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Orotidine 5′-Monophosphate Decarboxylase: Transition State Stabilization from Remote Protein−Phosphodianion Interactions

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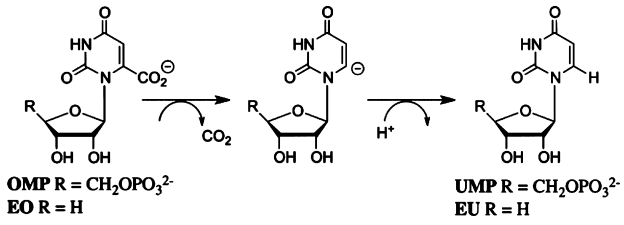
Supporting Information

ABSTRACT: Mutants of orotidine 5′-monophosphate decarboxylase containing all possible single (Q215A, Y217F, and R235A), double, and triple substitutions of the side chains that interact with the phosphodianion group of the substrate orotidine 5′-monophosphate have been prepared. Essentially the entire effect of these mutations on the decarboxylation of the truncated neutral substrate 1-(β-D-erythrofuranosyl)orotic acid that lacks a phosphodianion group is expressed as a decrease in the third-order rate constant for activation by phosphite dianion. The results are consistent with a model in which phosphodianion binding interactions are utilized to stabilize a rare closed enzyme form that exhibits a high catalytic activity for decarboxylation.

Orotidine 5′-monophosphate decarboxylase (OMPDC) effects an enormous 10^{17}-fold rate acceleration of the decarboxylation of orotidine 5′-monophosphate [OMP (Scheme 1)] to give uridine 5′-monophosphate (UMP).[1,2]

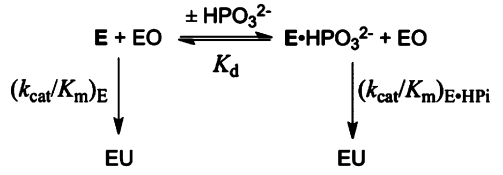
Scheme 1



The reaction proceeds through the UMP vinyl carbanion intermediate that is stabilized by at least 14 kcal/mol by interactions with the protein,[3,4] corresponding to a substantial fraction of the very large total substrate intrinsic binding energy[5] of 31 kcal/mol.[1]

We have shown that interactions between the phosphodianion group of OMP and OMPDC provide a 12 kcal/mol stabilization of the transition state for decarboxyation.[6,7] These protein−phosphodianion interactions do not simply anchor OMP to OMPDC, because ∼8 kcal/mol of this phosphodianion binding energy is recovered as stabilization of the transition state for OMPDC-catalyzed decarboxylation of the truncated substrate 1-(β-D-erythrofuranosyl)orotic acid [EO (Scheme 1)] by the binding of phosphite dianion [HPO_3^{2-} (Scheme 2)].[6,7]

Scheme 2



Here, the covalent connection between the ribosyl ring and the phosphodianion group is severed to give a "two-part substrate".[6,7] We are interested in understanding the mechanism by which binding interactions between OMPDC and the phosphodianion groups of bound OMP and HPO_3^{2-} are utilized to stabilize the transition state for decarboxylation at the remote pyrimidine ring of the substrate.

An examination of the X-ray crystal structure of yeast OMPDC liganded with 6-hydroxyuridine 5′-monophosphate (Figure 1) shows that the bound phosphodianion group

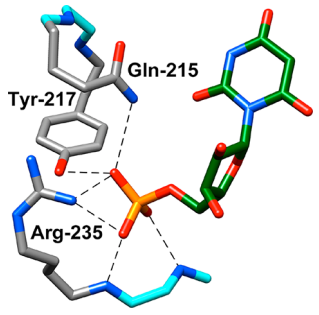


Figure 1. X-ray crystal structure of yeast OMPDC liganded with 6-hydroxyuridine 5′-monophosphate (Protein Data Bank entry 1DQX)[8] showing interactions of the protein with the bound phosphodianion group.

interacts directly with the side chains of Gln-215 and Tyr-217 that are part of a closed flexible loop, and with the guanidinium group of Arg-235.[8,9] The phosphodianion binding motif is completed by hydrogen bonds with the backbone NH groups of Gly-234 and Arg-235. A critical unknown is whether there are long-range interactions of these side chains with the

