Side Reactions Catalyzed by Ribulose-bisphosphate Carboxylase in the Presence and Absence of Small Subunits*

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The large subunit core of ribulose-bisphosphate carboxylase from Synechococcus PCC 6301 expressed in Escherichia coli in the absence of its small subunits retains a trace of carboxylase activity (about 1% of the kcat of the holoenzyme) (Andrews, T. J. (1988) J. Biol. Chem. 263, 12213–12219). During steady-state catalysis at substrate saturation, this residual activity diverted approximately 10% of the reaction flux to 1-deoxy-d-glycero-2,3-pentodiolose-5-phosphate as a result of β elimination of inorganic phosphate from the first reaction intermediate, the 2,3-enediol form of ribulose bisphosphate. This indicates that the active site's ability to stabilize and/or retain this intermediate is compromised by the absence of small subunits. Epimerization and isomerization of the substrate resulting from misprotonation of the enediol intermediate were not significantly exacerbated by lack of small subunits. The residual carboxylating activity partitioned product between pyruvate and 3-phosphoglycerate in a ratio similar to that of the holoenzyme, indicating that stabilization of the penultimate three-carbon aci-acid intermediate is not perturbed by lack of small subunits. The underlying instability of the five-carbon enediol intermediate was revealed, even with the holoenzyme, under conditions designed to lead to exhaustion of substrate CO2 (and O2). When carboxylation (and oxygenation) stalled upon exhaustion of gaseous substrate, both spinach and Synechococcus holoenzymes continued slowly to β eliminate inorganic phosphate from and to misprotonate the enediol intermediate. With carboxylation and oxygenation blocked, the products of these side reactions of the enediol intermediate accumulated to readily detectable levels, illustrating the difficulties attendant upon ribulose-P2 carboxylase's use of this reactive species as a catalytic intermediate.

The photosynthetic enzyme Rubisco1 (EC 4.1.1.39) catalyzes

1 The abbreviations used are: Rubisco, ribulose-P2 carboxylase/oxygenase; ribulose-P2, 5-ribulose-1,5-bisphosphate; xylulose-P2, 2-xylulose-1,5-bisphosphate; 3-ketoarabinitol-P2, 3-keto-D-arabinitol-1,5-bisphosphate; P-glycerate, 3-phospho-D-glycerate; deoxyxypentodiolose-P, the addition of CO2 to ribulose-P2, producing two molecules of P-glycerate using a multistep reaction sequence involving at least three enzyme-bound catalytic intermediates (Scheme 1) (for reviews, see Refs. 1–3). Two of these intermediates, the first and the third, are strong nucleophiles by virtue of their enediol character. Both are susceptible to side reactions that abort the carboxylation sequence and compromise the catalytic efficiency of the enzyme.

The first of these intermediates is the 2,3-enediol form of ribulose-P2 produced by removal, by an enzymatic base, of the proton attached to C-3 of ribulose-P2. There may be two or more differently protonated forms of this intermediate; only the enediol form is shown in Scheme 1. Collectively, we refer to all forms of this intermediate as “the enediol.” This is the species that must be attacked by CO2 at C-2 and (concertedly or sequentially) by H2O at C-3 to form the six-carbon intermediate and thus allow carboxylation to proceed (Scheme 1). Three different classes of side reactions are known for this intermediate. First, the enediol is also attacked by O2 at C-2, resulting in the formation of 2-phosphoglycolate which is, in turn, partially recycled to photosynthetic metabolism by the photosynthetic glycolate pathway (4–6). Second, the enediol can be reprotonated incorrectly, producing pentulose bisphosphate isomers of the substrate (xylulose-P2 and 3-ketoarabinitol-P2) (7–12) which are strong inhibitors and/or very weak substrates of the enzyme (13–15). Third, mutants of Synechococcus PCC 6301 and Rhodospirillum rubrum Rubiscos were discovered that were compromised in their ability to stabilize the enediol. These mutant enzymes catalyzed the production of varying amounts of deoxyxypentodiolose-P as a result of β elimination of the phosphoryl group attached to C-1 of the intermediate (P1) (16, 17).

