Multifaceted Roles of Lys166 of Ribulose-bisphosphate Carboxylase/Oxygenase As Discerned by Product Analysis and Chemical Rescue of Site-Directed Mutants†

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Received September 21, 2001; Revised Manuscript Received November 19, 2001

ABSTRACT: Ab initio calculations [King, W. A., et al. (1998) *Biochemistry 37*, 15414−15422] of an active-site mimic of D-ribulose-1,5-bisphosphate carboxylase/oxygenase suggest that active-site Lys166 plays a role in carboxylation in addition to its functions in the initial deprotonation and final protonation steps. To test this postulate, the turnover of 1-^{3}H-labeled D-ribulose 1,5-bisphosphate (RuBP) by impaired position-166 mutants was characterized. Although these mutants catalyze slow enolization of RuBP, most of the RuBP-enediol undergoes β-elimination of phosphate to form 2,3-pentodiulose 5-phosphate, signifying deficiencies in normal carboxylation and oxygenation. Much of the remaining RuBP-enediol is carboxylated but forms pyruvate, rather than 3-phospho-D-glycerate, due to incapacity in protonation of the terminal *aci*-acid intermediate. As a further test of the postulate, the effects of subtle perturbation of the Lys166 side chain on the carboxylation/oxygenation partitioning ratio (τ) were determined. To eliminate a chemically reactive site, Cys58 was replaced by a seryl residue without any loss of activity. The virtually inactive K166C-C58S double mutant was chemically rescued by aminoethylation or aminopropylation to reinsert a lysyl-like side chain at position 166. Relative to the wild-type value, τ for the aminoethylated enzyme was increased by ∼30%, and τ for the aminopropylated enzyme was decreased by ∼80%. Thus, two lines of experimentation support the theoretically based conclusion for the importance of Lys166 in the reaction of RuBP-enediol with gaseous substrates.

Rubisco[1] catalyzes the irreversible, rate-limiting reaction of overall photosynthetic carbon assimilation: the carboxylation of RuBP to form 2 molar equiv of PGA. This complex chemical transformation can be depicted as a succession of discrete partial reactions, inclusive of proton abstraction, enolization, carboxylation, hydration, carbon−carbon scission, stereochemical inversion, and proton addition (reviewed in refs *1*−*4*). The necessity to stabilize multiple transition states along the reaction coordinate likely represents the root cause of Rubisco's inefficiency: the enzyme is constrained not only by an inherently low k_{cat}, but also by a propensity to generate diversionary side products from reaction intermediates (Scheme 1). Primary among the side reactions is a pervasive oxygenation that competes directly with carboxylation and thus limits productive carbon assimilation (*5*, *6*).

Although extensive chemical modification (reviewed in ref *7*), site-directed mutagenesis (*2*, *8*, *9*), and crystallographic studies (*10*−*12*) of Rubisco have prompted functional assignment of various active-site residues, uncertainties remain. Clearly established as a key catalytic residue, Lys166[2] participates in both the initial step (deprotonation of RuBP) and the final step (protonation of the terminal *aci*-acid intermediate of carboxylation) of substrate turnover. The latter assignment is entirely compatible with both structural and functional observations. Lys166 is the only residue suitably located to serve as a proton donor to the *aci*-acid intermediate as required to complete formation of the second molar equivalent of PGA from RuBP (*13*). The K166G mutant of *Rhodospirillum rubrum* Rubisco, severely impaired as a carboxylase, nevertheless catalyzes forward processing (hydration and cleavage) of exogenously supplied carboxylated reaction intermediate (CKABP) (*14*). However, the major radiolabeled product derived from the processing of [2′-^{14}C]CKABP by K166G is not PGA but rather pyruvate (*8*). This product arises from β-elimination of phosphate from the terminal *aci*-acid, presumably due to the absence of the normal proton donor.

The means by which Lys166 facilitates deprotonation of RuBP is less clear. Based on the residue's unusually high

† Supported by the Chemical Sciences, Geosciences, and Biosciences Division, DOE, under Grant DE-FG02-99ER20348 to F.C.H.

