Structure of a Product Complex of Spinach Ribulose-1,5-bisphosphate Carboxylase/Oxygenase[†,‡]

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Received November 14, 1996; Revised Manuscript Received January 23, 1997[⊗]

ABSTRACT: The crystal structure of an activated complex of ribulose-1,5-bisphosphate carboxylase/oxygenase from spinach and its product 3-phosphoglycerate has been determined to 2.2 Å resolution. The structure is of the open form with the active site accessible to the solvent as observed in the structures of the activated ligand-free enzyme and the complex of the activated enzyme with the substrate ribulose-1,5-bisphosphate. Two molecules of 3-phosphoglycerate are bound per active site. The phosphates of both molecules bind approximately at the same position as the phosphates of ribulose-1,5-bisphosphate or the six-carbon intermediate analogue 2-carboxyarabinitol-1,5-bisphosphate, but one product molecule is swung out from the active site with its carboxylate group pointing toward solution. The present structure points to direct participation of the active site side chain of lysine 175 in later stages of catalysis. This possibility is discussed in the light of mutagenesis studies.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco)[1] catalyzes the carboxylation by CO_2 and hydrolysis of ribulose-1,5-bisphosphate to form two molecules of 3-phospho-D-glycerate (3PGA). Further processing of this product results in the net fixation of carbon into the food chain. Rubisco also catalyzes a side reaction, where O_2 competes with CO_2 for RuBP. This reaction yields only one molecule of 3PGA and in addition one molecule of 2-phosphoglycolate (Figure 1). The latter molecule is partly recirculated by photorespiration, an energy-requiring salvage route. The occurrence of the oxygenation reaction thus severely limits the efficiency of carbon fixation [for reviews, see e.g., Andrews and Lorimer (1987) and Hartman and Harpel (1994)].

Carbon dioxide plays a dual role in rubisco catalysis: apart from its function as a substrate in the carboxylation reaction it is also ultimately required as an activator in both reactions. The activation process consists of the carbamylation by non-substrate CO_2 of an active site lysine (lysine 201 in the spinach enzyme) and the subsequent stabilization of the carbamate by a magnesium ion (Lorimer et al., 1976). The activation proceeds almost without detectable changes in the structure of the enzyme (Taylor & Andersson, 1996).

Rubisco from all known higher plants and from a majority of microorganisms consist of 8 large subunits (MW 55 000) and 8 small subunits (MW 15 000), the entire L8S8 molecule having a molecular weight of about 550 000. Another type is found in rubisco from the photosynthetic bacterium *Rhodospirillum rubrum*, consisting of an L2 homodimer without S-subunits (Schneider et al., 1986). The L-subunit of the hexadecameric (L8S8) enzyme has close similarities with the dimeric (L2) enzyme from *R. rubrum* (Schneider et al., 1990a). The central structural motifs of the large subunits are very similar, as is the architecture of the active site.

X-ray structures of both types of rubisco have been determined in the non-activated as well as the activated state. Until relatively recently only one structure of the activated hexadecameric enzyme had been determined by X-ray crystallography, that of the complex with the transition state analogue 2-carboxy-D-arabinitol-1,5-bisphosphate (2CABP; Knight et al., 1990; Andersson, 1996; Schreuder et al., 1993; Newman et al., 1993).

The overall mechanism in terms of the chemical rearrangement of the substrates leading to product formation is well characterized, especially for the carboxylation reaction (Figure 1). In contrast much less was known about the exact involvement of the enzyme in each step and structural information on the different steps was missing for the L8S8 enzyme. Recently, the structures of several activated complexes of the hexadecameric (L8S8) rubisco from spinach have been determined, the unliganded Mg^{2+}-bound complex (Taylor & Andersson, 1996), the Ca^{2+}−RuBP complex (Taylor & Andersson, 1997) and the reaction intermediate analogue Mg^{2+}−2CABP complex to high resolution (Andersson, 1996). The structure of the substrate RuBP bound to the carbamylated Ca^{2+}-bound form of the spinach enzyme proved to be a useful complement to the 2CABP structure and has revealed more about the details of the reaction before carbon−carbon bond scission between C2 and C3 of the six-carbon intermediate (Taylor & Andersson, 1997). During the bond cleavage step a stereospecific protonation and inversion of a carbanion are required to form a stereochemically correct product (Andrews & Lorimer, 1987; Figure 2). We present here the structure of activated spinach rubisco complexed with the product 3PGA at a resolution of 2.2 Å. This structure provides the missing piece of structural information on the last step in

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[†] Supported by grants from the Swedish Research Council for Forestry and Agriculture and the Magnus Bergvall Foundation.

