Mechanism of Rubisco: The Carbamate as General Base[⊗]

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I. Introduction

Every year about 10^{11} metric tons of CO_2 are converted to organic material by the process of photosynthesis. Most of the carbon in the food we eat, in the fuel we burn, and in the clothes we wear has passed at one time or other through the active site of the world's most abundant enzyme, ribulose 1,5-bisphosphate carboxylase (Rubisco). The overall reaction catalyzed by Rubisco (Figure 1), the addition of CO_2 and H_2O to D-ribulose 1,5-bisphosphate (I) to yield one molecule each of *upper* 3-phospho-D-glycerate (IIa) and *lower* 3-phospho-D-glycerate (IIb), involves multiple discrete steps and associated transition states; enolization of ribulose 1,5-bisphosphate, subsequent reaction of $^{s}CO_2$ and H_2O with the 2,3-enediol(ate) (III) to yield the six-carbon hydrated ketone intermediate, 2C3KABP (IV), carbon−carbon cleavage between C-2 and C-3 to form *lower* 3-P-glycerate (IIb), and the *aci*-acid of *upper* 3-P-glycerate (V), inversion of configuration and stereospecific protonation of V to form *upper* 3-phospho-D-glycerate (IIa).

Rubisco also catalyzes the energy-wasteful biodegradative oxidation of RuBP (Figure 1), thereby compromising the efficiency of photosynthetic carbon assimilation that would otherwise prevail. The oxygenation pathway, strikingly similar to the carboxylation pathway, entails reaction of oxygen and water with the initial 2,3-enediol(ate) intermediate to form a hydrated peroxy ketone (VI), which undergoes C2−C3 scission to form *lower* 3-P-glycerate (IIb) and 2-phosphoglycolate (VII). At any given $[CO_2]/[O_2]$, the fractional partitioning of RuBP between the carboxylation and oxygenation pathways is dictated by the relative reactivity of the enzyme-bound 2,3-enediol(ate) toward CO_2 and O_2. Although evolutionary pressures have increased partitioning in favor of carboxylation, the feasibility of reducing the oxygenase activity, indigenous to Rubisco from all sources, to physiologically insignificant levels is problematic.

Because of its significance to agriculture, and biomass production generally, Rubisco has been the subject of considerable mechanistic and structural scrutiny, some of it previously reviewed.[1−4] There are now some 17 crystal structures of various forms of Rubisco (Table 1), some to very high resolution, containing a variety of ligands. These structures can be considered as snapshots taken at various points along the reaction coordinate. They impose severe mechanistic constraints. Other mechanistically revealing details have emerged as a result of site-directed mutagenesis. Exacting analyses of the

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[⊗] Abbreviations: *lower* 3-P-glycerate, the molecule of 3-phospho-D-glycerate formed from C-3, C-4, and C-5 of D-ribulose 1,5-bisphosphate and water; *upper* 3-P-glycerate, the molecule of 3-phospho-D-glycerate formed from substrate CO_2 and C-1 and C-2 of D-ribulose 1,5-bisphosphate; RuBP, D-ribulose 1,5-bisphosphate; Rubisco, ribulose 1,5-bisphosphate carboxylase-oxygenase [EC 4.1.1.39]; XuBP, D-xylulose 1,5-bisphosphate; $^{A}CO_2$, activator CO_2, the molecule of CO_2 which forms the carbamate on K201(191); $^{c}O_{nc}$, the carbamino oxygen atom not coordinated to the metal ion; $^{c}O_{c}$, the carbamino oxygen atom coordinated to the metal ion; ^{A}C, the carbamino carbon atom; $^{s}CO_2$, substrate CO_2; $^{s}O_{c}$, the atom of oxygen derived from CO_2 which becomes coordinated to the metal ion in 2C3KABP; $^{s}O_{nc}$, the atom of oxygen derived from CO_2 which is not coordinated to the metal ion in 2C3KABP; 2CABP, 2′-carboxy-D-arabinitol 1,5-bisphosphate; 2C3KABP, 2′-carboxy-3-keto-D-arabinitol 1,5-bisphosphate; 4CABP, 4′- carboxy-D-arabinitol 1,5-bisphosphate. There are two oligomeric forms of Rubisco. The simpler form, consisting of two large subunits, is referred to as L_2, whereas the more common form, consisting of eight large subunits and eight small subunits, is referred to as $(L_2S_2)_4$. The amino acid residues of Rubisco are numbered as found in the large subunit of hexadecameric spinach Rubisco, with the corresponding residue of dimeric *Rhodospirillum rubrum* Rubisco denoted in parentheses.

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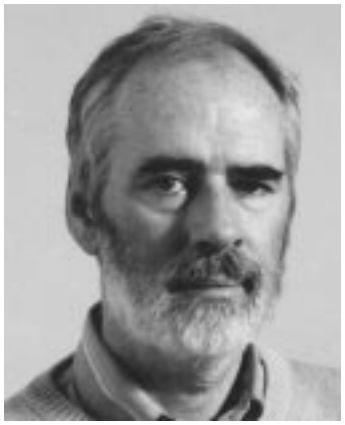
John Andrews is Professor of Molecular Plant Physiology in the Research School of Biological Sciences at the Australian National University. Before joining the National University in 1988, he was at the Australian Institute of Marine Science. A graduate of the University of Queensland, he had postdoctoral appointments at Michigan State University and the Australian National University. His main interests are in protein structure and function and in the manipulation of photosynthesis in transgenic plants.



Steven Gutteridge, a native of Tees-side, UK, spent his formative years in Glasgow, Scotland, acquiring both the vernacular and a B.Sc. in Biochemistry from Strathclyde University. Subsequently as a Carnegie Scholar, he received a Ph.D. with Don Robb, who first stimulated his interest in metalloenzymes. As a NATO scholar he studied copper-containing enzymes with Howard Mason at the University of Oregon Health Science Center, Portland, before joining Bob Bray at the University of Sussex to study Mo-hydroxylases. His first "proper job" was at the Rothamsted Experimental Station, accepting that Mg(II) bound to Rubisco really does catalyze an oxygenase reaction. He initiated site-directed mutagenesis at DuPont in 1983 with a view to improving Rubisco's specificity. This was combined with structural information resulting from a collaboration with the crystallographers in Uppsala, providing a detailed picture of the reaction mechanism. He is currently in the Agricultural Products Department of DuPont pursuing designed, intuitive and serendipitous means to new crop protection chemicals. He also supervises the Macromolecular Analysis Group in Central Research.

Fred C. Hartman is a senior investigator of the Life Sciences Division at the Oak Ridge National Laboratory and adjunct professor of biomedical sciences at the University of Tennessee. He received his B.S. in chemistry in 1960 from the University of Memphis and M.S. and Ph.D. in biochemistry in 1964 from the University of Tennessee. After completion of postdoctoral studies at the University of Illinois, he joined the Biology Division of the Oak Ridge National Laboratory in 1966 and served as its Director from 1988 through 1997. His research has centered on the design and use of affinity labels to map active sites of enzymes of carbohydrate metabolism and on the application of combined genetic and chemical approaches to elucidate precise roles of catalytic residues. Ongoing studies concern specificity determinants of Rubisco, structure–function relationships of phosphoribulokinase, and redox regulation of photosynthetic enzymes by thioredoxin.

reaction products formed by the mutant enzymes permit us to decipher the nature of the intermediates whence they are derived. Combined with structural information these results allow us to deduce the roles played by several active-site residues. A gratifyingly consistent picture of the mechanism of this key enzyme thus emerges.

It has been known for quite some time that Rubisco is activated for both carboxylation and oxygenation by formation of a lysyl carbamate in the active site.[5−11] It was further understood that the molecule of CO_2 which formed the carbamate was distinct from that which became fixed during carboxylation.[12,13] However, the precise role played by this lysyl carbamate in the carboxylation and oxygenation reactions remained obscure, although an undefined role in catalysis was invoked on the basis of noncovalent chemical rescue of the inactive mutant K201(191)C.[86] Now it is clear that the divalent metal ion coordinated lysyl carbamate plays the role of a cofactor. As such, it is central to our understanding of the Rubisco mechanism.

