SUPPORTING INFORMATION

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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Materials. Orotidine 5'-monophosphate trisodium salt and uridine 5'-monophosphate disodium salt (99%, from yeast) were purchased from Sigma. 6-aza-uridine 5'-monophosphate (free acid, > 99% by HPLC) was purchased from R. I. Chemical Inc. Glycylglycine (> 99%) and 3-(N-morpholino)propanesulfonic acid (MOPS, ≥ 99.5%) were purchased from Fluka. Imidazole was purchased from Aldrich and was recrystallized from benzene. Deuterium oxide (99.9% D), sodium deuteroxide (30% in D_2O, 99.5% D) and deuterium chloride (35% w/w, 99.9% D) were purchased from Cambridge Isotope Laboratories. Orotidine 5'-monophosphate decarboxylase from yeast (C155S mutant) was prepared as described below. All other chemicals were reagent grade or better and were used without further purification.

Cloning, Overexpression and Purification of C155S Mutant Yeast Orotidine 5'-Monophosphate Decarboxylase (OMPDC). The gene encoding the C155S mutant of OMPDC from *Saccharomcyes cerevisiae* was obtained from Dr. Steven Short (Glaxo-Smith-Kline) in the plasmid pBGM88.[1] The gene was subcloned into the pET-15b expression vector (Novagen; encoding an N-terminal His_6-tag) *via* the *Nde* I and *Bam* HI restrictions sites at the 5'- and 3'-ends of the gene, respectively, to produce the plasmid pScODC-15b. The sequence of the

gene encoding OMPDC contained in pScODC-15b was verified at the University of Illinois, Urbana-Champaign Core Sequencing Facility.

Escherichia coli strain BL21 (DE3) (Stratagene) was transformed with pScODC-15b and the transformed cells were grown in LB medium (4 L) for 48 hr at 20 ˚C, with the addition of 0.5 mM IPTG after 14 hours of growth to induce the expression of OMPDC. The cells were harvested by centrifugation for 10 min at 3000 x g and resuspended in binding buffer (5 mM imidazole, 0.5 M NaCl, 8 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.9). The cells were lysed by sonication with a 550 Sonic Dismembrator (Fisher Scientific) for 12 min (5 sec pulses separated by 10 sec), and cellular debris was removed by centrifugation for 45 min at 31,000 x g.

The N-terminal His_6-tagged OMPDC was purified using a Pharmacia BioPilot FPLC system. The cleared supernatant was loaded onto a column of Ni-Chelating Sepharose, Fast Flow (Pharmacia Biotech; 150 mL volume) that was equilibrated with the binding buffer. The column was washed with 300 mL of binding buffer, followed by 300 mL of wash buffer (60 mM imidazole, 0.5 M NaCl, 5 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.9). The N-terminal His_6-tagged OMPDC was eluted using a gradient (400 mL) of 60 mM to 1 M imidazole in the wash buffer. Fractions containing OMPDC were identified by SDS-PAGE, pooled and dialyzed twice at 5 ˚C against 4 L of 20 mM Tris-HCl, pH 7.9.

The N-terminal His_6-tag was cleaved by the action of thrombin (1 unit/mg OMPDC) in phosphate buffered saline at 25 ˚C for 16 hr, and the cleavage was confirmed by SDS-PAGE. The protein was dialyzed at 5 ˚C against 20 mM Tris-HCl, pH 7.9 and was loaded onto a Q Sepharose High Performance column (Pharmacia Biotech; 50 mL volume) that was equilibrated with 20 mM Tris-HCl, pH 7.9. OMPDC was eluted using a gradient (500 mL) of 0 to 500 mM NaCl in 20 mM Tris-HCl, pH 7.9. Fractions containing OMPDC were identified by SDS-PAGE,

pooled, and dialyzed three times at 5 ˚C against 4 L of 100 mM NaCl, 20% glycerol, 20 mM Tris-HCl, pH 7.9. The purified OMPDC was concentrated to ca. 25 mg/mL by ultrafiltration using an Amicon ultrafilter, flash frozen in liquid N_2 as ca. 25 μL pellets, and stored at -80 ˚C.