The final intermediate in the carboxylation sequence is produced following cleavage of the C-2/C-3 bond of the six-carbon intermediate (Scheme 1). This aci-acid form of the P-glycerate molecule, derived from carbons 1 and 2 of ribulose-P2 and the incoming CO2 molecule, requires stereospecific protonation at C-2 to form P-glycerate. Only a single kind of side reaction is known for this intermediate, β elimination of the phosphoryl moiety to produce enol-pyruvate and thus pyruvate (18). All wild-type Rubiscos studied so far partition approximately 0.7% of their aci-acid intermediate to pyruvate (18), and mutants are known in which this partitioning ratio is increased or decreased (14, 16, 17). Thus both of Rubisco’s enediol-like intermediates are prone to β elimination of the P1 phosphate group, 1-deoxy-d-glycero-2,3-pentodiolose-5-phosphate; carboxyarabinitol-P2, 2-carboxyarabinitol-1,5-bisphosphate; carboxypentitol-P2, unresolved isomeric mixture of carboxyarabinitol-P2 and 2-carboxyarabinitol-1,5-bisphosphate; Lsa, Lsb, large subunit octamer and holoenzyme of Rubisco from Synechococcus PCC 6301, respectively; kcat, substrate-saturated turnover rate; Epps, N-[2-hydroxyethyl]piperazine-N′-3-propanesulfonic acid; HPLC, high performance liquid chromatography.

This paper is available on line at http://www-jbc.stanford.edu/jbc/
and there may be a common reason (16, 18). Stereoelectronic considerations suggest that the β elimination tendency is least when the β substituent is maintained coplanar with the enediol double bond. However, a single disposition of the C-1/bridge O bond cannot be found which satisfies this requirement for both intermediates because the double bond is differently positioned in the two intermediates. In order to maintain co-planarity with the double bond, the C-1/bridge-O bond must change its dihedral angle with respect to the C-2/carboxyl-C bond of the six-carbon and aci-acid intermediates through 90° following hydrolysis of the six-carbon intermediate (Scheme 1). This movement may not occur rapidly or completely enough to stabilize the aci-acid intermediate fully.

The ability to produce a small subunit-free version of Synechococcus Rubisco by expression of the cyanobacterial rbcL gene in Escherichia coli without the companion rbcS gene presents an opportunity for study of the influence of the small subunits on catalytic activity. The large subunits assemble correctly into an octameric core that retains a divalent metal ion, normally Mg(II). R is –CHOH-CH₂OPO₄²⁻.

**SCHEME 1.** The catalytic sequence of Rubisco’s carboxylation reaction illustrating the intermediates involved, their stereochemistry, and their propensity for side reactions. **Solid arrows** indicate the productive pathway, and **dashed arrows** show the side reactions. Me⁺⁺ is the essential divalent metal ion, normally Mg(II). R is –CHOH-CH₂OPO₄²⁻.

**Experimental Procedures**

Materials—Ribulose-P₂ (25), [1-14C]ribulose-P₂ (26, and [carboxyl-14C]carboxypentitol-P₂ (27) were prepared as described. NaH¹⁴CO₃ was obtained from Amersham Corp. and o-phenylenediamine from Sigma. Rubisco holoenzyme (L₈S₈) expressed in E. coli from the rbcL and rbcS genes of Synechococcus PCC 6301 was purified as described (16). Spinach Rubisco was purified using a procedure involving polyethylene glycol precipitation followed by anion-exchange chromatography on a Waters Protein-Pak Q column, essentially as described by Edmondson et al. (7) but omitting the final gel filtration step.

Preparation of L₈ Rubisco—E. coli HB101 harboring plasmid pDB53, which bears the Synechococcus PCC 6301 rbcL gene under the transcriptional control of the lac promoter (28), was used to produce large subunit octamers (L₈) devoid of small subunits. L₈ was extracted from E. coli, purified, and stored using the same procedures developed for the Synechococcus L₈S₈ holoenzyme (16). Immediately before use, L₈ preparations were dialyzed overnight at 4°C against 20 mM potassium phosphate buffer, pH 7.6, containing 1 mM dithiothreitol and concentrated using a Centricon 30 unit (Amicon).

Carboxylase Assay—The carboxylase activity of L₈ Rubisco was routinely measured by ¹⁴CO₂ fixation both in the presence and in the absence of added small subunits (19, 29).

