# A Role for the ϵ-Amino Group of Lysine-334 of Ribulose-1,5-bisphosphate Carboxylase in the Addition of Carbon Dioxide to the 2,3-Enediol(ate) of Ribulose 1,5-Bisphosphate[†,ƒ]

George H. Lorimer,*[,‡] Yuh-Ru Chen,[§] and Fred C. Hartman*[,§]

*Central Research and Development Department, Dupont Company Experimental Station, Wilmington, Delaware 19880-0402, and Protein Engineering Program, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831-8077*

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ABSTRACT: Earlier structural and functional studies of ribulose-1,5-bisphosphate carboxylase/oxygenase imply that K334 facilitates the addition of gaseous substrate to the 2,3-enediol(ate) derived from ribulose 1,5-bisphosphate. Crystallographic analysis of the activated spinach enzyme [Knight et al. (1990) *J. Mol. Biol. 215*, 113–160] shows that the lysyl side chain is appropriately positioned to stabilize the transition state for the addition of $CO_2$ to the enediol(ate). Furthermore, despite total impairment of carboxylase and oxygenase activities, site-directed mutants of the *Rhodospirillum rubrum* enzyme with replacements for lysine K334 (formerly designated K329) retain the capacity to enolize ribulose bisphosphate, demonstrating that the primary catalytic lesion lies beyond this initial step [Soper et al. (1988) *Protein Eng. 2*, 39–44; Hartman & Lee (1989) *J. Biol. Chem. 264*, 11784–11789]. We now show that the K334C mutant is also competent in the latter stages of catalysis, whereby 2′-carboxy-3-keto-D-arabinitol 1,5-bisphosphate, the six-carbon intermediate of the carboxylation pathway, is correctly processed to 3-phosphoglycerate. Thus, the impairment of the mutant in overall catalysis can be attributed to preferential disruption of the reaction of $CO_2$ or $O_2$ with the enzyme-bound enediol(ate). Chemical rescue of the K334C mutant by aminoethylation and aminopropylation shows that this disruption reflects, at least in part, a failure to adequately stabilize the relevant transition state. With several simplifying assumptions, the $CO_2/O_2$ specificity factor $\tau$ can be reduced to the ratio of the fundamental second-order rate constants for the interaction of the gaseous substrates with the enzyme-bound 2,3-enediol(ate) of ribulose bisphosphate. The $\tau$-value is therefore a measure of the differential stabilization of the transition states that lead to the carboxylated and oxygenated intermediates. Our observations of altered $\tau$-values and hence differential perturbation of transition states, correlated with substitutions of K334 with *S*-(2-aminoethyl)cysteine and *S*-(3-aminopropyl)cysteine, lend strong credence to one assigned role for this lysyl residue.

Recent structural investigations (Schneider et al., 1990a; Knight et al., 1990; Curmi et al., 1992; Newman, 1992) of various forms of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco)[1] have added greatly to our understanding of the mechanism of this enzyme. Of particular relevance are the quaternary complexes of hexadecameric forms of the enzyme. These complexes contain the activator carbamate, the essential divalent metal ion, and 2′-carboxyarabinitol 1,5-bisphosphate (CA-$P_2$), an analog (Pierce et al., 1980; Schloss, 1988) of the carboxylated reaction intermediate 3-keto-2′-carboxyarabinitol 1,5-bisphosphate (KCA-$P_2$) (Schloss & Lorimer, 1982; Pierce et al., 1986a; Lorimer et al., 1986). This intermediate is generated during the carboxylation reaction by the direct addition of carbon dioxide to the *si* face of carbon 2 of the 2,3-*cis*-enediol(ate) of ribulose-$P_2$. Attempts to demonstrate the formation of a Michaelis complex involving the substrate $CO_2$, using techniques which should have revealed its presence if the complex existed, were unsuccessful (Pierce et al., 1986b). Thus, the 2′-carboxy group of the carboxylated reaction intermediate derives from the bimolecular reaction of $CO_2$ with the enediol(ate). In the crystal structure of the quaternary complex, the coordination of the 2′-carboxyl group of CA-$P_2$ is especially pertinent to our understanding of the mechanism of carboxylation, since this coordination presumably mimics that of the newly fixed $CO_2$ in the carboxylation reaction. The oxygen atoms of the 2′-carboxyl group of CA-$P_2$ are coordinated to the divalent metal ion and to the ϵ-amino group of K334 (Figure 1) (Knight et al., 1990; Newman, 1992). This strongly suggests that the transition state for the addition of $CO_2$ to the 2,3-enediol(ate) of ribulose-$P_2$ is jointly stabilized by the divalent metal ion and by the ϵ-amino group of K334.

The overall carboxylation reaction can be dissected into a series of three consecutive steps, enolization, carboxylation, and hydrolysis (Scheme I) [for reviews, see Andrews and Lorimer (1987), Hartman (1992), and Hartman and Harpel (1993)]. Enolization and hydrolysis can be monitored as independent partial reactions. Thus, the enolization of ribulose-$P_2$ can be assayed as the enzyme-catalyzed transfer of $^3H$ to water from [3-$^3H$]ribulose-$P_2$ (Sue & Knowles, 1982; Hartman & Lee, 1989), regardless of whether or not carboxylation occurs. The availability of small quantities of acid-labile but borohydride-stabilized [2′-$^{14}C$]KCA-$P_2$ (Schloss & Lorimer, 1982; Pierce et al., 1986a) provides an avenue

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[‡] Dupont Co. Experimental Station.

[§] Oak Ridge National Laboratory.

