Articles

Mechanistic Insights Provided by Deletion of a Flexible Loop at the Active Site of Ribulose-1,5-bisphosphate Carboxylase/Oxygenase†

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ABSTRACT: To evaluate the functions of flexible loop 6 at the active site of *Rhodospirillum rubrum* D-ribulose-1,5-bisphosphate carboxylase/oxygenase, the loop was truncated by cassette mutagenesis, whereby seven residues of the twelve-residue loop were excised and replaced by two glycyl residues. The purified loop-deletion mutant was totally devoid of carboxylase activity, but retained substantial catalytic competency in the enolization of ribulose bisphosphate (the initial step in the overall carboxylase pathway) and in normal processing of the six-carbon carboxylated intermediate (the terminal steps in the overall carboxylase pathway). Hence, catalytic impairment resides predominantly at the stage of carboxylation of the initial enediol(ate), a conclusion compatible with mechanistic deductions derived from crystallographic analyses. A critical role of loop 6 in the stabilization of the transition state for carboxylation is reinforced by the findings that the loop-deletion mutant displays preferentially compromised affinity for an analogue of the carboxylated intermediate relative to ribulose bisphosphate and that the mutant converts the substrate to a dicarbonyl compound as a consequence of β-elimination of phosphate from the initial enediol(ate).

Flexible loops that occlude active sites during catalysis are recognized as structural elements common to many enzymes (Clarke *et al.*, 1986; Gerstein & Chothia, 1991; Sampson & Knowles, 1992; First & Fersht, 1993; Tanaka *et al.*, 1993; Falzone *et al.*, 1994; Yüksel *et al.*, 1994). Such loops can serve in binding and orienting ligands, stabilizing transition states and reactive intermediates, excluding solvent from the active site, and ensuring proper throughput of substrate to product.

The interfacial active site of ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39), the CO_2 fixation enzyme ubiquitous to photosynthetic organisms, includes a flexible loop (loop 6) (residues 324−335[1]) that is positioned at the top of the eight-stranded β/α-barrel domain [for reviews of Rubisco,[2] see Andrews and Lorimer (1987), Schneider *et al.* (1992), Hartman and Harpel (1993, 1994), and Spreitzer (1993)]. The activated form of Rubisco (a Mg^{2+}-stabilized carbamate) exhibits avid affinity for the reaction intermediate analogue CABP, whereby the resulting quaternary complex is virtually inert to exchange (Miziorko & Sealy, 1980; Pierce *et al.*, 1980). Crystallographic analyses reveal that this exchange inertness is due in part to loop 6 adopting a closed conformation in which it partially covers the top of the barrel domain and approaches another flexible loop from the *N*-terminal domain of the adjacent subunit (Knight *et al.*, 1990; Schreuder *et al.*, 1993a,b; Newman & Gutteridge, 1993). In combination, these two loops completely sequester bound Mg^{2+}, activator CO_2, and CABP from external solvent (Figure 1, left panel). By contrast, either loop 6 is observed in an open conformation, as in the case of the nonactivated form of the tobacco enzyme (Curmi *et al.*, 1992), or it lacks electron density altogether, as in the case of *Rhodospirillum rubrum* Rubisco (Schneider *et al.*, 1990). Given the demonstrated flexibility of loop 6 and the fact that RuBP cannot access the active site when loop 6 is closed, loop movement is portrayed as a dynamic event during catalytic turnover. However, correlation of closing and opening of the loop with progression of the reaction coordinate is lacking.

The apical residue of loop 6, Lys329, is critical to catalysis. Crystallographic analysis of the activated enzyme•CABP complex shows that the ϵ-amino group interacts electrostatically with both the γ-carboxylate of Glu48 from the adjacent subunit and the carboxylate (derived from substrate CO_2) of the bound inhibitor (Knight *et al.*, 1990). Thus, this lysyl amino group appears uniquely positioned to contribute to the stabilization of the closed-loop conformation and concomitantly to the stabilization of transition states for carboxylation or oxygenation of the enediol(ate) of RuBP. Functional analyses of position 329 mutants of the *R. rubrum* enzyme are entirely supportive of these structure-based suppositions. Although severely impaired in carboxylase

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[1] Residue numbers refer to positions in the *Rhodospirillum rubrum* enzyme.

[2] Abbreviations: RuBP, D-ribulose 1,5-bisphosphate; XuBP, D-xylulose 1,5-bisphosphate; PGA, 3-phospho-D-glycerate; CABP, 2-carboxy-D-arabinitol 1,5-bisphosphate; PGyc, phosphoglycolate; Rubisco, D-ribulose-1,5-bisphosphate carboxylase/oxygenase.

