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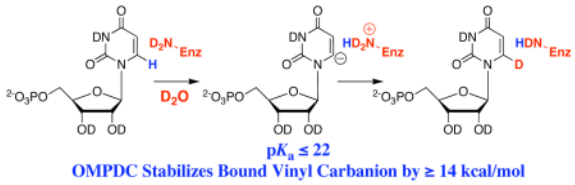
Formation and Stability of a Vinyl Carbanion at the Active Site of Orotidine 5′-Monophosphate Decarboxylase: pK_a of the C-6 Proton of Enzyme-Bound UMP

Tina L. Amyes[†], **Bryant M. Wood**[#], **Kui Chan**[#], **John A. Gerlt**[*,#], and **John P. Richard**[*,†]
Department of Chemistry, University at Buffalo, Buffalo, New York 14260 and Departments of Biochemistry and Chemistry, University of Illinois, Urbana, Illinois 61801

Abstract



We report that orotidine 5′-monophosphate decarboxylase (OMPDC) catalyzes exchange of the C-6 proton of uridine 5′-monophosphate (**UMP**) for deuterium from solvent in D_2O at 25 °C and pD 7.0 – 9.3. Kinetic analysis of deuterium exchange gives p$K_a \leq 22$ for carbon deprotonation of enzyme-bound **UMP**, which is at least 10 units lower than that for deprotonation of an analog of **UMP** in water. The observation of enzyme-catalyzed deuterium exchange via a *stabilized* carbanion provides convincing evidence for the decarboxylation of orotidine 5′-monophosphate (**OMP**) by OMPDC to give the same carbanion intermediate. The data show that yeast OMPDC stabilizes the bound vinyl carbanion by at least 14 kcal/mol. We conclude that OMPDC also provides substantial stabilization of the late carbanion-like transition state for the decarboxylation of **OMP**, and that this transition state stabilization constitutes a large fraction, but probably not all, of the enormous 10^{17}-fold enzymatic rate acceleration.

We report that orotidine 5′-monophosphate decarboxylase catalyzes exchange of the C-6 proton of uridine 5′-monophosphate (**UMP**) for deuterium from solvent in D_2O at 25 °C (Scheme 1). Kinetic analysis of deuterium exchange gives p$K_a \leq 22$ for carbon deprotonation of enzyme-bound **UMP**, which is at least 10 units lower than that for deprotonation of an analog of **UMP** in water.

Orotidine 5′-monophosphate decarboxylase (OMPDC) employs no metal ions or other cofactors but yet effects an enormous 10^{17}-fold acceleration of the decarboxylation of orotidine 5′-monophosphate (**OMP)** to give uridine 5′-monophosphate (**UMP**).[1] The X-ray structure of the yeast enzyme liganded with 6-hydroxyuridine 5′-monophosphate provides strong evidence that the C-6 proton of the product UMP is derived from the terminal NH_3^+ group of Lys-93.[2] The product isotope effect of unity for OMPDC-catalyzed decarboxylation of **OMP** in 50/50 (v/v) H_2O/D_2O eliminates a mechanism[3] in which proton transfer from Lys-93 to C-6 provides electrophilic *push* to the loss of CO_2 in a concerted reaction.[4] This

jrichard@chem.buffalo.edu; j-gerlt@uiuc.edu.
[†]University at Buffalo
[#]University of Illinois

result also provides strong evidence for the formation of a short-lived enzyme-bound carbanion intermediate that shows no discrimination between H and D in the proton transfer step (Scheme 2).[4]

The very large kinetic barrier to the nonenzymatic decarboxylation of **OMP** ($t_{1/2}$ = 78 million years)[5] arises mainly from the thermodynamic barrier to formation of the highly unstable C-6 vinyl carbanion. This activation barrier may be reduced by interactions with OMPDC that either destabilize bound **OMP** relative to the bound carbanion intermediate, or by interactions that stabilize the bound carbanion intermediate relative to bound **OMP**.[6] Computational studies support the proposal that binding of **OMP** to OMPDC induces either electrostatic *stress* between the protein and the bound substrate in the ground state Michaelis complex,[7] or conformational *stress* in the protein at this complex,[8] and that this stress is relieved in the transition state for enzyme-catalyzed decarboxylation. However, other calculations suggest that the enzymatic rate acceleration is due mainly to stabilization of the transition state for decarboxylation.[9] These results are difficult to evaluate because there are few experiments that address whether the rate acceleration for OMPDC is due mainly to ground state destabilization,[10] to transition state stabilization, or to both effects.

