Role of the Carboxylate in Enzyme-Catalyzed Decarboxylation of Orotidine 5′-Monophosphate: Transition State Stabilization Dominates Over Ground State Destabilization

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ABSTRACT: Kinetic parameters $k_{ex}$ ($s^{-1}$) and $k_{cat}/K_{d}$ (M$^{-1}$ s$^{-1}$) are reported for exchange for deuterium in D$_{2}$O of the C-6 hydrogen of 5-fluorooroticine 5′-monophosphate (FUMP) catalyzed by the Q215A, Y217F, and Q215A/Y217F variants of yeast orotidine 5′-monophosphate decarboxylase (ScOMPDC) at pH 8.1, and by the Q215A variant at pH 7.1–9.3. The pH rate profiles for wildtype ScOMPDC and the Q215A variant are identical, except for a 2.5 log unit downward displacement in the profile for the Q215A variant. The Q215A, Y217F and Q215A/Y217F substitutions cause 1.3–2.0 kcal/mol larger increases in the activation barrier for wildtype ScOMPDC-catalyzed deuterium exchange compared with decarboxylation, because of the stronger apparent side chain interaction with the transition state for the deuterium exchange reaction. The stabilization of the transition state for the OMPDC-catalyzed deuterium exchange reaction of FUMP is ca. 19 kcal/mol smaller than the transition state for decarboxylation of OMP, and ca. 8 kcal/mol smaller than for OMPDC-catalyzed deprotonation of FUMP to form the vinyl carbanion intermediate common to OMPDC-catalyzed reactions OMP/FOMP and UMP/FUMP. We propose that ScOMPDC shows similar stabilizing interactions with the common portions of decarboxylation and deprotonation transition states that lead to formation of this vinyl carbanion intermediate, and that there is a large ca. (19–8) = 11 kcal/mol stabilization of the former transition state from interactions with the nascent CO$_{2}$ of product. The effects of Q215A and Y217F substitutions on $k_{cat}/K_{m}$ for decarboxylation of OMP are expressed mainly as an increase in $K_{m}$ for the reactions catalyzed by the variant enzymes, while the effects on $k_{cat}/K_{d}$ for deuterium exchange are expressed mainly as an increase in $k_{ex}$. This shows that the Q215 and Y217 side chains stabilize the Michaelis complex to OMP for the decarboxylation reaction, compared with the complex to FUMP for the deuterium exchange reaction. These results provide strong support for the conclusion that interactions which stabilize the transition state for ScOMPDC-catalyzed decarboxylation at a nonpolar enzyme active site dominate over interactions that destabilize the ground-state Michaelis complex.

INTRODUCTION

Orotidine 5′-monophosphate decarboxylase (OMPDC) catalyzes the decarboxylation of OMP and 5-fluorooroticine 5′-monophosphate (FOMP) to form uridine 5′-monophosphate (UMP) and FUMP, respectively (Scheme 1). The enzyme mechanism of action has been a subject of intense interest because the protein catalys provides an enormous 31 kcal/mol stabilization of the decarboxylation transition state, and a large selectivity in binding the reaction transition state with a higher affinity than the 8 kcal/mol binding energy of substrate OMP.

OMPDC also catalyzes the exchange of the C-6 hydrogen of UMP, or 5-fluorooroticine 5′-monophosphate (FUMP), or the truncated substrate 1-(β-d-erythrofuranosyl)-S-fluororotic acid (FEO) with deuterium from solvent D$_{2}$O. A comparison of the kinetic parameters for enzyme-catalyzed decarboxylation and deuterium exchange shows that OMPDC provides strong stabilization of the common UMP vinyl carbanion reaction intermediate. This is consistent with the conclusion that the first step in the deuterium exchange reaction, deprotonation of enzyme-bound UMP or FUMP (Figure 1), is the reverse of protonation of the vinyl carbanion intermediate of the decarboxylation reaction.

There has been progress toward defining the roles of amino acid side chains at yeast OMPDC (ScOMPDC) in the...
stabilization of the rate-determining decarboxylation transition state.1,9−14 We have focused on the contribution to catalysis of binding interactions between ScOMPDC and the substrate phosphodianion, which drive the large protein conformational change shown in Figure 2, from an inactive flexible form of ScOMPDC to the stiff active form that provides an optimal stabilization of the decarboxylation transition state.13,15−17 This conformational change is driven by interactions of the OMP phosphodianion with the side chains of Q215 and Y217 from a gripper loop, with the guanidine cation side chain of R235 (Figure 2), and by an intraloop clamping interaction between the S154 side chain from the phosphodianion gripper and the pyrimidine umbrella loops.18 The contribution of these side chains to the enzymatic rate acceleration was determined for ScOMPDC-catalyzed decarboxylation of OMP,9,11 FOMP,9,10 and for decarboxylation of the phosphodianion truncated substrates 1-(β-D-erythrofuranosyl)uric acid (EO)13 and 1-(β-D-erythrofuranosyl)-5-fluorourorotic acid (FEO).19 The results show that the S154, Q215, and R235 side chains provide a total 10 kcal/mol stabilization of the transition state for decarboxylation of OMP, but a < 1 kcal/mol stabilization of the transition state for decarboxylation of EO.13 This shows that the observed transition state stabilization is from interactions of the Q215, Y217, and R235 side chains with bound dianions, and that there is no transition state stabilization from interactions with the distant pyrimidine ring.13 The results provide strong evidence that the side chains function to stabilize the closed form of ScOMPDC that shows a high reactivity toward decarboxylation of OMP.15,20