In 1998, after 19 years in the Central Research and Development Department at the DuPont Company's Experimental Station, latterly as DuPont Fellow, George H. Lorimer was appointed professor in the Department of Chemistry and Biochemistry at the University of Maryland, College Park. He received the B.Sc. degree with honors from the University of St. Andrews, Scotland, in 1965. After a period as high school teacher, he immigrated to the United States, gaining an M.S. from the University of Illinois in 1968 and a Ph.D. in Biochemistry from Michigan State University in 1972. After postdoctoral stints at the Max Planck (Berlin-Dahlem) and the Research School of Biological Sciences at the Australian National University, he joined the DuPont Company in 1978. Besides his interests in the role of carbamates in biology in general, he has contributed to our current understanding of chaperonin-assisted protein folding.

II. Carbamates

The reaction of CO_2 with an uncharged amine to form a carbamate (Figure 2) has long been recognized as a facile reaction.[8,14−16] However, the full functional significance of carbamate formation is seldom appreciated. This is due in part to the ephemeral nature of carbamates, to the fact that CO_2 is a universal contaminant of almost all biochemical assay systems and to the fact that the equilibria associated with carbamate formation are typically unfavorable under physiological conditions. But the p*K*s of amines are influenced by their environment. For example, in free solution valine has a p*K* of about 9.7 but as N-termini of the α- and *β*-chains of deoxyhemoglobin the valylamine p*K*s are 6.8 and 7.8, respectively.[17] This enables carbamate formation to occur with consequences for the affinity of hemoglobin for O_2 that are physiologically important.[18,19] In a positively charged environment, the p*K* of an amine is lowered toward the neutral pH range, thereby favoring carbamate formation. It is for this reason that carbamates form very readily on α,*β* diamines at neutral pH.[20]

Table 1. Crystal Structures of Rubisco

species	resolution (Å)	ref
Rhodospirillum rubrum		
L_2	1.7	92
L_2·2CABP	2.6	93
L_2·3-P-glycerate	2.9	94
L_2·^{A}CO_2·Mg^{II}	2.3	22
L_2·^{A}CO_2·Mg^{II}·RuBP	2.6	48
Synechococcus		
$(L_2S_2)_4$·^{A}CO_2·Mg^{II}·2CABP	2.2	37
$(L_2S_2)_4$·XuBP	2.3	95
Spinacia oleracea (spinach)		
$(L_2S_2)_4$·^{A}CO_2·Mg^{II}·2CABP	2.4	36
	1.6	28
$(L_2S_2)_4$·^{A}CO_2Mg^{II}	2.1	23
$(L_2S_2)_4$·^{A}CO_2·Mg^{II}·(3-P-glycerate)$_2$	2.2	89
$(L_2S_2)_4$·^{A}CO_2Ca^{II}·RuBP	2.1	30
$(L_2S_2)_4$·RuBP	2.4	30
$(L_2S_2)_4$·XuBP	2.3	96
$(L_2S_2)_4$·4CABP	2.3	96
Nicotiana tobacum (tobacco)		
$(L_2S_2)_4$·^{A}CO_2·Mg^{II}·2CABP	2.7	38
$(L_2S_2)_4$	2.0	90
$(L_2S_2)_4$·2CABP	2.7	97

When part of larger structures such as proteins, carbamates can be stabilized by noncovalent, intramolecular interactions. For example, the carbamate on the N-terminal valine of the *β*-chain of hemoglobin is stabilized by an intra-subunit salt bridge with a positively charged ϵ-amino group of K82.[21] Likewise divalent metal ions can stabilize protein-bound carbamates. For example, Rubisco assumes catalytic competence upon activation by CO_2 and Mg(II). This entails conversion of K201(191) in the active site to a carbamate, which is synergistically stabilized by monodentate coordination to a Mg(II). In turn this is coordinated to D203 (193) and E204(194).[5−7,11,22,23] The carbamate is additionally stabilized by hydrogen bonding between the carbamino nitrogen and the main chain carbonyl oxygen of D202(N192).[4,22]

Recently, the crystal structures of urease[24,25] and phosphotriesterase[26,27] were solved, revealing the presence of a lysyl carbamate as the bridging ligand between two divalent metal ions, Ni(II) in urease and Zn(II) in phosphotriesterase. The monodentate coordination of two metal ions to a single carbamate in phosphotriesterase and in urease points strongly to the involvement of a resonance form of the carbamate which has hitherto received little attention, namely the *aci*-carbamate (Figure 2). An *aci*-carbamate, coordinated monodentately to a metal ion, retains a negative charge on the other noncoordi-

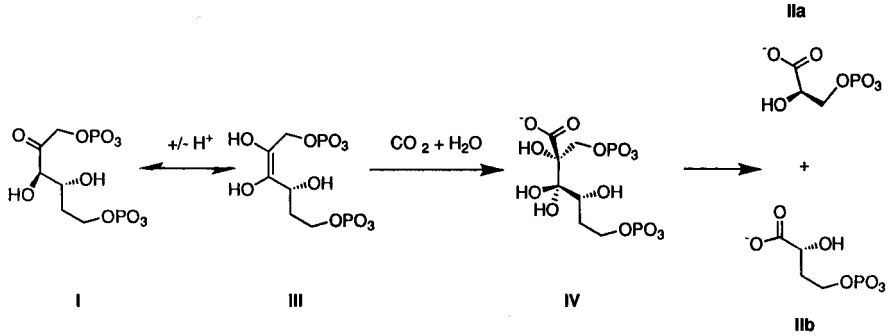


Figure 1. The carboxylation of ribulose 1,5-bisphosphate.

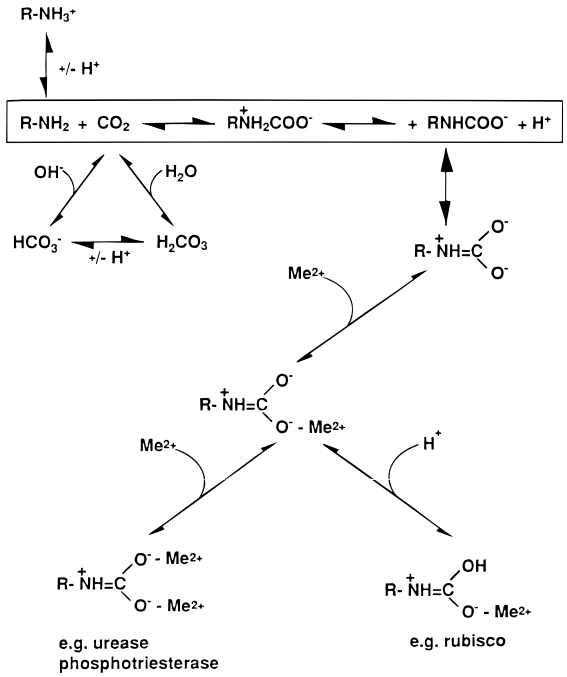


Figure 2. Carbamate formation. This involves the reaction of CO_2 with an unprotonated amine, proceeding through a zwitterionic species.[8,14−20] In proteins the *aci*-carbamate resonance form, $R-^{+}NH-COO^{2-}$ is stabilized by coordination of each of the oxygen atoms to different divalent metal ions in the case of urease and phosphotriesterase and by coordination of one divalent metal ion to one of the oxygen atoms in the case of Rubisco. It is unlikely that the metal-coordinated carbamino oxygen ($^{c}O_c$) senses the full +2 positive charge of the metal ion, in the case of Rubisco, as the metal ion is also liganded by two monodentate carboxylate oxygen atoms from D203 and E204. The carbamate in Rubisco is further stabilized by a hydrogen bond between the carbamino N and the main-chain carbonyl O of D202.

nated oxygen atom which can act as a general base. This feature is central to understanding the mechanism of the reaction catalyzed by Rubisco.[28]

III. Rubisco—Key Stereochemical, Kinetic, and Structural Constraints

A large number of crystal structures of Rubiscos from various sources, activated and nonactivated, containing a variety of ligands have been reported (Table 1). In addition, many kinetic, stereochemical, and structural investigations, employing very different techniques have been undertaken. The key mechanistic conclusions are consistent with one another. Here we summarize the most important points.

