Evidence for a Stepwise Mechanism of OMP Decarboxylase

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Orotidine 5′-monophosphate decarboxylase (ODCase, EC 4.1.1.23) catalyzes the conversion of orotidine 5′-monophosphate (OMP) to uridine 5′-monophosphate (UMP) in the final step of de novo pyrimidine biosynthesis (Figure 1). The enzyme catalyzes the most "proficient" enzymatic reaction described to date,[1] with the enzyme-catalyzed *V*/*K* value exceeding the spontaneous reaction by a factor of 10^{23}. The chemical mechanism of the enzyme-catalyzed reaction, however, has remained enigmatic, and a number of possible mechanisms have been proposed over the years. One mechanistic model for ODCase action invokes protonation of the C2 carbonyl to generate a zwitterionic nitrogen ylide intermediate (Figure 1) upon decarboxylation in a subsequent step.[2] This mechanism is consistent with the very strong inhibition ($K_i \approx 10^{-11}$ M) of ODCase by 1-β-D-ribofuranosylbarbituric acid 5′-phosphate.[3] The magnitude of the primary ^{13}C kinetic isotope effect (KIE) (1.0247 ± 0.0008 at pH 6.8, 25 °C)[4] of the yeast enzyme suggests that decarboxylation is the rate-limiting step.

Recently, Lee and Houk[5] have proposed a novel mechanism for ODCase on the basis of quantum mechanical calculations. They noted that the decarboxylation of C4-keto protonated OMP yields a carbene product that is more stable than the ylide which results from C2-keto protonated OMP. Analysis of gas-phase $\Delta H^{\ddagger}$ and proton affinities supported the suggestion that protonation at the C4-keto oxygen of OMP was more favorable. They further argued that protonation should be concerted with decarboxylation, since the departure of CO_2 would raise the effective pK_a of the C4 ketone such that it could accept a proton from a putative active-site lysine residue[6] with apparent p$K_a \approx 7$.[4]

The measurement of multiple KIEs can be used to distinguish whether an enzymatic reaction occurs via a stepwise or a concerted mechanism.[7] To test the proposal that the decarboxylation of OMP is concerted with protonation of the substrate, the D_2O solvent KIE and ^{13}C primary KIEs in H_2O and D_2O were measured for *Escherichia coli* ODCase[8,9] (Table 1). Solvent KIEs were measured in a standard spectrophotometric assay,[10] and yielded a $^{D_2O}(V/K)$ of 1.3 ± 0.2. Carbon isotope effects on *V*/*K* were determined by measuring the ratio of $^{13}C/^{12}C$ in product CO_2 by isotope ratio mass spectrometry.[11] The ^{13}C kinetic isotope effect is 1.043 ± 0.003 in H_2O, and decreases to 1.034 ± 0.002 in D_2O. This outcome is diagnostic of a mechanism in which decarboxylation and proton transfer[12] occur in separate steps. Proton transfer must occur prior to decarboxylation, since decarboxylation is

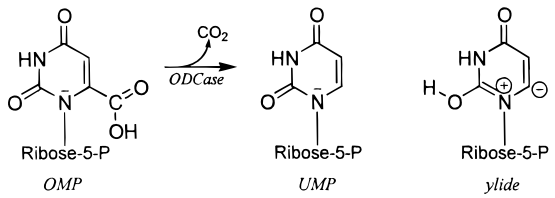

Figure 1. Reaction catalyzed by ODCase, and the putative ylide intermediate.

Table 1. Kinetic Isotope Effects for *E. coli* ODCase

isotope effect		value
$^{13}(V/K)_{H_2O}$	^{13}C KIE in H_2O	1.043 ± 0.003
$^{13}(V/K)_{D_2O}$	^{13}C KIE in D_2O	1.034 ± 0.002
$^{D_2O}(V/K)$	D_2O solvent KIE	1.3 ± 0.2

