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OMP Decarboxylase: Phosphodianion Binding Energy is Used to Stabilize a Vinyl Carbanion Intermediate

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Abstract

Orotidine 5′-monophosphate decarboxylase (OMPDC) catalyzes the exchange for deuterium from solvent D_2O of the C-6 proton of 1-(β-D-erythrofuranosyl)-5-fluorouracil (**FEU**), a phosphodianion truncated product analog. The deuterium exchange reaction of **FEU** is accelerated 1.8×10^4-fold by 1 M phosphite dianion (HPO_3^{2-}). This corresponds to a 5.8 kcal/mol stabilization of the vinyl carbanion-like transition state, which is similar to the 7.8 kcal/mol stabilization of the transition state for OMPDC-catalyzed decarboxylation of a truncated substrate analog by bound HPO_3^{2-}. These results show that the intrinsic binding energy of phosphite dianion is used in stabilization of the vinyl carbanion-like transition state common to the decarboxylation and deuterium exchange reactions.

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**, Scheme 1A) to give uridine 5′-monophosphate (**UMP**).[1–3] Several mechanisms utilizing different types of covalent or Brønsted acid catalysis by catalytic side chains have been proposed as pathways to avoid formation of the unstable **UMP** carbanion intermediate of a direct decarboxylation reaction (Scheme 1A). However, it is now known that OMPDC effectively catalyzes the exchange of the C-6 proton of **UMP** (Scheme 1B, X = H)[4] and of 5-fluorouridine 5′-monophosphate (**FUMP**, Scheme 1B, X = F)[5] for deuterium from solvent D_2O. The kinetic data for the enzymatic deuterium exchange reaction of **UMP** show that OMPDC stabilizes a bound **UMP** carbanion intermediate relative to **UMP** by at least 14 kcal/mol, compared to the proton transfer reaction in water.[4] This provides compelling evidence that the decarboxylation reaction also proceeds through the same enzyme-stabilized **UMP** carbanion intermediate (Scheme 1B).

The interactions between OMPDC and the phosphodianion group of **OMP** provide a large 12 kcal/mol stabilization of the transition state for enzyme-catalyzed decarboxylation.[6] A part of this total 12 kcal/mol of transition state binding energy is utilized in stabilization of the Michaelis complex. However, the binding of exogenous phosphite dianion (HPO_3^{2-}) to OMPDC results in an 8×10^4-fold increase in k_{cat}/K_m for enzyme-catalyzed decarboxylation of the truncated substrate 1-(β-D-erythrofuranosyl)orotic acid (**EO**) that lacks a 5′-phosphodianion moiety (Scheme 2A). This shows that the phosphodianion binding

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Supporting Information Available: Experimental procedures for the synthesis of **FEU** and the kinetic protocols and pD titrations, two Tables of values of k_{obsd} and $(k_{cat}/K_m)_{obsd}$ for the reactions of **FEU** and **EO**, and a graph similar to that in Figure 2 showing the phosphite activation of the OMPDC-catalyzed decarboxylation of **EO** in D_2O. This material is available free of charge via the Internet at http://pubs.acs.org.

interactions do not simply anchor **OMP** to OMPDC, but rather that they are also *utilized* to activate OMPDC towards catalysis of the decarboxylation reaction.[6] Phosphite dianion provides similar activation of the enzyme-catalyzed reactions of truncated substrates for the proton transfer reaction catalyzed by triosephosphate isomerase,[7] and the hydride transfer reaction catalyzed by glycerol 3-phosphate dehydrogenase.[8] This transmission of binding energy from a nonreactive binding determinant to a distant reaction center is a special property of enzymatic catalysis.[9,10] It has not yet been mimicked in the *de novo* design of protein catalysts, in part because the mechanisms for this utilization of binding energy are not fully understood.

