Chemical Rescue by Exogenous Amines of a Site-Directed Mutant of Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase That Lacks a Key Lysyl Residue†

Mark R. Harpel and Fred C. Hartman*

Protein Engineering Program, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831-8077

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ABSTRACT: Ligand binding to ribulose 1,5-bisphosphate carboxylase/oxygenase immobilizes the flexible loop 6 of the β/α barrel domain in its closed conformation. Lys329, located at the apex of this loop, interacts electrostatically with Glu48 of the adjacent subunit and with the CO_2-derived carboxylate of the carboxylated reaction intermediate [Knight et al. (1990) *J. Mol. Biol. 215*, 113–160]. Previous studies have implicated Lys329 in the addition of CO_2 to the initial enediol(ate) intermediate: mutants at position 329 catalyze enolization of ribulose 1,5-bisphosphate and processing of isolated carboxyketone intermediate, but are severely impaired in overall carboxylation and the tight-binding of the carboxylated intermediate analogue 2-carboxyarabinitol 1,5-bisphosphate. Using the chemical rescue method of Toney and Kirsch [(1989) *Science 243*, 1485–1488], we show that these defects are partially overcome by exogenous amines. For example, ethylamine enhances the carboxylation rate of K329A by about 80-fold and strengthens complexation of 2-carboxyarabinitol 1,5-bisphosphate. The CO_2/O_2 specificity of K329A is increased by amines, but remains lower than the wild-type value. Despite the pronounced enhancement of carboxylase activity, amines do not influence the rate at which ribulose 1,5-bisphosphate is enolized by K329A. Rescue of K329A follows an apparent Brønsted relationship with a β of 1, implying complete protonation of amine in the rescued transition state. Rate saturation with respect to amine concentration and the different steric preferences for amines between K329A and K329C suggest that the amines bind to the enzyme in the position voided by the mutation. Two side products, one formed via β-elimination of phosphate from enediol(ate) intermediate and the other linked preferentially to the oxygenase pathway, predominate in the absence of amines. Amines suppress these abortive products in favor of normal turnover products. Collectively, these results not only verify that Lys329 functions primarily in the gaseous substrate addition to the enediol(ate) but also demonstrate the residue's crucial role in stabilizing reaction intermediates.

Photosynthetic carbon assimilation is limited by the tendency of Rubisco[1] to catalyze the counterproductive oxygenation of RuBP, which forms 1 equiv each of PGA and PGyc, in addition to the productive carboxylation reaction, which forms 2 equiv of PGA [for reviews of Rubisco, see Hartman and Harpel (1993), Schloss (1990), and Andrews and Lorimer (1987)]. Oxygenation of RuBP appears to be endemic to the reactive enediol(ate) intermediate, the branch point between the competing pathways (Pierce et al., 1986; Lorimer & Andrews, 1973; Andrès et al., 1993). However, the enzyme actively influences partitioning between these pathways, as concluded from the observed species dependence of CO_2/O_2 specificity (Jordan & Ogren, 1981) and the perturbation of specificity caused by subtle active-site manipulations (Chen et al., 1988, 1991; Smith et al., 1990; Soper et al., 1992; Chène et al., 1992; Gutteridge et al., 1993; Lee, G. J., et. al., 1993; Lorimer et al., 1993; Lee, E. H., et al., 1993; Zhu & Spreitzer, 1994). Only in a few cases have programmed enhancements of selectivity for CO_2 utilization been realized (Harpel & Hartman, 1992; Read & Tabita, 1992).

The active sites of Rubisco are formed at the interfaces of adjacent subunits, and catalytically-functional residues are contributed by both interacting domains (Larimer et al., 1987; Lee et al., 1987; Chapman et al., 1988; Andersson et al., 1989; Knight et al., 1990). Among the conserved interfacial active-site residues, Lys329[2] was implicated in catalysis by chemical modification studies (Norton et al., 1975; Hartman et al., 1985). Subsequently, site-directed mutagenesis indicated that this residue is required for the addition of gaseous substrate to the enediol(ate) intermediate. Amino acid substitutions for Lys329 diminish carboxylation activity by 10^4-fold and abolish the ability to form a stable complex with the carboxylated intermediate analogue CABP (Soper et al., 1988). Despite these impairments, position 329 mutants retain catalytic competence in both enolization of RuBP (Hartman & Lee, 1989) and hydrolysis of isolated carboxyketone reaction intermediate (Lorimer et al., 1993). Carboxylation activity can be partially restored to the K329C[3] mutant by selective aminoethylation or aminopropylation of the newly-introduced sulfhydryl. However, the chemically-derivatized mutant proteins (which contain a lysyl analogue at position 329) display CO_2/O_2 specificity factors (τ) that are considerably lower than that of the wild-type enzyme. Hence, the precise location of the ϵ-amino group at position 329 appears crucial to the stabilization of the transition state for carboxylation of

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[1] Abbreviations: Rubisco, D-ribulose 1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39); RuBP, D-ribulose 1,5-bisphosphate; bicine, *N,N*-bis(2-hydroxyethyl)glycine; PGA, 3-phospho-D-glycerate; PGyc, 2-phosphoglycolate; CABP, 2-carboxy-D-arabinitol 1,5-bisphosphate; ν_c, carboxylation rate; ν_o, oxygenation rate.

[2] Unless otherwise stated, all residue numbers refer to the sequence position in *Rhodospirillum rubrum* Rubisco.

[3] The single-letter code for amino acids is used to designate mutants. The first letter denotes the amino acid present in the wild-type enzyme at the numbered position. The final letter denotes the amino acid present at the corresponding position in the mutant enzyme.

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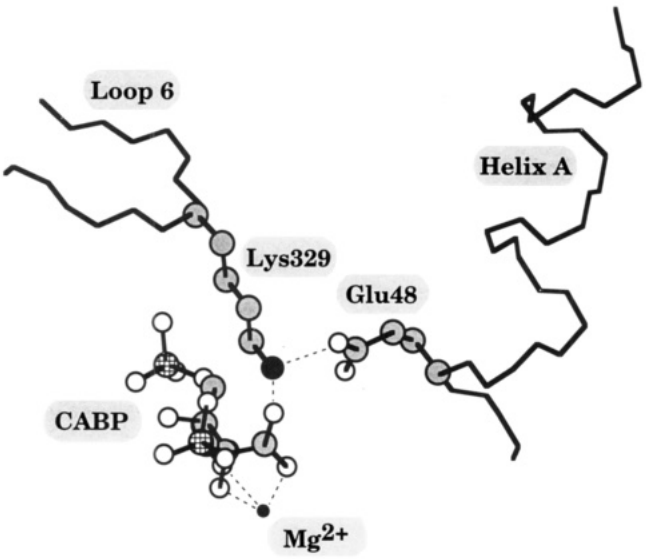

FIGURE 1: Ionic interactions involving Lys329 in the complex of Rubisco with bound CABP. CABP is depicted in an orientation with the C1-phosphate group closest to the reader and the C5-phosphate group distant. The carboxyl group of CABP is shown interacting with the ϵ-amino group of Lys329. The schematic was drawn using crystallographic coordinates for the complex of spinach Rubisco provided by Drs. C.-I. Brändén and G. Schneider.

enediol(ate) (Smith & Hartman, 1988; Lorimer et al., 1993).