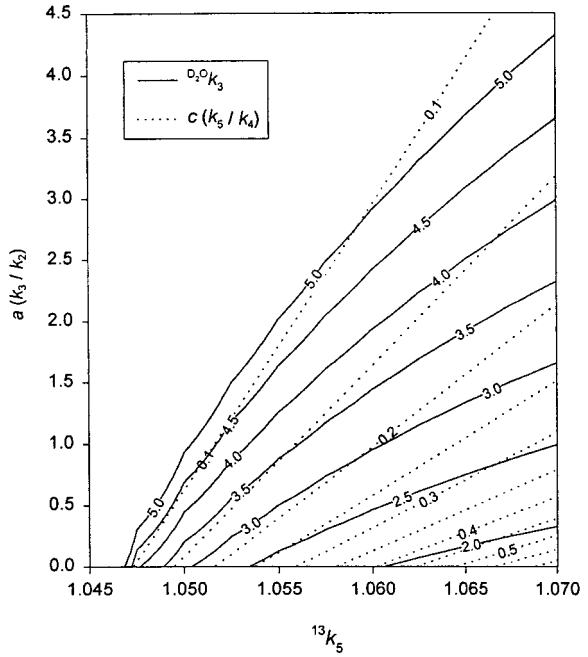

Figure 2. Contour plot representing the set of solutions for eqs 1−3 that satisfy the measured KIEs for ODCase. The axis ranges are abbreviated such that only the chemically reasonable solutions are displayed, as described in the text.

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(2) Beak, P.; Siegel, B. *J. Am. Chem. Soc.* **1976**, *98*, 3601−3606.
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(4) Smiley, J. A.; Paneth, P.; O'Leary, M. H.; Bell, J. B.; Jones, M. E. *Biochemistry* **1991**, *30*, 6216−6223.
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(8) The DNA sequence encoding *E. coli* ODCase (*pyrF* gene) was amplified from genomic DNA by PCR (Perkin-Elmer), digested with *Nde*I and *Bam*H1 (New England BioLabs), and ligated into the expression vector pET23a (Novagen). *E. coli* BL21(DE3) cells containing the resulting pET:pyrF plasmid were cultured in Luria Broth + 50 mg/L carbenicillin at 37 °C until A_{600} = 0.6−0.8. Isopropyl-β-D-thiogalactoside was added to a final concentration of 100 μM, and cells were harvested after an additional 3 to 4 h. The cell pellet was resuspended (1:1 weight:volume) in 20 mM TEA pH 7.8, 1 mM DTT, Complete protease inhibitors (Boehringer-Mannheim), 1 mg/mL lysozyme, frozen at −20 °C overnight and thawed at room temperature. The sample was disrupted by sonication and centrifuged. Streptomycin sulfate (pH 8) was added to the supernatant to a final concentration of 1% to precipitate nucleic acids. Following centrifugation, the supernatant (approximately 100 mL) was dialyzed 3−4 h against 12 L 20 mM TEA pH 7.8, 1 mM DTT, 50 mM NaCl, and ultracentrifuged (30K rpm). ODCase in the resulting supernatant was then purified to homogeneity by fast protein liquid chromatography (FPLC) on FastQ anion-exchange and phenyl-sepharose hydrophobic interaction columns (Pharmacia).

(9) *E. coli* ODCase was inactivated (90%) by overnight dialysis against 0.5 mM EDTA. This is consistent with the recent suggestion (Miller, B. G.; Traut, T. W.; Wolfenden, R. *J. Am. Chem. Soc.* **1998**, *120*, 2666−2667) that the enzyme requires a metal cofactor.

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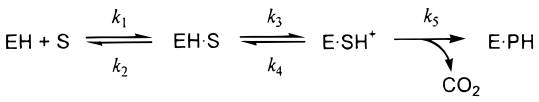
irreversible, and subsequent steps are not included in the expression for *V*/*K*. A minimal mechanism is thus



where substrate binding (k_1, k_2) is followed first by reversible substrate protonation (k_3, k_4),[13] and finally by irreversible decarboxylation (k_5). The isotope effects for this mechanism are described by the following equations:

$$^{D_2O}(V/K) = \frac{^{D_2O}k_3 + a + {}^{D_2O}K_{eq}/c}{1 + a + 1/c} \quad (1)$$

