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ARTICLE *in* JOURNAL OF THE AMERICAN CHEMICAL SOCIETY · MAY 2010

Impact Factor: 12.11 · DOI: 10.1021/ja102408k · Source: PubMed

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Published on Web 05/04/2010

Product Deuterium Isotope Effects for Orotidine 5′-Monophosphate Decarboxylase: Effect of Changing Substrate and Enzyme Structure on the Partitioning of the Vinyl Carbanion Reaction Intermediate

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Abstract: A product deuterium isotope effect (PIE) of 1.0 was determined as the ratio of the yields of [6-^{1}H]-uridine 5′-monophosphate (50%) and [6-^{2}H]-uridine 5′-monophosphate (50%) from the decarboxylation of orotidine 5′-monophosphate (OMP) in 50/50 (v/v) HOH/DOD catalyzed by orotidine 5′-monophosphate decarboxylase (OMPDC) from *Saccharomyces cerevisiae*, *Methanothermobacter thermautotrophicus*, and *Escherichia coli.* This unitary PIE eliminates a proposed mechanism for enzyme-catalyzed decarboxylation in which proton transfer from Lys-93 to C-6 of OMP provides electrophilic *push* to the loss of CO_2 in a concerted reaction. We propose that the complete lack of selectivity for the reaction of solvent H and D, which is implied by the value of PIE = 1.0, is enforced by restricted C−N bond rotation of the $-CH_2-NL_3^+$ group of the side chain of Lys-93. A smaller PIE of 0.93 was determined as the ratio of the product yields for OMPDC-catalyzed decarboxylation of 5-fluoroorotidine 5′-monophosphate (5-FOMP) in 50/50 (v/v) HOH/DOD. Mutations on the following important active-site residues of OMPDC from *S. cerevisiae* have no effect on the PIE on OMPDC-catalyzed decarboxylation of OMP or decarboxylation of 5-FOMP: R235A, Y217A, Q215A, S124A, and S154A/Q215A.

Introduction

Orotidine 5′-monophosphate decarboxylase (OMPDC) is a remarkable enzyme because it employs no metal ions or other cofactors, yet it effects an enormous ca. 30 kcal/mol stabilization of the transition state for the decarboxylation of orotidine 5′-monophosphate (OMP) to give uridine 5′-monophosphate (UMP, Scheme 1).[1−3] A significant fraction of this rate acceleration is obtained from utilization of the intrinsic phosphate binding energy in stabilization of the decarboxylation transition state.[4,5] The electrophilic substitution of $-CO_2$ by a proton may proceed by two reaction steps or stages: loss of CO_2 to form the putative vinyl carbanion reaction intermediate and protonation of this carbanion by an acidic catalytic residue to form UMP. It has also been suggested that these two steps might be coupled in a concerted electrophilic substitution reaction.[6−8]

OMPDC is assayed in the direction of decarboxylation of OMP, and mechanistic studies have naturally focused on the loss of CO_2 from substrate, which is the first stage in a stepwise reaction mechanism. The second stage of proton transfer between the enzyme and substrate has received much less consideration in experimental and theoretical studies.[2,9] This strong focus on the first reaction stage has resulted in a significant, but unappreciated, gap in our understanding of the enzymatic reaction mechanism, which can only be fully defined by studies that encompass the whole reaction.

Loss of CO_2 and the addition of a proton to carbon-6 of OMP may proceed by the fully stepwise reaction mechanism shown in Scheme 1, or by a concerted mechanism that avoids the formation of the vinyl carbanion reaction intermediate. The latter was suggested for OMPDC-catalyzed decarboxylation, in order to provide a mechanism for avoiding formation of the unstable vinyl carbanion intermediate, while at the same time providing for transition-state stabilization from electrophilic push by the transferred proton.[6−8] This distinction between stepwise and concerted reaction mechanisms for proton transfer from enzyme to substrate can only be made by a direct study of the OMPDC-catalyzed proton-transfer reaction.

The product deuterium isotope effect (PIE, Scheme 2), determined directly as the yield of products of reaction of -H

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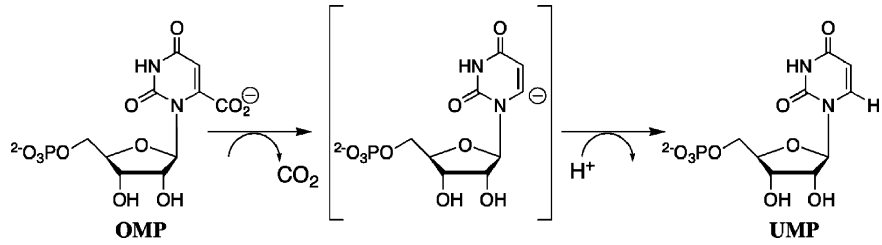
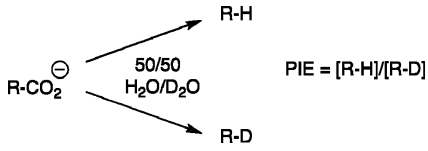
Scheme 1



Scheme 2



and of -D in 50/50 HOH/DOD, provides a measure of the relative electrophilic push of the transferred -H and -D at the transition state for chemical and enzymatic reactions. We have determined PIEs as large as 8 for thermoneutral proton transfer from carboxylic acids to ring-substituted α-methoxystyrenes, where there is apparently the full loss of zero-point energy at the transferred hydron at the rate-determining transition state.[10,11] The PIE decreases below this maximum value for either thermodynamically favorable or unfavorable proton-transfer reactions.[11−13] PIEs of 1.0 for nonenzymatic reactions in aqueous solution are almost never observed[14−18] but are possible for a complex reaction mechanism where proton transfer occurs after the step that determines the yields of hydrogen- and deuterium-labeled reaction products. In this case the deuterium enrichment of product will be determined by the initial deuterium enrichment of the catalytic acid.