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[1] Abbreviations: CA-$P_2$, 2′-carboxy-D-arabinitol 1,5-bisphosphate; KCA-$P_2$, 3-keto-2′-carboxy-D-arabinitol 1,5-bisphosphate; 2-P-glycolate, 2-phosphoglycolate; 3-P-glycerate, 3-phospho-D-glycerate; ribulose-$P_2$, D-ribulose 1,5-bisphosphate; Rubisco, D-ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39); K334C-EA, the *S*-(2-aminoethyl)cysteinyl derivative of the mutant K334C; K334C-PA, the *S*-(3-aminopropyl)-cysteinyl derivative of the mutant K334C.

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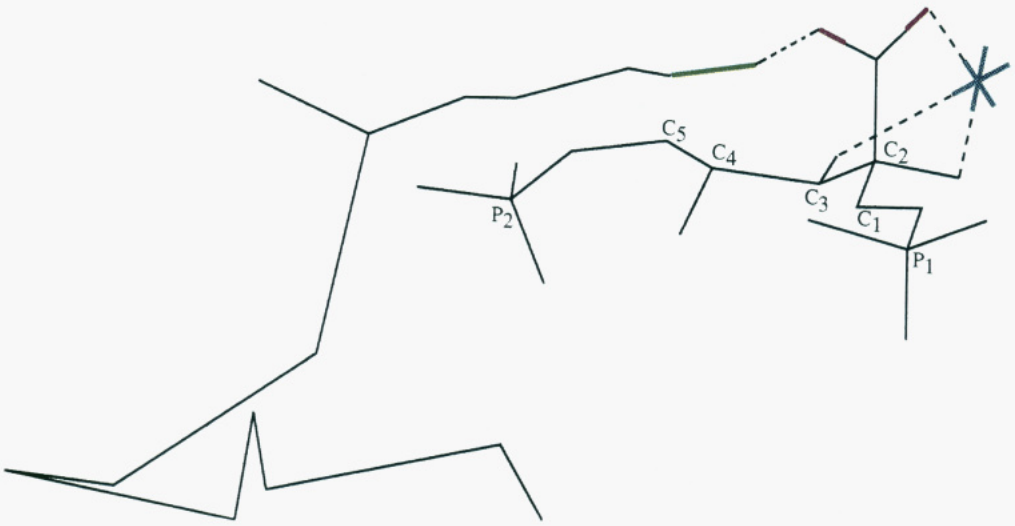
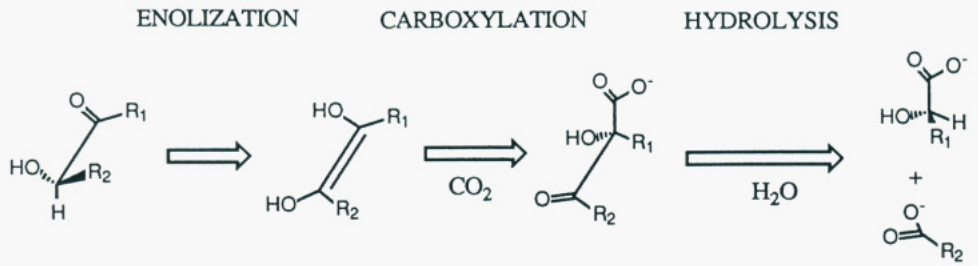



FIGURE 1: The active site of activated spinach Rubisco containing CA-$P_2$. The structure shows the interaction between one of the carboxyl oxygen atoms (red) and the $\epsilon$-amine of K334 (green) and between the other carboxyl oxygen atom and the $Mg^{2+}$ ion (blue). CA-$P_2$ is considered to be an excellent mimic of the *gem*-diol form of the real intermediate, KCA-$P_2$, the carboxyl group of which is derived from the substrate $CO_2$. Note that, with improved refinement of these structures, the hydroxyl groups at C2 and C3 are now displayed in the *cis* configuration (Newman, 1992). This implies that the 2,3-enediol(ate) of ribulose-$P_2$ is also in the *cis* configuration, stabilized by coordination to the divalent metal ion in the form of a five-membered ring. Addition of $CO_2$ creates the two fused five-membered rings shown in this representation. This structure is based on coordinates supplied by Dr. Stephen Knight, Uppsala, Sweden.

Scheme I[a]




[a] $R_1 = CH_2OPO_3^{2-}$; $R_2 = CH(OH)CH_2OPO_3^{2-}$.

for monitoring a second partial reaction, hydrolysis of the intermediate to the acid-stable product 3-P-glycerate.

The original assignment of K334 to the active site and deduction of catalytic functionality were based on its affinity labeling and its displaying of enhanced acidity and nucleophilicity (Norton et al., 1975; Hartman et al., 1985, 1986). Subsequently, these conclusions were reinforced and extended by site-directed mutagenesis; mutants of the *Rhodospirillum rubrum* enzyme with replacements for K334[2] are unable to carboxylate or oxygenate ribulose-$P_2$ at detectable rates but nevertheless catalyze the "wash out" of tritium from [3-$^3$H]-ribulose-$P_2$ at 2–5% of the wild-type rate (Soper et al., 1988; Hartman & Lee, 1989). Thus, these mutants are only moderately impaired as catalysts for enolization of ribulose-$P_2$, suggesting that the functional deficiency occurs at a later step or steps in the overall pathway. Satisfyingly then, crystallographic and functional analyses offer compatible views of the role of K334 in catalysis.

To define more precisely the catalytic contribution of K334, we have examined the competence of the K334C mutant in catalyzing the hydrolysis of KCA-$P_2$ to 3-P-glycerate. Our positive findings strengthen the crystallographic view (Knight et al., 1990) that at least one role of the $\epsilon$-amino group of K334 is to stabilize the transition state for the addition of $CO_2$.