X-ray crystallographic studies have defined interactions consistent with the catalytic roles of Lys329 as deduced from functional studies. In the models of the activated spinach and tobacco enzymes complexed with CABP (Andersson et al., 1989; Knight et al., 1990; Schreuder et al., 1993), the ϵ-amino group of Lys329 engages in two important ionic interactions—one with the CABP carboxylate, which mimicks substrate CO_2, and another with Glu48, which is contributed by the adjacent subunit and is necessary for the correct processing of enediol(ate) intermediate (Hartman et al., 1987; Lee, E. H., et al., 1993) (Figure 1). Lys329 is the apical residue of loop 6 of the β/α barrel domain of the active site. Electron density for this loop is ill-defined or missing in diffraction patterns of the nonactivated enzyme (Schneider et al., 1990) and the activated enzyme lacking ligand (Lundqvist & Schneider, 1991), but is well-defined in a "closed" conformation in the activated enzyme with bound CABP (Andersson et al., 1989; Knight et al., 1990; Schreuder et al., 1993). Thus, loop 6 is highly flexible, and its closure over the top of the β/α barrel, which renders the active site inaccessible to solvent, appears to be a discrete event during catalytic turnover. Functionally, the importance of loop-6 geometry has been demonstrated by the impact of mutations within this loop on the CO_2/O_2 specificity (Chen et al., 1988, 1991; Parry et al., 1992).

Given the importance of Lys329 to catalysis, as exemplified by the impact of even modest alterations of the lysyl side chain on the CO_2/O_2 partitioning ratio, a systematic exploration of structure–activity relationships at this site is warranted. Recently, Toney and Kirsch (1989, 1992) have provided a general avenue, denoted "chemical rescue," for elucidating such relationships in their studies of the ability of exogenous amines to restore activity to the catalytically-inactive K258A mutant of aspartate aminotransferase. A Brønsted relationship was observed between the pK_a of exogenous amines and the extent of restored activity, thereby providing information concerning rate limitation and charge distribution in the transition state of the proton transfer facilitated by Lys258. Since its initial application, chemical rescue has been applied to Lys296-replaced rhodopsin (Zhukovsky et al., 1991) and Lys80-replaced leucine dehydrogenase (Sekimoto et al., 1994) by use of *n*-alkylamines, Tyr14-substituted Δ^5-3-ketosteroid isomerase by use of phenols (Brooks & Benisek, 1992), and Arg127-replaced carboxypeptidase by use of guanidines (Phillips et al., 1992). In an earlier study of Rubisco, chemical rescue of a mutant with a substitution at Lys191 [the site of activation via condensation with CO_2 (Lorimer, 1981; Donnelly et al., 1983)] demonstrated a functional requirement of the carbamate nitrogen in catalysis (Smith & Hartman, 1991).

In this paper, we describe the application of chemical rescue to the K329A and K329C mutant Rubiscos by exogenous amines in order to provide additional insight into the facilitation of catalysis by Lys329 and into its interactions with various intermediates throughout the reaction coordinate.

EXPERIMENTAL PROCEDURES

Materials. RuBP [unenriched (Horecker et al., 1958), [1-^{3}H]-labeled (Kuehn & Hsu, 1978), and [3-^{3}H]-labeled (Hartman & Lee, 1989)] and CABP [unenriched and [2-^{14}C]-labeled (Pierce et al., 1980), both used as an epimeric mixture of the *arabino* and *ribo* isomers] were synthesized as previously described. Sodium [^{14}C]bicarbonate, sodium [^{14}C]cyanide, ^3H_2O, and [2-^{3}H]glucose were obtained from ICN. All amines were purchased from Aldrich Chemical Co. at the highest purity available (≥98%) and were used without further purification. The amines were prepared as 2 M aqueous stocks containing 1 M bicine and were adjusted to pH 8.0 with tetramethylammonium hydroxide or hydrochloric acid as necessary. Glyceraldehyde-3-phosphate dehydrogenase, PGA phosphokinase, glycerophosphate dehydrogenase/triosephosphate isomerase, alkaline phosphatase, protocatechuic acid, and protocatechuate 3,4-dioxygenase were purchased from Sigma.

Wild-type Rubisco from *Rhodospirillum rubrum* was prepared as previously described (Schloss et al., 1982). The mutant *R. rubrum rbc* genes encoding the K329A and K329C proteins (Soper et al., 1988) were transferred into vector pFL245 (Larimer et al., 1990) for more efficient expression in *Escherichia coli* MV1190. Transformed bacteria were cultured in 2X-YT media (Sambrook et al., 1989) containing 1% glycerol and 50 mg/mL ampicillin. Cultures were harvested 2.5 h after induction with 0.1 mM isopropyl β-D-thiogalactopyranoside. The mutant Rubiscos were purified either as previously described (Mural et al., 1990; Harpel et al., 1991) or by a modified procedure, which accomodates up to 25 g of cell paste. Cell-free extract, prepared by disruption of cells in a French pressure cell (Aminco), was adsorbed to a column of DEAE Sepharose fast-flow resin (1 mL of resin per 1 mL of extract). Following successive washes with pH 8.0 activation buffer (50 mM bicine, 10 mM $MgCl_2$, 1 mM EDTA, 66 mM $NaHCO_3$, 10 mM 2-mercaptoethanol, and 10% glycerol) containing 0 and 25 mM NaCl, respectively, Rubisco was eluted batchwise with buffer containing 150 mM NaCl. Both the NaCl-gradient and Tris/citrate-gradient elution anion-exchange steps that followed were carried out as described previously but were accomplished with a Waters Protein-Pak DEAE-8HR column (20 mm × 100 mm) in place of the MonoQ column. Flow rates and gradient mixing were controlled by a Pharmacia fast protein liquid chromatography system. All gradient volumes were increased proportionately from the previous methods. An additional anion-exchange step (Pharmacia MonoQ HR10/10, 10 mm × 100 mm) with a shallow gradient elution (12 column volumes from 0 to 0.3

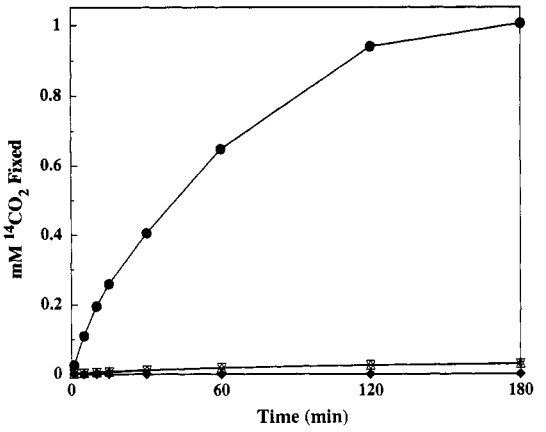

FIGURE 2: Time courses for the $^{14}CO_2$-fixation assay for RuBP turnover catalyzed by K329A (10 μM protomer) in the absence of exogenous amine (△) and in the presence of either 450 mM tetramethylammonium chloride (▽) or 450 mM ethylamine (●). The closed tilted squares (◆) correspond to an assay containing 450 mM ethylamine but lacking enzyme. The plateau approached at 180 min for the ethylamine-containing reaction reflects complete consumption of RuBP. The apparent discrepancy between the substoichiometric amount of CO_2 consumed (1 mM) relative to the starting concentration of RuBP (2 mM) is due to the decreased carboxylation/oxygenation specificity of the K329A mutant and the accumulation of stable reaction side products (see text).