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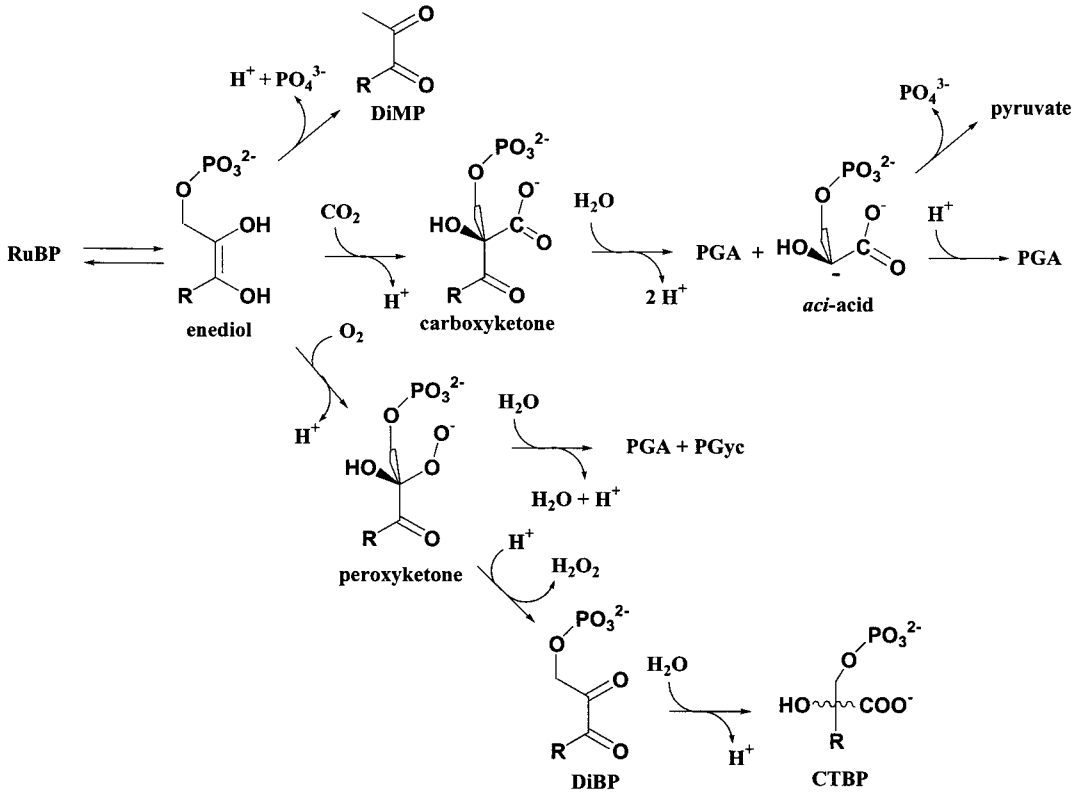
[1] Abbreviations: RuBP, D-ribulose 1,5-bisphosphate; PGA, 3-phospho-D-glycerate; PGyc, 2-phosphoglycolate; Rubisco, RuBP carboxylase/oxygenase (EC 4.1.1.39); CKABP, 2-carboxy-3-ketoarabinitol 1,5-bisphosphate; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; IEF, isoelectric focusing; DiMP, 1-deoxy-D-glycero-2,3-pentodiulose 5-phosphate; DiBP, 2,3-pentodiulose 1,5-bisphosphate; CTBP, 2-carboxytetritol 1,4-bisphosphate.

[2] Residue numbers refer to the *R. rubrum* enzyme.

10.1021/bi011828g CCC: $22.00
Published on Web 01/03/2002

Scheme 1: Reactions Catalyzed by Rubisco or Active-Site Mutants of Rubisco[a]



[a]Among the reactions depicted, reversibility has been demonstrated only for the enolization of RuBP, which initially forms an enediolate bearing a negative charge at O2 (not shown). Tautomerization via the depicted neutral enediol regenerates an enediolate bearing a negative charge at O3 (not shown) and thereby primed for reaction with gaseous substrate. Elimination of phosphate from the terminal *aci*-acid intermediate in the carboxylation pathway occurs to a very minor extent with the wild-type enzyme (*39*) and to a greater extent with some mutants (*8*). Elimination of phosphate from the enediol of RuBP to form DiMP has been observed only with certain mutants (*34*, *38*). Likewise, the elimination of peroxide from the oxygenation intermediate has not been detected with wild-type enzyme but only with certain mutants (*41*, *42*). The conversion of DiBP, the initial product of peroxide elimination, to CTBP is mechanistically similar to a benzylic acid rearrangement (*42*). R = $C(H,OH)CH_2OPO_3^{2-}$. The minor side reaction of misprotonation of the enediol to form D-xylulose 1,5-bisphosphate (*49*) is not shown.

acidity and nucleophilicity and the properties of the K166G mutant (severely impaired in deprotonation of RuBP and hence in overall carboxylation, but nevertheless active in turnover of CKABP), Lys166 was proposed as the essential base for enolization of RuBP (reviewed in ref *7*). Nonetheless, crystallographic models clearly show that the ϵ-amino group of Lys166 is too distant from C3 of bound RuBP and improperly oriented to serve as the initial proton acceptor but rather invoke the Lys191 carbamate in this role (*13*, *15*, *16*). Thus, facilitation of enolization by Lys166, even though pronounced, may be reflective of its serving as a secondary acceptor of the C3 proton of RuBP (*4*) or merely in an ill-defined, indirect role (*12*).

A different perspective on the role of Lys166 in enolization emerges from ab initio quantum−chemical analysis of an active-site mimic (*17*). These calculations suggest that a positively charged, but not a neutral, ϵ-amino group at position 166 substantially lowers the activation energy for RuBP enolization. In short, Lys166 serving as an acid catalyst would facilitate enolization through polarization of the C2 carbonyl of RuBP. Especially intriguing, the calculations also indicate that the lowest energy-level state of enyme-bound enolized RuBP is achieved by protonation (neutralization) of O2 by Lys166, thereby rendering C2 susceptible to electrophilic attack by CO_2. In this view, Lys166 (represented by an ammonium ion in the model) is critical to the carboxylation partial reaction in addition to its previously demonstrated roles in the initial deprotonation and final protonation steps of overall catalysis. By characterization of position-166 mutants and chemically rescued K166C mutants, we herein provide direct experimental evidence in support of this theoretically based postulate.

EXPERIMENTAL PROCEDURES

Materials. Unenriched, 1-^{3}H-, and 3-^{3}H-labeled RuBP were synthesized and purified as reported (*18*−*20*). ^{14}C-labeled $NaHCO_3$ (55 Ci/mol) was obtained from ICN. RuBP concentrations in stock solutions were determined as PGA produced by purified *R. rubrum* Rubisco (*21*). 2-Bromoethylamine hydrobromide and 3-bromopropylamine hydrobromide were obtained from Aldrich−Sigma Chemicals and utilized as freshly prepared 2 M stock solutions in 0.5 M Bicine, pH 8. Ethylenimine was prepared in situ by incubation of aqueous 2-bromoethylamine (2 M) with 3 molar equiv of NaOH for 1 h (*22*); the solution was then neutralized with HCl prior to use in protein modifications.