Additionally, we enumerate explicitly the assumptions needed to simplify the $CO_2/O_2$ specificity factor, $\tau$, to the ratio of the fundamental second-order rate constants for the interaction of the gaseous substrates with the enzyme-bound 2,3-enediol(ate) of ribulose-$P_2$. This mathematical treatment reveals that altered $\tau$-values can be interpreted as the differential perturbation of the transition states for carboxylation and oxygenation. As a corollary, subtle modification of a side chain that interacts with these transition states would be predicted to alter the wild-type $\tau$-values. Accordingly, we report substantial alterations in $\tau$-values by conversion of K334 to (2-aminoethyl)cysteine or (3-aminopropyl)cysteine through appropriate alkylation of the K334C mutant (Smith & Hartman, 1988).

## MATERIALS AND METHODS

*Enzymes.* Wild-type (K334) and mutant (K334C) Rubiscos were purified from a strain of *Escherichia coli* expressing a plasmid-encoded *rbcL* gene from *R. rubrum* as previously described (Pierce & Gutteridge, 1985). The construction of the K334C mutant has been previously reported (Soper et al., 1988). Rubisco protein was determined at 280 nm (Schloss et al., 1982).

*S-Aminoalkylated Mutants K334C-EA and K334C-PA.* To 150 $\mu$L of 140 $\mu$M K334 or K334C protomers in 50 mM Bicine–NaOH, 66 mM $NaHCO_3$, 10 mM $MgCl_2$, and 1 mM

[2] In numbering the amino acid residues of *R. rubrum* Rubisco we have adopted the convention suggested by Schneider et al. (1990b). In this unified numbering system K334 corresponds to the residue previously designated as K329.

EDTA, pH 8.0, was added 8 μL of either 2 M (2-bromoethyl)-amine or 2 M (3-bromopropyl)amine (pH 8.0). The solutions were incubated for 8 h in the dark at room temperature and then dialyzed at 4 °C in the same pH 8.0 Bicine buffer containing 10 mM 2-mercaptoethanol.

*KCA-$P_2$.* [2′-$^{14}$C]KCA-$P_2$ (4300 dpm nmol$^{-1}$) was prepared enzymatically from ribulose-$P_2$ and [$^{14}$C]$CO_2$ as previously described (Pierce et al., 1986a). Note that preparations of [2′-$^{14}$C]KCA-$P_2$ made by this method are unavoidably contaminated with unlabeled ribulose-$P_2$ and [1-$^{14}$C]-3-P-glycerate. KCA-$P_2$ was quantitated by the difference method of Pierce et al. (1986a). The ratio of $^{14}$C radioactivity in acid-labile but borohydride-stabilizable KCA-$P_2$ to $^{14}$C radioactivity in acid-stable 3-P-glycerate was about 1.4:1.

*Hydrolysis of KCA-$P_2$.* Reactions were begun by addition of 240 μL of 25.5 μM [2′-$^{14}$C]KCA-$P_2$ (4300 dpm nmol$^{-1}$) containing 39.2 μM [1-$^{14}$C]-3-P-glycerate (2150 dpm nmol$^{-1}$) and approximately 170 μM unlabeled ribulose-$P_2$ to 2.76 mL of either 251 nM wild-type Rubisco protomers or 640 nM K334C protomers in 87.0 mM Tris-HCl, pH 7.8, 54.3 mM $NaHCO_3$, and 10.9 mM $MgCl_2$ at 25 °C. Aliquots were withdrawn at the times indicated, and the amounts of 3-P-glycerate and KCA-$P_2$ were determined immediately afterward by the difference method of Pierce et al. (1986a).

*Product Analysis.* Fifty microliters of 25.5 μM [2′-$^{14}$C]-KCA-$P_2$ (4300 dpm nmol$^{-1}$) containing 39.2 μM [1-$^{14}$C]-3-P-glycerate (2150 dpm nmol$^{-1}$) and approximately 170 μM ribulose-$P_2$ was added to 450 μL of 80 mM Tris–formate, pH 7.7, 55.5 mM $NaHCO_3$, and 11.1 mM $MgAc_2$ containing either 2.35 μM K334C or 1.84 μM K334. The solutions were incubated at 23 °C for 10 min and then applied to 0.8 × 2 cm columns of Dowex 1-X8 (formate form). The columns were washed successively with 5 mL of $H_2O$, 3 mL of 10% formic acid, and 3 mL of 0.1 N HCl. The eluates were evaporated to dryness, and the residual $^{14}$C radioactivity was determined. Pyruvic acid is eluted from the column by the 10% formic acid wash, while 3-P-glycerate is eluted by the 0.1 N HCl wash.

## RESULTS

*Hydrolysis of KCA-$P_2$.* As shown in Figure 2, the K334C mutant catalyzes the hydrolysis of exogenously added KCA-$P_2$ to an acid-stable product. The rate of hydrolysis of KCA-$P_2$ by the mutant K334C is about 1.8 × 10$^{-2}$ s$^{-1}$ compared with a value of about 1.1 × 10$^{-1}$ s$^{-1}$ for the wild-type enzyme. In comparison, the wild-type enzyme turns over at a rate of 4.1 s$^{-1}$ (Table I). With an 8-fold molar excess of KCA-$P_2$ over mutant protomers, the measured partition coefficient (i.e., hydrolysis/hydrolysis plus decarboxylation) is 0.83. However, this value includes a contribution from nonenzymatic decarboxylation. When steps were taken to minimize nonenzymatic decarboxylation, by using a molar excess of mutant protomers over KCA-$P_2$, partition coefficients in excess of 0.9 are observed (data not shown), as has previously been reported for the wild-type enzyme (Pierce et al., 1986a). Thus both the mutant K334C and the wild-type enzymes are strongly committed to the hydrolysis of KCA-$P_2$ as opposed to its decarboxylation.