The enzyme conformational change has also been proposed to promote decarboxylation through the induction of electrostatic stress into the substrate carboxylate that is relieved at the decarboxylation transition state,21−23 and by distorting the bond to the substrate carboxylate ~36° out of the plane of the pyrimidine ring.24−27 The results of our earlier studies have provided no evidence for the utilization of phosphodianion binding energy to introduce interactions that destabilize

Figure 1. OMPDC-catalyzed decarboxylation of OMP and FOMP, and deuterium exchange reactions of UMP and FUMP, through common UMP or FUMP vinyl carbanion intermediates.

Figure 2. Representations of the X-ray crystal structures of ScOMPDC from Saccharomyces cerevisiae (ScOMPDC). The left-hand and middle surface structures show, respectively, the open unliganded form of ScOMPDC (PDB entry 1DQW) and the closed form with 6-hydroxyuridine 5′-monophosphate bound (PDB entry 1DQX). The phosphodianion gripper (residues 202−220) and pyrimidine umbrella loops (residues 151−165) are shaded blue, and the side chain from R235 is shaded green in both structures. The right-hand structure (PDB entry 1DQX) shows the interactions of Q215, Y217, and R235 with the phosphodianion of 6-hydroxyuridine 5′-monophosphate, and the clamping interaction between the Q215 side chain and the S154 side chain from the phosphodianion gripper and the pyrimidine umbrella loops.
enzyme-bound OMP at yeast OMPDC, but they have not focused on the evaluation of ground-state effects. The relative binding affinity to ScOMPDC reported for OMP, the product UMP, and for inhibitors are difficult to reconcile with the proposal that the Michaelis complex to OMP is destabilized by interactions with ScOMPDC. For example, the ca. 200-fold weaker binding affinity of product UMP compared with substrate OMP shows that the Michaelis complex to OMP is stabilized by interactions between the enzyme and substrate carboxylate group. The significance of these binding studies has been challenged, and the 50 year old proposal that enzyme-catalyzed decarboxylation is promoted by interactions that destabilize the reaction ground state remains entrenched, despite being strongly disputed. The disputes are largely theoretical in nature, and there have been few experimental studies on this problem.

The role of ground-state destabilization in decarboxylation catalyzed by ScOMPDC is examined here by comparing the effect of amino acid substitutions at diamin gripper side chains Q215 and Y217 (Figure 2) on the kinetic parameters for the enzyme-catalyzed decarboxylation of OMP, where ground state effects are proposed to be important, with kinetic parameters for the deuterium exchange reaction of FUMP (Figure 1) that lacks the $-\text{CO}_2^-$. Substitutions of diamin gripper side chains that introduce destabilizing interactions into bound OMP should reduce ground-state destabilization, and result in decreases in $k_{\text{cat}}$ and in $K_m$ for ScOMPDC-catalyzed decarboxylation, but there can be no ground-state effects on the corresponding kinetic parameters for the ScOMPDC-catalyzed deuterium exchange reactions of FUMP.

We report the effect of Q215A, Y217F and Q215A/Y217F substitutions on the kinetic parameters for ScOMPDC-catalyzed deuterium exchange reactions of FUMP. These results build upon an earlier study of the effect of the R235A substitution. A comparison of the effect of these substitutions on catalysis of the decarboxylation and deuterium exchange reactions shows that each protein substitution results in a (1.3–2.0)-kcal/mol larger increase in the activation barrier to the deuterium exchange compared to decarboxylation reaction. This is surprising, because the ca 19 kcal/mol smaller total stabilization of the deuterium exchange compared with the decarboxylation transition state is consistent with weaker transition state stabilization from interactions with the protein catalyst. Our analysis strongly supports the conclusion that the transition states for ScOMPDC-catalyzed decarboxylation of OMP/FOMP and for depropionation of UMP/FUMP show similar stabilizing interactions with the protein catalyst over the shared portions of these substrates, and differ because OMPDC shows robust binding interactions with the nascent CO$_2$ product at the decarboxylation transition state. This conclusion is generalized to other enzyme-catalyzed decarboxylation reactions, where the first step is transfer of the substrate $-\text{CO}_2^-$ from water to a hydrophobic protein binding pocket.

### EXPERIMENTAL SECTION

**Materials.** Glycglycine (GlyGly, >99%) was obtained from USB. 3-(N-Morpholino)propanesulfonic acid (MOPS, ≥99.5%) was purchased from Fluka. The following deuterium labeled compounds were purchased from Cambridge Isotope Laboratories: D$_2$O (99.9%), DCI (35 wt %, 99.9% D), and NaOD (30 wt %, 98% D). The water was distilled and purified on a Milli-Q water purification system. The triethylammonium salt of FUMP was synthesized as described in earlier work, and was converted to the free acid by passage over Amberlite IR120 resin (H$^+$-form) in methanol. All other chemicals were reagent grade and were used without further purification.

