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Rapid Report

Activation of R235A Mutant Orotidine 5′-Monophosphate Decarboxylase by the Guanidinium Cation: Effective Molarity of the Cationic Side Chain of Arg-235[†]

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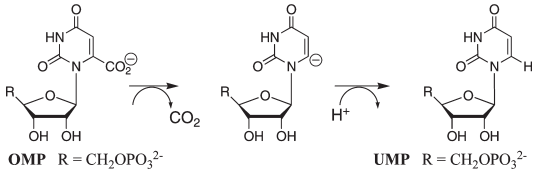
ABSTRACT: The R235A mutation at yeast orotidine 5′-monophosphate decarboxylase (OMPDC) results in a 1300-fold increase in K_m and a 14-fold decrease in k_{cat} for decarboxylation of orotidine 5′-monophosphate, corresponding to a 5.8 kcal/mol destabilization of the transition state. There is strong activation of this mutant enzyme by added guanidinium cation (Gua^+): 1 M Gua^+ stabilizes the transition state by ca. 3 kcal/mol. This stabilization is due to the binding of Gua^+ to the binary $E_{mut}\cdot OMP$ complex, with a K_d of 50 mM, to form the 9-fold more reactive ternary $E_{mut}\cdot OMP\cdot Gua^+$ complex. The "effective molarity" of the cationic side chain of Arg-235 at the wild-type enzyme is calculated to be 160 M.

Orotidine 5′-monophosphate decarboxylase (OMPDC)[1] is a remarkable enzyme because it employs no metal ions or other cofactors, yet it effects an enormous ca. 30 kcal/mol stabilization of the transition state for the decarboxylation of orotidine 5′-monophosphate (OMP) to give uridine 5′-monophosphate (UMP) (*1*) through an unstable vinyl carbanion intermediate (Scheme 1) (*2*).

Scheme 1



OMP R = $CH_2OPO_3^{2-}$ **UMP** R = $CH_2OPO_3^{2-}$
EO R = H **EU** R = H

The "intrinsic" binding energy of the phosphodianion group of OMP accounts for 40% (12 kcal/mol) of the total ca. 30 kcal/mol transition state binding energy (*3*). The substrate OMP has been partitioned into the "pieces" 1-(β-D-erythrofuranosyl)orotic acid (EO) and phosphite dianion (HPO_3^{2-}), each of which binds to OMPDC with low affinity ($K_d \approx 0.1$ M) (*3*). The EO piece undergoes slow OMPDC-catalyzed decarboxylation of its orotate base moiety to give 1-(β-D-erythrofuranosyl)uracil (EU) with a $(k_{cat}/K_m)_E$ of 0.021 M^{-1} s^{-1} (*3*). The separate binding of the HPO_3^{2-} piece strongly activates bound EO toward decarboxylation, with a $(k_{cat}/K_m)_{E\cdot HP_i}/(k_{cat}/K_m)_E$ of 80000 (*3*). Tethering the two pieces to give the whole substrate OMP places the orotate base and the oxydianion activator at a single molecule and results in a large entropic advantage over the binding and reaction of the individual pieces (*4*). Similar strong activation of the enzymatic reaction of a substrate piece by HPO_3^{2-} has been observed for proton transfer catalyzed by triosephosphate isomerase (*5*) and for hydride transfer catalyzed by glycerol 3-phosphate dehydrogenase (*6*).

X-ray crystallographic analysis of yeast OMPDC has shown that the guanidinium cation side chain of Arg-235 forms a bidentate ion pair with the phosphodianion group of the bound intermediate analogue 6-hydroxyuridine 5′-monophosphate (*7*). The R235A mutation at yeast OMPDC has been reported to result in a large 5.3 kcal/mol increase in the activation barrier to OMPDC-catalyzed decarboxylation of OMP (*8*). This suggests that the ionic and/or hydrogen bonding interactions between the cationic side chain of Arg-235 and the phosphodianion group of OMP account for ca. 5.3/12 (44%) of the total intrinsic phosphate binding energy of the substrate. The remaining phosphodianion binding interactions are principally with the flexible phosphate gripper loop that extends 19 residues from the strictly conserved Pro-202 to Val-220 (*7*, *9*), along with a hydrogen bond to the backbone NH group of Gly-234. The loop acts to stabilize both the Michaelis complex and the transition state for decarboxylation through formation of hydrogen bonds between the substrate phosphodianion group and the side chains of Gln-215 (*10*) and Tyr-217 (*7*). Since a large fraction of the total intrinsic phosphate binding energy is recovered when phosphite dianion binds to a wild-type $E_{wt}\cdot EO$ complex, we speculated that a large fraction of this binding energy might also be recovered in a two-part enzyme experiment in which the side chain of Arg-235 is truncated from OMPDC by site-directed mutagenesis, and the impaired activity for decarboxylation of OMP at the $E_{mut}\cdot OMP$ complex is rescued upon binding of the missing guanidinium cation (Gua^+) piece.

