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Product Deuterium Isotope Effect for Orotidine 5'-Monophosphate Decarboxylase: Evidence for the Existence of a Short-Lived Carbanion Intermediate

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We report that equal yields of [6-^{1}H]-uridine 5'-monophosphate (50%) and [6-^{2}H]-uridine 5'-monophosphate (50%) are obtained from the decarboxylation of orotidine 5'-monophosphate (**OMP**) catalyzed by orotidine 5'-monophosphate decarboxylase in a solvent of 50/50 (v/v) H_2O/D_2O. This observation of an unusually small product isotope effect of unity eliminates a proposed mechanism in which proton transfer from Lys-93[1] to C-6 provides electrophilic *push* to the loss of CO_2 from **OMP** in a concerted reaction.[2,3] It provides evidence that proton transfer from the ammonium cation side-chain of Lys-93 to a vinyl carbanion intermediate is *faster* than the bond rotation that exchanges the positions of the acidic N-L^+ hydrons of this side-chain.

Orotidine 5'-monophosphate decarboxylase (OMPDC) is a remarkable enzyme because it employs no metal ions or other cofactors but yet effects an enormous 10^{17}-fold acceleration of the chemically very difficult decarboxylation of **OMP** to give uridine 5'-monophosphate (**UMP**).[4,5] It has been shown that a large fraction of the enzymatic rate acceleration results directly from utilization of the intrinsic binding energy of th e remote nonreacting 5'-phosphodianion group of **OMP** in transition state stabilization.[6] The decarboxylation reaction is often proposed to proceed in two steps through a vinyl carbanion intermediate (Scheme 1). However, it has also been suggested that this unstable intermediate might be avoided in a concerted reaction in which decarboxylation and proton transfer to C-6 occur in a single step.[2,3]

Experimental and computational studies on OMPDC have focused largely on the partly rate-determining and highly unfavorable loss of CO_2 from **OMP**.[7-9] There are few data pertaining to the proton transfer to C-6 of the pyrimidine ring. Experimental characterization of this proton transfer step is essential for insight into the existence and lifetime of the putative enzyme-bound vinyl carbanion intermediate.

OMPDC catalyzes incorporation of a hydron from solvent into the **UMP** product and it has been reported that the decarboxylation of saturating **OMP** is 30% faster in H_2O than in D_2O.[7] While the origin of this solvent isotope effect on k_{cat} is unclear, it may represent a secondary

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solvent kinetic isotope effect (SKIE). By contrast, a product isotope effect (PIE) determined in experiments in which H and D in a mixed solvent of H_2O/D_2O compete for reaction with enzyme-bound **OMP** to form **UMP** labeled at C-6 (Scheme 2) would provide insight into the changes in bonding at the transferred hydron that occur on proceeding to the transition state for the product-determining step.[10] PIEs are more precise and easier to interpret than SKIEs determined as the ratio of rate constants for reactions in H_2O and D_2O because: (1) There are no complications from any secondary SKIE when the H- and D-labeled products are formed in the same mixed H_2O/D_2O solvent. (2) There are no errors due to differences in the conditions for separate reactions in H_2O and D_2O, such as enzyme concentration, temperature and pL.

The product distribution for the decarboxylation of **OMP** catalyzed by OMPDC in 50/50 (v/v) H_2O/D_2O was determined by ^1H NMR spectroscopy at 500 MHz. Figure 1 shows the partial ^1H NMR spectrum of **UMP** obtained from the decarboxylation of **OMP** (2 mM) catalyzed by OMPDC from *S. cerevisiae* (C155S mutant, 24 nM, 1 hr, >90% reaction) in 50/50 (v/v) H_2O/D_2O at pL 7.3 and 25 °C (I = 0.10, NaCl).[11,12] The value of PIE = 1.0 was calculated using eq 1, where A_H is the integrated area of the doublet due to the C-6 proton of [6-^{1}H]-**UMP** (7.990 ppm), and A_D is the integrated area of the singlet due to the C-5 proton of [6-^{2}H]-**UMP** (5.865 ppm).[13] By comparison, PIEs of 7.3 − 8.1 for proton transfer to ring-substituted aryl vinyl ethers from lyonium ion in 50/50 (v/v) H_2O/D_2O have been reported recently.[10]

$$\mathrm{PIE}=A_H/A_D \quad (1)$$

We used similar procedures to determine values of PIE = 1.0 for decarboxylation of **OMP** (2 mM) catalyzed by OMPDC from both *E. coli* (40 nM) and *M. thermoautotrophicum* (40 nM) in 50/50 (v/v) H_2O/D_2O at pL 7.3 and 25 °C (I = 0.10, NaCl). The essentially identical PIEs determined for OMPDC from different sources is significant, because these enzymes exhibit somewhat different architectures at their active sites.[2,9a,14,15]