M NaCl in activation buffer) was also introduced as a final purification step to remove trace levels of a general phosphatase derived from the *E. coli* expression host. The enzyme preparations were >98% homogeneous, as judged by Coomassie-Blue staining of gels following polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

Protein and Activity Assays. Protein concentrations of mutant and wild-type Rubiscos were determined by either a dye-binding assay (Bradford, 1976) with reagent obtained from Bio-Rad or by the absorbancy at 280 nm [1.2 AU/(mg/mL); Schloss et al., 1982].

Carboxylation activity was measured by a modified radiometric filter disk assay for the formation of acid-stable radioactivity from $NaH^{14}CO_3$ ($^{14}CO_2$) and unlabeled RuBP (Niyogi et al., 1986). Assays were carried out at 23 °C in a buffer (pH 8.0) containing 300 mM bicine, 10 mM $MgCl_2$, 1 mM EDTA, 30 mM $NaH^{14}CO_3$ (8 mCi/mmol), 10% glycerol, 1 mg/mL bovine serum albumin, and varied concentrations of RuBP and amines as indicated. Amine concentrations denote total species (free base plus protonated amine). Tetramethylammonium chloride, which does not stimulate carboxylase activity of the mutant enzymes, was added to assays for determinations of K_d values for primary amines so as to maintain the total amine concentration constant at 450 mM. The enzyme was first preincubated at 23 °C for 15–30 min in the assay solution lacking RuBP. Following initiation of the reaction by addition of RuBP, aliquots (25 μL) were periodically quenched by delivery onto Whatman 3MM filter disks (2.3 cm) soaked with 100 μL of 50% (v/v) trifluoroacetic acid (eight aliquots per assay during 3 min). After drying, the disks were subjected to scintillation counting (EcoLite liquid scintillant, ICN). In experiments with 2-fluoroethylamine, the carboxylation activity was monitored with the enzyme-coupled spectrophotometric assay for PGA formation (Schloss et al., 1982) due to a high background of acid-stable radioactivity formed from $NaH^{14}CO_3$ in the presence of this amine. In addition to the buffer components described above, the spectrophotometric assay mixtures also contained 0.2 mM NADH, 5 mM ATP, and the necessary coupling enzymes.

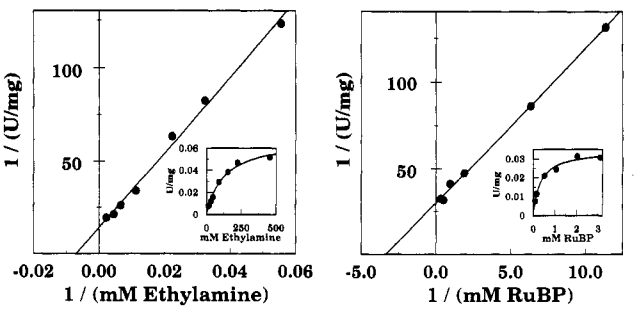

FIGURE 3: Saturation of K329A-catalyzed carboxylation rate as a function of RuBP concentration at 50 mM ethylamine (right) and as a function of ethylamine concentration at 2 mM RuBP (left).

The enolization partial reaction was assayed in the presence of 400 mM amine as the detritiation of [3-^{3}H]RuBP (Sue & Knowles, 1982; Hartman & Lee, 1989) under the same conditions as for the carboxylation assay. The reactions were initiated with [3-^{3}H]RuBP (3 mM, ~0.1 mCi/mmol); periodically, 15-μL aliquots were delivered into 100 μL of freshly-prepared 100 mM $NaBH_4$ to quench the reaction. The quenched samples were diluted with 0.5 mL of 10% acetic acid to decompose excess borohydride, evaporated to dryness, and resuspended in 0.5 mL of H_2O prior to counting in 10 mL of liquid scintillant.

Analysis of turnover products and determination of τ, the CO_2/O_2 specificity factor (Laing et al., 1974), followed the anion-exchange chromatographic method described by Harpel et al. (1993) with [1-^{3}H]RuBP as substrate. Various reaction conditions are given in the figure and table legends. Except where noted, all reactions included air-saturated concentrations of O_2 (255 μM). Freshly-prepared $NaBH_4$ (2 mM) was added to quench the reactions, followed by addition of glucose (4.5 mM) to consume excess borohydride, dilution (20-fold) with H_2O, and deproteination using Amicon Centricon-10 filters. Deproteinated reaction mixtures were applied to a column of MonoQ anion exchanger (Pharmacia HR5/5, 5 mm × 50 mm) and eluted with a gradient of NH_4Cl at pH 8.0 as indicated in the figures. Eluted radioactivity was detected either by scintillation counting of 0.5-mL fractions or by flow-through radiometric detection (IN/US β-RAM). Values for τ were calculated from the ratio of radioactive peak areas for PGA and PGyc [corresponding to the kinetic partitioning between carboxylation and oxygenation reactions in the relationship $\tau = (v_c/v_o)/([O_2]/[CO_2])$; Laing et al., 1974]. For analysis of products formed in the absence of oxygen, reactions were carried out in septum-sealed vessels in the presence of an O_2-depleting system comprised of protocatechuic acid and protocatechuate 3,4-dioxygenase (Harpel et al., 1993).

Binding of radiolabeled CABP by Rubisco was determined as protein-associated radioactivity following removal of unbound ligand by gel filtration (Smith & Hartman, 1988; Miziorko & Sealy, 1980) through a column (1 cm × 50 cm) of Sephadex G-50 (fine). Prior to gel filtration, the enzyme (10 μM protomer) was preincubated at 23 °C in pH 8.0 buffer (250 mM bicine, 1 mM EDTA, 10 mM $MgCl_2$, 66 mM $NaHCO_3$, 10% glycerol, ± 50 mM ethylamine) with [2-^{14}C]-CABP (100 μM, 1.8 mCi/mmol) for 1 h. Aliquots were chromatographed in equilibration buffers either containing or lacking amine as indicated.