We recently reported a fast and convenient method to determine the yields of [6-^{1}H]-uridine 5′-monophosphate ([6-^{1}H]-UMP) and [6-^{2}H]-uridine 5′-monophosphate ([6-^{2}H]-UMP) from the decarboxylation of OMP in 50/50 (v/v) HOH/DOD catalyzed by OMPDC. The ratio of the yields of these two products was determined to be equal to the isotopic enrichment of solvent, so that the PIE on this reaction is 1.0.[19] We now report (a) the full experimental details from our earlier preliminary communication and a significant modification of the protocol for ^{1}H NMR analysis of the deuterium enrichment of product UMP; (b) the PIE on OMPDC-catalyzed decarboxylation of 5-FOMP, a substrate that is activated for decarboxylation by the C-5 fluorine; and (c) PIEs on decarboxylation of OMP and 5-FOMP by several site-directed mutants of OMPDC. These results provide strong evidence that the step that determines the yields of H- and D-labeled products is decarboxylation of enzyme-bound substrate, and that protonation of the carbanion intermediate of decarboxylation is faster than steps that would exchange acidic hydrons and allow the intermediate to select between reaction with -H and -D. The observed PIE shows a remarkable insensitivity to changes in substrate reactivity and enzyme catalytic activity.

Experimental Section

Materials. Orotidine 5′-monophosphate trisodium salt (99%) was purchased from Sigma or prepared by chemical methods from uridine 5′-monophosphate using modifications of literature procedures.[20−22] 3-(*N*-Morpholino)propanesulfonic acid (MOPS, ≥99.5%) was purchased from Fluka. Water was from a Milli-Q purification system. Deuterium-labeled water (99.9% D), DCl (35 wt %, 99.9% D), and KOD (40 wt %, >98% D) were from Cambridge Isotope Laboratories. 5-FOMP was prepared by orotate phosphoribosyl transferase-catalyzed coupling of 5-fluoroorotate and 5′-phosphoribosyl-1′-pyrophosphate by following a literature procedure.[23] All other chemicals were reagent grade or better and were used without further purification.

The S155C mutant of OMPDC from *Saccharomyces cerevisiae* (ScOMPDC) was prepared as described in earlier work.[23,24] This enzyme was used as a model for the wild-type enzyme, because the mutant is more stable than but kinetically and structurally essentially identical with wild-type ScOMPDC. OMPDCs from *Methanothermobacter thermautotrophicus* (MtOMPDC) and from *Escherichia coli* (EcOMPDC) were also prepared and purified as described in earlier work.[23,25] The gene for the S155C mutant of ScOMPDC was used as the starting point for preparation of the S154A,[24] Q215A,[24] S154A/Q215A,[24] and R235A[26] mutant enzymes. Similar procedures were followed in preparing the Y217A mutant enzyme of ScOMPDC (see Supporting Information).

NMR Analyses. ^{1}H NMR spectra (ca. 30 transients) were recorded on a Varian Unity Inova-500 spectrometer using a sweep width of 6000 Hz, a 90° pulse angle, an acquisition time of 6 s, and a relaxation delay between pulses of 120 s (>7T_1), with suppression of the water peak. Baselines were subjected to first-order drift corrections before integration of the signals. Chemical shifts are reported relative to HOD at 4.67 ppm. ^{19}F NMR spectra (64 transients) were recorded on a Varian Unity Inova-500 spectrometer using a spectral width of 50 000 Hz, a 90° pulse angle,

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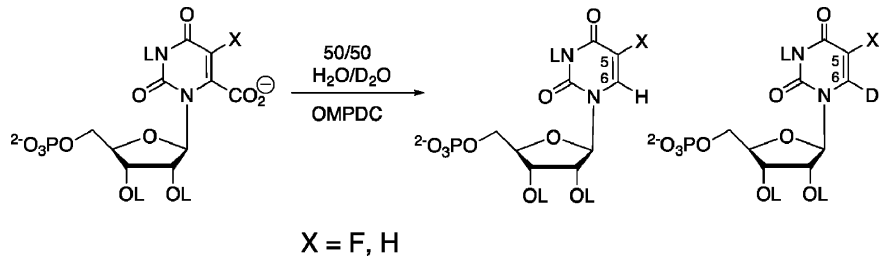
Scheme 3



a 2.6 s acquisition time, and a relaxation delay between pulses of 20 s (>7T_1). The receiver gating time was optimized to obtain a flat baseline. Baselines were subjected to first-order drift correction before integration of the signals. Chemical shifts are reported relative to a neat trifluoracetic acid external standard for which $\delta = -78.5$ ppm.[27]

