The Relationship between Side Reactions and Slow Inhibition of Ribulose-bisphosphate Carboxylase Revealed by a Loop 6 Mutant of the Tobacco Enzyme*

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The first directed mutant of a higher plant ribulose-bisphosphate carboxylase/oxygenase (Rubisco), constructed by chloroplast transformation, is catalytically impaired but still able to support the plant's photosynthesis and growth (Whitney, S. M., von Caemmerer, S., Hudson, G. S., and Andrews, T. J. (1999) Plant Physiol. 121, 579–588). This mutant enzyme has a Leu to Val substitution at residue 335 in the flexible loop 6 of the large subunit, which closes over the substrate during catalysis. Its active site was intact, as judged by its barely impaired competency in the initial enolization step of the reaction sequence, and its ability to bind tightly the intermediate analog, 2-carboxy-D-arabinitol-1,5-bisphosphate. Prompted by observations that the mutant enzyme displayed much less slow inhibition during catalysis in vivo than the wild type, its tendency to catalyze side reactions and its response to the slow inhibitor d-xylosulose-1,5-bisphosphate were studied. The lessening in slow inhibition was not caused by reduced production of inhibitory side products. Except for pyruvate production, these reactions were strongly enhanced by the mutation, as was the ability to catalyze the carboxylation of d-xylosulose-1,5-bisphosphate. Rather, reduced inhibition was the result of lessened sensitivity to these inhibitors. The slow isomerization phase that characterizes inhibition of the wild-type enzyme by d-xylosulose-1,5-bisphosphate was completely eliminated by the mutation, and the mutant was more adept than the wild type in catalyzing the benzylidene-acid-type rearrangement of d-glycero-2,3-pentodiulose-P that produces or oxidation of the substrate, d-ribulose-1,5-bisphosphate. These observations are consistent with increased flexibility of loop 6 induced by the mutation, and they reveal the underlying mechanisms by which the side reactions cause slow inhibition.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) catalyzes the carboxylation and oxygenation of ribulose-P2, in photosynthetic CO2 fixation and photosynthesis, in vivo. Despite excellent crystal structures of various liganded and unliganded Rubiscos from various sources (6) and intensive mutagenic study of algal (3) and bacterial (7) Rubiscos, progress toward understanding the catalytic mechanism has been hampered by an inability to express the dominant higher plant form of the enzyme in heterologous hosts. This precluded application of the power of directed mutagenesis to the higher plant enzyme until Whitney et al. (8) constructed the first such mutant by chloroplast transformation in the natural host, tobacco. Because it was desirable to maintain photosynthetic viability, a mutation (a Leu to Val substitution at residue 335 of the plastid-encoded large subunit) was chosen with the aim of changing the kinetic parameters (particularly the CO2/O2 specificity (9)) as much as possible without seriously disabling catalytic performance at elevated CO2 concentration. The substitution successfully reduced the CO2/O2 specificity of the wild type by a factor of 10, and the Michaelis constants for CO2, O2, and ribulose-P2 2-fold, while retaining sufficient substrate-saturated activity to allow growth under CO2 enrichment. The changes were readily apparent in the photosynthetic characteristics of the leaves of the mutant plant (8).

Leu-335 is located near the apex of loop 6 of the Rubisco large subunit, which closes over the active site after ribulose-P2 binds (6, 10). The adjacent residue, Lys-334, is catalytically essential, and it makes contacts with the P1 phosphate and the C2 carboxylate formed by addition of CO2 (10), which stabilizes the transition state for carboxylation (11). The Leu-335 residue makes van der Waals contacts with the P2 phosphate (attached to C5 of the substrate) (10). Thus the effects of the Val-335 substitution were interpreted in terms of its alteration of the position and orientation of the critical eN of Lys-334 (8).

In addition to carboxylation and oxygenation, Rubisco also catalyzes other reactions leading from the intermediates of the two central catalytic pathways (Scheme 1) (2). The initial step of catalysis is the formation of an enediol (VI) from ribulose-P2 (V). This enediol is subject to misprotonation to form xylulose-P2 (I) and perhaps other pentulose bisphosphate isomers and β elimination to form deoxypentolidulose-P (II). The peroxo ketone intermediate of the oxygenation pathway (X) eliminates H2O2, producing pentolidulose-P (XI), which rearranges to carboxytertol-P2 (XII). The aci-acid form of P-glyceraldehyde (VIII), produced following C2/C3 cleavage of the carboxyketone (VII), β eliminates its phosphogroup form, forming pyruvate (III). Some of these side reactions are enhanced by certain mutations (12–17) or when the small subunits are missing (18). In one case, pyruvate production was suppressed by a mutation (12). By virtue of their mimicry of ribulose-P2 or its enediolized form, the bisphosphate by-products are slow binding, competitive inhibitors of Rubisco catalysis. They have been implicated in the slow decline in Rubisco activity during in vivo assay (the...
so-called “fallover” phenomenon) that is always observed naturally with the higher plant enzyme (19–26) and can be induced mutationally in the Rhodospirillum rubrum enzyme (27). Although pentodiolulose-P₂ (XI) and carboxytetritol-P₂ (XII) are so far only known to be produced by mutant Rubiscos (14, 15), the former is particularly significant, because it is present in all ribulose-P₂ preparations by virtue of spontaneous oxidation and will further accumulate by this process during the usual aerobic Rubisco assays (25). To mitigate fallover inhibition of Rubisco in vivo, higher plants have Rubisco activase, a motor protein powered by ATP hydrolysis that apparently manipulates the active site of Rubisco to remove bound bisphosphates (3, 28).

Here we show that the perturbation of loop 6 caused by the Val-335 substitution affects catalysis more pervasively than can be explained by a simple misorientation of the adjacent Lys-334. The alteration exacerbates the inhibitor-producing side reactions, but the changed structure and dynamics of the loop permit the inhibitors to escape from, or be rearranged by, the active site. As a result, fallover inhibition during catalysis is suppressed or eliminated, depending on conditions, revealing the underlying causes of the phenomenon and suggesting how it may have evolved.

