# Carbon Isotope Effect Study on Orotidine 5′-Monophosphate Decarboxylase: Support for an Anionic Intermediate†

Jeremy L. Van Vleet,‡ Laurie A. Reinhardt,‡ Brian G. Miller,§,|| Annette Sievers,§,⊥ and W. Wallace Cleland*,‡

*Institute for Enzyme Research and Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53726, and Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599-7260*

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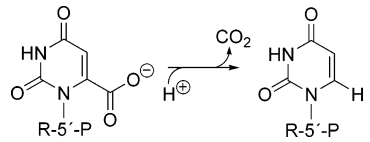
ABSTRACT: Orotidine 5′-monophosphate decarboxylase has been heavily examined in recent years due to its enzymatic proficiency, which provides a catalytic enhancement to a reaction rate $\sim 10^{17}$ times greater than that of the nonenzymatic reaction. Several mechanisms proposed to explain this catalytic enhancement have included covalent addition, ylide or carbene formation, and most recently concerted protonation. All of these mechanisms have circumvented the formation of a high-energy vinyl anionic intermediate. To investigate the presence of an anionic intermediate, $^{13}C$ isotope effect studies have been performed using the alternate substrate 5-fluoro-OMP (OMP = orotidine 5′-monophosphate). Isotope effects obtained for the wild-type enzyme with OMP and 5-fluoro-OMP are 1.0255 and 1.0106, respectively, corresponding to a decrease of ∼1.5% for 5-fluoro-OMP. With the K59A enzyme, the intrinisic isotope effects show a similar decrease of ∼1.9% from 1.0543 with OMP to 1.0356 with 5-fluoro-OMP. This decrease results from the inductive effect of the fluorine, which stabilizes the carbanion intermediate by electron withdrawal and produces a reaction with an earlier transition state. The isotope effect for the decarboxylation of the slow substrate 2′-deoxy-OMP produced a intrinsic isotope effect of nearly 1.0461.

The final step in the de novo biosynthesis of pyrimidine nucleotides is catalyzed by the enzyme orotidine 5′-monophosphate decarboxylase (EC 4.1.1.3) (ODCase).[1] In this enzymatic reaction uridine 5′-monophosphate (UMP) is produced from orotidine 5′-monophosphate by the removal of the carboxylate at the C6 position of the pyrimidine ring as shown in Scheme 1. An intriguing aspect of this enzyme was first presented by Radzicka and Wolfenden, who showed that ODCase is one of the most proficient enzymes with a rate enhancement as determined by $k_{cat}/k_{non}$ of $1.4 \times 10^{17}$ (*1*). Interest in how ODCase can catalyze such a large rate enhancement has increased with the finding that the enzyme performs such a feat in the absence of any cofactor requirement (*2*).

The chemical mechanisms by which decarboxylations occur require the rearrangement of the electronic structure to accommodate the electrons from the breaking bond. This is usually achieved either by providing an intramolecular electron sink or the departure of a leaving group (*3*). In enzyme-catalyzed decarboxylations a cofactor such as pyruvoyl, pyridoxal 5′-phosphate, or a metal ion is used to delocalize or stabilize the emerging negative charge (*3*). In the case of orotidine 5′-monophosphate (OMP), the emergent negative charge is formally in the $sp^2$ orbital at C6 (*4*). The $pK_a$ of the conjugate acid of this vinyl anion has recently been measured as 34−35 (*4*, *5*). Many mechanisms have been proposed over the past 30 years that avoid postulating such an intermediate.

Scheme 1




Beak and Siegel proposed a mechanism for ODCase based on their experimental results using 1,3-dimethylorotic acid as a model compound (*6*). They postulated that a zwitterion could form from protonation of the O2 of the pyrimidine ring, thereby stabilizing the negative charge formed upon decarboxylation. The credibility of a mechanism involving an ylide intermediate diminished when $^{15}N$ kinetic isotope effects (KIEs) demonstrated that there was no change in bond order of the N1 nitrogen during the course of the reaction (*7*). More recently, Lee and Houk, using computational studies of proton affinities of orotate and uracil in the gas phase, reported that protonation at the O4 position of the substrate is more favorable than at the O2 position (*8*). From this, they proposed the formation of a neutral carbene, which they suggest would be stabilized by an active site with a low dielectric constant.