Preparation of Solutions. Solution pH or pD was determined at 25 ˚C using an Orion Model 720A pH meter equipped with a Radiometer pHC4006-9 combination electrode that was standardized at pH 4.00 and 7.00 at 25 ˚C. Values of pD were obtained by adding 0.40 to the observed reading of the pH meter.[2]

The exchangeable protons of glycylglycine were exchanged for deuterium by dissolution in D_2O followed by evaporation and extensive drying at 55 ˚C in a vacuum oven. Buffered solutions of glycylglycine (125 mM, 80% or 20% free base) were prepared by dissolving exchanged glyglycine, and NaCl if needed, in D_2O followed by the addition of a measured amount of NaOD to give the desired acid/base ratio at I = 0.1. Buffered solutions of imidazole (60 mM, 50% or 20% free base) were prepared by dissolving the free base and NaCl in D_2O followed by the addition of a measured amount of DCl to give the desired acid/base ratio at I = 0.1. Buffered solutions in D_2O were stored in a dessicator.

The stock solution of uridine 5'-monophosphate (UMP, 100 mM) was prepared by dissolving the disodium salt in D_2O to give a solution at pD 8.2 (I = 0.3). The concentration of UMP was determined from the absorbance in 0.1 M HCl at 262 nm using ε = 10,000 M^{-1} cm^{-1}.[3] The stock solution of 6-aza-uridine 5'-monophosphate (6-aza-UMP, 12 mM) was prepared by dissolving the free acid in D_2O and the pD was adjusted to 7.9 by the addition of small aliquots of 4.3 M NaOD. Stock solutions of orotidine 5'-monophosphate (OMP) in H_2O were prepared by dissolution of the trisodium salt and the concentration of OMP was determined from the

absorbance in 0.1 M HCl at 267 nm using $\varepsilon = 9430\ M^{-1}\ cm^{-1}$.[4] The solutions of OMP, UMP and 6-aza-UMP were divided into small aliquots and stored at -20 ˚C.

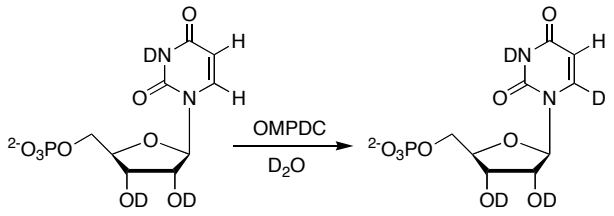
Samples of overexpressed and purified C155S mutant yeast OMPDC that had been stored at -80 ˚C were defrosted and extensively dialyzed at 4 ˚C against several changes of buffer in D_2O. The buffers used were: 125 mM glycylglycine 80% free base, pD 9.3; 125 mM glycylglycine 20% free base, pD 8.1; 60 mM imidazole 50% free base, pD 7.6; 60 mM imidazole 20% free base, pD 7.0. All buffers were at $I = 0.1$ (NaCl).

Enzyme Assays. C155S mutant yeast OMPDC is more stable than, but kinetically and structurally essentially identical with, the wildtype enzyme.[5] The activity of OMPDC in the dialyzed enzyme stock solutions in D_2O and in the deuterium exchange reaction mixtures was determined spectrophotometrically by monitoring the decrease in absorbance at 279 nm due to the decarboxylation of OMP ($\Delta\varepsilon = -2400\ M^{-1}\ cm^{-1}$ at 25 ˚C).[6] Assays in a total volume of 1 mL were conducted in 10 mM MOPS 50% free base, pH 7.1, at 25 ˚C and $I = 0.105$ (NaCl), with 40 - 50 μM OMP (25 - 30K_m). The reaction was initiated by the addition of 1 μL of a dilution of the stock solution of OMPDC or a dilution of the exchange reaction mixture to give a final enzyme concentration of 20 - 30 nM. The concentration of OMPDC in the stock solution or in the exchange reaction mixture was then determined from the value of V_{max} ($M\ s^{-1}$) using a value of $k_{cat} = 15\ s^{-1}$.[6]