Enzymes and Mutagenesis. Previously constructed mutant *rbc* genes encoding K166C, K166S, and K166G *R. rubrum* Rubiscos (*23*) were transferred into pFL260 (*24*) for expression in *Escherichia coli* strain MV1190. Plasmids pFL507 (containing the gene encoding the C58S mutant) and pFL436 (containing the gene for the C58S-K166C double mutant) were constructed by the Kunkel method of site-directed

mutagenesis (*25*) by use of the BioRad Mutagene kit in conjunction with deoxyuracil-replaced templates derived from plasmids that contained the genes for wild-type and K166C *R. rubrum* Rubiscos, respectively. The mutagenic primer (GibcoBRL Life Technologies) was 5′-pGTCGAGGTCAGTACTACCGACGAT, in which the underlined bases replace the normal codon for Cys58 and concurrently introduce a *Sca*I restriction site. Mutations were verified by double-stranded dideoxy sequencing (*26*). Transfection and culturing of MV1190 and purification of recombinant mutant Rubiscos were as described (*27*, *28*); recombinant wild-type Rubisco was purified according to the same protocol. Construction and purification of the K191A and K191C mutants were previously described (*29*).

Protein and Activity Assays. Protein concentrations were determined from the absorbance at 280 nm [1.2 AU for 1 mg/mL (*21*)]. Carboxylation activity at pH 8.0 and 23 °C was assayed spectrophotometrically by coupling PGA formation to NADH oxidation (*21*) or radiometrically as acid-stable counts derived from ^{14}C-labeled $NaHCO_3$ (*27*, *30*). In the latter case, assay mixtures contained 50 mM Bicine, 10 mM $MgCl_2$, 1 mM EDTA, 25 mM [^{14}C]$NaHCO_3$ (∼4.5 Ci/mol), 1 mg/mL bovine serum albumin, and variable levels of RuBP and enzyme. The otherwise complete reactions were preequilibrated at 23 °C for 15 min to ensure full activation [spontaneous carbamylation of active-site Lys191 (*31*)] prior to initiation of assays by addition of RuBP. K_m values for RuBP were determined by varying its concentration from 1 to 1000 μM at a fixed $NaHCO_3$ concentration of 25 mM. All assays were carried out at ambient concentrations (∼255 μM) of dissolved O_2.

Enolization activity was assayed as "wash-out" of label from [3-^{3}H]RuBP (*20*, *32*). Standard assay mixtures at pH 8 and 23 °C contained 50 mM Bicine, 10 mM $MgCl_2$, 1 mM EDTA, 25 mM $NaHCO_3$, and 2.1 mM [3-^{3}H]RuBP (∼0.1 Ci/mol). Periodically, aliquots were quenched with $NaBH_4$ (to reduce unconsumed RuBP), acidified with acetic acid (to consume excess $NaBH_4$), dried at 110 °C in an efficient fume hood, and scintillation-counted (EcoLite+ cocktail) as described previously (*28*).

Chromatographic profiles of product distributions were obtained by anion-exchange separation and radiometric detection of labeled compounds (*33*) produced by Rubisco-catalyzed turnover of [1-^{3}H]RuBP (5.6 Ci/mol) in combination with unlabeled $NaHCO_3$ or unlabeled RuBP in combination with ^{14}C-labeled $NaHCO_3$ (42 Ci/mol). Samples (25 μL) containing [1-^{3}H]RuBP were quenched with 3 μL of 10% (w/v) SDS, diluted with 1 mL of H_2O, and deproteinated by ultrafiltration (Amicon Centricon-10). Samples (50 μL) containing [^{14}C]$NaHCO_3$ were quenched by the addition of 175 μL of 10% (v/v) acetic acid, dried under a stream of dry N_2 (*caution*: requires an efficient fume hood), resuspended in 1 mL of H_2O, and deproteinated by ultrafiltration. Samples were chromatographed on a Mono Q column [Pharmacia HR 5/5 (5 mm × 50 mm)], which was eluted with a gradient of NH_4Cl (as defined in pertinent figures) in 10 mM sodium borate at pH 8.0. Column eluates were monitored by in-line radiometric detection (IN/US β-RAM; 4:1 ratio of IN-Flow ES cocktail to solvent). Product identification relied on elution positions, relative to standard reaction mixtures, and chromatographic shifts accompanying postreaction chemical or enzyme-catalyzed conversions (*8*, *33*−*35*).

PGA (derived from CO_2 plus C1 and C2 of RuBP during carboxylation) and PGyc (derived from O_2 and C1 and C2 of RuBP during oxygenation) retain tritium from [1-^{3}H]-RuBP. Thus, the partitioning of RuBP between carboxylation and oxygenation (commonly denoted as the CO_2/O_2 specificity factor or simply τ, where $\tau = V_cK_o/V_oK_c$) was calculated from the ratio of radioactivity associated with PGA and PGyc, respectively, in chromatographic profiles according to the relationship: $\tau = (v_c/v_o)([O_2]/[CO_2])$ (*36*). To ensure identical concentrations of CO_2 among samples being compared, the Rubisco stocks were all dialyzed in the same reservoir containing a known concentration of $NaHCO_3$ prior to their use in [1-^{3}H]RuBP turnover experiments. The reported τ values represent the average of multiple determinations with 2−9 mM $NaHCO_3$ in the reaction mixtures. Dissolved CO_2 concentrations were estimated from the Henderson−Hasselbach equation in conjunction with a pK_{a1} of 6.1; dissolved O_2 concentrations were assumed to be 255 μM. Sodium borate was excluded from chromatographic solvents for determination of τ in order to improve the resolution of PGA and PGyc.

