Effect of mutation of lysine-128 of the large subunit of ribulose biphosphate carboxylase/oxygenase from *Anacystis nidulans*

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The contribution of lysine-128 within the active site of *Anacystis nidulans* D-ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39) was investigated by the characterization of mutants in which lysine-128 was replaced with arginine, glycine, glutamine, histidine or aspartic acid. Mutated genes encoding the Rubisco large subunit were expressed in *Escherichia coli* and the resultant polypeptides assembled into active complexes. All of the mutant enzymes had a lower affinity for ribulose 1,5-bisphosphate (RuBP) and lower rates of carboxylation. Substitution of lysine-128 with glutamine, histidine or

aspartic acid decreased the specificity factor and led to the production of an additional monophosphate reaction product. We show that this product results from the loss of the phosphate from C-1 of RuBP, most probably by β -elimination from the 2,3-enediolate derivative of RuBP. The results confirm that lysine-128 is important in determining the position of the essential ϵ -amino group of lysine-334 within the active site and in loop dynamics. This further demonstrates that residues remote from the active site can be manipulated to modify catalytic function.

INTRODUCTION

Plant productivity can be reduced by up to 50% because the enzyme that initiates photosynthesis, ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39), is not specific [1]. In fact, Rubisco catalyses a variety of side-reactions which involve the enediol of ribulose 1,5-bisphosphate (RuBP), formed during the initial step of catalysis [2,3]. This enediol of RuBP is a potent nucleophile which can react with a range of electrophiles in addition to CO₂ (Scheme 1). In carboxylation catalysed by Rubisco, electrophilic attack by CO₂ on C-2 of the enediol results in the formation of a 2-carboxy,3-keto intermediate [4]. However, the enediol intermediate is also susceptible to reaction with molecular oxygen to produce a transient five-carbon hydroperoxy intermediate [5] (Scheme 1E), which is hydrolysed to yield 3-phosphoglycerate and 2-phosphoglycollate. This reaction not only consumes RuBP, but the recycling of 2-phosphoglycollate into useful metabolites is wasteful: it consumes both ATP and NADPH, and results in the loss of fixed carbon and of nitrogen (as ammonia). Furthermore, in abortive side-reactions, the enediol intermediate is the subject of misprotonation either at C-3 to generate xylulose 1,5-bisphosphate (Scheme 1B) or at C-2 to give 3-keto-arabinitol bisphosphate [6] (Scheme 1F). Both of these compounds are tight-binding inhibitors (K_D of about 0.2 μ M), and their release from Rubisco *in vivo* requires the presence of the ancillary protein Rubisco activase and ATP [7].

Partitioning between the carboxylase and oxygenase reactions is not constant, but can differ by more than an order of magnitude between Rubiscos isolated from different species [8–12]. Since the Rubisco large subunit carries all active-site residues, the fate of the enediolate intermediate depends primarily on the identity and disposition of the amino acid residues within the large subunit [13]. However, due to the apparent absence of a formal binding site for CO₂ or O₂ [3], the ability of different Rubiscos to

discriminate between these gaseous substrates is related to the stabilization of the carboxy or hydroperoxy intermediates by specific residues at the active site.

High-resolution crystal structures of Rubisco from *Anacystis nidulans* [14] and other species have shown that, in the reaction of CO₂ with RuBP, Lys-334 (positioned at the apex of loop 6) has electrostatic interactions with the phosphate group on C-1 and the 2¹-carboxy group of the transition-state intermediate. The position of the ϵ -amino group of Lys-334, together with an essential bivalent metal ion (Mg²⁺), largely determines the specificity of the enzyme for the gaseous substrates and stabilizes subsequent reaction intermediates [14]. During carboxylation, Lys-334 polarizes the two oxygens of CO₂, thereby enhancing the electrophilic status of the carbon atom of CO₂, promoting electrophilic attack on C-2 of the enediol to form the 2-carboxy,3-keto intermediate [4].

