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RUBISCO MECHANISM: DISSECTION OF THE ENOLIZATION PARTIAL REACTION

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K. ABSTRACT To test experimentally, the prior theoretical deduction that active-site residue Lys166 of ribulose-bisphosphate carboxylase participates in the carboxylation step of overall catalysis, site-directed mutants and chemically rescued site-directed mutants were characterized by kinetics and product analysis. Although position-166 mutants are able to catalyze normal enolization of ribulose bisphosphate, the enediol intermediate does not undergo carboxlation but rather eliminates phosphate. Furthermore, the chemically rescued mutant (aminoethylation of the severely impaired Lys66Cys mutant) generates a highly active mimic, which displays an enhanced carboxylation/ oxygenation partition ratio. These two distinct lines of experimentation document a crucial role of Lys166 in carboxylation and in discrimination between CO_2 and O_2. To ascertain whether Lys166 functions as an acid or base in facilitation of enolization, the chemically rescued mutant bearing ^{15}N was titrated by NMR. From pH 6.5-9.5, the amino group of Lys166 remains unprotonated, indicating that it promotes enolization by hydrogen bonding to the ketone group of the substrate.

Ribulose-bisphosphate carboxylase/oxygenase (Rubisco) catalyzes the irreversible, rate-limiting reaction of overall photosynthetic carbon assimilation: the carboxylation of D-ribulose 1,5-bisphosphate (RuBP) to form two molar equivalents of 3-phospho-D-glycerate (PGA). This complex chemical transformation reflects a succession of partial reactions, inclusive of proton abstraction, enolization, carboxylation, hydration, carbon-carbon scission, stereochemical inversion, and proton addition. The necessity to stabilize multiple transition states likely represents the root cause of Rubisco's inefficiency: not only is the enzyme constrained by an inherently low k_{cat}, but also by a propensity to generate diversionary side products from reaction intermediate. Primary among the side reactions is a pervasive oxygenation that competes directly with carboxylation and thus limits productive carbon assimilation.

Clearly established as a key catalytic residue, Lys166 (numbering according to the *Rhodospirillum rubrum* enzyme) participates in both the initial step (deprotonation of RuBP) and final step (protonation of the terminal *aci*-acid intermediate of carboxylation) of substrate turnover. The latter assignment is entirely compatible with structural, chemical, and mutagenesis studies.

The means by which Lys166 facilitates deprotonation of RuBP (the initial step) has been the subject of long-standing controversy. Compelling, albeit indirect, chemical modification and mutagenesis invoke the residue as the essential base for enolization of RuBP. 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. 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 or merely in an ill-defined, indirect role.

A different perspective on the role of Lys166 in enolization emerges from ab initio quantum chemical analysis of an active-site mimic. 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. These prior calculations from another laboratory 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.

The goals of this work were to test the postulate that Lys166 participates in the carboxylation step and to ascertain whether Lys166 functions as an acid or base the initial enolization step. The former entailed kinetic and product analysis of partial reactions catalyzed by position-166 mutants of the *R. rubrum* enzyme and by chemically rescued K166C mutants. A chemically rescued K166C mutant also provided avenues for distinguishing Lys166 as acid or base in promoting enolization of RuBP. These avenues entailed determination of the pH-dependence of kinetic parameters in conjunction with determination of the pKa of the ε-amino group of the rescued mutant by NMR.

Three different position-166 mutants (K166G, K166C, and K166S) were constructed and 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 at rates less than 0.1% that of wild-type), but the major product reflects β-elimination of phosphate from the enediol intermediate rather than normal formation of PGA.

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.

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 . 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. As carboxylases, and the aminoethylated and aminopropylated K166C-C58S are 30% and 5% as active, respectively, as wild-type enzyme. Particularly significant, the carboxylase/oxygenase partition ratio, relative to that of wild-type, is increased by 30% in the case of aminoethylated K166C-C58S and decreased by 80% with the aminopropylated enzyme. To my knowledge, the enhanced partition ratio of aminoethylated K166C-C58S is the largest ever reported as a consequence of structural alteration of a single active-site side chain.

Thus, the kinetic properties of position-166 mutants and the altered partition ratios of the chemically rescued K166C-C58S mutants provide convincing evidence that Lys166 does play a crucial role in the addition of gaseous substrates to the enediol intermediate.

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. The pK_a of the

original side chain should also be impacted; the ε-amino group of aminoethylcysteine is about 1 pKa unit lower than that of lysine. Thus, a comparison the pH activity profiles of aminoethylated K166C-C58S should exhibit a distinct difference in their ascending or descending limbs and thereby pinpoint the function of Lys166 as an acid or base. However, the two profiles superimposed, indicating that the ionization of Lys166 and its mimic do not contribute to the pH-dependence of catalysis. Subsequently, [^{15}N]aminoethylated K166C-C58S was prepared so that the pKa of the ε-amino group could be directly measured by NMR. Surprisingly, NMR titration showed that the ionization state of the [^{15}N]side-chain remains unchanged from pH 6.5 - 9.5 and accounts for the lack of perturbation of the pH-activity of aminoethylated K166C-C58S relative to that of the wild-type enzyme. Based on prior studies of the chemical reactivity of Lys166, the ionization state would appear to be neutral. Thus, the nonprotonated ε-amino group of Lys166 must be the species that facilitates enolization of RuBP. This likely occurs by hydrogen bonding of the amino group and the carbonyl group of RuBP. A precedent for this unusual type of acid-catalyzed enolization is provided by prior studies from another laboratory on triose phosphate isomerase, in which a neutral histidyl residue serves as an acid catalyst and proton transfer agent.

In summary, these studies prove that Lys166 contributes to the carboxylation step and CO_2/O_2 partitioning in overall catalysis and functions as an electrophile in facilitation of the initial enolization step. These new mechanistic insights serve as prerequisites to the long-term goal of engineering a more efficient Rubisco and thereby providing an avenue for enhancement of biomass yields.

Publications emanating from this grant:

Harpel, M. R., Larimer, F. W., and Hartman, F. C. (2002) "Multifaceted Roles of Lys166 of Ribulose-bisphosphate Carboxylase/Oxygenase As Discerned by Product Analysis and Chemical Rescue of Site-Directed Mutants", *Biochemistry* **41**, 1390 - 1397.

Hartman, F. C., and Serpersu, E. H. (2002) "An ^{15}N Probe for an Active-Site Residue of Rubisco", *FASEB Journal* **16(no.5)**, A905.