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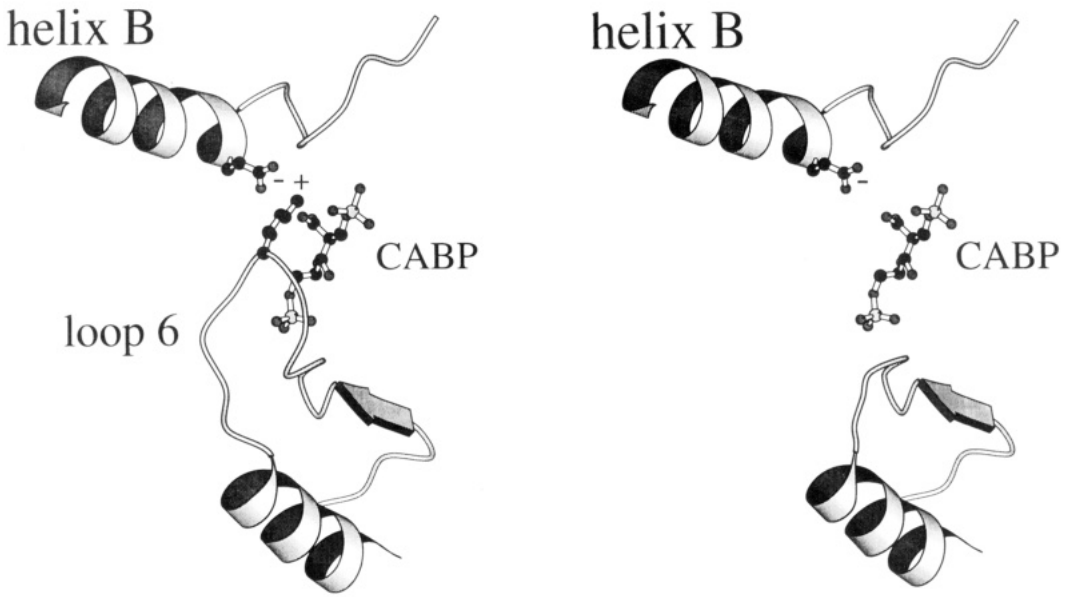

FIGURE 1: Computer graphics representation of mobile loops (loop 6 of one subunit and α-B from the adjacent subunit) at the solvent-accessible end of the β/α-barrel domain of the active site of Rubisco. The left panel depicts the closed-loop conformation of the CO_2/Mg^{2+}-activated enzyme with CABP bound; the interfacial salt bridge comprises Lys329 of loop 6 and Glu48 of α-B. The right panel illustrates the same structural elements, but with a truncated loop 6. Models are based on the atomic coordinates of the activated enzyme from tobacco (Schreuder *et al.*, 1993b) and are displayed using the program MOLSCRIPT (Kraulis, 1991).

activity, the K329G mutant proficiently catalyzes the enolization of RuBP (Hartman & Lee, 1989) and normal processing of the carboxylated intermediate to PGA (Lorimer *et al.*, 1993). Clearly, the mutant is preferentially disabled at the stage of carboxylation of the enediol(ate) of RuBP. Additionally, the carboxylation/oxygenation partitioning ratio is exquisitely sensitive to the length of the aminoalkyl side chain at position 329, which can be systematically varied by appropriate chemical modification of the K329C mutant (Smith & Hartman, 1988; Lorimer *et al.*, 1993). Formation of aberrant side products from RuBP by position 329 mutants apparently reflects destabilization of the closed-loop conformation and consequential escape of reactive intermediates from the active site. For example, when RuBP is incubated with K329A, the major products are 1-deoxy-D-*glycero*-2,3-pentodiulose 5-phosphate derived from β-elimination of phosphate from the enediol(ate) intermediate and an incompletely characterized compound derived from an intermediate of the oxygenase pathway (Harpel & Hartman, 1994). Neither of these side products is formed during RuBP turnover by wild-type enzyme.

Residues at the hinges of loop 6 also influence the carboxylation/oxygenation partitioning ratio (Chen *et al.*, 1991; Parry *et al.*, 1992; Gutteridge *et al.*, 1993), and Phe327 of the *R. rubrum* enzyme, although not a species-invariant residue, enhances the stability of the closed-loop conformation (Day *et al.*, 1993).