**EXPERIMENTAL PROCEDURES**

**Materials—**Wild-type tobacco (Nicotiana tabacum L. cv Petit Havana [N:N]) was grown in a naturally illuminated glasshouse. The plastome mutant that produces Rubisco with a Leu to Val mutation at residue 335 of the large subunit (8) was grown in an artificially illuminated growth cabinet under an atmosphere containing 0.3% (v/v) CO₂. Wild-type and L335V Rubiscos were purified either by crystallization as described previously (29) or by polyethylene glycol precipitation and anion-exchange chromatography (Mono Q, Amersham Biosciences) using a procedure similar to that described for spinach Rubisco (15). Tobacco Rubisco concentrations were estimated from the absorbance at 280 nm, assuming that the protein concentration in mg ml⁻¹ is given by 0.7 (30). 1-¹H- and 3-²H-labeled and unlabeled ribulose-P₂ were synthesized as described (25, 31). Unlabeled and carbonyl-¹³C-labeled carboxy-

"Scheme 1. Side reactions catalyzed by Rubisco. The rates for the wild-type (Leu-335) tobacco Rubisco (where known) are shown as fractions of the substrate-saturated rate of carboxylation. The analogous partitionings for the Val-335 mutant Rubisco are in parenthesis. Reactions where the partitioning (relative to carboxylation) is enhanced in the mutant are indicated by block arrows; the one where it is reduced is shown with a dashed arrow."
NMR spectrometry by observing the rate at which the deuterated [3-3H]ribulose-P2 was exchanged with a protein from solution. Assays were carried out at 25 °C in buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 1 mM EDTA, 10% (v/v) 2H2O, and 2.9 mM [3-3H]ribulose-P2. Reactions were initiated by the addition of Rubisco (to 3 μm sites) that had been activated as described above. Spectra were collected at 2-min intervals using a Varian INOVA 600-MHz NMR spectrometer. The data were suppressed by using a W5 WATER suppression pulse (36). The increasing intensity of the resonance at 4.37 ppm due to the 3-H proton with time was fitted to an exponential equation, and the initial rate was corrected for the 10% 2H2O present in the solvent. No exchange occurred in controls lacking enzyme.

**Reaction Products**—Assays were carried out at 25 °C in buffer containing 100 mM EPFS-NaOH, pH 8.0, 20 mM MgCl2, 1 mM EDTA, and 1 μM ribulose-P2. Assays under oxygenating conditions used buffer that had been sparged with O2 and had a final concentration of NaHCO3 (carried over with the preactivated enzyme solution) of 10 μM. Assays were initiated with preactivated enzyme (described earlier) to a final concentration of 3.7 μM active sites and terminated at 10 min after complete consumption of the ribulose-P2. To measure the total reaction products, the reaction was terminated by adding SDS to a final concentration of 2% (w/v). Denatured protein was removed using a Millipore Ultrafree-MC filter unit (10,000 cutoff) and the filtrate was loaded onto a Amersham Biosciences Mono Q 5/5 column equilibrated with 10 mM EPFS/10 mM boric acid/NaOH, pH 8.0, 2% (w/v) SDS and chromatographic analysis of the pentuloses at 85 °C on a Bio-Rad HPX-87C column. To measure products that remained bound to the enzyme, the reactions were terminated by ultrafiltration (as described above) before denaturation. The retentate was washed with four 400-μl aliquots of buffer before elution of the bound products with a 10 mM EPFS/10 mM boric acid/NaOH, pH 8.0, 2% (w/v) SDS and chromatographic analysis as described above. Radioactivity in the wash fractions was measured by scintillation counting.

Products that remained bound after complete consumption of oxidized ribulose-P2 were analyzed using a similar procedure. [1-3H]Ribulose-P2 (50 μM) was oxidized for 3 h with 1 mM CuSO4 as described (25). Oxidized [1-3H]ribulose-P2 (8 μM) was reacted at room temperature for 10 min with 10 μM preactivated Rubisco active sites in 50 mM EPFS-NaOH buffer, pH 8.0, 15 mM MgCl2, 1 mM EDTA, 10 mM NaHCO3, before separation of Rubisco-bound radioactivity from unbound by gel filtration on a 1 × 20 cm-column of Sephadex G-50 fine (Amersham Biosciences) equilibrated with the same buffer solution. The protein peak was collected, and the radioactivity it contained was measured and released by adding SDS to 2% (v/v). Denatured protein was removed by ultrafiltration, and an aliquot of the filtrate was chromatographed as described above. In some further experiments, 5 mM H2O2 was included with four 400-μl aliquots of buffer before elution of the bound products, the reaction was terminated by adding SDS to a final concentration of 2% (w/v). Denatured protein was removed using a Millipore Ultrafree-MC filter unit (10,000 cutoff) and the filtrate was loaded onto a Amersham Biosciences Mono Q 5/5 column equilibrated with 10 mM EPFS/10 mM boric acid/NaOH, pH 8.0, 15 mM MgCl2, 1 mM EDTA, 10 mM NaHCO3 before chromatographic analysis of the pentuloses at 85 °C on a Bio-Rad HPX-87C column. To measure products that remained bound to the enzyme, the reactions were terminated by ultrafiltration (as described above) before denaturation. The retentate was washed with four 400-μl aliquots of buffer before elution of the bound products with a 10 mM EPFS/10 mM boric acid/NaOH, pH 8.0, 2% (w/v) SDS and chromatographic analysis as described above. Radioactivity in the wash fractions was measured by scintillation counting.

Inhibition by Xyulose-P2—Equilibrium inhibition by xylulose-P2 (competitive with respect to ribulose-P2) was measured as described (38) by adding preactivated enzyme (final concentration, 12 μM sites) to initiate spectrophotometric assays containing mixtures of ribulose-P2 (2.5–320 μM) and xylulose-P2 (0–40 μM). Initial activities for each substrate/inhibitor combination were measured, and the data for all combinations were fitted simultaneously to the hyperbolic equation for competitive inhibition to estimate the K_i (ribulose-P2) and K_i (xylulose-P2).

