Role of Phenylalanine-327 in the Closure of Loop 6 of Ribulosebisphosphate Carboxylase/Oxygenase from *Rhodospirillum rubrum*†

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ABSTRACT: Phenylalanine-327 of ribulosebisphosphate carboxylase/oxygenase (rubisco) from *Rhodospirillum rubrum* was mutated to tryptophan, leucine, valine, alanine, and glycine, and was also deleted. The least active mutant, the deletion mutant, exhibits less than 0.5% of the carboxylase activity of the wild-type enzyme. Steady-state kinetic analysis of F327→Leu, Val, Ala, Gly mutant enzymes reveals that k_{cat} and the CO_2/O_2 specificity are unchanged while $K_{m(RuBP)}$ (RuBP = ribulose 1,5-bisphosphate) is drastically increased. The mutant enzyme with the highest value for $K_{m(RuBP)}$, Phe327→Gly, shows a 165-fold increase (1160 μM compared to 7 μM for the wild-type). The increase in $K_{m(RuBP)}$ suggests an alteration of the ratio k_{on}/k_{off} for RuBP. A longer hydrophobic lateral chain and/or the presence of an aromatic ring in the wild-type enzyme and the Phe327→Trp mutant enzyme could explain a better packing of loop 6 in the closed conformation and thus a tighter binding of RuBP at the active site.

The first step in the synthesis of saccharose and starch in photosynthetic organisms is the carboxylation of ribulose 1,5-bisphosphate (RuBP)[1] by ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) (Andrews & Lorimer, 1987). The yield of this reaction is diminished because rubisco competitively catalyzes the oxidation of RuBP. The oxidation reaction, which is up to 20% of the overall rubisco catalysis, is energetically wasteful for plants and reduces their growth. The study of rubisco by protein engineering should help us to understand the structural basis of the chemical mechanisms underlying catalysis.

Crystallographic data from both dimeric (L_2) (Schneider et al., 1990) and hexadecameric (L_8S_8) (Knight et al., 1990) rubisco variants and the use of a recombinant large subunit core of rubisco from *Synechococcus* (Gutteridge, 1991) reveal that the dimer L_2 is the minimal catalytic unit. In a dimer formed by two large subunits (L_2), the active site is located at the interface between the N-terminal domain of one subunit and the α/β barrel of the C-terminal domain of the other subunit. As in other α/β-barrel enzymes, the loops connecting the α helixes and the β sheets of the α/β barrel contain residues involved in catalysis (Farber & Petsko, 1990). One of these loops, loop 6, is very important for catalysis in rubisco. When no substrate is bound to the nonactivated (Schneider et al., 1990) or activated enzyme (Lundqvist & Schneider, 1991a), loop 6 is not defined in the crystal structure, presumably due to its high flexibility. However, when a transition-state analogue (CABP) is bound at the active site, loop 6 adopts a fixed conformation and is in contact with CABP (Knight et al., 1990). Loop 6 has to be in an open conformation to permit the access to the active site of substrates, and in a closed conformation, it may protect the reaction intermediate(s) and/or stabilize the transition state. A similar process occurs in triosephosphate isomerase, where the flexible loop 168–177 shifts during catalysis to bind tightly to the intermediate enediol (Pompliano et al., 1990; Joseph et al., 1990).

Mutations in loop 6 reveal the complex participation of this loop in catalysis. Mutation of valine-326[2] to alanine decreases the CO_2/O_2 specificity factor (V_cK_o/V_oK_c), τ and k_{cat}, but does not significantly change $K_{m(RuBP)}$ (Chen et al., 1991; Chen & Spreitzer, 1989, 1991). In comparison, mutation of alanine-334 to glutamic acid in rubisco from *Anacystis nidulans* decreases τ without a significant effect on either $K_{m(RuBP)}$ or k_{cat}, while mutation of valine-326 to alanine decreases k_{cat} but does not alter $K_{m(RuBP)}$ or τ (Parry et al., 1992). Mutation of methionine-330 of rubisco from *Rhodospirillum rubrum* to leucine decreases k_{cat} and increases $K_{m(RuBP)}$ but does not change τ (Terzaghi et al., 1986). In addition, when lysine-329 of rubisco from *R. rubrum* is mutated to cysteine, the mutant enzyme catalyzes the enolization of RuBP but is unable to catalyze the addition of gaseous substrates (Soper et al., 1988; Smith et al., 1988; Hartman & Lee, 1989).