Beyond structure-based inferences, the functional roles of loop 6 in its entirety have not been explored directly. Outstanding issues include the participation (or lack thereof) of loop 6 in subunit−subunit association, substrate binding and orientation, and partial reactions apart from the addition of CO_2 or O_2 to the enediol(ate) intermediate. To address these issues, we have constructed and characterized a truncated-loop mutant of the *R. rubrum* enzyme in which Gly326 is bridged to Ser334 with two glycyl residues (*i.e.*, a net deletion of five residues from the wild-type enzyme) (Figure 1, right panel).

EXPERIMENTAL PROCEDURES

Materials. Common laboratory reagents and biological compounds for enzyme assays were procured at the highest level of purity readily available. Unlabeled RuBP, [3-^{3}H]-RuBP, [1-^{3}H]RuBP, and [2-^{14}C]CABP were synthesized by literature procedures (Horecker *et al.*, 1958; Hartman & Lee, 1989; Kuehn & Hsu, 1978; Pierce *et al.*, 1980). Sodium [^{14}C]bicarbonate and sodium [^{14}C]cyanide were purchased from ICN. [2-^{14}C]-2-Carboxy-3-ketoarabinitol 1,5-bisphosphate was a generous gift from Dr. George H. Lorimer of DuPont. Wild-type Rubisco from *R. rubrum* and the recombinant K329A mutant were isolated as previously described (Schloss *et al.*, 1982; Harpel & Hartman, 1994). The loop-deletion mutant was isolated from *Escherichia coli* MV1190 that had been transformed with vector pFL372 (see the following) carrying the appropriately modified *rbc* gene. Transformed bacteria were cultured in 2X YT medium (Sambrook *et al.*, 1989) containing 1% glycerol and 50 mg/L ampicillin. Cultures were harvested 2.5 h after induction with 0.1 mM isopropyl β-D-thiogalactopyranoside. The loop-deletion mutant was purified by anion-exchange chromatography (Pharmacia Mono Q HR 10/10, 10 mm × 100 mm) as described earlier for other Rubisco mutants (Mural *et al.*, 1990; Harpel *et al.*, 1991). Both wild-type and mutant proteins were stored frozen at −80 °C in pH 8.0 activation buffer (50 mM bicine, 10 mM $MgCl_2$, 1 mM EDTA, and 66 mM $NaHCO_3$) containing 10 mM 2-mercaptoethanol and 20% glycerol. As needed, aliquots were thawed and freed of glycerol and 2-mercaptoethanol by dialysis against activation buffer. Protein concentrations were based on the $A_{280\ nm}$ and an extinction coefficient of 1.2 for 1 mg/mL (Schloss *et al.*, 1982).