Enzyme-Catalyzed Deuterium Exchange into UMP Monitored by ^{1}H NMR Spectroscopy. The deuterium exchange reactions of UMP in D_2O catalyzed by OMPDC at pD 7.0 - 9.3 were followed by ^{1}H NMR spectroscopy at 500 MHz (Scheme 1). Reactions in a volume of 1 - 4 mL were initiated in plastic ware by the addition of an aliquot of UMP in D_2O (100 mM stock, pD 8.2) to the reaction mixture at $I = 0.1$ that contained the appropriate buffer in D_2O, OMPDC in

buffered D_2O (final concentration 0.1 - 0.3 mM, 3 - 9 mg/mL) and NaCl in D_2O to give a final concentration of 2.5 - 10 mM UMP. The reaction mixture was divided into 1 mL aliquots that were placed in small screw-cap plastic centrifuge tubes equipped with O-rings and incubated in a water bath at 25 ˚C. After initiation of the reaction the concentration of OMPDC in the reaction mixture was determined by standard assay as described above. At various times an aliquot was withdrawn, the tube was lightly centrifuged to sediment any particulate matter, and OMPDC was assayed in order to monitor the activity of the enzyme during the reaction. The enzyme-catalyzed deuterium exchange was stopped by the addition of a 2 - 3-fold excess over enzyme of the tight-binding inhibitor 6-aza-UMP (12 mM stock, pD 7.9),[7] and the enzyme-inhibitor complex was removed by ultrafiltration using an Amicon Micron device (10K MWCO). The filtrate was transferred to an NMR tube and the extent of deuterium exchange was determined by ^1H NMR spectroscopy. After NMR analysis the sample was removed from the NMR tube and the pD of the mixture was recorded. There was no significant loss of enzyme activity or change in pD of the reaction mixture during these reactions at 25 ˚C which were followed for up to 12 days.

Scheme 1



***h*-UMP** ***d*-UMP**

Deuterium exchange of the C-6 proton of UMP at pD 7.6 - 9.3 was accompanied by up to 4% hydrolysis of the 5'-phosphoryl group to give uridine. The very slow reactions at pD 7.0 were accompanied by both hydrolysis of the 5'-phosphoryl group to give uridine (up to 19%) and of the glycosidic bond to give uracil (up to 24%). Control experiments conducted in the absence of OMPDC indicated that these side reactions are likely catalyzed by small contaminating enzyme

activities present in the preparation(s) of OMPDC. The rates of these hydrolysis reactions will be unaffected by the presence of deuterium at C-6 of UMP, so that they did not interfere with quantification of the extent of deuterium incorporation into UMP. However, at pD 7.0 in the presence of 2.5 mM UMP the depletion of *total* UMP by these side reactions resulted in an apparent *increase* in the velocity of deuterium exchange reaction (see kinetic analysis below). Therefore the kinetic analysis was restricted to the portion of the reaction that exhibited good first-order kinetics (see Figure S4).

NMR Analyses. ^{1}H NMR spectra at 500 MHz were acquired at 25 ˚C using a Varian Unity Inova 500 spectrometer with a sweep width of 6000 Hz, a 90˚ pulse angle, an acquisition time of 6 s and a total relaxation delay between pulses of 90 s. Values of T_1 for the C-5 and anomeric protons of the product *d*-UMP were determined to be ca. 9 s and 7 s, respectively. Chemical shifts were referenced to HOD at 4.67 ppm. The signals for the C-5 protons of the substrate *h*-UMP and the product *d*-UMP were greatly expanded, accurately phased and subjected to a first-order baseline drift correction before integration. The fractional extent of deuterium incorporation, *f*(*d*-UMP), was generally determined from the integrated area of the two downfield peaks of the double doublet (J = 8.1, 0.5 Hz, coupled to the C-6 and anomeric protons) due to the C-5 proton of *h*-UMP (A_H) and the *combined* integrated areas of the upfield peaks of this signal and the intervening broad doublet ($J \approx 0.5$ Hz, coupled to the anomeric proton) due to the C-5 proton of *d*-UMP (A_{D+H}), according to eq 1. A slightly different procedure was used at pD 8.1 where the signals for the anomeric and C-5 protons are only partially resolved. Separate integration of the small partially resolved doublet due to the C-5 proton of *d*-UMP results in large errors when the extent of deuterium exchange is small, and so this