The binding interactions between OMPDC and HPO_3^{2-} may be utilized to introduce destabilizing electrostatic *stress* between carboxylate side chains at OMPDC and the 6-CO_2^- group of bound OMP at the ground-state Michaelis complex, which is relieved at a product-like *unstressed* transition state for the decarboxylation reaction.[11,12] However, such ground state effects cannot explain the 8×10^4-fold difference in k_{cat}/K_m for the OMPDC-catalyzed reaction of **EO** in the absence and presence of bound phosphite dianion,[6] because this is a comparison of the activation barriers for conversion of *unstressed free* OMPDC to transition states in which any HPO_3^{2-}-induced stress has necessarily been relieved.[10]

We now consider this question: *Is the HPO_3^{2-} binding energy utilized in the stabilization of a UMP-carbanion intermediate, or in the stabilization of some other feature of the transition state for decarboxylation of **OMP**?* The removal of the 6-CO_2^- group from the pyrimidine ring of **EO** gives the truncated product analog 1-(β-D-erythrofuranosyl)uracil (**EU**). Now, the observation that the OMPDC-catalyzed deuterium exchange reaction of **EU** is also strongly activated by HPO_3^{2-} would provide compelling evidence that the dianion binding interactions are utilized in stabilization of an enzyme-bound vinyl carbanion intermediate common to both the enzyme-catalyzed decarboxylation and deuterium exchange reactions (Scheme 1). Conversely, the failure to observe strong phosphite activation of the deuterium exchange reaction of **EU** would suggest that the specificity in the binding of HPO_3^{2-} to the transition state also involves interactions between OMPDC and the 6-CO_2^- group of the pyrimidine ring of **OMP**.

The OMPDC-catalyzed deuterium exchange reaction of **EU** is calculated to be too slow to detect at room temperature. We therefore examined the OMPDC-catalyzed deuterium exchange reaction of the truncated substrate 1-(β-D-erythrofuranosyl)-5-fluorouracil (**FEU**, Scheme 2B), where the electron-withdrawing 5-F provides a large stabilization of the carbanion-like transition state.[5] The preparation of **FEU** is described in Supporting Information.

The exchange for deuterium of the C-6 proton of ***h*-FEU** to give ***d*-FEU** catalyzed by OMPDC from *S. cerevisiae* (C155S mutant) in D_2O was monitored by ^{19}F NMR spectroscopy at 470 MHz.[5,13] Figure 1 shows ^{19}F NMR spectra obtained during the OMPDC-catalyzed (360 μM) deuterium exchange reaction of **FEU** (4.2 mM) in the presence of 4.7 mM HPO_3^{2-} in D_2O buffered by 50 mM glycylglycine at pD 8.1, 25 °C and $I = 0.14$ (NaCl). Deuterium exchange results in a decrease in the area of the doublet at 46.87 ppm due to ***h*-FEU** and the appearance of an upfield shifted singlet at 46.58 ppm due to ***d*-FEU**. Observed first-order rate constants for OMPDC-catalyzed deuterium exchange were determined as the slopes of linear plots (not shown) of reaction progress against time over the first 5–10% reaction (eq 1), where A_H and A_D are the integrated areas of the signals for ***h*-FEU** and ***d*-FEU**, respectively (Figure 1). Observed second-order rate constants $(k_{cat}/K_m)_{obsd}$ for the OMPDC-catalyzed deuterium exchange reaction, determined in the absence and presence of phosphite dianion, were calculated according to eq 2. Details of the kinetic

protocols and the experimentally-determined rate constants are given in the Supporting Information.

$$\frac{A_D}{A_D+A_H}=k_{obsd}t \quad (1)$$

$$\left(\frac{k_{cat}}{K_m}\right)_{obsd}=\frac{k_{obsd}}{[E]} \quad (2)$$

A value of $(k_{cat}/K_m)_S = k_o/fND = 9.89 \times 10^{-5}$ M^{-1} s^{-1} (Table 1) was determined as the second-order rate constant for the OMPDC-catalyzed deuterium exchange reaction of **FEU** in the absence of HPO_3^{2-} at pD 8.1 (Scheme 3), where $k_o = 5.44 \times 10^{-5}$ M^{-1} s^{-1} is the *observed* second-order rate constant for enzyme-catalyzed deuterium exchange in the *absence* of phosphite, and $f_{ND} = 0.55$ is the fraction of **FEU** present in the reactive neutral N3-D form.[14] The value of f_{ND} was calculated from $pK_a = 8.19$ for ionization of the N3-D of the pyrimidine ring of **FEU** in D_2O at 25 °C and $I = 0.10$ (NaCl), determined by spectrophotometeric titration at 269 nm.