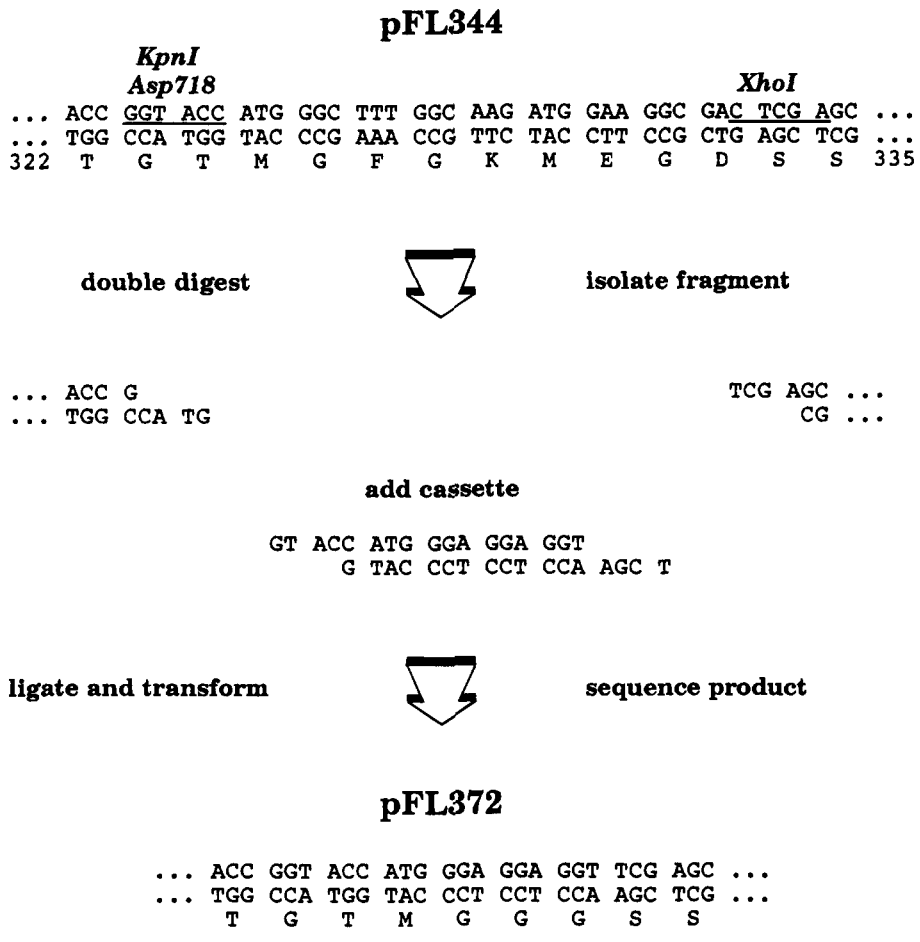

FIGURE 2: Diagrammatic representation of construction of the gene for the loop-deletion mutant.

Enzyme Assays. All assays were conducted at 23−25 °C in air-equilibrated buffers and thus included O_2 at 255 μM. Activation buffer, but with the bicarbonate concentration reduced to 25 mM (311 μM CO_2 based on a pK_{a1} for H_2CO_3 of 6.1) unless noted otherwise, was used in each type of assay and in reaction mixtures for determining product distributions, as will be described later.

$^{14}CO_2$ fixation activity was determined by a modified filter disk assay (Niyogi *et al.*, 1986).

The enolization partial reaction was measured as detritiation of [3-^{3}H]RuBP with slight modification (Hartman & Lee, 1989) of the original procedure (Sue & Knowles, 1982). Reactions were initiated by the addition of substrate to solutions of enzyme, and periodically, 15-μL aliquots were quenched with 100 μL of 100 mM $NaBH_4$. 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 H_2O prior to scintillation counting.

Hydrolysis of the six-carbon reaction intermediate, [2-^{14}C]-2-carboxy-3-ketoarabinitol 1,5-bisphosphate, was assayed as the difference between total borohydride-stabilized and acid-stable radioactivity (Pierce *et al.*, 1986). The reactions were carried out with either wild-type (0.1 μM active site) or loop-deletion (1.2 μM active site) protein. 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-^{14}C]PGA and unlabeled RuBP were also introduced at final concentrations of 2.31 and 10.4 μM, respectively. Periodically, duplicate aliquots (25 μL) were removed and quenched with either 10% formic acid or 0.1 M $NaBH_4$ containing 1% (w/v) sodium dodecyl sulfate. Following decomposition of excess $NaBH_4$ with 10% formic acid, both sets of samples were dried, and the residues were redissolved in 0.5 mL of H_2O prior to counting.

Product Determination. Analysis of turnover products from [1-^{3}H]RuBP was accomplished by anion-exchange chromatography (Harpel *et al.*, 1993). Reaction mixtures (250 μL) were deproteinated with Amicon Centricon-10 filters and were then chromatographed at pH 8.0 on a column of Mono Q (Pharmacia HR5/5, 5 × 50 mm) with a gradient of NH_4Cl (as depicted in Figure 5) containing a fixed concentration of sodium borate at 10 mM. Eluted radioactivity was monitored continuously by flow-through radiometric detection (IN/US β-RAM).

Construction of the Vector Encoding the Mutant rbc Gene. Comparisons of the crystal structures of activated and nonactivated Rubisco from tobacco (Schreuder *et al.*, 1993a) show that the α-carbons corresponding to residues 326 and 334 of the *R. rubrum* enzyme remain 5.7 Å apart, despite a complex reorientation of the intervening residues upon CO_2/Mg^{2+}-induced activation and subsequent binding of CABP. Thus, to minimize distortion of the β/α-barrel concomitant with excision of the mobile portion of loop 6, we chose to bridge the 5.7-Å gap with two glycyl residues. The desired plasmid was constructed by cassette mutagenesis as diagrammed in Figure 2. An *Xma*I−*Bgl*II restriction fragment (nucleotides 192−1341) of the *R. rubrum rbc* gene was excised from the mutagenesis vector pFL105 (Soper *et al.*, 1988) and subcloned into the *rbc* expression vector pFL245 (Larimer *et al.*, 1990), with concomitant replacement of the

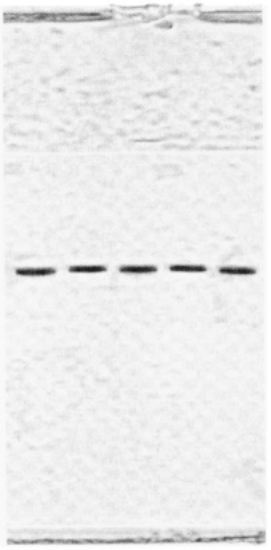

FIGURE 3: Computer reproduction of Coomassie Blue-stained nondenaturing polyacrylamide gel (20% Phast-Gel from Pharmacia) of the purified wild-type Rubisco (wt) and the loop-deletion mutant (del). The sample load for each lane was 200 ng of protein. The gel image was digitized at 600 dpi using an Apple Color OneScanner.