procedure was not generally used. The fraction of *h*-UMP remaining at time *t* was then calculated as *f*(*h*-UMP) = [1 - *f*(*d*-UMP)].

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

Kinetic Analysis. The *velocity* of formation of deuteriated UMP (*d*-UMP) is given by eq 2, derived for Scheme 2, where k_{ex} (s^{-1}) is the turnover number for the E•*h*-UMP complex to give E•*d*-UMP, $[UMP]_{total}$ = {[*h*-UMP] + [*d*-UMP]} is the *total* concentration of UMP, K_d is the dissociation constant for UMP, and [E] is the concentration of OMPDC. First-order *rate constants* for the deuterium exchange reactions of *h*-UMP, k_{obsd} (s^{-1}), were determined from the slopes of semi-logarithmic plots of the fraction of *h*-UMP remaining against time, according to eq 3 (Table 1 and Figures S1 - S4).

Scheme 2

$$\text{E} + \textbf{\textit{h}-UMP} \underset{K_d}{\rightleftharpoons} \text{E•}\textbf{\textit{h}-UMP} \xrightarrow{k_{ex}} \text{E•}\textbf{\textit{d}-UMP} \overset{K_d}{\rightleftharpoons} \text{E} + \textbf{\textit{d}-UMP}$$

$$d[d\text{-UMP}]/dt = -d[h\text{-UMP}]/dt = \frac{k_{ex}[\text{E}][h\text{-UMP}]_t}{[\text{UMP}]_{total} + K_d} \qquad (2)$$

$$\ln f(h\text{-UMP}) = \ln[1 - f(d - \text{UMP})] = -k_{obsd}t \qquad (3)$$

$$k_{obsd} = \frac{k_{ex}[\text{E}]}{[\text{UMP}]_{total} + K_d} \qquad (4)$$

The *rate constants* k_{obsd} (s^{-1}) for the disappearance of *h*-UMP determined at pD 7.0 - 9.3 and $[UMP]_{total}$ = 2.5 - 10 mM are inversely proportional to $[UMP]_{total}$, which shows that $[UMP]_{total} > K_d$ (eq 4) under these conditions. Rate constants k_{ex} (s^{-1}) for deuterium exchange into *saturating enzyme-bound* UMP at pD 7.0 - 9.3 were calculated from k_{obsd} (s^{-1}) using eq 5 (Table 1).

$$k_{ex} = \frac{k_{obsd}[\text{UMP}]_{total}}{[\text{E}]} \qquad (5)$$

Table S1. Observed First-Order Rate Constants k_{obsd} (s^{-1}) and Values of k_{ex} (s^{-1}) for Exchange for Deuterium of the C-6 Proton of Uridine 5'-Monophosphate Catalyzed by Yeast Orotidine 5'-Monophosphate Decarboxylase (C155S mutant) in D_2O at 25 °C and I = 0.1 (NaCl).[a]

Buffer	pD	$[UMP]_{total}$ (mM)	[E] (mM)	k_{obsd} (s^{-1})	k_{ex} [b] (s^{-1})
100 mM glycylglycine	9.34	2.5	0.110	4.90×10^{-7}	1.15×10^{-5}
		5	0.110	2.48×10^{-7}	
		10	0.166	2.02×10^{-7}	
100 mM glycylglycine	8.13	2.5	0.318	9.58×10^{-7}	7.53×10^{-6}
48 mM imidazole	7.58	2.5	0.284	3.52×10^{-7}	3.19×10^{-6}
		5	0.284	1.87×10^{-7}	
48 mM imidazole	7.03	2.5	0.284	1.22×10^{-7}	1.20×10^{-6}
		5	0.284	7.57×10^{-8}	

[a] The incorporation of deuterium into UMP was monitored by ^1H NMR spectroscopy. [b] The values of k_{ex} at each pD are the average values calculated from the values of k_{obsd}, $[UMP]_{total}$ and [E] using eq 5.