Figure 2 shows the dependence of $(k_{cat}/K_m)_{obsd}/k_o$ for the deuterium exchange reaction of **FEU** on the concentration of phosphite dianion in D_2O buffered by 50 mM glycylglycine at pD 8.1, 25 °C and $I = 0.14$ (NaCl), where $(k_{cat}/K_m)_{obsd}$ is the *observed* second-order rate constant for the exchange reaction (eq 3, derived for Scheme 3). The data were fit to eq 4, with $(k_{cat}/K_m)_S = 9.89 \times 10^{-5}$ M^{-1} s^{-1}, to give $(k_{cat}/K_m)_{E\cdot HPi}/K_d = 1.76$ M^{-2} s^{-1} as the *third-order* rate constant for OMPDC-catalyzed deuterium exchange into **FEU** activated by phosphite dianion (Table 1). In this nomenclature E·HPi denotes an enzyme that is saturated with phosphite dianion. Table 1 also reports the kinetic parameters for the OMPDC-catalyzed decarboxylation of the whole substrate **OMP** and of the truncated substrate **EO** in D_2O buffered by 20 mM glycylglycine at pD 8.1, 25 °C and $I = 0.14$ (NaCl) that were determined using published procedures.[6]

$$(k_{cat}/K_m)_{obsd}=(k_{cat}/K_m)_S f_{ND}+\left(\frac{(k_{cat}/K_m)_{E\bullet HPi}}{K_d}\right) f_{ND}[HPO_3^{2-}] \quad (3)$$

$$\left(\frac{(k_{cat}/K_m)_{obsd}}{k_o}\right)=1+\left(\frac{(k_{cat}/K_m)_{E\bullet HPi}/K_d}{(k_{cat}/K_m)_S}\right)[HPO_3^{2-}] \quad (4)$$

A value of $(k_{cat}/K_m)_{SPi} = 620$ M^{-1} s^{-1} for reaction of the *whole substrate* was determined for the OMPDC-catalyzed deuterium exchange reaction of **FUMP** in D_2O under the conditions used for the exchange reaction of **FEU** (Table 1).

The data in Table 1 show that 1 M HPO_3^{2-} is calculated to result in a 1.8×10^4-fold increase $(k_{cat}/K_m)_{obsd}$ for the OMPDC-catalyzed deuterium exchange reaction of **FEU**. This corresponds to a 5.8 kcal/mol stabilization of the vinyl carbanion-like transition state (Figure 3). By comparison, HPO_3^{2-} shows a 2.0 kcal/mol larger (7.8 kcal/mol) affinity for the transition state for *decarboxylation* of the truncated substrate **EO** (Table 1).[6] If OMPDC acts

similarly in catalysis of the deuterium exchange reactions of **EU** and **FEU**, and the only role of the 5-F is to provide electrostatic stabilization of a vinyl carbanion intermediate, then the binding of HPO_3^{2-} should likewise result in a 2.0 kcal/mol larger stabilization of the transition state for decarboxylation of **EO** compared with the deuterium exchange reaction of **EU** (Table 1).