corresponding segment of the latter. The resulting expression vector was designated pFL344. pFL105 was chosen as the parent plasmid, because it contains introduced *Asp*718/*Kpn*I and *Xho*I sites that bracket the coding region for loop 6 of Rubisco. The selection of pFL245 as the subcloning host was based on the previously reported high-yield expression of the wild-type *rbc* gene therein. Cleavage of pFL344 with *Asp*718 and *Xho*I excised the segment encoding Thr324-Ser334. A double-stranded oligonucleotide cassette, encoding Thr-Met-Gly-Gly-Gly-Ser, was ligated into the gapped plasmid by use of T4 DNA ligase. The sequence of the final construct, pFL372, was confirmed by dideoxy terminator sequencing (Sanger *et al.*, 1977).

RESULTS

General Characterization of the Mutant Protein. On the basis of recovery of the purified loop-deletion mutant from transformed *E. coli* (25 mg/13 g of cells), the gene encoding this protein is expressed from plasmid pFL372 with high efficiency similar to that of genes for the wild-type Rubisco and for single-residue substitutions as reported previously (Larimer *et al.*, 1990). This efficiency translates into 5−8% of the total soluble protein in cellular extracts represented as Rubisco. The electrophoretic mobilities of wild-type enzyme and loop-deletion mutant on nondenaturing polyacrylamide gels are virtually identical, signifying that mutant subunits properly associate as a dimer (Figure 3). Thus, gross conformational changes, which would likely be reflected by the proteolysis of mutant subunits during residence in *E. coli* and by their inability to form a stable dimer, do not appear to accompany the removal of loop 6.

Neither carboxylase activity nor complexation of CABP can be detected with the loop-deletion mutant. Given the inherently low k_{cat} (~5 s^{-1}) of wild-type enzyme, the threshold for detection and reliable measurement of carboxylase activity is about 0.001% of wild-type. Binding of CABP by a mutant Rubisco will escape detection if the complex is too weak to survive the 5 min required for rapid gel filtration.

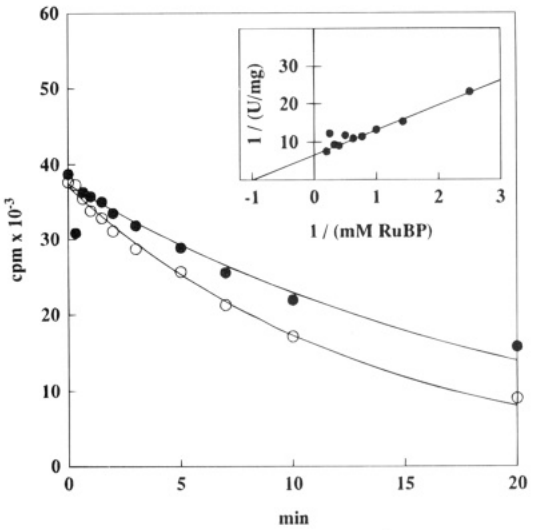

FIGURE 4: Time course of detritiation of [3-^{3}H]RuBP (3 mM) by wild-type Rubisco (40 μg/mL) (○) and by the loop-deletion mutant (1 mg/mL) (●). The rate dependence of the latter on the concentration of RuBP is shown in the inset.

Catalysis of Partial Reactions by the Mutant Protein. The enolization of RuBP by the loop-deletion mutant was assayed as the transfer of tritium from [3-^{3}H]RuBP to water (Figure 4). Despite its total impairment of carboxylase activity, the mutant catalyzes enolization at ~5% of the wild-type rate (specific activity of 0.15 *versus* 3.0 units/mg protein). The K_m for RuBP in the enolization reaction is 1 mM, in contrast to 11 μM with wild-type enzyme (Hartman & Lee, 1989). As the activators CO_2 and Mg^{2+} are required for the enolization of [3-^{3}H]RuBP by the mutant, it must undergo normal carbamate formation analogously to wild-type enzyme.