*Product Analysis.* The carboxylation of ribulose-$P_2$ predominantly (>99%) yields two molecules of 3-phospho-(2*R*)-glycerate, which requires that hydrolysis of KCA-$P_2$ occur with inversion of configuration about carbon 2. Protonation of the *si* face of the planar intermediate aci-carbanion form of the "upper"[3] 3-P-glycerate yields the product with the 2*R* stereo configuration. On the other hand, the nonenzymatic

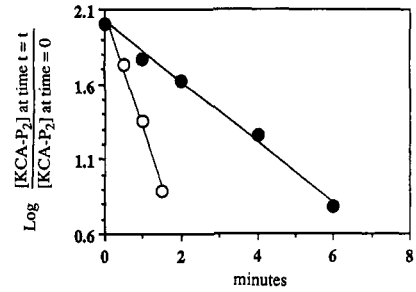



FIGURE 2: Hydrolysis of KCA-$P_2$ by wild-type and K334C mutant Rubiscos. Details of the hydrolysis of KCA-$P_2$ by wild-type (K334) (open circles) and mutant (K334C) (closed circles) Rubiscos can be found in the Materials and Methods section. The initial rates of hydrolysis of KCA-$P_2$ were 1.05 × 10$^{-1}$ s$^{-1}$ for the wild-type enzyme and 1.82 × 10$^{-2}$ s$^{-1}$ for the K334C mutant.

hydrolysis of KCA-$P_2$ at pH >12 results in the formation of pyruvate in addition to "lower" 3-P-glycerate (Lorimer et al., 1986). The pyruvate clearly arises as a consequence of β-elimination of $P_i$ from the aci-carbanion of upper 3-P-glycerate. More recently, Andrews and Kane (1991) have demonstrated that wild-type enzyme forms [$^{14}$C]pyruvate from [$^{14}$C]$CO_2$ and ribulose-$P_2$ approximately once in every 130 turnovers. With that result, the intermediacy of the aci-carbanion form of upper 3-P-glycerate has been unequivocally established. But how is the aci-carbanion of upper 3-P-glycerate stabilized within the active site so that protonation rather than β-elimination predominates?

The oxygen atoms of $CO_2$, involved in stabilizing the transition state for carboxylation through interaction with the divalent metal ion and the amino group of K334, eventually become the carboxyl oxygen atoms of the aci-carbanion of upper 3-P-glycerate. Thus, the notion arose that the aci-carbanion might be stabilized through interaction of its carboxyl group with the divalent metal ion and the amino group of K334. We therefore anticipated that the product of the hydrolysis of [2′-$^{14}$C]KCA-$P_2$ by K334C might be [1-$^{14}$C]-pyruvate rather than [1-$^{14}$C]upper 3-P-glycerate. However, chromatographic analyses of the product of the hydrolysis of [2′-$^{14}$C]KCA-$P_2$ by both K334 and K334C showed that the product was mostly (>99%) 3-P-glycerate. Only traces (0.8–1.0%) of a substance eluting from the ion-exchange column with 10% formic acid were observed. We assume that this is pyruvate.[4] Surprisingly, the partitioning of the terminal aci-carbanion of upper 3-P-glycerate is not influenced by the K334-to-K334C mutation.

*The Specificity Factor τ.* It has long been known (Laing et al., 1974) that the ratio of carboxylase/oxygenase activities is simply expressed by the ratio of $V_{max}/K_m$ values for the two reactions multiplied by the ratio of the gaseous substrates:

$$v_c/v_o = ([V_c/K_c]/[V_o/K_o])([CO_2]/[O_2]) = \tau[CO_2]/[O_2] \quad (1)$$

where τ, the specificity factor, is simply the ratio of the pseudo-second-order rate constants for the interaction of the respective gaseous substrates with the enzyme-bound 2,3-*cis*-enediol(ate). From the kinetic mechanism outlined in Scheme II, the $V_{max}/K_m$ terms can be dissected into elementary rate constants.

[3] The "upper" 3-P-glycerate refers to the product derived from $CO_2$ and carbon atoms 1 and 2 of ribulose-$P_2$. The "lower" 3-P-glycerate refers to the product derived from carbon atoms 3, 4, and 5 of ribulose-$P_2$.

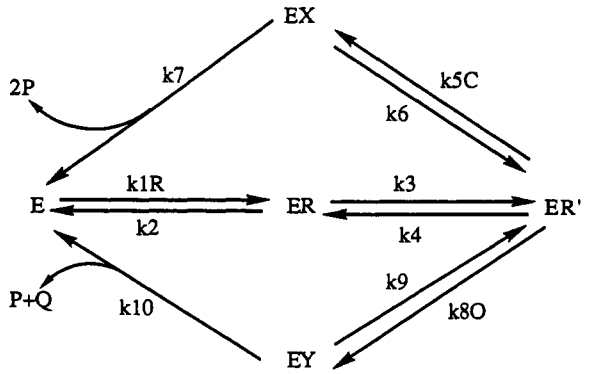
[4] The very limited quantities of $^{14}$C radioactivity associated with this material precluded further analyses.