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Table 1. Kinetic Parameters for Wild-Type and Mutant Yeast OMPDC-Catalyzed Decarboxylation of the Whole Substrate OMP, the Truncated Substrate EO, and the Two-Part Substrate [EO + HPO_3^{2-}] at pH 7 and 25 °C[a]

	OMP[b]		EO[c]		[EO + HPO_3^{2-}][c]	
enzyme	k_{cat}/K_m (M^{-1} s^{-1})	$\Delta\Delta G^{\ddagger}$ (kcal/mol)	$(k_{cat}/K_m)_E$ (M^{-1} s^{-1})	$\Delta\Delta G^{\ddagger}$ (kcal/mol)	$(k_{cat}/K_m)_{E\cdot HP_i}/K_d$ (M^{-2} s^{-1})	$\Delta\Delta G^{\ddagger}$ (kcal/mol)
WT	1.1×10^{7}[d]		2.6×10^{-2}		1.2×10^{4}[e]	
Q215A	2.6×10^{5}[f]	2.2	1.1×10^{-2}[g,h]	0.5	240[g]	2.3
Y217F	1.8×10^{5}[f]	2.4	1.2×10^{-2}	0.5	26	3.6
R235A	610[f]	5.8	2.6×10^{-2}	0	0.63	5.8
Q215A/Y217F	2300	5.0	4.6×10^{-3}	1.0	0.58	5.9
Q215A/R235A	13	8.1	4.2×10^{-3}	1.1	<0.04[i]	>7.4
Y217F/R235A	4.1	8.7	1.0×10^{-2}[h]	0.6	<0.10[i]	>6.9
Q215A/Y217F/R235A	0.036	11.5	3.0×10^{-3}[h]	1.3	<0.04[i]	>7.4

[a]The estimated error in the reported rate constants is ±10% for OMP and EO and ±20% for the two-part substrate [EO + HPO_3^{2-}]. [b]At pH 7.1 buffered by 10−30 mM MOPS (50% free base) and I = 0.105 (NaCl). [c]At pH 7.0 buffered by 10−25 mM MOPS (45% free base) and I = 0.14 (NaCl), unless noted otherwise. [d]Data from ref 7. [e]At pH 7.0 buffered by phosphite dianion (80% free base). Data from ref 6. [f]Data from ref 10. [g]Data from ref 9. [h]At pH 7.1 buffered by 30−50 mM MOPS (50% free base) and I = 0.14 (NaCl). [i]Upper limit calculated with the assumption that a 30% rate increase could have been detected at the highest concentration of phosphite dianion used.

pyrimidine ring of the substrate that result in stabilization of the transition state for decarboxylation. To probe this, we have prepared the Q215A, Y217F, and R235A mutants of yeast OMPDC, along with the mutants containing the three double and the triple substitutions of these side chains that interact with the bound phosphodianion group (see Table 1). The preparations of wild-type yeast OMPDC and the Q215A and R235A mutants were described previously;[9,10] similar procedures were used to prepare the other mutants described here (see the Supporting Information). Table 1 gives the following kinetic parameters for the decarboxylation of OMP, the truncated substrate EO, and the two-part substrate [EO + HPO_3^{2-}] catalyzed by the single, double, and triple mutants of yeast OMPDC (see the Supporting Information for details of the kinetic procedures): (1) the second-order rate constants k_{cat}/K_m for decarboxylation of OMP, determined from the dependence of the initial velocity of decarboxylation on substrate concentration[9] or from the observed first-order rate constant for the complete reaction of OMP determined at [OMP] ≪ K_m,[7] (2) the second-order rate constants $(k_{cat}/K_m)_E$ for decarboxylation of EO in the absence of phosphite (Scheme 2), determined by following the initial rate of formation of the product 1-(β-D-erythrofuranosyl)uridine (EU) by HPLC,[6,7,9] and (3) the third-order rate constants $(k_{cat}/K_m)_{E\cdot HP_i}/K_d$ for decarboxylation of EO activated by exogenous phosphite dianion in the two-part substrate experiments (Scheme 2), determined as the slopes of linear plots of the observed second-order rate constants for decarboxylation of EO versus HPO_3^{2-} concentration.[6,7,9]