The ability of the loop-deletion mutant to process [2-^{14}C]-2-carboxy-3-ketoarabinitol 1,5-bisphosphate (the carboxylated reaction intermediate) to PGA was examined as the conversion of acid-labile radioactivity to acid-stable radioactivity (Pierce *et al.*, 1986). In the presence of mutant protein (63 μg/mL, 1.2 μM subunit), 70% of the input intermediate (1.5 μM) is hydrolyzed to PGA, and 30% is decarboxylated during a 10-min incubation. This forward commitment factor of 0.7 contrasts to >0.9 for wild-type enzyme, consistent with the original report under similar conditions (Pierce *et al.*, 1986). Deactivated wild-type enzyme only catalyzes decarboxylation of the intermediate (Pierce *et al.*, 1986). Thus, the somewhat reduced commitment factor of the mutant compared to that of wild-type enzyme, both under activation conditions, could reflect incomplete carbamate formation of the former. Although our data do not allow a distinction between this possibility and actual catalysis of decarboxylation by the activated mutant protein, the mechanistically relevant observation is the predominant forward processing of the intermediate.

Product Analyses. Failure of RuBP, which has been enolized by the loop-deletion mutant, to undergo carboxylation as required for conversion to normal products raises the prospects of misprotonation and decomposition of the enediol(ate). Even with wild-type Rubisco from spinach, misprotonation of the enzyme-bound enediol(ate) at C3 to form XuBP occurs to a limited extent (Edmondson *et al.*,

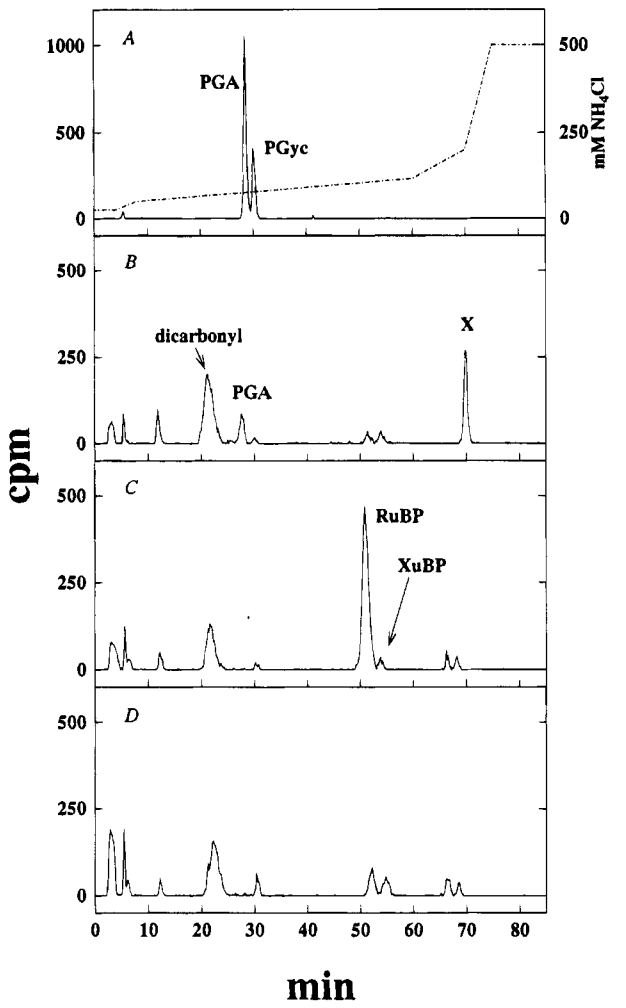

FIGURE 5: Anion-exchange chromatographic analyses of reaction mixtures initially containing 250 μM [1-^{3}H]RuBP and wild-type enzyme at 20 μg/mL (A), the K329A mutant at 1 mg/mL (B), and the loop-deletion mutant at 1 mg/mL (C and D). Incubation periods were 30 min for the wild-type enzyme, 2 h for the K329A mutant, and 2 (C) or 6 h (D) for the loop-deletion mutant. The standard concentration of 25 mM $NaHCO_3$ was lessened to 4 mM for the wild-type reaction mixture in order to elevate the amount of PGyc formed. The gradient depicted only in panel A was used throughout. Additional details are provided under Experimental Procedures.

1990), and decomposition of the enediol(ate) has been reported with the *R. rubrum* K329A mutant (Hartman & Harpel, 1994) and with those carrying substitutions in the binding site for the C1 phosphate group of RuBP (Larimer *et al.*, 1994; Morell *et al.*, 1994).