Crystallographic studies have yielded high-resolution structural information for Rubisco with a variety of ligands bound at the catalytic site (see Table 1 in [15]). These structures indicate that catalysis is accompanied by conformational changes involving at least five loops and flexible elements of both the N-terminal and α/β -barrel domains of the large subunits. These movements are timed to specific steps in the catalytic cycle [16] and thus position key catalytic residues (e.g. Lys-334) and restrict access to the catalytic site. Binding of RuBP induces the whole N-terminal domain to pivot relative to the α/β -barrel and, in addition, induces the N-terminal regions around Thr-65 and loop 6 of the α/β -barrel to close over the substrate [17]. Characterization of a loop 6 deletion mutant has demonstrated that loop 6 is vital for the normal processing of the enediolate intermediate [18]. These loops are stabilized by the repositioning of another N-terminal loop containing Lys-128, which results in the side chain of this residue being fully extended over loop 6 so that its ϵ -amino group is within hydrogen-bonding distance of

Abbreviations used: Rubisco, D-ribulose 1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39); RuBP, D-ribulose 1,5-bisphosphate; Ru5P, ribulose 5-phosphate; CABP, 2-carboxyarabinitol 1,5-bisphosphate.

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Scheme 1 Reactions involving the enediol of RuBP catalysed by Rubisco

(A) RuBP; (B) xylulose 1,5-bisphosphate; (C), proposed reaction product from β -elimination of phosphate from C-1; (D) the 2-carboxy,3-keto intermediate of carboxylation; (E) the 5-carbon hydroperoxy intermediate of oxygenation; (F) 3-keto-arabinitol bisphosphate.

the main-chain carbonyl groups of loop 6 (Val-331 and Gly-333) and of Phe-467 close to the C-terminus (Figure 1). These movements facilitate co-ordination of the active-site Mg^{2+} ion with the C-2 and C-3 oxygen atoms of RuBP, permitting the abstraction of the C-3 proton to form the enediol intermediate. In addition, with the loops closed over the substrate, only small molecules such as CO_2 and O_2 can gain access to the enediol intermediate.

The residues in the N-terminal loop around Lys-128 are highly conserved; for example, Phe-127 and Lys-128 are invariant in all sequences so far examined. In addition, Asn-123 at the N-terminal end of this loop interacts directly with the substrate analogue 2-carboxyarabinitol 1,5-bisphosphate (CABP) and is close to the activating Mg^{2+} ion. Mutation of the equivalent residue in *Rhodospirillum rubrum* Rubisco (Asn-111) to glycine decreased specificity for CO_2 by an order of magnitude and decreased the maximum rate of carboxylation to 1 % of the wild-type rate [19,20]. Although Lys-128 is not within the active site of the enzyme, inspection of the three-dimensional structure indicates the involvement of this residue with Lys-334, suggesting a possible role in establishing the correct active-site conformation (Figure 1). In order to determine the contribution of Lys-128 to stabilization of the carboxy or hydroperoxy intermediates (via Lys-334), we report the characteristics of *Anacystis nidulans* Rubiscos which contain substitutions for Lys-128. The rationale for the introduction of different amino acids at this position was to determine how the functional properties of the side-chain substituents contributed to catalysis, through their interaction with loop 6 and with the C-terminus.

MATERIALS AND METHODS

Materials

The plasmid pAn92 [21] contains a 2.2 kb fragment encoding the large and small subunits of Rubisco from *A. nidulans* cloned into pUC18. Restriction and DNA-modifying enzymes were pur-

Figure 1 Position of Lys-128 in relation to Phe-467 in the C-terminus, loop 6 residues 331–334 and CABP bound to the catalytic site of *A. nidulans* Rubisco large subunit

This stereo illustration is based on the structure determined from X-ray diffraction (co-ordinates supplied by S. Gutteridge) and generated using Bobscript [41] (an extension of Molscript [42]), then subsequently rendered with Raster3D [43,44].

chased from Gibco-BRL Life Technologies (Paisley, U.K.) or Promega; oligonucleotide primers were obtained from Severn Biotech Ltd; the mutagenesis kit was supplied by USB; and radionucleotides were from Amersham International. RuBP, [^{14}C]RuBP (52 Ci·mol $^{-1}$) and [^{14}C]CABP (1.0 Ci·mol $^{-1}$) were prepared as described previously [22–25]. Chemicals, supplementary enzymes and protease inhibitors were supplied by Sigma.