RESULTS

Restoration of Carboxylation Activity. Replacement of Lys329 by alanine decreases the carboxylase activity of *R.*

Table 1: Amine Rescue of $^{14}CO_2$-Fixation Activity for Lys329 Mutants

enzyme	amine	pK_a[a]	molecular volume[a] (Å^3)	K_d(amine)[b] (mM)[d]	k_{cat}[b] ($s^{-1} \times 10^3$)	rate enhancement[c]
(I) wild type	lysine	9.0[e]	79.8/65.7[f]		2400	3400–4000
(II) K329A	none				0.7	1
	methylamine	10.6	42.1	130	44.7	65
	ethylamine[g]	10.6	60.9	130	58.1	84
	propylamine	10.5	79.9	100	21.9	32
	butylamine	10.6	98.7	100	7.8	12
	ethanolamine	9.5	71.5	160	17.8	26
	2-methoxyethylamine	9.2	98.7	335	9.0	14
	ammonium	9.2	23.2	130	5.9	9
	2-fluoroethylamine[h]	9.0	64.4	40	43.8	64
	2-cyanoethylamine	7.7	70.5	40	5.1	8
	2,2,2-trifluoroethylamine	5.7	71.6	ND[i]	0[i]	1
	cyanomethylamine	5.3	51.1	ND[i]	0.3[i]	1
(III) K329C	none				0.6	1
	methylamine	10.6	42.1	170	13.0	23
	ethylamine	10.6	60.9	185	13.0	23
	propylamine	10.5	79.9	115	6.0	11
	butylamine	10.6	98.7	80	2.2	5

[a] Values from Toney and Kirsch (1989); the molecular volume of 2-methoxyethylamine was assumed to be the same as that for butylamine. [b] Determined at constant (2 mM) RuBP concentration and varied amine concentrations (5–450 mM) adjusted to 450 mM total with tetramethylammonium chloride. Other conditions are as described in the Experimental Procedures. The activity values for amine-containing reactions are corrected for the unassisted rate ($k_{cat} = k_{cat}(obsd) - k_0$, where k_0 = activity measured in the absence of exogenous amine). [c] Presented as $k_{cat}(obsd)/k_0$. The range for wild-type enzyme represents the enhancement of activity relative to K329A and K329C, respectively, in the absence of amine. [d] Refers to total amine concentration (free base plus protonated amine). [e] By analogy to the pK_a of Lys334 of spinach Rubisco determined by active-site labeling (Hartman et al., 1985). [f] Difference in average molecular volume for a buried lysine relative to alanine and cysteine, respectively (Chothia, 1975). [g] Using the spectrophotometric assay for PGA formation (Schloss et al., 1982), K_d(ethylamine) = 75 mM and k_{cat}(ethylamine) = 0.044 s^{-1}. [h] Determined solely by the spectrophotometric assay due to interference of 2-fluoroethylamine in the radiometric assay. [i] Insufficient rescue of activity for determination of kinetic parameters. The k_{cat} value refers to activity measured at 450 mM amine.

rubrum Rubisco, as measured by acid stabilization of $^{14}CO_2$, to <0.03% of the wild-type level. However, substantial activity is restored to this mutant by inclusion of primary amines in assay mixtures. For example, at 450 mM ethylamine and 2 mM RuBP, the mutant exhibits about 2% of the wild-type activity, representing ~80-fold stimulation compared to the sample in the absence of amine (Figure 2). The lack of enhanced activity with other salts, such as tetramethylammonium chloride or NaCl, excludes the possibility that rescue reflects an ionic-strength effect. Analogously to wild-type carboxylase, the activity displayed by the chemically-rescued mutant requires Mg^{2+} and is inhibited by CABP (Pierce et al., 1980). The rate of $^{14}CO_2$ fixation is stimulated to the same extent whether amine is added prior to or following initiation of the reaction with RuBP.

The rescued mutant obeys saturation kinetics with respect to both amine and RuBP concentrations (Figure 3). At 2 mM RuBP and variable ethylamine concentration (Figure 3, left) or 50 mM ethylamine and variable RuBP concentration (Figure 3, right) K329A exhibits an apparent K_d(ethylamine) of 130 mM and an apparent K_m(RuBP) of 300 μM. The corresponding k_{cat} values are 0.06 and 0.03 s^{-1}, respectively.[4] K_m(RuBP) and k_{cat} for wild-type rubisco are 12 μM and 2–3 s^{-1}.

Numerous amines restore activity to both the K329A and K329C mutants (Table 1). The extent of rescue is sensitive to molecular volume, as illustrated for amines with similar pK_a values (Figure 4, top panel). For K329A, the deviation of methylamine from the linear correlation (ethyl- > methyl- > propyl- > butyl-) suggests that the amine rescuer specifically binds in the site vacated by the alanyl substitution. The intrinsic volume parameter (V) relating this trend [derived from the slope of the line in Figure 4 (top) but disregarding the value for methylamine] is –0.02 Å^{-3}. In contrast, K329C exhibits a more direct correlation between rescue and chain length (methyl- ≥ ethyl- > propyl- > butyl-) and a lesser steric effect (V = –0.01 Å^{-3}).

A correlation between K329A rescue efficiency and amine pK_a is evident in the series of fluorinated ethylamines having similar molecular volumes but decreasing basicities (ethyl- > 2-fluoroethyl- > 2,2,2-trifluoroethyl-) (Table 1). This trend is quantified in a Brønsted plot [Figure 4 (bottom)] based on the data of Table 1, which have been volume-adjusted using V obtained from Figure 4 (top) (Toney & Kirsch, 1989). The β of 1 derived from the slope of this plot (R^2 = 0.72) implies full accumulation of positive charge in the transition state involving the amine. Note that the plots in Figure 4 utilize K_d values expressed as the free base concentration to normalize for a single ionic species.

Enolization Activities. Although drastically impaired in carboxylation activity, mutants containing substitutions at position 329 retain considerable activity in the enolization partial reaction as measured by the exchange of the C3 proton of RuBP with solvent (Hartman & Lee, 1989). Whether assayed in the absence of a primary amine (400 mM tetramethylammonium chloride) or in the presence of 400 mM methylamine, ethylamine, or propylamine, K329A exhibits an enolization activity of 0.35 ± 0.02 unit/mg (17% of the wild-type value). Thus, in contrast to the rescue of carbon-fixation activity, exogenous amines do not stimulate the inherent enolization activity of K329A. Negligible detritiation is observed in controls containing amines but lacking enzyme.

Product Analyses. The ethylamine-stimulated $^{14}CO_2$-fixation activity of K329A correlates with enhanced PGA formation as monitored by enzyme-coupled NADH oxidation (Table 1, footnote g). Chromatographic profiles of reaction

[4] At 2 mM RuBP and saturating [ethylamine], K329A exhibits a k_{cat} of 2.4% of the wild-type value (Table 1). This value increases with increasing [RuBP], but rate saturation with respect to [RuBP] is less apparent at high (450 mM) concentrations of ethylamine (data not shown), probably reflecting interactions among the amine, substrate, and other buffer components. Consequently, the true k_{cat} at saturating levels of both RuBP and amine has not been accurately determined.