OMPDC-Catalyzed Decarboxylation. Stock solutions of OMP and 5-FOMP were prepared in 50/50 HOH/DOD, and their concentrations were determined from the absorbance in 0.1 M HCl at 267 nm using $\epsilon = 9400$ and 10 200 M^{-1} cm^{-1} for OMP and 5-FOMP, respectively. The concentration of wild-type ScOMPDC, EcOMPDC, and MtOMPDC in stock solutions was determined from the absorbance at 280 nm using values of $\epsilon = 29\,900$, 10 100, and 6100 M^{-1} cm^{-1},[25] respectively, calculated using the ProtParam tool available on the ExPASy server.[28,29] The concentration of mutant OMPDCs in stock solutions was determined from the absorbance at 280 nm using $\epsilon = 29\,900$ or 28 420 M^{-1} cm^{-1} for the Y217A mutant, calculated using the ProtParam tool. All enzyme assays were carried out at 25 °C in 10 mM MOPS (50% free base) at pH 7.1 and $I = 0.105$ (NaCl). Standard assays described in earlier work were used to determine the activity of OMPDC for decarboxylation of OMP.[24] Decarboxylation of OMP (2 mM) catalyzed by wild-type and mutant OMPDCs at 25 °C was carried out in 50/50 HOH/DOD (2 mL) that contained 50 mM MOPS buffer (50% free base) at pL 7.4 with ionic strength of 0.1 (NaCl). The reactions were initiated by addition of OMPDC (0.01−30 μM). The reaction was allowed to proceed until ca. 95% complete, at which time (x min) an 800 μL aliquot was withdrawn and the enzyme was removed by ultrafiltration using Microcon centrifugal filter units (10K MWCO). After an additional reaction time of x min, a second 800 μL aliquot was withdrawn and treated in the same manner. The filtered samples were frozen until NMR analysis of the deuterium enrichment of the 6-H position. Prior to ^{1}H NMR analyses, the solution pL was adjusted to 9.5−10 using NaOD, in order to increase the resolution of the signals for the 5-H and 1′-H of UMP. The same procedures were followed in studies of OMPDC-catalyzed decarboxylation of 5-FOMP (2 mM), except that the pL was kept at the initial value of 7.4 for ^{19}F NMR analyses.

Results

The yields of H- and D-labeled products from decarboxylation of OMP catalyzed by OMPDC in 50/50 (v/v) HOH/DOD (Scheme 3, X = H) were determined by ^{1}H NMR spectroscopy at 500 MHz. Figure 1A shows a representative partial ^{1}H NMR spectrum at pL 7.4 of UMP obtained from the decarboxylation of OMP (2 mM) catalyzed by ScOMPDC (C155S mutant, 0.024 μM, 200 min reaction time) in 50/50 (v/v) HOH/DOD at pL 7.4 and 25 °C ($I = 0.10$, NaCl).[19] The product deuterium isotope effect of 1.0 (Table 1) was determined as the ratio of the integrated area A_H of the doublet due to the C-6 proton of [6-^{1}H]-UMP at 7.990 ppm and A_D of the collapsed singlet due to the C-5 proton of [6-^{2}H]-UMP at 5.865 ppm (eq 1).[19]

$$\mathrm{PIE} = \frac{[P_H]}{[P_D]} = \frac{A_H}{A_D} \quad (1)$$

The spectrum of UMP at pL 9.7 shows a better resolution of the signals for the C-5 pyrimidine and the C-1′ anomeric hydrogen, because of the effect of partial ionization of N-3 of UMP ($pK_a = 9.45$)[30] on chemical shift. Figure 1B shows a representative partial ^{1}H NMR spectrum, at pL 9.7, of the UMP product of decarboxylation of OMP (2 mM) catalyzed by the R235A mutant of ScOMPDC (13 μM) in 50/50 (v/v) HOH/

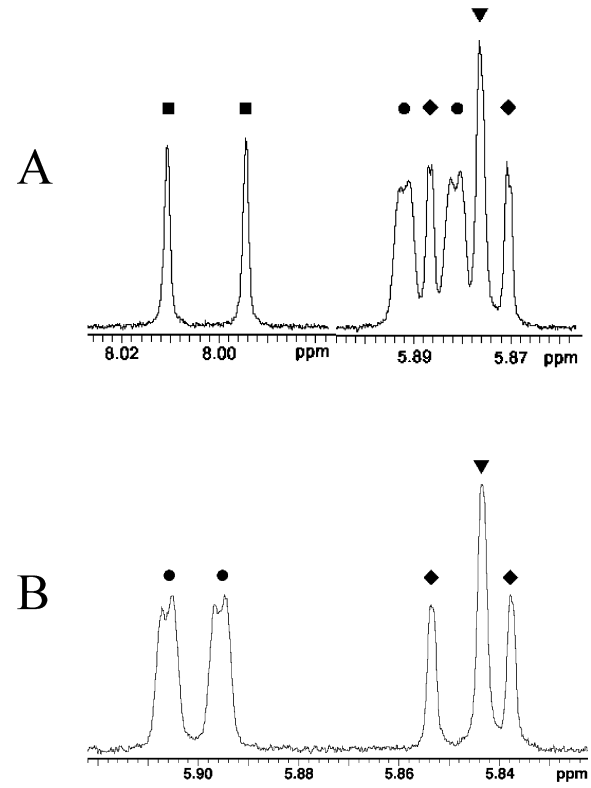


Figure 1. (A) Partial ^{1}H NMR spectrum (500 MHz) at pL 7.4 of UMP from decarboxylation of OMP (2 mM) catalyzed by ScOMPDC (0.024 μM) in 50/50 (v/v) HOH/DOD at 25 °C. Key: (■) doublet due to the C-6 proton of [6-^{1}H]-UMP; (●) double doublets (poorly resolved) due to the anomeric protons of [6-^{1}H]-UMP and [6-^{2}H]-UMP; (◆) doublet due to the C-5 proton of [6-^{1}H]-UMP; and (▼) collapsed singlet due to the C-5 proton of [6-^{2}H]-UMP. (B) Partial ^{1}H NMR spectrum at pL 9.7 of [6-^{1}H]-UMP and [6-^{2}H]-UMP. Key: (●) double doublets due to the anomeric protons of [6-^{1}H]-UMP and [6-^{2}H]-UMP; (◆) doublet due to the C-5 proton of [6-^{1}H]-UMP; and (▼) collapsed singlet due to the C-5 proton of [6-^{2}H]-UMP.

