Perturbation of Reaction-intermediate Partitioning by a Site-directed Mutant of Ribulose-bisphosphate Carboxylase/Oxygenase*

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To explore the roles of active-site Glu™ of ribulose-bisphosphate carboxylase/oxygenase from Rhodospirillum rubrum, the E48Q mutant has been characterized with respect to kinetics and product distribution. Although the kcat for carboxylase activity is only 0.8% of the wild-type value, the mutant retains full activity in catalyzing the conversion of the carboxylated reaction intermediate to 3-phosphoglycerate and retains 10% of the normal activity in catalyzing the enolization of ribulose bisphosphate. Thus, the mutant is preferentially impaired in the carboxylation step. Partitioning of the enediol(ate) intermediate during turnover of ribulose bisphosphate is perturbed dramatically in the case of the mutant protein. Whereas the wild-type enzyme displays a CO2/O2 specificity factor of 11, the corresponding parameter of the mutant is only 0.3, thereby signifying a shift of the relative reactivity of the enediol(ate) in favor of O2. The mutant protein is also unable to protect the enediol(ate) against misprotonation with consequential conversion of ribulose bisphosphate to xylulose bisphosphate. This side reaction, undetected with wild-type R. rubrum enzyme, proceeds as rapidly as carboxylation of d-ribulose 1,5-bisphosphate by the E48Q mutant. Formation of xylulose bisphosphate by the mutant does not appear to account for the decline in carboxylase activity that occurs during the course of an assay. These studies demonstrate the multiple functionalities of Glu™ in the facilitation of catalysis and in directing intermediate partition in the preferred direction.

As a primary determinant of overall efficiency of photosynthetic carbon assimilation, ribulose-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) has been studied extensively from perspectives of structure, mechanism, and regulation (for reviews, see Refs. 1–3). The active sites of the enzyme are located at subunit-subunit interfaces, as first indicated by chemical cross-linking studies (4). Direct evidence followed with the homodimeric enzyme from Rhodospirillum rubrum, in which catalytically active heterodimers were formed by in vivo hybridization of two distinct site-directed mutant proteins (K166G and E48Q)† that were severely deficient in carboxylase activity (5). Subsequently, x-ray crystallography confirmed interfacial active sites and showed that most active-site residues (including Lys™ and Lys™) are positioned in the eight-stranded β,α-barrel of the large C-terminal domain (6, 7). The smaller N-terminal domain from the adjacent subunit extends partially across the mouth of the barrel and includes active-site residues Glu™ and Asn™ (6, 8).

Catalytic competence of Rubisco is obligatorily dependent on the reversible derivatization of Lys™ by non-substrate CO2 to form a carbamate (9). In the nonactivated (noncarbamylated) form of the enzyme, Glu™ interacts electrostatically with Lys™ of the adjacent subunit (10). This interaction is disrupted and replaced by an intersubunit salt bridge between Glu™ and Lys™ in the carbamylated form of the enzyme when complexed with the reaction-intermediate analogue CABP (8) (Fig. 1). Lys™ is located at the apex of loop 6 of the β,α-barrel; based on ill defined electron density in diffraction patterns of nonactivated enzyme (10) and activated enzyme lacking bound phosphorylated ligand (11), this loop is concluded to be highly flexible. In its closed conformation, visualized directly in the three-dimensional structure of the activated enzyme-CABP complex (6, 8), the loop extends over the top of the barrel domain, thereby sequestering the bound inhibitor from bulk solvent and rendering it exchange inert. The electrostatic interactions of the ε-amino group of Lys™ with both the γ-glutamyl of Glu™ and the C2 carbonylate of bound inhibitor presumably assist in stabilizing the closed-loop conformation. RuBP can only gain entry to the active site when loop 6 is in its open position. Thus, loop 6 movement and consequential alterations of intersubunit electrostatic interactions at the active site appear to be dynamic aspects of catalytic turnover.

To explore the structural and functional significance of intersubunit salt bridges (and the ionic groups that comprise them) at the active site of Rubisco, we have turned to site-directed mutagenesis. In earlier studies with the R. rubrum enzyme, our laboratory has examined the consequences of various replacements of Lys™. Although a positive charge at position 329 is not required for subunit assembly as a stable dimer, all amino acid substitutions result in essentially total loss of overall carboxylase activity (12). However, these deficient mutant proteins retain the capacities to enolize RuBP and catalyze 1734 solely to indicate this fact. Since these residues are conserved in both wild-type and mutant enzymes, site-directed mutagenesis was not expected to alter active site structure.