Silverman and Groziak proposed a mechanism in which the Michael addition of an active site nucleophile at the C5

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* To whom correspondence should be addressed. E-mail: cleland@enzyme.wisc.edu. Phone: (608) 262-1373. Fax: (608) 265-2904.

‡ University of Wisconsin.

§ University of North Carolina.

|| Present address: Department of Chemistry and Biochemistry, The Florida State University, Tallahassee, FL 32360.

⊥ Present address: Department of Biochemistry, Brandeis University, Waltham, MA 02454.

[1] Abbreviations: OMP, orotidine 5′-monophosphate; UMP, uridine monophosphate; IE, isotope effect; ODCase, orotidine 5′-monophosphate decarboxylase; BMP, 6-hydroxyuridine 5′- monophosphate; 6-aza-UMP, 6-azauridine monophosphate.

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position would produce a covalent enzyme−substrate intermediate that could undergo acid−base catalysis to form the product (*9*). The basis for this mechanism was the observation that orotic acid analogues are susceptible to nucleophilic attack at C5. This proposal was later ruled out when Acheson and co-workers demonstrated a lack of any secondary deuterium isotope effect with the C5 hydrogens (*10*). Additionally, the authors observed no change in the $^{13}C$ NMR spectrum when using the potent inhibitor 6-hydroxyuridine 5′-phosphate (BMP) with a $^{13}C$ label at C5.

X-ray structures[2] of ODCase from several different species solved with BMP, UMP, or 6-aza-UMP, bound in the active site, showed a substantial number of favorable contacts between the phosphoryl, the ribofuranosyl, and the pyrimidine moieties and the enzyme (*4*, *11*, *12*). The phosphoribosyl group has up to 10 hydrogen bonds with the enzyme, giving rise to a binding energy for stabilization of the transition state of 16.6 kcal/mol (*13*). The contributions to this value by individual contacts of the phosphoribose moiety to the enzyme have been dissected. It has been shown that the contacts made by the 2′-OH and 3′-OH of the phosphoribose moiety with the enzyme contribute to the binding energy of the transition state, but have little to add to the binding energy of the ground state (*13*). The 2′-OH has a bifurcated hydrogen bond to Thr100 and Asp96 (yeast numbering), both of which are contributed from the opposite monomer. Removal of the 2′-OH from the substrate results in a 2250-fold reduction in $k_{cat}/K_m$ in comparison to that of OMP with ODCase. The 3′-OH is also hydrogen bonded to two residues, Lys59 and Asp37. The D37A mutation resulted in only a 300-fold decrease of the $k_{cat}/K_M$, while the mutation of Lys59 to alanine resulted in a decrease of $k_{cat}/K_M$ by over 5 orders of magnitude (*14*).In comparison to the phophoribosyl moiety, the crystal structures show relatively few hydrogen bonds to the pyrimidine ring. In several structures a glutamine forms a hydrogen bond to O2, N3 hydrogen bonds with a hydroxyl from either serine or threonine, and O4 forms a hydrogen bond with a backbone nitrogen. On the basis of the presence of these hydrogen-bonding interactions and lack of proton donors, mechanisms involving protonation at O2 and O4 seem unlikely. The structures also lack the presence of a suitable nucleophile near C5, which eliminates the covalent mechanism as a possibility (*4*, *11*, *12*, *15*).

In addition to the hydrogen bond network, the presence of a highly charged network was discovered near the C6 of the substrate in the active site. This network is composed of Lys93, Asp91, Lys59, and Asp96 (opposite monomer), with Lys93 located between the two aspartate residues. The residues of the charge network are interconnected by

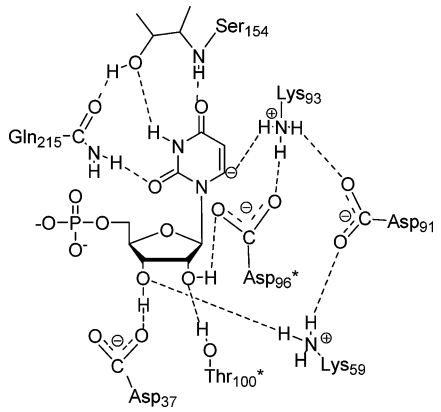



FIGURE 1: Active site showing the configuration of residues around the putative anionic intermediate (numbering based on the yeast sequence). An asterisk indicates the residue is from the second subunit.