Mutagenesis

Routine DNA manipulations were carried out as described in [26]. The 2.2 kb *Pst*I fragment bearing the Rubisco large and small subunit genes was excised from pAn92 and transferred to the *Pst*I site of M13mp18 to give M13An92. Mutations were introduced into the gene encoding the large subunit of Rubisco using the T7-GEN *in vitro* mutagenesis kit. The 2.2 kb *Pst*I fragment bearing the Rubisco large and small subunit genes was excised from mutated M13An92 and transferred to the *Pst*I site of pUC18. All mutations were confirmed by sequencing of double-stranded DNA templates through the region of mutation [27,28].

Expression and purification of Rubiscos

Cultures of *Escherichia coli* strain JM83 expressing wild-type or mutant genes were grown in L-broth to late-exponential phase. Cells were recovered by centrifugation, frozen in liquid nitrogen and stored at -20°C . Rubisco was purified from the frozen cells as described previously [29].

Phosphate release from RuBP

[γ - ^{32}P]ATP was synthesized by phosphorylation of ADP by [^{32}P]P $_i$ (5000 Ci·mmol $^{-1}$) using illuminated chloroplasts, then purified by anion-exchange chromatography, as described by Nelson [30]. RuBP was specifically labelled at C-1 or C-5 using this [γ - ^{32}P]ATP. In each case, at least 90% of the radiolabel supplied was incorporated into RuBP.

[1- ^{32}P]RuBP was synthesized by the phosphorylation of excess ribulose 5-phosphate (Ru5P) using 0.5 unit of phosphoribulokinase (EC 2.7.1.19) and 1 μCi of [γ - ^{32}P]ATP, which were added to buffer to give final concentrations of 50 mM Bicine (pH 8.2), 10 mM MgCl $_2$ and 5 mM HCO $_3$ in 167 μl , followed by incubation for 15 min at 25°C .

[5- ^{32}P]RuBP was produced by the phosphorylation of [5- ^{32}P]Ru5P using non-radiolabelled ATP. [5- ^{32}P]Ru5P was synthesized by incubating 1 μCi of [γ - ^{32}P]ATP with excess D-glucose and NADP $^{+}$ and 1 unit each of hexokinase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in 50 mM Bicine (pH 8.2), 10 mM MgCl $_2$, 5 mM dithiothreitol and 5 mM NaHCO $_3$ for 15 min at 25°C . This [5- ^{32}P]Ru5P was then phosphorylated using excess non-radiolabelled ATP and 0.5 unit of phosphoribulokinase, as described above, in a total volume of 167 μl . Both radiolabelled RuBP preparations were used immediately.

Release of P $_i$ from radiolabelled RuBP was initiated by adding 83 μl of activated Rubisco [3 mg·ml $^{-1}$ in 100 mM Bicine (pH 8.2), 20 mM MgCl $_2$ and 10 mM NaHCO $_3$] to the mixtures described above, which were then incubated for a further 70 min at 25°C . The reactions were quenched using 250 μl of ice-cold 0.5 M formic acid supplemented with 1 mM ATP, 0.5 mM RuBP, 1 mM P $_i$, 0.5 mM 3-phosphoglycerate and 1 mM 2-phosphoglycollate. Addition of an equal volume of 5% (w/v) ammonium molybdate in 2 M H $_2$ SO $_4$ was followed immediately by addition of 1 ml of isobutanol/toluene (1:1, v/v), and the mixture was vortexed for 20 s. The phases were separated by centrifugation

for 1 min at 700 g $_{\text{av}}$. The radioactivity in 100 μl of the upper organic phase (containing the complexed P $_i$) and 25 μl of the lower aqueous phase (containing the organically bound phosphate) was determined following addition to 2 ml of Ultima Gold scintillation cocktail (Packard).

Release of P $_i$ was also determined using the method of [31] (see Figure 3).