[‡] Coordinates and structure factor amplitudes have been deposited with the Brookhaven Protein Data Bank, accession numbers 1aa1 and 1aa1sf.

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[⊗] Abstract published in *Advance ACS Abstracts,* March 15, 1997.

[1] Abbreviations: rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, D-ribulose-1,5-bisphosphate; 3PGA, 3-phospho-D-glycerate; 2CABP, 2-carboxy-D-arabinitol-1,5-bisphosphate; XuBP, D-xylulose-1,5-bisphosphate; 4CABP, 4-carboxy-D-arabinitol-1,5-bisphosphate; CKABP, 2-carboxy-3-keto-D-arabinitol-1,5-bisphosphate; NCS non-crystallographic symmetry; rms, root mean square.

S0006-2960(96)02818-8 CCC: $14.00

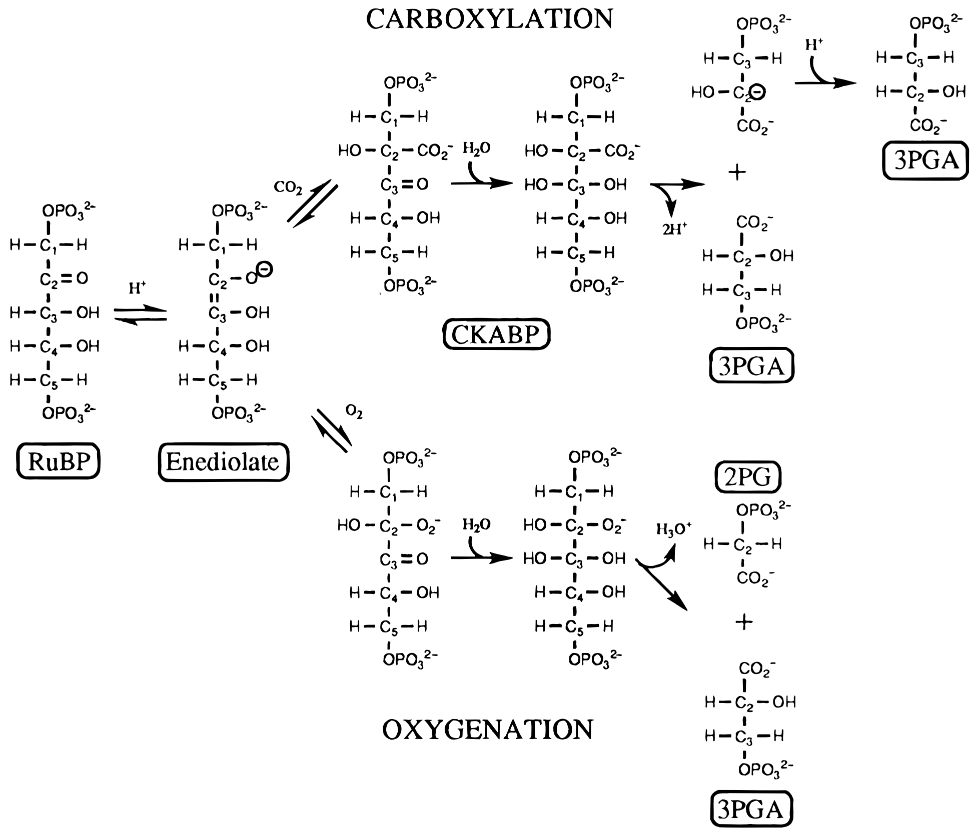

FIGURE 1: Reactions catalyzed by rubisco.

the carboxylation reaction. Together the structures of the activated spinach enzyme represent a series of snapshots along the reaction coordinate from free unliganded enzyme, through the first Michaelis complex with substrate and the stable six-carbon reaction intermediate, to the final complex of the enzyme with the two product molecules.