$$^{13}(V/K)_{H_2O} = \frac{^{13}k_5 + c(1 + a)}{1 + c(1 + a)} \quad (2)$$

$$^{13}(V/K)_{D_2O} = \frac{^{13}k_5 + c\left(^{D_2O}k_4 + a/^{D_2O}K_{eq}\right)}{1 + c\left(^{D_2O}k_4 + a/^{D_2O}K_{eq}\right)} \quad (3)$$

$$\frac{^{13}(V/K)_{H_2O} - 1}{^{13}(V/K)_{D_2O} - 1} = \frac{^{D_2O}(V/K)}{^{D_2O}K_{eq}} \quad (4)$$

where $^{D_2O}k_3$ and $^{D_2O}k_4$ are the intrinsic D_2O solvent KIEs in the forward and reverse directions, $^{13}k_5$ is the intrinsic ^{13}C KIE, $^{D_2O}K_{eq} = {}^{D_2O}k_3/{}^{D_2O}k_4$ is the D_2O solvent equilibrium isotope effect, $a = k_3/k_2$, and $c = k_5/k_4$.[14] These equations are not independent, and thus a unique analytical solution does not exist. However, the set of solutions can be computed[15] and represented as three-dimensional surfaces.

Inspection of contour plots of c and $^{D_2O}k_3$ over chemically reasonable ranges for $^{13}k_5$ and a (Figure 2) identified an upper limit of 0.63 for c and a lower limit of 1.8 for $^{D_2O}k_3$. When the intrinsic solvent KIE $^{D_2O}k_3$ is restricted to values ≤ 5.0, all solutions with $a \geq 4.3$ and $c \leq 0.10$ are excluded, and $^{13}k_5$ must be at least 1.047. This value compares favorably with the ^{13}C KIE of 1.0494 $\pm$ 0.0006 exhibited by the yeast enzyme at pH 4.0.[4] The limits on these mechanistic parameters are summarized in Table 2.

The multiple isotope effects measured for *E. coli* ODCase are thus consistent exclusively with a stepwise mechanism. As Figure 2 illustrates, only mechanisms in which decarboxylation is highly rate-limiting, such that the protonated intermediate preceding decarboxylation tends to deprotonate between 62 and 91% ($1.6 \leq 1/c \leq 10$) of the time, are possible. Either a partially or barely rate-limiting protonation step is compatible with these results.

Table 2. Mechanistic Parameters for *E. coli* ODCase

	parameter	lower limit	upper limit
a	k_3/k_2	0[m]	4.3[m]
c	k_5/k_4	0.1[m]	0.63[m]
$^{D_2O}k_3$	intrinsic D_2O KIE	1.8[m]	5.0[a]
$^{13}k_5$	intrinsic ^{13}C KIE	1.047[m]	1.07[a]

[a] Assumed constraint based upon chemical considerations. [m] Mathematical limit within the experimental results and chemical constraints.

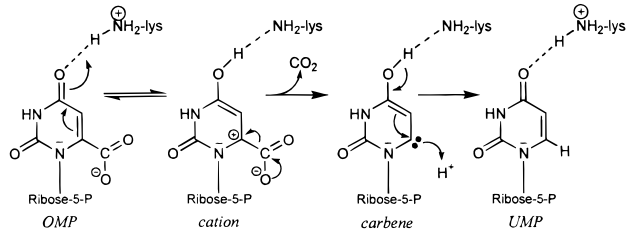


Figure 3. Proposed stepwise mechanism of ODCase, with protonation at O4 followed by decarboxylation to the carbene.

These data suggest a modification of the Houk mechanism[5] for ODCase as shown in Figure 3.[16] In this mechanism, initial C4 keto protonation by an essential enzymatic lysine residue generates the C6 cation. Since protonation concomitant with decarboxylation is excluded experimentally, we suggest that this thermodynamically unfavorable step may instead be promoted by a hydrogen bonding network to the C4 keto oxygen of the orotate moiety. For this substrate, such a network has been observed and implicated in the mechanism of the enzyme which immediately precedes ODCase in the pyrimidine biosynthetic pathway, orotate phosphoribosyl transferase.[17] Decarboxylation of the C6 cation then generates the carbene proposed by Lee and Houk in the rate-limiting step.