Slow inhibition of initially fully carbamylated Rubisco was monitored using a method based on that of Zhu and Jensen (23). Rubisco 0.05–1 μM active sites was fully carbamylated by incubation with 100 mM EPFS-NaOH buffer, pH 8.0, 20 mM MgCl2, 20 mM NaHCO3, 1 mM EDTA, and the other components required for the spectrophotometric assay (see above). Then 0.1–2.0 μM carboxyribulokinol-P2 was added and the mixture was incubated at 25 °C for various periods before assays were initiated by adding ribulose-P2 to 0.5 mM. The initial activities measured decayed exponenentially as a function of the duration of incubation with carboxyribulokinol-P2. The data were analyzed, and the various parameters were estimated as described below. Release of carboxyribulokinol-P2 was measured by observing the exchange of enzyme-bound [14C]carboxyribulokinol-P2 with unbound, unlabeled carboxyribulokinol-P2 using a gel-filtration procedure to separate bound and unbound label (32).

**Analysis of Slow Binding Inhibition**—Both xylulose-P2 and carboxyribulokinol-P2 are slow binding inhibitors of Rubisco. This has been shown previously as a two-step process (22). An initial rapid equilibrium interaction with the active site that competes with the substrate, ribulose-P2, followed by a slow isomerization of the initial complex to a much tighter complex that is likely to be associated with closure of flexible loops of the protein over the ligand within the active site (39).
The Val-335 Mutation Reduces Inactivation during Catalysis—As is always observed with higher plant Rubiscos (19, 24, 25, 43), the activity of the wild-type Leu-335 Rubisco from tobacco declined progressively after addition of ribulose-P₂. However, this fallover was much less marked with the Val-335 mutant enzyme (Fig. 1 and Table II). Under aerobic conditions (Fig. 1A), the mutant enzyme lost activity very slowly ($k_{ob}$ one-seventh of that of the wild type), losing only 14% of its initial activity in 500 s, compared with a 43% loss for the wild type. Moreover, the decline of the mutant appeared to be qualitatively different from that of the wild type. Consistent with previous reports (19, 25), the data for the wild type, when fitted to Equation 1, estimate a final steady-state rate ($v_f$) of 32% of the initial rate ($v_i$). Similar fitting of the mutant’s data project to a $v_i$ near zero, and this projection was confirmed in longer assays (up to 8000 s, not shown).

Under anaerobic conditions (Fig. 1B), shown previously to partially suppress fallover by limiting oxidation of ribulose-P₂ (25), the decline in activity of the wild-type enzyme was considerably reduced in extent ($v_f$ was 74% of $v_i$) but occurred at a similar rate (Table II). By contrast, exclusion of O₂ abolished fallover by the Val-335 mutant completely.

**Table I: Kinetic parameters (± S.D.) for inhibition by xylulose-P₂ and carboxyarabinitol-P₂**

| Products          | Xylulose-P₂ | Carboxyarabinitol-P₂ |
|-------------------|------------|----------------------|
| Leu-335           | Val-335    | Leu-335              | Val-335         |
| Rapid equilibrium inhibition $K_i$ (μM) | 4.8 ± 0.4 | 20.7 ± 4.6 | ND$^a$ | ND |
| Slow binding inhibition $K_i$ (μM) | 1.9 ± 0.5 | NA$^b$ | 2.3 ± 0.7 | 0.65 ± 0.14 |
| $k_3$ (s$^{-1}$) | 1.5 ± 0.2 × 10$^{-3}$ | NA | 0.17 ± 0.04 | 0.092 ± 0.010 |
| $k_4$ (s$^{-1}$) | 5.0 ± 0.1 × 10$^{-4}$ | NA | <10$^{-7}$ | <10$^{-7}$ |

$^a$ ND, not determined.

$^b$ NA, not applicable, no slow inhibition detected.

No detectable exchange of labeled carboxypentitol-P₂ in 9 days.

2 and, by iteration, to Equation 1. These relationships are analogous to those derived by Morrison (40) for slow isomerization binding.

Rubisco was incubated for various periods with various concentrations of carboxyarabinitol-P₂ or xylulose-P₂, and its activity was measured immediately after mixing with ribulose-P₂, as described above. To estimate $K_i$, $k_3$, and $k_4$, these data were fitted simultaneously to Equations 4 and 5, using multiple curve-fitting software (OriginLab, Northampton, MA).

Carbamylation of Lys-201 in the active site of Rubisco and subsequent binding of a divalent metal ion are prerequisites for catalysis (41). This introduces a complication into the model embodied in Equation 3, because some slow binding inhibitors, such as carboxyarabinitol-P₂ (32), require the carbamylated, metal-complexed active site for tight binding whereas others such as xylulose-P₂ (23) bind more tightly to the uncarbamylated, metal-free site. This may cause $k_3$ or $k_4$, and thus $K_i$, to depend on the concentrations of CO₂ and divalent metal, but, provided that these concentrations are kept constant and the binding and release of CO₂ and divalent metal are rapid compared with inhibitor release, the above relationships remain applicable.

The side-reaction product profile of the wild-type Leu-335 enzyme produced mostly P-glycerate, along with a trace of carboxytritol-P₂ (0.3%), some of which remained bound (Fig. 2, C and D). When the assay conditions...
promoted oxygenase activity, Leu-335 produced mostly P-glycolate and P-glycerate (76 and 23%, respectively). Because the \(^3\)H label was attached to C1 of the ribulose-P2, the P-glycerate molecule produced by oxygenation was not labeled. Again, these products were fully released. Some labeled xylulose-P2 was also produced (0.3%), most of it remaining bound to the wild-type enzyme (Fig. 3, A and B). The resistance of this trace of bound xylulose-P2 to consumption by carboxylation implies that it must be bound to inactive, uncatalyzed enzyme likely to be abundant in these assays at low CO2 concentration. The increased oxygenation, relative to carboxylation, catalyzed by Val-335 Rubisco (8) was reflected in the ratio of \[^3\text{H}\]P-glycolate (91%) to \[^3\text{H}\]P-glycerate (7%) produced and released. The mutant enzyme also produced several side products: pentodiulose-P2 (0.4%), xylulose-P2 (0.4%), and carboxytetritol-P2 (0.8%), the latter remaining substantially bound to the enzyme (Fig. 3, C and D). With the mutant, the xylulose-P2 produced did not remain bound.