The effect of the Q215A/Y217F/R235A triple mutation on $\Delta G^{\ddagger}$ for decarboxylation of OMP, 11.5 kcal/mol, is only 1.1 kcal/mol larger than the sum of the effects of the individual Q215A (2.2 kcal/mol), Y217F (2.4 kcal/mol), and R235A (5.8 kcal/mol) mutations. Therefore, the effect of the triple mutation on the catalytic activity of OMPDC toward OMP can be ascribed almost entirely to the loss of stabilizing interactions of the three excised side chains with the transition state for decarboxylation. There are no large synergistic effects of the multiple mutations that result in a decrease in catalytic activity.[9] The effects of the Q215A, Y217F, and R235A mutations on the activity of OMPDC toward decarboxylation of the truncated substrate EO are expressed almost entirely as changes in the third-order rate constant $(k_{cat}/K_m)_{E\cdot HP_i}/K_d$ for phosphite activation of this reaction. The large ≥8 kcal/mol effect of the double and triple mutations that include R235A on the activity toward OMP resulted in no significant increases in the rate of the decarboxylation of EO upon inclusion of 24−30 mM HPO_3^{2-}. $(k_{cat}/K_m)_{E\cdot HP_i}/K_d$ upper limits for these mutants were calculated with the assumption that a 30% rate increase could have been detected in these experiments (Table 1).

By contrast, the Q215A/Y217F/R235A triple mutation results in a relatively small 9-fold decrease in $(k_{cat}/K_m)_E$ for the unactivated decarboxylation of the truncated substrate EO, and there are only ≤6-fold effects of the single and double mutations (Table 1). The observation that multiple mutations at the phosphodianion binding site result in a ≤1.3 kcal/mol increase in $\Delta G^{\ddagger}$ for decarboxylation of the neutral truncated substrate EO shows that there is little or no *direct* stabilization of the transition state for this reaction by long-range interactions of the excised side chains with the pyrimidine ring. We conclude that the remote phosphodianion binding interactions are used almost entirely for the purpose of activating OMPDC for decarboxylation of the bound substrate: either OMP or the two-part substrate [EO + HPO_3^{2-}]. The very similar second-order rate constants for decarboxylation of EO catalyzed by wild-type OMPDC [$(k_{cat}/K_m)_E$ = 0.026 M^{-1} s^{-1}] and of OMP catalyzed by the Q215A/Y217F/R235A triple mutant (k_{cat}/K_m = 0.036 M^{-1} s^{-1}) show that the elimination of enzyme−phosphodianion interactions either by removal of the substrate phosphodianion group to give EO or by excision of the interacting side chains to give the triple mutant enzyme results in almost identical 11.7 and 11.5 kcal/mol increases, respectively, in $\Delta G^{\ddagger}$ for enzyme-catalyzed decarboxylation (Table 1). Therefore, the ablation of protein−phosphodianion interactions appears to result in a basal reactivity of ~0.03 M^{-1} s^{-1} for catalysis of decarboxylation of both OMP and EO. This can be compared to the estimated rate constant for the nonenzymatic decarboxylation of OMP (k_{non} = 2.8×10^{-16} s^{-1})[2] to give a proficiency[1] for decarboxylation of EO [$(k_{cat}/K_m)/k_{non}$] of 1×10^{14} M^{-1}. This corresponds to a transition state stabilization of 19 kcal/mol from interactions of OMPDC with the nucleoside portion of OMP.

The $[(k_{cat}/K_m)_{E\cdot HP_i}/K_d]/(k_{cat}/K_m)_E$ ratio for the decarboxylation of EO catalyzed by the wild-type enzyme (Scheme 2) shows that the binding of phosphite dianion to the transition state for decarboxylation results in a transition state

