CONF-9508202--1

CATALYTIC ROLES OF FLEXIBLE REGIONS AT THE ACTIVE SITE OF RIBULOSE-BISPHOSPHATE CARBOXYLASE/OXYGENASE (RUBISCO)

Fred C. Hartman, Mark R. Harpel, Yuh-Ru Chen, Eric M. Larson, and Frank W. Larimer. Protein Engineering Program, Biology Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831 USA

1. Introduction

Chemical and mutagenesis studies of Rubisco have identified Lys329 and Glu48 as active-site residues that are located in distinct, interacting domains from adjacent subunits (see Refs. 1 and 2 for reviews). Crystallographic analyses (3,4) have shown that Lys329 is the apical residue in a 12-residue flexible loop (loop 6) of the β,α-barrel domain of the active site and that Glu48 resides at the end of helix B of the *N*-terminal domain (also a flexible region) of the active site. When phosphorylated ligands are bound by the enzyme, loop 6 adopts a closed conformation and, in concert with repositioning of helix B, thereby occludes the active site from the external environment. In this closed conformation, the γ-carboxylate of Glu48 and the ε-amino group of Lys329 engage in intersubunit electrostatic interaction. By use of appropriate site-directed mutants of *Rhodospirillum rubrum* Rubisco, we are addressing several issues: (a) the catalytic roles of Lys329 and Glu48, (b) the functional significance of the intersubunit salt bridge comprised of these two residues, and (c) the roles of loop 6 and helix B in stabilizing labile reaction intermediates. Characterization of novel products derived from misprocessing of D-ribulose-1,5-bisphosphate (RuBP) by the mutant proteins have illuminated the structure of the key intermediate in the normal oxygenase pathway.

2. Procedures

2.1 Materials

Rubisco from *R. rubrum* was purified to homogeneity by chromatography on DEAE-cellulose (5). The genes encoding the K329A, E48Q, and loop-deletion mutants were expressed in *E. coli* MV1190; the mutant proteins were purified to homogeneity by anion-exchange chromatography on Mono Q (1 cm × 10 cm) (6–8). [2′-^{14}C]Carboxy-3-ketoarabinitol-1,5-bisphosphate (CKBP) was provided by Dr. George H. Lorimer of DuPont.

2.2 Assays

$^{14}CO_2$-fixation as 3-phospho-D-glycerate (PGA) was determined by a filter disk method. The enolization partial reaction was measured as detritiation of [3-^{3}H]RuBP. Hydrolysis of CKBP, the carboxylated reaction intermediate, was assayed as the difference between total borohydride-stabilized and acid-stable radioactivity. The total array of products derived from [1-^{3}H]RuBP turnover by wild-type and mutant Rubiscos was analyzed by chromatography of reaction mixtures on Mono Q (5 mm × 50 mm) equipped with on-line monitoring of radioactivity. By use of substrate labeled at C1, the ratio of radioactivity associated with the PGA and phosphoglycolate (PGyc) provides a direct measurement of the carboxylation/oxygenation partitioning ratio. Detailed procedures for each of these assays have been published (6–9).

3. Results and Discussion

The impacts of abolishing the intersubunit salt bridge and of truncation of loop 6 on overall catalysis and on discrete partial reactions are summarized in the table. Even

Characterization of Mutant Proteins

Protein	k_{cat}[a] (% w.t.)	Enolization[b] (% w.t.)	K_m(RuBP)[c] (μM)	Intermediate Turnover[d] (% w.t.)
Wild-type	100	100	10	100
E48Q	0.5	10	100	100
K329A	0.03	5	300	10
Loopless[e]	<0.001	5	1000	10

[a] For overall carboxylation
[b] Of RuBP
[c] Determined with assay for enolization
[d] Processing of CKBP
[e] Truncated-loop mutant in which Gly326 and Ser334 are bridged with two glycyl residues (*i.e.* a net deletion of five residues from the wild-type enzyme)

though each of the engineered changes severely impairs carboxylase activity, neutralization of the negative charge at position 48 is not as debilitating as neutralization of the positive charge at position 329. The loop-deletion mutant lacks detectable levels of carboxylase activity, demonstrating that loop 6 per se facilitates catalysis >10-fold independently of the contribution provided by Lys329. Each of the three mutants displays an increased K_m value for RuBP (presumably denoting weaker binding) but retains considerable activity in enolization of RuBP (the initial step of overall catalysis)

and in processing CKBP (the terminal steps in overall catalysis). Thus, disruption of the salt bridge or truncation of loop 6 leads to preferential impairment of the carboxylation of the enediol intermediate. A direct role of Lys329 in the carboxylation step is consistent with the crystal structure of the enzyme (3,4), which shows ionic interaction of the lysyl ε-amino with the carboxylate of bound 2-carboxyarabinitol-1,5-bisphosphate (a stable reaction-intermediate analogue of CKBP) (10). The differential impact of substitution for Glu48 on the carboxylation step may reflect lesser rigidity in the side chain of Lys329 in the absence of the usual interresidue electrostatic interaction.