Other experiments (not shown) revealed that pentodiulose-P2 was not fully retained on the enzyme during the long period (~2 h) required for the centrifugal ultrafiltration and washing procedure used to separate bound products in these experiments (Figs. 2 and 3). This caused variable retention of labeled pentodiulose-P2. Either this species is slowly released itself (consistent with the reported \(K_d\) of 0.13 \(\mu\)M (25)) or it might be slowly converted to monophosphate products on the active site before release. Therefore, the amounts of bound pentodiulose-P2 detected in those experiments must be considered to be minimum estimates. Carboxytetritol-P2 appeared to be better retained.

The rate of production of pyruvate, a by-product of the carboxylation reaction resulting from \(\beta\)-elimination of the aci-carbanion intermediate (Scheme 1) was measured spectrophotometrically under carboxylating conditions. In agreement with previous observations with other Rubiscos (37), the Leu-335 wild-type produced pyruvate at 0.69% of the rate at which it produced pairs of P-glycerate molecules. However, the analogous ratio for the Val-335 mutant was only one-fifth as great (0.13%).

**Binding and Processing of Ribulose-P2 Oxidation Products**—As reported previously (25), two of the side-reaction products seen with the Leu-335 mutant enzyme, pentodiulose-P2 and its product of benzylic acid-type rearrangement, carboxytetritol-P2 (Scheme 1), can be produced non-enzymatically by oxidation of ribulose-P2 in the presence of \(\text{Cu}^{2+}\) ions. Chromatographic resolution of the oxidation products (Fig. 4A) revealed the presence of both of these compounds, together with a variety of monophosphorylated and non-phosphorylated compounds arising from further degradation of the unstable pentodiulose-P2 (25). This mixture of oxidation/degradation products, including ~40% residual unoxidized ribulose-P2, was reacted with Leu-335 and Val-335 Rubiscos to completion under carboxylating conditions. Unbound compounds were then removed by gel filtration, as quickly as possible (within 20 min) to maximize retention of bound pentodiulose-P2. The unbound compounds were almost entirely monophosphate species (data not shown). With both Rubiscos, the fraction of radioactivity that remained bound correlated approximately with the total pentodiulose-P2 plus carboxytetritol-P2 in the oxidized mixture (Table III). Further information about the identity of the bound species was sought by adding \(\text{H}_2\text{O}_2\), which is known to cleave Rubisco-bound pentodiulose-P2 and release the cleaved products, P-glycolate and P-glycerate (25). With both wild-type and mutant enzymes, adding \(\text{H}_2\text{O}_2\) simultaneously with the oxidized ribulose-P2 preparation eliminated over 75% of the bound radioactivity. When the \(\text{H}_2\text{O}_2\) was added to the reaction mixture 5 min after the oxidized ribulose-P2 preparation, when conversion to products had been completed, the result was similar with the wild-type Rubisco but different with the Val-335 mutant, which retained 80% of the radioactivity bound in the absence of \(\text{H}_2\text{O}_2\) (Table III). Apparently, at least two compounds derived from oxidized ribulose-P2 must remain bound to Rubisco in these experiments. One is released from the enzyme by \(\text{H}_2\text{O}_2\), and the other is not, and the former is substantially converted to the latter by the mutant enzyme, but not by the wild type. This conclusion was confirmed and the two compounds were identified as pentodiulose-P2 and carboxytetritol-P2 by anion-exchange chromatography of the radioactivity bound in the absence of \(\text{H}_2\text{O}_2\) and released by treatment with SDS. Whereas the wild-type enzyme bound and retained pentodiulose-P2 and carboxytetritol-P2 approximately in the same proportions as they existed in the oxidized ribulose-P2 preparation (Fig. 4B), the proportions were reversed with the mutant, which had catalyzed the benzylic acid-type rearrangement of most of the pentodiulose-P2 present in the oxidized ribulose-P2 to carboxytetritol-P2 (Fig. 4C). Obviously, the mutation must alter the structure of the active site in a way that facilitates this rearrangement.
The Val-335 Mutation Promotes Side Reactions Originating from the Enediol Intermediate—Although the rates of “misfire” reactions originating from the enediol intermediate (Scheme 1 (12, 13, 18, 22, 23)) are small compared with the rate of CO2 addition to this intermediate, they can be measured in the absence of CO2 and O2 where consumption of ribulose-P2 by carboxylation and oxygenation is prevented and the fraction of the enzyme with enediol bound is maximized. Although CO2 is required to activate Rubisco by carbamylation, this requirement can be fulfilled by preincubation in a HCO3-/H2CO2-containing solution. When a small aliquot of preactivated enzyme is subsequently added to the assay mixture, the final HCO3- is small and rapidly exhausted by carboxylation. Decarbamylation of higher plant Rubisco in CO2-free medium occurs very slowly while ribulose-P2 persists (20). This device readily allows spectrophotometric measurement (see “Experimental Procedures”) of both β elimination of the enediol to produce deoxypentulose-P2 and misprotonation to produce xylulose-P2. Both assays showed an initial lag (presumably caused by the coupling systems and consumption of the last traces of CO2/HCO3-) followed by a linear phase in which the rate of product formation is first order in enzyme concentration (see “Experimental Procedures,” Table II). The reaction was stopped by addition of 1% (v/v) acetic acid. The products were chromatographed on an RP-HPLC column (ZorbAX ODS) with a sodium acetate gradient. The numbers in parentheses show the radioactivity recovered in each compound as a percentage of the total radioactivity eluting after 10 min in the total-product chromatogram.