Table I: Kinetic Properties of Rubiscos with Various Side Chains at Position 334

| enzyme | residue 334 | max $C_\alpha$–N distance (Å) | carboxylase activity ($s^{-1}$)[a] | specificity factor ($\tau$)[b] | |
|---|---|---|---|---|---|
| | | | | $Mg^{2+}$ | $Mn^{2+}$ |
| wild type | $C_\alpha$–$(CH_2)_4$–$NH_3^+$ | 6.42 | 4.1 ± 0.2 | 10.8 ± 1.0 | 1.7 ± 0.2 |
| wild type EA | $C_\alpha$–$(CH_2)_4$–$NH_3^+$ | 6.42 | 4.2 ± 0.2 | 11.0 ± 1.0 | |
| wild type PA | $C_\alpha$–$(CH_2)_4$–$NH_3^+$ | 6.42 | 4.2 ± 0.2 | 10.9 ± 1.0 | |
| K334C | $C_\alpha$–$CH_2$–$S^-$ | | <0.002 | | |
| K334C-EA | $C_\alpha$–$CH_2$–S–$(CH_2)_2$–$NH_3^+$ | 6.83 | 0.93 ± 0.05[c] | 6.2 ± 0.8 | 1.1 ± 0.2 |
| K334C-PA | $C_\alpha$–$CH_2$–S–$(CH_2)_3$–$NH_3^+$ | 8.06 | 0.21 ± 0.02 | 3.2 ± 0.4 | 0.6 ± 0.1 |

[a] Carboxylase activity was determined under standard conditions (25 mM $HCO_3^-$, 10 mM $MgCl_2$, 0.4 mM ribulose-$P_2$, pH 8.0). Considerably more activity was observed at pH 7.0 for K334-PA. This presumably reflects a decrease in the $pK_a$ of the terminal amine of K334C-PA relative to that of K334 (Hermann & Lenke, 1968; Planas & Kirsch, 1991). This phenomenon is being further investigated. [b] The specificity factor was determined by the method of Harpel et al. (1992). [c] If corrected for incomplete alkylation, this value would be ~1.9 $s^{-1}$.

Scheme II[a]




[a] E = enzyme; R = ribulose-$P_2$; R′ = 2,3-*cis*-enediol(ate) of ribulose-$P_2$; C = $CO_2$; O = $O_2$; X = KCA-$P_2$; Y = oxygenase intermediate; P = 3-P-glycerate; Q = 2-P-glycolate.

The assumptions involved in this analysis may be explicitly stated as follows:

(1) It is assumed that both carboxylation and oxygenation are kinetically ordered with the binding of and enolization of ribulose-$P_2$ preceding the addition of the gaseous substrates. In fact, this assumption has been experimentally verified (Pierce et al., 1986b; Lorimer & Pierce, 1989).

(2) Since the enzyme is unable to catalyze the reversal of either reaction, it is assumed that the hydrolyses of KCA-$P_2$ and of the corresponding hydroperoxide intermediate of the oxygenase reaction are both irreversible.

(3) The addition of the gaseous substrates is assumed to be kinetically irreversible, whereby the carboxylated and oxygenated reaction intermediates are committed to product formation. This assumption has been experimentally verified at least in the case of the carboxylase reaction (Pierce et al., 1986a). In Scheme II this is equivalent to setting the partition coefficient $[k_7/(k_6+k_7)]$ at unity. In the case of the oxygenase reaction intermediate, we must refer to the behavior of analogous hydroperoxides. Thus, when $H_2O_2$ adds to vicinal dicarbonyl compounds such as 2,3-butadione, the intermediate $\beta$-ketohydroperoxide undergoes hydrolytic carbon–carbon bond cleavage to yield two acids stoichiometrically (Frankvoort, 1978). Thus, we infer that the oxygenase reaction intermediate is also committed to product formation; i.e., the partition coefficient $[k_{10}/(k_9 + k_{10})]$ is equal to unity.

(4) Michaelis complexes for either gaseous substrate are assumed not to exist; i.e., both reactions proceed by a modified Theorell–Chance mechanism involving the bimolecular reaction of the gaseous substrates with the *si* face of the enzyme-bound 2,3-*cis*-enediol(ate). Such a mechanism is consistent with observed heavy isotope effects (van Dyck & Schloss, 1986). Additionally, attempts to demonstrate such Michaelis complexes, using techniques which should have revealed their existence, if they existed at all, have been unsuccessful (Pierce et al., 1986b).

With these assumptions the following kinetic relationships can be derived:

$$V_c/K_c = k_5[k_3/(k_3 + k_4)][k_7/(k_6 + k_7)] \quad (2)$$

and

$$V_o/K_o = k_8[k_3/(k_3 + k_4)][k_{10}/(k_9 + k_{10})] \quad (3)$$

With the partition coefficients $[k_7/(k_6 + k_7)]$ and $[k_{10}/(k_9 + k_{10})]$ set at unity, the specificity factor $\tau$ simplifies to the ratio of the fundamental second-order rate constants for the interaction of the gaseous substrates with the enzyme-bound 2,3-*cis*-enediol(ate):

$$\tau = [V_c/K_c]/[V_o/K_o] = k_5/k_8 \quad (4)$$

Introducing Eyring's theory of reaction rates [Eyring, 1935; see also Fersht (1985) and Chen and Spreitzer (1991)], it can be shown that

$$RT \ln \tau = (G_o^* - G_c^*) - (G_{O_2} - G_{CO_2}) = \Delta G_{o-c}^* \quad (5)$$