**Preparation of Solutions.** Solution pH and pD were determined at 25°C using an Orion model 720A pH meter equipped with a Radiometer pHC4006-9 combination electrode that was standardized at pH 7.00 and 10.00 at 25°C. The pD of buffers in D$_2$O was obtained by adding 0.4 to the reading on the pH meter. The acidic protons of GlyGly were exchanged for deuterium by dissolving the buffer in D$_2$O, followed by evaporation and drying under vacuum at 55°C. Buffed solutions of imidazole were prepared by dissolving the buffer base in D$_2$O and adjusting to the required pD using DCl. Buffed solutions of MOPS and GlyGly were prepared by dissolving the commercial buffer in D$_2$O and adjusting to the required pD using NaOD.

**Protein Variants of ScOMPDC.** The plasmid pScOMPDC-1bs containing the gene encoding ScOMPDC from Saccharomyces cerevisiae with a N-terminal His$_6$- or His$_{10}$-tag was available from previous studies. The procedures for the preparation of the Q215A, Y217F, and Q215A/Y217F variants were described in earlier work. In all cases the N-terminal His$_6$- or His$_{10}$-tag was removed by the action of thrombin (1 unit/mg ScOMPDC) at room temperature for ca. 16 h, as described in the Supporting Information to ref 14. The protein variants of ScOMPDC were stored at −80°C. These enzymes were thawed and then dialyzed at 7°C against 10 mM MOPS at pH 7.1 and $I = 0.10 (\text{NaCl})$. This was followed by exhaustive dialysis [at least 3 changes in dialysis buffer] at 7°C in D$_2$O against 5–10 mL of the following buffers ($I = 0.1, \text{NaCl}$): pH 7.1, 50 mM imidazole; pH 7.4, 50 mM MOPS; pH 7.7, 50 mM MOPS; pH 8.1, 50 mM GlyGly; pH 9.3, 50 mM GlyGly. The dialysis was with a D-tube dialyzer (10 kDa MWCO, Novagen) placed inside a narrow vessel that was isolated from atmospheric moisture using paraflin. The concentration of stock solutions of protein variants of ScOMPDC was determined from the absorbance at 280 nm using an extinction coefficient of 29900 M$^{-1}$ cm$^{-1}$, calculated using the ProtParam tool available on the ExPaSy server. The activity of these protein variants was determined by monitoring the decrease in absorbance at 279 nm during the enzyme-catalyzed decarboxylation of OMP. The exchange of the C-6 proton of FUMP for deuterium from solvent D$_2$O catalyzed by Q215A, Y217F, Q215A/Y217F variants of ScOMPDC at 25°C and I = 0.1 (NaCl) was monitored by following formation of deuterium labeled product (d-FUMP). The reaction mixtures (1–2 mL in D$_2$O) were prepared by mixing the stock enzyme solution with the appropriate buffer and NaCl to give the desired enzyme, buffer and salt concentrations. The reactions were initiated by the addition of FUMP in D$_2$O. The following are the final reaction solutions for the Q215A variant at (I = 0.1, NaCl): pH 7.1, 50 mM imidazole, 80–120 μM ScOMPDC at 1.0–10 mM FUMP; pH 7.4, 50 mM MOPS, 40–100 μM ScOMPDC at 0.75–7.5 mM FUMP; pH 7.7, 50 mM MOPS, 20–80 μM ScOMPDC at 0.50–7.1 mM FUMP; pH 8.1, 50 mM GlyGly, 20–60 μM ScOMPDC at 0.48–7.5 mM FUMP; pH 9.3, 50 mM GlyGly, 15–100 μM ScOMPDC at 0.25–7.5 mM FUMP. The final solutions for the other protein variants were at pH 8.1 (50 mM GlyGly, $I = 0.10 (\text{NaCl})$); Y217A variant, 10–20 μM ScOMPDC at 0.75–7.5 mM FUMP; Q215A/Y217A variant, 50–100 μM ScOMPDC at 2.5–5.0 mM FUMP. At timed intervals aliquots of 100–500 μL were withdrawn from the reaction solutions and quenched with 20 μL of neat HCO$_2$D. The ScOMPDC was removed by ultrafiltration using an Amicon Ultrafiltration device (10K MWCO). The volume of the filtrate was adjusted to 700 μL in D$_2$O and transferred to an NMR tube for analyses. The reactions were followed for up to several days, during which time no decrease (<10%) in enzyme activity was observed. For selected reactions at high [FUMP] the pD of the filtrate was determined, and in all cases was within 0.1 unit of the starting pD.