Quantification of pyruvate formed during RuBP turnover by Rubisco was achieved either spectrophotometrically by monitoring lactate dehydrogenase-catalyzed NADH oxidation at 340 nm or chromatographically, relative to pyruvate and lactate standards, before or after reduction with lactate dehydrogenase and NADH (*8*, *34*).

S-Aminoalkylation of Mutant Proteins. K166C and C58S-K166C Rubiscos were covalently modified with 2-bromoethylamine (0.1−0.3 M), ethylenimine (0.1 M), or 3-bromopropylamine (0.1−0.2 M), according to the general methodology described previously (*29*, *37*). Briefly, proteins (0.3−10 mg/mL) were incubated with the corresponding aminoalkylating reagent at 25 °C and pH 8 in a buffer containing 45−95 mM Bicine, 9 mM $MgCl_2$, 1 mM EDTA, and 60 mM $NaHCO_3$; the reactions also contained 1.5−5 mM 2-mercaptoethanol and 3−10% (v/v) glycerol carried over from enzyme stocks. Modifications were followed as time-dependent restoration of carboxylase activity to the inactive mutants by periodically assaying aliquots spectrophotometrically. After the activity levels plateaued (5−6 h for 0. 1 M 2-bromoethylamine, 4−5 h for 0.2 M 3-bromopropylamine, 1−2 h for 0.1 M ethylenimine), the reaction mixtures were quenched with an equal volume of reaction buffer containing 10−100 mM 2-mercaptoethanol and dialyzed exhaustively versus a pH 8 buffer containing 50 mM Bicine, 66 mM $NaHCO_3$, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 20% (v/v) glycerol. The dialyzed stocks were frozen in liquid N_2 and stored at −80 °C. Specific reaction times and conditions are given in the figure legends.

IEF. Denaturing-IEF analysis of Rubisco was accomplished with a Pharmacia Phastystem. Precast "Dry IEF" gels were rehydrated with 9.5 M urea containing 2% (v/v) Ampholine (pH 4−6. 5) (Aldrich−Sigma Chemicals) for 30 min at room temperature in the supplied PhastGel Cassette and prefocused according to the manufacturers' instructions prior to sample loading. Samples were prepared by diluting 5−25-fold in rehydration buffer containing 5% (v/v) 2-mercaptoethanol and were then loaded and focused for 600−

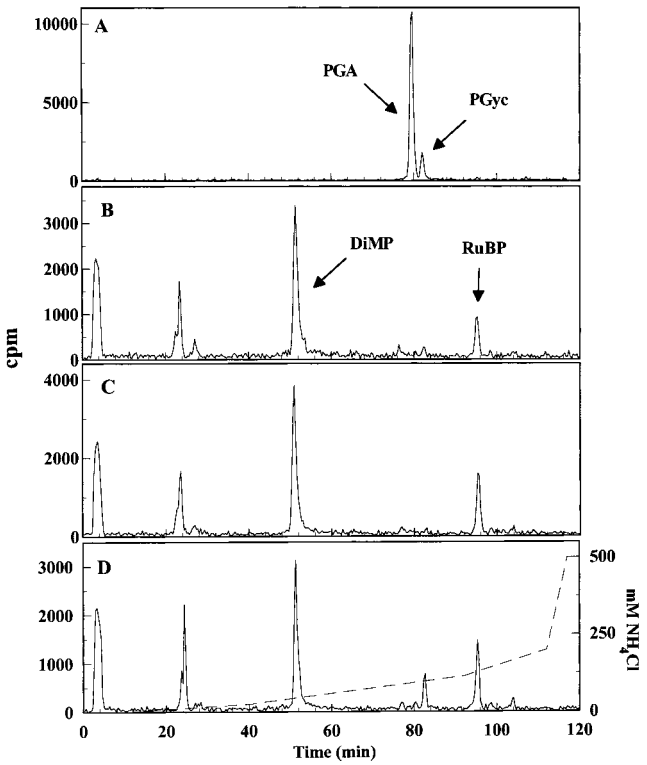


FIGURE 1: Radiolabeled products formed during turnover of [1-^{3}H]RuBP by wild-type (panel A), K166G (panel B), K166S (panel C), and K166C (panel D) Rubiscos. The reaction mixtures (pH 8.0, 25 °C) contained 50 mM Bicine, 10 mM $MgCl_2$, 1 mM EDTA, 250 μM [1-^{3}H]RuBP, and 36−39 mM $NaHCO_3$, and 0.2 mg/mL wild-type Rubisco (4 μM subunit) or 2.0 mg/mL mutant Rubisco (40 μM subunit). Reactions were quenched with 1% SDS (w/v) after 1 h (wild-type) or 4.75 h (mutants). The elution gradient as indicated by the dashed line in panel D was used throughout.

800 Vh; this time range was sufficient to completely focus samples, regardless of whether they were loaded at the anode, cathode, or middle of the gel. Gels were fixed with 2% (w/v) trichloroacetic acid for 10 min and soaked in a mixture of methanol−acetic acid−H_2O (3:1:6) (v/v/v) for 30 min prior to staining with Coomassie Blue.

RESULTS

Side Products Formed from RuBP by Rubisco Mutants. Replacement of Lys166 decreases the rate of enolization and carboxylation activities to undetectable levels in standard kinetic assays. To explore the possibility of residual activity of these mutants, we resorted to prolonged incubations with [1-^{3}H]RuBP at high enzyme concentrations followed by chromatographic analysis of products. As shown in Figure 1, K166G, K166S, and K166C mutants indeed process RuBP. However, the primary product of these reactions is DiMP, which results from β-elimination of phosphate from C1 of the enediol intermediate (*34*, *38*). Verification of DiMP as the major product of these reactions was provided by its coelution with a known standard and also alteration of its elution position after condensation with *o*-phenylenediamine (conversion to a characteristic diimino adduct) or reduction with $NaBH_4$ (conversion to a characteristic three-peak elution pattern) as described previously (*34*) (data not shown).