A primary sequence comparison of loop 6 of rubiscos L_2 and L_8S_8 indicates that 50% of the amino acids are completely conserved in both types of rubisco and that an aromatic residue (phenylalanine-327 and tyrosine-327 in rubiscos from *R. rubrum* and from *Rhodobacter sphaeroides* form II, respectively) is inserted between the highly conserved sequence GTxxGK in L_2 rubiscos (Figure 1). The presence of a large aromatic chain in the middle of loop 6 suggests an important function for this residue in the reaction catalyzed by dimeric rubiscos.

In this paper, we describe the result of the deletion and mutation of phenylalanine-327 to tryptophan, leucine, valine, alanine, and glycine in rubisco from *R. rubrum*.

EXPERIMENTAL PROCEDURES

Materials. Radiolabeled chemicals and the mutagenesis kit were purchased from Amersham International plc.,

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[1] Abbreviations: BSA, bovine serum albumin; CABP, 2-carboxy-D-arabinitol 1,5 bisphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, D-ribulose 1,5-bisphosphate; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; V_C and V_O, maximal rate for carboxylation and oxygenation, respectively; K_C and K_O, Michaelis constant for carboxylation and oxygenation, respectively; $K_{m(RuBP)}$, Michaelis constant for D-ribulose 1,5-bisphosphate; τ, specificity factor.

[2] Numbering refers to the primary sequence of rubisco from *Rhodospirillum rubrum*.

0006-2960/93/0432-1940$04.00/0

TGTMGFGKMEGE	*R. rubrum* (L_2)
.....Y.....	*R. sphaeroides* II (L_2)
S..VV-..L..D	*A. nidulans* (L_8S_8)
S..VV-..L...	*C. reinhardtii* (L_8S_8)
A..VV-..L...	Pea (L_8S_8)
S..VV-..L...	Tobacco (L_8S_8)
S..VV-..L...	Spinach (L_8S_8)

FIGURE 1: Comparison of the amino acid sequences of loop 6 of L_2 and L_8S_8 rubiscos. The sequences are aligned on the primary sequence of rubisco from *R. rubrum*. Identical residues are represented by a dot and deletions by a dash. Phenylalanine-327 is represented in boldface type.

molecular biology grade enzymes from Northumbria Biologicals Limited and Pharmacia LKB Biotechnology, and tryptone and yeast extract from Merck Sharp & Dohme Limited. All other commercially available chemicals were obtained from Sigma UK Limited.

CABP. Unlabeled and ^{14}C-labeled epimeric mixtures of carboxyarabinitol bisphosphate and carboxyribitol bisphosphate were synthesized according to the method of Pike and Berry (1989). The concentration of CABP given in the text is based on the assumption that there is an equal mixture of the two epimers.

Plasmid Construction and Site-Directed Mutagenesis. The wild-type enzyme and the mutant enzymes Phe327→Δ, Phe327→Trp, and Phe327→Gly were produced according to Chêne et al. (1992).