The $^{19}$F NMR spectra were obtained as described in previous work, and the chemical shifts referenced to the value of $-78.5$ ppm for a neat solution of the trifluoroacetic acid external standard. The integrated peak areas for the doublet with area (A$_D$) at −165.36 ppm for h-FUMP and the broad upfield-shifted apparent singlet with area (A$_S$) at −165.66 ppm for d-FUMP were recorded, and the initial reaction
velocity \( v_i \) for enzyme-catalyzed conversion of up to 10% of \( h\)-FUMP to \( d\)-FUMP was determined as the slopes of linear plots of 3 or 4 values of \( f_f/|FUMP|_o \) against time during disappearance of 3–10% of total \( h\)-FUMP, where \( f_f = A_f/(A_f + A_o) \) is the fraction of total FUMP labeled with deuterium at the C-6 position, and \(|FUMP|_o\) is the initial concentration of \( h\)-FUMP.

\[
v_i = \left( \frac{d(f_f/|FUMP|_o)}{dt} \right)
\]

(1)

## RESULTS

The ScOMPDC-catalyzed deuterium exchange reactions of FUMP were monitored by \(^{19}\)F NMR spectroscopy at 470 MHz, as described in an earlier study.\(^{14}\) Figure 3 shows the dependence of \( v_i/|E| \) on \(|FUMP|\) at several different pD and a constant ionic strength of 0.1 (NaCl). The data from Figure 4A,B show the dependence of \( v_i/|E| \) on \(|FUMP|\) at several different pD and ionic strengths of 0.1 (NaCl). The data from Figures 3 and 4A were fit to eq 2, derived for Scheme 2, to give values for the kinetic parameters \( k_{cat} \) and \( K_m \). The data from Figure 4B were fit to a linear form of eq 2 \((K_m > |FUMP|)\) to give the values of \( k_{cat}/K_m \). Table 1 reports these kinetic parameters at pD 8.1, and previously determined values of \( k_{cat} \) and \( K_m \) for wild type and variant forms of ScOMPDC-catalyzed decarboxylation of OMP at pH 7.1.\(^{14}\) The parameter \( K_d \) (Scheme 2) is treated as a thermodynamic dissociation constant, because formation of the Michaelis complex to \( h\)-UMP is effectively reversible with respect to its slow \((k_{ex} \leq 10^{-2} \text{ s}^{-1}, \text{Table 1})\) conversion to \( d\)-UMP.\(^{6}\) The binding of OMP to ScOMPDC is partly irreversible for decarboxylation catalyzed by the wild type enzyme \((k_{cat} = 16 \text{ s}^{-1})\).\(^{24}\) The parameter \( K_m \) is used here to describe the stability of the Michaelis complex. The values of the kinetic parameters for Q215A variant ScOMPDC-catalyzed deuterium exchange reactions of FUMP at several different pD are reported in Table 2, along with previously determined values of the kinetic parameters for the wild type ScOMPDC-catalyzed deuterium exchange reactions.\(^{6}\)

\[
v_i = \frac{k_{ex}[h-FUMP]_o}{|E|[h-FUMP]_o + K_d}
\]

(2)

## DISCUSSION

Figure 5A,B show pD rate profiles for \( k_{cat}/K_d \) (M\(^{-1}\) s\(^{-1}\)) and \( k_{ex} \) (s\(^{-1}\)), respectively, for wild type and Q215A variant ScOMPDC-catalyzed deuterium exchange reactions at C-6 of FUMP in D\(_2\)O, constructed using data from Table 2. There is a constant difference of \( \Delta \Delta G^\circ = 3.45 \pm 0.15 \text{ kcal/mol} \) (Table 2) between the activation barriers for the deuterium exchange reactions catalyzed by wild type and Q215A variant ScOMPDC.\(^{6}\) This shows that the Q215A substitution has no detectable effect on the apparent pK\(_a\) of the active site side chains that govern the shape of these pD profiles. However, the downward break at low pD is not well-defined for the pD profile of \( k_{cat} \) values for the Q215A variant, because we were unable to obtain kinetic parameters for this slow deuterium exchange reaction at pD = 6.45. The kinetic data for the pD rate profiles for wild type and Q215A variant ScOMPDC-catalyzed deuterium exchange reactions at C-6 of FUMP in D\(_2\)O reported in Figure 5A,B were fit to the kinetic Scheme previously described in a study on the exchange reaction catalyzed by wildtype OMPDC, to give the same apparent values for the ionization constants of enzyme catalytic side chains.\(^{6}\)

ScOMPDC-Catalyzed Decarboxylation and Deuterium Exchange Reactions: Relative Rate Acceleration and Transition State Stabilization. The rate acceleration for the decarboxylation of OMP catalyzed by ScOMPDC at a standard state of 1 M OMPDC is 4 \( \times \) 10\(^{22}\)-fold. This is the ratio of \( k_{cat}/K_m = 1.1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1} \) for decarboxylation at the pH optimum of 7.1,\(^{28,51}\) and an estimated \( k_{ex} = 2.8 \times 10^{-16} \text{ s}^{-1} \) for uncatalyzed

### Scheme 2. Kinetic Mechanism for OMPDC-Catalyzed Deuterium Exchange

\[
E + h-FUMP \quad \underset{K_d}{\overset{k_{ex}}{\rightleftharpoons}} \quad E \cdot h-FUMP \quad \overset{k_{cat}}{\rightarrow} \quad E \cdot d-FUMP
\]

### Figure 3. Dependence of \( v_i/|E| \) on \(|FUMP|\) for Q215A variant ScOMPDC-catalyzed deuterium exchange reactions at I = 0.1 (NaCl).