Kinetic measurements

To determine kinetic constants, lyophilized purified enzyme was dissolved in CO $_2$ -free assay buffer (100 mM Bicine, 20 mM MgCl $_2$, pH 8.2) and desalted by passing through a small column (6 cm $\times$ 0.6 cm) of Sephadex G25-Fine (Pharmacia) equilibrated with the same buffer to remove any CO $_2$. The enzyme was then incubated at 25°C following the addition of NaH $^{14}\text{CO}_3$ to 10 mM. The enzyme was fully activated after 10 min and could be stored at 25°C for up to 4 h without significant loss of activity.

For the K_m determinations, 10 μl aliquots of activated enzyme (1–5 mg·ml $^{-1}$) were assayed as described previously [21], except that the assays for the mutant K128G, K128Q, K128H and K128D enzymes were carried out in stoppered 1.5 ml glass vials (Edwards) and the assay time was extended up to 60 min in order to fix sufficient radiolabel to determine rates accurately. The concentration of Rubisco active sites, which is needed to calculate $V_{\text{carboxylation}}$, was determined from the binding of [^{14}C]CABP (1.0 Ci·mol $^{-1}$) to mutant and wild-type Rubiscos. [^{14}C]CABP was added to desalted enzyme that had first been incubated, for 10 min at 25°C , with unlabelled NaHCO $_3$ to generate the activated quaternary complex. After a further 30 min incubation, the enzyme complex was freed of excess inhibitor by gel filtration through a column (30 cm $\times$ 1 cm) of Superdex 75 (Pharmacia). To determine whether the mutations altered the rate of dissociation, the complex was challenged with a 10-fold molar excess of non-radiolabelled CABP, and the amount of radioactivity remaining with the enzyme fraction was measured at different times [32].

Specificity factor

Changes in the relative specificity for carboxylation and oxygenation were analysed using [^{14}C]RuBP. Immediately prior to initiating relative specific activity assays, purified Rubisco was desalted through a small column (6 cm $\times$ 0.6 cm) of Sephadex G25-Fine (Pharmacia) equilibrated with 100 mM triethanolamine HCl/20 mM MgCl $_2$, pH 8.2, to remove any CO $_2$, and was activated by the addition of NaHCO $_3$ to 10 mM. A 1 μl portion of [1- ^{14}C]RuBP was added to 450 μl of reaction buffer (100 mM triethanolamine HCl, 20 mM MgCl $_2$ and 100 units of carbonic anhydrase, pH 8.2) equilibrated with CO $_2$ -free air and then supplemented with NaHCO $_3$ to obtain the required CO $_2$ /O $_2$ ratio. The reaction was initiated by the addition of 50 μl of the freshly prepared enzyme solution and incubated for enough time to consume approx. 20% of the RuBP. The reaction was stopped by the addition of 100 μl of 10 M formic acid. The contents of the tube were passed through a small column (2 cm $\times$ 0.6 cm) of cation-exchange resin AG 50W-X8 (H $^{+}$ form; Bio-Rad) to remove excess cations, and then supplemented with 0.1 μmol each of RuBP, 3-phosphoglycerate and 2-phosphoglycollate before chromatography on a Mono Q HR 10/10 column (Pharmacia) equilibrated with 8 mM HCl and 32 mM NaCl. After isocratic chromatography for 25 min at a flow rate of 1 ml·min $^{-1}$, a linear gradient rising to 50 mM HCl and 200 mM NaCl was developed over 15 min. Post-column addition of Ultima-Flo M scintillation cocktail (Packard) at 3 ml·min $^{-1}$

facilitated the detection of radiolabel using a radioisotope detector model LB 507-A (EG&G Berthold). Fractions were subsequently collected at intervals of 30 s and radioactivity was re-counted in a liquid scintillation counter.

RESULTS

Purification of wild-type and mutant enzymes

Initially, the activity of Rubisco from cultures of *E. coli* expressing each of the mutant enzymes was determined after permeabilization of the cells with toluene [21]. All the mutants exhibited reduced activity compared with the wild-type enzyme. However, all of the mutant enzymes were purified to homogeneity using the same procedure developed for the wild-type enzyme.