† Mutants are designated by the amino acid and its position as found in the wild-type enzyme followed by the amino acid substitution. Irrespective of species of origin, residue numbers refer to the R. rubrum enzyme.

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FIG. 1. Computer graphics-generated model of the active site as visualized in the crystal structure of the quaternary complex of Rubisco spinach enzyme (6, 8). The $\beta_\alpha$-barrel domain of one subunit, viewed from the side with the tip of the barrel on the left, is shown in orange (excepting residues 326--337, which comprise flexible loop 6, in blue), and the N-terminal segment of the active site from the adjacent subunit that includes Glu$^\text{mut}$ is shown in yellow. Mg$^{2+}$ is illustrated in mauve. In the quaternary complex presented, Glu$^\text{mut}$ and Lys$^{329}$ form an intersubunit salt bridge. However, in the nonactivated form of the $R$. rubrum enzyme, these two residues are farther apart; and Glu$^\text{mut}$ is bridged with Lys$^{329}$ of the adjacent subunit. The model is based on atomic coordinates generously provided by Professors Carl Bränden and Gunter Schneider of the Swedish University of Agricultural Sciences, Uppsala.

The initial step in the overall pathway (13) and to process the 2-carboxy-3-keto reaction intermediate to PGA (14). These observations, reinforced by the three-dimensional structure of the enzyme-CABP complex, pinpoint a role for Lys$^{329}$ in facilitating the reaction of CO$_2$ with the enediol(ate) of RuBP. By contrast, little is known about the function of Glu$^\text{mut}$ other than its considerable contribution to carboxylase activity (15). To gain additional insight into the basis of catalytic stringency for this active-site residue, we have subjected the E48Q mutant protein to a variety of kinetic and product analyses, including a determination of the CO$_2$/O$_2$ specificity factor. The ability of the mutant protein to catalyze partial reactions in the productive carboxylation pathway and the propensity of the mutant protein to process abnormally
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the enediolate intermediate are also characterized and their mechanistic implications discussed.

EXPERIMENTAL PROCEDURES

Materials—Common laboratory reagents and biologicals for enzyme assays were procured at the highest level of purity readily available from conventional sources. 3-3H]RuBP (11) and [1-3H]RuBP were synthesized by literature procedures (12, 13). [2-14C]Carbonic anhydrase, 3-ke- toarabinitol-P, and XaBP were kindly provided by Dr. George H. Lorimer of DuPont. Wild-type Rubisco was isolated from R. rubrum as previously described (18). The E46Q mutant was expressed in E. coli (15) and purified by anion-exchange chromatography (Pharmacy 50-mm column) as described for other Rubisco mutants (19, 20); the final preparation exceeded 95% purity based on polyacrylamide gel electrophoresis under both non-denaturing and denaturing (sodium dodecyl sulfate) conditions.

Protein and Activity Assays—Concentrations of wild-type and mutant Rubisco were based on the A260 nm and an extinction coefficient of 1.2 for 1 mg/ml (18).

All assays were conducted at 23–25 °C. 14CO2-fixation activity was determined by a modified filter disk assay (21); carboxylase activity was also monitored spectrophotometrically by coupling PGA formation to NADH oxidation through P GA kinase, glyceraldehyde 3-phosphate dehydrogenase, triose phosphate isomerase, and glycerol phosphate dehydrogenase (18). The epimerase activity (conversion of [3-3H]RuBP with slight modification (13) of the original procedure (23). Reactions were initiated by addition of substrate to solutions of enzyme; and periodically, 15–mu aliquots were quenched with 100 μl of 100 mM NaBH4. Quenched samples were diluted to 0.5 ml with 10% (v/v) acetic acid, oven-dried at 110 °C, and redissolved in 0.5 ml of H2O prior to scintillation counting.