![Table II](image)

**Table II**

| Rubisco form | \( v_i \) | \( v_i/v_{i0} \) | \( k_{obs} \) |
|-------------|----------|----------------|--------|
| Ribulose-P2, aerobic (Fig. 1A) | | | |
| Leu-335     | 2.82 ± 0.01 | 0.32 | 1.93 ± 0.05 \( \times 10^{-3} \) |
| Val-335     | 0.477 ± 0.004 | -0.08 | 0.28 ± 0.18 \( \times 10^{-3} \) |
| Ribulose-P2, anaerobic (Fig. 1B) | | | |
| Leu-335     | 2.84 ± 0.01 | 0.74 | 2.25 ± 0.09 \( \times 10^{-3} \) |
| Val-335     | 0.463 ± 0.001 | 1 | NA |
| Xylulose-P2, aerobic (Fig. 1C) | | | |
| Leu-335     | 0.46 ± 0.02 \( \times 10^{-3} \) | 0.43 | 2.32 ± 0.40 \( \times 10^{-3} \) |
| Val-335     | 0.74 ± 0.02 \( \times 10^{-3} \) | 2.61 | 0.17 ± 0.71 \( \times 10^{-3} \) |

*NA, not applicable (no decline in activity).*

**Fig. 2.** Chromatography of reaction products under carboxylating conditions. Leu-335 (A and B) and Val-335 (C and D) Rubiscos consumed [\( 1^{-3H} \)]ribulose-P2 by carboxylation and the total (A and C) and enzyme-bound (B and D) products were chromatographed on an anion-exchange column with a NaCl gradient (indicated in B). See “Experimental Procedures” for details. The numbers in parentheses show the radioactivity recovered in each compound as a percentage of the total radioactivity eluting after 10 min in the total-product chromatogram.

**Fig. 3.** Chromatography of reaction products under predominantly oxygenating conditions. Leu-335 (A and B) and Val-335 (C and D) Rubiscos consumed [\( 1^{-3H} \)]ribulose-P2 under conditions where oxygenation predominated, and the total (A and C) and enzyme-bound (B and D) products were chromatographed on an anion-exchange column as for Fig. 2. See “Experimental Procedures” for details. The numbers in parentheses show the radioactivity recovered in each compound as a percentage of the total radioactivity eluting after 10 min in the total-product chromatogram.
rubisco-bound radioactivity was constituting 8.0% (Leu-335) and 6.4% (Val-335) of the radioactivity in and the Rubisco-bound products were isolated by gel filtration. These chromatography of the bound compounds released with SDS is shown in B (Leu-335) and C (Val-335).

**TABLE III**

Effect of H2O2 on binding of ribulose-P2 oxidation products to Rubisco

| H2O2 treatment | Rubisco-bound radioactivity (% of total supplied) |
|----------------|-----------------------------------------------|
|                | Leu-335 | Val-335 |
| None           | 8.0     | 6.4     |
| 0 time         | 1.2     | 1.5     |
| After 5 min    | 1.6     | 5.1     |

by a near linear period. When expressed as percentages of the carboxylation kcat, both activities were substantially increased by the Leu-335 mutation (10-fold for deoxypentodiulose-P and 4-fold for xylulose-P2, Table IV).

Inhibition by Xylulose-P2—Fully CO2/Mg2+-activated wild-type Rubisco was slowly inhibited by exposure to xylulose-P2 (Fig. 5A). Similar slow inhibition was seen with spinach Rubisco by Zhu and Jensen (23) who attributed it to the formation of a decarbamylated enzyme-xylulose-P2 complex. We analyzed xylulose-P2 inhibition in terms of the model for slow binding inhibition (Equation 3) (40) used previously to model inhibition of Rubisco by carboxyarabinitol-P2 (32, 42). The rate constants for slow binding and release, k3 and k−1, were obtained by fitting data for the rate of inhibition at varying xylulose-P2 concentrations to Equations 4 and 5. The results obtained show that, although the Ks values for the rapid-equilibrium steps are similar for xylulose-P2 and carboxyarabinitol-P2 binding, there are striking differences in the rate constants for the subsequent isomerization step (Table I). First, the isomerization step for xylulose-P2 binding is very slow (approximately one-hundredth of the rate of the analogous step for carboxyarabinitol-P2 binding); second, xylulose-P2 is re-
leased from its tight complex at least 5000-fold faster than is carboxyarabinitol-P₂, and third, although the Val-335 mutation perturbs the kinetics of carboxyarabinitol-P₂ only modestly, it completely abolishes the slow isomerization phase of xylulose-P₂ binding so that no slow inhibition was seen, even after prolonged incubation at xylulose-P₂ concentrations 20-fold greater than its rapid equilibrium $K_r$ (Fig. 5).

As predicted for slow isomerization binding where release from the tight complex occurs at an appreciable rate relative to isomerization (Equation 3), inhibition of the wild-type, Leu-335 Rubisco by xylulose-P₂ plateaued at less than complete inhibition. A fraction of activity was retained (25% in this case), even after prolonged incubation to equilibrium at saturating xylulose-P₂ concentrations (Fig. 5B). Although the data for $v/v_i$ versus xylulose-P₂ concentration may be fitted to Equations 5 and 6, the value of the parameter estimates obtained by this method is limited by strong interdependency between $k_i$ and $k_4$. Nevertheless, a curve drawn using the estimates of these parameters obtained from the data of Fig. 5A fits these data approximately (Fig. 5B).

The data reported in Fig. 5 are the initial activities seen immediately after 10-fold dilution of xylulose-P₂-treated ECM preparations into the assay solution containing saturating ribulose-P₂. Under these assay conditions, reactivation slowly occurred. In all cases, this recovery proceeded with a $k_{obs}$ of $-7 \times 10^{-4}$ s⁻¹ (data not shown), which is similar to the $k_4$ value estimated from the rate of xylulose-P₂ inhibition (Table I), as expected.

The action of xylulose-P₂ as a rapid equilibrium inhibitor, competitive with respect to ribulose-P₂, was assessed by measuring the activity of preactivated Rubisco immediately after addition to assay mixtures containing various ribulose-P₂ and xylulose-P₂ concentrations. Both wild-type and mutant enzymes showed classic, competitive inhibition. The $K_r$ for xylulose-P₂ was 4.8 $\mu$M for the Leu-335 form, which is consistent with the value reported for spinach Rubisco (38), and agrees approximately with the estimate obtained from the slow binding experiment (Fig. 5A). For the Val-335 form, the estimate increased to 20.7 $\mu$M (Table I).