### Figure 4. Dependence of \( v_i/|E| \) on \(|FUMP|\) for ScOMPDC-catalyzed deuterium exchange reactions at pD 8.1 (50 mM GlyGly) and I = 0.1 (NaCl). (A) Y217F variant; (B) Q215A/Y217A variant.
nonenzymatic decarboxylation of OMP in water. This corresponds to \((\Delta G^\circ_{\text{de}})^e = 31 \text{ kcal/mol}\) for the stabilization of the transition state for nonenzymatic decarboxylation. The second-order rate constant of \(k_{\text{iso}} = 0.71 \text{ M}^{-1} \text{s}^{-1}\) for HO\(^-\) catalyzed deprotonation of FUMP gives \(k_{\text{iso}} = 0.71 \text{ M}^{-1} \text{s}^{-1}[1.3 \times 10^{-5} \text{ M}] = 9 \times 10^{-8} \text{ s}^{-1}\) for nonenzymatic deprotonation of FUMP at pH 7.1. Combining this with \(k_{\text{ex}}/K_d = 33 \text{ M}^{-1} \text{s}^{-1}\) for the deuterium exchange reaction of FUMP catalyzed by ScOMPDC at pH 7.1, gives an enzymatic rate acceleration of \(4 \times 10^7\) fold and a transition state stabilization of \((\Delta G^\circ_{\text{ex}})^e = 12 \text{ kcal/mol}\). The S-F of FUMP results in similar 5.0 and 4.8 kcal/mol stabilization of the transition states for DO\(^+\) and ScOMPDC-catalyzed deprotonation of UMP, so that ScOMPDC provides a similar stabilization of the transition states for the two nonenzymatic reactions. This 12 kcal/mol transition state stabilization is 19 kcal/mol smaller than the 31 kcal/mol transition state stabilization estimated for ScOMPDC-catalyzed decarboxylation of OMP. [This 19 kcal/mol transition state stabilization is calculated from a comparison of rate constants for the enzymatic and nonenzymatic deprotonation reactions determined at pH 7.1. No attempt has been made to correct for the uncertain deuterium isotope effects on these reactions. By comparison, an estimate of 17 kcal/mol for this transition state stabilization was reported in an earlier paper, using the second order rate constant of \(k_{\text{ex}} = 2300 \text{ M}^{-1} \text{s}^{-1}\) for OMPDC with the catalytic side chains in their most reactive protonation states. The 2 kcal/mol difference between these two estimates does not affect the conclusion that OMPDC provides a substantially greater stabilization of the decarboxylation compared with the proton transfer transition state.]

This analysis demonstrates a large difference in the stabilization of the transition states for OMPDC-catalyzed reactions of FUMP at pH 7.1 and Decarboxylation Reactions of OMP at pH 7.1.

### Table 1. Kinetic Parameters at 25 °C for Wild Type and Variant Forms of ScOMPDC-Catalyzed Deuterium Exchange Reactions of FUMP at pD 8.1 and Decarboxylation Reactions of OMP at pH 7.1

| enzyme         | FUMP \(k_\text{ex} (s^{-1})\) | FUMP \(k_d (\text{mM})\) | FUMP \(k_{\text{ex}}/K_d (\text{M}^{-1} \text{s}^{-1})\) | OMP \(k_{\text{ex}} (s^{-1})\) | OMP \(K_d (\text{M})\) | OMP \(k_{\text{ex}}/K_d (\text{M}^{-1} \text{s}^{-1})\) |
|----------------|-------------------------------|--------------------------|-----------------------------------------------|-------------------------------|--------------------------|-----------------------------------------------|
| WT             | 4.40 × 10^{-2}                | 0.11                     | 400                                           | 15                            | 1.40 × 10^{-6}            | 1.10 × 10^{0}                                |
| Q215A          | (4.3 ± 0.08) × 10^{-4}        | 0.42 ± 0.03              | 1.0                                           | 24                            | 0.94 ± 10^{-4}            | 2.60 × 10^{0}                                |
| Y217F          | (2.3 ± 0.14) × 10^{-3}        | 5.8 ± 0.15               | 0.40                                          | 20                            | 1.10 ± 10^{-4}            | 1.80 × 10^{0}                                |
| Q215A/Y217F    | (3.9 ± 0.1) × 10^{-3}         | 1.90 × 10^{-3}           | 4.8                                           | 4.8                            | 1.40 × 10^{-3}            | 3.40 × 10^{0}                                |
| R235A          | 9.30 × 10^{-6}                | 5.00 × 10^{-3}           | 1.0                                           | 1.0                            | 1.10 × 10^{-3}            | 9.10                                        |

### Table 2. Kinetic Parameters at 25 °C for Deuterium Exchange Reactions of FUMP Catalyzed by Q215A Variant ScOMPDC at Several pD