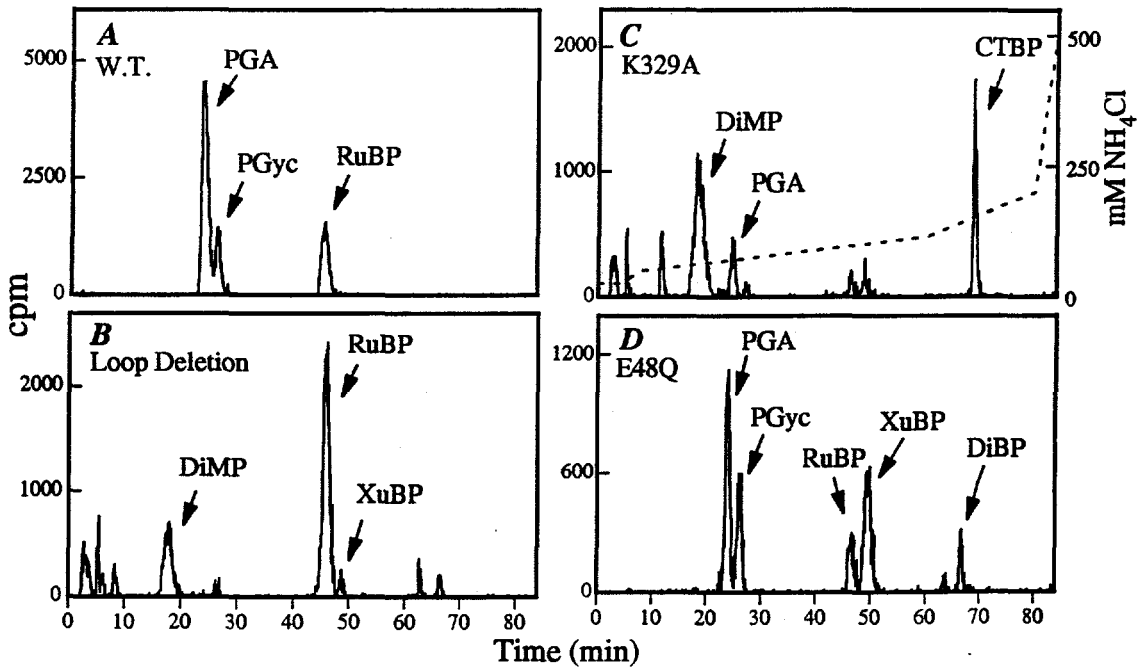

Figure 1: Anion-exchange chromatographic analyses on Mono Q of reaction mixures initially containing 250 μM [1-^{3}H]RuBP and wild-type enzyme at 20 μg/mL (A), loop-deletion mutant at 1 mg/mL (B), K329A at 1 mg/mL (C), or E48Q at 1 mg/mL (D). Incubation periods at room temperature were 8 min for wild-type enzyme, 2 hr for the loop-deletion and K329A mutants, and 10 min for E48Q. The concentration of $NaHCO_3$ was 5 mM with wild-type enzyme, 25 mM with the loop-deletion and K329A mutants, and 66 mM with E48Q. All reaction mixtures (pH 8.0) also contained 50 mM Bicine, 10 mM, $MgCl_2$, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 2% glycerol. The gradient depicted only in panel C was used throughout.

Normal turnover of RuBP by Rubisco entails several labile reaction intermediates (see Refs. 1 and 2 for detailed discussions of the reaction pathway). Thus, the impairment of the mutant proteins in overall catalysis, despite their catalytic competence in several partial reactions, could be due partially to their inability to properly stabilize or prevent

dissociation of the labile intermediates. To explore these possibilities, we have analyzed incubations of the mutants with [1-^{3}H]RuBP by Mono Q chromatography. An analysis of a wild-type reaction mixture, quenched prior to complete utilization of RuBP, is shown in Fig. 1A. As anticipated, the only products detected are PGA and PGyc, and transient intermediates do not accumulate.