Hydrolysis of the 6-carbon reaction intermediate, [2-14C]carboxy-3-keto-arabinitol 1,5-bisphosphate, was assayed as the difference between total borohydride-stabilized and acid-stable radioactivity as described by Pierce et al. (24). The reactions were carried out in a pH 8.0 buffer (50 mM Na+-Bicine, 1 mM EDTA, 10 mM MgCl2, 66 mM NaHCO3) using either wild-type (60 mM active site) or E48Q (110 mM active site) Rubisco. Turnover was initiated by the addition of intermediate (4300 dpm/nmol) at a final concentration of 1.55 μM. As contaminants of the intermediate preparation, [1,14C]PGA and unlabeled RuBP were also introduced at final concentrations of 2.31 and 10.4 μM, respectively. Periodically, duplicate aliquots were removed and quenched with either 10% formic acid or 1.0 M NaBH4. Following decomposition of excess NaBH4 with 10% formic acid, both sets of samples were dried, and the residues were redissolved in 0.5 ml of H2O prior to counting. Alternatively, aliquots could be quenched with 1% (w/v) sodium dodecyl sulfate prior to borohydride reduction (to ensure complete release of protein-bound intermediate), but the results were unchanged.

Determination of the CO2/O2 Specificity Factor (r) — Partitioning of RuBP between the carboxylase and oxygenase pathways catalyzed by Rubisco is referred to as the specificity factor (r), which is defined as the ratio of carboxylase and oxygenase efficiencies (Vc, Kc/Vo, Ko) (25). The r value may be determined in accordance with the relationship

\[ \frac{v_c}{v_o} = \frac{1}{1 + \left(\frac{[O_2]}{[CO_2]}\right)^r} \]  

(Eq. 1)

When [1-3H]RuBP is used as substrate, the relative amounts of radioactivity associated with PGA and FGyc then provide r directly (26).

Reaction mixtures at pH 8.0 and 23–25 °C under air routinely contained 50 mM Na+-Bicine, 10 mM MgCl2, 1 mM EDTA, NaHCO3 (66 mM for mutant enzyme or 10 mM for wild-type enzyme), 250 μM [1-3H]RuBP, and Rubisco (1 mg/ml of the mutant or 30 μg/ml of the wild type). In some experiments designed to monitor product distribution, concentrations of RuBP and mutant enzyme differed and are thus provided in relevant figure legends. At the desired time, reaction mixtures were quenched with sodium dodecyl sulfate (1% final concentration), deproteinized by ultrafiltration (Amicon Centricon-10), and chromatographed on Mono Q (Pharmacia, 5 × 50-mm column) in conjunction with a Pharmacia fast protein liquid chromatography system. The initial buffer was 10 mM sodium borate (pH 8.0), and the limit buffer for providing gradient elution was 10 mM sodium borate in 0.5 M NH4Cl (pH 8.0). The presence of borate in the elution buffers is crucial to the resolution of XaBP from RuBP. In-line monitoring of radioactivity was provided by a flow-through scintillation detector (IN/US 8-Ram) equipped with a 500-μl liquid scintillation cell.

Some quenched reaction mixtures, distinct from those subjected to chromatographic inspection, were analyzed specifically for concentrations of RuBP, XaBP, PGA, and pyruvate by NADH-based, enzyme-coupled assays (18, 22, 27). In these experiments, the input RuBP was not radiolabeled.

RESULTS

Time Course and Extent of RuBP Turnover Catalyzed by E48Q—At high concentrations of RuBP (3 mM) and [14C]NaHCO3 (66 mM), the extent of 14CO2 fixation catalyzed by wild-type Rubisco (2 μg/ml, 42 nM active sites) from R. rubrum is directly proportional to the reaction time as expected. By contrast, the activity of E48Q (0.21 mg/ml, 4.2 μM active sites) declines during the progression of RuBP turnover (Fig. 2A), reminiscent of the decay of spinach Rubisco reported under similar assay conditions (28–30). Based on the initial rate of 14CO2 fixation (the first 1-min interval), the specific activity of E48Q is 0.031 μmol/min/mg or only 0.6% of the value for wild type (5.2 units/mg). Although carboxylation virtually ceases during the 10-h period depicted, the total extent of reaction is less than 10% of the end-point reached with wild-type enzyme, the latter representing exhaustion of Rubisco. At the high concentrations of bicarbonate employed, the oxygenase activity of wild-type Rubisco is completely suppressed so that the observed end point of 14CO2
fixation reflects quantitative conversion of RuBP to PGA.