| pD  | \(k_{\text{ex}} (s^{-1})\) | \(K_d (\text{mM})\) | \(k_{\text{ex}}/K_d (\text{M}^{-1} \text{s}^{-1})\) | \(\Delta G^\circ_{\text{ex}}\) kcal/mol |
|-----|--------------------------|---------------------|-----------------------------------------------|-----------------------------------------------|
| 7.1 (7.03) | (2.7 ± 0.1) × 10^{-4} | 2.00 ± 0.2 | 0.13                                           | 1.70 × 10^{-2}                             | 0.51                           | 33                            | 3.30                                      |
| 7.4 (7.37) | (3.6 ± 0.1) × 10^{-4} | 0.70 ± 0.08 | 0.51                                           | 2.40 × 10^{-2}                             | 0.17                           | 140                           | 3.30                                      |
| 7.7 (7.64) | (4.1 ± 0.08) × 10^{-4} | 0.38 ± 0.04 | 1.08                                           | 3.20 × 10^{-2}                             | 0.11                           | 300                           | 3.30                                      |
| 8.1 (8.15) | (4.3 ± 0.08) × 10^{-4} | 0.42 ± 0.03 | 1.02                                           | 4.40 × 10^{-2}                             | 0.11                           | 400                           | 3.50                                      |
| 9.3 (9.33) | (3.0 ± 0.1) × 10^{-4} | 0.54 ± 0.03 | 0.56                                           | 3.80 × 10^{-2}                             | 0.19                           | 200                           | 3.60                                      |

Figure 5. Logarithmic rate profiles of kinetic parameters for ScOMPDC-catalyzed deuterium exchange for deuterium of the C-6 proton of FUMP in D\(_2\)O at 25 °C and I = 0.1 (NaCl). (A) Second-order rate constants \(k_{\text{ex}}/K_d (\text{M}^{-1} \text{s}^{-1})\) for reactions catalyzed by wild type ScOMPDC (○) and the Q215A variant (▲). (B) First order rate constants \(k_{\text{ex}} (s^{-1})\) for reactions catalyzed by wild type ScOMPDC (○) and the Q215A variant (▲). The solid lines show that fits of these data to the kinetic Scheme described previously for the reaction catalyzed by wild type OMPDC.
decarboxylation and deuterium exchange reactions. We view the 19 kcal/mol estimate for this difference as qualitative rather than quantitative because of uncertainties in the estimates of $k_{\text{non}}$ obtained by the long extrapolation of rate date to 25 °C. This difference in transition state stabilization for these two reactions reflects one interaction that provides specific stabilization of the transition state for decarboxylation of OMP, and a second interaction that destabilizes the transition state for the deuterium exchange reaction compared with direct deprotonation of DUMP.

(1) There is little or no activation barrier for addition of CO$_2$ to the vinyl carbanion intermediate of OMPDC-catalyzed decarboxylation, so that the reaction transition state is early for CO$_2$ addition and late for the reverse cleavage reaction. It is possible that the barrier for “CO$_2$ reversion” is so small that some type of motion of CO$_2$ away from the vinyl carbanion is rate determining for decarboxylation, in which case CO$_2$ will be fully formed at the rate-determining transition state. This late CO$_2$-like transition state for decarboxylation is stabilized by binding interactions with the CO$_2^--$CO$_2^-$ of OMP at the hydrophobic binding pocket described below, but CO$_2$ is not present during OMPDC-catalyzed deuterium exchange reaction of FUMP. The values of $K_d = 6-36$ μM for CO$_2$ that have been reported for fixation of carbon dioxide catalyzed by D-ribulose-1,5-bisphosphate carboxylase (RuBisCO) correspond to a 6-7 kcal/mol CO$_2$ binding energy. This observed CO$_2$ binding energy will underestimate the intrinsic CO$_2$ binding energy, by the binding energy utilized to reduce the translational and rotational entropy of solvated CO$_2$ upon transfer to the active site of RuBisCO. Entropic costs to the reaction of free CO$_2$ of (3-7) kcal/mol may be estimated from comparisons of the kinetic parameters for reactions of whole phosphodiantion substrates and [phosphate + truncated substrate] pieces for several enzymatic reactions. This sets 9-14 kcal/mol as the range of stabilization for the decarboxylation transition state from interactions between OMPDC and the nascent CO$_2$.

(2) The barrier to $k_{\text{rot}}$ for C–N bond rotation at $-CH_2-NL_3^+$ of the K93 side chain, which exchanges the positions of
ammonium cation hydrons, is added to the barrier for the deuterium exchange but not the decarboxylation reaction (Figure 7). This is demonstrated by the observation that the deuterium enrichment of the products of OMPDC-catalyzed decarboxylation of OMP or FOMP in 50/50 H2O/D2O is equal to the 50% enrichment of solvent. The observed deuterium isotope effect (PDIE) of 1.06 was expected selectivity for the reaction of the primary D with -D from a primary D is not observed, because bond rotation by k_{rot} which would allow for this selection is so slow relative to protonation of the deuterium side chain side. Note that k_f from Figure 7 is the rate constant for the microscopic reverse of substrate deprotonation (k_{dp}) and that the fractionation factor for hydron transfer between LOH - C-NL3 is 1.06. The position of the side chains for Asp-91, Lys-93, and Asp-96 revealed by the X-ray crystal structure of OMPDC-catalyzed reactions with BMP (Figure 6) provides strong evidence that the -CH2- group of Lys-93 is immobilized by hydrogen bonds to the carboxylate groups of Asp-91 and Asp-96 (Figure 7), so that k_{rot} ≪ 10^{11} s^{-1} for unhindered bond rotation and ΔΔG^m = ΔΔG^m_{dp} = ΔΔG^m_{rot} where ΔΔG^m_{dp} and ΔΔG^m_{rot} are the entropic stabilization of the transition states for OMPDC-catalyzed deprotonation and deuterium exchange reactions of FUMP, relative to a common barrier for nonenzymatic deprotonation of FUMP in water.