We have prepared R235A mutant yeast OMPDC and determined values for k_{cat} of 1.1 s^{-1} and K_m of 1.8 mM for decarboxylation of OMP at pH 7.1 (10 mM MOPS), 25 °C, and an ionic strength (I) of 0.105 (NaCl) (Table 1). The value of K_m for OMP is highly sensitive to ionic strength, and we have determined a K_m of 42 μM for this mutant at pH 7.1 (20 mM MOPS) and $I = 0.01$. By comparison, Wolfenden and co-workers reported a k_{cat} of 0.42 s^{-1} and a K_m of 49 μM for the R235A mutant at pH 7.4, 25 °C, and an unspecified ionic strength (*8*). Our data for the R235A mutant can be combined with those for the wild-type enzyme to give the effect of the R235A mutation on k_{cat}/K_m for turnover of OMP as 18000-fold (Table 1).

The Q215A mutation at OMPDC results in a small ca. 60% increase in k_{cat} for decarboxylation of OMP and a larger ca. 70-fold

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[1]Abbreviations: OMPDC, orotidine 5′-monophosphate decarboxylase; OMP, orotidine 5′-monophosphate; UMP, uridine 5′-monophosphate; EO, 1-(β-D-erythrofuranosyl)orotic acid; EU, 1-(β-D-erythrofuranosyl)uracil; Gua^+, guanidinium cation; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EM, effective molarity.

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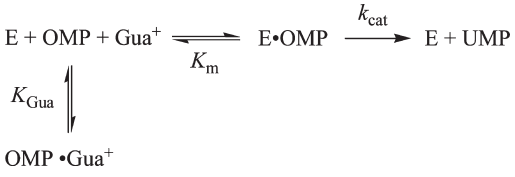
increase in K_m (*10*).[2] The apparent value of k_{cat}/K_m for decarboxylation of OMP catalyzed by Q215A OMPDC [$(k_{cat}/K_m)_{app}$] decreases from 2.4×10^5 to 1.5×10^5 M^{-1} s^{-1} as the Gua^+ concentration is increased from 0 to 0.10 M at pH 7.1 (10 mM MOPS), 25 °C, and I = 0.105 (NaCl) (data not shown). We attribute the decrease in $(k_{cat}/K_m)_{app}$ with an increase in Gua^+ concentration to the formation of an ion pair complex between OMP and Gua^+, OMP·Gua^+ (Scheme 2). This ion pair is not a substrate for the enzyme, and its formation leads to an increase in the apparent value of K_m for OMP. This analysis was not attempted for wild-type OMPDC because the tight binding of OMP to this enzyme (K_m = 1.4 μM) renders relatively small changes in K_m experimentally difficult to quantify accurately (*9*). The data for the Q215A mutant were fit to eq 1, derived for Scheme 2, to give a K_{Gua} of 0.18 M for breakdown of the OMP·Gua^+ ion pair. By comparison, a dissociation constant of 0.38 M has been reported for breakdown of the HPO_4^{2-}·Gua^+ ion pair (*11*).

Scheme 2



$$(k_{cat}/K_m)_{app} = \frac{k_{cat}/K_m}{1 + [Gua^+]/K_{Gua}} \quad (1)$$

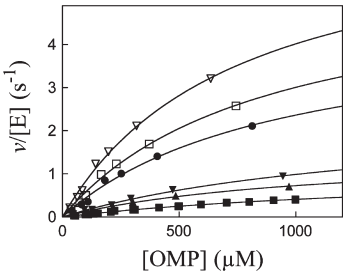


FIGURE 1: Dependence of v/[E] for decarboxylation of OMP catalyzed by R235A mutant OMPDC on the concentration of OMP in the presence of various concentrations of Gua^+ where [OMP] has been corrected for formation of the OMP·Gua^+ complex: (■) 0, (▲) 5, (▼) 10, (●) 40, (□) 60, and (▽) 100 mM Gua^+.