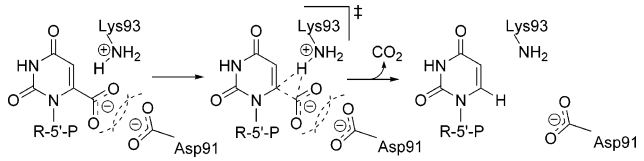
hydrogen-bonding interactions, and the loss of one member can have large effects on the functionality of the charge network (*4*, *11*, *12*, *15*). Mutants K93A, D91A, D96A, and K59A resulted in enzymes with activities reduced by at least 5 orders of magnitude as compared to that of the wild-type ODCase (*14*). Interestingly, the positively charged $\epsilon$-amino group of K93 lies adjacent to C6, suggesting its role in both stabilizing the formation of negative charge as decarboxylation proceeds and protonation of C6 (*16*, *17*).

When intact substrate is modeled into the active site of *Methanobacterium thermoautotrophicum* ODCase, it was found that a substantial amount of electrostatic repulsion and spatial overlap should occur between the carboxyl group of OMP and Asp70 (Asp91 in yeast numbering). This repulsion, coupled with the alternating conserved charges led Wu et al. to analyze the ground-state destabilization and transition-state stabilization contributions to catalysis (*12*). On the basis of these calculations, it was concluded that most of the energy that drives this reaction is from ground-state destabilization. They proposed that decarboxylation is accompanied by an enzyme conformational change and the resulting carbanion intermediate has its negative charge located further away from Asp70 (Asp91 yeast numbering). A more recent X-ray structure was determined of the K72A and D70A (K93 and D91 in yeast number) ODCase double mutant with OMP bound (*18*). This structure showed an OMP molecule where the carboxyl group attached to C6 was bent 14° below the plane of the pyrimidine ring, viewed with C6 on the right. The bending of the bond in this fashion is interpreted to be a result of the repulsive substrate carboxylate−enzyme interaction, causing a weakening of the bond. The multiple favorable hydrogen bond contacts of the phosphorybosyl moiety enable it to act as an anchor, ensuring the interaction of the substrate carboxylate and the enzyme.

On the basis of their crystal structure, Appleby and co-workers have recently proposed a bimolecular electrophilic substitution mechanism (Scheme 2) whereby C6 of the substrate undergoes protonation as decarboxylation occurs (*4*, *19*). They suggest that as soon as the C−C bond between C6 and the leaving carboxylate becomes sufficiently basic a proton can be transferred from the adjacent Lys93; thus, protonation and decarboxylation occur in a concerted manner. Although the mechanism avoids proposing a high-energy vinyl anion intermediate, it is disputed by a previous study

[2] Recently published papers have described crystallographic structures presenting evidence for two novel reactions found to occur in the ODCase active site. It was first discovered that the inhibitor 6-cyano-UMP was converted to the more potent inhibitor BMP during cocrystallization of the enzyme and 6-cyano-UMP [Fujihashi, M., Bello, A. M., Poduch, E., Wei, L., Annedi, S. C., Pai, E. F., and Kotra, L. P. (2005) *J. Am. Chem. Soc. 127*, 15048]. In a similar event, 6-iodo-UMP was found to form a covalent adduct to the $\epsilon$-amino group Lys93 (yeast numbering) of the enzyme, with the release of the iodo moiety, during cocrystallization. The relevance of these two reactions to the suggested mechanisms of ODCase action is not known at this time [Bello, A. M., Poduch, E., Fujihashi, M., Amani, M., Li, Y., Crandall, I., Hui, R.; Lee, P. I., Kain, K. C., Pai, E. F., and Kotra, L. P. (2007) *J. Med. Chem. 50*, 915].

Scheme 2




that shows that a proton is not transferred during the C−C bond-breaking step (*20*).