**Activation of Uncarboxylated Rubisco in the Presence of Xylulose-P₂**—When uncarboxylated, metal-free Rubisco (E) is added to an assay mixture containing saturating concentrations of CO₂, Mg²⁺, and ribulose-P₂, activity accelerates as the essential Mg²⁺-stabilized carbamate (ECM) forms in the active site. Although the rate of ECM formation is slowed by the presence of ribulose-P₂, which binds tightly to E (46), activity nevertheless increases from an initial zero to a final steady state where it matches that seen in controls where full CO₂/Mg²⁺ activation (ECM formation) was induced before addition of ribulose-P₂. The observed rate constant for this activation was increased slightly by the Val-335 mutation (Fig. 6), perhaps indicating that ribulose-P₂ binds a little less tightly to the E form of the mutant than the wild type.

The tendency of ligands, such as xylulose-P₂, to bind to the uncarboxylated E form of Rubisco may be gauged by observing the effect of preincubation of E with the ligand on the rate of activation in the above assay. The substantially reduced rate of activation caused by pre-exposure of the Leu-335 wild type to xylulose-P₂ (Fig. 6A) indicates that xylulose-P₂ binds to E and is released from it much more slowly than the rate of carbamylation under the same conditions without preincubation with xylulose-P₂. Similarly, slow release from the E-xylulose-P₂ complex was reported for spinach Rubisco (23, 44). The $k_{obs}$ for xylulose-P₂ release from the wild-type tobacco enzyme estimated by this means (Fig. 6A) is similar to the release parameter, $k_4$, estimated from the rate of xylulose-P₂ inhibition of the ECM form (Table I). However, in the experiment shown in Fig. 6A, we can be sure that the starting EI* species is uncarboxylated. Therefore, this observation lends support to the view that the tight complex with xylulose-P₂ is uncarboxylated, regardless of the carbamylation status of the starting, uninhibited enzyme (23, 44). In marked contrast, the effect of pre-exposure to xylulose-P₂ on subsequent activation of the Val-335 mutant was barely perceptible (Fig. 6B).

**DISCUSSION**

The Leu-335 → Val Substitution Does Not Grossly Distinguish the Active Site of Rubisco—Consistent with previous assessments (8), the Val-335 mutant of tobacco Rubisco is properly assembled and fully soluble. It crystallizes easily and can be purified in quantity by the same procedures used for the wild-type protein. Its apparently unimpaired tight binding of the reaction intermediate analog, carboxyarabinitol-P₂, and its only slightly impaired capacity for enolization of the substrate indicate that perturbation of the active by the mutation must be quite subtle.

**Slow Inhibition during Catalysis Is Less Severe and Different in Character with Val-335 Rubisco**—Consistent with the view that fallower inhibition of Rubisco during catalysis is caused by accumulation of slow binding inhibitors at the active site (21, 22), assay time-course data (Fig. 1) fit well to Equation 1, which can be derived from the two-step, slow isomerization model (40) of slow inhibition (see “Experimental Procedures”). However, interpretation of the meanings of the kinetic parameters estimated in this way is clouded by observations that at least two
different inhibitors are responsible and their concentrations vary during the assay because they are largely generated during the course of the assay itself, either by Rubisco-catalyzed misprotonation of the enediolate intermediate (22) (Scheme 1) or by non-enzymatic oxidation of ribulose-P₂ (25).

In the presence of atmospheric concentrations of O₂, the Val-335 mutation greatly reduced the severity of fallover inhibition and altered its character in the sense that the inhibition proceeded to completion (i.e. \( v_i \to 0 \)) rather than coming to a steady state where a finite fraction of the activity remained, as with the wild type (Fig. 1A). This suggests that the residual inhibition seen with the Val-335 mutant is caused by an inhibitor whose rate of release (\( k_i \); Equation 3) from the tight complex (\( E^* \)) is close to zero, unlike wild-type fallover where the finite steady-state \( v_i \) indicates that \( k_i \) must be appreciable compared with \( k_v \).

Exclusion of O₂ reduced the extent of fallover inhibition of the wild-type enzyme (Fig. 1B), consistent with previous observations that non-enzymatic oxidation of ribulose-P₂ to pentodiulose-P₂ occurring during the assay was one of the main causes of the fallover phenomenon (25). Total abolition of fallover inhibition of the Val-335 mutant enzyme under the same conditions (Fig. 1B) is consistent with pentodiulose-P₂ or a product derived from it, being the sole cause of the residual inhibition seen with the mutant under aerobic conditions.

All Abortive Side Reactions, Except Pyruvate Production, Are Exacerbated by the Val-335 Mutation — Obviously the reduced fallover inhibition seen with the mutant is not a result of reduced production of inhibitory by-products; production of all of these was increased by the mutation, except pyruvate. Another active site mutation, Thr-65 → Val in *Synechococcus* PCC6301 Rubisco, also partitioned less product toward pyruvate (12). Apparently, loosening the active site or reducing the catalytic flux can, in some circumstances, aid the stereospecific protonation that completes the carboxylation sequence (Scheme 1).

The increased tendency to catalytic misfire induced by the mutation was measured most quantitatively with the by-products arising from the enediolate intermediate, xylulose-P₂ and deoxypentodiulose-P, because spectrophotometric assays can be devised (Table IV). The 4- and 10-fold increases in the partitioning of product toward these compounds attest to the impairment of the ability of the mutant enzyme to control proton access to the *Re* face of C₃ of the enediolate and to maintain the planarity of the O₁, C₁, C₂, and C₃ atoms that is so essential for suppressing \( \beta \) elimination of the P₁ phosphate (2). The monophosphate deoxypentodiulose-P is not likely to be a strong inhibitor of Rubisco, but the decreased fallover inhibition, despite enhanced partitioning toward xylulose-P₂, suggests that the interaction of this biphosphate with the active site must be considerably loosened by the mutation.