Figure S1. Semi-logarithmic plots of reaction progress against time for the exchange for deuterium of the C-6 proton of uridine 5'-monophosphate catalyzed by yeast OMPDC (C155S mutant) in D_2O buffered by 100 mM glycylglycine at pD 9.34, 25 ˚C and I = 0.1 (NaCl).

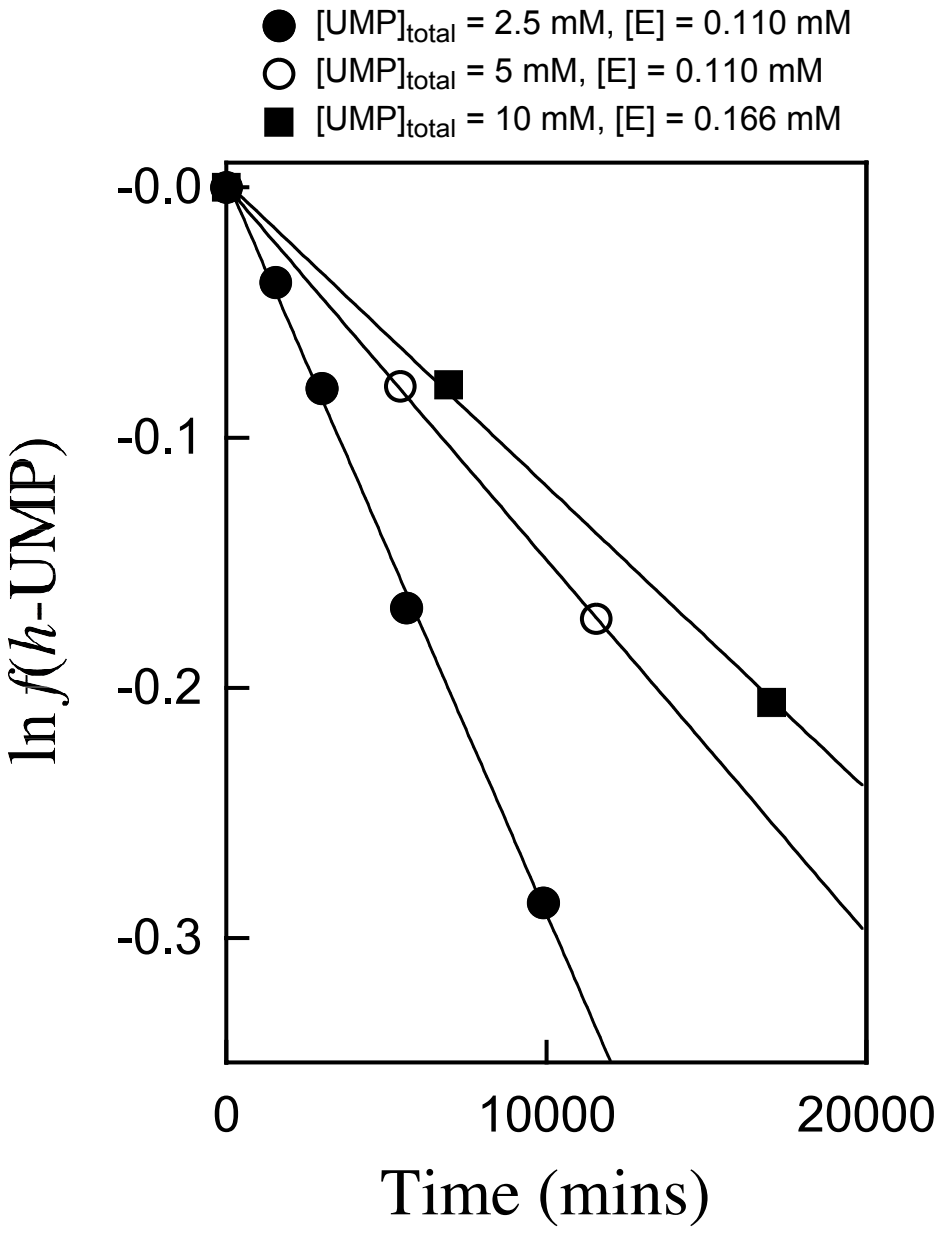


Figure S2. Semi-logarithmic plot of reaction progress against time for the exchange for deuterium of the C-6 proton of uridine 5'-monophosphate catalyzed by yeast OMPDC (C155S mutant) in D_2O buffered by 100 mM glycylglycine at pD 8.13, 25 ˚C and I = 0.1 (NaCl).