The exchange for deuterium of the C-6 proton of [6-^{1}H]-uridine 5′-monophosphate (***h*-UMP**) to give [6-^{2}H]-uridine 5′-monophosphate (***d*-UMP**) catalyzed by OMPDC from *S. cerevisiae* (C155S mutant) in D_2O (Scheme 1) was monitored by ^{1}H NMR spectroscopy at 500 MHz. Figure 1 shows partial ^{1}H NMR spectra in the region of the anomeric and C-5 protons of recovered **UMP** ($[\mathbf{UMP}]_{total}$ = 2.5 mM) obtained during deuterium exchange at 25 °C catalyzed by OMPDC (0.11 mM, 3.2 mg/mL, monitored for 7 days) in D_2O buffered by 100 mM glycylglycine at pD 9.34 (I = 0.1, NaCl). Deuterium exchange results in the disappearance of the double doublet (**a**) at 5.860 ppm due to the C-5 proton of ***h*-UMP** and the appearance of an upfield-shifted broad doublet (**b**) at 5.857 ppm ($\Delta\delta \approx 0.003$ ppm) due to the C-5 proton of ***d*-UMP**. The fractional extent of deuterium exchange was obtained from the integrated area of the two downfield peaks of the double doublet due to the C-5 proton of ***h*-UMP** (A_H) and the *combined* integrated areas of the upfield peaks of this signal and that of the intervening broad doublet due to the C-5 proton of ***d*-UMP** (A_{D+H}), according to eq 1. The observed first-order rate constant for deuterium exchange into **UMP**, k_{obsd} = 4.90 × 10^{-7} s^{-1}, was determined from the slope of a semi-logarithmic plot of the fraction of *h*-UMP remaining, {1 - *f*(*d*-UMP)}, against time.

$$f(d-\mathrm{UMP}) = \{A_{D+H} - A_H\} / \{A_{D+H} + A_H\} \quad (1)$$

The values of k_{obsd} (s^{-1}) determined for enzyme-catalyzed deuterium exchange in D_2O at pD 9.34 with $[\mathbf{UMP}]_{total}$ = 2.5 – 10 mM show a good fit to eq 2 that was derived for Scheme 3 (see Supporting Information), with $K_d \ll [\mathbf{UMP}]_{total}$. The data give the first-order rate constant for deuterium exchange into *saturating enzyme-bound* **UMP** at pD 9.34 as k_{ex} = 1.15 × 10^{-5} s^{-1}. Similar experiments using ca. 0.3 mM OMPDC (9 mg/mL) gave values of k_{ex} (s^{-1}) for the turnover of *saturating* **UMP** (2.5 – 5 mM) at pD 8.13 (100 mM glycylglycine buffer), and at pD 7.58 and 7.03 (48 mM imidazole buffer), at 25 °C and I = 0.1 (NaCl).[11]

$$k_{obsd} = \frac{k_{ex}[\mathrm{E}]}{[\mathrm{UMP}]_{total} + K_d} \quad (2)$$

Figure 2 shows the pD-rate profile of the values of k_{ex} (s^{-1}) for turnover of *enzyme-bound* ***h*-UMP** to give ***d*-UMP** by yeast OMPDC in D_2O at 25 °C and I = 0.1 (NaCl). The large increase

in k_{ex} (s^{-1}) with increasing pD and the leveling off at pD > 8 shows that deuterium exchange is promoted by the basic form of an amino acid side chain at the active site of OMPDC.[12] We suggest that the catalytic base is the neutral form of Lys-93,[13] so that deuterium exchange arises from the reverse of the proton transfer "half reaction" that occurs in the active site during the physiological decarboxylation of **OMP** to give **UMP** (Scheme 2). Analysis of the data in Figure 2 gives $p(K_a)_{Lys} = 8.0$ for the catalytic base in D_2O at $I = 0.1$, and $(k_{ex})_{max} = 1.24 \times 10^{-5}\ s^{-1}$ for proton transfer from bound ***h*-UMP** to the *neutral* side chain of Lys-93 to give the enzyme-bound vinyl carbanion (Scheme 4).