Since a sharp end point was not observed at a substrate:active site molar ratio of 715 as shown in Fig. 2A, the time course of carboxylation was also examined at a substrate:active site molar ratio of 12.5 (250 \mu M RuBP and 20 \mu M in E48Q subunit). Under these conditions, a stable plateau is reached; the total amount of CO₂ fixed is still only 40% of the control value (Fig. 2B), but nevertheless substantially greater than observed at the higher substrate:enzyme ratio. The smaller amount of CO₂ fixed, relative to the wild-type control, is not due to enzyme inactivation during turnover, because fixation resumes upon the addition of fresh RuBP to a reaction mixture that has already reached the plateau (Fig. 2B). With the assumptions of total utilization of RuBP and formation of PGA and PGyc as the sole products, the difference in end points observed with wild-type and mutant proteins equates to a \( \tau \) value of \( \approx 0.3 \) for E48Q in contrast to 11 for wild-type enzyme.

**Determination of \( \tau \) Value for E48Q**—To determine the \( \tau \) value of E48Q directly, rather than to rely on differential end points in the \(^{14} \text{CO}_2\)-fixation assay, \([1-^3\text{H}]\text{RuBP}\) was used as substrate, and the turnover products were resolved and quantified by ion-exchange chromatography. Conditions for these incubations were identical to those described in the legend for Fig. 2B, except NaHCO₃ was reduced to 10 mM for the wild-type control so that enough PGyc would be formed to permit calculations of partitioning between the carboxylase and oxygenase pathways. Chromatographic profiles of reaction mixtures are shown in Fig. 3; based on peak areas of PGA and PGyc, the \( \tau \) values are 0.3 and 11 for the E48Q mutant and wild-type enzyme, respectively.

As predicted by the sharp end point in carboxylation at low [substrate]/[enzyme] observed in Fig. 2B, utilization of \([1-^3\text{H}]\text{RuBP}\) by E48Q is complete. Thus, the difference in end point between wild-type and mutant enzymes (Fig. 2B) is fully explained by the skewed partitioning in favor of oxygenation as shown by the chromatographic analysis.

**Side Products of RuBP Turnover Catalyzed by E48Q**—The decline in carboxylase activity of E48Q during the course of assaying and the inverse correlation between end point of CO₂ fixation and [substrate]/[enzyme] ratio could be due to generation of inhibitors. In the case of spinach Rubisco, the time dependent decay of activity during substrate turnover, denoted as fallover, has been attributed to build-up of XUbP and KABP (22, 29, 30). These inhibitors reflect catalytic imperfections of Rubisco, whereby the initial enedioleolate intermediate occasionally undergoes misprotonation at C3 (net epimerization of RuBP to XUbP) or at C2 (net isomerization of RuBP to KABP). Although not inhibitory, pyruvate is also a minor side product formed by \( \beta \)-elimination of phosphate from the terminal PGA carbanion intermediate as opposed to its normal protonation (27).

To gauge the total array of RuBP-derived products, two separate 20-h E48Q reaction mixtures, prepared under conditions which apply to Fig. 2A (i.e. a [substrate]/[active site] ratio of 715), were subjected either to several enzyme-coupled assays (reaction mixture with unlabeled RuBP) or to chromatographic analysis (reaction mixture with \([1-^3\text{H}]\text{RuBP}\)). The quantities of RuBP remaining and PGA, XUbP, and pyruvate formed are compiled in Table I. In the case of wild-type enzyme, RuBP is completely consumed with PGA appearing as the sole major product; the small amount of pyruvate (representing an equivalent per 125 equivalents of RuBP processed) agrees well with published observations (27). In contrast to XUbP formation as occurs with spinach Rubisco, this side product cannot be detected with the wild-type enzyme from *R. rubrum*. As expected from the modest extent of carboxylation as displayed in Fig. 2A, the E48Q reaction mixture contains substantial unused RuBP in addition to PGA (produced in both the carboxylation and oxygenation pathways). Strikingly, the epimerization of RuBP to XUbP is almost as prevalent as normal turnover. Although still a minor side product, pyruvate (as a percentage of RuBP processed) is somewhat elevated in the E48Q reaction mixture as compared to the control.

To verify the values of Table I and to determine the fate of RuBP unaccounted by the summation of analyzed components, an E48Q reaction mixture was also examined by anion-exchange chromatography (Fig. 4A). Of the input RuBP, 33% remains as RuBP, 19% is converted to XUbP, and 26% (the sum of PGA and PGyc) is processed via carboxylation or oxygenation; these amounts are very similar to those provided by the enzyme-coupled assays depicted in Table I. The minor peak (1.5% of starting material) with a retention time of 6 min coincides with authentic pyruvate. An additional 18% of the input RuBP is accounted for by the broad peak at the front and the sharp peak immediately thereafter. These apparently represent spontaneous decomposition products based on their presence in a RuBP control that lacks protein (Fig. 4B). The very small doublet centered at 67 min (1% of starting material) apparently includes KABP, because subsequent to its isolation and reduction with sodium borohydride, rechromatography shows arabinitol 1,5-bisphosphate to be the principal product (data not shown) as reported earlier for KABP derived with the spinach enzyme (31). Among the various isomers of pentulose bisphosphate, only KABP should yield a single pentitol bisphosphate upon reduction; hence, the
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Table I
Enzyme-coupled assays for products of RuBP turnover as catalyzed by wild-type and E48Q Rubisco