Similarly, there is a 2.0 kcal/mol difference in the intrinsic phosphodianion binding energies determined for the OMPDC-catalyzed decarboxylation reaction of **OMP** (11.2 kcal/mol) and the deuterium exchange reaction of **FUMP** (9.2 kcal/mol, Table 1). In other words, ca. 80% of the intrinsic phosphodianion binding energy in the decarboxylation reaction is utilized in stabilization of the carbanion-like transition state common to both the decarboxylation and the deuterium exchange reactions. These results provide strong evidence that the interactions between bound HPO_3^{2-} and the flexible phosphate gripper loop of OMPDC are directed towards effecting thermodynamic stabilization of a vinyl carbanion intermediate.[4]

We are unsure of the explanation for the 2.0 kcal/mol larger dianion binding energy for the transition state of the decarboxylation compared to the deuterium exchange reactions. The D70N mutation at OMPDC from *M. thermautotrophicus* results in a 200-fold decrease in k_{cat} for decarboxylation of **OMP**, but only a 2-fold decrease in the rate of the deuterium exchange reaction of **FUMP**.[5] This provides evidence that interactions between the carboxylate groups of D70 and enzyme-bound **OMP** promote decarboxylation of **OMP**, but not deuterium exchange of **FUMP**. However, the results from Table 1 are not relevant to the question of whether phosphite- driven loop closure induces destabilizing electrostatic interactions between carboxylate groups, which are relieved at the transition state for decarboxylation of **OMP**. This is because the rate constants reported in Table 1 provide a measure of the relative barriers to conversion of the *free* enzyme and reactants in the ground state to the respective transition states (see Figure 3). Destabilizing interactions at the intermediate Michaelis complexes cannot lead to a rate-enhancing reduction in the barrier for formation of these transition states from *free enzyme and substrates*.[10] We note that one difference between the decarboxylation and deuterium exchange reactions is the presence of neutral CO_2 at the product complex for the decarboxylation reaction. We suggest that the absence of CO_2 from the transition state and product complex for the deuterium exchange reaction may lead to small changes in the position of other catalytic side chains that result in the observed 2.0 kcal/mol smaller phosphite activation of the deuterium exchange reaction of **EU**, compared with the decarboxylation of **EO**. This is consistent with the notion that a precise orientation of these side chains relative to reactant is required to observe the full catalytic power of OMPDC.

In conclusion, the substrate phosphodianion and the substrate piece phosphite dianion interact strongly with catalytic side chains at the flexible gripper loop of OMPDC.[16] These interactions drive loop closure over the ligand and this leads to a large stabilization of carbanion-like transition states for both the decarboxylation and deuterium exchange reactions. The results may be rationalized by the model shown in Scheme 4, where OMPDC in the open form ($\mathbf{E_O}$) is inactive and the rare unliganded closed enzyme ($\mathbf{E_C}$) and the HPO_3^{2-}-liganded enzyme ($\mathbf{E_C}\cdot HPO_3^{2-}$) exhibit essentially equal high reactivities towards both carbon deprotonation and decarboxylation of the truncated substrates **FEU** and **EO**, respectively, so that $k_{cat}/K_m = (k_{cat}/K_m)'$. The intrinsic binding energy of HPO_3^{2-} is utilized to drive the unfavorable conformational change from $\mathbf{E_C}$ to give $\mathbf{E_C}$.[17,18] This model suggests that an important remaining challenge in understanding the mechanism of action of OMPDC is to provide a physical explanation for the proposed large effect of loop closure on the stability of the vinyl carbanion intermediate that is common to the OMPDC-catalyzed decarboxylation and deuterium exchange reactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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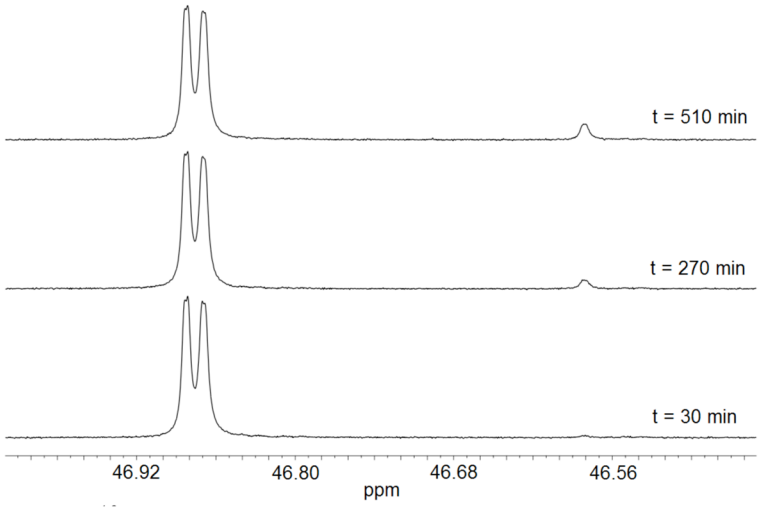