The prominent peak at the solvent front observed with the mutant Rubiscos is due to slight phosphatase contamination, which becomes significant at the high protein concentrations

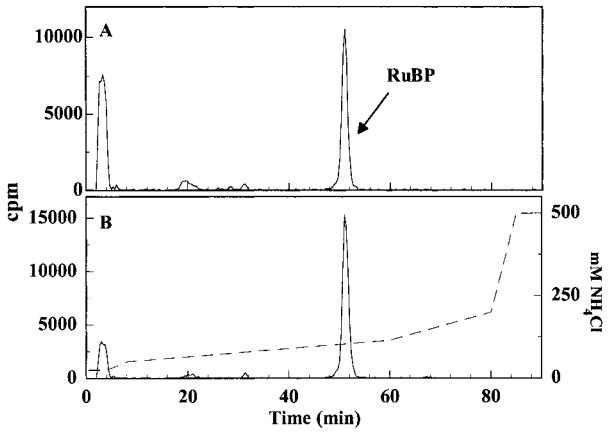


FIGURE 2: Radiolabeled products formed during incubation of position-191 mutants with [1-^{3}H]RuBP (250 μM). Conditions and buffer compositions were as defined in the legend to Figure 1, except for a higher bicarbonate concentration of 50 mM. Protein concentrations were 2.2 mg/mL (44 μM subunit) for K191A (panel A) and 1.3 mg/mL (26 μM subunit) for K191C (panel B). Reactions were quenched with 1% (w/v) SDS after 7 h. The elution gradient indicated by the dashed line in panel B was used for each sample.

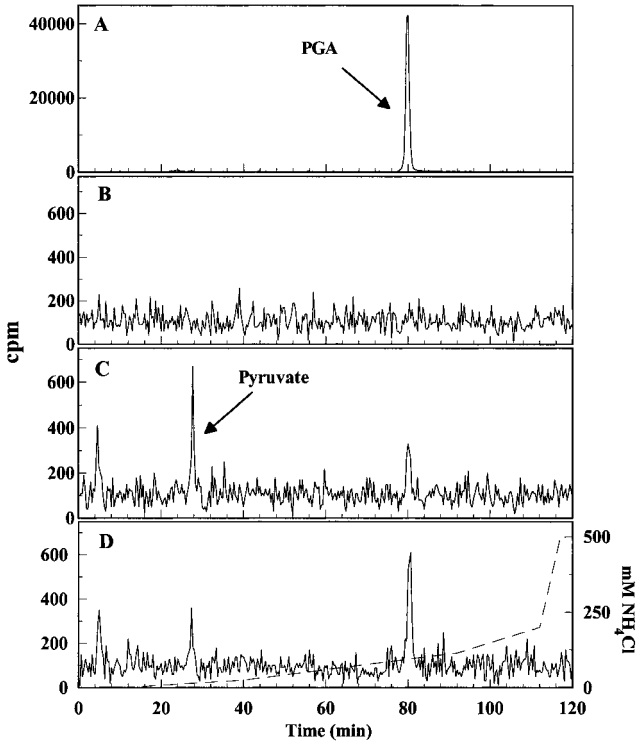


FIGURE 3: $^{14}CO_2$-derived products formed during turnover of RuBP by wild-type (panel A), K166G (panel B), K166S (panel C), and K166C (panel D) Rubiscos. Buffer compositions and protein concentrations were identical to those described in the legend to Figure 1, except for the use of [^{14}C]$NaHCO_3$ (36−39 mM) and unlabeled RuBP (250 μM). After 1 h (wild type) or 4.75 h (mutants), samples were quenched with acetic acid and treated as described under Experimental Procedures.

and long reaction times used. The smaller doublet centered at about 24 min may be attributed to pyruvate in part (see Figure 3) but also to phosphatase-catalyzed hydrolysis of one of the two phosphate groups of RuBP.

To verify that DiMP formation is a consequence of enzyme-catalyzed enolization of RuBP, we also inspected incubations of the labeled substrate with K191A and K191C, mutants that lack the carbamate (the putative primary base)

and should thus be totally incapacitated in enolization activity. As seen in Figure 2, these incubations, at similarly high protein concentrations and even longer reaction times in comparison to the experiments with position-166 mutants, contain only traces of DiMP. Similar trace levels of DiMP were observed in RuBP controls in the absence of any Rubisco mutant (data not shown). Note that the lack of correspondence of elution positions of compounds depicted in Figures 1 and 2 is due to differences in the steepness of gradients applied.

Not surprisingly, given the kinetic impairment of position-166 mutants as judged by the CO_2-fixation assay (*23*), PGA is not observed in the product profiles derived from [1-^{3}H]RuBP (Figure 1). To increase the sensitivity for detection of PGA and to label concurrently any pyruvate formed, incubations of the mutant enzymes with unlabeled RuBP and $^{14}CO_2$ were carried out. As can be seen in Figure 3, the K166S and K166C mutants generate pyruvate and PGA at molar ratios of 1.6 and 0.2, respectively. These ratios greatly exceed the value of 0.01 as observed with wild-type enzyme (*34*, *39*); the apparent absence of pyruvate in Figure 3A, depicting the wild-type reaction mixture, is due simply to such a small amount being formed relative to PGA. The lack of detectable radiolabeled products in the profile for K166G (Figure 3B) reflects the lower turnover rate of this mutant, in comparison to K166S and K166C, and the detection limit of the assay.