A new expression vector, pFTF2000, was used for the expression of the mutant enzymes Phe327→Leu, Phe327→Ala, and Phe327→Val. The expression vector pFTF2000 was constructed from pSELECT 1 (Promega) and pMAG221190 (Chêne et al., 1992). Following removal of the *Pvu*II restriction site distal to the lac region of pSELECT 1 by site-directed mutagenesis, the resulting plasmid, pSELECT-, was digested with *Pvu*II to release the polylinker region. Plasmid pMAG221190 was digested with *Pvu*II, and the resulting ~2400 bp fragment, containing the rubisco gene from *R. rubrum*, was gel-purified and ligated into the *Pvu*II-cut pSELECT-vector. A clone containing the fragment such that the promoter region was recreated in the same sense as pMAG221190 was identified by restriction analysis and the plasmid designated pFTF2000. pFTF2000 has the same promoter region and expresses the same recombinant protein as pMAG221190 and pRR2119 (Somerville & Somerville, 1984). The advantages of this plasmid over pMAG221190 and pRR2119 are 3-fold. pFTF2000 is a phagemid with an M13 origin of replication which allows the production of ssDNA using a helper phage (Maniatis et al., 1989). It is a high copy number pUC-based plasmid unlike pMAG221190 and pRR2119 which are pBR322 derivatives. In addition to a tetracycline resistance gene, it contains an ampicillin resistance gene which has been inactivated by a single point mutation. By using an oligonucleotide to reconstitute a functional ampicillin gene in addition to the oligonucleotide for rubisco mutagenesis, it is possible to select for mutagenesis events by growth on ampicillin (Altered Sites Technical manual, Promega). Mutagenesis was carried out by the method of Kunkel (1985) as described in Maniatis et al. (1989) in combination with the ampicillin selection described above.

DNA Sequencing. DNA sequencing was carried out by the dideoxy method of Sanger (1977) as described in Maniatis et al. (1989). All mutants were sequenced throughout their entire coding region.

Expression of Wild-Type and Mutant Proteins. The wild-type enzyme and the mutant enzymes Phe327→Trp, Phe327→Δ, and Phe327→Gly were expressed in *Escherichia coli* strain TG2 and the mutants enzymes Phe327→Leu, Phe327→Ala, and Phe327→Val in *E. coli* strain DH5 F'. Growth and purification were carried out according to Chêne et al. (1992) except that strains harboring pFTF2000 derivatives were grown on tetracycline.

Active-Site Titration and Determination of Kinetic Parameters. The determination of $K_{m(RuBP)}$ and k_{cat} and the active titration were carried out as described in Chêne et al. (1992).

Specificity Determination. The CO_2/O_2 specificity was determined at 30 °C by the total consumption of RuBP (Parry et al., 1989). The CO_2 concentrations were estimated according to Schloss (1990).

CABP Exchange. Wild-type and position 327 rubisco mutant enzymes were adjusted to 2.5 mg/mL in binding buffer [100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 30 mM $NaHCO_3$, 1 mM EDTA, and 1 mM DTT]. A 1 mM solution of ^{14}C-labeled CABP (25 μL) was added to the enzyme solution (75 μL) and incubated overnight at 25 °C. The unbound CAPB was separated from the complex by passage through two 0.9-mL Sephadex G-50M spin columns (Chêne et al., 1992). The solution was adjusted to 500 μL with binding buffer, and at time zero, 275 μL of unlabeled 0.85 mM CABP was added. The mixture was incubated at 25 °C, and 100-μL aliquots were passed through Sephadex G-50M spin columns periodically. The eluent was adjusted to 100 μL, and 50 μL was put into 6-mL scintillation vials containing 4 mL of scintillation cocktail (OptiPhase "HiSafe" II Pharmacia) and counted for 2 min. The protein concentration in the eluent was measured by assaying the remaining 50 μL using a Bio-Rad Bradford assay. The specific radioactivity of each sample was calculated by dividing the numbers of counts by the protein concentration. These values were then normalized by a factor such that the sample with the highest specific radioactivity was set equal to 1.

A parallel experiment was carried out with wild-type enzyme in which magnesium was omitted from the binding buffer.

RESULTS

Integrity and Purity of the Mutant Enzymes. The comigration of mutant and wild-type enzymes under nondenaturing PAGE chromatography reveals that all the mutant enzymes form stable dimers (data not shown). The purity of the preparations is greater than 90% as determined by SDS–PAGE (data not shown).

Carboxylase Activity of Mutant Enzymes. All position 327 mutant enzymes exhibit a carboxylase activity indicating that phenylalanine-327 is not essential for catalysis. Nevertheless, the Phe327→Δ mutant enzyme exhibits less than 0.5% of the carboxylase activity of the wild-type enzyme.