We have shown that proton transfer from protonated Lys-93 to the vinyl carbanion must be *faster* than any molecular motion that exchanges the positions of the $N\text{-}L^+$ hydrons of Lys-93, so that $k_p \gg k_{rot}$ (Scheme 4).[4] Therefore, the observed deuterium exchange reaction consists of the pre-equilibrium *reversible* chemical step of proton transfer from **UMP** to Lys-93, followed by the *occasional* rotation of the terminal $CH_2\text{-}ND_2H^+$ group of Lys-93 into a position to deliver a deuteron to the vinyl carbanion (k_{rot}, Scheme 4). The $CH_2\text{-}NH_3^+$ group of Lys-93 is hydrogen-bonded to the carboxylate groups of Asp-91 and Asp-96,[2] and the barrier to $CH_2\text{-}ND_2H^+$ bond rotation and hydron exchange is expected to be at least 5 kcal/mol, so that $k_{rot} \leq 10^9\ s^{-1}$.[14] The limit of $k_{rot} \leq 10^9\ s^{-1}$ can be substituted into eq 3, derived for the mechanism shown in Scheme 4, with $(k_{ex})_{max} = 1.24 \times 10^{-5}\ s^{-1}$ and $(K_a)_{Lys} = 10^{-8}$ M to give $p(K_a)_{UMP} \leq 22$ for the C-6 proton of enzyme-bound **UMP**.

$$(k_{ex})_{max} = \left(\frac{k_{-p}}{k_p}\right) k_{rot} = \left(\frac{(K_a)_{UMP}}{(K_a)_{Lys}}\right) k_{rot} \tag{3}$$

The observation of enzyme-catalyzed deuterium exchange via formation of a *stabilized* carbanion provides convincing evidence for decarboxylation of **OMP** by yeast OMPDC to give the same carbanion.[15] The value of $pK_a \leq 22$ for the C-6 proton of enzyme-bound **UMP** determined here is at least 10 units lower than the estimated values of $pK_a = 30 – 34$ for the C-6 proton of 1,3-dimethyluracil in water.[16–18] Therefore, yeast OMPDC stabilizes the bound vinyl carbanion by *at least* 14 kcal/mol. We conclude that OMPDC also provides substantial stabilization of the late carbanion-like transition state for the decarboxylation of **OMP**, and that this transition state stabilization constitutes a large fraction, but probably not the entire, enzymatic rate acceleration. Further experimental studies directed at elucidating the *origin* of the transition state stabilization for OMPDC will provide insight into its so far unexplained extraordinary catalytic power.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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12. The exchange reaction does not occur in the presence of a saturating amount of the competitive inhibitor 6-aza-uridine 5′-monophosphate.
13. The pH-dependence of k_{cat} for turnover of OMP indicates the involvement of the acidic form of a catalytic residue of $pK_a = 8.8$ [Porter DJT, Short SA. Biochemistry 2000;39:11788–11800. [PubMed: 10995247]
14. $k_{rot} = 10^{11}\ s^{-1}$ for unhindered rotation within an ion pair in water [Richard JP, Tsuji Y. J Am Chem Soc 2000;122:3963–3964.
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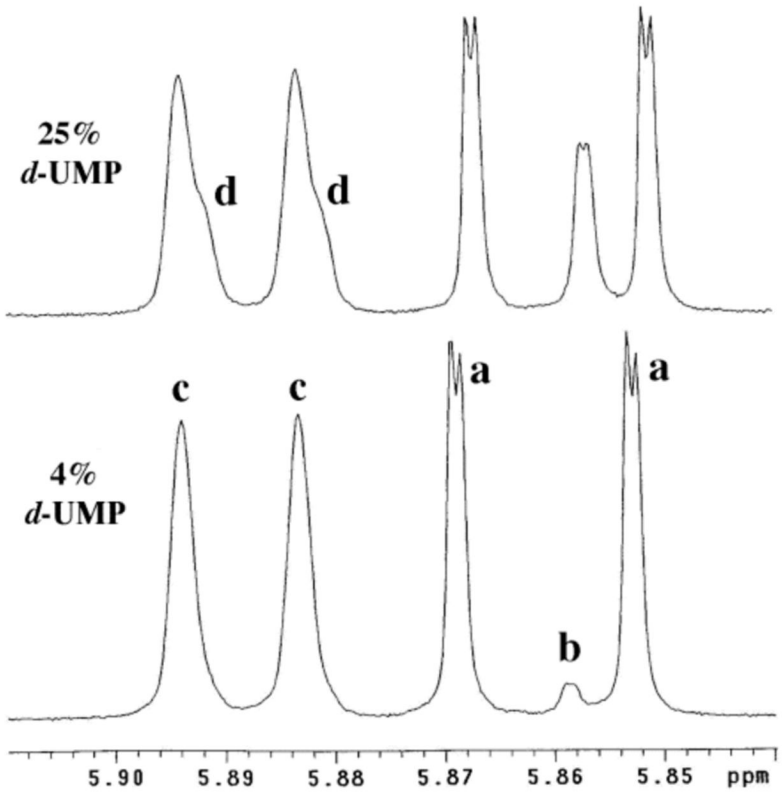


Figure 1.