1. RuBP is oriented in the active site with the *Si* face of C-2 accessible to the bulk solution.[29,30]

2. The reaction is kinetically ordered with $^{s}CO_2$ adding to the enzyme−RuBP enediol(ate) complex.[31−33]

3. Consistent with the above, $^{s}CO_2$ adds to the *Si* face of C-2 of the enediol(ate) to form the six-carbon β-keto acid intermediate, 2C3KABP[34] (Figures 3 and 4F,G).

4. The crystal structure of the quaternary $(L_2S_2)_4 \cdot {}^{A}CO_2 \cdot Mg \cdot 2CABP$ shows that the carboxyl group of 2CABP, which substitutes for the carboxyl group of

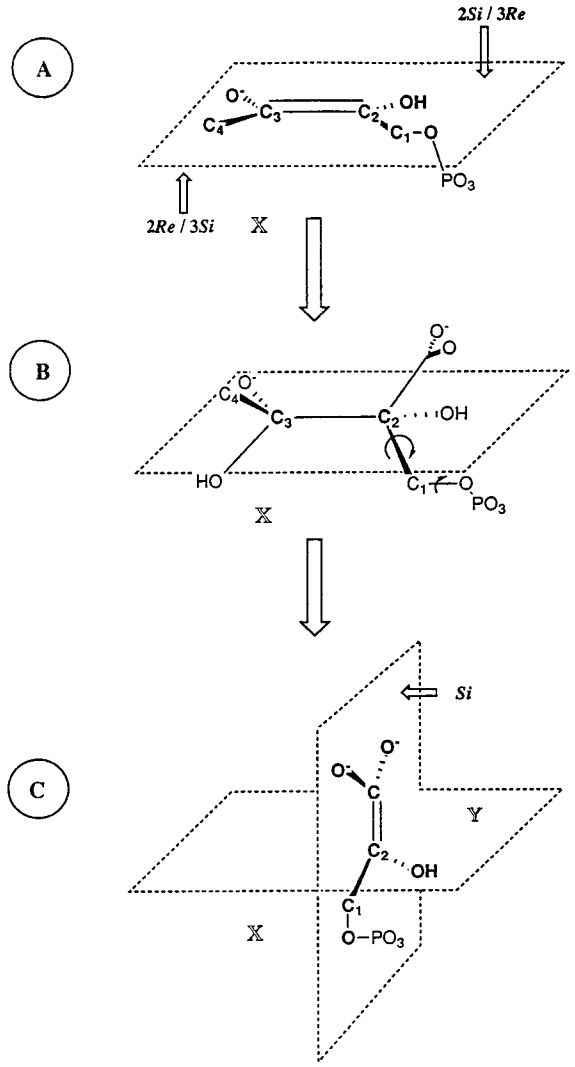


Figure 3. Stereoelectronic control. The carboxylase reaction involves two *cis*-enediolate phosphates, the 2,3-*cis*-enediolate of RuBP (A) and the *aci*-carboxylate form of *upper* 3-P-glycerate (C). The undesired β-elimination of the 1-phosphate from the 2,3-*cis*-enediolate is suppressed by holding the C-1/O-1 bond in the plane of the *cis*-enediolate. Atoms describing this plane are shown in bold letters in (A). Carbon bond cleavage of the hydrated, six-carbon reaction intermediate (B) creates the second *cis*-enediolate (C), the plane of which is defined by the bold letters in (C). Note particularly that the respective planes of these *cis*-enediolates are orthogonal. If the C-1/O-1 bond remained in the plane of the first *cis*-enediolate, orthogonal to the plane of the *aci*-carboxylate, β-elimination of the P1 phosphate and the formation of pyruvate would result. In wild-type Rubisco, β-elimination of the P-1 phosphate from the *aci*-carboxylate is suppressed in 993 out of 1000 turnovers.[71] This can be accomplished if, during cleavage of the C-2/C-3 bond, clockwise rotations of the C-1/C-2 and C-1/O-1 bonds bring the C-1/O-1 bond into the plane of the *aci*-carboxylate (see also Figure 6). This change in the planarity as the reaction proceeds from one *cis*-enediolate (A) to the other (C), can be accomplished with minimal movement of the P1 phosphate. Note also that the general acid/base groups (X and Y) which transfer a proton to and from these *cis*-enediolates are stereochemically remote from one another and must therefore be distinct. They are also ideally situated for the task in stereoelectronic terms. Thus, the $^{c}On_c$ of the *aci*-carbamate (X) on K201(191) and the ϵ-amino group of K175(166) (Y) are respectively orthogonal to the 2*Re*/3*Si* face of the *cis*-enediolate (A) and the *Si* face of C-2 of the *cis*-enediolate (C).

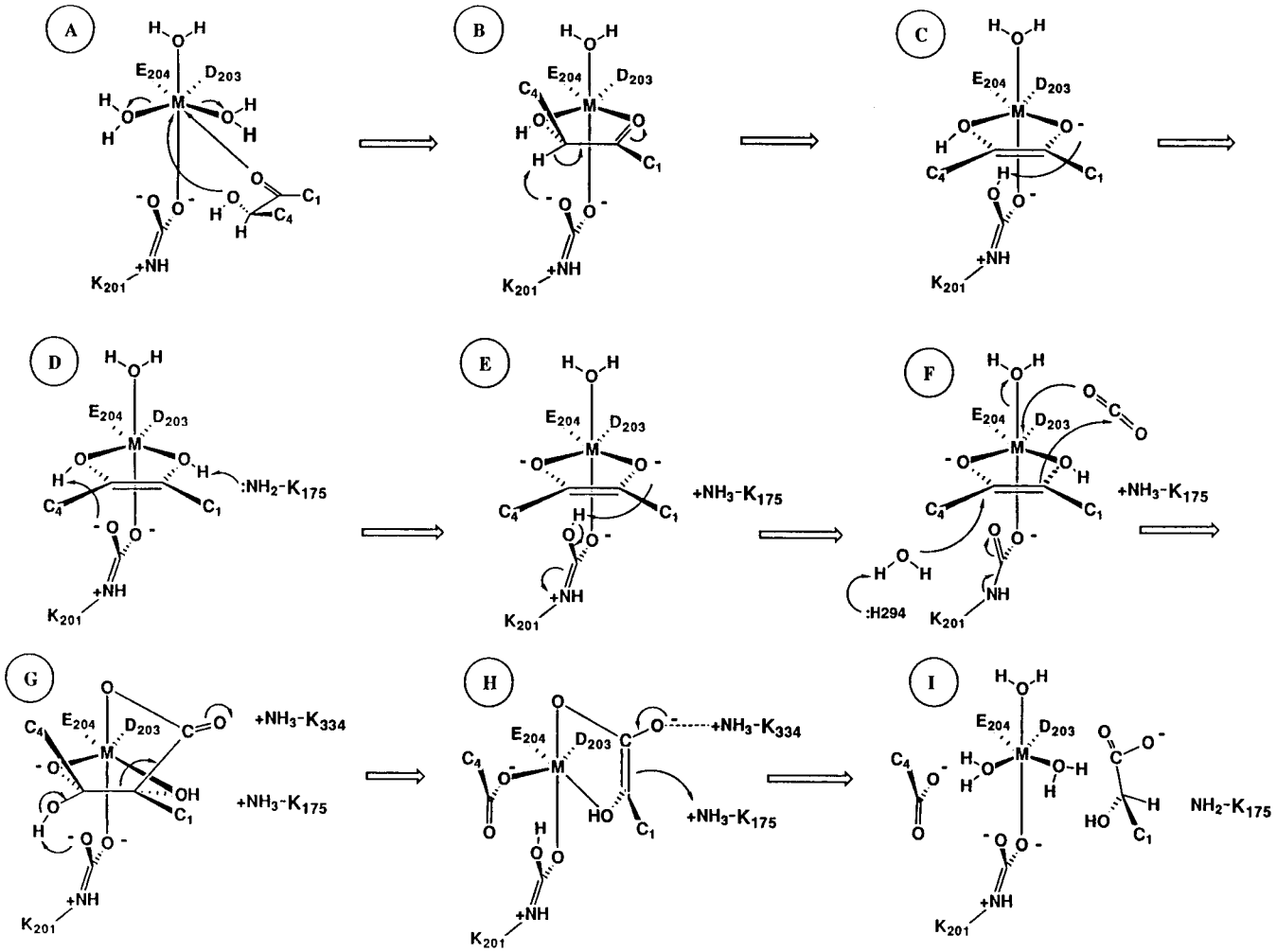


Figure 4. The mechanism of carboxylation of RuBP. See text for a detailed description.

the six-carbon reaction intermediate that is derived from $^{s}CO_2$, points toward the bulk solution.[28,35−38] This is entirely consistent with the ordered addition of $^{s}CO_2$ to the *Si* face of C-2 of the enzyme-bound 2,3-enediol(ate) of RuBP.