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Table 1. Product Deuterium Isotope Effects for Catalysis of Decarboxylation of OMP and 5-FOMP by Wild-Type and Mutants of Orotidine 5-Monophosphate Decarboxylase[a]

enzyme		k_{cat}/K_m (M^{-1} s^{-1})	substrate	[E]/μM	reaction time (min) [b]	PIE[c]
E. coli	wild-type	6.4×10^5	OMP	0.044	200, 400	1.05 ± 0.04 [d]
M. thermautotrophicus	wild-type	3.3×10^6	OMP	0.042	600, 1200	1.01 ± 0.07 [d]
S. cerevisiae	wild-type	1.1×10^7	OMP	0.024	200	1.0 [d]
				0.47	17, 33	1.02 ± 0.01 [e]
		1.2×10^7	5-FOMP	0.13	8, 16	0.93 ± 0.02 [f]
	R235A	610	OMP	13	15, 30	1.03 ± 0.01 [e]
		1.3×10^5	5-FOMP	0.054	15, 30	0.92 ± 0.04 [f]
	Q215A	2.5×10^5	OMP	0.026	200, 400	1.06 ± 0.01 [e]
		2.6×10^6	5-FOMP	0.010	40, 80	0.95 ± 0.01 [f]
	S154A	630	OMP	28	55, 110	1.02 ± 0.02 [e]
		2.7×10^5	5-FOMP	1.6	5, 10	0.95 ± 0.02 [f]
	S154A/Q215A	380	OMP	12	240, 480	1.04 ± 0.01 [e]
		7.7×10^4	5-FOMP	0.38	150, 300	0.92 ± 0.01 [f]
	Y217A	2500	OMP	0.59	50, 100	1.03 ± 0.02 [e]
		1.3×10^4	5-FOMP	0.40	45, 90	0.93 ± 0.02 [f]

[a] For reactions in 50/50 (v/v) HOH/DOD at pL 7.4 and 25 °C ($I = 0.10$, NaCl). [b] The product yields were first determined after ca. 95% completion of the decarboxylation reaction (see text). [c] The ratio of the yields of the H- and D-labeled products for reactions in 50/50 (v/v) HOH/DOD. In all cases but one, the reported PIEs are the average of the values determined at two different reaction times. The quoted uncertainty gives the range of these two values. [d] The ratio of the area from ^{1}H NMR of the doublet for the C-6 hydrogen of [6-^{1}H]-UMP and of the collapsed singlet for the C-5 hydrogen of [6-^{2}H]-UMP determined from spectral data at pL 7.4 (Figure 1A). [e] The ratio of the area from ^{1}H NMR of the doublet for the C-5 hydrogen of [6-^{1}H]-UMP and of the collapsed singlet for the C-5 hydrogen of [6-^{2}H]-UMP determined from spectral data at pL 9.5−10 (Figure 1B). [f] The ratio of the area from ^{19}F NMR of the doublet for the C-5 fluorine of [6-^{1}H]-UMP and of the singlet for the C-5 fluorine of [6-^{2}H]-UMP (Figure 2).

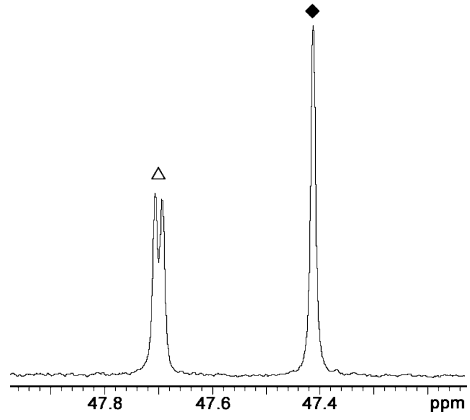

Figure 2. ^{19}F NMR spectrum of 5-FUMP, which is the product of decarboxylation of 5-FOMP catalyzed by R235A ScOMPDC (0.054 μM) at pL 7.4. The reported chemical shifts are relative to a neat trifluoracetic acid external standard for which $\delta = -78.5$ ppm.[27] Key: (Δ) doublet at $\delta = 47.70$ ppm ($J_{HF} = 6$ Hz) due to the fluorine at [6-^{1}H]-5-FUMP; (◆) downfield-shifted singlet at $\delta = 47.41$ ppm due to the fluorine at [6-^{2}H]-5-FUMP.

DOD at pL 7.4 and 25 °C ($I = 0.10$, NaCl) and a reaction time of 30 min. The PIE of $A_H/A_D = 1.03 \pm 0.01$ was calculated from the ratio of the integrated area of the doublet at 5.846 ppm for the C-5 hydrogen of [6-^{1}H]-UMP and of the collapsed singlet at 5.843 ppm for the C-5 hydrogen of [6-^{2}H]-UMP. This PIE is the average of the values determined at two different reaction times, and the quoted uncertainty gives the range of the two experimental values.

