

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

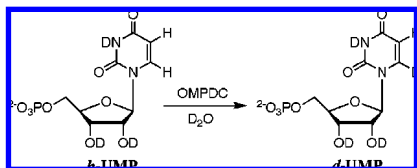
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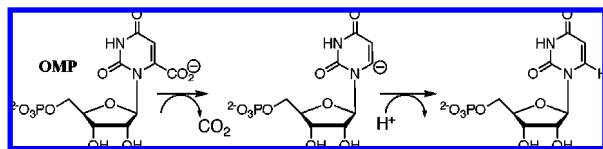
We report that orotidine 5'-monophosphate decarboxylase catalyzes exchange of the C-6 proton of uridine 5'-monophosphate (UMP) for deuterium from solvent in D₂O at 25 °C (Scheme 1). Kinetic analysis of deuterium exchange gives $pK_a \leq 22$ for carbon deprotonation of enzyme-bound UMP, which is at least 10 units lower than that for deprotonation of an analogue of UMP in water.

Scheme 1.



Orotidine 5'-monophosphate decarboxylase (OMPDC) employs no metal ions or other cofactors but yet effects an enormous 10¹⁷-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 NH₃⁺ group of Lys-93.² The product isotope effect of unity for OMPDC-catalyzed decarboxylation of OMP in 50/50 (v/v) H₂O/D₂O eliminates a mechanism³ in which proton transfer from Lys-93 to C-6 provides electrophilic *push* to the loss of CO₂ in a concerted reaction.⁴ This 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

Scheme 2.



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 either by interactions with OMPDC that 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 complex⁷ or conformational *stress* in the protein at this complex⁸ and that this stress is relieved in the transition state for enzyme-catalyzed

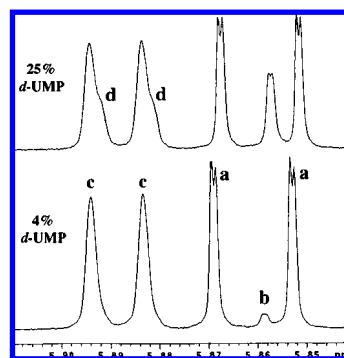


Figure 1. Partial ¹H NMR spectra (500 MHz) of recovered UMP obtained during exchange for deuterium of the C-6 proton catalyzed by OMPDC (0.11 mM) in D₂O at pD 9.34 and 25 °C ($I = 0.1$, NaCl). Disappearance of the double doublet (a) due to the C-5 proton of *h*-UMP at 5.860 ppm ($J = 8.1$, 0.5 Hz, coupled to the C-6 and anomeric protons) is accompanied by the appearance of an 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.

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 [6-¹H]-uridine 5'-monophosphate (*h*-UMP) to give [6-²H]-uridine 5'-monophosphate (*d*-UMP) catalyzed by OMPDC from *S. cerevisiae* (C155S mutant) in D₂O (Scheme 1) was monitored by ¹H NMR spectroscopy at 500 MHz. Figure 1 shows partial ¹H NMR spectra in the region of the anomeric and C-5 protons of recovered UMP ([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 D₂O 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, $k_{\text{obsd}} = 4.90 \times 10^{-7} \text{ s}^{-1}$, was determined from the slope of a semilogarithmic plot of the fraction of *h*-UMP remaining, $\{1 - f(d\text{-UMP})\}$, against time.

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

The values of k_{obsd} (s^{-1}) determined for enzyme-catalyzed deuterium exchange in D_2O at pD 9.34 with $[\text{UMP}]_{\text{total}} = 2.5\text{--}10$ mM show a good fit to eq 2 that was derived for Scheme 3 (see Supporting Information), with $K_d \ll [\text{UMP}]_{\text{total}}$. The data give the first-order rate constant for deuterium exchange into *saturating enzyme-bound* UMP at pD 9.34 as $k_{\text{ex}} = 1.15 \times 10^{-5} \text{ s}^{-1}$. 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

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

Scheme 3.



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 $\text{p}(K_a)_{\text{Lys}} = 8.0$ for the catalytic base in D_2O at $I = 0.1$, and $(k_{\text{ex}})_{\text{max}} = 1.24 \times 10^{-5} \text{ s}^{-1}$ for proton transfer from bound *h*-UMP to the *neutral* side chain of Lys-93 to give the enzyme-bound vinyl carbanion (Scheme 4).

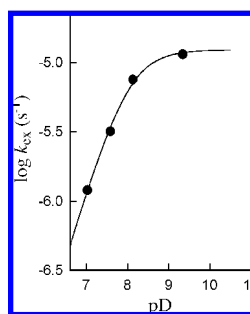


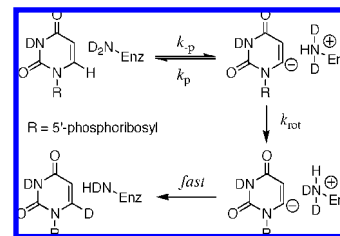
Figure 2. The pD-rate profile of k_{ex} (s^{-1}) for turnover of enzyme-bound *h*-UMP to give *d*-UMP by OMPDC from *Saccharomyces cerevisiae* (C155S mutant) in D_2O at 25 °C and $I = 0.1$ (NaCl). The solid line shows the calculated profile for a catalytic base of $\text{p}K_a = 8.0$.

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-L^+ hydrons of Lys-93, so that $k_p \gg k_{\text{rot}}$ (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 $\text{CH}_2\text{-ND}_2\text{H}^+$ group of Lys-93 into a position to deliver a deuteron to the vinyl carbanion (k_{rot} , Scheme 4). The $\text{CH}_2\text{-NH}_3^+$ group of Lys-93 is hydrogen-bonded to the carboxylate groups of Asp-91 and Asp-96,² and the barrier to $\text{CH}_2\text{-ND}_2\text{H}^+$ bond rotation and hydron exchange is expected to be at least 5 kcal/mol, so that $k_{\text{rot}} \leq 10^9 \text{ s}^{-1}$.¹⁴ The limit of $k_{\text{rot}} \leq 10^9 \text{ s}^{-1}$ can be substituted into eq 3, derived for the mechanism shown in Scheme 4, with $(k_{\text{ex}})_{\text{max}} = 1.24 \times 10^{-5} \text{ s}^{-1}$ and $(K_a)_{\text{Lys}} = 10^{-8} \text{ M}$

to give $\text{p}(K_a)_{\text{UMP}} \leq 22$ for the C-6 proton of enzyme-bound UMP.

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

Scheme 4.



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 $\text{p}K_a \leq 22$ for the C-6 proton of enzyme-bound UMP determined here is at least 10 units lower than the estimated values of $\text{p}K_a = 30\text{--}34$ 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 of, 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.

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: Experimental procedures, kinetic analysis, semilogarithmic plots, table of values of k_{obsd} and k_{ex} . This material is available free of charge via the Internet at <http://pubs.acs.org>.

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JA710384T