In the case of the loop-deletion mutant (Fig. 1B), the only significant turnover product is 1-deoxy-D-*glycero*-2,3-pentodiulose 5-phosphate (DiMP), formed by β-elimination of phosphate from the 2,3-enediol of RuBP (Fig. 2). Although never observed with wild-type Rubisco, DiMP is generated by mutants with replacements for residues that constitute the binding pocket for the C1 phosphate group of RuBP (11,12). Given the absence of forward processing of the enediol and its compromised binding due to the truncated loop 6, this intermediate is relegated to dissociation from the active site and subsequent decomposition. Trace amounts of xylulose-1,5-bisphosphate (XuBP), signifying misprotonation of the enediol intermediate at C2 (Fig. 2), as occurs with wild-type spinach Rubisco (13) but not the wild-type *R. Rubrum* enzyme (7), also appear. The small peaks preceding elution times of 10 min are due to slight phosphatase contamination in the enzyme preparation. Upon more prolonged incubation, the RuBP is completely consumed, but DiMP remains as the only significant product (data not shown).

DiMP is also generated by the action of K329A on RuBP; however, substantial amounts of 2-carboxytetritol-1,4-bisphosphate (CTBP) appear as well (Fig. 1C). The structure of this novel compound (Fig. 2) was solved by the techniques of NMR, MS, and periodate oxidation (14). CTBP is not formed in the absence of oxygen and thus appears to reflect misprocessing of an oxygenase intermediate. The small amount of PGA detected by chromatography is consistent with residual carboxylase activity of K329A as determined by the CO_2-fixation assay.

Neither DiMP nor CTBP is found in the E48Q reaction mixture, but, as expected from the relatively greater retention of carboxylase activity, PGA and PGyc appear in quantifiable amounts (Fig. 1D). Substantial peaks of XuBP and the novel D-*glycero*-2,3-pentodiulose-1,5-bisphosphate (DiBP) are also observed. Both of these aberrant products (Fig. 2) must be classified as transients, because ultimately they are converted to PGA and PGyc exclusively (15,16). Although K329A and E48Q both lack the same intersubunit salt bridge, the latter mutant displays a much greater propensity to misprotonate the enediol of RuBP. Perhaps E48Q (with a normal loop 6) remains effective in stabilizing the bound enediol (hence, β-elimination is minimized), but because of slowed forward processing of enediol and consequently a longer residence time, the opportunity for misprotonation is increased.

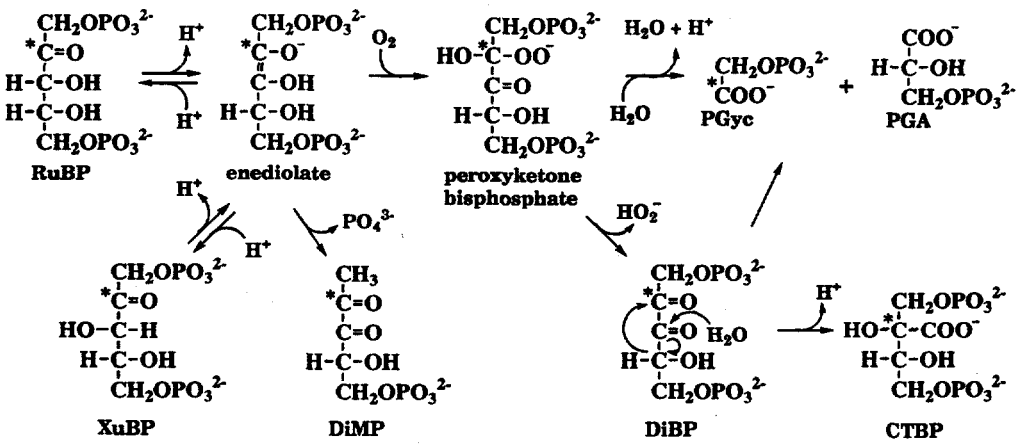

Figure 2: Fates of the enediol and peroxyketone reaction intermediates during RuBP turnover by wild-type or mutant Rubiscos.