Partial ^{1}H NMR spectra (500 MHz) of recovered **UMP** obtained during exchange for deuterium of the C-6 proton catalyzed by OMPDC (0.11 mM) in D_2O at pD 9.34 and 25 °C (I = 0.1, NaCl). Disappearance of the double doublet (**a**) due to the C-5 proton of ***h*-UMP** at 5.860 ppm (J = 8.1, 0.5 Hz, coupled to the C-6 and anomeric protons) is accompanied by the appearance of a upfield-shifted broad doublet (**b**) due to the C-5 proton of ***d*-UMP** at 5.857 ppm ($J \approx 0.5$ Hz, coupled to the anomeric proton). The broad doublet (**c**) due to the anomeric proton of ***h*-UMP** at 5.889 ppm (J = 5.3 Hz, coupled to the C-2′ proton) exhibits "shoulders" (**d**) due to the slightly upfield-shifted doublet for the anomeric proton of ***d*-UMP**.

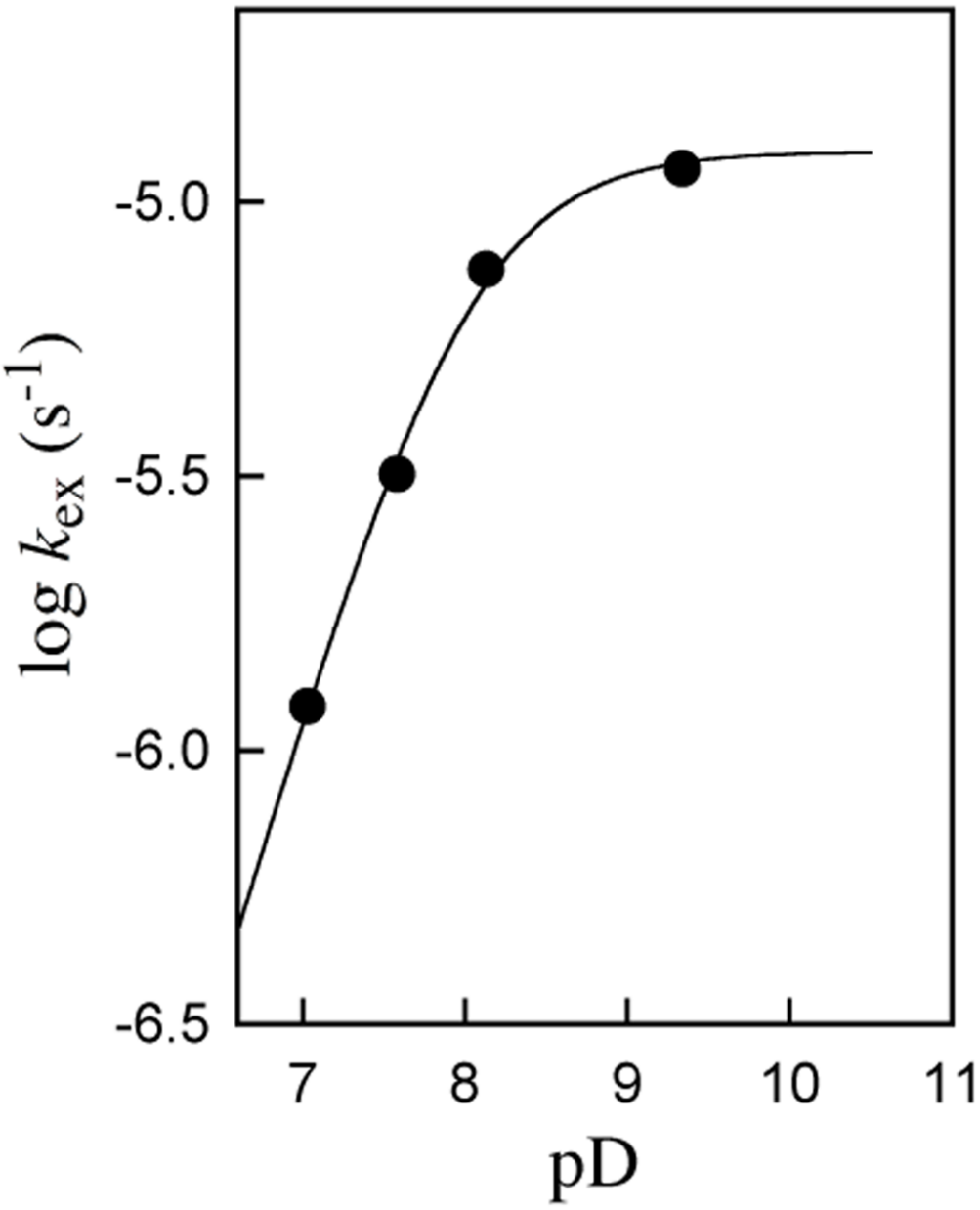
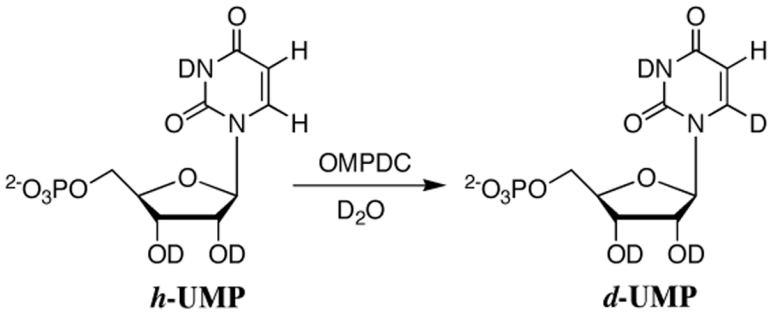
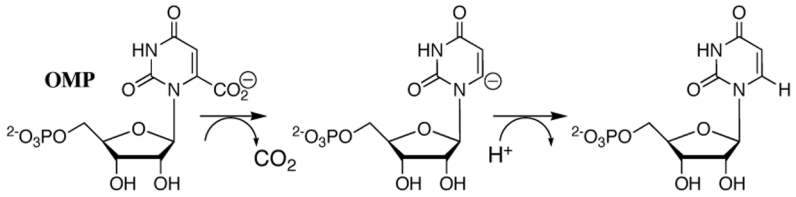