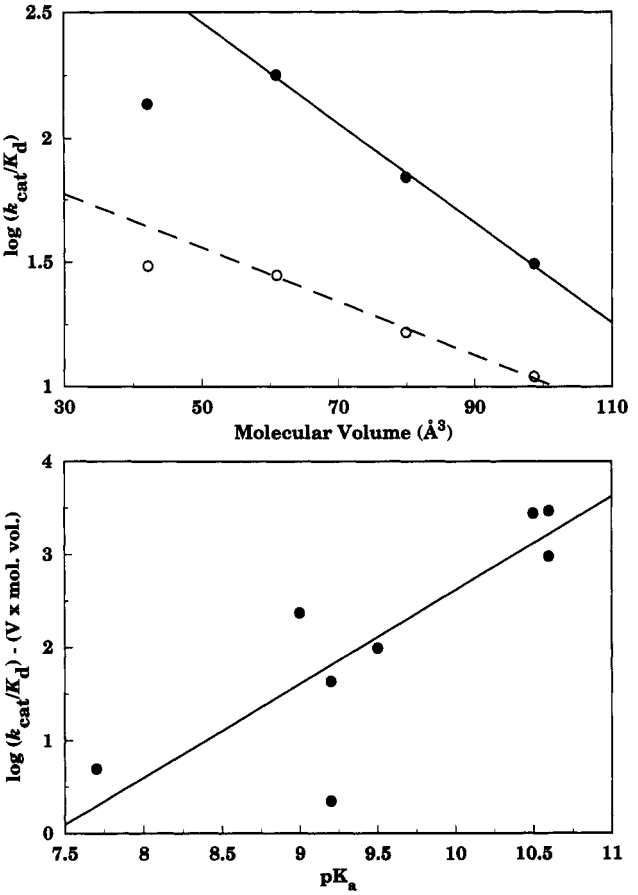

FIGURE 4: (Top) Effect of solvent-excluded amine molecular volume for amines with similar pK_a values (methyl-, ethyl-, propyl-, butyl-) on catalytic efficiencies of amine-rescued K329A (●) and K329C (○). The values for V, the intrinsic volume parameter, were obtained from the slopes of these plots. The data points for methylamine were not used in the calculation of V, because they deviate significantly from the data trend. (Bottom) Volume-adjusted Brønsted plot for the chemical rescue of K329A by the amines listed in Table 1. The Brønsted parameter, β, was obtained from the slope of the line determined by linear regression of all data points. The K_d values used in these plots are expressed as free base concentrations.

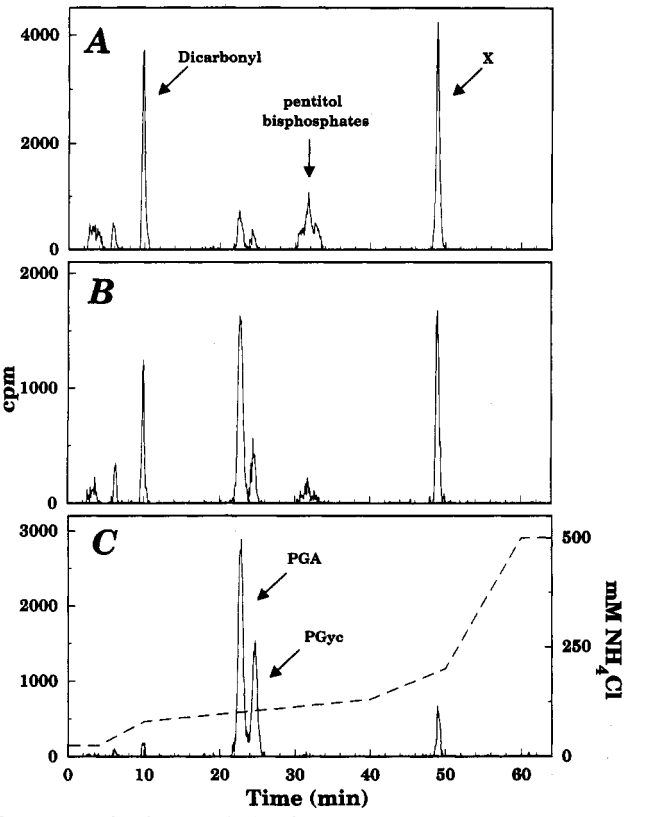

FIGURE 5: Product analysis of K329A turnover reactions in the absence of amine (A) or in the presence of 20 mM (B) or 400 mM (C) ethylamine. Reaction constituents at 23 °C and pH 8.0 were 1 mg/mL K329A, 1 mM EDTA, 10 mM $MgCl_2$, 415 mM bicine, 19.6 mM $NaHCO_3$, 10% glycerol, ethylamine as indicated, and 250 μM [1-^{3}H]RuBP. The total amine concentration was held constant throughout at 400 mM with compensating tetramethylammonium chloride. Reactions were quenched after 4 h by reduction with borohydride.

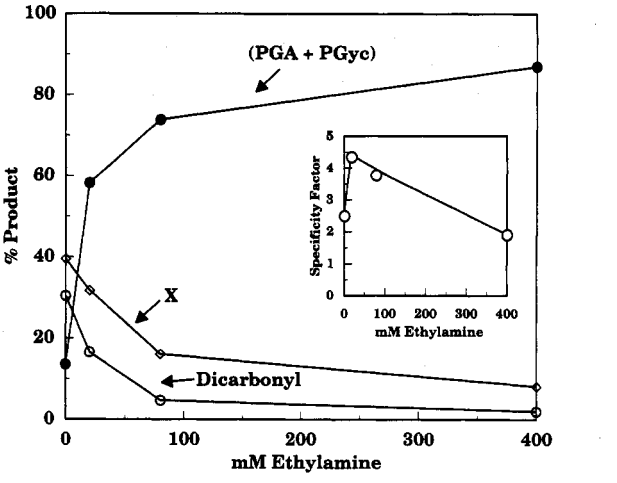

FIGURE 6: Product distributions in K329A turnover reactions as a function of ethylamine concentration. All values are normalized for extent of turnover as estimated by the amount of pentitol bisphosphates remaining in product profiles. Inset: Effect of ethylamine concentration on the specificity factor, τ, for K329A. The data are derived from reactions, including those shown in Figure 5, adjusted to a constant total amine concentration of 400 mM with tetramethylammonium chloride.

mixtures of K329A with [1-^{3}H]RuBP at varied ethylamine concentrations also verify that the amine-stimulated activity of K329A represents a restoration of normal carboxylation activity (Figure 5). In the absence of amine, two novel side products accumulate (dicarbonyl and X in Figure 5 and discussed further below). In contrast to previous findings at lower [enzyme]/[RuBP] ratios (Hartman & Lee, 1989), these results demonstrate that K329A can process RuBP, but with the predominant formation of aberrant side products. In the presence of increasing ethylamine concentrations, PGA and PGyc (the normal reaction products) predominate over these side products (Figure 6).