Aminoalkylation of Rubisco Variants. To gauge a possible influence of Lys166 on the carboxylation partial reaction, aminoethylation and aminopropylation of the K166C mutant were used to generate Rubisco variants with lysyl mimics at position 166. Consistent with previous results (*37*), treatment of inactive K166C with 2-bromoethylamine restored carboxylation activity to ~10−15% of the untreated wild-type level, while wild-type enzyme showed ~25% loss of activity over a similar incubation time with the reagent. The extent of chemical modification is readily determined by denaturing IEF. Due to the loss of one charge contributed by the lysyl ϵ-amino group, unmodified K166C migrates as a less basic protein than wild-type Rubisco, but becomes indistinguishable from wild type upon aminoalkylation (sample not shown, see ref *37*). However, as also noted previously, the aminoalkylation is incomplete, as gauged by the remaining starting material, and extends to a second site, as gauged by an additional new band. The latter cofocuses with the major modified species observed in the treated wild-type control (sample not shown, see ref *37*). In the case of aminopropylation (not previously reported), the reaction with the newly introduced sulfhydryl goes to completion, but alkylation at a second site is still prominent (Figure 4, sample PA-K166C). Aminopropylation of K166C restores only ~5% of wild-type activity, while a counterpart wild-type control loses ~25% of its original activity.

To mitigate potential complications in interpretation of kinetic and product analyses of the chemically rescued mutants due to incomplete and nonspecific aminoalkylations, we pursued two avenues. To improve selectivity of modification of Cys166, we replaced Cys58 in the wild-type enzyme with a seryl residue to give a fully active mutant and then proceeded to construct the K166C-C58S double mutant. A consideration of the three-dimensional structure of *R. rubrum* Rubisco (*40*) had indicated that solvent-exposed

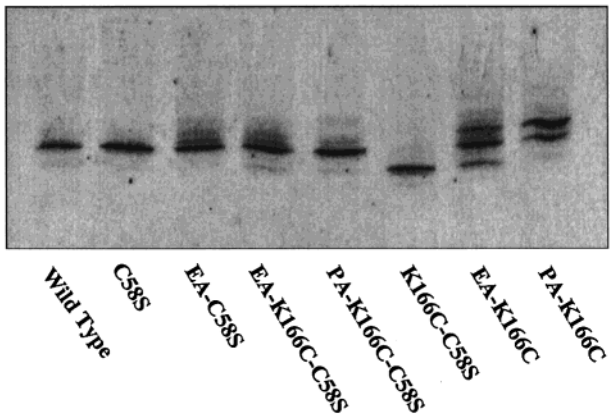


FIGURE 4: IEF of aminoalkylated Rubisco variants. Note that C58S co-focuses with wild-type enzyme; likewise, the double mutant K166C-C58S co-focuses with K166C (not shown), which are well separated from wild type. EA denotes aminoethylation, and PA denotes aminopropylation. EA-C58S, EA-K166C, and EA-K166C-C58S are products of protein modifications by ethylenimine. All samples were applied at 1 μg/lane. The pH gradient within the gel decreases from top to bottom.

Cys58 was the most likely secondary target of aminoalkylation. To improve the efficiency of aminoethylation, ethylenimine instead of bromoethylamine was used as the modification reagent. Indicative of more complete modification, 35% of wild-type (or C58S) activity is restored to K166C-C58S upon incubation with ethylenimine. Companion C58S controls lose ~25% of their original activity during treatment. Although alkylation of K166C with ethylenimine proceeds nearly to completion, the extent of second-site modification is increased (Figure 4, sample EA-K166C). However, both ethylenimine and 3-bromopropylamine fully modify K166C-C58S with far less accumulation of additional species (Figure 4, samples EA-K166C-C58S and PA-K166C-C58S). The identity of Cys58 as the predominant secondary site of modification is also indicated by the relative homogeneity of C58S after treatment with ethylenimine (Figure 4, sample EA-C58S).

Enzymatic Properties of Aminoalkylated Rubiscos. Given the wild-type activity levels of the C58S mutant, the kinetic properties of fully S-aminoalkylated K166C-C58S should reflect the unique attributes of the modified side chain at position 166. The $^{14}CO_2$-fixation assay gave k_{cat} values of 35% and 5% of wild-type for the S-aminoethylated and S-aminopropylated K166C-C58S variants, respectively. The apparent K_m values for RuBP were 5, 10, and 30 μM with C58S, S-aminoethylated K166C-C58S, and S-aminopropylated K166C-C58S, respectively. The S-aminoalkylated variants are nearly as impacted in their ability to catalyze the enolization of RuBP as in net carboxylation (45% and 5% of C58S enolization activity) (data not shown).

The CO_2/O_2-specificity factors (τ) for chemically rescued K166C-C58S were determined by chromatographic analysis of the ratio of labeled PGA to PGyc formed during turnover of [1-^{3}H]RuBP. As expected, the C58S profile is qualitatively indistinguishable from wild type (Figure 5A) with PGA and PGyc as the only detectable products; from multiple determinations, the ratio of these products equates to a τ value of 11 for C58S relative to the value of 10 determined in parallel for the wild-type enzyme. The profile for aminoethylated K166C-C58S (Figure 5B) shows an elevated PGA/PGyc