Table 1 reports product deuterium isotope effects on the decarboxylation of OMP (2 mM) at pL 7.4 catalyzed by wild-type OMPDC from several different organisms and by several mutant forms of OMPDC from *S. cerevisiae*. These PIEs were calculated as the ratio of the integrated area of the C-5 hydrogen of [6-^{1}H]-UMP and of the C-5 hydrogen of [6-^{2}H]-UMP determined for spectra at pL 9.5−10, similar to the spectrum shown in Figure 1B. There is good agreement (±5%) between the PIEs calculated from spectral data at pL 7.4 (Figure 1A) and at pL 9.5−10. We have used data from NMR spectra determined at the higher pL because of the better resolution of peaks for the relevant protons. Table 1 reports PIEs that are the average of values determined at two reaction times. The first time (x min) was after ca. 95% completion of the decarboxylation reaction. A second analysis at $2x$ min was carried out to verify that the reaction product UMP was stable under the reaction conditions to further OMPDC-catalyzed hydron exchange,[31,32] and to provide an estimate for the uncertainty in these product isotope effects. In every case but one, the agreement between the PIEs determined from the products obtained at these two reaction times is better than ±5%.

Figure 2 shows a representative ^{19}F NMR spectrum (500 MHz) at pL 7.4 of 5-FUMP obtained from the decarboxylation of 5-FOMP (2 mM) catalyzed by R235A mutant ScOMPDC (0.054 μM in 50/50 (v/v) HOD/DOD) at pL 7.4 and 25 °C ($I =$ 0.10, NaCl) and a reaction time of 30 min. Note that the deuterium at C-6 of [6-^{2}H]-5-FUMP perturbs the ^{19}F chemical shift of the C-5 fluorine by 0.29 ppm. The product deuterium isotope effect of 0.92 was determined as the ratio of the integrated area of the doublet at $\delta = 47.70$ ppm ($J_{HF} = 6$ Hz) due to the fluorine at [6-^{1}H]-5-FUMP and of the downfield-shifted singlet at $\delta = 47.41$ due to the C-5 fluorine at [6-^{2}H]-5-FUMP (Scheme 2, X = F).

Table 1 reports the PIEs on decarboxylation of 5-FOMP (2 mM) at pL 7.4 catalyzed by wild-type ScOMPDC and by several mutant forms of ScOMPDC. These PIEs were calculated as the ratio A_H/A_D of the integrated area of C-5 fluorine of [6-^{1}H]-5-FUMP and of the C-5 fluorine of [6-^{2}H]-5-FUMP determined for spectra similar to the spectrum shown in Figure 2. The PIEs reported in Table 2 are the average of values determined at two reaction times. The first time (x min) was after ca. 95% completion of the decarboxylation reaction. A second analysis

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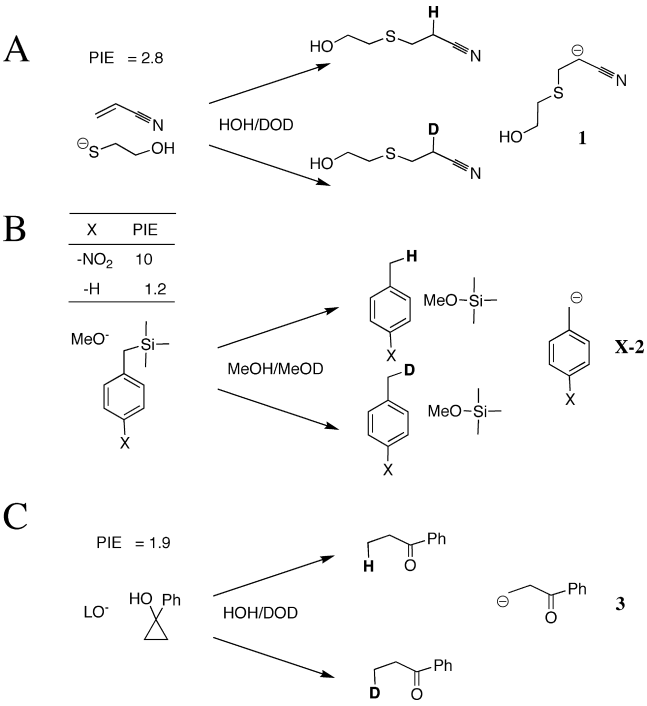
Chart 1



at 2x min was carried out to verify that the product 5-FUMP was stable under the reaction conditions to further OMPDC-catalyzed hydron exchange.[31,32] The agreement between the PIEs determined for the products obtained at these two reaction times is better than ±5%.