5. The crystal structure of $(L_2S_2)_4$·$^{A}CO_2$·Mg·2CABP shows that the C-3 proton of 2CABP, which substitutes for one of the hydroxyl groups of the hydrated six-carbon reaction intermediate, is shielded from the bulk solution.[28,35−38] Likewise, in the crystal structure of $(L_2S_2)_4$·$^{A}CO_2$·Ca·RuBP the C-3 proton of RuBP is directed away from the bulk solution.[30]

6. High-resolution crystal structures of the quaternary $(L_2S_2)_4$·$^{A}CO_2$·Mg·2CABP complex show that the oxygen atoms attached to C-2 and C-3 of 2CABP are in the cis conformation, coordinated to Mg(II) as part of two, fused five-membered rings.[28,37] Likewise the oxygen atoms attached to C-2 and C-3 of RuBP are in the cis conformation, coordinated to Mg(II) in the crystal of the $(L_2S_2)_4$·$^{A}CO_2$·Ca·RuBP complex.[30]

7. Hydrolysis of the six-carbon intermediate, 2C3KABP, to produce a molecule each of *lower* 3-P-glycerate and *upper* 3-P-glycerate, involves inversion of configuration about C-2 and the addition of a proton to the *Si* face of the *aci*-carboxylate form of *upper* 3-P-glycerate, which is stereochemically remote from the position occupied by the C-3 proton of RuBP[1] (Figures 3 and 4G−I).

Two mechanistically important conclusions may be drawn from the above, which are germane to the roles played by various amino acid residues in the acid−base chemistry of the reaction.

1. Since the oxygen atoms at C-2 and C-3 of enzyme-bound RuBP and 2CABP are in the cis conformation, we may deduce that the $^{s}CO_2$ adds to the *cis*-2,3-enediol(ate) of RuBP. Since the *Si* face of C-2 of RuBP faces upward toward the bulk solution we may deduce that the proton at C-3, which is removed during enolization, projects downward, away from the bulk solution[28,30,37] (Figure 4B). Consequently, the enzyme base responsible for abstracting the C-3 proton of RuBP is constrained to occupy this sector of the active site; i.e., beneath the plane created by the five-membered metal ion coordinated *cis*-enediol ring, and shielded by this ring from the bulk solution.

2. Since the *Si* face of the *aci*-carboxylate of *upper* 3-P-glycerate is stereochemically remote from the position occupied by the C-3 proton of RuBP, the group which donates the proton to C-2 to terminate the reaction must be different from the group which abstracts the C-3 proton of RuBP to initiate the reaction;[1] i.e., a minimum of two acid−base groups, X and Y, are needed to satisfy the constraints imposed by the inversion of stereoconfiguration about C-2 (Figure 3).

IV. Acid–Base Chemistry of Rubisco

Calvin[39] was the first to recognize that the reaction catalyzed by Rubisco most likely proceeds via an enediol(ate) intermediate of RuBP. Hydrogen isotope exchange experiments[40,41] provided the first evidence for this. Furthermore, the proton abstracted from C-3 ultimately exchanges with the medium since it is not transferred to C-2 of *upper* 3-P-glycerate.[42] It was subsequently shown that the reaction is kinetically ordered with RuBP binding first and that $^{s}CO_2$ is not required for enolization.[31,43] There has been an intense search to identify the general base that facilitates enolization.[2,3] Extensive chemical and mutagenesis data are compatible with K175(166) serving such a role. The ϵ-amino group of this residue exhibits enhanced acidity and nucleophilicity relative to prototypical counterparts[44] and its p*K* of 7.9 closely approximates that of an ionizable group observed in the pH profile of the deuterium isotope effect with 3-deuterated RuBP as substrate.[33] Replacement of K175(166) with glycine abolishes carboxylase and enolization activities but does not severely impair processing of 2C3KABP;[45,46] i.e., the role of K175(166) appears singularly suited as the base catalyzing the enolization step. Further, replacement of K175(166) by a less basic aminoethylcysteinyl residue reduces k_{cat} 5-fold, demonstrating a correlation of catalytic turnover and the p*K* of the amino group at this position.[47]

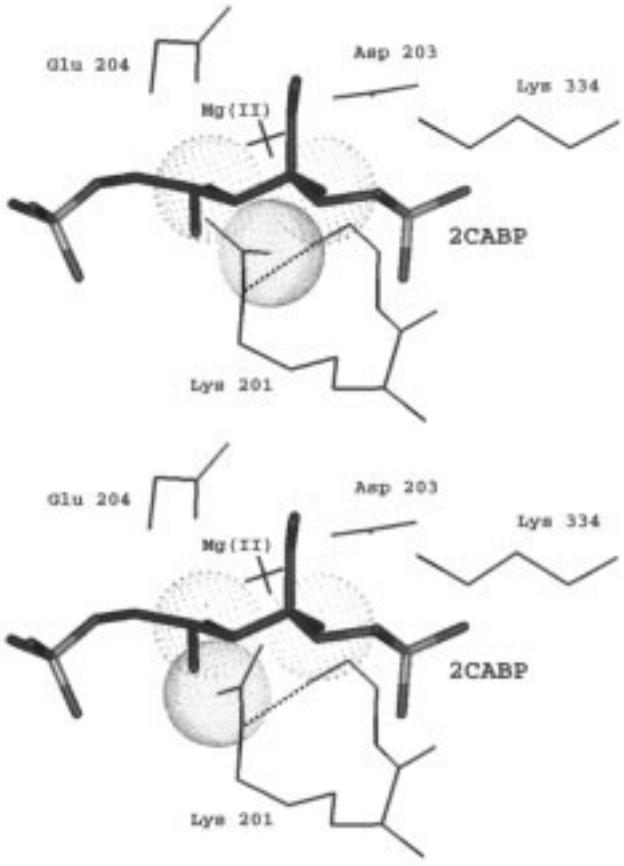


Figure 5. Carbamate-assisted proton relay. Proton transfer to O-2 from either C-3 of RuBP (Figure 4B−D), or the metal ion coordinated oxygen atom, O-3, of the enediolate form of RuBP (Figure 4D−F), can proceed via $^{c}O_{nc}$. In both panels O-3, O-2, both coordinated to Mg(II), and $^{c}O_{nc}$ are represented with the appropriate van der Waals radii. In panel A (top), the $^{c}O_{nc}$ occupies the position closest to the C-3 groups of the bisphosphate at the point of accepting a proton. In panel B (bottom), the carbamate has rotated to the position where it can donate the proton to O-2. On the basis of coordinates for the *Synechococcus* $(L_2S_2)_4{\cdot}^{A}CO_2{\cdot}Mg^{II}{\cdot}$2CABP complex.[37]

Subsequent X-ray structures of activated Rubisco with bound 2CABP showed that K175(166) is too distant from the C-3 position of the bisphosphate to act as the acceptor of the C-3 proton in enolization, but it is exquisitely positioned to act as the general acid that stereospecifically protonates the terminal *aci*-acid intermediate and thereby form *upper* 3-P-glycerate at the terminal step of carboxylation[35−38,48] (Figures 4 and 6).