| Enzyme | Treatment time (h) | Product | mM |
|--------|-------------------|---------|----|
|        |                   | RuBP    | PGA | XuBP | Pyruvate |
| Wild type | 0                  | 9 (100)* | ND  | 0.02 (0.22)c | ND |
|          | 2                  | ND      | 17.5 (98) | 0.02 (0.22) | 0.075 (0.8) |
| E48Q    | 0                  | 3 (100) | ND  | <0.01 | ND |
|          | 20                 | 0.9 (30) | 1.3 (29)d | 0.6 (20) | 0.033 (1.1)e |

* Numbers in parentheses denote percent of initial RuBP concentration.
† Not detected.
‡ Reflects slight contamination of the RuBP preparation with XuBP.
§ To equate the amount of PGA formed with the amount of RuBP consumed, the former is divided by 1.5, because oxygenation and carboxylation proceed at similar rates under the conditions used.
¶ If based on the amount of RuBP that undergoes carboxylation, this percentage becomes 3.8.

Fig. 4. Anion-exchange chromatographic analyses of a 20-h reaction mixture of E48Q at 0.2 mg/ml and [1-3H]RuBP at 3 mM (Panel A) and an identical 20-h control lacking E48Q (Panel B). Additional details are provided under "Experimental Procedures."

Identity of the second component of the doublet is not known.

The confirmed incomplete utilization of RuBP, as well as its substantial conversion to XuBP, by E48Q at high [RuBP]/[enzyme] accounts for the dependence of the CO2-fixation end point on this ratio (Fig. 2).

Formation and Utilization of XuBP by E48Q—Since the only major end products observed at the low [substrate]/[enzyme] ratio were PGA and PGyC, XuBP must be formed as a transient. For verification, aliquots of a reaction mixture were inspected by anion-exchange chromatography during the progression of turnover of [1-3H]RuBP. As seen in Fig. 5, XuBP appears in substantial amounts in the early stages of RuBP utilization, but eventually is processed completely to PGA and PGyC. The small doublet, which presumably includes KABP, also appears to be converted to normal products.

XuBP, but not RuBP, is a good substrate for aldolase (22). Hence, if aldolase, glycerol phosphate dehydrogenase, and NADH are included in the E48Q reaction mixture, XuBP will be processed by aldolase concomitant with its formation from RuBP by the action of the mutant carboxylase, thereby revealing the maximal extent of epimerization. A product distribution profile of an aldolase-coupled reaction mixture (conditions were the same as those for the reaction mixture depicted in Fig. 5) is shown in Fig. 6. About 32% of the input radioactivity from [1-3H]RuBP chromatographs as glycerol phosphate (from aldolase cleavage of XuBP and subsequent reduction of the resulting dihydroxyacetone phosphate) and about 34% is associated with PGA and PGyC combined. Thus, misprotonation of the RuBP enediol intermediate and its
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FIG. 6. Anion-exchange chromatographic analysis of a 15-min reaction mixture of E48Q at 1 mg/ml and [1-3H]RuBP at 250 µM. The reaction mixture also contained aldolase (0.2 mg/ml), glycerol phosphate dehydrogenase (0.02 mg/ml), and NADH (0.3 mM) so that [3H]XuBP would be processed to [3H]glycerol phosphate and unlabeled glycoaldehyde phosphate as it was formed. Additional details are provided under "Experimental Procedures."

forward processing to PGA and PGyc occur at similar rates.

Under V_max conditions, wild-type R. rubrum Rubisco carboxylates XuBP at 0.1–0.2% the rate observed with RuBP as substrate (0.01 versus 5.2 units/mg). This value is about 10-fold greater than reported earlier for spinach Rubisco (30). By contrast, XuBP is carboxylated by the E48Q mutant 60% as rapidly as RuBP (0.018 versus 0.03 unit/mg) (data not shown). Hence, the mutant is lesser compromised in the processing of the alternate substrate than the normal substrate.