Figure 1 shows the dependence of v/[E] (s^{-1}) for decarboxylation of OMP catalyzed by R235A mutant OMPDC on the concentration of OMP, where v ($M\ s^{-1}$) is the initial velocity and [E] is the concentration of the enzyme, in the presence of various concentrations of Gua^+ at pH 7.1 (10 mM MOPS), 25 °C, and I = 0.105 (NaCl). The data show that exogenous Gua^+ strongly activates this mutant OMPDC for catalysis of decarboxylation of OMP. The activation by Gua^+ is reflected in an increase both in $(k_{cat})_{app}$ (s^{-1}) for decarboxylation at saturating OMP levels and in the apparent affinity of OMPDC for OMP [decrease in $(K_m)_{app}$].

Figure 2 shows kinetic data for the activation of R235A mutant OMPDC toward decarboxylation of OMP by added

[2]Our consensus kinetic parameters for turnover of OMP by Q215A mutant yeast OMPDC are as follows: $k_{cat} = 24 \pm 1\ s^{-1}$, and $K_m = 94 \pm 6\ \mu$M. They differ slightly from the previously published values (*10*).

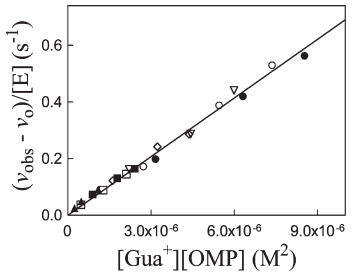
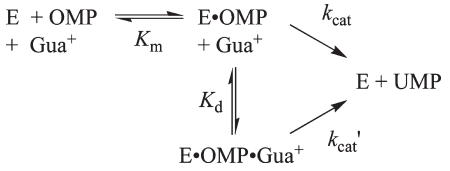


FIGURE 2: Activation of R235A mutant OMPDC toward decarboxylation of OMP by added Gua^+: (▲) 5, (□) 10, (■) 20, (◇) 40, (▽) 60, (○) 80, and (●) 100 mM Gua^+.

Gua^+ at low [Gua^+][OMP], where effectively all of the enzyme is present in the free unliganded form (Scheme 3). The activation is quantified as $(v_{obs} - v_o)$/[E], where v_{obs} ($M\ s^{-1}$) is the observed initial velocity of decarboxylation at a particular [Gua^+] and v_o ($M\ s^{-1}$) is the initial velocity of decarboxylation of the *same* concentration of OMP in the absence of Gua^+. The slope of the linear correlation according to eq 2, derived for Scheme 3, gives a k_{cat}'/K_mK_d of 69000 M^{-2} s^{-1}. The ratio of this third-order rate constant and the k_{cat}/K_m of 610 M^{-1} s^{-1} for the unactivated mutant enzyme [$(k_{cat}'/K_mK_d)/(k_{cat}/K_m) = 110\ M^{-1}$] is the hypothetical activation of R235A mutant OMPDC by 1 M Gua^+.

Scheme 3



$$(v_{obs} - v_o)/[E] = (k_{cat}'/K_mK_d)[OMP][Gua^+] \quad (2)$$

$$(k_{cat})_{app} = \frac{k_{cat} + k_{cat}'[Gua^+]/K_d}{1 + [Gua^+]/K_d} \quad (3)$$

Table 1: Kinetic Parameters for Decarboxylation of OMP Catalyzed by Wild-Type and R235A Mutant Yeast OMPDC[a]

kinetic parameter[b]	wild type[c]	R235A	R235A with Gua^+
k_{cat} or k_{cat}' (s^{-1})	15	1.1	9.7
K_m (μM)	1.4	1800	
k_{cat}/K_m (M^{-1} s^{-1})	1.1×10^7	610	
k_{cat}'/K_mK_d (M^{-2} s^{-1})			6.9×10^4
K_d (mM)			50

[a]At pH 7.1 (10 mM MOPS), 25 °C, and I = 0.105 (NaCl). [b]Kinetic parameters are defined in Scheme 3 (see the text). [c]Data from ref *9*.

Figure 3 shows the dependence of the apparent value of k_{cat} for the decarboxylation of OMP catalyzed by R235A mutant OMPDC on [Gua^+], where $(k_{cat})_{app}$ was obtained from the fits of the data in Figure 1 to the Michaelis−Menten equation. The solid line in Figure 3 shows the fit of the data to eq 3, derived for Scheme 3, which gave a K_d of 50 mM for the binding of Gua^+ to the binary E_{mut}·OMP complex and a k_{cat}' of 9.7 s^{-1} for decarboxylation of OMP at the ternary E_{mut}·OMP·Gua^+ complex. Table 1 summarizes the kinetic parameters obtained in these experiments. The data show the following about activation of the R235A mutant OMPDC-catalyzed decarboxylation of OMP by Gua^+.