Discussion

The product deuterium isotope effect on electrophilic substitution reactions of the proton in aqueous solution depends strongly on the lifetime of the putative carbanion intermediate of the stepwise reaction.[33] A PIE of 2.8 was determined for addition of the $HOCH_2CH_2S^-$ to acrylonitrile in water (Chart 1A).[18] This reaction proceeds through the α-cyano-stabilized carbanion **1**, which shows a modest selectivity for protonation by HOL compared with DOL.[34] The sharp decease from 10 to 1.2 in the PIE on methoxide ion-catalyzed cleavage of substituted benzyltrimethylsilanes in 50/50 MeOH/MeOD (Chart 1B) is due to the large decrease in the selectivity of the strongly resonance-stabilized carbanion **4-NO$_2$-2** compared to the much more unstable unsubsituted benzylic carbanion **H-2**.[17] Finally, the PIE of 1.9 determined for specific base-catalyzed retroaldol cleavage of 1-phenylcyclopropanol reflects the stronger electrophilic push of HOL compared with DOL for C−C bond cleavage by a concerted reaction mechanism. This concerted mechanism is enforced because the putative primary carbanion intermediate **3** of the stepwise mechanism is far too unstable to exist for the time of a bond vibration in water.[33,35]

X-ray crystallographic analysis of the complex between ScOMPDC and the transition-state analogue 6-hydroxyuridine 5′-monophosphate (BMP) shows that the acidic ϵ-NH_3^+ group of the Lys-93 appears to be positioned to protonate the putative C-6 carbanion reaction intermediate of decarboxylation of OMP.[31] The PIE on OMPDC-catalyzed decarboxylation is equal to $1/\Phi_{TS}$ (eq 2, Scheme 4), where Φ_{TS} is the fractionation factor for partitioning of -H and -D between solvent LOL and the ϵ-NL_3^+ group of the Lys-93 at the transition state for decarboxylation.[36−38] The kinetic deuterium isotope effect (KIE = k_{EH}/k_{ED}, Scheme 4) on OMPDC-catalyzed decarboxylation of OMP in 50/50 HOH/DOD is controlled by the fractionation factor Φ_{EL} for partitioning of -H and -D between solvent L-OL and the ϵ-NL_3^+ group of the Lys-93, and by Φ_{TS} (eq 3, Scheme 4). Values of $\Phi_{EL} \approx 1.0$ have been reported for H/D fractionation between L_2O and R-NL_3^+,[36,39] so that the PIE of 1.0 is equal to the KIE.

$$\mathrm{PIE} = 1/\Phi_{TS} \tag{2}$$

$$\mathrm{KIE} = \left(\frac{k_{EH}}{k_{ED}}\right) = \left(\frac{K^{\pm}_{EH}}{K^{\pm}_{ED}}\right) = \frac{\Phi_{EL}}{\Phi_{TS}} = \Phi_{EL}(\mathrm{PIE}) \tag{3}$$

The values of PIE = KIE = 1.0 for decarboxylation of OMP catalyzed by wild-type ScOMPDC, EcOMPDC, and MtOMPDC show that there is no selectivity between the reaction of -H and -D at the transition state for the rate-determining step. Therefore, the zero-point energy of the bond to the transferred hydron does not change significantly on proceeding first from the ground state of 50/50 HOH/DOD to the ϵ-NL_3^+ of Lys-93, and then to the transition state for the rate-determining step for the OMPDC-catalyzed decarboxylation reaction. The absence of *any* significant primary deuterium isotope effect shows that the rate-limiting step for OMPDC catalyzed decarboxylation is strongly *uncoupled* from the proton-transfer step.

It is interesting that essentially the same PIE is observed for OMPDCs from *S. cerevisiae*, *M. thermautotrophicus*, and *E. coli* (Table 1). These three OMPDCs show striking differences in the size and structure of a flexible loop that interacts with the phosphodianion of bound substrate and provides binding energy that is utilized in catalysis of decarboxylation of OMP,[4,25] but they otherwise exhibit a high degree of structural homology.[6] The results reported here show that the PIEs on these OMPDC-catalyzed reactions are not controlled by the size of the enzyme flexible phosphate gripper loops.

The PIE of 1.0 for decarboxylation of OMP is inconsistent with a transition state for a concerted mechanism in which there is significant electrophilic "push" by the transferred hydron to displacement of CO_2.[6−8] The observations that OMPDC catalyzes the slow exchange of the C-6 hydrogen of UMP for deuterium from solvent[31] and of the ca. 1000-fold faster exchange of the C-6 hydrogen of 5-FUMP[32] show that OMPDC provides strong stabilization of a bound UMP carbanion. They provide strong support for formation of the UMP carbanion as an intermediate of the decarboxylation reaction.[31] The PIEs reported in this work cannot be easily rationalized by a stepwise reaction mechanism in which the lifetime of a UMP carbanion reaction intermediate is sufficiently long to allow for it to "select" between reaction with -H or -D from the enzyme. This is because there is a significant discrimination between proton-