The structure of DiBP was deduced on the basis of product analysis subsequent to its reaction with *o*-phenylenediamine, hydrogen peroxide, or sodium borohydride (15). As dictated by DiBP representing oxidation of the C3 hydroxyl of RuBP to a ketone, the formation of DiBP by E48Q requires O_2. Oxidation of RuBP by molecular oxygen to form a 2,3-pentodiulose must entail a C2 peroxy intermediate as invoked in the initial characterization of the Rubisco oxygenase pathway that leads to PGA and PGyc (17) (Fig. 2). As predicted by a peroxy precursor, hydrogen peroxide is formed concomitantly and stoichiometrically with DiBP (data not shown). The discovery of DiBP provides a direct signature of the postulated, but heretofore unproven, oxygenase intermediate in the oxidative cleavage of RuBP as catalyzed by wild-type Rubisco.

Armed with the information that both DiBP and CTBP, although generated by different mutants, were derived from the oxidation of RuBP, we suspected an interrelationship. Based on analogies in organic chemistry, CTBP would be the product of DiBP having undergone a benzylic-acid-type rearrangement (Fig. 2). As shown in the companion paper (16), both K329A and wild-type *R. Rubrum* Rubisco catalyze the conversion of purified DiBP to CTBP.

These studies not only illustrate the importance of the intersubunit salt bridge and the conformational integrity of loop 6 in the stabilization of labile reaction intermediates, they unexpectedly provide confirmation of a peroxyketone intermediate in the normal oxygenase pathway. Significantly, stabilization of this intermediate is required to direct its cleavage to PGA and PGyc. Although this oxidation pathway is energy wasteful, the normal products are partially shunted back into the Calvin cycle. In the absence of such stabilization (*i.e.* as witnessed with K329A and E48Q), the peroxyketone intermediate would yield DiBP and/or CTBP. As likely dead-end products, these would impact the energy economy of the plant cell far more severely.

4. Acknowledgement

This research was sponsored by the Office of Health and Environmental Research, United States Department of Energy, under Contract DE-AC05-84OR21400 with Lockheed Martin Energy Systems, Inc.

References

1 Hartman, F.C. and Harpel, M.R. (1993) *Adv. Enzymol.* **67**, 1–75
2 Hartman, F.C. and Harpel, M.R. (1994) *Annu. Rev. Biochem.* **63**, 197–234
3 Knight, S., Andersson, I. and Brändén, C.-I. (1990) *J. Mol. Biol.* **215**, 113–160
4 Schneider, G., Lindqvist, Y. and Brändén, C.-I. (1992) *Annu. Rev. Biophys. Biomol. Struct.* **21**, 119–143
5 Schloss, J.V., Phares, E.F., Long, M.V., Norton, I.L. Stringer, C.D. and Hartman, F.C. (1982) *Methods Enzymol.* **90**, 522–528
6 Harpel, M.R. and Hartman, F.C. (1994) *Biochemistry* **33**, 5553–5561
7 Lee, E.H., Harpel, M.R., Chen, Y.-R. and Hartman, F.C. (1993) *J. Biol. Chem.* **268**, 26583–26591
8 Larson, E.M., Larimer, F.W. and Hartman, F.C. (1995) *Biochemistry* **34**, 4531–4537
9 Harpel, M.R., Lee, E.H. and Hartman, F.C. (1993) *Anal. Biochem.* **209**, 367–374
10 Pierce, J., Tolbert, N.E. and Barker, R. (1980) *Biochemistry* **19**, 934–942
11 Larimer, F.W., Harpel, M.R. and Hartman, F.C. (1994) *J. Biol. Chem.* **269**, 11114–11120
12 Morell, M.K., Paul, K., O'Shea, N.J., Kane, H.J. and Andrews, T.J. (1994) *J. Biol. Chem.* **269**, 8091–8098
13 Edmondson, D.L., Kane, H.J. and Andrews, T.J. (1990) *FEBS Lett.* **260**, 62–66
14 Harpel, M.R., Serpersu, E.H., Lamerdin, J.A., Huang, Z.-H., Gage, D.A. and Hartman, F.C. (1995) *Biochemistry* **34**, in press
15 Chen, Y.-R. and Hartman, F.C. (1995) *J. Biol. Chem.* **270**, 11741–11744
16 Harpel, M.R., Chen, Y.-R. and Hartman, F.C. these proceedings
17 Lorimer, G.H., Andrews, T.J. and Tolbert, N.E. (1973) *Biochemistry* **12**, 18–23