Determination of Kinetic Parameters. The values of $K_{m(RuBP)}$ and k_{cat} for the wild-type enzyme and the Phe327→Trp, Phe327→Gly, Phe327→Leu, Phe327→Ala, and Phe327→Val mutant enzymes are summarized in Table I. None of the mutations drastically alter k_{cat}, revealing that the transition-state stabilization is not altered to any great degree. Even the removal of the lateral chain at position 327 (Phe327→Gly) does not destabilize the transition state. The wild-type and mutant enzymes may be divided into three categories according to the values of $K_{m(RuBP)}$ found. The first group consists of Phe327→Trp and wild-type enzyme, which have values of $K_{m(RuBP)}$ close to 10 μM, the second consists of Phe327→Leu, Phe327→Ala, and Phe327→Val, which have values of $K_{m(RuBP)}$ between 200 and 300 μM, and the third group consists of Phe327→Gly, which has a $K_{m(RuBP)}$

Table I: Kinetic Properties of Purified Wild-Type Enzyme and Phenylalanine-327 Mutant Enzymes[a]

enzymes	$K_{m(RuBP)}$ (μM)[b]	k_{cat} (s^{-1})[b]	τ
wild type	7 ± 1	2.9 ± 0.3	9.9 ± 1.0
Phe327 → Trp	8 ± 1	1.9 ± 0.1	10.2 ± 1.0
Phe327 → Leu	183 ± 18	3.0 ± 0.1	11.5 ± 0.5
Phe327 → Ala	198 ± 21	1.9 ± 0.2	10.9 ± 0.3
Phe327 → Val	277 ± 17	1.7 ± 0.3	9.0 ± 0.3
Phe327 → Gly	1160 ± 40	2.2 ± 0.1	10.4 ± 0.6

[a] Carboxylase activity was determined at 30 °C by measurement of $^{14}CO_2$ fixation in the absence of oxygen. [b] The values of $K_{m(RuBP)}$ and k_{cat} for RuBP and their standard deviations were determined with the program ENZFITTER (Leatherbarrow, 1987).

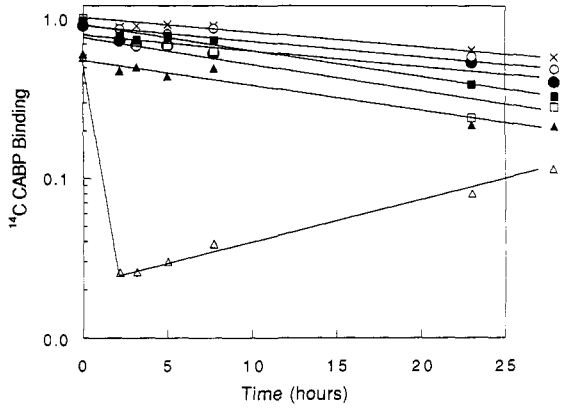

FIGURE 2: Plot showing exchange of ^{14}C-labeled CABP from wild-type and mutant enzymes. Symbols have been placed outside the plot against each line. [^{14}C]CABP binding on the ordinate is defined under Experimental Procedures. Closed circles, wild type; crosses, Phe327→Trp; open circles, Phe327→Leu; open squares, Phe327→Ala; closed squares, Phe327→Val; closed triangles, Phe327→Gly.

close to 1200 μM. Each group corresponded to a different type of residue at position 327: Phe327→Trp and wild-type enzyme have an aromatic lateral chain, Phe327→Leu, Phe327→Ala, and Phe327→Val have a hydrophobic lateral chain, and Phe327→Gly has no lateral chain. The low carboxylase activity of Phe327→Δ appears to be due, at least in part, to a k_{cat} rather than a K_m effect because an increase in RuBP concentration from 2.5 to 5 mM does not give rise to an increase in the rate of the very low but measurable carboxylation (data not shown).

Specificity Determination. The determination of the specificity factor (V_cK_o/V_oK_c) for Phe327→Trp, Phe327→Gly, Phe327→Leu, Phe327→Ala, and Phe327→Val mutant enzymes reveals that the mutations do not change specificity toward the gaseous substrates. A value close to 10 is found for the mutant and for the wild-type enzymes (Table I).