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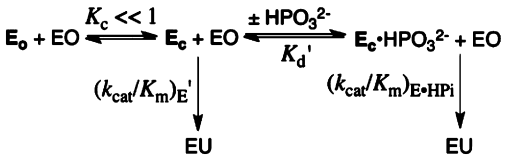
stabilization of ~8 kcal/mol.[6,7] Therefore, the interactions between HPO_3^{2-} and the side chains of Gln-215, Tyr-217, and Arg-235 serve to increase the transition state stabilization for decarboxylation of EO from 19 to 27 kcal/mol. This is smaller than the total transition state stabilization of 31 kcal/mol for the decarboxylation of OMP,[1] because the covalent connection between the nucleoside and phosphodianion portions of the substrate reduces the entropic cost of ligand binding,[11] resulting in an ~4 kcal/mol catalytic advantage for the decarboxylation of the whole substrate OMP over the pieces [EO + HPO_3^{2-}].[6]

The results reported here provide strong evidence for our proposed mechanism of activation of OMPDC by phosphite dianion, shown in Scheme 3. Here the free enzyme exists largely in an inactive open form ($\mathbf{E_O}$) and undergoes an energetically unfavorable conformational change to a rare active loop-closed form $\mathbf{E_C}$ ($K_c \ll 1$), the concentration of which is increased upon the binding of phosphite dianion ($1/K_d' \gg 1$). In this model, the rare unliganded closed enzyme $\mathbf{E_C}$ and the phosphite-liganded enzyme $\mathbf{E_C}$·HPO_3^{2-} have essentially equally high activities for decarboxylation, so that $(k_{cat}/K_m)_E' = (k_{cat}/K_m)_{E\cdot HP_i}$. Equations 1 and 2 define the relationships between the experimental rate constants $(k_{cat}/K_m)_E$ for the unactivated decarboxylation of EO and $(k_{cat}/K_m)_{E\cdot HP_i}/K_d$ for the phosphite-activated reaction (Scheme 2) and the terms in Scheme 3. We propose that (1) the single, double, and triple Q215A, Y217F, and R235A mutations result in only small changes in $(k_{cat}/K_m)_E$ (Table 1) because their effect on the reactivity of the closed enzyme, $(k_{cat}/K_m)_E'$, and on the equilibrium constant K_c for the unfavorable conformational change in the absence of ligand is small (eq 1) and (2) the major effect of these mutations is to strongly decrease the affinity of $\mathbf{E_C}$ for HPO_3^{2-}, resulting in an increase in K_d' and a decrease in $(k_{cat}/K_m)_{E\cdot HP_i}/K_d$ (eq 2).

$$(k_{cat}/K_m)_E = K_c(k_{cat}/K_m)_E' \quad (1)$$

$$(k_{cat}/K_m)_{E\cdot HP_i}/K_d = (K_c/K_d')(k_{cat}/K_m)_{E\cdot HP_i} \quad (2)$$

Scheme 3



The existence of OMPDC in an open form in which the active site is exposed to bulk solvent is necessary to allow access for substrate binding.[12] However, there is an important imperative for the desolvation and shielding of the active site from bulk solvent because this decreases the effective dielectric constant of the active site that will in turn greatly enhance stabilizing electrostatic protein–ligand interactions.[13,14] We propose the following. (1) The thermodynamic barrier to formation of the productive catalytic complex $\mathbf{E_C}$·S from the inactive enzyme $\mathbf{E_O}$ arises largely from the desolvation of the active site accompanying the conformational change and sequestration of the substrate from bulk solvent. The barrier may also include strain introduced into the protein that is subsequently relieved at the transition state for decarboxylation.[15] (2) The energetically unfavorable conformational change and desolvation of the active site are "paid for" by the binding energy available from the formation of strong phosphodianion–protein interactions in the desolvated environment present at the $\mathbf{E_C}$·S complex. (3) The phosphodianion binding energy is recovered as transition state stabilization via the enhanced electrostatic and hydrogen bonding interactions at the transition state in the desolvated active site. There is good evidence that the closed conformation of OMPDC from *Methanobacter thermoautotrophicus* is stabilized by interactions of the hydrophobic side chain of Val-182 in the active site loop with the side chains of Ile-199, Val-201, and Ile-218.[16] This suggests that there is evolutionary pressure to attenuate the barrier to the conversion of $\mathbf{E_O}$ to $\mathbf{E_C}$ so that it does not adversely affect catalysis.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Procedures for the preparation of mutant OMPDCs and details of the kinetic protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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