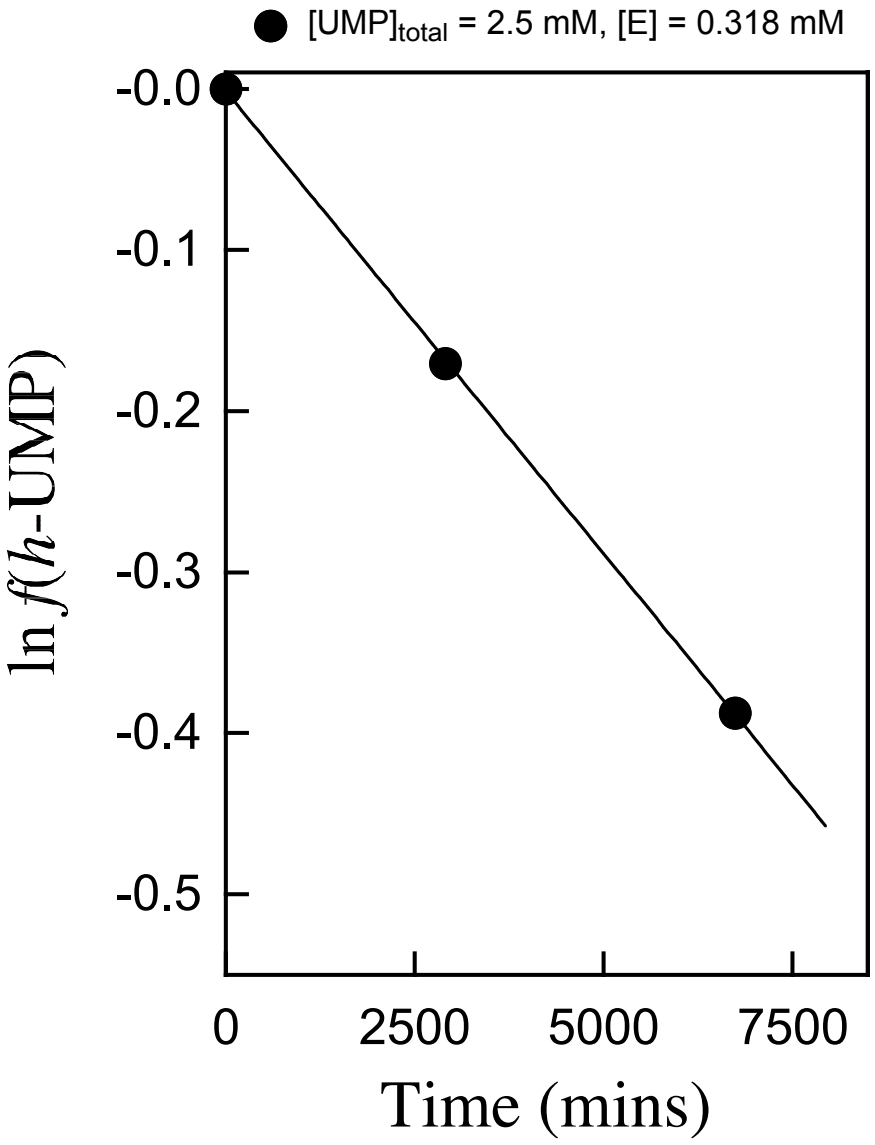


Figure S3. Semi-logarithmic plots of reaction progress against time for the exchange for deuterium of the C-6 proton of uridine 5'-monophosphate catalyzed by yeast OMPDC (C155S mutant) in D_2O buffered by 48 mM imidazole at pD 7.58, 25 ˚C and I = 0.1 (NaCl).