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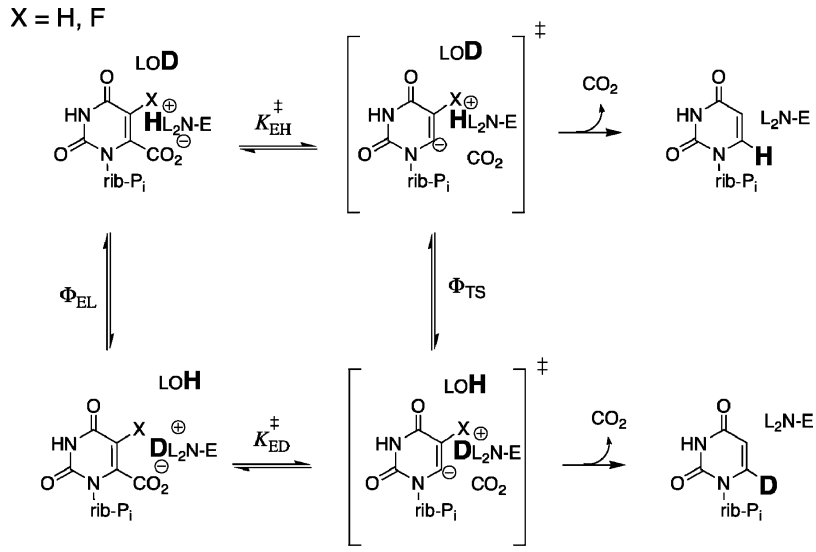
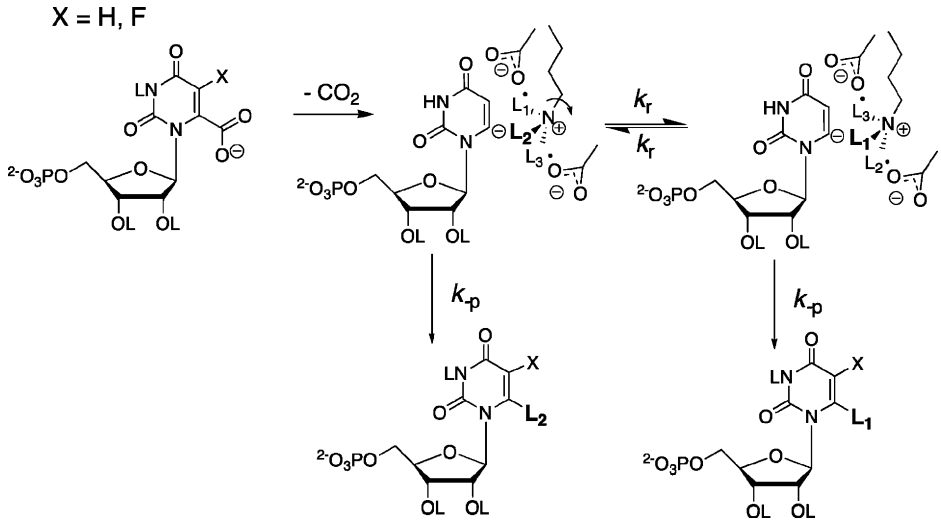
Scheme 4



Scheme 5



ation of carbanions by -H and -D, even when proton transfer is strongly thermodynamically favorable.[18]

We propose that breakdown of OMP to form a C-6 UMP carbanion intermediate is the rate-determining step for OMPDC-catalyzed decarboxylation, and that hydron transfer from the side chain of Lys-93 to this vinyl carbanion is faster than movement that exchanges the positions of the N−L$^+$ hydrons and allows the carbanion to *select* for reaction with H or D ($k_{-p} \gg k_r$, Scheme 5). The yield of [6-^{1}H]-UMP and [6-^{2}H]-UMP products is therefore determined by the initial enrichment of this acid in -H and -D (Φ_{EL}, Scheme 4).

These results are consistent with the observed -$^{13}CO_2^-$ kinetic isotope effect of 1.0255 on k_{cat}/K_m for OMPDC-catalyzed decarboxylation of OMP,[21] which shows that C−C bond cleavage partly limits the *overall* rate of this decarboxylation reaction.[21,40] The decrease to 1.0106 in the [^{13}C]-KIE on k_{cat}/K_m for OMPDC-catalyzed decarboxylation of [6-$^{13}C_2^-$]-5-FOMP is consistent with a partial change in rate-determining step, from C−C bond cleavage to substrate binding, for decarboxylation of this activated substrate.[21] The value of the product isotope effect is determined by the rate constants for partitioning of the carbanion intermediate between reaction with -H and with -D, and it is not affected by this change in rate-determining step for the overall decarboxylation reaction.

The rate constant for C−NL$_3^+$ bond rotation in water is similar to the value of 10^{11} s^{-1} estimated for *reorganization* that exchanges the relative positions of -O and -S at a thiobenzoate·carbocation ion pair in water.[41] The X-ray crystal structure of yeast OMPDC complexed with BMP shows that the −CH$_2$−NH$_3^+$ group of Lys-93 is fixed by hydrogen bonds to the carboxylate groups of Asp-91 and Asp-96 (Scheme 5).[42] We propose that these two hydrogen bonds restrict rotation about the carbon−nitrogen bond of the CH$_2$−NL$_3^+$ group of Lys-93, so that $k_r \ll 10^{11}$ s^{-1}, and that they direct the single unliganded hydron toward the C-6 UMP carbanion. Decarboxylation of bound OMP (Scheme 5) then results in a deuterium enrichment of product UMP that is essentially the same as the enrichment

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of $CH_2-NL_3^+$ in 50/50 HOH/DOD, because of the strong forward commitment for transfer of the non-hydrogen-bonded hydron of Lys-93 to the C-6 UMP carbanion ($k_{-p} \gg k_r$, Scheme 5).

Effect of Changing Reactivity of Substrate and Enzyme. The PIE of 0.93 for OMPDC-catalyzed decarboxylation of 5-FOMP by wild-type ScOMPDC is ca. 10% smaller than the value of 1.02 determined for wild-type OMPDC-catalyzed decarboxylation of OMP (Table 1). Similar 5−10% differences in PIEs for OMPDC-catalyzed decarboxylation of 5-FOMP and OMP are also observed for the reactions catalyzed by several mutant enzymes. These differences are reproducible and larger than the uncertainty in the individual PIEs.

