation for the loss of ribulose-P_2 carboxylase activity is the reaction of enzyme with II, which is generated spontaneously from I.

If it is assumed that enzyme inactivation in a solution of I is due to the presence of II (generated by the spontaneous elimination of HF from I), it is perhaps surprising that deviations from linearity were not observed in the inactivation rates as the concentration of II increased. It is possible though that since inactivation was carried out under pseudo-first-order conditions, the concentration of I was sufficient to rapidly generate a significant amount of II. Consequently, pseudo-first-order inactivation of enzyme by II was observed. The similarity of the parameters k_2 and K_I for I and II reflects the fact that in both cases II was the inhibitor species.

Inactivation was not reversible by dialysis or protein precipitation to remove excess inhibitor, nor was there a differential effect on the loss of carboxylase or oxygenase activity. The presence of 6-P-gluconate protected against inactivation, suggesting the inhibitor was combining with a group at the enzyme active site. In addition, inactivation at a range of concentrations of II demonstrated rate saturation, providing evidence for the formation of a dissociable enzyme–inhibitor complex prior to inactivation. Since II is structurally similar to ribulose-P_2, the implication may be made that the inhibitor binds at the enzyme active site. In fact, the structure of 1-hydroxy-3-buten-2-one phosphate (II) is very similar to the affinity label 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate. This reagent alkylates two active-site lysyl residues that are essential for catalytic activity (Lys-175 and Lys-334), although only one of the two residues is modified per catalytic subunit (Stringer & Hartman, 1978). Modification of some nonessential sulfhydryls by 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate was also observed, reflecting the high reactivity of this reagent for sulfhydryl groups (Hartman et al., 1973).

Reactivity studies showed that II reacts rapidly with 2-mercaptoethanol, so it was proposed that II might react with sulfhydryl groups on the enzyme. Ribulose-P_2 carboxylase from spinach contains a total of 96 sulfhydryl groups, 12 per protomer (large and small subunit) (Sugiyama et al., 1968). Two cysteine residues have been identified in the active site region of spinach ribulose-P_2 carboxylase with the affinity label *N*-(bromoacetyl)ethanolamine phosphate when Mg^{2+} is omitted from the inactivation condition (Schloss et al., 1978). It appears that II reacts with sulfhydryl groups since the quantitation of exposed sulfhydryl groups on ribulose-P_2 carboxylase demonstrated fewer groups were available after treatment with II. The fact that little incorporation of II was observed outside of the active site when the active sites were blocked with bound CPBP suggest II may preferentially react with active site residues. It is known that II generated in situ reacts with a cysteine residue at the active site of aldolase (Motiu-DeGrood et al., 1979).

Although early reports postulated that cysteine residues were involved in the catalytic mechanism through a thiohemiketal intermediate (Rabin & Trown, 1964), this possibility has been ruled out since the oxygen atom on C-2 of ribulose-P_2 is retained during the enzyme reaction (Lorimer, 1978). Cysteine residues are present at the active site of spinach ribulose-P_2 as evidenced by affinity labeling (Schloss et al., 1978); however, they appear to play no essential role in structure or catalysis. This is further evidenced by the lack of homology between the position of cysteine residues in the primary structure of ribulose-P_2 carboxylase from higher plants (McIntosh et al., 1980) and *Rhodospirillum rubrum*, a purple, non-sulfur bacterium (Hartman et al. 1982). Therefore, it is likely that II reacts with cysteine residues at the active site that are not essential for activity. The loss of activity that is observed upon reaction of ribulose-P_2 carboxylase with II may be the result of an increase in steric bulk caused by the covalent attachment of II. Alternatively, another nucleophilic group, such as a lysine residue, which is essential for activity, may be modified in addition to cysteine groups.

Registry No. I, 102922-48-5; I (Na salt), 102922-49-6; II, 65116-94-1; II (Na salt), 102922-50-9; III, 461-56-3; IV, 503-62-8; V, 102922-51-0; VI, 102922-52-1; CPBP, 27442-42-8; $(PhCH_2O)_2PO_2H$, 1623-08-1; ribulose-P_2 carboxylase, 9027-23-0; ribulose-P_2 oxygenase, 39335-11-0.

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