Measurement of Pyruvate Production—Pyruvate was determined by measuring [¹⁴C]lactate formed during complete consumption of ribulose-P₃ by Rubisco in the presence of CO₂, lactate dehydrogenase, and NADH. The assay solution contained purified Synechococcus Rubisco (final concentrations, 150 μg ml⁻¹ of L₈ or 5 μg ml⁻¹ of L₈S₈), 100 mM Epp’s-NaOH buffer, pH 8.0, 20 mM MgCl₂, 0.1 μM o-phenylenediamine (Merck, spectroscopic grade), pH 1.6, as the mobile phase. Fractions of 0.1 ml were collected and mixed with 3 ml of scintillant for scintillation counting. Pyruvate production was calculated as the ratio of ¹⁴C in lactate to the total ¹⁴C in lactic, glycic, and 3-phosphoglyceric acids.

Analysis of the Products from [¹⁴C]Ribulose-P₃—Experiments were conducted with Synechococcus L₈S₈ and L₈ under anaerobic and CO₂-saturating conditions. The enzymes were placed in the main compartment of a Warburg flask in 2 (L₈S₈) or 0.5 (L₈) ml (final volume) of a solution containing 50 mM trithioanilamide acetic acid buffer, pH 8.3, 20 (L₈S₈) or 8 (L₈) mM magnesium acetate, and 40 mM NaHCO₃. The L₈ reaction also contained 10 mM K₂HPO₄ and 1 mM diithiothreitol. The phosphate was necessary to maintain solubility of L₈ and its presence also necessitated the use of a lower Mg²⁺ concentration to avoid pre-
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cipitation. Controls lacking enzyme and with decarbamylated L₈S₈ (minus Mg²⁺, plus 1 mM EDTA) were also included using the same conditions as for L₈S₈. The side arm of the flask contained 0.59 μmol of (1⁻¹⁴C)ribulose-P₂ (5.9 Bq nmol⁻¹⁻¹). The flask were placed in a Warburg apparatus, and an atmosphere of humidified 1% (v/v) CO₂ in N₂ was passed continuously throughout the assay period. After pre-equilibration with shaking for 30 min at 25 °C, the contents of the side arm were mixed with those of the main compartment to initiate the reaction. Shaking was stopped 20 s afterward, and incubation was continued for 2 h. To terminate the reaction, 1 volume of 0.5 N NaBH₄ in 0.1 N NaOH (equilibrated under N₂) was added via a septum-capped injection port, and the mixture was shaken for a further 30 min. Borohydride was discharged with Bio-Rad AG 50W-X8 resin (H⁺ form), and the sample prepared for anion-exchange chromatography as described (16). A sample was applied to a 5-μm SpheriSorb SAX column (0.46 × 25 cm, Alltech) and eluted with a phosphate gradient as described previously (16). Fractions comprising individual radioactive peaks were pooled, incubated with alkaline phosphatase, passed through Bio-Rad AG 1-X8 (formate) resin, and chromatographed on an Aminex HPX-87C column as described previously (16).

Additional experiments were conducted with Rubisco holoenzymes from spinach and Synechococcus under conditions designed to lead to exhaustion of CO₂/HCO₃⁻ (and any traces of O₂) while still leaving an excess of unreacted ribulose-P₂. In this case, the enzyme or subunits were placed in the main compartment of the Warburg flask in 1 (spinach) or 0.5 (Synechococcus) ml (final volume) of a solution containing 92 mM Epps-NaOH, pH 8.0, 18 mM MgCl₂, 0.2 mM NADH, 50 mM NaHCO₃, 0.6 mM EDTA, 20 mM phosphocreatine, 12 units of rabbit muscle creatine phosphokinase, 12 units of rabbit muscle glyceraldehyde-phosphate dehydrogenase, 36 units of rabbit muscle triose-phosphate isomerase, and 12 units of rabbit muscle glyceraldehyde-phosphate dehydrogenase (all enzymes from Boehringer Mannheim). P-glycerate content was measured from the reduction in absorbance at 340 nm which followed addition of ATP to 50 μM. Experiments with deoxypentodiulose-P produced by prolonged room temperature storage of ribulose-P₂ at pH 8 (30) showed that the quinoxaline adduct is formed with a half-time of approximately 60 s under these assay conditions. Therefore, although adduct formation does induce a lag in these assays, it is not an unacceptably long one, given the slow rate of deoxypentodiulose-P formation and the long assay periods used. The amounts of deoxypentodiulose-P produced were calculated using the molar absorbivity of the quinoxaline adduct determined by measuring inorganic phosphate (31) released from chromatographically isolated adduct by alkaline phosphatase. The value obtained (9300 M⁻¹ cm⁻¹ in aqueous solution at pH 8) approximates those reported for other quinoloxines (32).