The goal of the research presented in this paper has been to address the presence of an anionic intermediate through the use of kinetic isotope effects using the alternate substrate 5-fluoro-OMP. Here we also report results of kinetic isotope effect experiments performed using the slow substrate 2′-deoxy-OMP and a K59A mutant ODCase.

## EXPERIMENTAL PROCEDURES

*Materials.* Orotic acid, 5-fluoroorotic acid, 2′-deoxyuridine monophosphate, potassium cyanide, orotate phosphoribosyl transferase, inorganic pyrophosphatase, MES, MOPS, and Amberlite IR120 were from Sigma-Aldrich. Dowex AG1-X8 was from BioRad. Preparation of ODCase and the K59A mutant was described previously (*14*). All other compounds were of the highest purity available.

*Synthesis of Orotidine 5′-Monophosphate and 5-Fluoro-orotidine 5′-Monophosphate.* Orotate (5-fluoroorotate) (68 mg) and 5′-phosphorybosyl-2′-pyrophosphate (200 mg), in a 1.1:1 mole ratio, were dissolved in 35 mL of 100 mM Tris containing 50 mM $MgCl_2$ at pH 8.0. To this were added 75 units of orotate phosphoribosyl transferase and 150 units of inorganic pyrophosphatase, followed by incubation of the solution at 30 °C in a water bath. After 6 h, an additional 25 units of the transferase and 100 units of pyrophosphatase were added, and the solution was incubated overnight. The following day, the enzyme was removed from the solution using an Amicon stirred cell device with a YM10 filter (Millipore, Billerica, MA). The filtered OMP (5-fluoro-OMP) solution (∼50 mL) was diluted to 750 mL with deionized $H_2O$, loaded onto a Dowex AG1-X8 anion-exchange column (2.5 × 30 cm, $Cl^-$ form), and washed with 200 mL of deionized $H_2O$. The column was eluted with a 4 L gradient of 0−500 mM $NH_4HCO_3$ for OMP and a gradient of 400−650 mM $NH_4HCO_3$ for 5-fluoro-OMP. Two large peaks were eluted from the column, with OMP (5-fluoro-OMP) located in the second peak as determined by UV−vis spectroscopy. The $\lambda_{max}$ for OMP and 5-fluoro-OMP are 266 and 272 nm, respectively. Fractions containing OMP (5-fluoro-OMP) were pooled and evaporated to a small volume. Residual $NH_4HCO_3$ was removed by addition of Amberlite IR120 ($H^+$ form), until no further evolution of gas was observed. The resin was removed by filtration and the resulting solution titrated to pH 7 with $NH_4OH$ and evaporated to dryness.

*Synthesis of 2′-Deoxyorotidine 5′-Monophosphate.* 2′-Deoxy-OMP was prepared as reported by Miller et al. (*14*). 2′-Deoxy-UMP was converted to 5-bromo-2′-deoxy-UMP with $Br_2$. The brominated product was converted to its proton form using Dowex ($H^+$) resin and brought to pH 7 with tetraethylammonium hydroxide. Under anhydrous conditions, the vacuum-dried (48 h) syrup was converted to 6-cyano-2′-deoxy-UMP with KCN in the presence of 18-crown-6. Basic hydrolysis yielded 2′-deoxy-OMP. The purification procedure was modified from the literature procedure as follows. The isolated compound (355 μmol) was purified on an AG-1 ($^-$OH form) resin column (2.5 × 30 cm) with a gradient of 0.1−0.4 M LiCl (5 L). The desired fractions, as determined by UV absorption, were pooled and lyophilized. The precipitate was washed several times (3 × 100 mL) with acetone/methanol (95:5) to dissolve excess LiCl. The solid was dissolved with sufficient $H_2O$, and then acetone/methanol was added to precipitate pure 2′-deoxy-OMP. UV: $\lambda_{max}$ 265 nm. $^1H$ NMR ($D_2O$, 200 MHz): 5.94 (dd, 1H, 1′-H), 5.69 (s, 1H, 5-H), 4.54 (m, 1H), 4.05−3.95 (series of m, 3H), 2.90 (m, 1H), 2.26 (m, 1H) ppm.