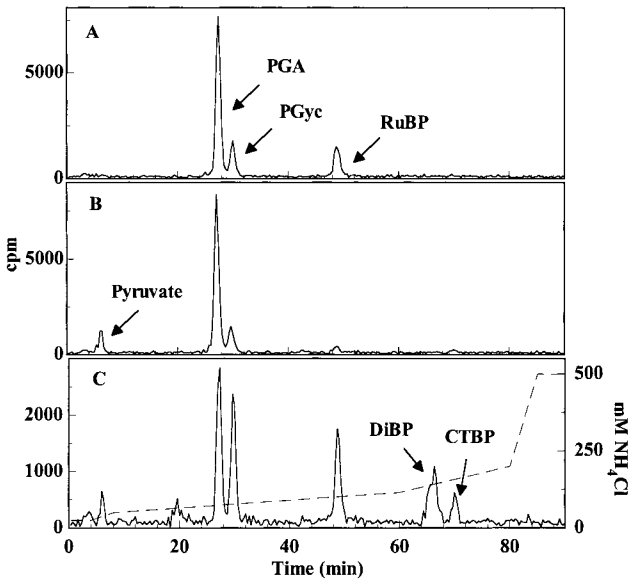


FIGURE 5: Radiolabeled products formed during turnover of [1-^{3}H]RuBP (240 μM) by wild-type enzyme at 0.03 mg/mL (panel A), by aminoethylated K166C-C58S at 0.10 mg/mL (panel B), and by aminopropylated K166C-C58S at 0.13 mg/mL (panel C). Except for a lower concentration of $NaHCO_3$ (9.7 mM), reaction conditions and buffer compositions were as defined in the legend to Figure 1. Reactions were quenched with 2% (w/v) SDS after 2 h reaction times.

ratio, which equates to a τ of 14 (27% greater than that for C58S in multiple determinations). Pyruvate is also apparent in the profile, signifying a greater than normal extent of β-elimination of phosphate from the terminal *aci*-acid intermediate. In the case of aminopropylated K166C-C58S (Figure 5C), PGyc is substantially increased relative to PGA, corresponding to a τ of only 2.1. Pyruvate is also detected, but more striking is the appearance of DiBP and CTBP as observed with some other site-directed mutants (*41*, *42*). These two compounds arise from elimination of peroxide from the hydroperoxide intermediate of the oxygenation pathway; this partitioning does not occur with wild-type enzyme.

In separate experiments, we used the standard spectrophotometric assays to quantify the amounts of pyruvate and PGA formed from [1-^{3}H]RuBP by the two aminoalkylated proteins; in both cases, the pyruvate/PGA ratio was about 0.1. After conversion of PGA to phosphoglycerol by the coupling enzymes used for spectrophotometric analysis, chromatographic analysis verified that all of the radioactivity that had been equated to PGA in profiles as depicted in Figure 5 was indeed PGA; i.e., the PGA peak was eliminated and replaced by a peak that eluted earlier in the gradient coincident with a phosphoglycerol standard (data not shown).

DISCUSSION

Prior to elucidation of complete sequences and three-dimensional structures, Lys166 of Rubisco had been pinpointed as an active-site residue by affinity labeling (reviewed in ref *7*). Subsequent site-directed mutagenesis studies showed that this residue facilitates enolization of RuBP by about 1000-fold (*14*, *20*) and is also necessary for protonation of the terminal *aci*-acid intermediate (*8*). In this latter role, Lys166 may well serve directly as a proton donor, but in the former role it cannot serve as the crucial base that abstracts the C3 proton from RuBP to initiate overall catalysis. Such a possibility, invoked by seemingly compelling but nevertheless indirect evidence, was rigorously excluded by crystallographic analysis (*10*−*12*, *15*, *16*). Thus, a dilemma arose as to the stringent requirement for Lys166 in enolization despite its inability to abstract the C3 proton from RuBP. Although the unusually strong nucleophilicity and high acidity of Lys166 (pK_a of 7.9) (*43*) suggest that it functions as a general base, its ϵ-amino nitrogen is 6.5 Å from C3 but only 3.1 Å from O2 of bound RuBP (*12*). If the ϵ-amino group is protonated within this complex, as deduced from quantum chemical analysis (*17*), it is ideally positioned to facilitate abstraction of the C3 proton by the carbamate via polarization of the C2 carbonyl group as emphasized in this same study. Thus, the troubling conflicts between functional and structural studies will have been resolved.

Deprotonation of RuBP initially forms an enediolate in which the O2 carries a negative charge. Electronic considerations argue that this charge must be neutralized as a prerequisite to eletrophilic carboxylation at C2. Indeed, a rate-limiting step attributed to tautomerization, interposed between abstraction of the C3 proton of RuBP and subsequent carboxylation, was detected by pre-steady-state kinetic analyses (*44*). In one mechanistic model (*4*), tautomerization is effected by the carbamate carboxylate of Lys191 and Lys166 in concert: the carbamate-abstracted C3 proton is transferred to Lys166 via the O2 oxyanion of the initial enediolate, thereby accounting for the dependence of enolization on Lys166 and for rendering this residue positively charged for participation in the protonation of the terminal *aci*-acid intermediate. Entirely compatible with X-ray structures and the stereochemical course of substrate turnover, the carbamate is then used a second time as a base to transfer the proton from the C3 hydroxyl group to the O2 oxyanion of the enediolate. Intuitively satisfying, the deduced mechanism for a single, complete substrate turnover returns the carbamate to its negative state and Lys166 to its neutral state for initiation of the next round of substrate turnover. Although the quantum chemical analysis (*17*) also invokes the carbamate in the abstraction of the C3 proton of RuBP, it implicates Lys166 as the proton donor for generation of the neutral enediol, which must then undergo deprotonation of the C3 hydroxyl by an unspecified active-site base prior to the carboxylation step. In this mechanism, the avenues by which Lys166 and the carbamate are returned to their original charge states are unclear, but several possibilities are allowed by structural considerations (*17*). We also note that the ab initio calculations were applied to a 29-atom mimic of the active site that included Mg^{2+}, RuBP, and fragments of several key side chains. However, Lys168 was not represented in the mimic. This residue not only facilitates enolization of RuBP by >10-fold (*45*) but also probably accounts for the low pK_a of proximal Lys166 (*46*), reminiscent of repulsive electrostatic interplay between adjacent lysyl residues of acetoacetate decarboxylase (*47*). Thus, the calculations may not exclude a neutral Lys166, at initiation of catalysis, which nevertheless could serve as a proton donor due to its close association with Lys168.