**Protein Determination**—Protein was determined with the Cu-bicinchoninic acid procedure (33) using Pierce reagents or by measuring the absorbance at 280 nm (34, 35). L₈ concentrations were calculated from determinations of its carboxylase activity, assuming a specific activity of 50 nmol/min ¹⁴C/mg⁻¹ under the standard assay conditions where the bicarbonate concentration (25 mM) is approximately half-saturating for L₈ (19).

**RESULTS**

**Pyruvate Production by L₈**—The partitioning of Rubisco’s aci-carbanion intermediate between its alternative products, P-glycerate and pyruvate (measured as lactate), was determined for both the L₈ core and the L₈S₈ holoenzyme forms of *Synechococcus* Rubisco. In this study, a reverse-phase HPLC method was used to separate the lactate from the other labeled products. Three radioactive peaks were observed, corresponding to 3-phosphoglyceric acid, glyceric acid, and lactic acid (Fig. 1). Glycerate presumably is formed by dephosphorylation of a portion of the major product P-glycerate by traces of phosphatases contaminating the enzyme preparations.

The large enzyme concentrations and extended assay periods necessitated by the feeble activity of L₈ amplifies the effects of even tiny traces of phosphatases. No pyruvate (retention time 4.3 min) was apparent, confirming the efficacy of the coupling
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FIG. 2. Chromatography of the NaBH₄-reduced products derived from [1-¹⁴C]ribulose-P₂ in the presence of L₈S₈ (dashed lines) and L₈ (solid lines) Synechococcus Rubiscos. See “Experimental Procedures” and Table I for details. A, anion-exchange HPLC of the reduced products. The peaks labeled mono-P, P-glycerate, and bis-P were identified by their co-chromatography with d-ribulose-5-phosphate, P-glycerate, and ribulose-P₂, respectively. These standards were detected by their absorbance at 214 nm (not shown). B and C, Aminex HPX-87C chromatography of the pooled radioactivity eluting in the mono-P (B) and bis-P (C) fractions of the L₈ chromatogram in A, after dephosphorylation. The refractive index traces indicate the elution of ribitol, arabinitol, and xylitol standards, labeled R, A, and X, respectively. U indicates unidentified radioactive peaks.

THE DATA WERE OBTAINED FROM A SERIES OF CHROMATOGRAMS SIMILAR TO THOSE SHOWN IN FIG. 2. THE NUMBERS IN PARENTHESES ARE THE PERCENTAGES THAT EACH PRODUCT REPRESENTS OF THE TOTAL PRODUCTS. SEE “EXPERIMENTAL PROCEDURES” FOR FURTHER DETAILS.

| Conditions   | Holoenzyme (L₈S₈) | L₈ |
|--------------|-------------------|-----|
| Rubisco proteomes (µM) | 0.4 | 20 |
| Initial HCO₃⁻ (mM)  | 40 | 40 |
| Initial ribulose-P₂ (µM) | 293 | 1170 |
| Final bisphosphate (µM)⁶ | 5 | 59 |
| Reaction time (min) | 120 | 120 |
| Product analysis |                  |
| Deoxypentodiulose-P (µM)⁶ | 0 (0) | 68 (6) |
| P-glycerate (µM)⁶ | 279 (99) | 966 (89) |
| Unknown phosphorylated acid⁶ | 0 (0) | 41 (4) |
| Xylulose-P₂ (µM)⁶ | 1 (0) | 12 (1) |
| 3-Ketoarabinolit-P₂ (µM)⁶ | 1 (0) | 1 (0) |

*L* Fully carbamylated L₈S₈ in the presence of 20 mM Mg²⁺. Similar data were obtained with L₈S₈ under the experimental conditions required to maintain L₈ solubility (8 mM Mg²⁺, 10 mM K₂HPO₄, 1 mM dithiothreitol, see “Experimental Procedures”). A control with decarbamylated L₈S₈ in the absence of Mg²⁺ gave results similar to those for the enzyme-free control.