CABP Exchange. We measured the exchange of enzyme-bound radioactive CAPB with added nonradioactive CABP (Figure 2). In the absence of magnesium, CABP is exchanged very rapidly (data not shown). We observed that the rate of exchange of CAPB in the Phe327→Δ mutant is rapid, and was reduced to background levels by 2 h. This is not surprising as CAPB is a transition-state analogue and transition-state binding is severely compromised in this mutant. The Phe327→Ala, Phe327→Val, and Phe327→Gly mutants appear to have similar, or possibly slightly increased, exchange rates. These mutants have increased values of $K_{m(RuBP)}$ relative to wild type. However, Phe327→Leu also has an increased value of $K_{m(RuBP)}$ relative to wild type, but, within experimental error, an unaltered CAPB exchange rate. It is not possible to interpret CABP exchange in terms of loop closure because CABP is a transition-state analogue with two-phase binding kinetics such that

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^*$$

where EI* is the final complex with a $K_D \sim 10^{-11}$ M and EI has a $K_D \sim 4 \times 10^{-7}$ M. The conversion of EI to EI* is slow, $k_3 \sim 0.04$ s^{-1} (Pierce et al., 1980), and probably a measure of events which take place after the closure of loop 6. CABP exchange is thus a measure of k_4 which may or may not reflect loop 6 packing.

DISCUSSION

Loops are commonly located on the surface of proteins. This special location explains why they are very often implicated in protein–protein and protein–substrate interactions (Lesk, 1991). The location of the loops at the surface of proteins allows them a certain degree of structural flexibility (Karplus & Schulz, 1985). Enzymes often use this structural feature to bring functional groups to the active site and allow an induced fit (Fersht et al., 1988; Pompliano et al., 1990; Fry et al., 1986; Tanaka et al., 1992; Yang & Miles, 1992). Loops can also act as a "shield" to protect reaction centers or reaction intermediates from degradation by bulk solvent (Joseph et al., 1990; Tanaka et al., 1992; Knowles, 1991).

Most of the loops in rubisco containing residues involved in catalysis are part of the α/β barrel (Knight et al., 1990). One of them, loop 6, has been intensively studied. Crystallographic data reveal that loop 6 is very flexible and has a fixed conformation only when interacting with a transition-state analogue (CABP) (Schneider et al., 1990; Lundqvist & Schneider, 1991a; Knight et al., 1990). Site-directed mutagenesis experiments indicate that mutations in loop 6 can alter $K_{m(RuBP)}$, k_{cat} and/or τ (Chen et al., 1991; Chen & Spreitzer, 1989, 1991; Parry et al., 1992; Terzaghi et al., 1986). One of the most fascinating results has been described by Hartman and his co-workers (Soper et al., 1988; Smith et al., 1988; Hartman & Lee, 1989). When lysine-329 in loop 6 is mutated to a cysteine residue, the mutant enzyme is able to catalyze the enolization of RuBP but is unable to catalyze the CO_2 fixation. In other words, the enzyme creates the reactive center, but this does not react with CO_2. Hartman and Lee (1989) advanced that lysine-329 facilitates the addition of gaseous substrates and most of the effects due to other mutations in loops 6 have been attributed to misalignment of lysine-329 at the active site (Chen et al., 1991; Parry et al., 1992).

Despite a low homology in their primary sequence (≈25%), both L_2 and L_8S_8 rubiscos have a similar three-dimensional structure, and the regions located at the active site are homologous. The primary sequence of loop 6 is highly conserved (Figure 1), in particular, the stretch GTxxGK is conserved in all L_8S_8 rubiscos. A comparison with L_2 rubiscos reveals the insertion of an aromatic amino acid in this conserved region in both dimeric rubiscos sequenced (Figure 1). In this work, we have used site-directed mutagenesis to investigate the role of phenylalanine-327 in rubisco from *R. rubrum* in catalysis.