Further information about misfire products was obtained by chromatographic analysis of total and enzyme-bound products after complete conversion of labeled ribulose-P₂ (Figs. 2 and 3). Here the gradient of the anion-exchange chromatography was optimized to focus attention on the biphosphate and carboxylated-monophosphate products. The experiment under predominantly oxygenating conditions (Fig. 3) was the most informative, because the low CO₂ concentration maximized the fraction of active sites with enediol bound, and thus the side reactions that stem from this intermediate. Furthermore, the high \( \text{O}_2/\text{CO}_2 \) ratio promotes flux through the oxygenation pathway, allowing detection of the pentodiulose-P₂ and carboxytetritol-P₂ products that derive from the peroxyketone intermediate (Scheme 1). Xylulose-P₂ was the only biphosphate by-product arising from the Leu-335 wild type (Fig. 3A), much of it remaining bound to the enzyme (Fig. 3B). The Val-335 mutant also produced pentodiulose-P₂ and carboxytetritol-P₂ (Fig. 3C) with only the latter remaining bound (Fig. 3D). This supports the view that xylulose-P₂ is not bound tightly by the mutant enzyme. It also demonstrates that the mutant does not fully suppress \( \text{H}_2\text{O}_2 \) elimination from the peroxyketone intermediate but is able to promote the benzyl acid-type rearrangement of the elimination product, pentodiulose-P₂, to carboxytetritol-P₂ (Scheme 1), which remains tightly bound at the active site. Enhanced production of pentodiulose-P₂ and carboxytetritol-P₂ was observed previously with mutants of *R. rubrum* Rubisco (14, 15).

The Val-335 Mutation Promotes Rearrangement of Pentodiulose-P₂ to Carboxytetritol-P₂ within the Active Site — Exposure to Cu-oxidized ribulose-P₂, which contains pentodiulose-P₂ and carboxytetritol-P₂ (25), provided a more direct demonstration of the ability of the active site to bind pentodiulose-P₂ and, in the case of the mutant, to rearrange it to carboxytetritol-P₂. These experiments benefited from the recognition that, unlike carboxytetritol-P₂, pentodiulose-P₂ is not stable when bound to Rubisco and, therefore, rapid isolation of the complex by gel filtration is required for optimal detection. These experiments also exploited the ability of added \( \text{H}_2\text{O}_2 \) to return pentodiulose-P₂ to the catalytic pathway for oxygenation, leading to its release from the enzyme as P-glyceraldehyde (25). The results (Table III and Fig. 4) demonstrate clearly that both wild-type and mutant enzymes bound both pentodiulose-P₂ and carboxytetritol-P₂ and that the mutant converted the former to the latter with much greater facility than the wild type. Similar facilitation of this benzyl acid-type rearrangement was observed previously with the Lys-329 → Ala (analogous to residue 334 in the higher plant enzyme) mutant of *R. rubrum* Rubisco (15).

Slow Binding Inhibition of Rubisco — The data (Figs. 5 and 6 and Table I) are consistent with xylulose-P₂ being a slow binding inhibitor following the classic two-step, slow isomerization model (Equation 3) (40). Its inhibition is analogous to the more thoroughly studied inhibition by 2’-carboxyarabinitol-P₂ and 4’-carboxyarabinitol-P₂ (32, 42) but differs in that it is a two orders of magnitude slower and that it has an appreciable release rate, so that it comes to an equilibrium where 25% of the activity remains uninhibited even at saturating xylulose-P₂ concentrations. Whether the slow rate of inhibition is related to the preference of xylulose-P₂ for binding to the uncarbamylated E form of the active site (23, 44) remains to be determined. This could be assessed by comparison with another slow binding inhibitor, such as 2’-carboxyarabinitol-1-phosphate, which is known to prefer the carbamylated site (ECM) (47) but binds less tightly than carboxyarabinitol-P₂ (48). Measurement of the rate of slow inhibition by xylulose-P₂ commencing with E rather than ECM (as in Fig. 5) also might shed light on this question.

Saturation of inhibition by xylulose-P₂ at less than complete inhibition was observed previously (23, 38), and this phenomenon is shared by one or more inhibitors remaining in Rubisco assays after complete consumption of ribulose-P₂ (21), 2’-carboxyarabinitol-1-phosphate (48, 49), and pentodiulose-P₂ (25). Although this might suggest anti-cooperativity (i.e. binding of the ligand to one or more sites on a Rubisco octamer makes binding to subsequent sites less favored) (21), it is more economically explained as being simply the expected consequence of the model for slow inhibition embodied in Equation 3, where a rate of inhibition that becomes saturated at high inhibitor concentrations is coupled with an appreciable rate of release (see “Experimental Procedures”).
Alterations to Fallover Inhibition Caused by the Val-335 Mutation—Two different slow binding inhibitors have been definitively implicated in fallover inhibition: xylulose-P2 produced by misprotonation of the enediolate (22) and pentodiulose-P2 produced by non-enzymatic oxidation of ribulose-P2 during its synthesis and storage and also under the conditions of the Rubisco assay (25). Another pentulose bisphosphate misprotonation product, 3-keto-D-arabinitol-1,5-bisphosphate, has been hypothesized to contribute to fallover inhibition (18, 22, 24, 50). However, its identification as its arabinitol product after dephosphorylation and borohydride reduction is compromised by observations that pentodiolose-P2 can generate predominantly the same product after similar treatment (14), presumably because of stereochemical bias during reduction. Therefore, we do not consider that the contribution of 3-keto-D-arabinitol-1,5-bisphosphate to fallover inhibition has been established securely. The observation, that production of putative 3-keto-D-arabinitol-1,5-bisphosphate depended on the presence of O2 (50), heightens suspicion that the species detected was really the ribulose-P2 oxidation product, pentodiolose-P2. If so, xylulose-P2 and pentodiolose-P2 may be the only significant causes of fallover inhibition.

One of the central observations of this study is the total abolition of the slow isomerization phase of xylulose-P2 inhibition by the Val-335 mutation. Rapid equilibrium inhibition by this species still persists in weakened form (Table I), but no sign of the second step of the two-step binding mechanism (Equation 3) was evident. Apparently, the minimal perturbation of loop 6 in the mutant (effectively, removal of one methylene carbon) was sufficient to loosen its interactions with the active site and allow the rapid release of the ligand. The binding of other slow binding bisphosphates, such as pentodiolose-P2, might be similarly affected, but the instability of the latter compound precludes detailed study.