The limited resolution of the earliest crystal structures did not permit a precise description of the groups coordinated to the metal ion. For example, in the 2.4 Å structure of the spinach $(L_2S_2)_4{\cdot}^{A}CO_2{\cdot}$Mg·2CABP complex[36] and in the 2.6 Å structure of the *R. rubrum* $L_2{\cdot}^{A}CO_2{\cdot}$Mg·RuBP complex[48] the carbamino oxygens were thought to be bidentately coordinated to the metal ion. Additionally, O-2 and O-3 of 2CABP were assigned the trans conformation, with O-2 and O-4 being coordinated to the metal ion.[36] Prospective candidates for the base that promotes enolization, as deduced by crystallography, were systematically discounted by mutagenesis.[49−53]

Higher resolution structures of both the cyanobacterial Rubisco[37] and subsequently of the spinach enzyme[23,28,30] resolved many of these uncertainties. The carbamate on K201(191) is quite clearly monodentately coordinated to the metal ion. The O-2 and O-3 atoms of 2CABP are in the cis conformation, coordinated in the equatorial plane of the metal ion as part of a five-membered ring (Figures 4 and 5). With the O-3 atom cis to O-2, the C-3 proton of 2CABP, which corresponds to the one in RuBP removed during enolization, is only 3.1 Å from $^{c}O_{nc}$. The carbamate on K201(191) was thus proposed as the most likley group to act as the general base at the initial and subsequent steps of the reaction.[28,30,37]

V. The Basicity of $^{c}O_{nc}$

The question thus arises, is the carbamate the elusive base for enolization? Hitherto, the general view has been that a carbamate could not simultaneously coordinate to a metal ion, albeit with a rather long Me−O bond, and also act as a base. This view is based upon the behavior of isosteric carboxylates. As noted above, metal ion coordination lowers the p*K* of a carboxylate so greatly that it can no longer act as a general base. However, the properties of a carbamate are quite different. The fact that the single lysyl carbamate in urease[24,25] and in phosphotriesterase[26,27] can serve as monodenate ligands to not one, but two divalent metal ions, argues strongly that considerable negative charge can be localized on both oxygen atoms of the carbamate (Figure 2). In Rubisco, one oxygen of the carbamate is coordinated to the Mg(II) but the noncoordinated oxygen of the carbamate can retain a negative charge. The resultant *aci*-carbamate has net positive charge localized on the carbamino N that is further stabilized by a

hydrogen bond to the main chain carbonyl oxygen of D202(N192) (Figure 5).[4,22]

An important but unresolved problem concerns the basicity of $^{c}O_{nc}$ and thus its ability to act as a general base. Several factors are likely to influence its pK_a. It is difficult to quantitatively assess the magnitude of each of these factors although their probable contributions can be qualitatively considered. Binding of RuBP displaces two water molecules from the inner coordination sphere of Mg(II) and sequesters the carbamate from the bulk solution. A single molecule of water is observed near C-3 in the *R. rubrum* L_2·$^{A}CO_2$·Mg^{II}·RuBP complex—perhaps the substrate water.[48] In the spinach $(L_2S_2)_4$·$^{A}CO_2$·Mg^{II}·2CABP complex no water is seen near the carbamate, indicating that the carbamate experiences a substantially nonaqueous environment.[28] The carbamates of simple alkylamines have a p*K* of about 5.8 in water, about one pH unit more basic than acetic acid in water. However, there are several polar groups within hydrogen-bonding distance of the carbamate, including His 294 and the hydroxyl oxygens of the substrate. These, in concert with the large number of peptide dipoles, should render the environment nonaqueous but somewhat polar. As pointed out elsewhere,[54] polar but nonaqueous solvents such as methanol and dimethyl sulfoxide increase the p*K* of a carboxylate by 8 units or more, and a carbamate should respond similarly.

If RuBP is bound to the activated enzyme in the same orientation as is the inhibitor 2CABP, the proton at C-3 of RuBP would be transferred to the syn orbital of $^{c}O_{nc}$. Gandour[55] has suggested that the syn orbital of a carboxylate oxygen should be many orders of magnitude more basic than the anti orbital. For enolization reactions a difference in p*K* between the syn and anti orbitals of 1−2 units has been experimentally observed.[56] Both of the above factors suggest that the p*K* of the syn orbital of $^{c}O_{nc}$ in the Rubisco·$^{A}CO_2$·Mg^{II}·RuBP complex would likely be substantially greater than 5.8.

However, there are other factors to consider, especially the influence of Mg(II) and its coordinating ligands. If there were no other ligands to consider, the presence of a divalent cation coordinated to one of the oxygens of the carbamate would be expected to increase the acidity of the noncoordinated oxygen. This is especially true when the proton and the metal ion compete for the syn orbitals of the oxygen atoms, as appears to be the case in Rubisco. However, this effect is likely ameliorated by the unusually long $^{c}O_{c}$−Mg^{II} bond[37] and the carboxylate ligands D203 and E204 shield the $^{c}O_{c}$ from the full influence of the positive charge on the divalent metal ion.

Another way to estimate the p*K* of the Mg(II)-bound carbamate is to consider the p*K*s of the enol form of mandelic acid. The first p*K* was measured by Kresge[57] as 6.6, and the second p*K* (the one corresponding to the carbamate p*K* of interest) was estimated by Gerlt and Gassman[58] as 10. Since the positively charged nitrogen in the *aci*-carbamate would be more electron withdrawing than the hydroxyl group in enol-mandelate, the second p*K* of the carbamate should be at least several pH units lower than 10.

We therefore suggest that the p*K* of $^{c}O_{nc}$ is approximately neutral, entirely compatible with functioning as a general base. Andersson[28] has also suggested that the p*K* and electronic state of the carbamate on K201(191) is fine-tuned by the other ligands to Mg(II), in particular D203(193) and E204(194) and their hydrogen bonding to other active-site residues. Thus, we suggest that the carbamate formed on K201(191) of Rubisco during activation is the general base that removes the C-3 proton of RuBP to effect enolization. We further suggest that the carbamate is part of a proton relay involving the metal-coordinated O-3 and O-2 of RuBP and the ϵ-amino group of K175(166). The mechanism we envision for the carboxylation of RuBP is shown in Figure 4 and is described in detail below.

VI. Binding of RuBP

Activation of the enzyme requires the reaction of K201(191) with CO_2 which is then stabilized by monodentate coordination to Mg(II).[5−7,11,22,28,30,35−37] The other key Mg(II) ligands in the activated enzyme are D203(193) and E204(194), both with monodenate coordination. Three water molecules complete the octahedral coordination sphere around the metal ion[23] (Figure 4A). When RuBP binds, O-2 and O-3 become coordinated to Mg(II), displacing two of the three water molecules and forming a five-membered ring (Figure 3B). The coordination of O-2 and O-3 by the metal ion almost certainly increases the acidity of the protons attached to C-3 and O-3, thus assisting with their subsequent removal.

VII. Enolization

The combined influence of the metal on the C-3 proton and the proximity of the syn orbital of $^{c}O_{nc}$ suggests that it is ideally positioned to fulfill its function as the acceptor of the proton and thereby initiate enediol formation. $^{c}O_{nc}$ is orthogonal to the planar, five-membered, metal ion coordinated, *cis*-2,3-enediolate, thus satisfying stereoelectronic constraints (Figure 4B,C).[59,60] The O-2 of the enediolate has negative charge that should be neutralized so as not to interfere with subsequent steps in catalysis. Proton transfer from $^{c}O_{nc}$ to the syn orbital on O-2 can be accomplished by rotation of the carbamate beneath the plane of the enediolate (Figures 4C,D and 5). At the optimum position for transfer from $^{c}O_{nc}$ to O-2, both carbamino oxygens are equidistant to the Mg(II), suggesting that they are at least transiently bidentate to the metal. Transfer of the proton to O-2 would allow the double bond to re-form between the carbamino C and N atoms, "snapping" the $^{c}O_{nc}$ back to its original position.

We presume that normally the proton on O-2 is transferred to K175(166), given the proximity of the ϵ-amino group to O-2[28] and the low p*K* of this residue. However, K175(166) ultimately donates a proton to the *Si* face of the *aci*-carboxylate of *upper* 3-P-glycerate to complete the reaction (Figures 3, 4G−I, and 6). The C-3 proton of RuBP is not conserved in

upper 3-P-glycerate,[31,61] so it must exchange with the medium at some point before the last step of the reaction. The paucity of enolization activity with the K175(166)G mutant suggests that solvent exchange of the C-3 proton normally occurs via K175(166). Thus, in the absence of this key lysyl residue, the C-3 proton may reversibly shuttle between the carbamate and RuBP but be unable to exchange directly with the solvent.