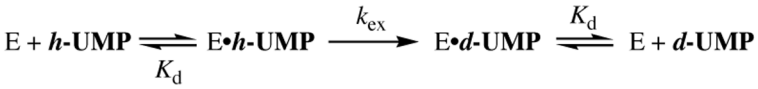
Figure 2.
pD-Rate profile of k_{ex} (s^{-1}) for turnover of enzyme-bound ***h*-UMP** to give ***d*-UMP** by OMPDC from *Saccharomcyes cerevisiae* (C155S mutant) in D_2O at 25 °C and I = 0.1 (NaCl). The solid line shows the calculated profile for a catalytic base of pK_a = 8.0.



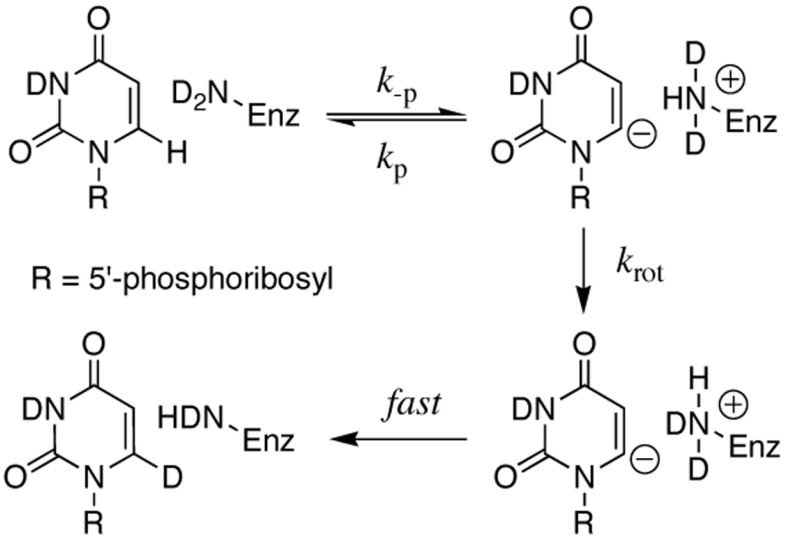
Scheme 1.



Scheme 2.



Scheme 3.



Scheme 4.