MATERIALS AND METHODS

Rubisco was purified from spinach according to Andersson et al. (1983). Crystals of the activated enzyme in complex with 3PGA were grown as described in Taylor and Andersson (1996). Crystals grew within a few days at 4 °C. During all subsequent transfer and mounting procedures the 3PGA concentration was maintained at 100 mM to ensure that it remained bound to the enzyme. The crystals are approximately isomorphous with those of the spinach-activated 2CABP complex, belong to space group $C222_1$ with cell dimensions $a = 157.6$ Å, $b = 158.7$ Å, $c = 203.3$ Å, and contain one-half of a rubisco L8S8 hexadecamer in the asymmetric unit.

Data were collected from a total of seven crystals at station 9.5, SRS, Daresbury, U.K., and at beamline 4 at the ESRF, Grenoble, France. These data were processed with the DENZO data reduction suite (Otwiniwski, 1993). A total of 799 161 measurements were reduced to 107 965 unique reflections between 20 and 2.2 Å with an overall R_{merge} of 0.103 and completeness 85% (75% in the highest resolution bin).

Refinement was performed with X-PLOR (Brünger, 1992) using the refined protein coordinates of the unliganded activated form (1AUS; Taylor & Andersson, 1996) as a starting model. 3% of the reflections were randomly chosen as a test set to monitor R_{free} (Brünger, 1992b). The initial R-factor for the starting model was 0.272. After an initial round of rigid body refinement non-crystallographic symmetry (NCS) operators were calculated and 4-fold NCS restraints were applied during subsequent cycles of refinement. Both simulated annealing and conventional positional refinement were performed using the parameter files of Engh and Huber (1991). Individual B factors were refined. Two 3PGA molecules were located and built into 4-fold averaged density, and 253 solvent molecules were added per LS unit using a combination of the CCP4 package (1994) and O (Jones et al., 1991). The final R-factor of the model is 0.207 ($R_{\text{free}} = 0.224$). Data processing and refinement statistics are shown in Table 1.

RESULTS

The structure is of the open form of rubisco as observed in the activated unliganded enzyme (Taylor & Andersson, 1996) and the activated Ca^{2+}−RuBP complex (Taylor & Andersson, 1997). A comparison using O between the 3PGA complex structure and the structures of the activated unliganded complex, the activated Ca^{2+}−RuBP complex, and the activated 2CABP complex results in root mean square (rms) deviations of 0.179, 0.241, and 0.936 Å, respectively, for all the C^{α} atoms of an L2S2 unit. Two 3PGA molecules are bound at the active site. The two phosphates bind at the same sites as the P1 and P2 phosphate groups of the 2CABP molecule (Andersson, 1996), but their positions are shifted by 1.1 and 1.5 Å, respectively, relative to their positions in the 2CABP complex. As a result the phosphate−phosphate distance is 9.7 Å in the 3PGA complex as opposed to 8.9 Å in the closed 2CABP complex (Andersson, 1996). One 3PGA molecule, corresponding to the P1 position of 2CABP, has two oxygen atoms ligated to the magnesium ion, O2, and one of the carboxylate oxygens as predicted by electron paramagnetic resonance spectroscopy (Styring & Brändén,