*A* Measured as the radioactivity eluting in the relevant regions of the anion-exchange chromatograms (Fig. 2), after subtraction if appropriate for the traces of radioactivity seen in the analogous regions in controls lacking enzyme.

*calculated as described under “Experimental Procedures.”

alcohol moiety derived from the monophosphate fraction co-chromatographing with the d-ribulose 5-phosphate standard. The middle peak was broader and larger than the flanking peaks, consistent with it being composed of two overlapping peaks (Fig. 2B). None of the peaks coeluted with either ribitol, arabinitol, or xylitol, the expected products of reduction of pentuloses. The elution pattern was similar to that obtained for the reduced sugar moiety of the monophosphate by-product seen with the T65V mutant form of Synechococcus Rubisco. This by-product was identified as deoxypentodiulose-P (16). The unknown peak eluting near P-glycerate (Fig. 2A) co-chromatographs with a peak produced nonenzymatically from [1-¹⁴C]ribulose-P₂ under strongly alkaline conditions (data not shown) that we have tentatively identified as being composed of both isomeric, branched, carboxylic acids produced by benzyl oxazolidine rearrangement of deoxypentodiulose-P (30). If this assignment is correct, then the radioactivity in this peak should be summed with that in the deoxypentodiulose-P peak. Since the combined total concentration of deoxypentodiulose-P and its putative rearrangement products at quenching was many fold greater than the concentration of enzyme protomers (Table I), these compounds must be genuine products, not merely the consequence of release of enzyme-bound enediol intermediate on quenching.

Reduction and dephosphorylation of the bisphosphate fraction remaining after exposure of [¹⁴C]ribulose-P₂ to L₈ yielded all three pentitols together with a small unknown peak frequently seen in controls lacking enzyme (Fig. 2C). Ribitol and arabinitol predominated, suggesting that the consumption of ribulose-P₂ was incomplete as might be expected from the poor affinity of L₈ for ribulose-P₂ (19). Furthermore, the arabinitol/ribitol ratio was similar to that observed following reduction of ribulose-P₂ in the minus-enzyme control, suggesting that little if any 3-ketoarabinolit-P₂ had been produced (Table I). A small amount of xylitol was also observed, but its amount indicates that only a trivial amount of xylulose-P₂ had been produced (Table I).

By-products of Catalysis by Rubisco Holoenzymes Under
Conditions Leading to Exhaustion of Gaseous Substrates—Under the usual CO₂-saturating conditions, the *Synechococcus* holoenzyme produced negligible traces of by-products derived from the endiul intermediate, even when the reaction continued until the ribulose-P₂ had been almost exhausted (Fig. 2A, Table I). In order to maximize the possibility of detecting endiul by-products with *L₅S₅* holoenzymes, assay conditions were designed so that the gaseous substrates were exhausted well before complete consumption of ribulose-P₂. The steady-state pool size of enzyme-bound endiul would be expected to be maximal under these conditions and, since carboxylating and oxygenating activities are suppressed by lack of substrates, the remaining ribulose-P₂ would be exposed to large concentrations of enzyme for extended periods, amplifying the chance of detecting by-products. The data obtained (Table II) show that P-glycerate production ceased, as expected, when its concentration became approximately equal to the concentration of bicarbonate originally supplied. Given the high concentration of enzyme present, this would have occurred only seconds after initiation of the reactions. Subsequently, both spinach and *Synechococcus* holoenzymes converted the remaining ribulose-P₂ to deoxypentodiulose-P and pentulose bisphosphate isomers of the substrate. Although production of these by-products was slow, readily measurable amounts had accumulated by the end of the experiments (Table II). While xylulose-P₂ and 3-ketoarabinitol-P₂ have been detected previously as trace products of spinach Rubisco (10–12), this is the first demonstration of the production of deoxypentodiulose-P by a wild-type Rubisco. In the case of the spinach enzyme, it is clear that deoxypentodiulose-P production represents true catalytic turnover because the amount of deoxypentodiulose-P produced far exceeded the concentration of active sites present. It is also possible that the deoxypentodiulose-P detected underestimates that actually produced because of further conversion of some of the deoxypentodiulose-P to other compounds, such as the rearrangement products mentioned above. These were not always detected as a discrete peak following the P-glycerate peak, as in Fig. 2A (solid line), and sometimes occurred as an unquantifiable tail on the P-glycerate peak (Fig. 2A, dashed line). For the *Synechococcus* holoenzyme, the deoxypentodiulose-P detected did not exceed the concentration of active sites present despite the longer incubation period.