The CO_2/O_2 specificity (τ) for K329A determined in the absence of amine rescuer is 2.6, relative to a wild-type value of 13. The specificity of K329A is enhanced at low [ethylamine] (Figure 6, inset) in parallel with the increased commitment to forward catalysis. This effect is integral to rescue, because the specificity factor of wild-type enzyme is unaltered in the presence of any of these amines at 20 mM. The reduction of τ that occurs at very high concentrations of ethylamine presumably reflects either depletion of substrate CO_2 via carbamate formation or an otherwise indirect disruption of the HCO_3^-/CO_2 equilibrium, since similar relative decreases in specificity were also observed for the wild-type enzyme at these same high concentrations (data not shown). The increase in specificity for amine-rescued K329A is sensitive to the molecular volume of the amine side chain, as demonstrated by the different τ values observed at a single fixed concentration of unsubstituted aliphatic amines of similar basicity (propyl- > ethyl- > methyl- > butyl-) (Table 2).

Table 2: Influence of Amines upon the Carboxylation/Oxygenation Specificity (τ) of K329A[a,b]

amine	τ 5 mM amine	τ 20 mM amine	amine	τ 5 mM amine	τ 20 mM amine
methylamine	4.9	4.0	propylamine	6.8	6.5
ethylamine	5.9	4.9	butylamine	3.1	2.7

[a] Reaction constituents buffered at pH 8.0 were 2 mg/mL K329A, 300 mM bicine, 10 mM $MgCl_2$, 1 mM EDTA, 7.5 mM $NaHCO_3$, 10% glycerol, ● amine, and 250 μM [1-^{3}H]RuBP. After a 3-h incubation, the reactions were quenched with borohydride prior to chromatographic analysis of τ as described in the Experimental Procedures. [b] In the absence of amine, the τ value for the K329A mutant was 2.6.

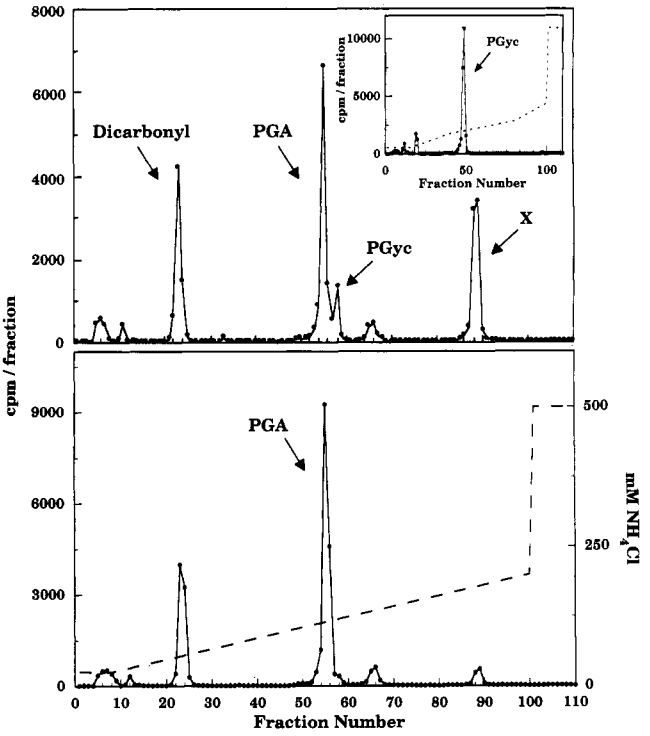

FIGURE 7: Product profiles for [1-^{3}H]RuBP consumption by K329A in the presence (top) and absence (bottom) of O_2 (255 μM). O_2-scrubbing was accomplished enzymatically (Harpel et al., 1993). Reaction constituents at 23 °C and pH 8.0 were 1 mg/mL K329A, 1 mM EDTA, 10 mM $MgCl_2$, 250 mM bicine, 10% glycerol, 20 mM ethylamine, 12 mM $NaHCO_3$, 0.3 unit protocatechuate 3,4-dioxygenase, ● 2 mM protocatechuate, and 250 μM [1-^{3}H]RuBP. Reactions were quenched after 1.5 h by reduction with borohydride. The O_2-containing reaction lacked only protocatechuate. Inset: Reaction of Mn^{2+}-substituted K329A with [1-^{3}H]RuBP. Reaction constituents at 23 °C and pH 8.0 were 0.6 mg/mL K329A and the same buffer as described above, but with 5 mM $NaHCO_3$, 10 mM $MnCl_2$ instead of $MgCl_2$, no exogenous amine, and no O_2-scrubbing system. The total reaction time was 3 h.

Reaction Side Products. The two stable side products of K329A reactions (denoted dicarbonyl and X in Figure 5) are detected radiometrically whether [1-^{3}H]RuBP or [5-^{3}H]RuBP (data not shown) is supplied as substrate. Therefore, both carbons 1 and 5 are conserved in these products. Neither compound accumulates from RuBP in the absence of K329A, with deactivated mutant enzyme, or with activated mutant enzyme in the presence of inhibitory levels of CABP. Reaction profiles for the wild-type enzyme also lack these peaks. Thus, both products derive from the misprocessing of RuBP by the mutant enzyme.

The peak in Figure 5 labeled dicarbonyl represents a mixture of stereoisomers of pentitol monophosphates produced by borohydride reduction of 1-deoxy-D-glycero-2,3-pentodiulose

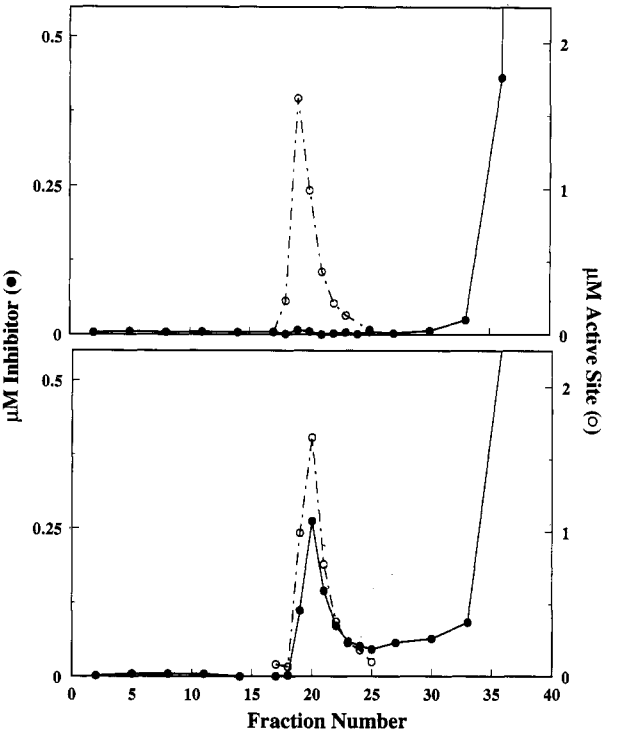

FIGURE 8: Gel-filtration analysis of CABP binding by K329A in the absence (top) and presence (bottom) of 50 mM ethylamine in the column buffer. Unbound CABP (total included volume) begins to elute at fraction 35.