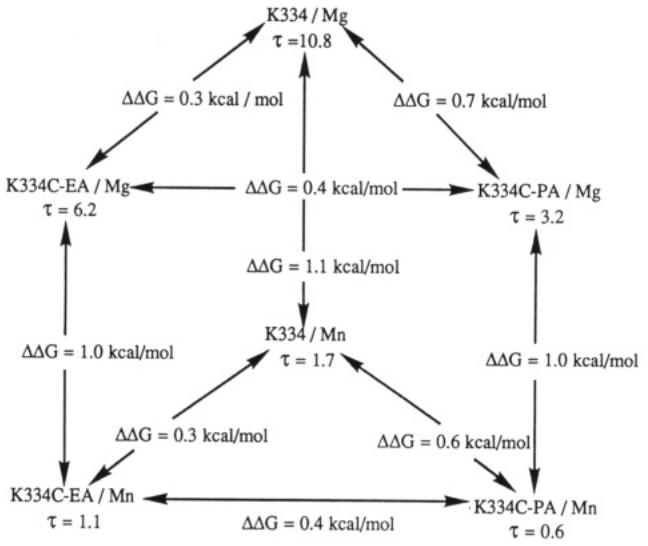
where $R$ is the gas constant, $T$ is the absolute temperature, $G_o^*$ and $G_c^*$ are the free energies of the transitions states for oxygenation and carboxylation of the enzyme-bound 2,3-enediol(ate), respectively, $G_{O_2}$ and $G_{CO_2}$ are the free energies of the $O_2$ and $CO_2$, respectively, and $\Delta G_{o-c}^*$ is the difference between the two activation energies.[5]

The task of stabilizing the transition states for the addition of the gaseous substrates to the enzyme-bound 2,3-*cis*-enediol(ate) does not fall to K334 alone. On the basis of crystallographic studies and the influence of the divalent metal ion on the partitioning of the *cis*-enediol(ate) between the carboxylation and oxygenation reactions (Jordan & Ogren, 1983), the divalent metal ion clearly contributes to the stabilization of the transition states. An inorganic "mutation", the substitution of $Mn^{2+}$ for $Mg^{2+}$, reduces the specificity factor, $\tau$ (Table I and Scheme III), reflecting destabilization of the transition state for carboxylation, relative to that for oxygenation, by about 1.0 kcal/mol.

*Chemical Rescue of K334C.* To explore the influence of the side chain at position 334 on the specificity factor, $\tau$, the K334C mutant was alkylated with (2-bromoethyl)amine and (3-bromopropyl)amine, respectively. The former *S*-aminoethylation, which results in the net replacement of the

[5] This analysis is predicated upon the assumption that the free energy (or energies) of the form (or forms) of the 2,3-enediol(ate) with which the gaseous substrates interact is (are) the same. If $O_2$ only reacts with the triplet state of the 2,3-enediol(ate) and $CO_2$ only with the singlet state, as has recently been suggested on theoretical grounds (Andrés et al., 1992), then this assumption may not be valid.

Scheme III: Free Energy Relationships ($\Delta\Delta G$) between the Transition States for Addition of $CO_2$ and $O_2$ to the 2,3-Enediol(ate) of Ribulose-$P_2$




$\beta$-methylene of a lysyl residue with a sulfur atom and an increase of 0.4 Å in the maximum distance between $C_\alpha$ and the side-chain N, was shown previously to partially restore carboxylase activity to K334C (Smith & Hartman, 1988); however, the $\tau$-value of the chemically rescued mutant was not reported. The corresponding *S*-aminopropylation, as now carried out, displaces the $\epsilon$-amino group of K334 by 1.6 Å in the fully extended side chain.

Extents and selectivity of alkylation were determined by isoelecric focusing of subunits on polyacrylamide gels under denaturing conditions (Figure 3). Both reagents target a single group in the wild-type enzyme; under the conditions used, reaction with (3-bromopropyl)amine is virtually complete, while about half of the protein remains unmodified in the case of (2-bromoethyl)amine. Alkylation of K334C is also highly selective and results in modified species with isoelectric points matching those of the treated wild-type controls. On the basis of these focusing patterns, we conclude that the mutant protein is alkylated at two sites: C334 and the residue which is also attacked in wild-type enzyme. Once again, the alkylation proceeds to completion with (3-bromopropyl)amine, but considerable unmodified (25%) and singly substituted (50%) protein remains after treatment with (2-bromoethyl)amine.

When assayed under standard conditions, both K334C-EA and K334C-PA showed substantial carboxylase activity but $\tau$-values that were lower than wild type (Table I). Perturbation of $\tau$ was greater with the longer side chain at position 334. The level of activity associated with K334C-EA agreed well with that in an earlier report (Smith & Hartman, 1988). As expected, the $\tau$-values of the novel mutant proteins were also sensitive to the nature of the divalent metal ion.

The catalytic properties of the chemically rescued proteins reported in Table I must reflect specific replacements of K334 by *S*-(2-aminoethyl)cysteine and *S*-(3-aminopropyl)cysteine, respectively for the following reasons. (1) Alkylation of C334 is required for restoration of activity, because K334G cannot be rescued by (2-bromoethyl)amine (Smith & Hartman, 1988). (2) Alkylation of wild-type enzyme by either reagent does not lead to significant inactivation or alteration of the $\tau$-value. (3) Any remaining starting material in the preparations examined would be without influence, because K334C is devoid of catalytic activity.