If indeed proton transfer from C-3 to $^{c}O_{nc}$ to O-2 reaches K175(166), an enediolate is created in which O-2 is unprotonated, while the O-3 is protonated. We suggest that the proton on O-3 is below the plane of the enediolate and syn to the carbamate. Note that in this metal ion coordinated ring, the nonbonding orbitals on O-2 and O-3 point away from one another at a distance of 3.1 Å. In such circumstances a large barrier exists to the direct transfer of the proton from O-3 to O-2.[62] However, the carbamate can again facilitate the transfer of the proton on O-3 to O-2 (Figure 4D−F). Using the carbamate to facilitate proton transfer among C-3, O-3, and O-2 is similar in some respects to the mechanism of proton transfer suggested for the H95Q mutant of triose phosphate isomerase.[63] In that instance E165 of the triose phosphate isomerase mutant, like the carbamate in Rubisco, is similarly located orthogonal to the enediol, and the syn orbital of the carboxylate oxygen is also thought to facilitate proton transfer.

VIII. Addition of CO_2 and H_2O: A Sequential, Two-Step Process or a Concerted Reaction?

Once the substrate has been converted to the *cis*-2,3-enediolate with ionized oxygen at C-3 and protonated oxygen at C-2, the stage is set for reaction with $^{s}CO_2$ and water. The crystal structure of *R. rubrum* Rubisco·$^{A}CO_2$·Mg·RuBP complex[48] has a molecule of water within 4 Å of C-3 of RuBP and it is attractive to consider that this is the substrate water molecule, ideally positioned to add to C-3 below the plane of the enediolate (Figure 3F). Addition of $^{s}CO_2$ to the *Si* face of C-2 and water to the *Si* face of C-3 yields the hydrated six-carbon intermediate, 2C3KABP (Figures 2B and 3F−G). This can be isolated from reaction mixtures rapidly quenched with acid, and when added back to fresh enzyme, is converted to two molecules of 3-P-glycerate.[32,34,64,65] In free solution this intermediate exists almost entirely as the dehydrated free ketone.[32] However, within the active site of Rubisco, this intermediate is predominantly (perhaps only) hydrated.[66] The conversion of this intermediate to product by fresh enzyme is slow (about 3% of the normal k_{cat}). Cleland[67] has suggested that this is because hydration of the keto group on the enzyme is a slow process. Alternatively, the binding of the dehydrated intermediate may involve a slow conformational change in the protein,[32,68] such as the loop movements that close the active site.[36,37] In any event, the hydrolysis of exogenously supplied intermediate is rate determined by a process that is not on the direct catalytic pathway.

The precise details of the reaction of the enediolate with $^{s}CO_2$ and water are unclear. Hitherto, it has been assumed that this is a sequential, two-step reaction, with the electrons from the enediolate oxygen at C-3 forming a double bond and permitting attack of C-2 on $^{s}CO_2$. On the basis of the crystal structures of bound 2CABP (which closely resembles the six-carbon hydrated ketone intermediate but lacks one hydroxyl group at C-3), catalysis of attack on $^{s}CO_2$ is provided by having the newly formed carboxyl group coordinated to Mg(II) (thus displacing a molecule of water) and hydrogen bonded to K334-(329). Detailed characterization of site-directed mutants provides functional proof of the specific role of K334(329) in facilitating $^{s}CO_2$ addition to the enediolate and in stabilizing the carboxylated intermediate.[99,100] In this formulation of the mechanism, attack of water below the plane of the enediolate would occur on the newly formed keto group at C-3, with general base catalysis by H294(287).

While this mechanism explains the events and roles of catalytic groups, it has a major problem. In a sequential, two-step mechanism, Mg(II) coordination to the ionized hydroxyl group at C-3 will partially neutralize its negative charge and thereby hinder the reaction of $^{s}CO_2$ at C-2. An alternative mechanism is a concerted one in which attack of water at C-3 occurs in the same transition state as attack on $^{s}CO_2$ by C-2[65] (Figure 4F,G). Such a mechanism is likely not synchronous, however, and bond formation at C-2 probably leads bond formation at C-3. But in such a concerted mechanism the oxygen at C-3 does not lose as much negative charge, and the reaction will presumably have a lower activation barrier than a fully stepwise one. The electron density on the oxygen at C-3 certainly permits attack on $^{s}CO_2$, but the developing positive charge at C-3 provides the conditions for water attack as part of the same transition state. A concerted reaction avoids the necessity for generating a high-energy intermediate (the unhydrated ketone) during a reaction which is essentially irreversible; there is no evidence that the reaction with $^{s}CO_2$ is reversible, as would be the case if the unhydrated ketone were an intermediate.

IX. Carbon–Carbon Cleavage

Once the six-carbon hydrated ketone intermediate is formed, the stage is set for cleavage between C-2 and C-3. The Mg(II)-coordinated oxygen at C-3 is still ionized, although its p*K* of 12−13 as a *gem*-diol is now much less than that of the hydroxyl group at C-2 (p*K* of ∼15), so that the proton on O-2 will have no tendency to move to O-3. Steric strain may be involved in activating this step, but in any case bond cleavage between C-2 and C-3 now occurs, with electrons in the C-2/C-3 bond moving to form an *aci*-carboxylate of *upper* 3-P-glycerate (Figures 3G,H and 5).

Cleavage of the C-2/C-3 bond is facilitated by proton removal from the noncoordinated OH group at C-3, so that the coordinated oxygen at C-3 can remain negatively charged. The base for this deprotonation again appears to be the carbamate on K201-(191), so that it plays a multiple role as a general base during the reaction. This formulation of the reaction is also stereoelectronically favorable, in that

the double-bond formation to the noncoordinated oxygen at C-3 is antiperiplanar to the double bond forming in the *aci*-carboxylate.[69,70] If it were the coordinated oxygen at C-3 that became double bonded, the electrons from the C-2/C-3 bond would tend to form a double bond between C-1 and C-2 (thus eliminating phosphate), rather than between C-2 and the carboxyl carbon that came from $^{s}CO_2$.

X. Stereospecific Protonation

To complete the reaction, the *aci*-carboxylate of *upper* 3-P-glycerate is protonated on the *Si* face of C-2 (Figures 2 and 3H,I). This face is anti to the newly formed molecule of *lower* 3-P-glycerate. Protonated K175(166) is ideally positioned to act as the general acid for this step (Figures 2 and 5). The rotation of C-1 and C-2 leading to *aci*-carboxylate formation brings C-2 in contact with K175(166) and should facilitate the protonation that completes the reaction. The speed of this rotation and the subsequent protonation are clearly critical in order to avoid pyruvate formation by elimination of phosphate. [The protonation shows an isotope effect of 3, since the amount of pyruvate produced rises to 2.2% in D2O.[71]] Since mutants lacking the K175(166) side chain are disabled in enolization, the role of this residue can best be gauged by characterizing the products of 2C3KABP hydrolysis. Consistent with the crystallographic deduction that K175(166) serves as the proton donor in the final step of overall carboxylation, a K166G mutant of the *R. rubrum* enzyme generates pyruvate plus *lower* 3-P-glycerate, rather than *upper* 3-P-glycerate plus *lower* 3-P-glycerate, as the predominant products derived from exogenously supplied 2C3KABP.[52]

XI. The Oxygenation Reaction

As intermediates unique to the oxygenase pathway have not been isolated nor have detection methods to monitor their flux during RuBP turnover been reported, this pathway has not been dissected as rigorously as the carboxylation counterpart. However, on the basis of similar chemical transformations, many of the intricate features described for carboxylation seemingly extend to oxygenation as well.

Substantial evidence for the key intermediate 2-peroxy-3-ketoarabinitol 1,5-bisphosphate has accumulated. Shortly after the discovery of the oxygenase activity,[72] isotopic-labeling experiments showed that one oxygen atom from $^{18}O_2$ substrate is incorporated into the carboxylate of phosphoglycolate and the other into water, leading to the proposition of a peroxy ketone intermediate.[73] Spin inversion, as required for such a reaction between triplet-state O_2 and singlet-state substrate, might be effected through a caged radical pair (superoxide radical anion and enediol(ate) radical cation)[1] or by activation of the carbanionic enediol(ate) to the triplet state by geometric deformation.[74,75] Subsequent C2−C3 scission of the peroxy ketone (akin to that of the carboxylation intermediate) with concomitant heterolytic O−O cleavage of the peroxy group yields directly one molecule each of *lower* 3-P-glycerate and 2-phosphoglycolate; formation of the latter does not entail a terminal proton transfer as required to complete carboxylative formation of *upper* 3-P-glycerate.