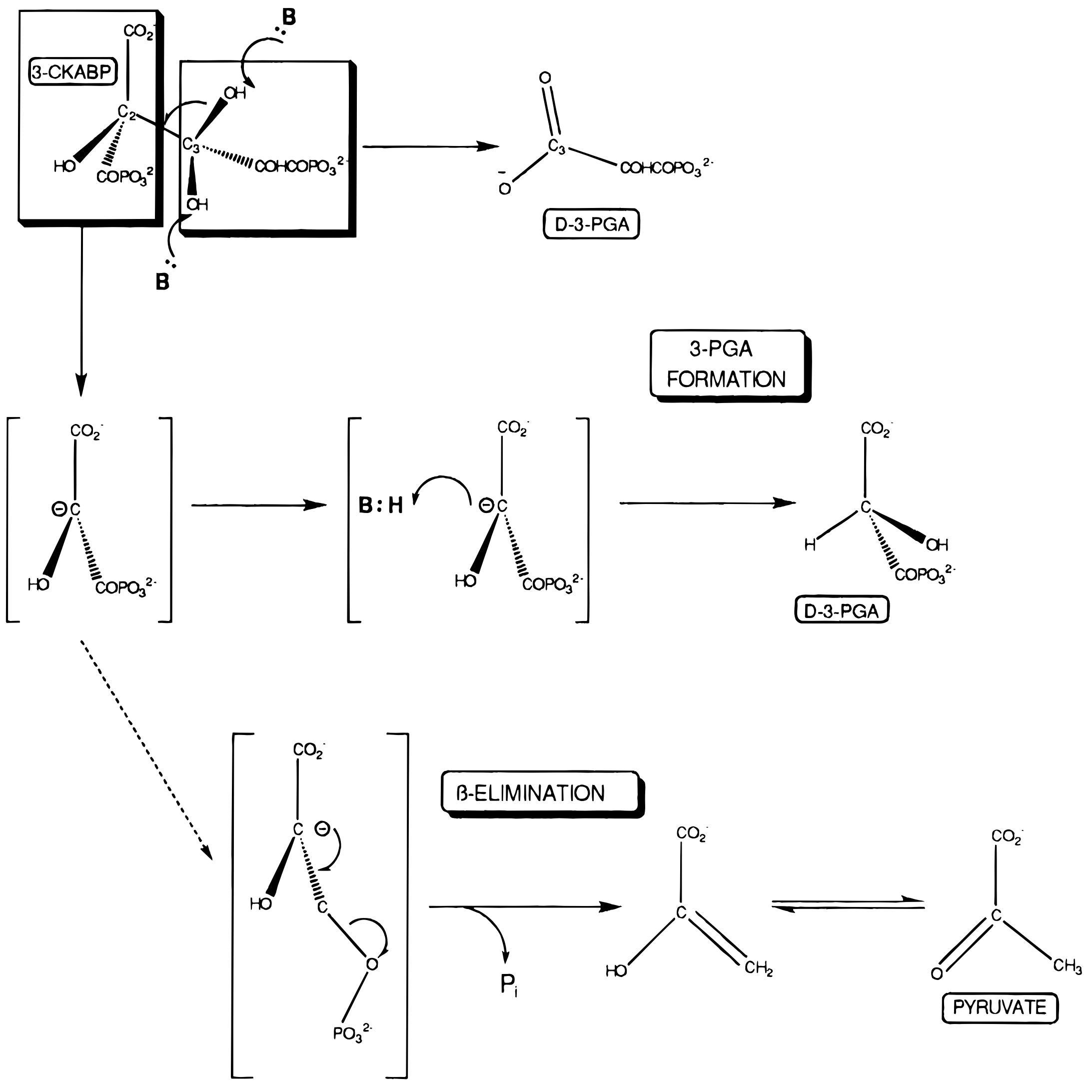

FIGURE 2: Fate of the six-carbon intermediate CKABP in the carboxylation reaction catalyzed by rubisco. One molecule of D-3PGA is immediately yielded, whereas the second molecule is produced by stereospecific protonation of the resulting carbanion. β-Elimination of the carbanion instead leads to formation of pyruvate.

Table 1: Statistics for Data Collection and Refinement of the Spinach Rubisco−3PGA Complex

no. of crystals	7
maximum resolution (Å)	2.2
space group	$C222_1$
no. of measurements	799 161
no. of unique reflections	107 965
completeness of data (%)	85
R_{merge}[a]	0.103
no. of protein non-hydrogen atoms	4 741
no. of solvent molecules	252
R_{cryst}[b]	0.209
R_{free}[b]	0.224
rms deviation	
bond lengths (Å)	0.007
bond angles (deg)	1.667

[a] $R_{\text{merge}} = \Sigma_j\Sigma_h|I_{h,j} - \langle I\rangle|/(\Sigma_j\Sigma_h\langle I_h\rangle)$. [b] $R_{\text{cryst}} = \Sigma||F_o| - |F_c||/\Sigma|F_o|$.

1985). The carbon atoms are numbered in Figure 1, and O2 corresponds to the hydroxyl oxygen of the 3PGA molecule derived from carbons C1−C2 of RuBP. Comparison with the 2CABP complex shows that the equivalent oxygens are also coordinated to the magnesium ion. The other 3PGA molecule, corresponding to the P2 position of 2CABP, is shifted with respect to the corresponding 2CABP atoms such that the phosphate is centered at approximately the same position but the molecule is rotated away from the metal ion with its carboxyl group pointing toward the outside solvent (Figure 3A,B).