The addition of the 5-F to OMP causes only small changes in k_{cat}/K_m (from 1.3×10^7 to 2.6×10^7 M^{-1} s^{-1}) and in k_{cat} (from 20 to 130 s^{-1}) for ScOMPDC-catalyzed decarboxylation.[23] However, the full effect of the 5-fluorine substituent on the microscopic rate constant for decarboxylation of bound substrate is not observed, because binding of 5-FOMP to OMPDC is strongly rate-determining for k_{cat}/K_m, and release of product 5-FUMP is strongly rate-determining for k_{cat}.[23] By comparison, addition of the 5-fluorine to OMP causes a much larger, 200-fold increase in k_{cat}/K_m (from 610 to 1.2×10^5 M^{-1} s^{-1}, Table 1) and 270-fold increase in k_{cat} (from 1.2 to 320 s^{-1})[43] for decarboxylation of OMP catalyzed by the R235A mutant of ScOMPDC. This shows that the 5-fluorine provides ca. 3 kcal/mol stabilization of the *transition state* for OMPDC-catalyzed decarboxylation when the breakdown of enzyme-bound 5-FOMP is the rate-determining step. This substituent provides a similar stabilization of the UMP carbanion intermediate of the OMPDC-catalyzed deuterium exchange reaction of UMP.[32]

Stabilization of the OMPDC-bound UMP carbanion by the 5-fluorine substituent should lead to a decrease in k_{-p} (Scheme 5) for protonation of this intermediate.[44] The PIE of 0.93 for decarboxylation of 5-FOMP catalyzed by wild-type ScOMPDC (Table 1) shows not only that this decrease in k_{-p} is not sufficiently large to affect the inequality $k_{-p} \gg k_r$ (Scheme 5) that was proposed for partitioning of the UMP vinyl carbanion, but also that the PIE has become smaller than the limiting KIE of 1.0 for a reaction where there the is *no* movement of hydrogen on moving from reactant to transition state. We propose that the PIE of 0.93 is observed because the deuterium enrichment of the product 5-FUMP is the same as the enrichment of $CH_2-NL_3^+$ at the OMPDC·5-FOMP Michaelis complex. This enrichment is measured by the fractionation factor Φ_{EL} (PIE = KIE/Φ_{EL}, Scheme 4). The smaller PIE on wild-type ScOMPDC-catalyzed decarboxylation of 5-FOMP (0.93 ± 0.02) compared to decarboxylation of OMP (1.02 ± 0.02, Table 1) is consistent with an increase in Φ_{EL} from 1.0 for decarboxylation of OMP to 1.1 for decarboxylation of 5-FOMP. This increase in Φ_{EL} provides evidence that the effect of the 5-fluorine at enzyme-bound 5-FOMP is to strengthen the N−L bonds of the $-CH_2-NL_3^+$ group of Lys-93.[37,38] This may be due to an interaction between the *negative* end of the C−F dipole and the *positively* charged $-CH_2-NL_3^+$ that *stabilizes* the alkylammonium ion relative to the basic amine.

The PIEs on decarboxylation of OMP and 5-FOMP catalyzed by OMPDC show a remarkable insensitivity to mutation of amino acid residues that are essential for the observation of robust enzymatic activity (Table 1). In other words, these mutations affect the stability of the rate-determining transition state for decarboxylation but do not affect the relative yield of H- and D-labeled reaction products. The results are consistent with the mechanism shown in Scheme 5, where $k_{-p} \gg k_r$ for all mutant enzyme-catalyzed reactions. This is simply rationalized. The active-site residues examined all lie distant from Lys-93, so their mutation is not expected to affect the barrier to k_r for rotation about the carbon−nitrogen bond of the $-CH_2-NL_3^+$ group of Lys-93. The mutations all cause an increase in the barrier for decarboxylation of OMP to form the C-6 UMP carbanion. This presumably reflects an increase in the barrier to formation of the carbanion intermediate, which should be accompanied by a decrease in the activation barrier to carbanion protonation (increase in k_{-p}, Scheme 5)[44] that will favor observation of a unitary PIE.

There is a striking contrast between the large conformational flexibility of OMPDC that is suggested by the differences in the structures for the free and liganded enzyme[42] and the rotational rigidity of the $-CH_2-NL_3^+$ group of Lys-93 that is hydrogen-bonded to carboxylate groups of Asp-91 and Asp-96 (Scheme 5).[42] We note that the *most* efficient catalysis of decarboxylation will be observed when there is the *maximum* increase in *stabilizing* electrostatic interactions between the $-CH_2-NL_3^+$ group of OMPDC and the bound substrate/intermediate on proceeding from the Michaelis complex to the C-6 UMP carbanion reaction intermediate. Therefore, one role for "clamping" the $-CH_2-NL_3^+$ side chain of Lys-93 may be to position this amino acid side chain to *minimize* its electrostatic interactions with the $-CO_2^-$ of OMP in the Michaelis complex and to *maximize* the interactions with the developing C-6 UMP carbanion at the transition state for decarboxylation.[45,46] This may involve a shift in the position of the side chain that is *coupled* in some way to decarboxylation.

Acknowledgment. We acknowledge the National Institutes of Health (Grant GM39754 to J.P.R. and Grant GM65155 to J.A.G.) for generous support of this work.

Supporting Information Available: Protocol for preparation of Y217A mutant OMPDC. This information is available free of charge via the Internet at http://pubs.acs.org.

JA102408K

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