Specificity factors

The specificity factor for the K128R mutant had a value close to that of the wild-type, but all the other mutant Rubiscos had decreased specificity factors (Table 1). The K128G mutant had a specificity factor of 83 % of the wild-type value, whereas the remaining mutants showed much greater decreases in specificity factor; the value for K128D mutant was the lowest, at only 11 % of the wild-type value.

The radiolabelled reaction products were resolved by anion-exchange HPLC at acidic pH with continuous radiometric detection of the column effluent. As expected, the chromatograms used to determine the specificity factor showed that 3-phosphoglycerate and 2-phosphoglycollate were the only reaction products of wild-type Rubisco. However, the reaction products of the mutants K128Q, K128H and K128D revealed an additional peak with a retention time of 15 min (Figure 2), consistent with a monophosphate product. The addition of stoichiometric amounts of CABP to inhibit the enzyme prior to assay not only prevented the production of 3-phosphoglycerate and 2-phosphoglycollate, but also greatly decreased production of the additional product. Similarly, the peak was not produced when the mutant K128Q was incubated with ^{14}C -labelled 3-phosphoglycerate and ^{14}C -labelled 2-phosphoglycollate, and so is unlikely to be a derivative of these compounds. Neither did the additional radiolabelled product appear when the substrates consisted of unlabelled RuBP and $^{14}\text{CO}_2$.

Phosphate release from RuBP

Since the retention time of the unexpected compound suggested that it was a monophosphate, the appearance of P_i during catalysis by the wild-type and K128Q forms of Rubisco was

Figure 2 Distribution of ^{14}C among products derived from $[1-^{14}\text{C}]\text{RuBP}$ in various reaction mixtures containing CO_2 and O_2

Products were separated using a Mono Q HR10/10 column (Pharmacia). (A) Without enzyme; (B) wild-type (K128) Rubisco; (C) K128Q Rubisco; (D) K128Q Rubisco inhibited by bound CABP. Abbreviations: PGA, 3-phosphoglycerate; PG, 2-phosphoglycollate.

investigated (Figure 3). After 4 h, 12.1 nmol of P_i had been released in the reaction catalysed by the K128Q mutant, whereas release from the wild-type enzyme was not above background levels.

In order to determine whether the release of P_i is confined to a specific phosphate group in RuBP, the wild-type and K128Q forms of Rubisco were incubated with $[^{32}\text{P}]\text{RuBP}$ specifically labelled at either C-1 or C-5 and the release of radiolabelled P_i was measured (Table 2). Whereas very little $[^{32}\text{P}]\text{P}_i$ was released (less than 2 % of the radiolabelled substrate supplied) from either position using wild-type Rubisco, the K128Q mutant liberated more than 15-fold more phosphate from C-1 than from C-5.

Table 1 Kinetic parameters of *A. nidulans* wild-type (K128) and Lys-128 variant Rubiscos

Specificity factors (τ) were normalized to the value for the *A. nidulans* wild-type Rubisco to give the change (%). Values are means $\pm$ S.E.M. Units for $V_{\text{carboxylation}}$ are $\text{mol of CO}_2 \cdot (\text{mol Rubisco active site})^{-1} \cdot \text{s}^{-1}$.

Rubisco	Specificity factor (τ)	Change in τ (%)	K_m RuBP (μM)	K_m CO_2 (μM)	$V_{\text{carboxylation}}$ ($\text{mol} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$)
K128 (wild-type)	46.3 ± 0.9	—	35.5 ± 3.1	124.3 ± 10.8	7.2 ± 0.15
K128R	42.5 ± 1.4	—8	194.3 ± 8.8	86.4 ± 16.7	2.9 ± 0.30
K128G	38.3 ± 1.7	—17	135.8 ± 12.3	204.5 ± 9.9	0.15 ± 0.01
K128Q	6.7 ± 0.7	—85	225.4 ± 43.0	204.4 ± 5.9	0.23 ± 0.05
K128H	6.1 ± 0.9	—87	143.2 ± 2.5	215.5 ± 28.0	0.22 ± 0.01
K128D	5.1 ± 1.1	—89	232.7 ± 2.6	109.6 ± 12.6	0.13 ± 0.08

Figure 3 Amount of P_i released in reactions catalysed by wild-type (●) and K128Q (○) Rubiscos

Table 2 Specificity of P_i release from $[^{32}P]RuBP$ labelled at C-1 or C-5, using wild-type and K128Q Rubiscos

Values are expressed relative to the most active combination (K128Q with $[1-^{32}P]RuBP$), for which a relative value of 100% corresponds to 41% of the available radiolabel (approx $0.4 \mu Ci$). When present, CABP (100 nmol) exceeded the concentration of Rubisco active sites by a factor of 28. Values are means $\pm$ S.D. ($n = 3$).