The value of PIE = 1.0 for the OMPDC-catalyzed decarboxylation of **OMP** in 50/50 (v/v) H_2O/D_2O shows that the deuterium enrichment of the hydron used to protonate **OMP** or an intermediate carbanion at the reaction transition state (50%) is the same as that of the 50/50 (v/v) H_2O/D_2O solvent. The product-determining step is thought to be proton transfer from the NL_3^+ group of the side-chain of Lys-93 to **OMP** or to a reaction intermediate (Scheme 3).[15] Values of $ø_{NL3+} \approx 1.0$ have been reported for the H/D fractionation between L_2O and R-NL_3^+, so that the deuterium enrichment of the NL_3^+ group of Lys-93 should be similar to that of the solvent L_2O.[16] Therefore the PIE of 1.0 is essentially equal to the primary kinetic isotope effect for reaction of the H- and D-labeled NL_3^+ group of Lys-93 to form [6-^{1}H]-**UMP** and [6-^{2}H]-**UMP**.

A significant primary product isotope effect is expected for a reaction in which there is *movement* of the proton in the transition state for the product-determining step,[17a] and there is no precedent for PIEs as small as 1.0 when carbanion protonation is the product-determining step.[17a,18] The observed PIE of 1.0 requires that all of the zero point energy present in the N-L^+ bonds of Lys-93 be maintained at the transition state for the step that determines whether the **UMP** product is labeled at C-6 with H or D. This PIE is not consistent with a mechanism 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 that avoids formation of an unstable vinyl carbanion intermediate (bottom pathway, Scheme 3).[2,3,19]

We suggest that the essentially statistical yields of [6-^{1}H]-**UMP** and [6-^{2}H]-**UMP** from the OMPDC-catalyzed decarboxylation of **OMP** are established at a step that occurs prior to hydron transfer to a vinyl carbanion intermediate. This could be the decarboxylation step, if an N-L^+ bond of Lys-93 is already correctly positioned to deliver a hydron to a vinyl carbanion (k_{dc}, Scheme 3). Alternatively it may be a step that orients an N-L^+ bond of Lys-93 into a

"reactive position" where hydron transfer to a vinyl carbanion intermediate can occur. In both cases the PIE of 1.0 *requires* that the chemical step of hydron transfer to the carbanion be *faster* than any molecular motion that allows its discrimination between reaction with H and D at the NL_3^+ group of Lys-93.[17] We therefore propose that hydron transfer from the side-chain of Lys-93 to a vinyl carbanion intermediate (k_p) is faster than any movement that exchanges the positions of the N-L^+ hydrons and which would allow the carbanion to *select* for reaction with H or D.[17] In water, the rate constant for such a step is ca. 10^{11} s^{-1}.[20]

The X-ray crystal structure of yeast OMPDC complexed with 6-hydroxyuridine 5'-monophosphate shows that the CH_2-NH_3^+ group of Lys-93 is anchored by two hydrogen bonds to the carboxylate groups of Asp-91 and Asp-96 that are proposed to direct the third ammonium hydron of Lys-93 towards the putative vinyl carbanion intermediate.[15] These hydrogen bonds should also restrict rotation about the carbon-nitrogen bond of the terminal CH_2-NL_3^+ group of Lys-93 ($k_{rot} << 10^{11}$ s^{-1}). This would favor the observed unselective proton transfer from the remaining free (non-hydrogen-bonded) hydron to a vinyl carbanion intermediate.

Acknowledgement

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7], which shows that decarboxylation is *less* rate-determining in D2O than in H_2O. This result is not easily rationalized by a mechanism in which proton transfer from Lys-93 to C-6 of **OMP** is concerted with the loss of CO^2 because the change from H_2O to D_2O should raise the barrier to a reaction in which proton transfer is concerted with loss of CO_2, as a result of a normal primary KIE. This would cause the loss of CO_2 to become more rate-determining in a multistep enzymatic reaction in D_2O and would result in an increase, rather than the observed decrease, in the ^{13}C isotope effect.