K_m of E48Q for RuBP—Whether based on the direct 14CO2-fixation assay, the enzyme-coupled assay for PGA, or the enzyme-coupled assay for XuBP, the mutant protein exhibited a K_m for RuBP of 90–140 µM. By comparison, the K_m of wild-type Rubisco (R. rubrum) for RuBP is only 10–15 µM.

Catalysis of Rubisco Partial Reactions by E48Q—The enolization of RuBP, independent of overall carboxylation, was monitored as the transfer of tritium from [3-3H]RuBP to water (Fig. 7). Based on initial rates of detritiation of 0.5 mM [3-3H]RuBP, the mutant catalyzes enolization at 10–12% of the wild-type rate (specific activity of 0.3 unit/mg versus 2.8 units/mg).

The ability of E48Q to process the 6-carbon reaction intermediate to PGA was also determined, independent of overall carboxylation, as the conversion of acid-labile radioactivity of [2-14C]carboxy-3-ketoarabinitol 1,5-bisphosphate (the intermediate) to acid-stable radioactivity of PGA. The data in Fig. 8 show that E48Q is virtually as effective as wild-type enzyme in the processing of this reaction intermediate with both proteins exhibiting specific activities of approximately 0.04 unit/mg.

DISCUSSION

This study was prompted by two overriding questions: (1) What is the catalytic significance of the Glu48-Lys329 intersubunit salt bridge at the active site of Rubisco? (2) What is the catalytic role of Glu48? These questions were addressed by characterization of the E48Q mutant in which the salt bridge is abolished by neutralization of the carboxylate with the isosteric amide. Despite this exceedingly modest structural alteration, the consequential catalytic effects are striking. The k_cat is diminished 200-fold; the τ-value is diminished 35-fold (signifying poorer discrimination between CO2 and O2); the K_m (RuBP) is increased 10-fold; and even at saturating levels of RuBP, the time course of CO2-fixation is clearly nonlinear.

Keen interest surrounds the issues of determinants and
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potential modulation of the \( r \) value of Rubisco because of its relevance to efficiency of photosynthetic carbon assimilation and hence biomass yield. Discrimination between \( CO_2 \) and \( O_2 \) occurs at the stage of the enediol(ate) of RuBP, the initial reaction intermediate common to both the carboxylation and oxygenation pathways. Since Rubisco lacks binding sites for the gaseous substrates (32), discrimination must reflect their relative reactivity with the enediol(ate) and the forward commitment factors for the oxygenated and carboxylated intermediates. In the case of the latter (2-carboxy-3-ketoarabinitol bisphosphate), which can be isolated and used as alternate substrate, the forward commitment factor approaches unity (24). However, whether the putative hydroperoxy intermediate is also highly committed in the forward direction or rather can be shunted to the carboxylation pathway by enzyme-catalyzed reversal to the enediol(ate) has not been established. In either event, differential stabilization at the transition states leading to the carboxylated and oxygenated intermediates, respectively, is fundamental to defining the partitioning of enediol(ate) between the two pathways and to altering that partitioning by external intervention.

Mechanistically, the most revealing Rubisco structure is that of the activated spinach enzyme complexed with the reaction-intermediate analogue CABP (6, 8). This structure shows that active-site Lys on the apical residue of mobile loop 6, interacts electrostatically with the carboxylate (derived from substrate \( CO_2 \)) of the bound inhibitor. A logical implication of this interaction is a role of Lys in transition-state stabilization and hence facilitation of the reaction of gaseous substrate with enediol(ate). Similar conclusions emerged independently from site-directed mutagenesis studies, in which enolization activity was retained by position 329 mutants of \( R. \ rubrum \) Rubisco despite loss of carboxylase activity to immeasurable levels (13). More recently (33), the sensitivity of the \( r \) value to the nature of the side chain at position 329 was shown by analysis of a site-directed mutant of \( Synecho-

uccus \ rubiscus \) with an arginyl replacement for the lysyl residues; in this case, the wild-type \( r \) value plunges nearly 200-fold from 56 to 0.3.

In addition to its interaction with the C2 carboxylate of C1BP, the \( \gamma \)-amino group of Lys on the adjacent subunit, as also gleaned from the three-dimensional structure of the activated Rubisco-C1BP complex. Thus, we suggest that the decreased \( r \) value of the E48Q mutant reflects, to some extent, less precise alignment of the Lys on the side chain as a consequence of elimination of the intersubunit electrostatic interaction. This imprecise alignment would then be argued to impact preferentially the stabilization of the transition state for the carboxylated intermediate.