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Supporting Information Available: Experimental data for the determination of $^{D_2O}(V/K)$ and $^{13}(V/K)_{L_2O}$; 3-Dimensional and contour plots of c and $^{D_2O}k_3$ vs $\{^{13}k_5, a\}$; a plot of decarboxylation commitment factors vs $^{13}k_5$ (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) (a) ODCase was assayed by monitoring the decrease in A_{280} ($\epsilon = 1350$ M^{-1} cm^{-1}) as OMP is converted to UMP.[10b] Reactions contained OMP, enzyme, 20 mM Hepes pL 7.5, and 0.5 mM DTT at room temperature. (b) Brody, R. S.; Westheimer, F. H. *J. Biol Chem.* **1979**, *254*, 4238−4244.

(11) (a) The method for measuring $^{13}(V/K)$ by collection and isotope ratio analysis of product CO_2 is described by O'Leary.[11b] All reactions contained 2 mM OMP, 20 mM Hepes pL 7.5, and 0.5 mM DTT at room temperature in sealed vacuum flasks, and were sparged overnight with N_2 to remove all contaminating CO_2 prior to addition of enzyme. D_2O was previously sparged with CO_2 in the presence of carbonic anhydrase to exchange excess ^{18}O. Two 100% conversion (20 mL) and three partial conversion (60 mL) reactions were performed in H_2O and D_2O. Fractional conversion of OMP to UMP was monitored in aliquots spectrophotometrically,[10] and reactions were quenched by the addition of concentrated sulfuric acid to pH < 1. Product CO_2 was purified by three cycles of high-vacuum distillation though dry ice−ethanol and liquid N_2 traps prior to analysis by isotope ratio mass spectrometry. (b) O'Leary, M. H. *Methods Enzymol.* **1980**, *64*, 83−104.

(12) While other schemes (e.g., solvent-sensitive conformational change preparatory to decarboxylation) are also consistent with these data, we retain the pre-protonation of substrate model suggested by previous studies.[2−7]

(13) In this derivation, the terms k_3 and k_4 represent "net" rate constants that describe the entire solvent-sensitive portion of the reaction coordinate, which may formally consist of a single or multiple microscopic steps.

(14) The forward commitment factor (partition ratio for the enzyme form competent to undergo decarboxylation), $(k_5/k_4)(1 + k_3/k_2)$, is divided into an internal commitment factor (k_5/k_4) and an external commitment factor (k_5k_3/k_3k_2).

(15) (a) According to eq 4 and the experimental results, $^{D_2O}K_{eq} = 1.03 \pm 0.16$. This suggests that the solvent equilibrium isotope effect is, in fact, unity. This value is not unexpected, since the fractionation factors for N−L and O−L are unity;[15b] therefore, $^{D_2O}K_{eq} = 1$ was used in the calculations. An array of values for $^{13}k_5$ and a, representing the complete range of chemically reasonable solutions, was generated. A minimum value of 1.043 was selected for $^{13}k_5$, corresponding to the observed KIE (the observation of partially rate-limiting D_2O effects requires that $^{13}k_5$ be higher than this). The upper limit given for $^{13}k_5$ was 1.07, approximating the largest known values for enzymatic decarboxylations.[15c,d] The forward commitment factor a was given an initial range of 0 to 5. Equation 2 was used to calculate c for each point in the array, and $^{D_2O}k_3$ was calculated with eq 1 from the resulting $\{^{13}k_5, a, c\}$ array. The final $\{^{13}k_5, a, c, {}^{D_2O}k_3\}$ array satisfied eqs 1, 2, and 3, and yielded values for the forward commitments for decarboxylation smaller than unity, as expected. (b) Quinn, D. M.; Sutton, L. D. In *Enzyme Mechanism from Isotope Effects*; Cook, P. F., Ed.; CRC Press: Boca Raton, Florida, 1991; 73−126. (c) Karsten, W. E.; Gavva, S. R.; Park S. H.; Cook P. F. *Biochemistry* **1995**, *34*, 3253−60. (d) Grissom, C. B.; Cleland, W. W. *Biochemistry* **1988**, *27*, 2927−34.

(16) The Beak and Siegel mechanism[2] with protonation occurring at the C2 carbonyl is also consistent with these results. We elect to interpret the data in the context of the Houk mechanism on the basis of its more favorable predicted thermodynamics.

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