Rubisco	Relative release of $[^{32}P]P_i$ (%) from:	
	$[1-^{32}P]RuBP$	$[5-^{32}P]RuBP$
K128 (wild-type)	4.2 ± 0.1	2.50 ± 0.04
K128 (wild-type) + CABP	0.30 ± 0.02	0.20 ± 0.02
K128Q	100 ± 1.3	6.4 ± 0.2
K128Q + CABP	23.9 ± 2.2	3.2 ± 0.2

Phosphate release from C-1 by the K128Q mutant represented 41% of the radiolabelled RuBP supplied. Thus, not only is P_i liberated specifically from C-1, but also the resulting monophosphate derivative retains the phosphate on C-5. In all cases, the inclusion of CABP during incubation with Rubisco substantially reduced the release of $[^{32}P]P_i$. Since CABP is a specific inhibitor of Rubisco, the phosphate release observed must be catalysed by Rubisco, rather than by any other components in the mixture.

The radiolabelled products were further analysed by anion-exchange HPLC, as described above (results not shown). The additional ^{32}P -labelled monophosphate product was detected in samples that contained $[5-^{32}P]RuBP$, but not $[1-^{32}P]RuBP$. As expected, the monophosphate product derived from $[5-^{32}P]RuBP$ had the same retention time as that observed using $[1-^{14}C]RuBP$ (Figure 2), and its appearance was greatly diminished when CABP was included in the reaction mixture.

Kinetic parameters

The kinetic characteristics of the enzymes were determined from their response to various substrate concentrations, and are given in Table 1. When the amount of CO_2 was varied in the presence of a saturating concentration of RuBP, the affinity for CO_2 of

Figure 4 Affinity of wild-type and K128 mutant Rubiscos for CABP

●, Wild type (K128); ▽, K128R; ■, K128G; ◇, K128Q; ▲, K128H; ○, K128D. The amount of radioactivity bound to Rubisco, as a percentage of the amount of radioactivity in the starting material, is shown over a 20 h period.

mutants K128G, K128Q and K128H was found to be lower than that of the wild-type enzyme. The affinity for CO_2 of mutants K128R and K128D was not significantly different from that of the wild-type. However, when RuBP was varied at a constant, saturating CO_2 concentration, the affinity of the mutant Rubiscos for RuBP was found to be 4–6-fold lower than that of the wild-type enzyme.

Apart from the K128R mutant, in which the $V_{\text{carboxylation}}$ was only reduced by 60%, the $V_{\text{carboxylation}}$ values were found to be severely impaired relative to the wild-type enzyme (Table 1).

Wild-type Rubisco has a high affinity for CABP, as demonstrated by the exchange of $[^{14}C]CABP$ with non-radiolabelled CABP, which was limited to just 5% after 20 h (Figure 4). In contrast, the majority of the exchange of $[^{14}C]CABP$ with CABP occurred within 2 h for the K128Q, K128H and K128D enzymes. However, after 2 h, 95% and 70% of the radiolabelled CABP was still bound to the K128R and 70% K128G mutants respectively (Figure 4).

DISCUSSION

Each of the mutant large subunits assembled with the small subunit into hexadecameric complexes which were purified satisfactorily by the same procedure that was used for the wild-type enzyme. Therefore the substitutions of Lys-128 considered here do not cause major disruption to the tertiary structure of the large subunit, and so alterations in kinetic parameters are likely to result from disruption in the local environment of residue 128 rather than from gross structural changes. However, in every case, substitution of Lys-128 loosened the binding of CABP within the quaternary complex (Figure 4).