To gauge turnover of RuBP by the loop-deletion mutant, reaction mixtures with [1-^{3}H]RuBP were analyzed by anion-exchange chromatography on Mono Q. Wild-type enzyme and the K329A mutant were used for comparisons. As seen in Figure 5A, the sole products observed with the wild-type enzyme are PGA and PGyc. Consistent with an earlier report (Hartman & Harpel, 1994), the K329A mutant converts RuBP primarily to a dicarbonyl (1-deoxy-D-*glycero*-2,3-pentodiulose 5-phosphate) and compound X (Figure 5B), which was previously shown by partial characterization to be derived from an oxygenase intermediate. The peaks appearing early (3−12 min) in the chromatogram represent hydrolysis products due to trace contamination of the enzyme preparation by phosphatases. The slight carboxylase activity, as detected in the standard $^{14}CO_2$ fixation assay, is verified by the presence of PGA in the chromatogram. The major product derived from the turnover of RuBP by the loop-deletion mutant is the dicarbonyl; a smaller amount of XuBP is clearly discernible (Figure 5C, D). Despite its appearance in small quantities, XuBP must be enzyme-derived, because the labeled RuBP used in these experiments lacks detectable XuBP. Slow, mutant-catalyzed turnover of XuBP, subsequent to its formation, presumably accounts for the lack of accumulation. The small doublet, eluting just prior to 70 min, has also been observed in reaction mixtures with the E48Q mutant (Lee *et al.*, 1993). Although these were tentatively identified as 3-pentulose bisphosphates (derived from misprotonation of the enediol(ate) at C2), proof of structure has not been obtained. In stark contrast to the chromatographic profile for the K329A mutant, those for the loop-deletion mutant are totally lacking in PGA and compound X.

DISCUSSION

This study was prompted by our desire to gain insights into the function of the flexible loop 6 of Rubisco (residues 324−335), apart from its serving as a vehicle for properly positioning the apical active-site residue (Lys329) during catalytic turnover. The catalytic roles of Lys329 have been rather well defined, as reviewed in the introduction. The avenue chosen for pursuing our goals was loop truncation by site-directed mutagenesis, which proved highly successful in earlier studies of triosephosphate isomerase (Pompliano & Knowles, 1990). This enzyme, like Rubisco, includes an eight-stranded β/α-barrel with a flexible loop that folds over the entry to the active site. Furthermore, both enzymes catalyze reaction pathways that entail enolizations of α-hydroxy ketones. In the case of triosephosphate isomerase, deletion of four residues from the 10-residue loop resulted in a precipitous 10^5-fold drop in k_{cat} and differential weakening of the binding of a transition-state analogue relative to substrate. This indication of destabilization of the enediol(ate) intermediate was strongly reinforced by its decomposition via β-elimination to methylglyoxal and inorganic phosphate, presumably due to dissociation of the intermediate from the active site. Although β-elimination occurs to a minor extent with the wild-type isomerase [see Richard (1991) and references cited therein], the mutant enzyme actually catalyzed the decomposition of glyceraldehyde 3-phosphate to methylglyoxal 5 times faster than normal isomerization to dihydroxyacetone phosphate.

Characterization of the loop-deletion mutant described herein, in comparison to the properties of mutants with substitution for Lys329 reported earlier (Hartman & Lee, 1989; Harpel & Hartman, 1994), allows dissection of loop functions *per se* from those of the active-site lysyl within the loop. The K329A mutant retains demonstrable carboxylase activity (0.01−0.03% wild-type), resulting in the formation of PGA (Figure 5B). However, the loop-deletion mutant is incapable of catalyzing the carboxylative cleavage of RuBP to any discernible extent. Since the threshold for detecting carboxylase activity is 0.001% that of wild-type, we conclude that the loop (independent of Lys329) contributes at least 10-fold to the total rate enhancement achieved by Rubisco.

Remarkably, the loop is not required for the enolization of RuBP nor for hydrolysis of the carboxylated intermediate (2-carboxy-3-ketoarabinitol 1,5-bisphosphate). The V_{max} for enolization, as catalyzed by the mutant protein, is about 5%

of the wild-type value. This impairment of the first step of overall catalysis is no worse than what occurs with some of the single amino acid substitutions for Lys329 (Hartman & Lee, 1989). A similar picture emerges with respect to processing the carboxylated intermediate. Although the scarcity of this compound has precluded detailed product[3] and kinetic analyses, we observe a rate of processing that is akin to that with the K329A mutant (Lorimer *et al.*, 1993) or ~10% of wild-type (data not shown). In view of the three-dimensional structure of the closed-loop form of Rubisco (Knight *et al.*, 1990), which shows an electrostatic interaction between Lys329 and the carboxylate of bound CABP (a close structural analogue of the actual reaction intermediate), the forward processing of intermediate, even at a suppressed rate, by the mutant is rather striking. The mutant lacks not only a key interaction with the intermediate but also an entire loop that assists in sequestration of the intermediate from solvents and mitigation of its dissociation.