No density is observed for the N-terminus (residues 9−20), loop 6 (residues 333−337), or for the C-terminus (residues 464−475). This is also the case in the unliganded activated complex. The disordering of these residues opens the active site to the bulk solvent.

In terms of the protein the active site is very similar to the activated unliganded form, with the exception of three residues (Lys 175, Leu 402, and Gln 401) whose side chains adopt slightly different conformations. Leu 402 is situated near Lys 175 whereas Gln 401 is located on the opposite side of the bound 3PGA molecule with respect to Lys 175. Contacts made to the 3PGA molecules are listed in Table 2. The 3PGA molecule at the P2 site is much more loosely bound than the one in the P1 site. The position of the P2 phosphate could be located by contouring of electron density maps at 0.8σ (where σ represents the rms electron density for the unit cell), but 4-fold NCS averaging of the $2|F_o| - |F_c|$ map was required in order to build the remainder of the molecule. The carboxylate group interacts with the protein only via interactions with solvent molecules. The 3PGA molecule at the P1 site, on the other hand, is much more tightly bound and makes close interactions to the magnesium.

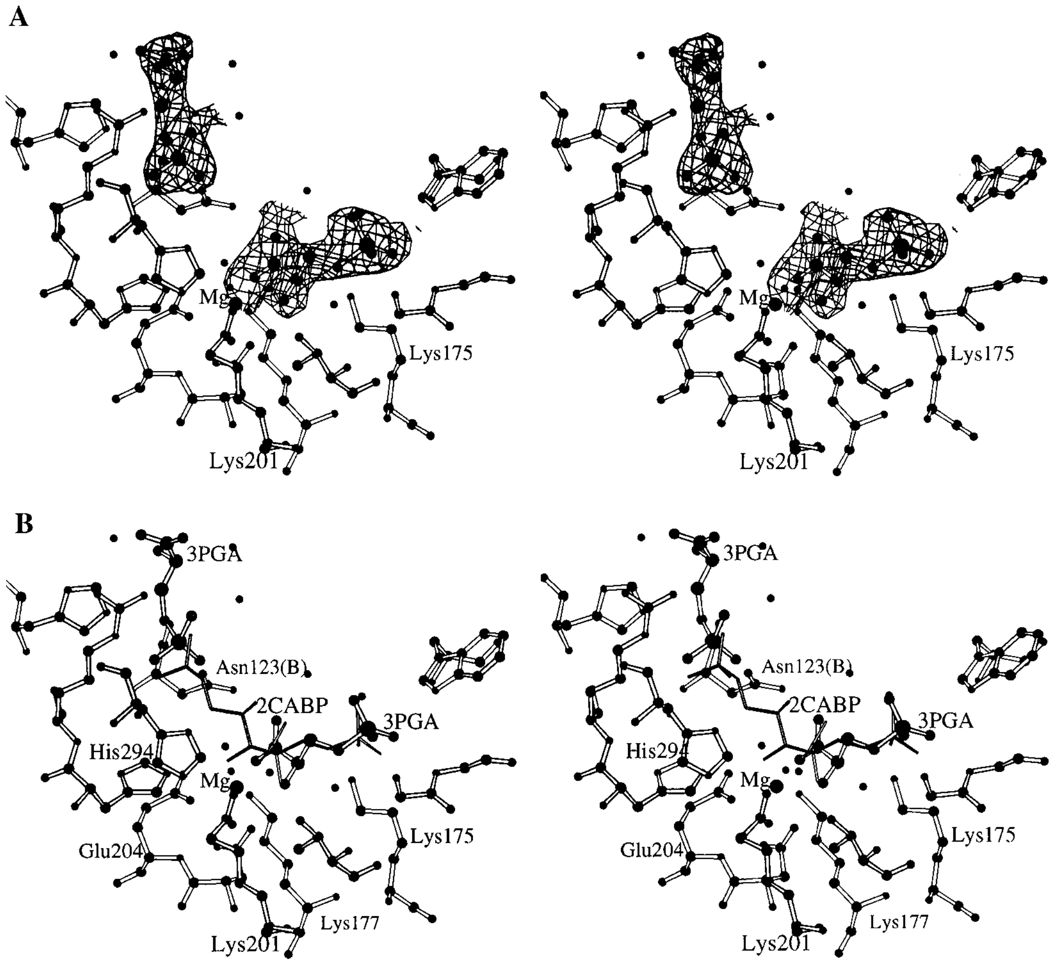

FIGURE 3: Structure of the active site of the complex of activated spinach rubisco with 3PGA. Two molecules of 3PGA are bound. (A) The $2|F_0| - |F_c|$ map is contoured at 1σ, where σ represents the root mean square (rms) electron density for the unit cell. Solvent molecules are shown as filled circles. (B) The position of 2CABP (Andersson, 1996) is indicated by the thin black line for comparison.