Figure 1.

^{19}F NMR spectra obtained during the OMPDC-catalyzed deuterium exchange reaction of **FEU** (4.2 mM) in the presence of 4.7 mM phosphite dianion in D_2O at pD 8.1 and 25 °C.

$$\frac{A_D}{A_D + A_H} = k_{obsd}t \qquad (1)$$

$$\left(\frac{k_{cat}}{K_m}\right)_{obsd} = \frac{k_{obsd}}{[E]} \qquad (2)$$

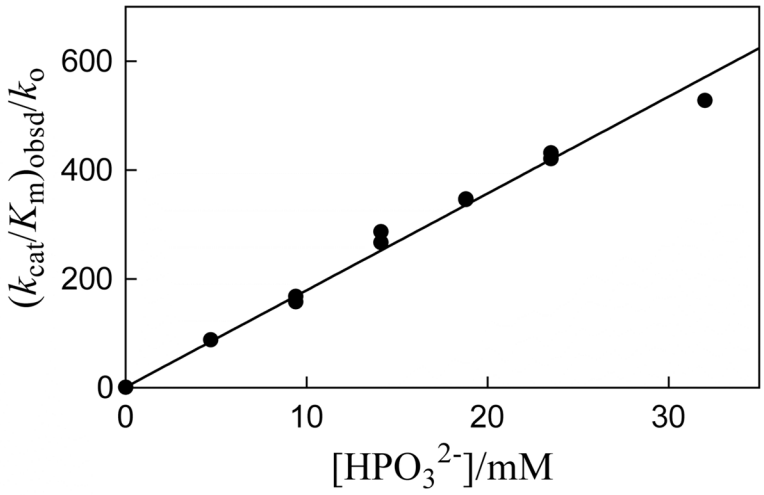


Figure 2.
The dependence of $(k_{cat}/K_m)_{obsd}/k_o$ for the OMPDC-catalyzed deuterium exchange reaction of **FEU** on the concentration of phosphite dianion in D_2O at pD 8.1, 25 °C and I = 0.14 (NaCl).