*Kinetic Assays.* The rate of decarboxylation of OMP or 5-fluoro-OMP by ODCase or the K59A mutant was determined by measuring the decrease in absorbance at 285 nm for OMP or 290 nm for 5-fluoro-OMP using a Cary 300 UV−vis spectrophotometer (Varian Inc., Palo Alto, CA) with extinction coefficients of $\Delta\epsilon_{285} = -1650\ M^{-1}\ cm^{-1}$ or $\Delta\epsilon_{290} = -750\ M^{-1}\ cm^{-1}$. To permit accurate determination of the kinetic parameters for this substrate, reactions carried out at concentrations less than 15 μM necessitated the use of 5 cm cylindrical cuvettes to increase the sensitivity, and those carried out at concentrations greater than 0.25 mM necessitated the use of a 0.1 cm path length. Steady-state kinetic parameters were determined at 25 °C in 1−3 mL volumes containing 25 mM MOPS, pH 7.2, 5 mM DTT, and varying concentrations of 5-fluoro-OMP (2−168 μM)/ODCase, 5-fluoro-OMP (0.25−4 mM)/K59A-ODCase, OMP (70−135 μM)/ODCase, and OMP (0.28−5 mM)/K59A-ODCase. The assays were initiated by addition of enzyme. Initial velocities were used to determine the specific activity at each concentration and were fitted to the Michaelis−Menton equation using the program HYPERO (*21*).

*$^{13}C$ Isotope Effects with Either wt-ODCase or K59A-ODCase.* For high-conversion reactions ∼10 μmol of substrate was dissolved in 3 mL of 100 mM MES containing 5 mM DTT at pH 6.5 in a sealed flask with a side arm and stopcock. The solution was sparged with nitrogen gas at room temperature for 3 h, and then 100 μL of wt-ODCase (44 μM) was added via an airtight needle and syringe to the solution. A second addition of 100 μL of enzyme was made after 6 h, and the reactions were incubated at room temperature overnight. A further 50 μL of wt-ODCase was added, and the reactions were incubated for 30 min. The reactions were quenched with the addition of 300 μL of concentrated $H_2SO_4$, releasing the $CO_2$ produced. $CO_2$ was distilled through two dry ice/2-propanol traps (−128 °C) and one liquid nitrogen trap (−196 °C) on a high-vacuum line and collected in a sample chamber using liquid nitrogen. The ratio of $^{12}C$ to $^{13}C$ in the gas was determined using a Finnegan MAT Delta E isotope ratio mass spectrometer. The remaining solution was checked for unreacted substrate by enzymatic assay to confirm that the reaction went to 100% completion. When ODCase (5 μL) was added to a solution of neutralized sample (MES, 100 mM, pH 6.5 DTT, 5 mM), no change in absorbance was observed at 285 or 290 nm, indicating the absence of OMP or 5-fluoro-OMP.

Low-conversion reactions containing ∼ 20 μmol of substrate were prepared as above. The reactions were initiated by addition of either wt-ODCase or the K59A-ODCase by a needle and syringe. The reactions were quenched with 300 μL of concentrated $H_2SO_4$ after an experimentally determined

Table 1: Kinetic Parameters for 5-Fluoro-OMP and OMP

| enzyme | substrate | $k_{cat}$, $s^{-1}$ | $K_m$, $\mu M$ | $k_{cat}/K_m$, $M^{-1}\ s^{-1}$ |
|---|---|---|---|---|
| wt | OMP[a] | 44 | 0.7 | $6.3 \times 10^7$ |
| wt | OMP[b] | 19 | 1.5 | $1.27 \times 10^7$ |
| wt | OMP[c] | 22 | nd[d] | nd[d] |
| wt | 5-fluoro-OMP[b] | 570 | 56 | $1.02 \times 10^7$ |
| wt | 5-fluoro-OMP[c] | 242 | 8.2 | $3.0 \times 10^7$ |
| wt | 2-deoxy-OMP[a] | 0.15 | 5.4 | $2.8 \times 10^4$ |
| K59A | OMP[c] | 0.52 | 730 | $7.1 \times 10^2$ |
| K59A | 5-fluoro-OMP[c] | 29 | 410 | $7.1 \times 10^4$ |