Deoxypentodiulose-P formation during catalysis by spinach and *Synechococcus* holoenzymes under conditions designed to lead to rapid exhaustion of gaseous substrate was also measured by observing the absorbance at 330 nm of the quinoxaline adduct formed between deoxypentodiulose-P and α-phenylenediamine (Fig. 3). For this experiment, it was important to exclude the possibility that other α-dicarbonyl species might also contribute to the absorbance at this wavelength. Two other α-dicarbonyl species are known to be produced by Rubisco side reactions. One is pyruvate which, like other α-keto acids (32), could form a quinoxaline adduct under acidic conditions. However, this possibility was excluded by a control experiment which showed that negligible increase in absorbance at 330 nm occurred when 0.5 mM pyruvate was added to this assay system at pH 7.9 (Fig. 3). Another is the α-dicarbonyl by-product of Rubisco’s oxygenase reaction, detected with some mutants of *R. rubrum* Rubisco (23, 24). Use of strictly anaerobic conditions avoided this second potential complication in the present experiments.

For comparison, P-glycerate production was measured in small aliquots withdrawn at intervals from the cuvettes. For both enzymes, P-glycerate production was initially rapid but ceased abruptly when the P-glycerate concentration approached twice the concentration of NaHCO₃ initially present, confirming CO₂/HCO₃⁻ exhaustion (Fig. 3). This indicates that α-phenylenediamine did not seriously inhibit the carboxylase activity of either enzyme. The continuing rise in absorbance at 330 nm confirmed that deoxypentodiulose-P was indeed produced by both enzymes, supporting the conclusions from the analysis of 14C-labeled products (Table II) and establishing beyond doubt that this compound is a true by-product of both enzymes and not solely the result of degradation of endiul intermediate released at quenching. Using the molar absorptivity estimated for the quinoxaline adduct (see “Experimental Procedures”), it may be calculated from the absorbance increase that approximately 1% of the added ribulose-P₂ had been converted to deoxypentodiulose-P by the spinach enzyme in 20 min. Allowing for differences in enzyme concentration and reaction time, this is in approximate agreement with the amount of this by-product seen in the product analysis experiments (Table II). No sign of a lag in the rate of absorbance increase was observed with either enzyme during the early period before CO₂/HCO₃⁻ exhaustion. CO₂/HCO₃⁻ obviously caused little suppression of deoxypentodiulose-P production, at

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### Table II

| Conditions | Spinach | Synechococcus |
|------------|---------|---------------|
| Rubisco protomers (mM) | 0.18 | 0.22 |
| Approximate initial HCO₃⁻ (mM) | 4 | 3 |
| Initial ribulose-P₂ (mM) | 6.43 | 6.44 |
| Final bisphosphate (mM) | 1.87 | 3.28 |
| pH | 8.3 | 7.8 |
| Reaction time (min) | 60 | 180 |

| Product analysis | Spinach | Synechococcus |
|------------------|---------|---------------|
| Deoxypentodiulose-P (mM) | 0.43 (8.0) | 0.091 (2.3) |
| P-glycerate (mM) | 4.13 (76) | 3.07 (77) |
| Xylulose-P₂ (mM) | 0.64 (12) | 0.50 (13) |
| 3-Ketoarabinitol-P₂ (mM) | 0.21 (3.8) | 0.31 (7.8) |

* Measured as the radioactivity eluting in the relevant regions of the anion-exchange chromatograms (Fig. 4), after subtraction if appropriate of the traces of radioactivity seen in the analogous regions in controls with fully carboxypentitol-P₂-inhibited Rubisco.