When slow inhibition by xylulose-P2 (and perhaps 3-keto-D-arabinitol-1,5-bisphosphate, if it exists) was prevented by the Val-335 mutation and pentodiolose-P2 production was blocked by anoxia, fallover was abolished completely (Fig. 1B), a situation never encountered previously with any higher plant Rubisco. Four pieces of circumstantial evidence collectively mount a strong case that carboxytetritol-P2 is the inhibitor responsible for the reduced degree of fallover inhibition retained by the mutant under aerobic conditions (Fig. 1A). First, unlike the wild-type enzyme, the mutant catalyzes the production of significant amounts of pentodiolose-P2, the precursor of carboxytetritol-P2, when O2 is present (Figs. 2 and 3). This substantially augments the basal amounts of this dicarbonyl compound produced by non-enzymatic oxidation during the course of the assay, the lack of which causes the moderate alleviation of fallover inhibition of the wild-type enzyme in anoxia (Fig. 1, A and B) (25). Second, the mutation greatly facilitates the benzylic acid-type rearrangement of pentodiolose-P2 to carboxytetritol-P2 (Fig. 4 and Table II). Third, bound carboxytetritol-P2 must be released from the active site imperceptibly slowly, despite the loosening of loop 6 induced by the mutation. This is shown by the recovery of enzyme-bound carboxytetritol-P2 even after four-times repeated washing by centrifugal ultrafiltration over a 2-h period, a procedure that led to a loss of most of the bound pentodiolose-P2 (Figs. 2 and 3). Fourth, consistent with this, the residual fallover inhibition displayed by the mutant projects to complete inhibition, suggesting that it is caused by an inhibitor whose release rate (k4, Equation 3) is close to zero.

The Val-335 mutation therefore illustrates the relative contributions of the two main causes of fallover inhibition by eliminating one (by facilitating rapid release of any pentulose bisphosphates produced within the active site by misprotonation) and exacerbating the other when O2 is present (by permitting rearrangement of pentodiolose-P2 to carboxytetritol-P2, which binds even more tightly). Reduced fallover inhibition of spinach Rubisco observed after chemical modification of some Arg residues with phenylglyoxal (26) might reflect similar facilitation of pentulose bisphosphate release from active sites indirectly perturbed by modification of remote Arg residues.

Alterations to Xylulose-P2 Carboxylation—The increased capacity to catalyze xylulose-P2 carboxylation induced by the mutation (Fig. 1C and Table II) presumably is the consequence of looser packing of loop 6 against the substrate. By some means, this apparently allows an increase in the rate of abstraction of the C3 proton from xylulose-P2, which, if xylulose-P2 binds exactly analogously to ribulose-P2, would not be positionable appropriately for abstraction by carbamylated Lys-201 (1, 2). The fallover-like decline in xylulose-P2 carboxylase activity of the wild-type enzyme during assay (Fig. 1C) parallels the time course of xylulose-P2 inhibition of ribulose-P2 carboxylase activity measured on subsequent exposure to ribulose-P2 (Fig. 5A). This presumably reflects the progressive decarbamylation of the active site induced by xylulose-P2 (23, 44). Nor is the lack of fallover-type inhibition with the mutant enzyme’s xylulose-P2 carboxylase activity (Fig. 1C) surprising, given the mutant’s lack of the slow isomerization, second step of xylulose-P2 binding likely to be required for such decarbamylation. What is surprising is the progressive increase in the apparent xylulose-P2 carboxylase activity of the Val-335 mutant that occurs during the assay (Fig. 1C). This observation might be consistent with a finite amount of 3-epimerization of xylulose-P2 (correct reprotonation of the enediol intermediate to produce ribulose-P2, Scheme 1) occurring even at saturating CO2. Any traces of ribulose-P2 so produced and released from the enzyme initially would compete very poorly with the vastly higher xylulose-P2 concentrations present but the competition would become more equal as ribulose-P2 accumulated, leading to an increasing rate of product formation. In the case of the Val-335 mutant lacking xylulose-P2-induced decarbamylation, this process was apparently more than sufficient to offset any residual inhibition caused by production of pentodiolose-P2 and carboxytetritol-P2 in the active site. This inhibition might be expected to be slight, because, although pentodiolose-P2 should be produced in the same fraction of catalytic turnovers under aerobic conditions regardless of whether xylulose-P2 or ribulose-P2 was the substrate, turnover with xylulose-P2 was 600-fold slower (Table II).

The rates of carboxylation of xylulose-P2 were much slower than the rates of xylulose-P2 synthesis from ribulose-P2 under CO2/O2-free conditions (5% for Leu-335, 14% for Val-335; Tables II and IV). However, under the CO2-saturated conditions of the usual assays, the steady-state concentrations of the enzyme-enediol complex will be lower and therefore xylulose-P2 production should be reduced. Indeed, fallover inhibition was observed to be lessened at CO2 saturation (19). Under these conditions, carboxylation of xylulose-P2 may make a significant contribution to the total rate of release of xylulose-P2.

What Is the Adaptive Advantage of Fallover Inhibition?—The insights into the mechanism of fallover inhibition provided by the Val-335 mutant prompt questions about its adaptive significance. Slow isomerization inhibition of Rubisco, according to Equation 3, whether directed at the free E form or the carbamylated ECM form, provides an elegant device for regulating Rubisco activity that has an inbuilt limit that prevents total inhibition, always permitting a basal level of activity. Coupled with the Rubisco activase system for ATP-powered
release of the inhibitor, itself regulated by energy charge and redox conditions (28), it becomes a sophisticated and effective mechanism for regulating Rubisco activity according to the prevailing light intensity, temperature, and requirement for photosynthetic. However, the many examples of algal and bacterial Rubiscos that lack fallover inhibition (27, 43, 51, 52) suggest additional significance. Like the Val-335 mutant, fall-over-free Rubiscos generally have lower CO2/O2 specificity and lower catalytic effectiveness (k_cat/K_m for carboxylation), than the higher plant enzyme (53). They also have differences in the sequence of loop 6 and its environs. Perhaps the attainment of greater carboxylase specificity and efficacy, by evolving better complementarity between the active site and the transition state for CO2 addition, has come at the cost of making the closure of loop 6 over the substrate so precise and so tight that close analogs of the substrate or its enediolized version are unable to escape without external assistance. In co-opting the motor protein, Rubisco activase, as the release agent, and further co-opting the release mechanism for regulatory purposes, natural selection has made an elegant virtue out of an ugly necessity.

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