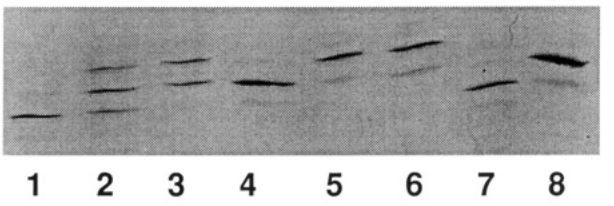



FIGURE 3: Isoelectric focusing with a Phast System (Pharmacia) under denaturing conditions of chemically rescued K334C mutant Rubiscos. Gels (Phast Gel IEF 5–8) were soaked in 9.5 M urea containing 2% (v/v) Pharmalyte (pH 5–8) for 10 min, dipped momentarily in $H_2O$ to remove excess urea, and drained for 5 min prior to prefocusing according to the manufacturer's instructions. Protein samples were diluted 3-fold with 9.5 M urea–2% (v/v) Pharmalyte (pH 5–8)–5% (v/v) 2-mercaptoethanol and applied (1 $\mu$g in 1 $\mu$L) to the prefocused gel. Focusing of proteins was then achieved at 650 V h for 1 h. Gels were fixed in 20% (v/v) trichloroacetic acid for 10 min and soaked in destain solution [MeOH–HOAc–$H_2O$, 3:1:6 (v/v)] for 30 min prior to staining (Commassie blue) and destaining as described by the manufacturer. Samples are K334C (lane 1), K334C-EA (lane 2), wild-type EA (lane 3), wild type (lane 4), wild-type PA (lane 5), K334C-PA (lane 6), K334C (lane 7), and wild type (lane 8).

## DISCUSSION

The rate of hydrolysis of KCA-$P_2$ by the mutant K334C is about 17% of the value for the wild-type enzyme. These rates of hydrolysis of KCA-$P_2$ by K334C and K334 are 10–100 times faster, respectively, than nonenzymatic hydrolysis that can be observed at pH 14 (Lorimer et al., 1986). Moreover, at pH 8 in the absence of enzyme KCA-$P_2$ undergoes decarboxylation with a rate of $2 \times 10^{-4}$ $s^{-1}$ (Pierce, 1986a) rather than hydrolysis. Pertinent to the slower rate of hydrolysis by the mutant enzyme, it is important to note that the wild-type enzyme only hydrolyzes exogenously supplied KCA-$P_2$ at a rate that is 5% of $k_{cat}$ for turnover of ribulose-$P_2$. Thus, even in the case of the wild-type enzyme the hydrolysis of exogenously supplied KCA-$P_2$ is limited by an event not on the direct catalytic pathway. As suggested earlier, this slow event may be the isomerization of the enzyme following the initial binary complex formation, in a manner analogous to that observed in the two-stage binding of the analog, CA-$P_2$ (Pierce et al., 1980; Schloss, 1988; Lorimer et al., 1986).[6] [For an alternative viewpoint, see Cleland (1990)]. Regardless of what that rate-determining event may be in the hydrolysis of exogenously added intermediate, it is clear that the K334C mutant can also catalyze these reactions. For both wild-type and K334C mutant enzymes it could very well be the case that, after this rate-determining event has occurred, the processing of KCA-$P_2$ to product occurs with rates equaling or even exceeding overall $k_{cat}$.

Given these considerations, we judge the rate at which K334C hydrolyzes KCA-$P_2$ to product to be of lesser mechanistic significance. More importantly and by analogy with the wild-type enzyme, K334C processes this intermediate in a forward, productive direction, thereby overcoming the propensity of this intermediate to undergo spontaneous decarboxylation. Previous work (Soper et al., 1988; Hartman & Lee, 1989) has shown that the K334C mutant cannot

[6] The binding of KCA-$P_2$ or CA-$P_2$ to activated enzyme–carbamate–Mg is a complex process. It involves the breakage of *at least* three bonds between the metal ion and the enzyme and/or water molecules, present in the activated enzyme–carbamate–Mg complex, the closure of loop 6 over the active site, and the formation of *at least* four new bonds. These new bonds include three between the metal ion and the 2′-carboxyl oxygen atom and the 2- and 3-hydroxyl oxygen atoms of KCA-$P_2$, as well as the bond between the $\epsilon$-amino group of K334 and the other 2′-carboxyl oxygen atom of KCA-$P_2$.

catalyze the overall carboxylation or oxygenation of ribulose-$P_2$ but remains catalytically competent in the first step common to each reaction sequence, the enolization of ribulose-$P_2$. Since K334C can catalyze the terminal hydrolytic step, the functional deficiency of K334C can be pinpointed primarily to its failure to catalyze the carboxylation step of the reaction.

Two diverse observations demonstrate that mere enolization of ribulose-$P_2$ is insufficient to effect reaction with gaseous substrate. Despite catalytic competency in enolization, K334C lacks measurable carboxylase and oxygenase activity (Hartman & Lee, 1989). Furthermore, pre-steady-state kinetics invoke a rate-limiting step between deprotonation at C-3 and carboxylation at C-2 of ribulose-$P_2$ (Schloss, 1990). Thus, beyond a role in transition-state stabilization, K334 may promote polarization of the enediol(ate) (development of nucleophilic character at C-2) or polarization of $CO_2$ (development of electrophilic character at the carbon atom) and thereby further facilitate catalysis.