5-phosphate, one of the actual side products. This dicarbonyl was invoked as a product of β-elimination of phosphate from the 2,3-enediol(ate) of RuBP, generated under alkaline or acidic conditions (Paech et al., 1978; Jaworowski et al., 1984). More recently, 1-deoxy-D-glycero-2,3-pentodiulose 5-phosphate was observed as a reaction side product in studies of Rubisco mutants altered at the C1-phosphate binding site (Larimer et al., 1994) and as a product of carboxyketone intermediate degradation by deactivated wild-type enzyme (Pierce et al., 1986). Proof of the structure of the dicarbonyl was provided in these previous studies by its reduction with borohydride, oxidation with hydrogen peroxide, and ^{13}C-NMR spectroscopy.

The other side product (X) that accumulates in K329A reactions lacking amine elutes in nearly the same position as CABP, which carries a net charge of −5 at pH 8.0. Despite this chromatographic similarity, compound X does not contain CO_2-derived carbon on the basis of inspection of a reaction mixture with unlabeled RuBP and $NaH^{14}CO_3$. Rather, formation of this compound requires O_2 (Figure 7), suggestive of a byproduct of the oxygenase reaction. In contrast, formation of the dicarbonyl compound is insensitive to O_2 concentration. Also, X is not found in reaction mixtures in which Mg^{2+} has been replaced by Mn^{2+} (Figure 7, inset), which is known to increase the oxygenation preference of wild-type enzyme (Christeller & Laing, 1979). A shift in elution position, corresponding to a net loss of four negative charges, occurs subsequent to treatment of isolated X with alkaline phosphatase. Thus, X appears to retain both phosphate groups of RuBP. Radiolabel is not incorporated into X in reactions quenched with NaB^3H_4, thereby discounting the presence of an aldehyde or ketone group.

Reaction Intermediate Analogue Binding. In contrast to activated wild-type Rubisco, K329A fails to form a stable complex with CABP (Soper et al., 1988). Likewise, a complex of K329A with CABP is not observed following preincubation

with ethylamine and gel filtration in buffer lacking amine (Figure 8, top). However, an enzyme–inhibitor complex is apparent when ethylamine is included in the column buffer (Figure 8, bottom), but the substoichiometric binding of CABP (~0.12 per active site) and trailing of radioactivity indicate rather weak complexation relative to the wild-type enzyme. Weak binding persists even when the column buffer contains higher ethylamine concentration (200 mM) (data not shown). The presence of ethylamine does not impact the characteristic stoichiometric tight-binding of the inhibitor by the wild-type enzyme.

DISCUSSION

The multifaceted, but incompletely defined, roles of active-site Lys329 of Rubisco prompted us to pursue structure–activity profiles at position 329 by noncovalent chemical rescue of the severely impaired K329A and K329C mutants. Although these mutants retain considerable activity with respect to enolization of RuBP (Hartman & Lee, 1989) and also catalyze normal processing of the intermediate 2-carboxy-3-ketoarabinitol bisphosphate to PGA at rates that approximate that of the wild-type enzyme (Lorimer et al., 1993), their overall carboxylase activities barely exceed the threshold for detection (Soper et al., 1988). Our results clearly show that position 329 mutants are amenable to rescue, whereby their carboxylase activity is restored in the presence of aliphatic amines to more than 2% of the wild-type level.[4] Since the mutants are preferentially impaired in the reaction of CO_2 with the enediol(ate) intermediate, the exogenous amines must serve as surrogates for the side chain of Lys329 at this particular step in the overall reaction. Indeed, the effective facilitation of the carboxylation step by exogenous amines may be more substantial than indicated by the restoration of 2% of the wild-type carboxylase activity, because amines do not enhance the inherent enolization activity of the mutant proteins. Thus, whereas carboxylation of enediol(ate) is rate-limiting in the absence of amines, enolization may become rate-limiting in their presence.

Efficacy of a chemical rescuer does not necessarily require its discrete complexation with the disabled mutant. For example, the extent of rescue of aspartate aminotransferase by exogenous amines observed by Toney and Kirsch (1989, 1992) is directly proportional to the concentration of amine without any hint of rate saturation. Furthermore, the K258A and K258M mutants of this enzyme are similarly amenable to rescue by short aliphatic amines even though the former mutant contains a potential binding pocket for the amine (space created by removal of the lysyl side chain) and the latter mutant does not (the methionyl and lysyl side chains are isosteric). Thus, the amine appears to attack bimolecularly an enzyme-bound intermediate from the solvent face of the active site.

In contrast to these results with aspartate aminotransferase, several lines of evidence indicate that formation of a ternary complex of amine, RuBP, and protein is required for the rescue of the position 329 mutants of Rubisco. Perhaps the most direct of these is the observed preferential binding of CABP by the K329A mutant in the presence of amines. Involvement of a specific equilibrium complex between K329A and exogenous amine is also indicated by the steric preference among amines. For this mutant, ethylamine is more effective than larger (propyl-, butyl-) or smaller (methyl-) amines, despite the fact that the lysine-to-alanine mutation is more akin to the molecular volume of propylamine (80 Å^3) (Chothia, 1975). Thus, steric factors other than the simple loss of a linear side chain determine the interactions of amines with the enzyme. Furthermore, the lower enhancement of activity attained with the K329C mutant [net loss of 65 Å^3 (Chothia, 1975)] and the different preferences for amine shown in k_{cat}/K_d values (methyl- ≥ ethyl- > propyl- > butyl-) relative to K329A disclose unique selectivities of the two mutant sites for amines. The importance of amine orientation within the productive complex is also indicated by the sensitivity of the CO_2/O_2 specificity to side chain length observed in this study and in previous studies with the covalently modified K329C mutant (Lorimer et al., 1993) and with a mutant of *Anacystis* Rubisco containing an arginyl substitution for the corresponding lysyl residue (Gutteridge et al., 1993).

A third observation consistent with complexation of amine by the K329A mutant is the rate saturation of rescued carboxylation activity with respect to amine concentration. This criterion is not definitive, however, because the amine could condense with RuBP via a Schiff base and thereby limit the availability of substrate for carboxylation. In any event, the binding of amine within the putative ternary complex must be considered quite weak on the basis of the high K_d (120 mM for ethylamine at 2 mM RuBP), the substoichiometric binding of CABP as supported by amines, and the absence of an order-of-addition effect (relative to RuBP and amine) on the kinetics of rescue. Combined with the lack of enhancement of the enolization partial reaction by amines, the latter point suggests that the catalytically-competent enzyme involves an equilibrium complex of RuBP- (or enediolate-) bound K329A with readily-dissociable amine rather than a preformed complex between K329A and amine.