Substitutions of Lys-128 are detrimental to carboxylation (Table 1). The decreased affinity for bisphosphate substrate, decreased catalytic activity, decreased specificity factor and generation of an additional product following such substitutions all suggest that the interactions of this residue may be instrumental in positioning the ϵ -amino group of Lys-334. Examination of the three-dimensional structure of the large subunits

Table 3 Efficiencies of carboxylation and oxygenation of *A. nidulans* wild-type (K128) and Lys-128 variant Rubiscos

Carboxylation efficiency = $V_{\text{carboxylation}}/(K_m \text{ CO}_2)$; oxygenation efficiency = (carboxylation efficiency)/(specificity factor). Efficiencies were calculated using data from Table 1. The values in parentheses are the calculated percentages of the value for K128 (wild type).

Rubisco	Carboxylation efficiency	Oxygenation efficiency
K128 (wild-type)	5.8×10^4 (100)	1.3×10^3 (100)
K128R	3.4×10^4 (58.6)	8.0×10^2 (61.5)
K128G	7.3×10^2 (1.3)	1.9×10^1 (1.5)
K128Q	1.1×10^3 (1.9)	1.7×10^2 (13.1)
K128H	1.0×10^3 (1.8)	1.7×10^2 (13.1)
K128D	1.2×10^2 (2.1)	2.3×10^2 (17.7)

[14] reveals that the ϵ -amino group of Lys-128 is capable of forming hydrogen bonds with the backbone carbonyls of loop 6 (Val-331 and Gly-333) and with Phe-467 (Figure 1). Disruption of these hydrogen bonds may cause the ϵ -amino group of Lys-334 to assume a different position within the catalytic site, which is less favourable for catalysis.

The electrostatic interactions of the ϵ -amino group of Lys-334 with the phosphate at C-1 of RuBP in wild-type Rubisco may be disrupted in all of the mutants which, irrespective of size, charge or shape of the substitute residue, showed a decreased affinity for RuBP. However, this decreased affinity does not explain the decrease in $V_{\text{carboxylation}}$ shown by all the Lys-128 substitution mutants, which must reflect changes in the subsequent catalytic steps. Site-directed mutants containing substitutions of Lys-334 catalysed enediolate formation, but were unable to catalyse the reaction of the enediolate with CO_2 or to form a stable complex with CABP [32–34]. The substitutions of Lys-128 may impair the ability of Lys-334 to promote electrophilic attack by CO_2 at C-2 of the enediol by disrupting the ability of Lys-334 to polarize the two oxygens of CO_2 , thereby decreasing its potency as an electrophile. In *A. nidulans* the ϵ -amino group of Lys-334 may not be optimally positioned within the active site, since mutation of four other residues at the C-terminal end of loop 6 resulted in a 3–7% increase in the specificity factor. In that instance, the increased specificity factor was attributed to a repositioning of the ϵ -amino group of Lys-334 [29,35]. All of our substitutions of Lys-128 impaired the ability of the enzyme to discriminate in favour of CO_2 as gaseous substrate. However, the K128R substitution, which retains the capacity to hydrogen-bond to the main-chain carbonyls of loop 6 and the C-terminus, caused only slight impairment. In contrast, the more radical substitutions K128G, K128Q, K128H and K128D decreased the specificity factor by 17, 85, 87 and 89% respectively.

In none of the mutants did the decrease in the specificity factor result from an increase in the oxygenation efficiency (Table 3). Whereas the carboxylation and oxygenation efficiencies for the K128R and K128G mutants decreased by similar amounts, for the remaining mutants the decrease in oxygenation efficiency was less than the decrease in carboxylation efficiency.