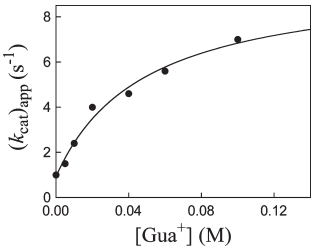


FIGURE 3: Dependence of the apparent value of k_{cat} for decarboxylation of OMP catalyzed by R235A mutant OMPDC on the concentration of added Gua^+.

(1) The transition state for decarboxylation of OMP catalyzed by R235A mutant OMPDC is stabilized by 2.8 kcal/mol by 1 M Gua^+. By comparison, the R235A mutation destabilizes the transition state for decarboxylation of OMP by 5.8 kcal/mol, so that 50% of the transition state stabilization due to the side chain of Arg-235 would be restored by a level of its Gua^+ analogue of 1 M. An even more impressive 66% of the transition state stabilization due to the phosphodianion group of OMP would be recovered when decarboxylation of EO by wild-type OMPDC is conducted in the presence of 1 M HPO_3^{2-} (*3*).

(2) The affinity of the $E_{mut}\cdot$OMP complex for Gua^+ [$K_d = 50$ mM (Scheme 3)] is ~4-fold higher than the affinity of OMP for Gua^+ in water [$K_{Gua} = 180$ mM (Scheme 1)]. This shows that the enzyme scaffold has a significant effect on the interactions between OMP and Gua^+.

(3) Binding of Gua^+ to the $E_{mut}\cdot$OMP complex of R235A mutant OMPDC causes a 9-fold increase in the apparent value of k_{cat} from 1.1 to 9.7 s^{-1}. This corresponds to nearly complete recovery of the wild-type activity ($k_{cat} = 15\ s^{-1}$). Similarly, we find that the binding of HPO_3^{2-} to the wild-type $E_{wt}\cdot$EO complex results in an increase in k_{cat} for decarboxylation of EO to a value estimated to be greater than or equal to that for the decarboxylation of OMP (*3*).

There have been many reports of chemical rescue of the activity of mutant enzymes by small molecules that are functionally equivalent to the excised amino acid side chain (*12*). These side chains are often proposed to play a chemical role in Brønsted acid−base or nucleophilic catalysis that is replicated in rescue by small molecule analogues (*12*). Arg-127 stabilizes the oxyanion of the tetrahedral intermediate formed during peptide hydrolysis catalyzed by carboxypeptidase A, and chemical rescue of the R127A mutant by guanidine has been reported (*13*). By contrast, the cationic side chain of Arg-235 at OMPDC is too distant from the orotic acid moiety of OMP to participate *directly* in decarboxylation (*7*). Thus, rather than chemical rescue, exogenous Gua^+ *rescues* a critical electrostatic interaction that is absent in the $E_{mut}\cdot$OMP complex.

The R235A mutation at OMPDC presumably creates a cleft at the enzyme surface that can be occupied by Gua^+ to give the $E_{mut}\cdot$OMP$\cdot Gua^+$ complex that undergoes decarboxylation at nearly the same rate as the wild-type $E_{wt}\cdot$OMP complex. Wild-type and R235A mutant OMPDC would show the same value of k_{cat}/K_m if the reaction of the mutant enzyme could be conducted in the presence of 160 M Gua^+. This is the "effective molarity" (EM) (*14*) of the Arg-235 side chain at wild-type OMPDC, calculated as $(k_{cat}/K_m)/(k_{cat}'/K_mK_d) = 160$ M (Table 1). By comparison, data for the two-part substrate experiment in which HPO_3^{2-} activates wild-type OMPDC toward decarboxylation of EO (*3*) give an EM of 920 M for the phosphodianion group of OMP. These large EMs probably result from the entropic price paid to freeze the translational and rotational motions of HPO_3^{2-} in the two-part substrate experiment or Gua^+ in the two-part enzyme experiment (*4*).

This novel rescue by Gua^+ of a critical enzyme−phosphodianion electrostatic interaction that is excised by the R235A mutation at OMPDC raises questions about the requirements for the observation of the recovery of wild-type enzyme activity in the reaction of a mutant enzyme and its missing part. Efficient rescue of the activity of R235A mutant OMPDC is observed because the cationic side chain of Arg-235 sits in a solvent-exposed cleft. This leads to fast, reversible, binding of Gua^+ in the empty cleft in the R235A mutant. Additional work is needed to establish the generality of such a rescue of electrostatic interactions that provide strong stabilization of the transition state for enzymatic reactions.

SUPPORTING INFORMATION AVAILABLE

Kinetic protocols and the preparation of R235A mutant OMPDC. This material is available free of charge via the Internet at http://pubs.acs.org.

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