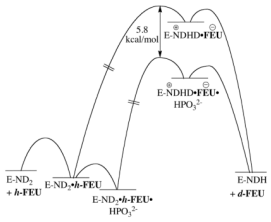
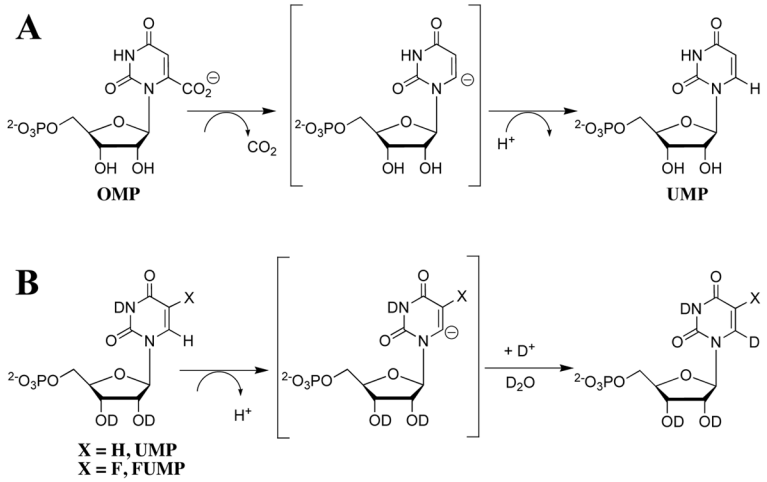
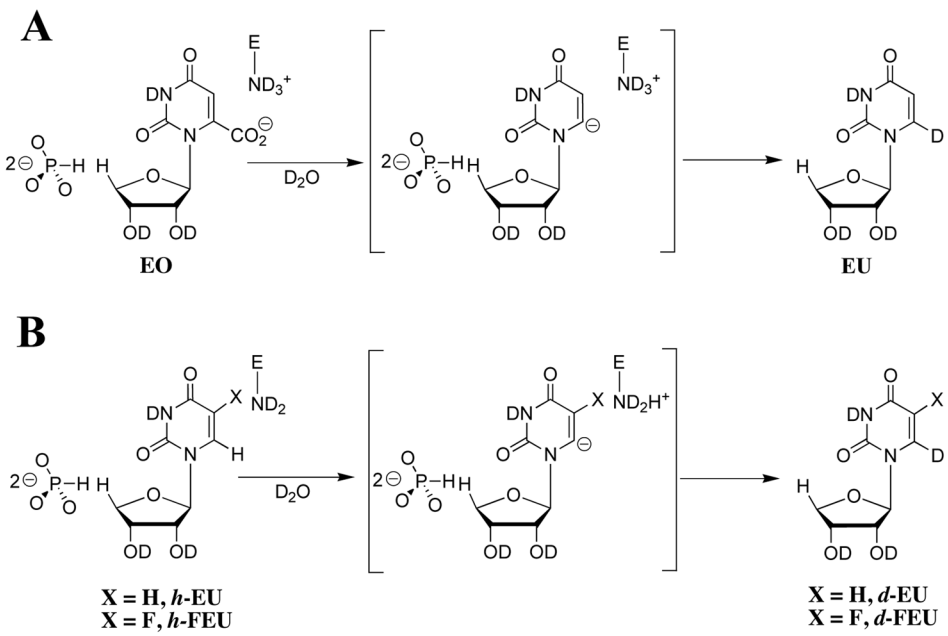


Figure 3.

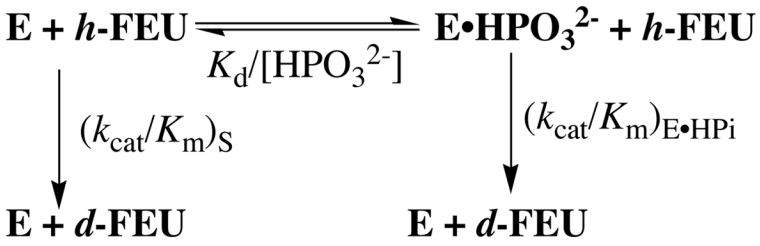
Partial free energy profiles for the unactivated and phosphite-activated OMPDC-catalyzed deuterium exchange reactions of the truncated substrate ***h*-FEU** in D_2O. The *slowest* step for the enzyme-catalyzed reaction is deprotonation of substrate by the alkyl amino side chain of Lys-93 to form the enzyme bound carbanion. The carbanion-like transition state is stabilized by 5.8 kcal/mol by the binding of phosphite dianion. In this figure, the barrier to formation of the enzyme-bound carbanion also includes rotation of the CH_2-ND_2H^+ bond at Lys-93 that is required to exchange the positions of the substrate derived –H and the solvent derived –D prior to hydron transfer to form ***d*-FEU**.[4,13,15]



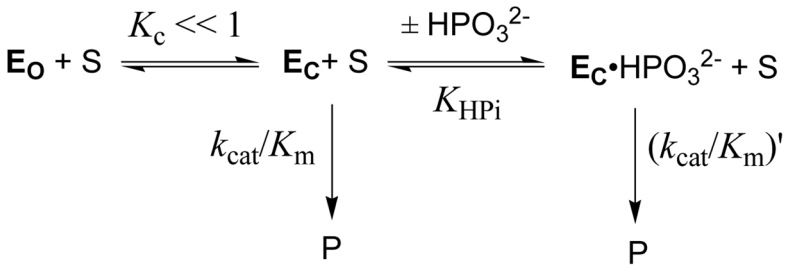
Scheme 1.