The comparative efficacy of the numerous aliphatic amines was examined by Brønsted analysis in order to gain additional insight into the catalytic role of Lys329. Classically, the Brønsted coefficient (β) embodies both the transition-state stabilization effected by acid or base groups and the influence of these groups upon equilibria for complete proton transfer (Jencks, 1987). As an approximation, β denotes the extent of charge development (or transfer) for the proton acceptor (or donor) in the transition state. The observed β of 1 for the chemical rescue of K329A is consistent with the amine being fully protonated in the rescued transition state(s), regardless of whether the amine is formally involved in proton transfer. On the basis of essentially complete protonation of the amine in the transition state, the protonated amine is implicated as the ionic species which actually effects rescue. However, rigorous distinction between the free base and conjugate acid as the active species would require an evaluation of the influence of pH on the kinetics of rescue, which has been precluded by technical difficulties.[5] Thus, our limited Brøn-

[5] In contrast to the amine rescue of aspartate aminotransferase (Toney & Kirsch, 1989, 1992), we have not identified by pH-dependence studies the ionic species of the amine that is responsible for chemical rescue of the mutant Rubisco. Such studies are technically difficult and likely to be compromised by the pH dependence of several parameters other than the ionic nature of the rescuer. These include the degree of enzyme activation (carbamylation), the K_m values of all three substrates (RuBP, CO_2, and O_2), and the K_d value for interaction of rescuer with enzyme. Additional complications include maintaining constant ionic strength simultaneously with constant concentrations of CO_2 and O_2 at variable pH. The apparent requisite binding of amines by K329A imposes another barrier to meaningful pH-dependence studies. In contrast to previous cases, in which rate saturation is not observed and rescue occurs by bimolecular attack rather than formal binding of amine (Toney & Kirsch, 1989, 1992; Sekimoto et al., 1993), the chemical rescue of K329A may be inhibited by competitive binding of the unproductive form of the amine. Such inhibition could potentially impact the apparent k_{cat} and K_d values and compromise the quantitative interpretation of the Brønsted β value.

sted analysis, in conjunction with a β value that approaches the theoretical limit, only allows the general conclusions that Lys329 could act as a general-acid–base catalyst or solely as a structural component through the ionic interactions with bound intermediate as deduced crystallographically (Andersson et al., 1989; Knight et al., 1990; Schreuder et al., 1993). Polarization of substrate CO_2 in the transition state for forming the carboxylated reaction intermediate mimicked by CABP is one obvious role for the protonated Lys329 consistent with both the structural and chemical rescue studies. The possibility of additional catalytic roles for Lys329 is not excluded by these results, because β may reflect only a single rate-limiting step or a single transition state.

The chemical rescue of K329A by amines clearly illuminates the importance of Lys329 in normal forward processing of this intermediate. Our laboratory previously showed that little or no decomposition or forward processing of enediol(ate) occurs during the minimal time necessary for K329C to completely detritiate 3 mM [3-^{3}H]RuBP in the presence of 66 mM $NaHCO_3$ (Hartman & Lee, 1989); we have confirmed these results during the course of the present study (data not shown). By contrast, incubation of RuBP with relatively high concentrations of K329A, which support multiple enolizations, leads to substantial accumulation of aberrant end products. If amines are included in these incubations, RuBP is processed normally to PGA and PGyc with concomitant suppression of the side products. One of these products is a dicarbonyl, 1-deoxy-D-glycero-2,3-pentodiulose 5-phosphate, resulting from β-elimination of phosphate from enediol(ate), in analogy with properties of triosephosphate isomerase (Pompliano et al., 1990; Richard, 1991). Proof of the structure of the dicarbonyl was provided in recent studies of Rubisco mutants, altered at the C1-phosphate binding site, which generate this same dicarbonyl (Larimer et al., 1994). The other side product (denoted X), although not completely characterized, is clearly linked to the oxygenase pathway, because it is not observed under anaerobic conditions or when Mg^{2+} is replaced by Mn^{2+}. We postulate that this unknown arises from rearrangement of an unstable oxygenation intermediate to form the stable compound detected chromatographically.[6,7] The lack of X formed by K329A in the presence of Mn^{2+} presumably reflects more efficient stabilization of the oxygenation intermediate(s) by this alternate activator ion. Therefore, both 1-deoxy-D-glycero-2,3-pentodiulose 5-phosphate and X may be viewed as aborted reaction intermediates.

Whether dicarbonyl and X are formed at the active site of K329A or free in solution can not be distinguished by the available data. The absence of stabilizing interactions between Lys329 and reaction intermediates could allow diversionary chemistry while the intermediates are bound by the enzyme. However, the absence of Lys329 and the associated intersubunit salt bridge with Glu48 is also likely to destabilize the closed conformation of loop 6, thereby increasing the chances of dissociation of intermediates from the active site. In fact, the weak binding of CABP by these mutants is consistent with destabilization of loop 6. We have also observed that the enediol(ate) misprotonation products xylulose 1,5-bisphosphate and 3-ketoarabinitol 1,5-bisphosphate (Edmondson et al., 1990; Zhu and Jensen, 1991) transiently accumulate to low levels during the turnover of RuBP by K329A (data not shown). Eventual turnover of these compounds by the mutant enzyme is demonstrated by the predominance of only PGA, PGyc, dicarbonyl, and X as reaction end products.

Recent ab initio modeling of the Rubisco carboxylation and oxygenation reactions has suggested that deformation of the planar enediol(ate) intermediate is required for its attack by CO_2. Relatively small geometric alterations of the deformed enediol(ate) are envisioned to render an O_2-reactive enediol(ate) triplet state energetically accessible from the ground-state singlet, thereby permitting the formally spin-forbidden reaction with triplet-state O_2 (Andrés et al., 1992, 1993; Tapia & Andrés, 1992). Deformation and concomitant activation of the enediol(ate), which perhaps equate with the rate-limiting step interposed between enolization and gaseous substrate addition as detected by pre-steady-state kinetics (Schloss, 1990), could be mediated by Lys329. The increase in τ noted at increasing amine concentrations with K329A presumably reflects either a direct role of the amine in promoting productive carboxylation or an indirect role in enhancing the stability of loop 6 in the conformation conducive to carboxylation, in analogy with the partial restoration of specificity observed for spatially-compensating second-site mutations within loop 6 (Chen et al., 1991).

Given the importance of both conformational dynamics of loop 6 and Lys329 within this loop to catalysis, the requirements for chemical rescue of position 329 mutants are particularly stringent. The surrogate for the lysyl side chain must not only mimick the catalytic roles of Lys329 but also stabilize the closed conformation of loop 6 at the appropriate stage of the reaction coordinate. Thus, our results extend the application of chemical rescue to enzymes that are impaired due to substitutions of active-site residues located in flexible loops.

[6] We have not attempted to factor contributions from the amount of X produced as an oxygenation byproduct into our calculations of the CO_2/O_2 specificity. If X is considered in these calculations, τ would be significantly lower for reactions lacking amines (in whch X predominates) but much less impacted for reactions containing amines (in which the normal products predominate). Such a correction would even further dramatize the effectiveness of amines in restoring normal intermediate processing.

[7] Preliminary NMR and mass spectral analyses of isolated X suggest a structure arising from rearrangement of the initial hydroperoxy intermediate of the oxygenase pathway.

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