Our laboratory previously reported (34) that a Rubisco variant in which Glu is replaced by a carboxymethyllysineyl residue (achieved by site-directed mutagenesis followed by chemical modification), exhibits a \( r \) value of 2. This was, in fact, the first documented example of a major perturbation of the specificity factor by a desired alteration of an active-site residue. We did not determine the \( r \) value of the non-alkylated mutant (E48C), because of its very feeble activity, but rather emphasized the compromised selectivity in CO2 utilization by the chemically rescued protein. However, with the present characterization of the E48Q mutant, a more complete structure-activity profile emerges. Charge neutralization of the Glu \( \gamma \)-carboxylate (i.e. replacement by an amide group) decreases the \( r \) value from 11 to 0.3; reintroduction of the negative charge, albeit \( \sim 1 \) A farther removed from the peptide backbone, via carboxymethylation of Cys48 proves partially compensatory with a resulting \( r \) value of 2.

In addition to its large influence on enediol(ate) partitioning, the Glu \( \gamma \)-carboxylate enhances carboxylase efficiency by \( >1000 \)-fold \( \left( k_{\text{cat}}/\text{Km} = 2.3 \times 10^4 \text{ m}^{-1} \text{s}^{-1} \right) \) for wild-type enzyme and \( k_{\text{cat}}/\text{Km} = 1.4 \times 10^4 \text{ m}^{-1} \text{s}^{-1} \) for the E48Q mutant. Despite severe impairment of the E48Q mutant in overall carboxylase activity \( \left( k_{\text{cat}} \text{ of } 0.014 \text{ s}^{-1} \text{ compared to } 2.3 \text{ s}^{-1} \text{ for wild type} \right) \), relatively more activity is retained in two key partial reactions. Based on exchange of the C3 proton (tritium label) of RuBP with solvent, the mutant protein is about 10% as effective as wild-type enzyme in facilitating the enolization reaction. This value may, in fact, understate the true rate, because the kinetic isotope effect has not been determined. The E48Q protein also catalyzes the forward processing of 2-\( [\text{C}] \) carboxy-3-ketoarabinitol bisphosphate (the intermediate formed by the reaction of CO2 with the enediol(ate) of RuBP) to an acid-stable product (presumably PGA), and the rate of this conversion is the same as observed with wild-type enzyme. Thus, this mutant, like mutants with replacements for Lys, is preferentially deficient in the formation of carboxylated intermediate from enolized RuBP, leading to the deduction that the catalytic role of the \( \gamma \)-carboxylate of Glu is to facilitate, either directly or indirectly, this discrete step. However, whereas the ionic interaction of Lys on the C2 carboxylate of protein-bound C1BP provides a rather compelling case for a direct involvement in carboxylation, the side chain of Glu is not within direct contact of C1BP. The decrease of activity associated with the Glu to Gln substitution may be an indirect effect of diminished rigidity of the side chain of Lys. Consistent with this viewpoint, the \( k_{\text{cat}} \) of E48Q is 0.6% of wild type in contrast to undetected carboxylase activity with the position 329 mutants.

The level of carboxylase activity displayed by the E48Q mutant is 10-fold greater than our laboratory reported in preliminary characterization (15). This disparity can be reconciled by our earlier failure to appreciate the rapid decline in activity that occurs during the course of an assay. Such a decline or "fallover" is an inherent characteristic of Rubisco from higher plants (e.g. spinach) and in that case is due to partial conversion of RuBP to the potent inhibitors XuBP and KABP (22, 28-31). In addition to direct competition with RuBP in binding to the activated enzyme, these compounds may bind preferentially to noncarbamylated Rubisco, thereby limiting the amount of catalyst available for RuBP turnover (29, 30). The side reactions, like the oxygenase pathway, reflect imperfections in the productive carboxylation of the enediol(ate) intermediate (Fig. 9). Reaction of O2, rather than CO2, with C2 of the enediol(ate) results in oxidative cleavage of RuBP. Protonation at C2, which occurs about once per 400 turnovers, yields the isomerization product KABP; and misprotonation at C3 (on the opposite face of the planar enediol(ate) as required to regenerate RuBP), which also occurs about once per 400 turnovers, yields the epimerization product XuBP (22).