Scheme 2.



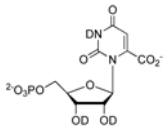
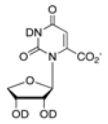
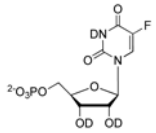
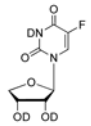
Scheme 3.



Scheme 4.

Table 1

Kinetic parameters for the OMPDC-catalyzed decarboxylation and deuterium exchange reactions of the whole substrates **OMP** and **FUMP** and of the truncated substrate pieces **EO** and **FEU** and the calculated intrinsic phosphodianion and phosphite binding energies.[a]

Reaction	Whole Substrate	$(k_{cat}/K_m)_{SPi}$[b] $M^{-1}s^{-1}$	Substrate Piece	$(k_{cat}/K_m)_S$[c] $M^{-1}s^{-1}$	ΔG_{ROPi}[d]	$(k_{cat}/K_m)_{E\cdot HPi}/K_d$[e] M^{-2} s^{-1}	ΔG_{HPi}[f]
OMP $\xrightarrow[\text{OMPDC}]{D_2O}$ UMP		$(5.50 \pm 0.55) \times 10^6$		0.030 ±0.003	−11.2 kcal/mol	$(1.73 \pm 0.35) \times 10^4$	−7.8 kcal/mol
h − FUMP $\xrightarrow[\text{OMPDC}]{D_2O}$ *d* − FUMP		620 ± 62 [g]		$(9.89 \pm 0.99) \times 10^{-5}$ [h]		1.76 ± 0.35 [h]	
					−9.2 kcal/mol		−5.8 kcal/mol
					$\Delta\Delta G_{ROPi}$ [i]		$\Delta\Delta G_{HPi}$ [j]
					2.0 kcal/mol		2.0 kcal/mol

[a] In D_2O buffered by either 20 or 50 mM glycylglycine at pD 8.1, 25 °C and I = 0.14 (NaCl). Quoted errors are the range of uncertainty based on multiple determinations.

[b] Second-order rate constant for OMPDC-catalyzed reaction of the whole substrate.

[c] Second-order rate constant for OMPDC-catalyzed reactions of **EO** or **FEU** in the absence of phosphite dianion.

[d] The intrinsic phosphodianion binding energy, calculated from the ratio $(k_{cat}/K_m)_{SPi}/(k_{cat}/K_m)_S$.

[e] Third-order rate constant for the phosphite-activated OMPDC-catalyzed reactions of the substrate pieces **EO** or **FEU** (Scheme 3).

[f] The intrinsic phosphite binding energy, calculated from the ratio $[(k_{cat}/K_m)_{E\cdot HPi}/K_d]/(k_{cat}/K_m)_S$.

[g] Calculated from $(k_{cat}/K_m)_{obsd}$ = 400 M^{-1} s^{-1} at pD 8.1 and pK_a = 8.37 for ionization of N3-D of **FUMP.** The ionized substrate is assumed to be unreactive towards OMPDC-catalyzed deuterium exchange.

[h] Calculated from $k_O = 5.44 \times 10^{-5}$ M^{-1} s^{-1} at pD 8.1 and pK_a = 8.19 for ionization of N3-D of **FEU** (see text).

[i] The difference in the phosphodianion binding energies for the decarboxylation and deuterium exchange reactions of the whole substrate.

[j] The difference in the phosphite binding energies for the decarboxylation and deuterium exchange reactions of the truncated substrate pieces.