We estimate, crudely, that the requirement for cleavage of hydrogen bonds to allow side-chain rotation results in an increase in the barrier to deuterium exchange of ca. 4 kcal/mol for the transition state stabilization for all interactions is only (4–50)-fold smaller than K_e for variant OMPDC-catalyzed reactions (Table 1). We conclude that these protein substitutions act to destabilize the Michaelis complex to OMP compared to FUMP, instead of the relative stabilization of the Michaelis complex to OMP predicted for amino acid substitutions that relieve destabilizing interactions between the protein and the carboxylate of substrate OMP.

| enzyme | ΔΔG^m_{OMP} kcal/mol | ΔΔG^m_{FUMP} kcal/mol | [ΔΔG^m_{FUMP} − ΔΔG^m_{OMP}] kcal/mol |
|--------|----------------------|-----------------------|----------------------------------------|
| Q215A | 2.2                  | 3.5                   | 1.3                                    |
| Y217F | 2.4                  | 4.1                   | 1.7                                    |
| Q215A/| 4.8                  | 6.8                   | 2.0                                    |
| Y217F |                                                    |
| R235A | 5.6                  | 7.2                   | 1.6                                    |

*Data from ref 12. The effect of the amino acid substitutions on the activation barrier ΔG^b for k_{rot}/K_e for OMPDC-catalyzed decarboxylation of OMP, or on k_{rot}/K_e for OMPDC-catalyzed exchange of the C-6 H of FOMP for deuterium, calculated from data in Table 1.*

The protein catalyst over the shared portions of these substrates (Figure 6), and differ largely or entirely because OMPDC shows robust binding interactions with nascent CO2 at the decarboxylation transition state.

Table 3. Effect of Q215A, Y217F, and R235A Substitutions on the Activation Barriers for OMPDC-Catalyzed Decarboxylation of OMP at pH 7.1 and for Exchange of the C-6 Hydrogen of FUMP for Deuterium in D2O at pH 8.1
side chains of ScOMPDC over the shared portions of these reactants.

We suggest the following explanation for the larger effects of amino acid substitutions on \( \Delta \Delta G_{\rm FUMP}^\text{Sc} \) compared with \( \Delta \Delta G_{\text{OMP}}^\text{Sc} \) (Table 3). The single Q215A, Y217F and R235A substitutions at wildtype OMPDC result mainly in the loss of stabilizing interactions with the excised side chain, and in minimal changes in the interactions of peripheral side chains at the structured decarboxylation transition state,\(^{9,11}\) so that \( \Delta \Delta G_{\text{UMP}} \) provides an estimate for the excised interactions. (ii) Elimination of tightly bound CO\(_2^-\)/CO\(_2\) from this transition state loosens interactions of the remaining side chains at the transition state for the D-exchange reaction of FUMP. Single amino acid substitutions of ScOMPDC further erode these interactions, and this results in elevated values for \( \Delta \Delta G_{\text{FUMP}}^\text{Sc} \) compared with \( \Delta \Delta G_{\text{OMP}}^\text{Sc} \). In other words, the value of \( \Delta \Delta G_{\text{FUMP}}^\text{Sc} \) for the single variants reflects both the loss of the stabilizing interactions of the excised side chain, plus a weakening of transition state stabilization by the remaining side chains.\(^{12}\)

**Interactions between OMPDC and \(-\text{CO}_2^-/\text{CO}_2\).** The CO\(_2\) binding pocket at OMPDC was identified by analysis of the X-ray crystal structure of human OMPDC (HsOMPDC) liganded by UMP.\(^{27}\) This structure of HsOMPDC shows good superposition (Figure 9), of active site side chains, with the structure of ScOMPDC complexed to 6-aza uridine 5'-monophosphate (6-azaUMP) and small differences in the orientation of the pyrimidine rings.\(^{69}\) The hydrophobic CO\(_2\) binding pocket lies on the opposite side of the pyrimidine ring from the essential K314 (K93 at ScOMPDC) and is lined by the hydrophobic side chains of F310, I401, and I448 (F89, I183, and I232 at ScOMPDC).\(^{6,27}\) The carboxylate side chain of D312/D91 is close to the position of a hypothetical OMP carboxylate, so that the interactions of these carboxylates might destabilize the Michaelis complex to substrate. However, there is an overall 3 kcal/mol stabilization of the Michaelis complex to OMP compared to UMP\(^{30}\) and, as discussed above, an even larger ca. 11 kcal/mol stabilization of the transition state for decarboxylation of OMP (\(k_{dp}\)) compared with deprotonation of UMP (\(k_{dp}\)) to form enzyme-bound UMP vinyl carbanions (Figure 8).