Given the substantial enolization activity of the mutant, its K_m for RuBP could be accurately determined. A value of 1 mM is observed, in comparison to 11 μM for the wild-type enzyme and 180 μM for the K329C mutant (Hartman & Lee, 1989). Thus, loop removal does not preclude binding of RuBP, but it weakens binding considerably more than does elimination of the cationic side chain at position 329. Interestingly, the F327G mutant, having undiminished carboxylase activity, also displays an elevated K_m (RuBP) of ~1 mM (Day *et al.*, 1993). Inasmuch as RuBP binding is concerned, the equivalent impact of loop deletion is exerted by the single-residue substitution of position 327. However, in stark contrast to the loopless mutant, the enediol(ate) generated by the F327G mutant (with Lys329 in place) is processed normally.

By inference, the catalytic competence of the loop-deletion mutant in intermediate turnover argues for competence in binding of the reaction intermediate analogue CABP. However, binding of CABP could not be detected in the usual gel filtration assay. Since this assay would have readily revealed 10^4-fold weaker binding than that displayed by wild-type enzyme, we conclude that loop deletion differentially impairs the binding of CABP (and probably the transition state for the carboxylated intermediate) relative to RuBP. Such a situation is entirely analogous to that described for the loopless mutant of triosephosphate isomerase (Pompliano *et al.*, 1990).

The propensity of the loopless mutant to convert RuBP to the dicarbonyl, 1-deoxy-D-*glycero*-2,3-pentodiulose 5-phosphate (Figure 5C,D), most likely reflects dissociation of the enediol(ate) from the active site followed by β-elimination of phosphate. Since the enzyme-bound enediol(ate) cannot undergo carboxylation, its only possible fate is protonation or decomposition. The modest misprotonation of enediol(ate) at C3 to form XuBP suggests a rather short residence time for enediol(ate) relative to its rate of dissociation. By comparing the rate of enolization of RuBP with the rate of dicarbonyl formation, we estimate a reprotonation/decomposition partitioning ratio of about 10. In our earlier studies of RuBP processing by the K329A mutant, we observed substantial formation of a decomposition product derived from an oxygenase intermediate (denoted X in Figure 5B). The absence of this compound from reaction mixtures of the loop-deletion mutant affirms the refractiveness of the enediol(ate) to O_2, as well as to CO_2.

The K329A mutant is subject to chemical rescue; the addition of aliphatic amines to reaction mixtures of mutant protein and RuBP suppresses the formation of decomposition products (the dicarbonyl and X) and restores normal processing of substrate to PGA and PGyc (Harpel & Hartman, 1994). However, carboxylase activity could not be restored to the loop-deletion mutant by a wide variety of amines at concentrations up to 1 M. Hence, the accessibility of the enzyme-bound enediol(ate) to amines in solution is insufficient to promote carboxylation; rather, the loop must serve to bind and orient the incoming amine in order for it to mimic the role of Lys329.

In summary, truncation of loop 6 of *R. rubrum* Rubisco does not interfere with subunit−subunit association as a dimer nor with normal activation chemistry (carbamate formation), as proven by its retention of enolization activity. Even though competent in both enolization and processing of the carboxylated intermediate, the loop-deletion mutant appears totally incapacitated at the stage of addition of gaseous substrate to the enediol(ate) derived from RuBP. Since the extent of incapacitation exceeds that of the K329A mutant, loop 6 contributes to catalysis beyond the rate enhancement attributable to Lys329. The prevalent decomposition of the enediol(ate), which occurs with the loop-deletion mutant, but not with wild-type enzyme, emphasizes the role of loop 6 in mitigating dissociation of unstable intermediates during catalytic turnover.

[3] On the basis of the conversion of acid-labile radioactivity of the carboxylated intermediate ([2-^{14}C]-2-carboxy-3-oxoarabinitol 1,5-bisphosphate) to acid-stable radioactivity, we assume that the labeled product is PGA, derived from the carboxyl group of the intermediate and the carbon atoms corresponding to C1 and C2 of RuBP. This molecule of PGA arises from stereospecific protonation of the precursor *aci*-carbanion, formed by hydrolysis of the carboxylated intermediate. If the *aci*-carbanion undergoes β-elimination of P_i [a rare event with wild-type enzyme (Andrews & Kane, 1991)] rather than protonation, the product will be pyruvate rather than PGA. Although we have not attempted to distinguish the two, both products reflect hydrolysis (*i.e.*, processing) of the carboxylated intermediate.

ACKNOWLEDGMENT

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