Although lability of the putative peroxy ketone intermediate precludes its isolation and direct proof of structure, two different site-directed mutants provide a distinctive chemical signature for its occurrence during oxidative turnover of RuBP. E60-(48)Q and K334(329)A, both severly impaired in carboxylase activity, catalyze oxidation of RuBP to the diketone D-*glycero*-2,3-pentodiulose 1,5-bisphosphate with equimolar amounts of hydrogen peroxide being formed as a byproduct.[76,77] The latter mutant further processes the diketone to the branched-chained 2-carboxytetritol 1,4-bisphosphate by a benzylic acid type rearrangement.[77] As the diketone can only have arisen by heterolytic C−O cleavage (expulsion of hydrogen peroxide) of the proposed peroxy ketone intermediate, proof of structure is thereby provided. These studies also demonstrate that wild-type Rubisco plays an active role in stabilizing the oxygenase intermediate and directing its cleavage exclusively to 3-P-glycerate and phosphoglycolate. At first consideration, stabilization of an intermediate in a nonprodutive pathway might appear incongruous. However, partial mitigation of the negative consequences of RuBP oxidation by ensuring the formation of products that can be partially recyled to RuBP would be preferable to the formation of metabolic dead-end products (i.e., the diketone or the branched-chain compound).

Ultimately, the partitioning of RuBP between the carboxylation and oxygenation pathways must reflect the difference between the free energies of the transition states for carboxylation and oxygenation.[81,99] Hence, the partitioning ratio would be expected to be sensitive to the active-site microenvironment. Indeed, several active-site substitutions and chemical manipulations have been reported that alter partitioning, inclusive of slight increases in favor of carboxylation.[78−80] However, these increases have only been observed with species of Rubisco (e.g., bacterial and alga) that exhibit low specificity for CO_2 and do not even approach the levels of CO_2 specificity inherent to native Rubisco from higher plants.

XII. Stereoelectronic Suppression of β-Elimination

cis-Enediol phosphates are especially prone to β-elimination.[82] The overall Rubisco-catalyzed reaction involves two *cis*-enediol(ate) phosphates as intermediates, the *cis*-2,3-enediolate of RuBP and the *aci*-carboxylate form of *upper* 3-P-glycerate. In free solution, both undergo rapid β-elimination of P1 phosphate to yield 1-deoxy-D-*glycero*-2,3-pentodiulose 5-phosphate and pyruvate, respectively.[66,83] However, within the active site, β-elimination of the P1 phosphate is largely (although not completely) suppressed.

The P1 phosphate is hydrogen bonded by the NH groups of three glycines and the OH group of T65-(53) (see Table 2 for identities), while the 5-phosphate is hydrogen bonded to a histidine and an arginine. Thus, the P1 phosphate is bound by induced dipolar

Table 2. Groups Involved in the Rubisco Reaction

amino acid	role in catalysis
K201(191)	forms carbamate which provides mondentate ligand to Mg(II); the non-metal-coordinated oxygen of the carbamate ($^{c}O_{nc}$) serves as the general base for removing the C-3 proton of RuBP, transferring it via the O-2 of RuBP to K175(166)
	facilitates proton transfer between O-3 and O-2 of the enediolate; finally $^{c}O_{nc}$ serves as the general base to remove the non-metal-coordinated hydroxyl proton at C-3 of the *gem*-diol form of 2C3KABP to initiate C-2/C-3 bond cleavage
D202(N192)	carbonyl oxygen accepts H-bond from carbamate NH, increasing the positive charge on this nitrogen
K175(166)	must be neutral for activity (pK ~7.9); accepts proton after enolization; protonates *aci*-carboxylate in last step
H294(287)	general base for water attack at C-3
K334(329)	located at apex of flexible loop which acts like a lid on the active site; assists carboxylation by stabilizing the transition state for addition of $^{s}CO_2$; stabilizes *aci*-carboxylate by hydrogen bonding to $^{s}O_{nc}$ of carboxyl group; also hydrogen bonds to the P1 phosphate; forms intersubunit salt bridge with E60(48)
E60(48)	holds K334(329) in place; assists loop 6 closure; involved in binding
D203(193)	monodentate Mg(II) ligand
E204(194)	monodentate Mg(II) ligand
G403(393)	binding of P1 phosphate
G404(394)	binding of P1 phosphate
G381(370)	binding of P1 phosphate
T65(53)	binding of P1 phosphate
H327(321)	binding of P5 phosphate
R295(288)	binding of P5 phosphate
S379(368)	binding of O-4 of RuBP
N123(111)	NlllQ is active in enolization, but k_{cat} in overall reaction is reduced by 10^4; N111 hydrogen bonds to carboxyl group of 2C3KABP *gem*-diol intermediate
K177(168)	K168Q carries out enolization, but k_{cat} in overall reaction is reduced by 10^4; controls pK of K175(166)?

hydrogen bonding, while the 5-phosphate is bound by ionic hydrogen bonding. The C4-hydroxyl group of the enediol hydrogen bonds to S379(368). These interactions freeze the geometry of the bisphosphate, and anchor the P1 phosphate, particularly the bridging "O" in the plane of C-1, C-2, C-3, so that β-elimination from the enediolate intermediate is suppressed. A similar situation occurs with triose phosphate isomerase and its enediolate intermediate.[84−86] The elimination reaction requires the phosphate to rotate to an out-of-plane position, which in the case of Rubisco leads to 1-deoxy-D-*glycero*-2,3-pentodiulose 5-phosphate. This reaction is not detected with the native enzyme but does occur 13% of the time in a T65V mutant of the cyanobacterial enzyme[87] and 36% of the time in a G393A mutant of the *R. rubrum* enzyme.[88]

The changes in the geometry of the top half of the substrate which accompany bond cleavage between C-2 and C-3 leading to the second enediol(ate) are critical to its productive processing to *upper* 3-P-glycerate. As the C-2/C-3 bond breaks, C-1, C-2 and, to a lesser extent, O-1 must move so that they and the carboxyl carbon all become coplanar. The *aci*-carboxylate formed would have a double bond between C-2 and the carboxyl carbon, and both carboxyl oxygens, $^{s}O_{nc}$ and $^{s}O_{c}$, would be single bonded and negatively charged. Planarity of this system is critical to mitigating the elimination of the P1 phosphate from the *aci*-carboxylate. In fact, pyruvate, the product of this elimination, is formed in 0.7% of turnovers by all Rubiscos studied.[71] Compared to its angle in the *cis*-2,3-enediolate intermediate, the C-1/O-1 bond must rotate by 90° on the axis provided by the C-1/C-2 bond. The fragment of hydrated 2C3KABP that becomes the *aci*-carboxylate is tethered within the active site by coordination of O-2 and one of the carboxyl oxygens with the Mg(II), by hydrogen bonding between K334(329) and the other carboxyl oxygen, and by hydrogen bonding between various residues and the P1 phosphate oxygens (Table 2). The movement to coplanarity must occur within the restraints imposed by these tethers. The necessary rotation can be achieved most economically if, upon cleavage of the C-2/C-3 bond, C-2 moves into the plane defined by C-1, O-1, and atoms of the carboxyl group. This requires some modest changes in the O-2/Mg(II) and $^{s}O_{c}$/ Mg(II) bonds and some movement of the K334(329) side chain to preserve the hydrogen bond to $^{s}O_{nc}$. A smaller movement of O-1 is also needed to bring it into the same plane. Rotations of the C-1/C-2 bond and of the C-1/O-1 bond (clockwise as viewed from P-1) bring the bridge oxygen into the plane of the *aci*-carboxylate so that O-1 and O-2 are syn to each other (Figures 2 and 5). The structure of the spinach $(L_2S_2)_4{\cdot}^{A}CO_2{\cdot}Mg^{II}{\cdot}(3\text{-P-glycerate})_2$ complex shows the *upper* 3-P-glycerate displayed in a manner consistent with these movements.[89]

Suppression of the tendency to partition the product to pyruvate in a mutant of the cyanobaterial enzyme which lacks one of the hydrogen bonds to the phosphate oxygens might be indicative of faster movement to planarity by the *aci*-carboxylate fragment within the mutant active site.[87] If so, this implies that some disturbance to the hydrogen-bonded network around the P1 phosphate group may occur at this stage of the reaction.