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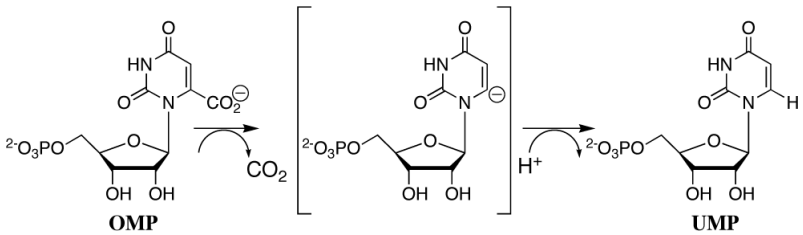
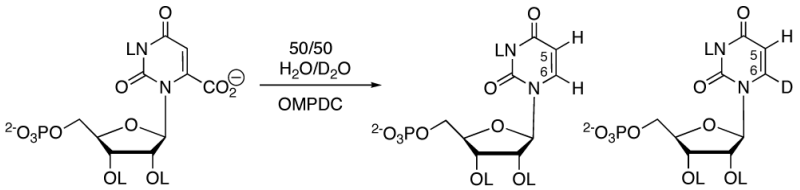


Scheme 1.



Scheme 2.

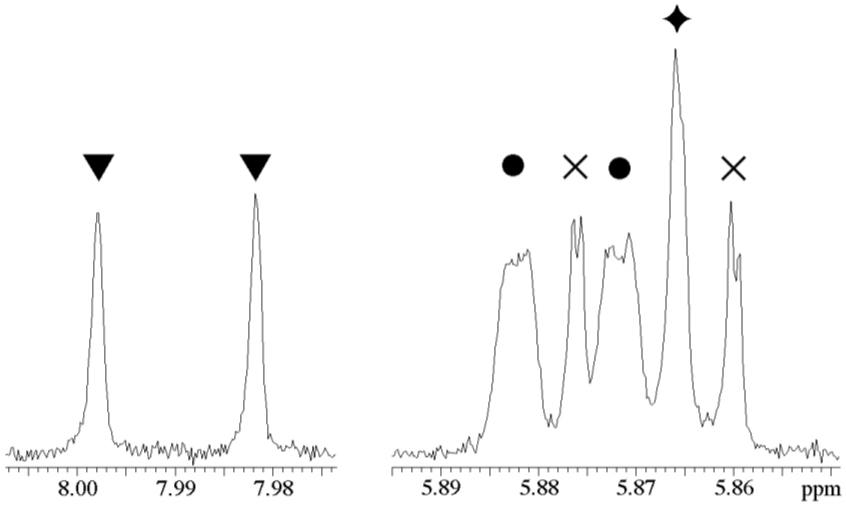
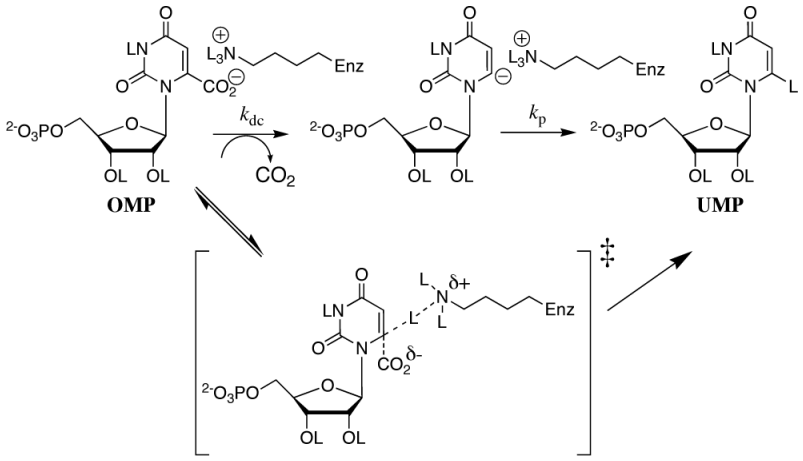


Figure 1.

Partial ^{1}H NMR spectrum (500 MHz) of **UMP** from decarboxylation of **OMP** (2 mM) catalyzed by OMPDC from *S. cerevisiae* (24 nM) in 50/50 (v/v) H_2O/D_2O at pL 7.3 and 25 ° C. Key: (▼) Doublet due to the C-6 proton of [6-^{1}H]-**UMP**; (•) Doublets (not 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**; (✦) Singlet due to the C-5 proton of [6-^{2}H]-**UMP**.



Scheme 3.