Eimerization of RuBP to XuBP becomes a major pathway catalyzed by the E48Q mutant and proceeds at a rate comparable to carboxylation. Lesser, yet significant, amounts of KABP are also formed during RuBP turnover. Accumulation of these poor alternate substrates, which compete with binding of RuBP, would appear to provide an obvious explanation for the pronounced fallover observed with the mutant protein. However, fallover is not significantly alleviated in the presence of aldolase coupling (data not shown); similarly, aldolase coupling had no effect on fallover of spinach enzyme (28). Thus, unless KABP is bound by the protein much tighter than XuBP, the basis of fallover in the case of the mutant
remains elusive. Irrespective of the basis of fallover, it clearly accounts for the reduced utilization of CO\textsubscript{2} at high molar ratios of RuBP:E48Q (Fig. 2).

The prevalence of side products generated with the mutant protein prompted closer scrutiny of product distributions with wild-type \textit{R. rubrum} Rubisco. At high initial concentrations of RuBP (9 mm) and reaction times sufficient for complete turnover of RuBP by the wild-type enzyme, neither XuBP nor KABP could be found. The chromatographic analyses and additionally the aldolase-coupled assay (for the detection of XuBP) would have readily revealed the side products even if present at levels below those observed with spinach Rubisco. Thus, wild-type Rubisco from \textit{R. rubrum} is more efficient than the spinach enzyme in protecting the enediol(ate) intermediate from misprotonations.

As pointed out in the introduction, an N-terminal segment of the polypeptide (which includes Glu\textsuperscript{48}) and loop 6 (which includes Lys\textsuperscript{329}) fold across the top of the \(\beta\)-\textalpha-barrel, whereby these structural features likely limit ingress and egress of ligands and solvent to and from the active site. Increased flexibility of the N-terminal segment due to elimination of the intersubunit salt bridge between Glu\textsuperscript{48} and Lys\textsuperscript{329} could result in greater access of solvent to the active site and hence more prevalent misprotonation of the enediol(ate) intermediate as occurs with the E48Q mutant. Similarly, the moderate elevation of pyruvate formation by this mutant might reflect increased dissociation of the terminal acyl acid from the enzyme relative to normal protonation. Precedence for this speculation is provided by mutagenesis of triose phosphate isomerase, a prototypical \(\beta\)-\textalpha-barrel protein, which also includes a flexible loop at the top of the barrel. Truncation of the loop results in extensive release of the triose phosphate enediol intermediate from the active site and consequent \(\beta\)-elimination of phosphate to yield methyl glyoxal (35).

Interestingly, disruption of the Lys\textsuperscript{329}-Glu\textsuperscript{48} linkage by replacement of the lysyl residue does not result in significant stimulation of epimerase activity (13) as occurs with the E48Q mutant. How might these contrasting properties be reconciled even though both classes of mutants lack the same intersubunit interaction? We suggest that positioning of the N-terminal segment at the top of the barrel domain is crucial to minimizing misprotonations of the enzyme-bound enediol(ate). Perhaps this positioning is relatively unperturbed in position 329 mutants due to an alternate interaction of Glu\textsuperscript{48} with Lys\textsuperscript{168}. Of course, when Glu\textsuperscript{48} is replaced, all opportunity for intersubunit electrostatic interaction is lost.

Stabilization of the closed conformation of loop 6 may be only marginally dependent on the Lys\textsuperscript{329}-Glu\textsuperscript{48} bridge due to other key interactions. For example, site-directed mutagenesis has shown that hydrophobic interactions involving Phe\textsuperscript{207} of loop 6 favor the closed conformation (36). Another consideration is the lack of reactivity toward CO\textsubscript{2} of the enzyme-bound enediol(ate) in the case of position 329 mutants, whereas the E48Q mutant retains slight carboxylase activity. This difference suggests a correlation between reactivity of enediol(ate) and the potential for its misprotonation.

In summary, our studies illuminate the multifaceted role of Glu\textsuperscript{48} at the active site of Rubisco and dramatize the differences in functional consequences between replacement of the anionic versus the cationic group of the Lys\textsuperscript{329}-Glu\textsuperscript{48} intersubunit interaction. The \(\gamma\)-carboxyglycate of Glu\textsuperscript{48} contributes not only to the overall carboxylase efficiency but also influences the carboxylation-oxygenation partitioning ratio and mitigates against misprotonation of enediol(ate) intermediate.

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