[a] From ref *14*. [b] From ref *22*. [c] Present work. The enzyme used in this work appears to have been ~50% active. [d] Not determined.

amount of time. The $CO_2$ produced during the reaction was distilled and analyzed as above. To determine the fraction of reaction, the reactions containing OMP or 5-fluoro-OMP were titrated to pH 7. An aliquot of this solution was assayed as above, and the overall change in absorbance was used to determine the amount of OMP or 5-fluoro-OMP present. For reactions containing 2′-deoxy-OMP, an aliquot of the reaction before quenching was removed and the absorbance at 285 nm was immediately measured. The fraction of reaction was determined by comparing this absorbance to that of an equal-volume aliquot of the initial reaction solution, since this compound is labile in acid.

*Data Analysis.* The isotope effect for each substrate/enzyme pair was calculated from the natural abundance of $^{13}C$ in high-conversion samples ($R_{100}$) and low-conversion samples ($R_p$) using the following equation:

$$\text{isotope effect} = \frac{\log(1-f)}{\log\left[1 - f\left(\frac{R_p}{R_{100}}\right)\right]}$$

where $f$ is the fraction of reaction, determined as described above.

## RESULTS

*Kinetic Parameters for wt-ODCase and K59A-ODCase.* The kinetic parameters for the enzymatic decarboxylation of substrate to product, determined in this and previous studies, are listed in Table 1. In the present study, kinetic assays were performed to measure the activity of ODCase for 5-fluoro-OMP, and results similar to those first reported by Shostak and Jones for wt-ODCase from yeast (*22*) were obtained. Both results clearly showed that ODCase is able to utilize this substrate at a faster rate than OMP at saturating substrate concentrations, but with lower efficiency, as determined by $k_{cat}/K_m$. The same result is true in reactions catalyzed by the K59A mutant where decarboxylation of 5-fluoro-OMP occurs 60 times faster than that with OMP as the substrate at saturating substrate concentrations. The loss of efficiency in the wt-ODCase-catalyzed reaction with 5-fluoro-OMP is a result of the increased $K_m$ that is ~10-fold greater than that of OMP. Interestingly, the value of $k_{cat}$ for 5-fluoro-OMP as catalyzed with the mutant enzyme is almost as high as the $k_{cat}$ for the conversion of OMP catalyzed by wt-ODCase, although the $K_m$ is almost 600-fold higher.

*$^{13}C$ Isotope Effects with Either wt-ODCase or K59A-ODCase.* A summary of kinetic isotope effects determined in the present study is listed in Table 2. The $^{13}C$ kinetic isotope effects determined in this study for OMP and ODCase are comparable to those determined by Smiley et al. (*23*). In that study, kinetic isotope effects of 2.5% and 2.7% at pH 6.8 and 6.0, respectively, were observed. These values bracket the value of 2.6% at pH 6.5 determined in the present study. The isotope effect on the decarboxylation of OMP by K59A was 1.054, which is in good agreement with the value of 1.049 at pH 4.0 determined by Smiley et al. with the wild-type enzyme and is presumably the intrinsic isotope effect for the decarboxylation of OMP (*23*). The reaction of the alternate substrate 2′-deoxy-OMP catalyzed by ODCase produced an isotope effect of 1.046, close to the intrinsic value obtained for OMP. An interesting result obtained in this study is the reduction of the isotope effect from 2.6% to 1.0% upon switching from OMP to the faster substrate 5-fluoro-OMP in reactions catalyzed by ODCase. This is a decrease in the isotope effect of ~1.5%, which is similar to the decrease of 1.9% for the two substrates in reactions catalyzed by the K59A mutant enzyme.