Table 2: Distances Less than 3.3 Å between the Products and Active Site Residues

3PGA atom	protein/water atom distance to P1 3PGA molecule (Å)		protein/water atom distance to P2 3PGA molecule (Å)	
O1P	G404 N	2.9	R295 NH2	3.0
	W66 NE1	3.0	WAT O	2.5
O2P	G403 N	2.7		
	WAT O	2.6		
	WAT O	2.9		
	WAT O	3.1		
O3P	G381 N	2.9	R295 NE	2.7
			WAT O	2.7
O1			H298 NE2	2.8
			WAT O	3.0
C1			H298 NE2	2.9
			H298 CE1	3.2
			R295 NH2	3.1
C2	K175 NZ	2.9		
O2	Mg^{2+}	2.4		
C3	Mg^{2+}	3.0		
O31	WAT O	2.6	WAT O	3.0
			WAT O	3.1
O32	Mg^{2+}	2.4	WAT O	2.4

Coordination to the magnesium ion is almost perfectly octahedral (Figure 4).

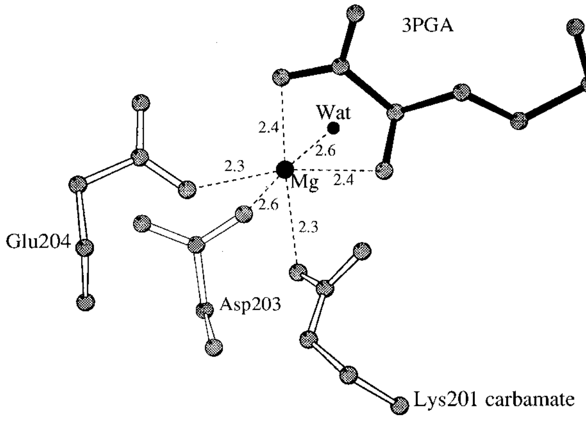

FIGURE 4: Coordination of the magnesium ion in the 3PGA complex.

DISCUSSION

Analysis of a number of structures of rubisco reveal three disordered regions of the large subunit: the N-terminal segment (residues 9−20), the C-terminal segment (residues 465−475), and loop 6 (residues 333−337) situated between strand 6 and helix 6 of the α/β-barrel domain. This was observed in the structure of unliganded activated spinach rubisco (Taylor & Andersson, 1996), but also similarly in the structure of the activated spinach enzyme in complex with RuBP (Taylor & Andersson, 1997) and in the structures of the nonactivated enzyme from *R. rubrum* (Schneider et al., 1990b) and tobacco (Curmi et al., 1992). The flexibility of these regions results in an open conformation where the active site is accessible to the solvent. In the structures of

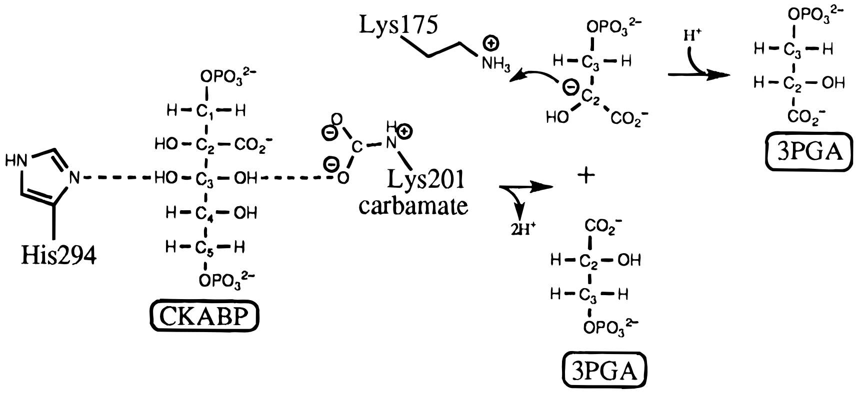

FIGURE 5: The potential mechanism for the hydrolysis of the six-carbon intermediate CKABP.