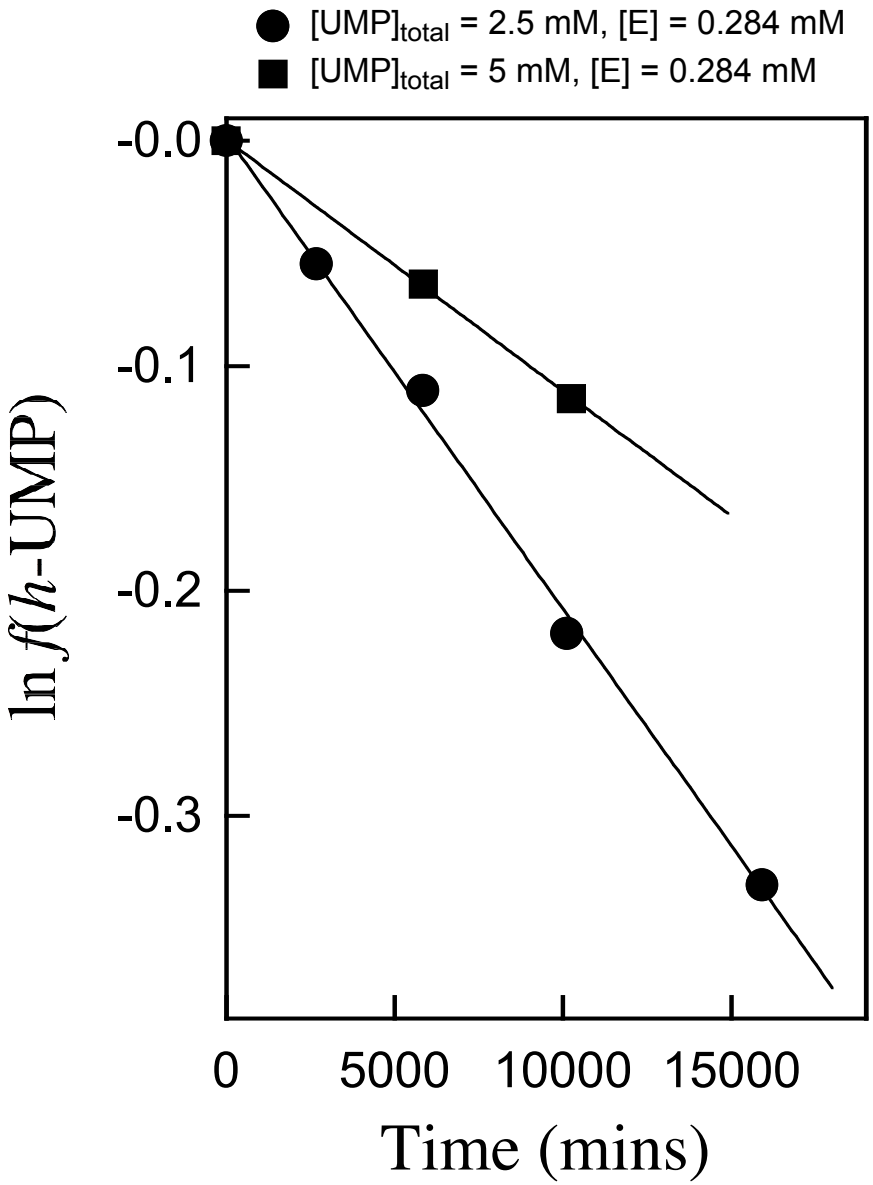
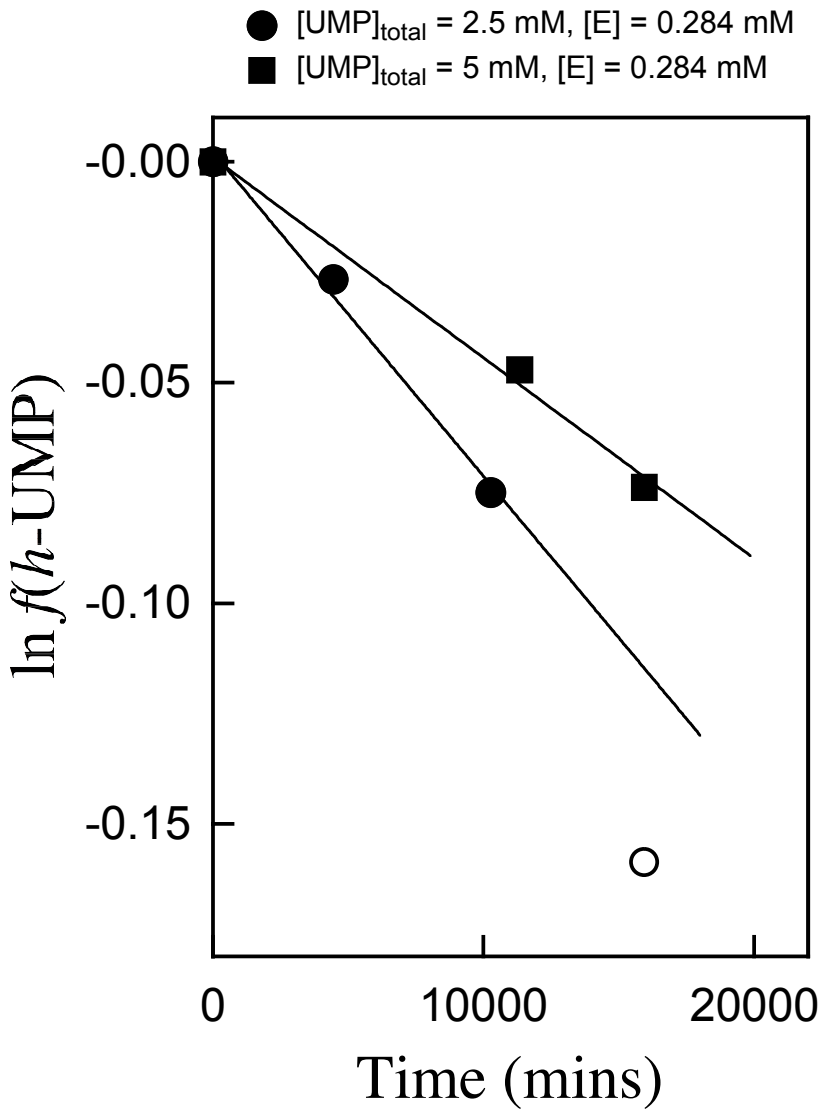


Figure S4. Semi-logarithmic plots of reaction progress against time for the exchange for deuterium of the C-6 proton of uridine 5'-monophosphate catalyzed by yeast OMPDC (C155S mutant) in D_2O buffered by 48 mM imidazole at pD 7.03, 25 ˚C and I = 0.1 (NaCl). The open circle represents an apparent *increase* in the velocity of deuterium exchange as the reaction proceeds. We attribute this to the relatively large extents of hydrolysis of the 5'-phosphoryl group (~ 19%) and of the glycosidic bond (~ 24%) which deplete the pool of total UMP and hence increase the velocity of deuterium exchange (see eq 2).



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