The transfer of carboxylate substrates from an aqueous to a nonpolar solvent results in large increases in the rate constants for nonenzymatic decarboxylation.\(^{33,34,36,37}\) These rate accelerations have been attributed to ground-state effects, where
desolvation of the carboxylate group that accompanies this transfer results in an increase in the substrate reactivity toward decarboxylation.\textsuperscript{35} The transfer from an aqueous to an organic solvent is mimicked by the binding of decarboxylation substrates, including OMP, to hydrophobic binding pockets at enzymes which catalyze decarboxylation reactions.\textsuperscript{36,42–46,60,71}

In one case the polarity of the active site of a thiamine pyrophosphate dependent yeast pyruvate decarboxylase was estimated from the medium-dependent fluorescence wavelength maximum of bound thiochrome diphosphate to be similar to solvents with dielectric constants of 13–15.\textsuperscript{72}

We propose an alternative model, where the dominant effect of the change in medium polarity on the activation barrier for enzymatic and nonenzymatic decarboxylation reactions is stabilization of the late CO\textsubscript{2}-like decarboxylation transition state by interactions with the hydrophobic solvent for nonenzymatic decarboxylation, or with the hydrophobic enzyme binding cavity. In the case of OMPDC, any increase in \( K_m \) for decarboxylation of OMP and FOMP from the requirement for desolvation of CO\textsubscript{2} during substrate binding is smaller that the increase in \( k_{cat} \) from transition state stabilization by interactions with the nascent CO\textsubscript{2} at the hydrophobic CO\textsubscript{2} binding pocket (Figure 9). We estimate that the net effect of these interactions at the hydrophobic binding pocket is a 10\textsuperscript{4}-fold increase in the kinetic parameter \( k_{cat}/K_m \) from the 11 kcal/mol stabilizing interactions between OMPDC and the nascent CO\textsubscript{2}.

The binding of OMP to inactive protein variants of OMPDC results in distortion of the bond to substituents at C-6 from planarity with respect to the pyrimidine ring.\textsuperscript{25–27,73,74} For example, the D312N variant of HsOMPDC shows a 36° distortion of the bond to the substrate carboxylate. Similar out of plane distortions have been reported for the –CN group of 6-cyanouridine S'-monophosphate bound to ScOMPDC and for C-6 substituents at other pyrimidine nucleotides bound to HsOMPDC.\textsuperscript{25,73,74} However, there is no apparent destabilization of this Michaelis complex by substituents at C-6.\textsuperscript{28} We suggest that relatively little bond angle strain energy is associated with these distortions. The distortions appear to track the early stages of a decarboxylation reaction coordinate, where the acidic K93 side chain approaches one face of the pyrimidine ring as CO\textsubscript{2} is lost from the opposite face. However, the product deuterium isotope effect of 1.0 for OMPDC-catalyzed decarboxylation of OMP and FOMP in 50/50 H\textsubscript{2}O/D\textsubscript{2}O shows that the hydron provides no electrophilic push to the loss of CO\textsubscript{2} that is characteristic of a fully coupled-concerted electrophilic displacement reaction.\textsuperscript{62,63}

**Evolution of Hydrophobic Binding Pockets at Decarboxylases.** Hydrophobic binding pockets at decarboxylases mimic hydrophobic solvents that strongly accelerate non-enzymatic decarboxylation relative to water.\textsuperscript{35,34,37} The observation of these binding pockets promoted the proposal that enzymatic rate accelerations for decarboxylation are promoted by destabilization of the enzyme-bound carboxylate, which moves the energy of the reaction ground state closer to the decarboxylation transition state.\textsuperscript{35} This proposal has been criticized\textsuperscript{10,14} and has not received a large amount of direct experimental support.

It is important to emphasize that there is no pressure early in enzyme evolution to select for catalysts that introduce destabilizing interactions into decarboxylation substrates, which are relieved at the decarboxylation transition state, since such ground state interactions have no effect on the kinetic parameter \( k_{cat}/K_m \) for the overall efficiency of the catalyst.\textsuperscript{35} Pressure for enzymatic specificity in transition state binding will only be observed as enzymatic catalysis becomes so efficient that the expression of the large transition state binding energy in substrate or product binding results in rate determining ligand binding steps.\textsuperscript{7,20,75} We propose that the evolutionary pressure results in the selection of nonpolar binding pockets that provide optimal stabilization of late CO\textsubscript{2}-like decarboxylation transition states. Once this transition state stabilization from CO\textsubscript{2} binding interactions has been optimized, it becomes impossible to select for destabilizing interactions between the protein and the substrate carboxylate that do not result in a decrease in \( k_{cat}/K_m \) by perturbing the preoptimized hydrophobic interactions. In the case of OMPDC, rate-determining substrate binding or product release is avoided through utilization of a substantial fraction of the 31 kcal/mol intrinsic substrate binding energy to drive a thermodynamically favorable enzyme conformational change from the flexible open form, to the stiff Michaelis complex.\textsuperscript{7,6,77}

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**ABBREVIATIONS**

OMPDC, orotidine 5'-monophosphate decarboxylase; OMP, orotidine 5'-monophosphate; UMP, uridine 5'-monophosphate; FOMP, 5-fluoroorotidine 5'-monophosphate; FUMP, 5-fluorouridine 5'-monophosphate; E0, 1-(β-D-erythrofuranosyl)orotic acid; EU, 1-(β-D-erythrofuranosyl)-uracil; FEO, 1-(β-D-erythrofuranosyl)-5-fluoroorotic acid; FEU, 1-(β-D-erythrofuranosyl)-5-fluouracil; MOPS, 3-(N-morpholino)propanesulfonic acid; GlyGly, glycyl glycine; AP, alkaline phosphatase.

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