Table 2: $^{13}C$ Kinetic Isotope Effects for ODCase and K59A-ODCase[a]

| enzyme[b] | substrate | $^{13}(V/K) \pm$ SE | $n$[c] |
|---|---|---|---|
| wt | OMP | $1.0255 \pm 0.0005$ | 5 |
| wt | 5-fluoro-OMP | $1.0106 \pm 0.0001$ | 4 |
| wt | 2-deoxy-OMP | $1.0461 \pm 0.0005$ | 6 |
| K59A | OMP | $1.0543 \pm 0.0002$ | 6 |
| K59A | 5-fluoro-OMP | $1.0356 \pm 0.0001$ | 5 |

[a] In 100 mM MES, 5 mM DTT (pH 6.5) at 23 °C. [b] Wild-type (wt) or mutant ODCases. [c] Number of determinations.

## DISCUSSION

The latest proposed models for the mechanism of ODCase are based on transition-state stabilization and ground-state destabilization and have key roles for the conserved quartet of charged residues. Asp91 and Asp96 provide steric and electronic repulsion to the substrate's carboxylate group. This point is emphasized in the crystal structure of ODCase where, even in the less crowded double mutant D91A/K93A, there is a strained C6−C7 bond in OMP with the carboxyl group bent out of the plane of the pyrimidine ring and away from Lys93 (*18*). This positioning enables Lys93 to interact preferentially with the negative charge forming on C6 in the transition state. A measure of the destabilization can be determined from evaluating the extremely tight binding of inhibitor BMP, whose $-O^-$ replaces OMP's C6-carboxylate group and places the charge closer to where it resides in the transition state for decarboxylation. The energy of destabilization (10 kcal/mol) of OMP can be estimated by comparing the $K_i$ ($10^{-11}$ M) of BMP and the $K_m$ ($10^{-6}$ M) of OMP (*24*). Lys93 provides transition-state stabilization by placing a positive charge close to the developing negative charge of the carbanion intermediate. Thus, both transition-state stabilization and ground-state destabilization accelerate this enzymatic reaction.

Previously, a multiple isotope effects study was performed to determine whether a protonation occurred concertedly with decarboxylation for the ODCase reaction (*20*). $^{13}C$ isotope effects measured were $1.043 \pm 0.003$ in $H_2O$ and 1.034 in $D_2O$. The $^{13}C$ isotope effects did not increase when the

solvent for the reaction was replaced with $D_2O$, showing that no protonation occurs as the C−C bond is cleaved. If protonation had occurred concertedly, then the bond-breaking step would become more rate limiting, raising the observed $^{13}C$ isotope effects (*25*). In the same study, the deuterium solvent isotope effect measured was 1.3 ± 0.2. While this small effect was attributed to a protonation important to the chemical mechanism, it was pointed out that there were alternative interpretations such as a solvent-sensitive conformational change prior to the catalysis. Since O2 and O4 preprotonation now has been ruled out on the basis of X-ray structure data, this second suggestion seems most likely. The *V*/*K* pH profiles showing two p$K_a$ values, 6.5 and 7.5, can be attributed to the deprotonation and protonation of enzymic residues Asp96 and Lys93, respectively. Therefore, the reaction must be stepwise, with the protonation of C6 occurring after the irreversible loss of $CO_2$ (*20*). Utilizing the information now known about ODCase, a minimal kinetic model can be written:

$$E + O \rightleftarrows EO \rightarrow EU^- + CO_2 \xrightarrow{H^+} E + U$$

where O is OMP, EO is the Michaelis complex, and $EU^-$ is the deprotonated vinyl anion of UMP. This vinyl anion is stabilized by the positive charge on Lys93.

The H-bonds to the conserved residues Lys59 and Asp96 have been shown to be important. Removal of the 2′-OH group of OMP results in a loss of the two H-bonding interactions between T100 and D96. This loss has a profound effect on substrate turnover, which is dependent on dimerization of enzyme subunits (*13*). Since both D96 and T100 are contributed by the opposite subunit, their interaction with the 2′-OH of OMP is important for enzyme dimerization.

The effect of the K59A mutation is more profound on catalysis. The replacement of this lysine with alanine results in a decrease in binding energy by 6.9 kcal/mol (*13*). The overall effect of this mutation is reduced with 5-fluoro-OMP as a substrate in place of OMP. The change in substrate reduces the decrease in binding energy by nearly half, from 6.9 to 3.1 kcal/mol. In addition, with the replacement of lysine with alanine the charge network goes from being charge neutral to being negatively charged (*26*). This results in a reduction in the enzyme's affinity for OMP. When H-bonds to key enzyme residues are disrupted using either the slow substrate analogue 2′-deoxy-OMP or the K59A mutant, the nearly intrinsic or the intrinsic $^{13}C$ isotope is measured (1.046 or 1.054). The $k_{cat}$ values for these reactions are 200−300 times slower than that of OMP with ODCase, and the chemistry is rate limiting.