* Calculated as described under “Experimental Procedures.”

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![Fig. 3. Continuous spectrophotometric measurement of deoxypentodiulose-P formation with wild-type spinach (solid line) and *Synechococcus* (dashed line) Rubiscoes. Deoxypentodiulose-P and P-glycerate (,), spinach (●, *Synechococcus*) formation were measured as described under “Experimental Procedures” under conditions designed to lead to rapid exhaustion of gaseous substrates (total initial concentration of CO₂ plus HCO₃⁻ plus O₂, approximately 3 mM) while still leaving an excess of unreacted ribulose-P₂ (initial concentration, 6.1 mM). Controls lacking enzyme (dotted line) and with ribulose-P₂ replaced by 0.5 mM pyruvate (dashed-dotted line) were also included.](image-url)
least at the moderately low concentrations initially present in these experiments.

The rate of deoxypentodiulose-P production slowed progressively for both enzymes throughout the experiment (Fig. 3). For the spinach enzyme, this might be a result of inhibition caused by accumulating misprotonation by-products. Indeed, the kinetics of the decline for the spinach enzyme appeared quite reminiscent of the decline usually seen during catalysis which has been attributed to that cause (7–10). *Synechococcus* Rubisco, however, is not subject to progressive inactivation during catalysis, at least at CO₂ saturation (16), and it showed a more pronounced decline such that, after 15–20 min, the rate of absorbance increase had fallen to approach the basal rate seen in the enzyme-free control. Therefore, accumulation of misprotonation products is not likely to be the cause of the inactivation in this case. Decarbamylation in the CO₂-free conditions may be a more plausible reason.

The very slow increase in absorbance at 330 nm in the control lacking enzyme (Fig. 3) is consistent with known rate of spontaneous production of deoxypentodiulose-P from ribulose-P₂ (30). Rapid conversion of the expected amount of ribulose-P₂ to P-glycerate also engenders confidence that α-phenylenediamine does not quickly cause extensive ribulose-P₂ degradation.

**DISCUSSION**

β Elimination of the Enediol Intermediate Occurs Even with Wild-type Rubiscos—The discovery that mutagenic perturbation of Rubisco’s active site in the region that binds the P₁ phosphate of the substrate resulted in a tendency of the enediol intermediate to β eliminate the P₁ group (16, 17) raised a question about whether this tendency existed at all with wild-type Rubiscos and, if so, whether it varied between different Rubiscos. Morell et al. (16) speculated that the improvement in catalytic effectiveness under limiting CO₂ conditions that accompanied the evolution of higher plant Rubiscos might have been achieved by increasing the fraction of enzyme active sites in the enediol-bound form during steady-state catalysis. If so, any tendency to β eliminate the enediol intermediate would be more obvious with higher plant Rubisco than with bacterial or cyanobacterial enzymes which are adapted to higher CO₂ concentrations. Experiments at saturating CO₂ with wild-type Rubisco showed little sign of the β elimination product, deoxypentodiulose-P (Table I). Therefore, to address this question, it was necessary to devise experimental conditions where the wild-type enzyme could be exposed to ribulose-P₂ for extended periods but prevented from disposing of it rapidly via carboxylation or oxygenation. This was achieved under anaerobic conditions by the simple expedient of ensuring that the starting concentration of ribulose-P₂ was well in excess of the CO₂/HCO₃⁻ concentration. With sufficient Rubisco present to exhaust the inorganic carbon supply (and any traces of O₂) in seconds, residual ribulose-P₂ would be left at a still saturating concentration for the remainder of the experiment. With the main routes of conversion of the enediol blocked by lack of gaseous substrates, the active site would thus be expected to remain maximally charged with enediol, maximizing its vulnerability to side reactions. This approach will only be successful if inactivation by decarbamylation does not occur quickly in the absence of CO₂. For the spinach enzyme at least, the data of Edmondson et al. (8) provide confidence that decarbamylation is very slow under these conditions, providing ribulose-P₂ remains saturating. This confidence is sustained by data which showed that deoxypentodiulose-P appeared in reaction mixtures containing both spinach and *Synechococcus* Rubiscos in easily detectable quantities under these conditions (Table II, Fig. 3) but not in a control with decarbamylated enzyme (Table I, footnote). Deoxypentodiulose-P formation was established both chromatographically, by resolving the products from [1-¹⁴C]ribulose-P₂ (Table II), and spectrophotometrically, by measuring the formation of the quinoxaline adduct with α-phenylenediamine (Fig. 3). Both methods agreed that spinach Rubisco produced larger amounts of deoxypentodiulose-P than *Synechococcus* Rubisco. Either spinach Rubisco has a larger fraction of its active sites occupied by the enediol under these conditions, as speculated by Morell et al. (16), or it stabilizes or retains the enediol less effectively than *Synechococcus* Rubisco. An idea of the scale of deoxypentodiulose-P production can be obtained by comparing the amounts observed with those of xylulose-P₂, previously shown to be produced by spinach Rubisco once in every 400 catalytic turnovers while a constant supply of CO₂ was maintained (10). In the present experiments, spinach Rubisco produced approximately two-thirds as much deoxypentodiulose-P as xylulose-P₂, whereas for the *Synechococcus* enzyme the ratio was approximately one-fifth (Table II).