XIII. Loop Dynamics

Conformational changes induced by the binding of ligands to Rubisco were first invoked to account for the slow, tight-binding behavior of the reaction intermediate analogue, 2CABP.[9,68] Comparisons of the crystal structures of the enzyme without ligand and those forms with the active site occupied by phosphorylated analogues has provided some definition of these changes in terms of the structural elements involved.[22,23,28,30,37,38,48,89,90] It is clear from

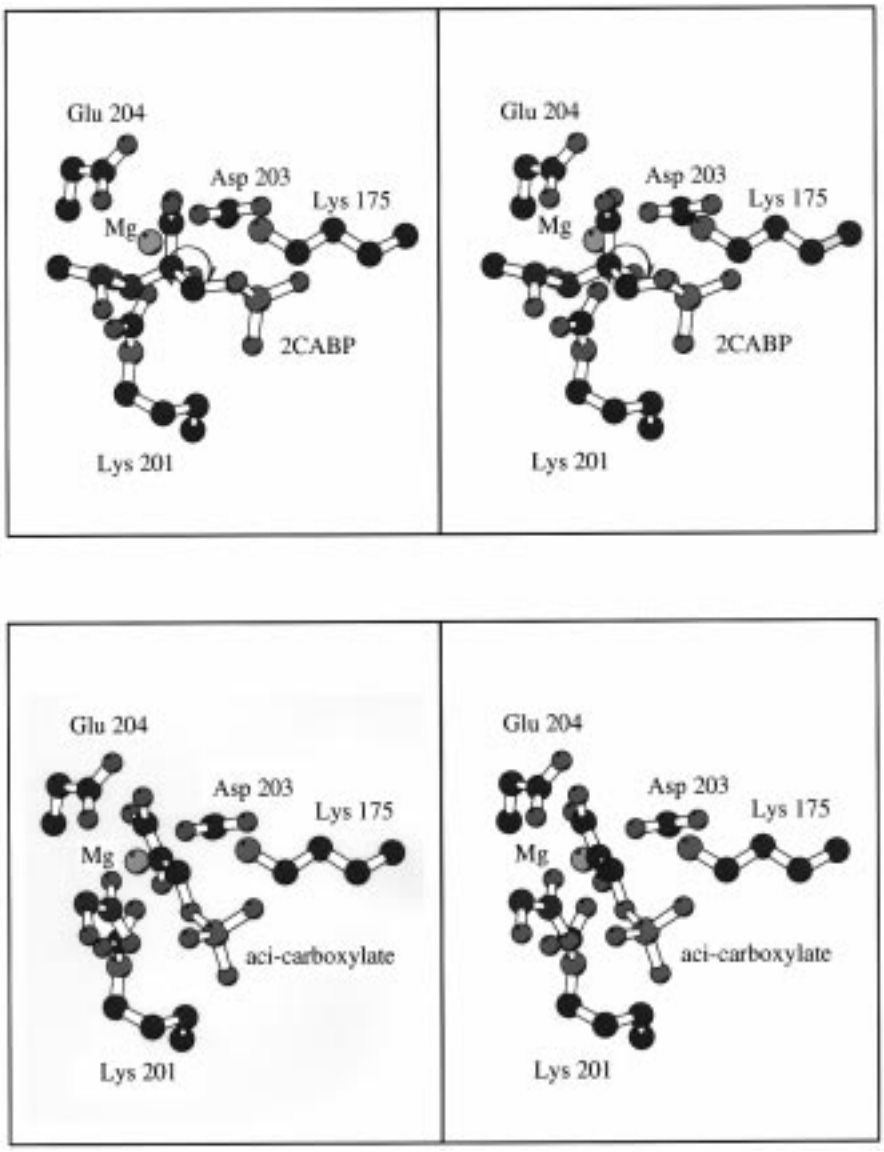


Figure 6. The formation of the *aci*-carboxylate of *upper*-3-P-glycerate. Stereoviews of the positions of the six-carbon intermediate, represented here by 2CABP (upper panel) and the *aci*-carboxylate of *upper*-3-P-glycerate following C2−C3 cleavage (lower panel). Stereoelectronic considerations indicate that to minimize β-elimination of the P1 phosphate from the *aci*-carboxylate, the bridge oxygen moves into the plane of the *aci*-carboxylate during carbon−carbon cleavage. This can be accomplished with minimal motion by rotation (indicated by blue arrows) of the P1 phosphate around the C1−C2 axis as C-2/C-3 bond cleavage occurs, bringing the C-2 close to the ϵ-amino group of K175(166).

these structures that the active site lies at the interface of the N-terminal domain of one large subunit and the C-terminal barrel domain of the second subunit of the large subunit dimer. The intersubunit location of the active site was first deduced by the formation of catalytically active heterodimers by in vivo hybridization of inactive site-directed mutants of the *R. rubrum* enzyme.[101] Without ligand, the site is open to external solvent, and RuBP can gain access. Binding of the substrate is accompanied by changes in the position of both domains.[30]

The most dramatic movement involves residues 331−337 (326−332) encompassing loop 6 of the C-terminal domain, which in conjunction with a short loop at the end of helix B of the N-terminal domain, interacts with the bisphosphate, restricts access to the active site and positions key catalytic residues for action. K334(329) at the apex of loop 6 moves some 10 Å and interacts with E60(48) of the N-terminal loop as well as with the P-1 phosphate. T65-(53) also moves to restrain the P-1 phosphate group.[28,30,36,37]

The importance of loop 6 to normal processing of the enediolate intermediate has been demonstrated by the characterization of a loop deletion mutant.[100] Although the mutant retains substantial activity in the enolization of RuBP and in the forward processing of 2C3KABP, it is totally incompetent in catalyzing the carboxylation of the enediolate intermediate. Rather RuBP is converted primarily to 1-deoxy-D-*glycero*-2,3-pentodiulose 5-phosphate, the product of the β-elimination of the P-1 phosphate from the enediolate. Thus, in the absence of loop 6, the labile enediolate dissociates from the active site and undergoes spontaneous decomposition.

In the $(L_2S_2)_4$ form of the enzyme, three other elements show significant movement. Two in the N-terminal domain around Y20 and residues 123−

128 alter their positions, along with the C-terminal tail to stabilize the movements of the first loops and further restrict access to the active site. In this closed conformation, the bisphosphate is buried 15 Å from the bulk solution and premature release of unstable intermediates or undesired reaction with the solution is minimized.

The signal event that triggers opening of the loops is most probably C2−C3 bond separation; after all, the enzyme has little affinity for the products of catalysis. Relaxation of the protein back to the open conformation accompanying product release must also be controlled. It is likely that, as the active site opens, the rotations around the bridge O of the P-1 phosphate in the *aci*-carboxylate occur (see Figure 6). This is accompanied by the movement of the C-2 toward K175(166), its stereospecific protonation by the ϵ-ammonium group and release of the *upper* 3-P-glycerate. The recently solved structure of the spinach enzyme containing two 3-P-glycerates is in the open conformation and shows the *upper* 3-P-glycerate in the expected position shown in Figures 2 and 3H.[89] While the phosphate of the *lower* 3-P-glycerate is bound in the position it has occupied during the reaction, the carboxyl group the *lower* 3-P-glycerate has moved to a position out of the active site.

XIV. Concluding Remarks

This formulation of the Rubisco mechanism accounts for all of the proton transfers and geometry changes that occur around the metal in this very elegant reaction. It is particularly impressive how the 1-phosphate is kept in plane both with the *cis*-2,3-enediolate intermediate in the early stages of the reaction, and with the *aci*-carboxylate in the last part of the reaction, despite the fact that the two planes are orthogonal to one another. It is hard to imagine any other way in which such a reaction could be catalyzed with apparently only minimal movements of the various intermediates!

Equally impressive is exploitation of the resonance properties of the carbamate in the dual roles of metal ion coordination and general base catalyst. To our knowledge, such bifunctionality is unprecedented. Twenty years ago Hanson and Rose[91] suggested that "natural selection for catalytic efficiency has led to: (1) the use of the minimal number of acidic and basic groups; (2) the maximal separation of catalytic groups and/or replacing groups; (3) minimal motion of the substrate". The mechanism of Rubisco which we propose is a striking example of those principles.

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