Our results reveal that a lateral chain is not essential at position 327; Phe327→Gly exhibits carboxylase activity. However, the absence of a residue at position 327 drastically reduces the enzyme activity. The Phe327→Δ mutant has less than 0.5% of the carboxylase activity of the wild-type enzyme. Surprisingly, the other mutations (except Phe327→Trp) have an influence only on $K_{m(RuBP)}$. The absence of any effect on k_{cat} or τ indicates that despite being

only two residues from lysine-329, mutations at position 327 do not alter the CO_2/O_2 specificity or destabilize the transition state. This result reveals that all the residues involved in the stabilization of the transition state are in the correct orientation. Terzaghi et al. (1986) have also reported a large increase in $K_{m(RuBP)}$ mutating methionine-330 to leucine in loop 6. However, the 25-fold increase in K_m is accompanied by a 5-fold decrease in k_{cat}, suggesting that methionine-330 is also involved in the stabilization of the transition state either directly or indirectly. In contrast, phenylalanine-327 appears to be the only residue in loop 6 investigated so far which is involved in the binding of RuBP but which does not participate in the stabilization of the transition state or the addition of the gaseous substrates.

The loss of 2.8 kcal mol^{-1} of binding energy when the aromatic side chain is removed is partially recovered when linear lateral hydrophobic chains are introduced at position 327 (leucine and alanine). The gain in stability of 1 kcal mol^{-1}, compared with Phe327→Gly, may be explained by these residues being able to make some hydrophobic interactions with the body of the protein to increase the stability of loop 6 in a closed conformation. The higher $K_{m(RuBP)}$ value for the valine substitution [leucine or alanine-327, $K_{m(RuBP)} \approx 200$ μM; valine-327, $K_{m(RuBP)} \approx 300$ μM] may reveal that the branched side chain of valine-327 makes unfavorable steric interactions compared with leucine or alanine. The low activity of the Phe327→Δ mutant suggests that the additional residue in loop 6 of the dimeric rubiscos is absolutely required for correct packing of the loop to give effective transition-state stabilization.

One explanation for the changes in $K_{m(RuBP)}$ associated with mutations at position 327 is as follows. The loss of binding energy between phenylalanine-327 in loop 6 and the body of the protein in the mutant enzymes will lead to loop 6 spending more time in an open conformation. This open conformation will cause k_{off} for RuBP to increase without having a major effect upon k_{on}, thus leading to an increase in $K_{m(RuBP)}$. Nevertheless, the lack of any alteration in k_{cat} suggests that when the loop is closed, it is packed in a similar manner to the wild type for the transition-state stabilization. Confirmation of this hypothesis awaits further experiments.

The contacts made by phenylalanine-327 are not known in the absence of a three-dimensional structure of rubisco from *R. rubrum* in which loop 6 has a closed conformation (Lundqvist & Schneider, 1991b). However, by looking at residues in close proximity (<4 Å) to residues VVGK in loop 6 of rubisco from spinach when CABP is bound (Knight et al., 1990) and comparing these with the equivalent residues in rubisco from *R. rubrum*, it is possible to suggest some hydrophobic/aromatic residues in contact with phenylalanine-327 in the dimeric enzyme. Two of these are phenylalanine-379 and isoleucine-447. The observation that the kinetic parameters for both Phe327→Trp and the wild-type enzyme are identical and the presence of a tyrosine in loop 6 of the dimeric enzyme from *R. sphaeroides* suggest that an aromatic side chain is important at this position. The presence of phenylalanine-379 in the vicinity of phenylalanine-327 in a closed conformation indicates that aromatic–aromatic interactions between these two residues may be important in the closure of loop 6. In support of this idea, phenylalanine-379 is conserved in dimeric rubiscos but is an invariant leucine in 13 L_8S_8 rubiscos. This hypothesis could be tested by constructing double mutants of phenylalanine-377 and phenylalanine-379 and using thermodynamic cycles (Carter et al., 1984).

In conclusion, we have shown that replacing phenylalanine-327 of rubisco from *R. rubrum* with leucine, valine, alanine, and glycine alters the binding of RuBP at the active site. The lower $K_{m(RuBP)}$ obtained with phenylalanine and tryptophan at position 327 (wild-type and Phe327→Trp) suggests that aromatic lateral chains may be required for a correct packing of loop 6 in a closed conformation.

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