K334 is part of loop 6, a flexible region, which is thought to close over the active site during catalysis (Knight et al., 1990; Newman, 1992). The positioning of the positively charged amine group of K334 during the addition of the gaseous substrates is presumably critical for stabilizing the negative charge that develops on the incipient carboxylate or hydroperoxide oxygen atom in the transition states. In the case of crystals of spinach and *Anacystis* Rubiscos containing the reaction-intermediate analog, CA-$P_2$, in the active site, the aminoalkyl side chain of K334 is maximally extended, the $C_\alpha$–N distance being 6.42 Å. To ascertain whether transition-state stability is indeed sensitive to the positioning of the terminal amine, we sought subtle displacements through chemical modifications. For this purpose we exploited the earlier observation that the inactive K334C mutant can be rendered catalytically competent by aminoalkylation (Smith & Hartman, 1988). This derivitization generates an *S*-(2-aminoalkyl)cysteine residue which differs from the lysyl residue only in the replacement of the $\beta$-methylene with a sulfur atom and an increase in the maximum $C_\alpha$–N distance of 0.4 and 1.6 Å for the *S*-aminoethyl and *S*-aminopropyl derivatives, respectively (Table I). The substitution of –S– for $-CH_2-$ (the K334 to K334C-EA transformation) destabilizes the transition state for carboxylation, relative to that for oxygenation, by some 0.3 kcal/mol. The insertion of an additional $-CH_2-$ (the K334C-EA to K334C-PA transformation) is destabilizing to the extent of a further 0.4 kcal/mol. These results suggest that the distance between the amine N atom of residue 334 and the oxygen atoms of the incipient carboxylate and hydroperoxide in the transition states is of critical importance. Shortening this distance, as would occur if the K334C-EA and K334C-PA were fully extended in the transition state, clearly favors oxygenation. A similar pattern has also been observed with the K334R mutant of *Anacystis* Rubisco (Gutteridge et al., 1993). The specificity factor, $\tau$, of *Anacystis* Rubisco is reduced from 56 for the wild type to 0.3 for K334R. When fully extended, the positively charged guanidium ion of K334R could be up to 1.4 Å closer to the incipient carboxyl or hydroperoxide oxygen atoms of the transition states than the $\epsilon$-amino nitrogen of K334.

Less direct evidence for the involvement of K334 in stabilizing the transition states for the addition of the gaseous substrates comes from an analysis of random mutations in this region of the *Chlamydomonas reinhardtii* enzyme (Chen et al., 1991). A single mutation in this loop, V331A, reduces the specificity factor, $\tau$, by some 37%. A second, suppressor mutation, T342I, partly restores the specificity. Knight et al. (1990) have pointed out that the side chains of residues 331 and 342 make van der Waals contact with one another and have suggested that the cavity in the hydrophobic core created by the V331A mutation is compensated by being partly filled by the $\gamma$-methylene group supplied by the suppressor mutation T342I. In light of the effects of K334C-EA and K334C-PA on the specificity factor, $\tau$, reported here, the consequences of the *Chlamydomonas* mutations can also be attributed to subtle alterations in the manner in which the terminal amine function of K334 interacts with the transition states for the addition of the gaseous substrates.

Table II: Differences in the Specificity Factor ($\tau$), in the Carboxylase:Oxygenase Activity ($v_c/v_o$) Ratio in Air at 25 °C, and in the Free Energies of the Transition States ($\Delta G_{o-c}^{\ddagger}$) for Two Functionally Divergent Rubiscos

| species | $\tau$ | $v_c/v_o$[a] | $\Delta G_{o-c}^{\ddagger}$ (kcal/mol) | $\Delta\Delta G$ (kcal/mol) |
|---|---|---|---|---|
| *S. oleracea* | 80[b] | 3.25 | 2.6 | 1.2 |
| *R. rubrum* | 10.8 | 0.44 | 1.4 | |

[a] Calculated from $v_c/v_o = \tau[CO_2]/[O_2]$, taking the solution concentrations of $CO_2$ and $O_2$ in equilibrium with air at 25 °C and normal pressure to be 10.5 and 258 of $\mu$M, respectively. Assuming a stoichiometry of photorespiration of 0.5 mol of $CO_2$/mol of ribulose-$P_2$ oxygenated, growth (i.e., net carbon gain) occurs when $v_c/v_o > 0.5$. [b] Data from Jordan and Ogren (1983).

*Biological Perspective.* The modest perturbations that we have made to the transition states for carboxylation and oxygenation amount to less than 1 kcal/mol, which is equivalent to the energy contributed by a single hydrogen bond between uncharged groups in a protein (Fersht et al., 1985). However, a comparison of the most taxonomically divergent Rubiscos (Table II) reveals the importance of even such small differences in free energy. We have compared the hexadecameric, higher plant Rubisco from *Spinacia oleracea* with the dimeric, bacterial Rubisco from *R. rubrum*. Structural analyses have revealed that both active sites are highly similar (Schneider et al., 1990b). However, the specificity factor, $\tau$, for the spinach enzyme is some 8-fold larger than that for the bacterial enzyme. This corresponds to an increase of about 1.2 kcal/mol in the differential activation energies of the two transition states. The biological significance of this small difference can best be appreciated if we assess the performance of these two enzymes in air. From eq 1, the ratio of the carboxylase/oxygenase activities ($v_c/v_o$) can be computed for any given ratio of $CO_2$ and $O_2$ by substituting the appropriate value for $\tau$. Since the oxygenation of ribulose-$P_2$ leads, via photorespiration, to the loss of 0.5 mol of $CO_2$/mol of ribulose-$P_2$ oxygenated (Lorimer & Andrews, 1981), it follows that autotrophs which grow (i.e., have a positive carbon balance) by using Rubisco to fix $CO_2$ can only do so when $v_c/v_o$ exceeds 0.5. When $v_c/v_o$ is solved for the concentrations of $CO_2$ and $O_2$ in solution equilibrium with air at 25 °C, only the higher plant enzyme has a value in excess of 0.5. In other words, if one were to replace the Rubisco of spinach with dimeric Rubisco from *R. rubrum*, the resultant plant would be incapable of growing in air. Experiments in which the Rubisco of *Anacystis* was replaced by Rubisco from *R. rubrum* (Pierce et al., 1989) have confirmed this point. Photosynthesis, and hence life on this planet, consequently depends on these small differences in the free energies associated with the transition states for the addition of $CO_2$ and $O_2$ to the 2,3-enediol(ate) of ribulose-$P_2$.

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