the 2CABP complex, however, these regions are ordered over the intermediate analogue 2CABP; in addition, the N-terminal region is brought closer into the active site providing tighter contact with 2CABP (Andersson et al., 1989; Schreuder et al., 1993; Newman et al., 1993; Taylor & Andersson, 1996). These movements result in a closed conformation where the active site is shielded from the solvent. A similar conformation is also observed in nonactivated complexes with the inhibitors D-xylulose-1,5-bisphosphate (XuBP) and 4-carboxy-D-arabinitol-1,5-bisphosphate (4CABP) (Newman & Gutteridge, 1994; Taylor et al., 1996).

The ordering of loop 6 over the active site is effected by a series of hydrogen bonds made by residues in the loop and also from the N-terminal domain (Taylor & Andersson, 1997). Following formation of the product, the active site needs to be able to reopen to allow the product molecules to diffuse away to complete the reaction cycle. The open structure of rubisco with bound product 3PGA, which is almost identical to the structure of the activated ligand-free enzyme in terms of which elements are flexible (rms deviation between the two structures is 0.179 Å for all C^{α} atoms of an L2S2 unit), is consistent with a view of the enzyme just prior to release of the two product molecules. Both 3PGA molecules are easily accessible from the solvent region. The driving force behind the opening of the active site after cleavage of the six-carbon intermediate 2-carboxy-3-ketoarabinitol-1,5-bisphosphate (CKABP) could be provided by the additional entropic freedom of motion gained by the cleavage.

The structure of the bacterial enzyme from *R. rubrum* has been determined in complex with the product 3PGA but in the absence of the metal ion and the carbamate (Lundqvist & Schneider, 1988). In this structure one 3PGA molecule is bound with its phosphate near the P2 phosphate binding site (the weaker binding site in the spinach structure), and its carbohydrate moiety points toward instead of away from the active site.

The six-carbon intermediate of the reaction catalyzed by rubisco, CKABP, is bound in the rubisco active site predominantly in its hydrated C3 gemdiol form (Lorimer et al., 1986; Figure 1). Carbon−carbon bond cleavage to yield 3PGA is initiated by abstraction of one or both of the protons from the C3 hydroxyl groups of CKABP. Likely candidates for this abstraction are the carbamate on Lys 201 and His 294 (Figure 5). To enable the carbamate to accept protons while being coordinated to the magnesium ion, the carbamate was postulated to adopt a resonance form in which a negative charge is maintained on each of the carboxylate oxygens (Andersson, 1996). In the 2CABP structure the free oxygen of the carbamate is about 2.8 Å from the position of one of the C3 hydroxyl oxygens. His 294 is situated on the other side of 2CABP in this complex, 2.9 Å from the O3 hydroxyl oxygen of 2CABP. Thus it is ideally positioned to abstract a proton from the gemdiol form of the intermediate CKABP. A role for the histidine in this step in accordance with biochemical evidence showing that mutants at position 287 in *R. rubrum* rubisco (the equivalent of His 294 in spinach) are incapable of processing the six-carbon intermediate (Lorimer et al., 1987). In the present 3PGA complex, the lower 3PGA molecule has rotated out with its carboxyl group pointing toward solution and the position occupied by C4 of 2CABP is now occupied by a water molecule. This movement, however, does not perturb the rest of the structure in the vicinity, and the positions of the side chains of His 294 and the carbamylated Lys 201 are identical to those in the 2CABP complex.