The K128Q mutant catalysed the production of an additional reaction product (Figure 2C). This additional product was not derived from the normal reaction products 2-phosphoglycollate and 3-phosphoglycerate, and neither did it contain the co-substrate CO_2 . Although the generation of this additional product was the major reaction catalysed by the K128Q mutant, the low level of activity (100-fold lower than wild-type) precluded full characterization. Nevertheless, our results show that the product is a monophosphate resulting from the loss of the phosphate

moiety at C-1 (Table 2). The 4-fold decrease in the release of such a phosphate in the presence of CABP shows that the process is dependent on the interaction of radiolabelled substrate with Rubisco catalytic sites. Owing to the decreased affinity with which the K128Q mutant Rubisco binds CABP (Figure 4), it is not surprising that a low level of dephosphorylation persisted in the presence of CABP (Table 2). Given the identical retention times of the monophosphate product derived from either $[1-^{14}\text{C}]\text{RuBP}$ (Figure 2) or $[5-^{32}\text{P}]\text{RuBP}$ (results not shown), this compound is likely to incorporate all five carbon atoms of RuBP as well as the phosphate from C-5.

These results are consistent with β -elimination of phosphate from an enzyme-bound intermediate. β -Elimination is a well known reaction for enediols. Catalysis by Rubisco involves two enzyme-bound enediol intermediates: the 2,3-enediolate of RuBP (Scheme 1) and an *aci*-carbanion form of 3-phosphoglycerate. The production of pyruvate, by the β -elimination of phosphate from the *aci*-carbanion form of 3-phosphoglycerate, is reported to occur once in every 150 carboxylation reactions [36,37], but the chromatographic properties of the additional product preclude pyruvate (which is not phosphorylated). A very low level of β -elimination of phosphate from the 2,3-enediolate of RuBP has been reported for both spinach and *A. nidulans* Rubisco [38]. In agreement with this, a small but significant amount of CABP-sensitive liberation of $[^{32}\text{P}]\text{P}_i$ using wild-type Rubisco and $[1-^{32}\text{P}]\text{RuBP}$ as substrate is also evident from Table 2. In addition, β -elimination of P_i from the 2,3-enediolate of RuBP has also been reported following mutation of another large-subunit residue, Thr-65 [39] (Scheme 1F). Like Lys-334, Thr-65 is implicated in a network of hydrogen bonds to the phosphate of C-1 of RuBP. Since the very small amount of β -elimination of phosphate from the 2,3-enediolate of RuBP by wild-type *A. nidulans* Rubisco is not enhanced by limiting the supply of CO_2 and O_2 , the generation of this unidentified product by the K128Q mutant is also unlikely to result from an increase in the abundance of the enediolate in the steady state as a result of impaired carboxylation and oxygenation. However, it could result from the release of the enediolate into solution, followed by β -elimination. Consistent with this hypothesis is the lower-affinity binding of CABP within the quaternary complex shown by all K128 mutants, relative to the wild-type enzyme. However, while a non-enzymic β -elimination of phosphate from RuBP and xylulose biphosphate in free solution has been observed [40], this cannot be the source of the additional product identified in the current experiments, since its production was greatly decreased by inhibiting the enzyme with CABP, as was the liberation of $[^{32}\text{P}]\text{P}_i$ from $[1-^{32}\text{P}]\text{RuBP}$ (Table 2).

The decreases in specificity and affinity for CABP in the mutant Rubiscos followed the order: aspartate = histidine = glutamine > glycine > arginine > lysine (wild type). The most conservative substitution, K128R, shows the smallest change relative to the wild type. Omission of a substituent side chain (in K128G) is more deleterious, but substituents that alter the position and nature of hydrogen bonding (histidine, glutamine and aspartate) and/or the overall charge (aspartate) at this position have an even greater effect. In addition to their effect on the positioning of Lys-334, it is probable that the loop closures that normally follow RuBP binding are impaired or even prevented by these substitutions; most noticeably with aspartate, histidine or glutamine, less so with glycine and only minimally with arginine. Impaired loop closure may result in release of a reaction intermediate, which may account for the appearance of both P_i and the unusual monophosphate product using the K128Q, K128D and K128H mutants, and for their lower affinity for both RuBP and CABP. In this event, loop closure must

normally be an early event in the catalytic cycle, accompanying or following immediately after the formation of the enediol.

These results demonstrate that both the steric and functional properties of Lys-128 are important for catalysis. This residue influences the position of the ϵ -amino group of Lys-334 within the active site and also facilitates correct loop closure (Figure 1). This work demonstrates how residues remote from the active site can be manipulated to modify catalytic function.

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