It occurred to us that if Lys166, as a base or an acid, facilitates efficient tautomerization of the enediolate and hence its subsequent carboxylation, perturbation of this

critical side chain might have consequences heretofore not uncovered. Specifically, if position-166 mutants do retain slight enolization activity as suggested in prior studies (*14*, *20*), what is the fate of the generated enediolate? Additionally, might very subtle manipulation of the position-166 side chain alter the CO_2/O_2 partitioning of the enediolate?

Three different position-166 mutants (K166G, K166C, and K166S) were examined to be sure that any novel properties observed reflected the absence of the lysyl side chain and were not unique to the particular side chain introduced. Each of these mutants is able to process RuBP, but the major product is DiMP derived from β-elimination of phosphate from the enediol (Figure 1). Thus, even though position-166 mutants retain slight enolization activity, they cannot catalyze subsequent carboxylation or oxygenation of the enediol at rates competitive with that of the β-elimination reaction. In companion experiments, RuBP was refractive to position-191 mutants (Figure 2), reinforcing the structure-based deduction that the lysyl-191 carbamate abstracts the C3 proton from RuBP (*15*, *16*). These contrasting results also verify the conclusion that formation of the enediol in the presence of position-166 mutants is truly enzyme-catalyzed. However, there is no need to suggest that the subsequent β-elimination reaction is enzyme-catalyzed. Since the enediol cannot be processed forward, it either eliminates phosphate due to increased residence time at the active site or dissociates from the enzyme and eliminates phosphate free in solution. A small percentage of the enediol generated by the mutants does undergo normal carboxylation, but a disproportionately high level of pyruvate is ultimately formed (Figure 3), consistent with prior studies of the turnover of the carboxylated intermediate by K166G (*8*).

To examine the influence of Lys166 on CO_2/O_2 partitioning, we replaced the residue with aminoethylcysteine or aminopropylcysteine by combining chemical modification with site-directed mutagenesis (*37*). The inactive K166C that was catalytically resurrected by aminoalkylation also included a seryl substitution for Cys58, which otherwise would have undergone unwanted modification. By elimination of the sulfhydryl group at position 58, we were able to selectively and completely modify the newly introduced sulfhydryl at position 166 (Figure 4). As carboxylases, aminoethylated K166C-C58S and aminopropylated K166C-C58S are 30% and 5% as active, respectively, as C58S. Of direct relevance to the issue of the role of Lys166 in influencing CO_2/O_2 partitioning, the τ value for the aminoethylated protein is increased by nearly 30%, while the τ value for the aminopropylated protein is decreased by 80% relative to that of C58S (Figure 5). Both enzymes generate elevated levels of pyruvate during processing of RuBP, and the aminopropylated enzyme also generates substantial levels of side products from the oxygenation intermediate. To our knowledge, the enhanced τ of aminoethylated K166C-C58S is the largest ever reported as a consequence of structural alteration of a single active-site side chain.

The striking differences in catalytic properties and productive substrate throughput of the aminoalkylated enzymes, in comparison to wild-type Rubisco, are due to very minor manipulation of the Lys166 side chain. Aminoethylation results in net replacement of the β-methylene with a sulfur atom, which increases the maximum length of the side chain by only 0.4 Å. Insertion of an additional methylene group, as occurs upon aminopropylation, increases that length by 1.6 Å. The pK_a of the original side chain may also be impacted; the relevant amino groups of aminoethylcysteine and aminopropylcysteine are about 1 and 0.5 pK_a units lower, respectively, than that of lysine (*48*). Whether these enhanced acidities prevail at the active site is not known. The slight increase in side-chain length effected in the aminoethylated protein could allow the ϵ-amino group to approach within van der Waals' radii of O2 of bound RuBP and perhaps account for the improved CO_2/O_2 partitioning. By contrast, the additional length of the aminopropyl side chain may impose conformational distortions that preferentially compromise carboxylation.

As noted earlier, tautomerization of the RuBP enediolate intermediate formed initially in the Rubisco reaction must occur prior to carboxylation (*44*). Although our studies do not distinguish between base-catalyzed (*4*) and acid-catalyzed tautomerization (*17*) by Lys166, they clearly demonstrate a stringent dependence for Lys166 in both the carboxylation partial reaction per se and gaseous substrate partitioning. If the pK_a of the aminoethylated cysteinyl residue is indeed lowered relative to its lysyl counterpart, a comparison of pH−activity profiles for the rescued mutant vs wild-type enzyme should show whether Lys166 functions as an acid or a base in facilitating enolization of RuBP. However, in preliminary experiments, we do not observe significant differences between the two profiles. Therefore, we plan to directly determine the pK_a by NMR after alkylation of the K166C-C58S mutant with [^{15}N]ethylenimine.

NOTE ADDED IN PROOF

In a continuation of the quantum chemical study cited (*17*), steps after enolization of RuBP in the overall carboxylation pathway of Rubisco have been subjected to computational analyses (*50*). These results indicate the likelihood of additional involvement of Lys166 in the C2−C3 scission step, further reinforcing the multifaceted roles of this catalytic residue.

ACKNOWLEDGMENT

We are grateful to Dr. Y.-R. Chen for purifying the C58S mutant and to T.-Y. S. Lu and A. Hardigree for assisting with genetic manipulations and bacterial growths.

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BI011828G