As expected from previous studies (10–12), spinach Rubisco also produced considerable amounts of pentulose bisphosphate isomers under these conditions. Our data show that the wild-type *Synechococcus* holoenzyme produces these isomers also, and in approximately similar amounts (Table II). The method for calculating the contributions of xylulose-P₂ and 3-ketоШarabinitol-P₂ to the pool of substrate isomers assumes that these are the only two isomers produced. A third isomer, 3-keto-D-ribitol-1,5-bisphosphate, is also a theoretically possible misprotonation product although not shown so far to be the product of any Rubisco. If it is present, the method for calculating the amounts of individual pentulose bisphosphates collapses because it provides a potential second source of ribitol and xyitol. Nevertheless, the conclusion that pentulose bisphosphate isomers of the substrate, in general, are produced remains unassailed.

Absence of Small Subunits Exacerbates β Elimination of the Enediol Intermediate, But Not of the Aci-Acid Intermediate—The intrinsic tendency of the *Synechococcus* Rubisco holoenzyme to catalyze β elimination of its enediol intermediate is small enough to be overlooked in experiments at saturating CO₂ because of very rapid conversion of all of the ribulose-P₂ supplied to P-glycerate (Table I). In the absence of small subunits, however, the tendency becomes very large (Table I), rivalling that seen when Thr-65 in the P₁ binding site of the large subunit was mutated (16). Thus it must be concluded that lack of small subunits also alters active-site geometry in ways that impair Rubisco’s ability to bind or stabilize the enediol. This observation might provide some support for the idea that binding of the small subunits indirectly influences the arrangement of the P₁ binding site within the active site (22). Alternatively, since lack of small subunits is known to weaken the affinity of the active site for ligands such as 2-carboxyarabinitol-1,5-bisphosphate and to increase the *Kₘ* (ribulose-P₂) (19), increased β elimination might simply reflect a weakening of the active site’s grip on the enediol with consequent decay of this intermediate after release into solution.

The impairment of the active site’s ability to stabilize the enediol intermediate induced by absence of small subunits was not accompanied by a similar degradation of the ability to protect this intermediate from misprotonation (Table I). Nor was the stability of the aci-acid intermediate compromised by lack of the small subunits because *Lₚ* partitioned its carboxylated product between P-glycerate and pyruvate in a ratio similar to that of *LₚSₚ* (Fig. 1). Thus, although enolization and processing of the six-carbon intermediate are impaired to approximately equal extents by lack of small subunits (36), the
impairment is quite specific in its effect on side reactions; only $\beta$ elimination of the enediol is selectively stimulated. Similar specificity is not apparent with mutant Rubiscos with enhanced enediol $\beta$ elimination. Where examined, these mutants of both Synechococcus and R. rubrum Rubiscos also show either enhanced misprotonation of the enediol and/or alterations in the amount of pyruvate produced (16, 17, 24, 37). The $L_{99}$ data thus provide evidence that different features within the active site must be involved in stabilizing the enediol and aci-acid intermediates and that, even in the case of the enediol intermediate, different features are involved in suppressing its $\beta$ elimination and its misprotonation.

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