The observed kinetic isotope effect in an enzyme-catalyzed reaction is indicative of both the structure of the transition state for the isotopically sensitive step and the magnitude of the commitments. Using the following formula, the commitment to catalysis for OMP can be measured:

$$\text{observed IE} = \frac{\text{intrinsic IE} + c_f}{1 + c_f}$$

$$1.0255 = \frac{1.0543 + c_f}{1 + c_f}$$

This yields a $c_f$ equal to 1.13 and indicates the substrate is just as likely to dissociate as to continue along the reaction pathway.

The following equation shows the commitment for 5-fluoro-OMP, assuming the value with the K59A mutant is the intrinsic one:

$$1.0106 = \frac{1.0356 + c_f}{1 + c_f}$$

yielding a $c_f$ equal to 2.36, so the reaction is twice as likely to proceed than to dissociate the substrate. The intrinsic $^{13}C$ isotope effect with 5-fluoro-OMP of 3.56% vs a value of 5.43% with OMP suggests an earlier transition state, likely resulting from the inductive effect of the fluorine, which stabilizes the carbanion intermediate by electron withdrawal and increases the exothermicity of the reaction.[3] The $k_{cat}$ for the reaction with 5-fluoro-OMP is 2.5 times faster than that for OMP with wt-ODCase and 60 times faster with the K59A mutant. In fact, the fluorine substitution gives a $k_{cat}$ with the K59A mutant that is almost as high as that of OMP and the wild-type enzyme. Presumably, 5-fluoro-OMP does not require a full catalytic tetrad because anion stabilization is an intrinsic property of this substrate. Gas-phase calculations on the acidity of $\beta$-fluorovinyl anion were similar to the experimentally determined acidity of α-fluorovinyl anion, and both are ∼22 kcal more acidic than ethylene anion (*27*). Accordingly, the $k_{cat}$ for 5-fluoro-OMP with ODCase could be up to $10^4$ times faster than with OMP only if the reaction is solely dependent on the p$K_a$ of the conjugate acid of OMP or 5-fluoro-OMP. Other factors, such as solvation in the active site, influence the rate enhancements. These results all support a mechanism with a carbanion intermediate stabilized by the positive charge of Lys93. The recent finding that the enzyme catalyzes the exchange of the hydrogen at C6 of UMP also supports the presence of a carbanion intermediate in the reaction (T. Amyes and J. Richard, personal communication)

Taking these results in combination with those previously reported, one can conclude that the mechanism of this enzyme is fairly well understood. Charge repulsion and out of plane bending of the carboxyl (as seen by X-ray crystallography) produces ground-state destabilization (5 orders of magnitude, comparing the $K_m$ of OMP and $K_i$ for BMP). In addition, the lysine that protonates the carbanion at C6 provides transition-state stabilization in the same way that the positive charge at N1 stabilizes the negative charge in the ylide intermediates of nonenzymatic reactions of this type (*6*, *28*).

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[3] The $^{13}C$ isotope effect on a decarboxylation is smaller for early transitions states and increases as the transition state becomes later. This is shown clearly by the change in the $^{13}C$ isotope effect on the formate dehydrogenase reaction from 1.036 to 1.042 as the redox potential of the nucleotide substrate changes from −0.258 V for acetylpyridine NAD to −0.320 V with NAD [Hermes, J. D., Tipton, P. A., Fisher, M. A., O'Leary, M. H., Morrison, J. F., and Cleland, W. W. (1984) *Biochemistry 23*, 6263]. The intrinsic $^{13}C$ isotope effect with prephenate dehydrogenase, which has an early transition state, is only 1.023 [Hermes, J. D., Morrical, S. W., O'Leary, M. H., and Cleland, W. W. (1984) *Biochemistry 23*, 5479].

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