Deprotonation of the gemdiol initiates carbon−carbon bond cleavage (Figures 1 and 2). One 3PGA molecule, corresponding to carbon atoms C3 to C5 of the intermediate, is directly formed whereas a C2 carbanion is derived from C1 to C2 of the intermediate. Formation of the second product molecule requires the stereospecific protonation of the carbanion, leading to reversal of the stereochemistry around C2 (Andrews & Lorimer, 1987). It is likely that rapid protonation of the carbanion following bond cleavage is required in order to avoid non-stereospecific product formation. This would mean that this step occurs before the opening of the active site by disordering of loop 6 and the N- and C-terminal regions which is likely to be a slow process relative to the capture of a proton by the carbanion. There is no evidence for the formation of L-3PGA by rubisco (Andrews & Kane, 1991), suggesting that this protonation indeed is highly specific.

Analysis of the structure of the 2CABP complex revealed that Lys175 is ideally situated to donate a proton to the resulting carbanion (Knight et al., 1990; Taylor & Andersson, 1996, Figure 5). This is also the case in the present 3PGA product complex. In this structure, rotation of C1 and C2 leading to the change of the stereochemistry around C2 brings C2 within hydrogen bonding distance to Lys 175 (the distance between C2 and the ϵ-amino group of Lys 175 is 2.9 Å). There is no other residue close to C2 in either the 2CABP or 3PGA structures, and the unusually low pK_a (7.9) of this residue (Hartman et al., 1985; VanDyk & Schloss, 1986) would favor proton exchange during catalysis. However, mutagenesis experiments provide conflicting evidence. The mutation of the equivalent lysine in *R. rubrum* rubisco to a glycine does not appreciably affect the ability of the enzyme to process CKABP (Lorimer & Hartman 1988). This indicates that Lys 175 is not involved in carbon−carbon bond cleavage; however, its involvement in reprotonation of the carbanion cannot be discounted. The ^{14}C difference method used to monitor CKABP processing during kinetic analysis of the mutant (Pierce et al., 1986; G. Lorimer, personal communication) did not specifically determine the nature of the product. If the rate-limiting step in the formation of product from CKABP is proton abstraction then the K166G mutation will not affect carbon−carbon bond cleavage. Besides D-3PGA, there are at least two other possible products that can result during processing of the six-carbon intermediates by the mutant (Taylor, 1996). Firstly, the rate of misprotonation of the carbanion may be increased, resulting in L-3PGA formation. Analysis of the structures of the spinach enzyme suggests that the carbamate is in a position to be able to protonate the carbanion from the opposite side. The result of this misprotonation would then be L-3PGA. Although this does not seem to happen to any detectable extent in wild-type enzyme we argue that it could occur in a mutant devoid of the Lys 175 side chain.

Another possible fate of the carbanion would be to undergo β-elimination of the phosphate group to form pyruvate as a side reaction (Andrews & Kane, 1991; Figure 2). The rate at which β-elimination occurs is dependent on the orientation of the phosphate group with respect to the carbon−carbon bond (Rose, 1981) and happens approximately once every 125−150 turnovers in the wild-type enzyme (Andrews & Kane, 1991; Morell et al., 1994). Mutations in *R. rubrum* rubisco of residues constituting the P1 phosphate binding site result in increased pyruvate formation (Larimer et al., 1993), whereas mutations of a corresponding residue in *Synechococcus* instead seem to protect the three-carbon carbanion from β-elimination (Morell et al., 1994). This apparent contradiction stresses the importance of these residues for maintaining the phosphate in the correct orientation to minimize the chance of β-elimination. It seems likely that the absence of the side chain of Lys 175 will increase the life time of the carbanion and promote the β-elimination reaction. In line with this argument it has recently been corroborated (Harpel & Hartman, 1996) during the review of this manuscript that pyruvate is the major labeled product formed by *R. rubrum* position-166 mutants. The coordinates and structure factor amplitudes have been deposited with the Brookhaven Protein Data Bank, entry numbers 1aa1 and 1aa1sf.

ACKNOWLEDGMENT

We thank SRS Daresbury (Daresbury, U.K.) for use of data collection facilities at station 9.5 and B. Rasmussen and A. Åberg for assistance at beamline 4 of the ESRF (Grenoble, France). The use of the facilities at the Laboratory of Molecular Biophysics, Oxford, and Oxford Centre for Molecular Sciences is gratefully acknowledged.

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